Compendium of International Methods of Wine and Must Analysis

VOLUME 1

Foreword · Annex A: Sections 1, 2 and 3.1.1 to 3.1.4

INTERNATIONAL ORGANISATION
OF VINE AND WINE

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Compendium

of International Methods

of Wine and Must Analysis

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Foreword · Annex A: Sections 1, 2 and 3.1.1 to 3.1.4

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176	Conductivity (OIV-OENO 419A-2011)	OIV-MA-F1-01	IV
177	Hydroxymethylfurfural (HMF) by High-Performance Liquid Chromatography (OIV-OENO 419A-2011)	OIV-MA-F1-02	IV
178	Determination of the acquired alcoholic strength by volume (ASV) of concentrated musts (CM) and grape sugar (or rectified concentrated musts, RCM) (OIV-OENO 419A-2011)	OIV-MA-F1-03	IV
179	Sucrose by high-performance liquid chromatography (OIV-OENO 419A-2011)	OIV-MA-F1-04	IV
180	Total acidity (OIV-OENO 419A-2011)	OIV-MA-F1-05	IV
181	pH (OIV-OENO 419A-2011)	OIV-MA-F1-06	IV
182	Sulphur dioxide (OIV-OENO 419A-2011)	OIV-MA-F1-07	IV
183	Chromatic properties (OIV-OENO 419A-2011)	OIV-MA-F1-08	IV

N°	Method	Sheet	Type
184	Total cations (OIV-OENO 419B-2012)	OIV-MA-F1-09	I
185	Heavy metals by ETAAS (OIV-OENO 419B-2012)	OIV-MA-F1-10	IV
186	Heavy metals by ICP-MS (OIV-OENO 419B-2012)	OIV-MA-F1-11	IV
187	Determination of meso-inositol, scyllo-inositol and sucrose (OIV-OENO 419C-2012)	OIV-MA-F1-12	II
188	Folin-Ciocalteu Index (OIV-OENO 419D-2012)	OIV-MA-F1-13	IV

ANNEX G

Grape juice, reconstituted grape juice, concentrated grape juice and grape nectar

N°	Method	Sheet	Type
189	Measuring Ochratoxin A in grape juice, reconstituted grape juice, concentrated grape juice, and grape nectar, after going through an immunoaffinity column and high liquid performance chromatography with fluorescence detection (OIV-OENO 662A-2022)	OIV-MA-G1-01	IV
190	Determination of volatile acidity in grape juice, reconstituted grape juice, concentrated grape juice, and grape nectar (OIV-OENO 662C-2022)	OIV-MA-G1-02	IV
191	Method for $^{18}O/^{16}O$ isotope ratio determination of water in grape juice (OIV-OENO 662H-2022)	OIV-MA-G1-03	IV
192	Soluble solids in grape juice, reconstituted grape juice, concentrated grape juice and grape nectar (OIV-OENO 662F-2023)	OIV-MA-G1-04	I
193	Horizontal method for the enumeration of coliforms — Colony-count technique (OIV-OENO 662M-2023)	OIV-MA-G1-05	
194	Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli (OIV-OENO 662N-2023)	OIV-MA-G1-06	
195	Horizontal method for the enumeration of coagulase-positive staphylococci (OIV-OENO 662O-2023)	OIV-MA-G1-07	

N°	Method	Sheet	Type
196	Horizontal method for the enumeration of microorganisms (OIV-OENO 662P-2023)	OIV-MA-G1-08	
197	Determination of mineral elements in grape juices, reconstituted grape juice, concentrated grape juices and grape nectars using ICP-AES (OIV-OENO 662K-2024)	OIV-MA-G1-09	IV
198	Determination of total acidity in grape juice, reconstituted grape juice, concentrated grape juice and grape nectar by titration (OIV-OENO 662G-2025)	OIV-MA-G1-10	IV

Foreword

The *Compendium of International Methods of Wine Analysis* was first published in 1962 and re-published in 1965, 1972, 1978, 1990 and 2000; each time it included additional material as approved by the General Assembly and produced each year by the Sub-Commission.

This edition of *Compendium of International Methods of Wine and Must Analysis* includes all material as approved by the General Assembly of representatives of the member governments of the OIV, revised and amended since 2000.

The Compendium plays a major part in harmonising methods of analysis. Many vine-growing countries have introduced its definitions and methods into their own regulations.

Regulation (EC) No 479/2008 lays down that *the analysis methods for establishing the composition of the products covered by that Regulation and the rules for checking whether those products have been subjected to processes in violation of authorised oenological practice are those recommended and published by the OIV in the Compendium of International Methods of Analysis of Wines and Musts*. In Regulation (EC) No 606/2009 to ensure greater transparency, it was stated to publish at Community level (C Series of the *Official Journal of the European Union*) the list and description of the analysis methods described in the Compendium of International Methods of Analysis of Wines and Musts of the International Organisation of Vine and Wine and applicable for the control of vitivinicultural products.
In this way the European Union recognises all of the methods in the Compendium and makes them binding in all Member States, confirming the close collaboration established between the EU and the OIV.

Thus, through its leading role in the harmonisation of methods of analysis, the Compendium contributes to facilitating international trade. With the *International Code of Oenological Practices* and the *International Oenological Codex,* it constitutes a body of considerable scientific, legal and practical benefit.

Layout and wording of OIV method of analysis

Extract of ISO 78-2:1999 standard

1. Title

2. Introduction

optional

3. Scope

This clause shall state succintly the method of chemical analysis and specifically the product to which applies.

4. Définitions

5. Principle

This optional clause indicates the essential steps in the method used, the basic principles.

6. Reagents and materials

This clause shall list all the reagents and materials used during the test, together with their essential characteristics, and shall specify, if necessary, their degree of purity.
Shall be given :
Products used in their commercially available form
Solutions of defined concentration
Standard volumetric solution
Standard reference solution
Standard solution
Standard matching solution
Note : each reagent shall be mentioned by a specific reference number

7. Apparatus

This cluse shall list the names and significant characteristics of all the apparatus and equipment to be used during the analysis or test.

8. Sampling (Preparation of the sample)

Shall be given :
Sampling procedure
Preparation of the test sample

9. Procedure

Each sequence of operations shall be described unambiguously and concisely.
This clause shall normally include the following subclauses :
Test portion (this subclause shall give all the information necessary for the preparation of the test portion from the test sample).
Determination(s), or test(s) (this subclause shall be described accurately in order to facilitate the description, the understanding and the application of the procedure).
Calibration (if necessary).

10. Calculation (Results)

This clause shall indicate the method for calculating the results. Shall be precised the units, the equation used, the meanings of the algebraic symbols, the number of decimal places to which the results is to be given.

11. Precision (if interlaboratory validation)

The precision data shall be indicated:
The number of laboratoriese
The mean value of the concentration
The repeatability and the reproducibility
The repeatability and reproducibility standard deviation
A reference to the document containing the published results of the interlaboratory tests.

12. Annex

Annex related to precision clauses
Annex concerning statistical and other data derived from the results of interlaboratory tests.

13. Bibliography

Annex related to precision clauses

This annex shall indicate in particular
- repeatability statements
- reproducibility statements

Annex concerning statistical and other data derived from the results of interlaboratory tests.

Statistical and other data derived from the results of interlaboratory tests may be given in an informative annex.

Example of table giving statistical results

Sample identification	A	B	C
Number of participating laboratories			
Number of accepted test results			
Mean values (g/100g sample)			
True or accepted value (g/100g)			
Repeatability standard deviation (S_r)			
Repeatability coefficient of variation			
Repeatability limit (r) (2,8 x S_r)			
Reproducibility standard deviation (S_R)			
Reproducibility coefficient of variation			
Reproducibility limit (R) (2,8 x S_R)			

Whilst it may not be considered necessary to include all the data shown in the table, it is recommended that at least the following data be included:

- The number of laboratories
- The mean value of the concentration
- The repeatability standard deviation
- The reproducibility standard deviation
- A reference to the document containing the published results of the interlaboratory tests.

Annex A

Methods of analysis of wines and musts

General Remarks

1/ Clear wine or must, must be used for chemical and physical analysis. If the wine or the must is cloudy, it is first filtered through filter paper in a covered funnel or centrifuged in a closed container. This operation must be stated on any required documentation.

2/ The reference of the method employed for each determination must be on any required documentation.

3/ Units of measure for the various magnitudes (volume, mass, concentration, temperature, pressure, etc.) shall be in accordance with the recommendations of the IUPAC (International Union for Pure and Applied Chemistry).

4/ In respect of reagents and titration solutions used, unless otherwise required in the text, the chemicals used are to be of "analytical grade" and the water is to be distilled or of equivalent purity.

5/ Enzyme methods, and the determination of a number of parameters, are to be based on absolute measurements of absorbance, which requires spectrophotometers to be calibrated for wavelengths and absorbance. Wavelength may be calibrated by use of Hg lines: 239.94, 248.0, 253.65, 280.4, 302.25, 313.16, 334.15, 365.43, 404.66, 435.83, 546.07, 578.0, and 1014.0 nm. Absorbance may be calibrated by means of commercial reference solutions, obtained from suitable suppliers, or neutral density filters.

6/ The essential bibliographical references are given. The references to working documents of the Sub-Commission are marked '*F.V., O.I.V.*' (feuillets verts or 'green pages'), followed by the year of publication and the number of the document.

Classification of analytical methods

(Resolution OENO 9/2000)

CATEGORY I* (CRITERION BENCHMARK METHOD): A method which determines a value that can be arrived at only by implementing the method *per se* and which serves, by definition, as the only method for establishing the accepted value of the parameter measured (e.g., alcoholometric content, total acidity, volatile acidity).

CATEGORY II* (BENCHMARK METHOD): A category II method is designated as the Benchmark Method in cases where category I methods cannot be used. It should be selected from category III methods (as defined below). Such methods should be recommended for use in cases of disputes and for calibration purposes. (e.g., potassium, citric acid).

CATEGORY III* (APPROVED ALTERNATIVE METHODS): A category III Method meets all of the criteria specified by the Sub-Committee on Methods of Analysis and is used for monitoring, inspection and regulatory purposes (e.g., enzymatic determinations of glucose and fructose).

CATEGORY IV (AUXILIARY METHOD): A category IV Method is a conventional or recently-implemented technique, with respect to which the Sub-Committee on Methods of Analysis has not as yet specified the requisite criteria (e.g., synthesized coloring agents, measurement of oxidation-reduction potential).

* Methods requiring formal approval in accordance with the procedures in force at the Sub-Commission of Methods of Analysis.

Matrix effect for metals content analysis using atomic absorption

(Resolution OENO 5/2000)

The GENERAL ASSEMBLY,

In consideration of Article 5, Paragraph 4 of the International Standardization Convention on Methods of Wine Analysis and Rating of October 13, 1954,

Action on the proposal of the Sub-Committee on International Methods of Analysis and Rating of Wines,

CONSIDERING that the methods described in the Compendium of International Methods of Wine and Must Analysis and entailing the use of reference solutions are implemented for dry wines,

DRAWS the attention of users to the fact that deviations may be observed in other cases involving the presence of sugars or sugar derivatives,

DECIDES that it is therefore necessary to undertake analyses using the quantified additions method. A minimum of three aliquot portions of the sample containing various additions should be used.

DECIDES to supplement the methods for analyzing metals (iron, lead, zinc, silver, cadmium) and arsenic with a description of the quantified additions technique, when the matrix effect so requires.

Provisions on the use of proprietary methods

(Resolution OIV-OENO 526-2016)

Definition of a Proprietary Method of Analysis

For OIV purposes, a proprietary method of analysis is one that contains protected intellectual property preventing full disclosure of information about the method and/or where the intellectual property owner restricts the use or distribution of the method or materials for its performance such that no alternative source of these would be available. It does not extend to a method which is subject only to copyright.

Requirements

The OIV requires the method sponsors to provide relevant data to enable the SCMA ("Methods of Analysis" Sub-Commission) or another expert group to carry out an assessment. Following assessment, the SCMA, or another expert group, may submit methods of analysis that are proprietary, or are based on proprietary aspects, to the OIV General Assembly, for their approval, according to the following procedures.

a) A proprietary method should not be endorsed if a suitable non-proprietary method of analysis is available that has been or could be endorsed and that has similar or better performance characteristics. This should ensure that no approach is taken that could suggest that a proprietary method is endorsed by the OIV to the detriment of other potential methods; where possible preference should be given to adopting appropriate method criteria rather than endorsing a specific proprietary method of analysis.

b) Whilst respecting the necessity for reasonable protection of intellectual property, sufficient information should be available to enable reliable use of the method by analysts and to enable evaluation of the performance of the method by the SCMA or another expert group. In particular cases this may extend beyond performance data, for example, including details of the operating principle, at the sole discretion of the SCMA or another expert group.

c) Preference should be given to endorsing those methods of analysis where the reagents and/or apparatus are described in the method, to the extent that either laboratories or other manufacturers could produce these themselves;

alternatively, details enabling them to acquire these themselves would also suffice.

d) Method performance criteria required for proprietary methods are the same as those for non-proprietary methods. The performance criteria should be those stipulated above. If appropriate, information about the effect of variability of the reagents used should be provided.

e) After endorsing, any changes that may influence performance characteristics must be reported to the SCMA or another expert group for consideration.

f) A method with some parts that constitute protected proprietary information should be fully and collaboratively validated according to the OIV standards appearing in Annex A of the Compendium of International Methods of Analysis of Wines and Musts. The results of such studies will be made available for the SCMA or another expert group.

g) The manufacturer or the party submitting for evaluation a proprietary method should demonstrate to the satisfaction of the SCMA or another expert group that the fundamental principles and characteristics for the execution of the method may be made available to all interested parties.

h) The SCMA or another expert group may decline to assess a proprietary method if intellectual property restrictions unduly limit research into determining the method properties, scope and validity or development of improvements to the technology.

i) If suitable non-proprietary methods become available and endorsed, the status of the previously endorsed proprietary method should be reviewed and revised if necessary.

Method OIV-MA-AS2-01 Type I and IV Methods

Density and Specific Gravity at 20°C

(A1 revised by OIV-OENO 601A-2021)

1. Scope of application

This resolution is applicable for determining the density and specific gravity at 20 °C of wines and musts, using any of the following:

A.	Pycnometry	Type I Method,
B.	Electronic densimetry using a frequency oscillator	Type I Method,
C.	Densimetry using a hydrostatic balance	Type I Method,
D.	Hydrometry	Type IV Method.

2. Definition

Density is the quotient of the mass of a certain volume of wine or must at 20 °C by this volume. It is expressed in g/cm^3 and its symbol is $\rho_{20\,°C}$.

The specific gravity is the ratio of the density of a substance to the density of a reference material. For the analysis of wine or must, it is typically expressed as the ratio of the density of the wine or must at 20 °C to the density of water at 20 °C. Its symbol is: $d^{20°C}_{20°C}$

Note: It is possible to obtain the specific gravity from the density ρ_{20} at 20 °C:

ρ_{20} = 0.998203 x $d^{20°C}_{20°C}$ or $d^{20°C}_{20°C} = \rho_{20}$ /0.998203 (where 0.998203 is the density of water at 20 °C in g/ cm^3)

3. Principle of the methods

The principle of each method is detailed in the following parts:

Method A: Pycnometry

Method B: Electronic densimetry using a frequency oscillator

Method C: Densimetry using a hydrostatic balance

Method D: Hydrometry

Note: For very precise determinations, the density should be corrected to account for sulphur-dioxide action.

ρ_{20} (g/cm^3) = ρ'_{20} - 0.0006 × S
ρ_{20} (g/cm^3) = corrected density
ρ'_{20} (g/cm^3) = observed density
S (g/L) = total sulphur dioxide

4. Preliminary sample preparation

If the wine or must contains notable quantities of carbon dioxide, remove the grand majority by, for example, mixing 250 mL of sample in a 1000-mL vial, or by filtering under reduced pressure on 2 g of cotton placed in an extension tube, or by any other suitable method.

Method A: Density at 20 °C and specific gravity at 20 °C measured by pycnometry (Type method)

A.1 *Principle*

The density of the wine or must is measured for a specific temperature using a glass pycnometer. This comprises a flask of known capacity, onto which a hollow ground-glass stopper is fitted equipped with a capillary tube. When the flask is closed, the overflow rises in the capillary. The volumes of the flask and the capillary being known, the density is determined by weighing using precision balances before and after filling of the pycnometer.

A.2 *Reagents and products*

A.2.1 Type II water for analytical use (ISO 3696 standard), or of equivalent purity

A.2.2 Sodium chloride solution (2% m/v)

To prepare 1 litre, weigh out 20 g of sodium chloride and dissolve to volume in water.

A.3 *Apparatus and materials*

Current laboratory apparatus, including the following:

A.3.1 Pyrex-glass pycnometer of around 100 mL capacity with a removable thermometer, with ground-glass joint and 10^{th}-of-a-degree graduations, from 10 °C to 30 °C. This thermometer should be calibrated (Fig. 1).

Any pycnometer of equivalent characteristics may be used.

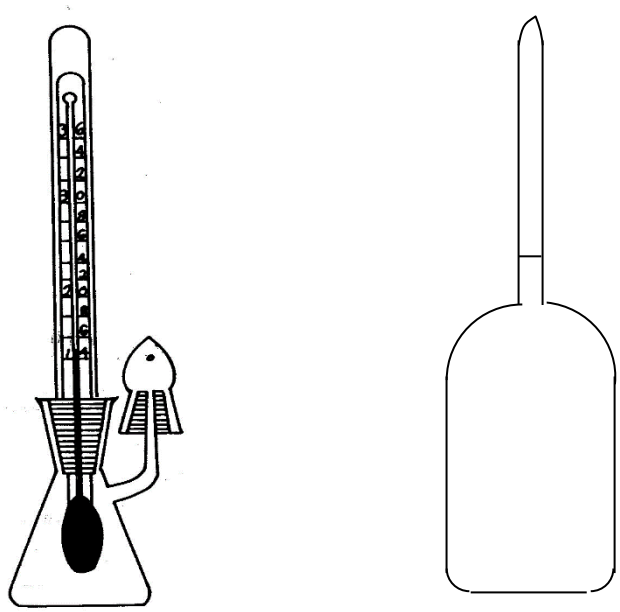


FIGURE 1: Pycnometer and its tare bottle

This pycnometer includes a side tube of 25 mm in length and an inside diameter of at most 1 mm, terminated by a ground-glass conical joint. This side tube may be capped by a 'reservoir stopper' composed of a ground-glass conical tube, terminated by a tapered joint. This stopper serves as an expansion chamber.

The two joints of the apparatus should be prepared with great care.

A.3.2 Tare bottle of the same external volume (to within 1 mL) as the pycnometer and with a mass equal to the mass of the pycnometer filled with a liquid of a density of 1.01 g/mL (sodium chloride solution at 2% m/v).

A.3.3 Thermally insulated jacket that fits the body of the pycnometer exactly.

A.3.4 Twin-pan balance accurate to the nearest 0.1 mg
or
single-plate balance accurate to the nearest 0.1 mg.

A.3.5 Masses calibrated by an accredited body

A.4 ***Procedure***

A.4.1 Pycnometer calibration

The calibration of the pycnometer comprises the determination of the following characteristics:
- tare weight,
- volume at 20 °C,
- water mass at 20 °C.

A.4.1.1 *Using a twin-pan balance*

Place the tare bottle on the left-hand pan and the clean, dry pycnometer with its 'reservoir stopper' on the right-hand pan. Balance them by placing weights of known mass on the pycnometer side: *p* grams.

Fill the pycnometer carefully with water (A.2.1) at room temperature and fit the thermometer.

Carefully wipe the pycnometer dry and place it in the thermally insulated jacket.

Shake by inverting the container until the thermometer's temperature reading is constant, accurately adjust the level to the upper rim of the side tube, wipe the side tube clean and fit the reservoir stopper.

Read the temperature, *t* °C, carefully and if necessary correct for any inaccuracies in the temperature scale.

Weigh the water-filled pycnometer, with the weight in grams, *p'*, making up the equilibrium.

Calculations:

- Tare of the empty pycnometer:

Tare weight = $p + m$ where m (g) = mass of the air contained in the pycnometer

$$m \text{ (g)} = 0.0012 \, (p - p')$$

- Volume at 20 °C in mL:

$V_{20\,°C}$ (mL) = $(p + m - p') \times F_t$

F_t = factor for temperature, *t* °C, taken from Table I

$V_{20\,°C}$ should be known to ± 0.001 mL

- Water mass at 20 °C:
  $M_{20\,°C}$ (g) = $V_{20\,°C}$ x 0.998203

0.998203 (g/cm^3) = water density at 20 °C

A.4.1.2 *Using a single-pan balance*

Determine:

- the mass of the clean, dry pycnometer: P,
- the mass of the water-filled pycnometer at *t* °C: P_1 following the instructions outlined in A.4.1.1,
- the mass of the tare bottle, T_0.

- Calculations:

Tare of the empty pycnometer:

Tare weight: P – *m* where *m* (g) = mass of the air contained in the pycnometer

$$m\ (g) = 0.0012\ (P_1 - P)$$

- Volume at 20 °C in mL:
  $V_{20\,°C}$ (mL) = [P1 - (P - *m*)] x F_t

F_t = factor for temperature, *t* °C, taken from Table I

$V_{20\,°C}$ should be known to ± 0.001 mL

- Water mass at 20 °C:
  $M_{20\,°C}$ (g) = $V_{20\,°C}$ x 0.998203
  0.998203 = water density at 20 °C (g/cm^3)

A.4.2 Determination of the density:

A.4.2.1 *Using a twin-pan balance*

Weigh the pycnometer filled with the test sample following the instructions outlined in A.4.1.1.

Where *p*" represents the mass in grams that makes up the equilibrium at *t* °C, taking into account that the liquid mass contained in the pycnometer = $p + m - p''$, the apparent density at *t* °C, in g/cm^3, is given by the following equation:

$$\rho_{t°C} = \frac{p + m - p''}{V_{20°C}}$$

Calculate the density at 20 °C using one of the following correction tables in Annex I, according to the nature of the liquid to be analysed and the type of

pycnometer to be used: dry wine and dealcoholized wine (Table II or V), natural or concentrated must (Table III or VI), or liqueur wine (Table IV or VII).

A.4.2.2. *Using a single-pan balance*

Weigh the tare bottle, where T_1 is its mass in g.
Calculate $dT = T_1 - T_o$
Mass of the empty pycnometer at the time of measurement = $P - m + dT$ in g
Weigh the pycnometer filled with the test sample following the instructions outlined in A.4.1.1.
Where P_2 represents its mass at t °C,
the liquid mass contained in the pycnometer at t °C = P_2 ($P - m + dT$) in g
and the apparent density at t °C, in g/cm^3, is as follows

$$\rho_{t°C} = \frac{P_2 - (P - m + dT)}{V_{20°C}}$$

Calculate the density at 20 °C of the liquid to be analysed: dry wine, natural or concentrated must, or liqueur wine, as indicated in A.4.2.1.

A.5 ***Expression of results***

The density is expressed in g/cm^3 to 5 decimal places.

A.6 ***Precision***

A.6.1 Repeatability in terms of density:

- for dry and sweet wines, except liqueur wines: r = 0.00010 g/cm^3,
- for liqueur wines: r = 0.00018 g/ cm^3.

A.6.2. Reproducibility in terms of density:

- for dry and sweet wines, except liqueur wines: R = 0.00037 g/cm^3,
- for liqueur wines: R = 0.00045 g/cm^3.

A.7 ***Numerical example***

A.7.1 Measurement by pycnometer on a twin-pan balance

A/ Calibration of the pycnometer

1. Weighing of the clean, dry pycnometer:

Tare = pycnometer + p
p = 104.9454 g

2. Weighing of the water-filled pycnometer at the temperature t °C:
Tare = pycnometer + water + p'
p' = 1.2396 g for t = 20.5 °C

3. Calculation of the mass of the air contained in the pycnometer:
$m = 0.0012\ (p - p')$
$m = 0.0012\ (104.9454 - 1.2396)$
$m = 0.1244$

4. Parameters to be kept:
Tare of the empty pycnometer: $p + m$
$p + m = 104.9454 + 0.1244$
$p + m = 105.0698$ g

Volume at 20 °C = $(p + m - p') \times F_{t\ °C}$

$F_{20.50\ °C}$ = 1.001900
$V_{20\ °C}$ = (105.0698 – 1.2396) x 1.001900
$V_{20\ °C}$ = 104.0275 mL

Water mass at 20 °C = $V_{20\ °C}$ x 0.998203
$M_{20\ °C}$ = 103.8405 g

B/ Determination of the density at 20 °C and the 20 °C/20 °C specific gravity of a dry wine:

p' = 1.2622 g at 17.80 °C

$$\rho_{17.80\ °C} = \frac{105.0698 - 1.2622}{104.0275}$$

$\rho_{17.80\ °C}$ = 0.99788 g/cm^3

Table II makes it possible to calculate $\rho_{20\ °C}$ from $\rho_{t\ °C}$ using the following formula:

$$\rho_{20\ °C} = \rho_{t\ °C} \pm \frac{c}{1000}$$

For t = 17.80 °C and for an alcoholic strength of 11% vol., c = 0.54:

$$\rho_{20\ °C} = 0.99788 - \frac{0.54}{1000}$$

$\rho_{20\,°C} = 0.99734\ g/cm^3$

$$d_{20\,°C}^{20\,°C} = \frac{0.99734}{0.998203} = 0.99913$$

A.7.2 Measurement by pycnometer on a single-pan balance

A/ Establishment of the pycnometer constants

1. Weighing of the clean, dry pycnometer:
   P = 67.7913 g
2. Weighing of the water-filled pycnometer at *t* °C:
   P_1 = 169.2715 g at 21.65 °C
3. Calculation of the mass of the air contained in the pycnometer:
   m = 0.0012 (P1 - P)
   m = 0.0012 x 101.4802
   m = 0.1218 g
4. Characteristics to be retained:
   Tare of the empty pycnometer: P - *m*
   P - *m* = 67.7913 – 0.1218
   P - *m* = 67.6695 g

   Volume at 20 °C = [P_1 - (P - *m*)] x $F_{t\ °C}$
   $F_{21.65\,°C}$ = 1.002140
   $V_{20\,°C}$ = (169.2715 – 67.6695) x 1.002140
   $V_{20\,°C}$ = 101.8194 mL

   Water mass at 20 °C: $V_{20\,°C}$ x 0.998203
   $M_{20\,°C}$ = 101.6364 g
   Mass of the tare bottle: T_0
   T_0 = 171.9160 g

B/ Determination of the density at 20 °C and 20 °C/20 °C specific gravity of a dry wine:

T_1 = 171.9178
*d*T = 171.9178 – 171.9160 = 0.0018 g
P - *m* + *d*T = 67.6695 + 0.0018 = 67.6713 g
P_2 = 169.2799 at 18 °C

$$\rho_{18\,°C} = \frac{169.2799 - 67.6713}{101.8194}$$

$$\rho_{18\,°C} = 0.99793\ \text{g/cm}^3$$

Table II makes it possible to calculate $\rho_{20\,°C}$ from $\rho_{t\,°C}$ using the following formula:

$$\rho_{20°C} = \rho_{t\,°C} \pm \frac{c}{1000}$$

For t = 18 °C and for an alcoholic strength of 11% vol., *c* = 0.49:

$$\rho_{20\,°C} = 0.99793 - \frac{0.49}{1000}$$

$$\rho_{20\,°C} = 0.99744\ \text{g/cm}^3$$

$$d_{20\,°C}^{20\,°C} = \frac{0.99744}{0.998203} = 0.99923$$

Method B: Density at 20 °C and specific gravity at 20 °C measured by electronic densimetry using a frequency oscillator (Type I method)

B.1 *Principle*

The density of the wine or must is measured by electronic densimetry using a frequency oscillator. The principle consists of measuring the period of oscillation of a tube containing the sample undergoing electromagnetic stimulation. The density is related to the period of oscillation by the following formula:

$$\rho = T^2 \times \left(\frac{C}{4\pi^2 V} \right) - \left(\frac{M}{V} \right) \qquad (1)$$

ρ = density of the sample
T = period of induced vibration
M = mass of empty tube
C = spring constant
V = volume of vibrating sample

This relationship is in the form $\rho = A\ T^2 - B$ (2), so there is a linear relationship between the density and the period squared. The constants A and B are specific to each oscillator and are estimated by measuring the period of fluids of known density.

B.2. *Reagents and products*

B.2.1 Reference fluids

Two reference fluids are used to adjust the densimeter. The densities of the reference fluids should encompass the densities of the wines or musts to be analysed. A spread of greater than 0.01000 g/cm^3 between the densities of the reference fluids is recommended.

The reference fluids used to measure the density of the wines or musts by electronic densimetry are as follows:

- dry air (unpolluted),
- Type II water for analytical usage (ISO standard 3696), or of equivalent analytical purity,

- hydro-alcoholic solutions, wines or musts whose densities have been determined by a different Type I method, for which the uncertainty does not exceed 0.00005 g/cm^3 at the temperature of 20.00 ± 0.05 °C,
- solutions calibrated with traceability to the International System of Units, with viscosities of less than 2 mm^2/s, for which the uncertainty does not exceed 0.00005 g/cm^3 at the temperature of 20.00 ± 0.05 °C.

B.2.2 Cleaning and drying products

Use products that ensure the perfectly clean and dried state of the measuring cell, according to the residues and manufacturer's indications. For example:

- detergents, acids, etc.,
- organic solvents: 96% vol. ethanol, pure acetone, etc.

B.3 ***Apparatus and equipment***

B.3.1. Electronic densimeter with frequency oscillator

The electronic densimeter consists of the following elements:

- a measuring cell consisting of a measuring tube and a temperature controller,
- a system for setting up an oscillation tube and measuring the period of oscillation,
- a digital display and possibly a calculator,
- sample injector syringe, autosampler or other equivalent system.

The densimeter is placed on a perfectly stable support isolated from all vibrations.

B.3.2 Temperature control of the measuring cell

Locate the measuring tube in a temperature-controlled system. Temperature stability should be better than ± 0.02 °C.

It is necessary to control the temperature of the measuring cell when the densimeter makes this possible, because this strongly influences the determination results. The density of a hydro-alcoholic solution with an alcoholic strength by volume (ABV) of 10% vol. is 0.98471 g/cm^3 at 20 °C and 0.98447 g/cm^3 at 21 °C, equating to a spread of 0.00024 g/cm^3.

The test temperature is 20 °C. Measure the cell temperature with a resolution thermometer accurate to less than 0.01 °C and with traceability to national standards. This should enable a temperature measurement with an uncertainty of better than ± 0.07 °C.

B.3.3 Calibration of the apparatus

The apparatus should be calibrated before using it for the first time, then periodically or if the verification is not satisfactory. The objective is to use two reference fluids to calculate the constants A and B [see formula (2), B.1]. To carry out the calibration in practice, refer to the user manual of the apparatus. In principle, this calibration is carried out with dry air (taking into account the atmospheric pressure) and very pure water (B.2.1).

B.3.4 Calibration verification

In order to verify the calibration, the density of the reference fluids is measured.

- Every day of use, a density check of the air is carried out. A difference between the theoretical density and observed density of more than 0.00008 g/cm^3 may indicate that the tube is clogged. In that case, it should be cleaned. After cleaning, verify the air density again. If the verification is not conclusive, adjust the apparatus.
- Check the density of the water; if the difference between the theoretical density and the density observed is greater than 0.00008 g/cm^3, adjust the apparatus.
- If verification of the cell temperature is difficult, it is possible to directly check the density of a hydro-alcoholic solution of comparable density to those of the samples analysed.

B.3.5 Checks

When the difference between the theoretical density of the reference solution (known with an uncertainty of ± 0.00005 g/cm^3) and the measured density is above 0.00008 g/cm^3, the calibration of the apparatus should be checked.

B.4 ***Procedure***

Before measuring, if necessary, clean and dry the cell with acetone or absolute alcohol and dry air. Rinse the cell with the sample.

Inject the sample into the cell (using a syringe, autosampler or other equivalent system) so that it is filled completely. While filling, check that all air bubbles have been removed. The sample should be homogenous and not contain any solid particles. Where necessary, filter to remove any suspended matter before analysis.

If there is a lighting system available that makes it possible to verify the absence of bubbles, turn it off quickly after checking because the heat generated by the lamp can influence the measuring temperature (for apparatus with a permanent lighting system, this statement is not applicable).

The operator should ensure that the temperature of the measuring cell is stable.

Once the reading has been stabilised, record the density, $\rho_{20°C}$.

If the apparatus only provides the period, the density can be calculated from the A and B constants (refer to the instructions for the equipment or Annex I of the method OIV-MA-AS312-01A).

B.5. ***Expression of results***

The density is expressed in g/cm^3 to 5 decimal places.

B.6. ***Precision parameters***

The precision parameters are detailed in Table 4 of Annex II.

Repeatability: $r = 0.00011\ g/cm^3$
Reproducibility: $R = 0.00025\ g/cm^3$

Method C: Density at 20 °C and specific gravity at 20 °C measured using a hydrostatic balance (Type I Method)

C.1 ***Principle***

The density of wine or musts can be measured by densimetry with a hydrostatic balance following the Archimedes principle, by which any body immersed in a fluid experiences an upwards force equal to the weight of the displaced fluid.

C.2 ***Reagents and products***

C.2.1 Type II water for analytical usage (ISO 3696 standard), or of equivalent purity

C.2.2 Floater-washing solution (sodium hydroxide, 30 % m/v)
To prepare a 100-mL solution weigh 30 g of sodium hydroxide and fill using 96% vol. ethanol.

C.3 ***Apparatus and materials***

Normal laboratory apparatus, particularly:

C.3.1 Single-pan hydrostatic balance accurate to the nearest 1 mg

C.3.2 Floater with at least 20 mL volume, specifically adapted for the balance, suspended by a thread with a diameter of less than or equal to 0.1 mm

C.3.3 Cylindrical test tube with level indicator. The floater should be able to fit entirely within the test tube volume below the level indicator; only the hanging thread should break the surface of the liquid. The cylindrical test tube should have an inside diameter at least 6 mm greater than that of the floater.

C.3.4 Thermometer (or temperature-measurement probe) with degree and 10^{th}-of-a-degree graduations, from 10 °C to 40 °C, calibrated to ± 0.06 °C

C.3.5 Masses calibrated by an accredited body.

C.4 ***Procedure***

After each measurement, the floater and the test tube should be cleaned with distilled water, wiped with soft laboratory paper that does not lose its fibres and rinsed with solution whose density is to be determined. These measurements should be carried out once the apparatus has reached a stable level in order to limit alcohol loss through evaporation.

C.4.1 Calibration of the apparatus

C.4.1.1 *Balance calibration*

While balances usually have internal calibration systems, hydrostatic balances should be calibrated with weights with traceability to the International System of Units.

C.4.1.2 *Floater calibration*
Fill the cylindrical test tube up to the level indicator with water (C.2.1) whose temperature is between 15 °C and 25 °C, but preferably at 20 °C.
Plunge the floater and the thermometer into the liquid, shake, note down the density on the apparatus and, if necessary, adjust the reading in order for it to be equal to that of the water at the measurement temperature.

C.4.1.3 *Verification using a solution of known density*
Fill the cylindrical test tube up to the level indicator with a solution of known density at a temperature of between 15 °C and 25 °C, preferably at 20 °C.

Immerse the floater and the thermometer in the liquid, stir, read the density of the liquid indicated by the apparatus and record the density and the temperature where the density is measured at *t* °C (ρ_t).

If necessary, correct ρ using a ρt density table of hydro-alcoholic mixtures (Table II in Annex I).
The density determined in this way should be identical to the previously determined density.

Note: This solution of known density can also replace water for floater calibration.

C.4.2 Determination of the density
Pour the test sample into the cylindrical test tube up to the level indicator.

Plunge the floater and the thermometer into the liquid, shake and note down the density on the apparatus. Note the temperature if the density is measured at *t* °C (ρt).

Correct ρt using a ρt density table of hydro-alcoholic mixtures (Table II in the Annex).

C.4.3 Cleaning of the floater and cylindrical test tube
Plunge the floater into the washing solution in the test tube.
Allow to soak for one hour while turning the floater regularly.

Rinse with tap water, then with distilled water.
Wipe with soft laboratory paper that does not lose its fibres.

Carry out these operations when the floater is used for the first time and then on a regular basis when necessary.

C.5 ***Expression of results***
The density is expressed in g/cm^3 to 5 decimal places.

C.6 ***Precision parameters***
The precision parameters are detailed in Table 4 of Annex II.

r = 0.00025 g/cm^3
R = 0.00067 g/cm^3

Method D: Density measured by hydrometry (Type IV Method)

D.1 ***Principle***

The density and specific gravity at 20 °C are determined for the test sample by hydrometry following the Archimedes principle. A weighted cylinder equipped with a graduated stem is more or less immersed into the liquid sample whose density is to be determined. The density of the liquid is read directly on the graduation of the stem at the level of the meniscus.

D.2 ***Apparatus***

D.2.1 Hydrometer

Hydrometers should meet ISO requirements relating to their dimensions and graduations.

They should have a cylindrical body and a circular stem with a cross-section of at least 3 mm in diameter. For dry wines, they should be graduated in g/cm^3 from 0.983 to 1.003, with graduation marks at every 0.001 and 0.0002 interval. All of the marks at 0.001 intervals should be separated from the next by at least 5 mm. For the measurement of the specific gravity of dealcoholized wines, liqueur wines and musts, a set of 5 hydrometers are to be used, graduated (in g/cm^3) from 1.000-1.030; 1.030-1.060; 1.060-1.090; 1.090-1.120; 1.120-1.150. These hydrometers are to be graduated for density at 20 °C by marks and intervals of no greater than 0.001 and 0.0005, with all the marks at the 0.001 intervals being separated from the next by at least 3 mm.

These hydrometers should be graduated so that they can be read at 'top of the meniscus'. The indication of the graduation in density at 20 °C or specific gravity at 20 °C, and of the reading at the top of the meniscus, is to be given either on the graduated scale, or on a strip of paper attached to the bulb.

This apparatus should be calibrated with traceability to the International System of Units.

D.2.2 Thermometer graduated to intervals of no greater than 0.5 °C, calibrated with traceability to the International System of Units.

D.2.3 Measuring cylinder with dimensions that allow for the immersion of the thermometer and the hydrometer without contact with the sides, held vertically.

D.3 ***Measurement method***

Place 250 mL of the test sample (4) in the measuring cylinder (D.2.3) and insert the hydrometer and thermometer. Stir the sample and wait 1 minute to allow temperature equilibration, then read the thermometer. Remove the thermometer and, after 1 minute of rest, read the apparent density at *t* °C on the stem of the hydrometer.

Correct the apparent density as read at *t* °C for the effect of the temperature, using the tables in Annex I applying to dry wines (Table V), natural and concentrated musts (Table VI) and liqueur wines (Table VII).

D.4 ***Expression of results***

The density is expressed in g/cm^3 to 4 decimal places.

5. Annexes

ANNEX I
Tables

TABLE I

F factors
by which the mass of the water in the *Pyrex pycnometer* at *t* °C has to be multiplied to calculate the volume of the pycnometer at 20 °C

t °C	F	*t* °C	F	*t* °C	F	*t* °C	F	*t* °C	F	*t* °C	F	*t* °C	F
10.0	1.000398	13.0	1.000691	16.0	1.001097	19.0	1.001608	22.0	1.002215	25.0	1.002916	28.0	1.003704
.1	1.000406	.1	1.000703	.1	1.001113	.1	1.001627	.1	1.002238	.1	1.002941	.1	1.003731
.2	1.000414	.2	1.000714	.2	1.001128	.2	1.001646	.2	1.002260	.2	1.002966	.2	1.003759
.3	1.000422	.3	1.000726	.3	1.001144	.3	1.001665	.3	1.002282	.3	1.002990	.3	1.003797
.4	1.000430	.4	1.000738	.4	1.001159	.4	1.001684	.4	1.002304	.4	1.003015	.4	1.003815
10.5	1.000439	13.5	1.000752	16.5	1.001175	19.5	1.001703	22.5	1.002326	25.5	1.003041	28.5	1.003843
.6	1.000447	.6	1.000764	.6	1.001191	.6	1.001722	.6	1.002349	.6	1.003066	.6	1.003871
.7	1.000456	.7	1.000777	.7	1.001207	.7	1.001741	.7	1.002372	3	1.003092	.7	1.003899
.8	1.000465	.8	1.000789	.8	1.001223	.8	1.001761	.8	1.002394	.8	1.003117	.8	1.003928
.9	1.000474	.9	1.000803	.9	1.001239	9	1.001780	.9	1.002417	.9	1.003143	.9	1.003956
11.0	1.000483	14.0	1.000816	17.0	1.001257	20.0	1.001800	23.0	1.002439	26.0	1.003168	29.0	1.003984
.1	1.000492	.1	1.000829	.1	1.001273	.1	1.001819	.1	1.002462	.1	1.003194	.1	1.004013
.2	1.000501	.2	1.000842	.2	1.001286	.2	1.001839	.2	1.002485	1	1.003222	2	1.004042
3	1.000511	3	1.000855	3	1.001306	.3	1.001959	.3	1.002508	.3	1.003247	.3	1.004071
.4	1.000520	.4	1.000868	.4	1.001323	.4	1.001880	.4	1.002531	.4	1.003273	.4	1.004099
11.5	1.000530	14.5	1.000882	17.5	1.001340	20.5	1.001900	23.5	1.002555	26.5	1.003299	29.5	1.004128
.6	1.000540	.6	1.000895	.6	1.001357	.6	1.001920	.6	1.002578	.6	1.003326	.6	1.004158
.7	1.000550	.7	1.000909	.7	1.001374	.7	1.001941	3	1.002602	.7	1.003352	.7	1.004187
.8	1.000560	.8	1.000923	.8	1.001391	.8	1.001961	.8	1.002625	.8	1.003379	.8	1.004216
.9	1.000570	.9	1.000937	.9	1.001409	.9	1.001982	.9	1.002649	.9	1.003405	.9	1.004245
12.0	1.000580	15.0	1.000951	18.0	1.001427	21.0	1.002002	24.0	1.002672	27.0	1.003432	30.0	1.004275
.1	1.000591	.1	1.000965	.1	1.001445	.1	1.002023	.1	1.002696	.1	1.003459		
.2	1.000601	.2	1.000979	.2	1.001462	.2	1.002044	.2	1.002720	.2	1.003485		
.3	1.000612	.3	1.000993	.3	1.001480	.3	1.002065	.3	1.002745	.3	1.003513		
.4	1.000623	.4	1.001008	.4	1.001498	.4	1.002086	.4	1.002769	.4	1.003540		
12.5	1.000634	15.5	1.001022	18.5	1.001516	21.5	1.002107	24.5	1.002793	27.5	1.003567		
.6	1.000645	.6	1.001037	.6	1.001534	.6	1.002129	.6	1.002817	.6	1.003594		
.7	1.000656	.7	1.001052	.7	1.001552	.7	1.002151	.7	1.002842	.7	1.003621		
.8	1.000668	.8	1.001067	.8	1.001570	.8	1.002172	.8	1.002866	.8	1.003649		
.9	1.000679	.9	1.001082	.9	1.001589	.9	1.002194	.9	1.002891	.9	1.003676		

TABLE II

Temperature corrections, c, required for the density of dry wines and dealcoholised wines, measured using a Pyrex-glass pycnometer at *t* °C, in order to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

\− if *t* ºC is lower than 20 °C
\+ if *t* ºC is higher than 20 °C

Temperature in °C	Alcoholic strength																								
	0	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
10	1.59	1.64	1.67	1.71	1.77	1.84	1.91	2.01	2.11	2.22	2.34	2.46	2.60	2.73	2.88	3.03	3.19	3.35	3.52	3.70	3.87	4.06	4.25	4.44
11	1.48	1.53	1.56	1.60	1.64	1.70	1.77	1.86	1.95	2.05	2.16	2.27	2.38	2.51	2.63	2.77	2.91	3.06	3.21	3.36	3.53	3.69	3.86	4.03
12	1.36	1.40	1.43	1.46	1.50	1.56	1.62	1.69	1.78	1.86	1.96	2.05	2.16	2.27	2.38	2.50	2.62	2.75	2.88	3.02	3.16	3.31	3.46	3.61
13	1.22	1.26	1.28	1.32	1.35	1.40	1.45	1.52	1.59	1.67	1.75	1.83	1.92	2.01	2.11	2.22	2.32	2.44	2.55	2.67	2.79	2.92	3.05	3.18
14	1.08	1.11	1.13	1.16	1.19	1.23	1.27	1.33	1.39	1.46	1.52	1.60	1.67	1.75	1.94	1.93	2.03	2.11	2.21	2.31	2.42	2.52	2.63	2.74.
15	0.92	0.96	0.97	0.99	1.02	1.05	1.09	1.13	1.19	1.24	1.30	1.36	1.42	1.48	1.55	1.63	1.70	1.78	1.86	1.95	2.03	2.12	2.21	2.30
16	0.76	0.79	0.80	0.81	0.94	0.86	0.89	0.93	0.97	1.01	1.06	1.10	1.16	1.21	1.26	1.32	1.38	1.44	1.51	1.57	1.64	1.71	1.78	1.85
17	0.59	0.61	0.62	0.63	0.65	0.67	0.69	0.72	0.75	0.78	0.81	0.85	0.88	0.95	0.96	1.01	1.05	1.11	1.15	1.20	1.25	1.30	1.35	1.40
18	0.40	0.42	0.42	0.43	0.44	0.46	0.47	0.49	0.51	0.53	0.55	0.57	0.60	0.63	0.65	0.68	0.71	0.74	0.77	0.81	0.84	0.87	0.91	0.94
19	0.21	0.21	0.22	0.22	0.23	0.23	0.24	0.25	0.26	0.27	0.28	0.29	0.30	0.32	0.33	0.34	0.36	0.37	0.39	0.41	0.42	0.44	0.46	0.47
20																								
21	0.21	0.22	0.22	0.23	0.23	0.24	0.25	0.26	0.27	0.28	0.29	0.30	0.31	0.32	0.34	0.36	0.37	0.38	0.40	0.41	0.43	0.44	0.46	0.48
22	0.44	0.45	0.46	0.47	0.48	0.49	0.51	0.52	0.54	0.56	0.59	0.61	0.63	0.66	0.69	0.71	0.74	0.77	0.80	0.83	0.87	0.90	0.93	0.97
23	0.68	0.70	0.71	0.72	0.74	0.76	0.78	0.80	0.83	0.86	0.90	0.93	0.96	1.00	1.03	1.08	1.13	1.17	1.22	1.26	1.31	1.37	1.41	1.46
24	0.93	0.96	0.97	0.99	1.01	1.03	1.06	1.10	1.13	1.18	1.22	1.26	1.31	1.36	1.41	1.47	1.52	1.58	1.64	1.71	1.77	1.84	1.90	1.97
25	1.19	1.23	1.25	1.27	1.29	1.32	1.36	1.40	1.45	1.50	1.55	1.61	1.67	1.73	1.80	1.86	1.93	2.00	2.08	2.16	2.24	2.32	2.40	2.48
26	1.47	1.51	1.53	1.56	1.59	1.62	1.67	1.72	1.77	1.83	1.90	1.96	2.03	2.11	2.19	2.27	2.35	2.44	2.53	2.62	2.72	2.81	2.91	3.01
27	1.75	1.80	1.82	1.85	1.89	1.93	1.98	2.04	2.11	2.18	2.25	2.33	2.41	2.50	2.59	2.68	2.78	2.88	2.98	3.09	3.20	3.31	3.42	3.33
28	2.04	2.10	2.13	2.16	2.20	2.25	2.31	2.38	2.45	2.53	2.62	2.70	2.80	2.89	3.00	3.10	3.21	3.32	3.45	3.57	3.69	3.82	3.94	4.07
29	2.34	2.41	2.44	2.48	2.53	2.58	2.65	2.72	2.81	2.89	2.99	3.09	3.19	3.30	3.42	3.53	3.65	3.78	3.92	4.05	4.19	4.33	4.47	4.61
30	2.66	2.73	2.77	2.81	2.86	2.92	3.00	3.08	3.17	3.27	3.37	3.48	3.59	3.72	3.84	3.97	4.11	4.25	4.40	4.55	4.70	4.85	4.92	5.17

Note: This table can be used to convert the density d_{20}^{t} to d_{20}^{20}

TABLE III

Temperature corrections, *c*, required for the density of natural or concentrated musts, measured using a Pyrex-glass pycnometer at *t* °C, in order to correct to 20 °C

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

− if *t* °C is lower than 20 °C
+ if *t* °C is higher than 20 °C

Temperature in °C	Density																					
	1.05	1.06	1.07	1.08	1.09	1.10	1.11	1.12	1.13	1.14	1.15	1.16	1.18	1.20	1.22	1.24	1.26	1.28	1.30	1.32	1.34	1.36
10	2.31	2.48	2.66	2.82	2.99	3.13	3.30	3.44	3.59	3.73	3.88	4.01	4.28	4.52	4.76	4.98	5.18	5.42	5.56	5.73	5.90	6.05
11	2.12	2.28	2.42	2.57	2.72	2.86	2.99	3.12	3.25	3.37	3.50	3.62	3.85	4.08	4.29	4148	4.67	4.84	5.00	5.16	5.31	5.45
12	1.92	2.06	2.19	2.32	2.45	2.58	2.70	2.92	2.94	3.04	3.15	3.26	3.47	3.67	3.85	4.03	4.20	4.36	4.51	4.65	478	4.91
13	1.72	1.84	1.95	2.06	2.17	2.27	2.38	2.48	2.58	2.69	2.78	2.89	3.05	3.22	3.39	3.55	3.65	3.84	3.98	4.11	4.24	4.36
14	1.52	1.62	1.72	1.81	1.90	2.00	2.09	2.17	2.26	2.34	2.43	2.51	2.66	2.82	2.96	3.09	3.22	3.34	3.45	3.56	3.67	3.76
15	1.28	1.36	1.44	1.52	1.60	1.67	1.75	1.82	1.89	1.96	2.04	2.11	2.24	2.36	2.48	2.59	2.69	2.79	2.88	2.97	3.03	3.10
16	1.05	1.12	1.18	1.25	1.31	1.37	1.43	1.49	1.55	1.60	1.66	1.71	1.81	1.90	2.00	2.08	2.16	2.24	2.30	2.37	2.43	2.49
17	0.80	0.86	0.90	0.95	1.00	1.04	1.09	1.13	1.18	1.22	1.26	1.30	1.37	1.44	1.51	1.57	1.62	1.68	1.72	1.76	1.80	1.84
18	0.56	0.59	0.62	0.66	0.68	0.72	0.75	0.77	0.80	0.83	0.85	0.88	0.93	0.98	1.02	1.05	1.09	1.12	1.16	1.19	1.21	1.24
19	0.29	0.31	0.32	0.34	0.36	0.37	0.39	0.40	0.42	0.43	0.44	0.45	0.48	0.50	0.52	0.54	0.56	0.57	0.59	0.60	0.61	0.62
20																						
21	0.29	0.30	0.32	0.34	0.35	0.37	0.38	0.40	0.41	0.42	0.44	0.46	0.48	0.50	0.53	0.56	0.58	0.59	0.60	0.61	0.62	0.62
22	0.58	0.61	0.64	0.67	0.70	0.73	0.76	0.79	0.81	0.84	0.87	0.90	0.96	1.03	1.05	1.09	1.12	1.15	1.18	1.20	1.22	1.23
23	0.89	0.94	0.99	1.03	1.08	1.12	1.16	1.20	1.25	1.29	1.33	1.37	1.44	1.51	1.57	1.63	1.67	1.73	1.77	1.80	1.82	1.94
24	1.20	1.25	1.31	1.37	1.43	1.49	1.54	1.60	1.66	1.71	1.77	1.82	1.92	2.01	2.10	2.17	2.24	2.30	2.36	2.40	2.42	2.44
25	1.51	1.59	1.66	1.74	1.81	1.88	1.95	2.02	2.09	2.16	2.23	2.30	2.42	2.53	2.63	2.72	2.82	2.89	2.95	2.99	3.01	3.05
26	1.84	1.92	2.01	2.10	2.18	2.26	2.34	2.42	2.50	2.58	2.65	2.73	2.87	3.00	3.13	3.25	3.36	3.47	3.57	3.65	372	3.79
27	2.17	2.26	2.36	2.46	2.56	2.66	2.75	2.84	2.93	3.01	3.10	3.18	3.35	3.50	3.66	3.80	3.93	4.06	4.16	4.26	4.35	4.42
28	2.50	2.62	2.74	2.85	2.96	3.07	3.18	3.28	3.40	3.50	3.60	3.69	3.87	4.04	4.21	4.36	4.50	4.64	4.75	4.86	4.94	5.00
29	2.86	2.98	3.10	3.22	3.35	3.47	3.59	3.70	3.82	3.93	4.03	4.14	4.34	4.53	4.72	4.89	5.05	5.20	5.34	5.46	5.56	5.64
30	3.20	3.35	3.49	3.64	3.77	3.91	4.05	4.17	4.30	4.43	4.55	4.67	4.90	5.12	5.39	5.51	5.68	5.94	5.96	6.09	6.16	6.22

Note: This table can be used to convert the density d_{20}^{t} to d_{20}^{20}

TABLE IV

Temperature corrections, *c*, required for the density of liqueur wines,
measured using a Pyrex-glass pycnometer at *t* °C, in order to correct to 20 °C

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ – if *t* °C is lower than 20 °C
+ if *t* °C is higher than 20 °C

		13% vol. wines							15% vol. wines							17% vol. wines						
		Density							Density							Density						
		1.000	1.020	1.040	1.060	1.080	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120
Temperature in °C	10	2.36	2.71	3.06	3.42	3.72	3.96	4.32	2.64	2.99	3.36	3.68	3.99	4.30	4.59	2.94	3.29	3.64	3.98	4.29	4.60	4.89
	11	2.17	2.49	2.80	2.99	3.39	3.65	3.90	2.42	2.73	3.05	3.34	3.63	3.89	4.15	2.69	3.00	3.32	3.61	3.90	4.16	4.41
	12	1.97	2.25	2.53	2.79	3.05	3.29	3.52	2.19	2.47	2.75	3.01	3.27	3.51	3.73	2.42	2.70	2.98	3.24	3.50	3.74	3.96
	13	1.78	2.02	2.25	2.47	2.69	2.89	3.09	1.97	2.21	2.44	2.66	2.87	3.08	3.29	2.18	2.42	2.64	2.87	3.08	3.29	3.49
	14	1.57	1.78	1.98	2.16	2.35	2.53	2.70	1.74	1.94	2.14	2.32	2.52	2.69	2.86	1.91	2.11	2.31	2.50	2.69	2.86	3.03
	15	1.32	1.49	1.66	1.82	1.97	2.12	2.26	1.46	1.63	1.79	1.95	2.10	2.25	2.39	1.60	1.77	1.93	2.09	2.24	2.39	2.53
	16	1.08	1.22	1.36	1.48	1.61	1.73	1.84	1.18	1.32	1.46	1.59	1.71	1.83	1.94	1.30	1.44	1.58	1.71	1.83	1.95	2.06
	17	0.83	0.94	1.04	1.13	1.22	1.31	1.40	0.91	1.02	1.12	1.21	1.30	1.39	1.48	1.00	1.10	1.20	1.30	1.39	1.48	1.56
	18	0.58	0.64	0.71	0.78	0.84	0.89	0.95	0.63	0.69	0.76	0.83	0.89	0.94	1.00	0.69	0.75	0.82	0.89	0.95	1.00	1.06
	19	0.30	0.34	0.37	0.40	0.43	0.46	0.49	0.33	0.37	0.40	0.43	0.46	0.49	0.52	0.36	0.39	0.42	0.46	0.49	0.52	0.54
	20																					
	21	0.30	0.33	0.36	0.40	0.43	0.46	0.49	0.33	0.36	0.39	0.43	0.46	0.49	0.51	0.35	0.39	0.42	0.45	0.48	0.51	0.54
	22	0.60	0.67	0.73	0.80	0.85	0.91	0.98	0.65	0.72	0.78	0.84	0.90	0.96	1.01	0.71	0.78	0.84	0.90	0.96	1.01	1.07
	23	0.93	1.02	1.12	1.22	1.30	1.39	1.49	1.01	1.10	1.20	1.29	1.38	1.46	1.55	1.10	1.19	1.29	1.38	1.46	1.55	1.63
	24	1.27	1.39	1.50	1.61	1.74	1.84	1.95	1.37	1.49	1.59	1.72	1.84	1.95	2.06	1.48	1.60	1.71	1.83	1.95	2.06	2.17
	25	1.61	1.75	1.90	2.05	2.19	2.33	2.47	1.73	1.87	2.02	2.17	2.31	2.45	2.59	1.87	2.01	2.16	2.31	2.45	2.59	2.73
	26	1.94	2.12	2.29	2.47	2.63	2.79	2.95	2.09	2.27	2.44	2.62	2.78	2.94	3.10	2.26	2.44	2.61	2.79	2.95	3.11	3.26
	27	2.30	2.51	2.70	2.90	3.09	3.27	3.44	2.48	2.68	2.87	3.07	3.27	3.45	3.62	2.67	2.88	3.07	3.27	3.46	3.64	3.81
	28	2.66	2.90	3.13	3.35	3.57	3.86	4.00	2.86	3.10	3.23	3.55	3.77	3.99	4.20	3.08	3.31	3.55	3.76	3.99	4.21	4.41
	29	3.05	3.31	3.56	3.79	4.04	4.27	4.49	3.28	3.53	3.77	4.02	4.26	4.49	4.71	3.52	3.77	4.01	4.26	4.50	4.73	4.95
	30	3.44	3.70	3.99	4.28	4.54	4.80	5.06	3.68	3.94	4.23	4.52	4.79	5.05	5.30	3.95	4.22	4.51	4.79	5.07	5.32	5.57

TABLE IV (continued)

Temperature corrections, *c*, required for the density of liqueur wines, measured using a Pyrex-glass pycnometer at *t* °C, in order to correct to 20 °C

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ − if *t* °C is lower than 20 °C; + if *t* °C is higher than 20 °C

Temperature in °C	19% vol. wines							21% vol. wines						
	Density							Density						
	1.000	1.020	1.040	1.060	1.000	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120
10	3.27	3.62	3.97	4.30	4.62	4.92	5.21	3.62	3.97	4.32	4.66	4.97	5.27	5.56
11	2.99	3.30	3.61	3.90	4.19	4.45	4.70	3.28	3.61	3.92	4.22	4.50	4.76	5.01
12	2.68	2.96	3.24	3.50	3.76	4.00	4.21	2.96	3.24	3.52	3.78	4.03	4.27	4.49
13	2.68	2.96	3.24	3.50	3.76	4.00	4.21	2.96	3.24	3.52	3.78	4.03	4.27	4.49
14	2.11	2.31	2.51	2.69	2.88	3.05	3.22	2.31	2.51	2.71	2.89	3.08	3.25	3.43
15	1.76	1.93	2.09	2.25	2.40	2.55	2.69	1.93	2.10	2.26	2.42	2.57	2.72	2.86
16	1.43	1.57	1.70	1.83	1.95	2.08	2.18	1.56	1.70	1.84	1.97	2.09	2.21	2.32
17	1.09	1.20	1.30	1.39	1.48	1.57	1.65	1.20	1.31	1.41	1.50	1.59	1.68	1.77
18	0.76	0.82	0.88	0.95	1.01	1.06	1.12	0.82	0.88	0.95	1.01	1.08	1.13	1.18
19	0.39	0.42	0.45	0.49	0.52	0.55	0.57	0.42	0.46	0.49	0.52	0.55	0.58	0.61
20														
21	0.38	0.42	0.45	0.48	0.51	0.54	0.57	0.41	0.45	0.48	0.51	0.54	0.57	0.60
22	0.78	0.84	0.90	0.96	1.02	1.07	1.13	0.84	0.90	0.96	1.02	1.08	1.14	1.19
23	1.19	1.28	1.38	1.47	1.55	1.64	1.72	1.29	1.39	1.48	1.57	1.65	1.74	1.82
24	1.60	1.72	1.83	1.95	2.06	2.18	2.29	1.73	1.85	1.96	2.08	2.19	2.31	2.42
25	2.02	2.16	2.31	2.46	2.60	2.74	2.88	2.18	2.32	2.47	2.62	2.76	2.90	3.04
26	2.44	2.62	2.79	2.96	3.12	3.28	3.43	2.53	2.81	2.97	3.15	3.31	3.47	3.62
27	2.88	3.08	3.27	3.42	3.66	3.84	4.01	3.10	3.30	3.47	3.69	3.88	4.06	4.23
28	3.31	3.54	3.78	4.00	4.22	4.44	4.64	3.56	3.79	4.03	4.25	4.47	4.69	4.89
29	3.78	4.03	4.27	4.52	4.76	4.99	5.21	4.06	4.31	4.55	4.80	5.04	5.27	5.48
30	4.24	4.51	4.80	5.08	5.36	5.61	5.86	4.54	4.82	5.11	5.39	5.66	5.91	6.16

TABLE V

Temperature corrections, *c*, required for the density of dry wines and dealcoholised dry wines, measured using an ordinary-glass pycnometer or hydrometer at *t* °C, in order to correct to 20 °C

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

– if *t* °C is lower than 20 °C
+ if *t* °C is higher than 20 °C

Temperature in °C	Alcoholic strength																								
	0	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
10	1.45	1.51	1.55	1.58	1.64	1.76	1.78	1.89	1.98	2.09	2.21	2.34	2.47	2.60	2.75	2.93	3.06	3.22	3.39	3.57	3.75	3.93	4.12	4.31
11	1.35	1.40	1.43	1.47	1.52	1.58	1.65	1.73	1.83	1.93	2.03	2.15	2.26	2.38	2.51	2.65	2.78	2.93	3.08	3.24	3.40	3.57	3.73	3.90
12	1.24	1.28	1.31	1.34	1.39	1.44	1.50	1.58	1.66	1.75	1.84	1.94	2.04	2.15	2.26	2.38	2.51	2.63	2.77	2.91	3.05	3.19	3.34	3.49
13	1.12	1.16	1.18	1.21	1.25	1.30	1.35	1.42	1.49	1.56	1.64	1.73	1.82	1.91	2.01	2.11	2.22	2.33	2.45	2.57	2.69	2.81	2.95	3.07
14	0.99	1.03	1.05	1.07	1.11	1.14	1.19	1.24	1.31	1.37	1.44	1.52	1.59	1.67	1.75	1.84	1.93	2.03	2.13	2.23	2.33	2.44	2.55	2.66
15	0.86	0.89	0.90	0.92	0.95	0.98	1.02	1.07	1.12	1.17	1.23	1.29	1.35	1.42	1.49	1.56	1.63	1.71	1.80	1.88	1.96	2.05	2.14	2.23
16	0.71	0.73	0.74	0.76	0.78	0.81	0.84	0.87	0.91	0.95	0.99	1.05	1.10	1.15	1.21	1.27	1.33	1.39	1.45	1.52	1.59	1.66	1.73	1.80
17	0.55	0.57	0.57	0.59	0.60	0.62	0.65	0.67	0.70	0.74	0.77	0.81	0.84	0.88	0.92	0.96	1.01	1.05	1.10	1.15	1.20	1.26	1.31	1.36
18	0.38	0.39	0.39	0.40	0.41	0.43	0.44	0.46	0.48	0.50	0.52	0.55	0.57	0.60	0.62	0.65	0.68	0.71	0.74	0.78	0.81	0.85	0.88	0.91
19	0.19	0.20	0.20	0.21	0.21	0.22	0.23	0.24	0.25	0.26	0.27	0.28	0.29	0.30	0.32	0.33	0.34	0.36	0.38	0.39	0.41	0.43	0.44	0.46
20																								
21	0.21	0.22	0.22	0.23	0.23	0.24	0.25	0.25	0.26	0.27	0.28	0.29	0.31	0.32	0.34	0.35	0.36	0.38	0.39	0.41	0.43	0.44	0.46	0.48
22	0.43	0.45	0.45	0.46	0.47	0.49	0.50	0.52	0.54	0.56	0.58	0.60	0.62	0.65	0.68	0.71	0.73	0.77	0.80	0.83	0.86	0.89	0.93	0.96
23	0.67	0.69	0.70	0.71	0.72	0.74	0.77	0.79	0.82	0.85	0.88	0.91	0.95	0.99	1.03	1.07	1.12	1.16	1.21	1.25	1.30	1.35	1.40	1.45
24	0.91	0.93	0.95	0.97	0.99	1.01	1.04	1.07	1.11	1.15	1.20	1.24	1.29	1.34	1.39	1.45	1.50	1.56	1.62	1.69	1.76	1.82	1.88	1.95
25	1.16	1.19	1.21	1.23	1.26	1.29	1.33	1.37	1.42	1.47	1.52	1.57	1.63	1.70	1.76	1.83	1.90	1.97	2.05	2.13	2.21	2.29	2.37	2.45
26	1.42	1.46	1.49	1.51	1.54	1.58	1.62	1.67	1.73	1.79	1.85	1.92	1.99	2.07	2.14	2.22	2.31	2.40	2.49	2.58	2.67	2.77	2.86	2.96
27	1.69	1.74	1.77	1.80	1.83	1.88	1.93	1.98	2.05	2.12	2.20	2.27	2.35	2.44	2.53	2.63	272	2.82	2.93	3.04	3.14	3.25	3.37	3.48
28	1.97	2.03	2.06	2.09	2.14	2.19	2.24	2.31	2.38	2.46	2.55	2.63	2.73	2.83	2.93	3.03	3.14	3.26	3.38	3.50	3.62	3.75	3.85	4.00
29	2.26	2.33	2.37	2.41	2.45	2.50	2.57	2.64	2.73	2.82	2.91	2.99	3.11	3.22	3.34	3.46	3.58	3.70	3.84	3.97	4.11	4.25	4.39	4.54
30	2.56	2.64	2.67	2.72	2.77	2.83	2.90	2.98	3.08	3.18	3.28	3.38	3.50	3.62	3.75	3.88	4.02	4.16	4.30	4.46	4.61	4.76	4.92	5.07

Note: This table can be used to convert the density d_{20}^{t} to d_{20}^{20}

TABLE VI

Temperature corrections, *c*, required for the density of concentrated musts, measured using an ordinary-glass pycnometer or hydrometer at *t* °C, in order to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000\ 000}$$ – if *t* °C is lower than 20 °C; + if *t* °C is higher than 20 °C

		Density																					
		1.05	1.06	1.07	1.08	1.09	1.10	1.11	1.12	1.13	1.14	1.15	1.16	1.18	1.20	1.22	1.24	1.26	1.28	1.30	1.32	1.34	1.36
Temperature in °C	10	2.17	2.34	2.52	2.68	2.85	2.99	3.16	3.29	3.44	3.58	3.73	3.86	4.13	4.36	4.60	4.82	5.02	5.25	5.39	5.56	-5.73	5.87
	11	2.00	2.16	2.29	2.44	2.59	2.73	2.86	2.99	3.12	3.24	3.37	3.48	3.71	3.94	4.15	4.33	4.52	4.69	4.85	5.01	5.15	5.29
	12	1.81	1.95	2.08	2.21	2.34	2.47	2.58	2.70	2.82	2.92	3.03	3.14	3.35	3.55	3.72	3.90	4.07	4.23	4.37	4.52	4.64	4.77
	13	1.62	1.74	1.85	1.96	2.07	2.17	2.28	2.38	2.48	2.59	2.68	2.77	2.94	3.11	3.28	3.44	3.54	3.72	3.86	3.99	4.12	4.24
	14	1.44	1.54	1.64	1.73	1.82	1.92	2.00	2.08	2.17	2.25	2.34	2.42	2.57	2.73	2.86	2.99	3.12	3.24	3.35	3.46	3.57	3.65
	15	1.21	1.29	1.37	1.45	1.53	1.60	1.68	1.75	1.82	1.89	1.97	2.03	2.16	2.28	2.40	2.51	2.61	2.71	2.80	2.89	2.94	3.01
	16	1.00	1.06	1.12	1.19	1.25	1.31	1.37	1.43	1.49	1.54	1.60	1.65	1.75	1.84	1.94	2.02	2.09	2.17	2.23	2.30	2.36	2.42
	17	0.76	0.82	0.86	0.91	0.96	1.00	1.05	1.09	1.14	1.18	1.22	1.25	1.32	1.39	1.46	1.52	1.57	1.63	1.67	1.71	1.75	1.79
	18	0.53	0.56	0.59	0.63	0.65	0.69	0.72	0.74	0.77	0.80	0.82	0.85	0.90	0.95	0.99	1.02	1.05	1.09	1.13	1.16	1.18	1.20
	19	0.28	0.30	0.31	0.33	0.35	0.36	0.38	0.39	0.41	0.42	0.43	0.43	0.46	0.48	0.50	0.52	0.54	0.55	0.57	0.58	0.59	0.60
	20																						
	21	0.28	0.29	0.31	0.33	0.34	0.36	0.37	0.39	0.40	0.41	0.43	0.44	0.46	0.48	0.51	0.54	0,56	0.57	0.58	0.59	0.60	0.60
	22	0.55	0.58	0.61	0.64	0.67	0.70	0.73	0.76	0.78	0.81	0.84	0.87	0.93	0.97	1.02	1.06	1.09	1.12	1.15	1.17	1.19	1.19
	23	0.85	0.90	0.95	0.99	1.04	1.08	1.12	1.16	1.21	1.25	1.29	1.32	1.39	1.46	1.52	1.58	1.62	1.68	1.72	1.75	1.77	1.79
	24	1.15	1.19	1.25	1.31	1.37	1.43	1.48	1.54	1.60	1.65	1.71	1.76	1.86	1.95	2.04	2.11	2.17	2.23	2.29	2.33	2.35	2.37
	25	1.44	1.52	1.59	1.67	1.74	1.81	1.88	1.95	2.02	2.09	2.16	2.22	2.34	2.45	2.55	2.64	2.74	2.81	7.87	2.90	2.92	2.96
	26	1.76	1.84	1.93	2.02	2.10	2.18	2.25	2.33	2.41	2.49	2.56	2.64	2.78	2.91	3.03	3.15	3.26	3.37	3.47	3.55	3.62	3.60
	27	2.07	2.16	2.26	2.36	2.46	2.56	2.65	2.74	2.83	2.91	3.00	3.07	3.24	3.39	3.55	3.69	3.82	3.94	4.04	4.14	4.23	4.30
	28	2.39	2.51	2.63	2.74	2.85	2.96	3.06	3.16	3.28	3.38	3.48	3.57	3.75	3.92	4.08	4.23	4.37	4.51	4.62	4.73	4.80	4.86
	29	2.74	2.86	2.97	3.09	3.22	3.34	3.46	3.57	3.69	3.90	3.90	4.00	4.20	4.39	4.58	4.74	4.90	5.05	5.19	5.31	5.40	5.48
	30	3.06	3.21	3.35	3.50	3.63	3.77	3.91	4.02	4.15	4.28	4.40	4.52	4.75	4.96	5.16	5.35	5.52	5.67	5.79	5.91	5.99	6.04

Note: This table can be used to convert the density d^{t}_{20} to d^{20}_{20}

TABLE VII

Temperature corrections, *c*, required for the density of liqueur wines,
measured using an ordinary-glass pycnometer or hydrometer at *t* °C, in order to correct to 20 °C

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

– if *t* °C is lower than 20 °C
+ if *t* °C is higher than 20 °C

Temperature in °C	13% vol. wines							15% vol. wines							17% vol. wines						
	Density							Density							Density						
	1.000	1.020	1.040	1.060	1.080	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120
10	2.24	2.58	2.93	3.27	3.59	3.89	4.18	2.51	2.85	3.20	3.54	3.85	4.02	4.46	2.81	3.15	3.50	3.84	4.15	4.45	4.74
11	2.06	2.37	2.69	2.97	3.26	3.53	3.78	2.31	2.61	2.93	3.21	3.51	3.64	4.02	2.57	2.89	3.20	3.49	3.77	4.03	4.28
12	1.87	2.14	2.42	2.67	2.94	3.17	3.40	2.09	2.36	2.64	2.90	3.16	3.27	3.61	2.32	2.60	2.87	3.13	3.39	3.63	3.84
13	1.69	1.93	2.14	2.37	2.59	2.80	3.00	1.88	2.12	2.34	2.56	2.78	2.88	3.19	2.09	2.33	2.55	2.77	2.98	3.19	3.39
14	1.49	1.70	1.90	2.09	2.27	2.44	2.61	1.67	1.86	2.06	2.25	2.45	2.51	2.77	1.83	2.03	2.23	2.42	2.61	2.77	2.94
15	1.25	1.42	1.59	1.75	1.90	2.05	2.19	1.39	1.56	1.72	1.88	2.03	2.11	2.32	1.54	1.71	1.87	2.03	2.18	2.32	2.47
16	1.03	1.17	1.30	1.43	1.55	1.67	1.78	1.06	1.27	1.40	1.53	1.65	1.77	1.88	1.25	1.39	1.52	1.65	1.77	1.89	2.00
17	0,80	0.90	1.00	1.09	1.17	1.27	1.36	0.87	0.98	1.08	1.17	1.26	1.35	1.44	0.96	1.06	1.16	1.26	1.35	1.44	1.52
18	0.54	0.61	0.68	0.75	0,81	0.86	0.92	0.60	0.66	0.73	0.80	0.85	0.91	0.97	0.66	0.72	0.79	0.86	0.92	0,97	1.03
19	0.29	0.33	0.36	0.39	0.42	0.45	0.48	0.32	0.36	0.39	0.42	0.45	0.48	0.51	0.35	0.38	0.41	0.45	0.48	0.51	0.53
20																					
21	0.29	0.32	0.35	0.39	0.42	0.45	0,47	0.32	0.35	0.38	0.42	0.45	0.48	0.50	0.34	0.38	0.41	0.44	0.47	0,50	0.53
22	0.57	0.64	0.70	0.76	0.82	0.88	0.93	0.63	0.69	0.75	0.81	0.87	0.93	0.99	0.68	0,75	0.81	0.87	0.93	0.99	1.04
23	0.89	0,98	1.08	1.17	1.26	1.34	1.43	0,97	1.06	1.16	1.25	1.34	1.42	1.51	1.06	1.15	1.25	1.34	1.42	1.51	1.59
24	1.22	1.34	1.44	1.56	1.68	1.79	1.90	1.32	1.44	1.54	1.66	1.78	1.89	2.00	1.43	1.56	1.65	1.77	1.89	2.00	2.11
25	1.61	1.68	1.83	1.98	2.12	2.26	2.40	1.66	1.81	1.96	2.11	2.25	2.39	2.52	1.80	1.94	2.09	2.24	2.39	2.52	2.66
26	1.87	2.05	2.22	2.40	2.56	2.71	2.87	2.02	2.20	2.37	2.54	2.70	2.85	3.01	2.18	2.36	2.53	2.71	2.86	3.02	3.17
27	2.21	2.42	2.60	2.80	3.00	3.18	3.35	2.39	2.59	2.78	2.98	3.17	3.35	3.52	2.58	2.78	2.97	3.17	3.36	3.54	3.71
28	2.56	2.80	3.02	3.25	3.47	3.67	3.89	2.75	2.89	3.22	3.44	3.66	3.96	4.07	2.97	3.21	3.44	3.66	3.88	4.09	4.30
29	2.93	3.19	3.43	3.66	3.91	4.14	4.37	3.16	3.41	3.65	3.89	4.13	4.36	4.59	3.40	3.66	3.89	4.13	4.38	4.61	4.82
30	3.31	3.57	3.86	4.15	4.41	4.66	4.92	3.55	3.81	4.10	4.38	4.66	4.90	5.16	3.82	4.08	4.37	4.65	4.93	5.17	5.42

TABLE VII (continued)

Temperature corrections, *c*, required for the density of liqueur wines,
measured using an ordinary-glass pycnometer or hydrometer at *t* °C, in order to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

– if *t* °C is lower than 20 °C
+ if *t* °C is higher than 20 °C

		19% vol. wines							21% vol. wines						
		Density							Density						
		1.000	1.020	1.040	1.060	1.080	1.100	1.120	1.000	1.020	1.040	1.060	1.080	1.100	1.120
Temperature in °C	10	3.14	3.48	3.83	4.17	4.48	4.78	5.07	3.50	3.84	4.19	4.52	4.83	5.12	5.41
	11	2.87	3.18	3.49	3.78	4.06	4.32	4.57	3.18	3.49	3.80	4.09	4.34	4.63	4.88
	12	2.58	2.96	3.13	3.39	3.65	3.88	4.10	2.86	3.13	3.41	3.67	3.92	4.15	4.37
	13	2.31	2.55	2.77	2.99	3.20	3.41	3.61	2.56	2.79	3.01	3.23	3.44	3.65	3.85
	14	2.03	2.23	2.43	2.61	2.80	2.96	3.13	2.23	2.43	2.63	2.81	3.00	3.16	3.33
	15	1.69	1.86	2.02	2.18	2.33	2.48	2.62	1.86	2.03	2.19	2.35	2.50	2.65	2.80
	16	1.38	1.52	1.65	1.78	1.90	2.02	2.13	1.51	1.65	1.78	1.91	2.03	2.15	2.26
	17	1.06	1.16	1.26	1 .35	1.44	1.53	1.62	1.15	1.25	1.35	1.45	1.54	1.63	1.71
	18	0,73	0.79	0.85	0.92	0.98	1.03	1.09	0.79	0.85	0.92	0.98	1.05	1.10	1.15
	19	0.38	0.41	0.44	0.48	0.51	0.52	0.56	0.41	0.44	0.47	0.51	0.54	0.57	0.59
	20														
	21	0.37	0.41	0.44	0.47	0.50	0.53	0.56	0.41	0.44	0.47	0.51	0.54	0.57	0.59
	22	0.75	0.81	0.87	0.93	0.99	1.04	1.10	0.81	0.88	0.94	1.00	1.06	1.10	1.17
	23	1.15	1.30	1.34	1.43	1.51	1.60	1.68	1.25	1.34	1.44	1.63	1.61	1.70	1.78
	24	1.55	1.67	1.77	1.89	2.00	2.11	2.23	1.68	1.80	1.90	2.02	2.13	2.25	2.36
	25	1.95	2.09	2.24	2.39	2.53	2.67	2.71	2.11	2.25	2.40	2.55	2.69	2.83	2.97
	26	2.36	2.54	2.71	2.89	3.04	3.20	3.35	2.55	2.73	2.90	3.07	3.22	3.38	3.54
	27	2.79	2.99	3.18	3.38	3.57	3.75	3.92	3.01	3.20	3.40	3.59	3.78	3.96	4.13
	28	3.20	3.44	3.66	3.89	4.11	4.32	4.53	3.46	3.69	3.93	4.15	4.36	4.58	4.77
	29	3.66	3.92	4.15	4.40	4.64	4.87	5.08	3.95	4.20	4.43	4.68	4.92	5.15	5.36
	30	4.11	4.37	4.66	4.94	5.22	5.46	5.71	4.42	4.68	4.97	5.25	5.53	5.77	6.02

ANNEX II

Comparison of results for the methods of measurement of density using a frequency oscillator (Method B) and using a hydrostatic balance (Method C)

Using samples with densities between 0.992 and 1.012 g/cm^3, the repeatability and reproducibility were measured using an inter-laboratory test. The densities of the different samples as measured using a hydrostatic balance and using electronic densimetry were compared, including the repeatability and reproducibility values derived from the multi-year inter-comparison tests performed on a large scale.

1 Samples
Wines with different densities and alcoholic strengths prepared monthly on an industrial scale, taken from a stock of bottles stored under normal conditions, and supplied anonymously to the laboratories.

2. Laboratories
Laboratories participating in the monthly tests organised by *Unione Italiana Vini* (Verona, Italy) according to ISO 5725 (UNI 9225) regulations and the International Harmonized Protocol for the Proficiency Testing of Analytical Chemical Laboratories produced by the AOAC, ISO and IUPAC, and ISO 43 and ILAC G13 guidelines. An annual report is provided by the above-mentioned organisation to all participants.

3 Apparatus

3.1 An electronic hydrostatic balance (with precision to 5 decimal places), equipped if possible with a data-processing device.

3.2 An electronic densimeter, equipped if possible with an autosampler.

4 Analyses
According to the rules for the validation of methods of analysis, each sample was analysed twice consecutively to determine the alcoholic strength.

5 Results

Table 1 shows the results of the measurements obtained by the laboratories using a hydrostatic balance.
Table 2 shows the results obtained by the laboratories using an electronic densimeter.

6 Evaluation of results
6.1 The test results were examined for evidence of individual systemic error ($p<0.025$) using Cochran's and Grubbs' tests successively, according to the procedures described in the internationally accepted Protocol for the Design, Conduct and Interpretation of Method-Performance Studies.

6.2 *Repeatability (r) et reproducibility (R)*
Calculations for repeatability (r) and reproducibility (R) as defined by the protocol were carried out on the results remaining after the removal of outliers. When assessing a new method, there is often no validated reference or statutory method to compare precision criteria; 'predicted' levels of precision and therefore used to compare the precision data obtained from collaborative tests. These predicted levels are calculated from the Horwitz formula. Comparison of the test results and the predicted levels give an indication as to whether the method is sufficiently precise for the level of analyte being measured. The Horwitz predicted value is calculated from the Horwitz equation.
$RSD_R = 2^{(1-0.5 \log C)}$
where C is the measured concentration of analyte expressed as a decimal (e.g. 1 g/100 g = 0.01).
The Horrat value gives a comparison of the actual precision measured with the precision predicted by the Horwitz formula for the method and at the particular level of concentration of the analyte. It is calculated as follows:
$HoR = RSD_R(\text{measured})/RSD_R(\text{Horwitz})$

6.3 *Inter-laboratory reproducibility*
A Horrat value of 1 usually indicates satisfactory reproducibility, whereas a value of more than 2 usually indicates unsatisfactory reproducibility, i.e. reproducibility that is too variable for analytical purposes or where the variation obtained is greater than that predicted for the type of method employed. Hor is also calculated and used to measure intra-laboratory reproducibility, using the following approximation:

RSD_r(Horwitz) = 0.66 RSD_R(Horwitz) (this assumes the approximation that r = 0.66 R)

CrD95 is the critical difference for a 95% probability level. It is calculated according to Resolution OIV-MA-AS1-08.

Table 3 shows the differences between the measurements obtained by laboratories using an electronic densimeter and those using a hydrostatic balance.

6.4 *Precision parameters*
Table 4 shows the overall averages for the precision parameters calculated from all monthly tests carried out between January 2008 and December 2010

Table 1: Results obtained by laboratories that conducted tests using a hydrostatic balance (HB)

Sample	Average	Total no. of values	No. of selected values	Repeatability	s_r	RSD_r	Hor	Reproducibility	s_R	RSDRcalc	HoR	No. of repet.	CrD95
01/08	0.995491	130	120	0.0001701	0.0000607	0.0061016	0.0046193	0.0005979	0.0002135	0.0214502	0.0107178	2	0.0004141
02/08	1.011475	146	125	0.0004714	0.0001684	0.0166457	0.0126320	0.0008705	0.0003109	0.0307366	0.0153947	2	0.0005686
03/08	0.992473	174	161	0.0001470	0.0000525	0.0052898	0.0040029	0.0004311	0.0001540	0.0155140	0.0077482	2	0.0002959
04/08	0.993147	172	155	0.0002761	0.0000986	0.0099274	0.0075130	0.0005446	0.0001945	0.0195839	0.0097818	2	0.0003595
05/08	1.004836	150	138	0.0001882	0.0000672	0.0066905	0.0050723	0.0007495	0.0002677	0.0266373	0.0133283	2	0.0005215
06/08	0.993992	152	136	0.0001486	0.0000531	0.0053391	0.0040411	0.0005302	0.0001894	0.0190506	0.0095167	2	0.0003675
07/08	0.992447	162	150	0.0002660	0.0000950	0.0095709	0.0072424	0.0006046	0.0002159	0.0217575	0.0108664	2	0.0004063
08/08	0.992210	162	151	0.0002619	0.0000935	0.0094281	0.0071341	0.0006309	0.0002253	0.0227108	0.0113420	2	0.0004265
09/08	1.002600	148	131	0.0001093	0.0000390	0.0038920	0.0029496	0.0007000	0.0002500	0.0249341	0.0124719	2	0.0004919
10/08	0.994482	174	152	0.0001228	0.0000439	0.0044105	0.0033385	0.0004250	0.0001518	0.0152645	0.0076259	2	0.0002942
11/08	0.992010	136	125	0.0000909	0.0000325	0.0032742	0.0024775	0.0004256	0.0001520	0.0153217	0.0076516	2	0.0002975
01/09	0.994184	174	152	0.0001655	0.0000591	0.0059435	0.0044987	0.0005439	0.0001942	0.0195384	0.0097606	2	0.0003756
02/09	0.992266	118	101	0.0001742	0.0000622	0.0062682	0.0047431	0.0005210	0.0001861	0.0187534	0.0093658	2	0.0003580
03/09	0.991886	164	135	0.0001850	0.0000661	0.0066603	0.0050395	0.0004781	0.0001707	0.0172136	0.0085963	2	0.0003251
04/09	0.993632	180	150	0.0001523	0.0000544	0.0054754	0.0041440	0.0004270	0.0001525	0.0153476	0.0076664	2	0.0002922
05/09	1.011061	116	100	0.0003659	0.0001307	0.0129234	0.0098067	0.0008338	0.0002978	0.0294527	0.0147508	2	0.0005605
06/09	0.992063	114	105	0.0002923	0.0001044	0.0105238	0.0079631	0.0005257	0.0001877	0.0189240	0.0094507	2	0.0003418
07/09	0.992708	172	155	0.0002892	0.0001033	0.0104040	0.0078732	0.0006156	0.0002199	0.0221478	0.0110617	2	0.0004106
08/09	0.993064	136	127	0.0002926	0.0001045	0.0105224	0.0079632	0.0007520	0.0002686	0.0270446	0.0135081	2	0.0005112
09/09	1.005285	118	110	0.0002946	0.0001052	0.0104661	0.0079352	0.0007226	0.0002581	0.0256704	0.0128454	2	0.0004892
10/09	0.992905	150	132	0.0002234	0.0000798	0.0080358	0.0060812	0.0004498	0.0001607	0.0161803	0.0080815	2	0.0002978
11/09	0.994016	142	127	0.0001896	0.0000677	0.0068114	0.0051555	0.0004739	0.0001693	0.0170278	0.0085062	2	0.0003214
01/10	0.994734	170	152	0.0002125	0.0000759	0.0076288	0.0057748	0.0005406	0.0001931	0.0194104	0.0096975	2	0.0003672
02/10	0.993177	120	110	0.0002210	0.0000789	0.0079467	0.0060140	0.0005800	0.0002071	0.0208565	0.0104175	2	0.0003950
03/10	0.992799	148	136	0.0002277	0.0000813	0.0081923	0.0061995	0.0015157	0.0005413	0.0545262	0.0272335	2	0.0010657
04/10	0.995420	172	157	0.0002644	0.0000944	0.0094866	0.0071819	0.0006286	0.0002245	0.0225542	0.0112693	2	0.0004244
05/10	1.002963	120	108	0.0007086	0.0002531	0.0252330	0.0191244	0.0013667	0.0004881	0.0486677	0.0243447	2	0.0008991
06/10	0.992546	120	113	0.0001737	0.0000620	0.0062506	0.0047300	0.0005435	0.0001941	0.0195567	0.0097673	2	0.0003744
07/10	0.992831	174	152	0.0003003	0.0001073	0.0108031	0.0081753	0.0006976	0.0002492	0.0250959	0.0125344	2	0.0004699
08/10	0.993184	144	130	0.0001799	0.0000642	0.0064674	0.0048945	0.0005951	0.0002125	0.0213984	0.0106882	2	0.0004111
09/10	1.012293	114	103	0.0002265	0.0000809	0.0079907	0.0060647	0.0014586	0.0005209	0.0514596	0.0257772	2	0.0010251
10/10	0.992289	154	136	0.0006386	0.0002281	0.0229860	0.0173933	0.0007033	0.0002512	0.0253124	0.0126415	2	0.0003812
11/10	0.994649	130	112	0.0002902	0.0001036	0.0104200	0.0078876	0.0005287	0.0001888	0.0189830	0.0094838	2	0.0003445

Table 2: Results obtained by laboratories that conducted tests using an electronic densimeter (ED)

Sample	Average	Total no. of values	No. of selected values	Repeatability	s_r	RSD_r	Hor	Reproducibility	s_R	RSDRcalc	HoR	No. of repet.	CrD95
01/08	0.995504	114	108	0.0000755	0.0000270	0.0027085	0.0020505	0.0001571	0.0000561	0.0056361	0.0028162	2	0.0001045
02/08	1.011493	132	125	0.0001921	0.0000686	0.0067837	0.0051480	0.0004435	0.0001584	0.0156582	0.0078426	2	0.0002985
03/08	0.992491	138	118	0.0000746	0.0000266	0.0026830	0.0020303	0.0002745	0.0000980	0.0098776	0.0049332	2	0.0001905
04/08	0.993129	132	120	0.0001230	0.0000439	0.0044247	0.0033486	0.0002863	0.0001023	0.0102965	0.0051429	2	0.0001929
05/08	1.004892	136	116	0.0000926	0.0000331	0.0032893	0.0024937	0.0004777	0.0001706	0.0169785	0.0084955	2	0.0003346
06/08	0.994063	142	123	0.0000558	0.0000199	0.0020051	0.0015177	0.0001776	0.0000634	0.0063791	0.0031867	2	0.0001224
07/08	0.992498	136	125	0.0000822	0.0000294	0.0029576	0.0022381	0.0002094	0.0000748	0.0075368	0.0037641	2	0.0001423
08/08	0.992270	130	115	0.0000515	0.0000184	0.0018537	0.0014027	0.0001665	0.0000595	0.0059940	0.0029935	2	0.0001149
09/08	1.002603	136	121	0.0000821	0.0000293	0.0029236	0.0022157	0.0003328	0.0001189	0.0118565	0.0059306	2	0.0002318
10/08	**0.994493**	**128**	**117**	**0.0000667**	**0.0000238**	**0.0023954**	**0.0018132**	**0.0001429**	**0.0000510**	**0.0051309**	**0.0025633**	**2**	**0.0000954**
11/08	0.992017	118	104	0.0000842	0.0000301	0.0030309	0.0022933	0.0001962	0.0000701	0.0070644	0.0035279	2	0.0001322
01/09	0.994216	148	131	0.0000830	0.0000297	0.0029832	0.0022580	0.0001551	0.0000554	0.0055712	0.0027832	2	0.0001015
02/09	0.992251	104	88	0.0000947	0.0000338	0.0034097	0.0025801	0.0002846	0.0001017	0.0102451	0.0051165	2	0.0001956
03/09	0.991875	126	108	0.0001271	0.0000454	0.0045777	0.0034637	0.0002067	0.0000738	0.0074421	0.0037165	2	0.0001316
04/09	0.993654	134	114	0.0001166	0.0000416	0.0041899	0.0031711	0.0002043	0.0000730	0.0073417	0.0036673	2	0.0001322
05/09	1.011035	128	104	0.0002388	0.0000853	0.0084361	0.0064016	0.0003554	0.0001269	0.0125542	0.0062875	2	0.0002211
06/09	0.992104	116	106	0.0001005	0.0000359	0.0036178	0.0027375	0.0003169	0.0001132	0.0114088	0.0056976	2	0.0002184
07/09	0.992720	144	140	0.0001579	0.0000564	0.0056815	0.0042995	0.0002916	0.0001042	0.0104923	0.0052404	2	0.0001905
08/09	0.993139	110	102	0.0001175	0.0000420	0.0042242	0.0031969	0.0003603	0.0001287	0.0129577	0.0064721	2	0.0002479
09/09	1.005276	112	108	0.0001100	0.0000393	0.0039070	0.0029622	0.0003522	0.0001258	0.0125134	0.0062617	2	0.0002429
10/09	0.992912	122	111	0.0000705	0.0000252	0.0025365	0.0019195	0.0002122	0.0000758	0.0076315	0.0038117	2	0.0001458
11/09	0.994031	128	118	0.0000718	0.0000256	0.0025784	0.0019516	0.0001639	0.0000585	0.0058883	0.0029415	2	0.0001102
01/10	0.994752	144	136	0.0000773	0.0000276	0.0027765	0.0021017	0.0001787	0.0000638	0.0064144	0.0032046	2	0.0001203
02/10	0.993181	108	98	0.0001471	0.0000525	0.0052893	0.0040029	0.0001693	0.0000605	0.0060884	0.0030410	2	0.0000945
03/10	0.992665	140	127	0.0001714	0.0000612	0.0061683	0.0046678	0.0002378	0.0000849	0.0085559	0.0042732	2	0.0001447
04/10	0.995502	142	128	0.0001175	0.0000419	0.0042138	0.0031901	0.0002320	0.0000829	0.0083248	0.0041596	2	0.0001532
05/10	1.002851	130	119	0.0001195	0.0000427	0.0042555	0.0032253	0.0002971	0.0001061	0.0105815	0.0052930	2	0.0002014
06/10	0.992607	106	99	0.0001228	0.0000438	0.0044172	0.0033427	0.0002226	0.0000795	0.0080092	0.0040001	2	0.0001449
07/10	0.992871	160	150	0.0001438	0.0000513	0.0051712	0.0039134	0.0003732	0.0001333	0.0134258	0.0067057	2	0.0002539
08/10	0.993235	104	93	0.0000895	0.0000320	0.0032182	0.0024356	0.0002458	0.0000878	0.0088399	0.0044154	2	0.0001680
09/10	1.012328	112	105	0.0000870	0.0000311	0.0030692	0.0023295	0.0003395	0.0001213	0.0119781	0.0060001	2	0.0002361
10/10	0.992308	128	115	0.0000606	0.0000216	0.0021811	0.0016504	0.0001635	0.0000584	0.0058845	0.0029388	2	0.0001116
11/10	0.994683	120	108	0.0001127	0.0000402	0.0040450	0.0030620	0.0001597	0.0000570	0.0057339	0.0028647	2	0.0000979

Table 3: Comparison of results from the hydrostatic balance (BH) and from the electronic densimeter (ED)

Density – Hydrostatic balance				**Density – Frequency oscillator**				**Comparison**
Sample	Average value	Total values	Selected values	Sample	Average value	Total values	Selected values	Δ(BH-DE)
01/08	0.995491	130	120	01/08	0.995504	114	108	-0.000013
02/08	1.011475	146	125	02/08	1.011493	132	125	-0.000018
03/08	0.992473	174	161	03/08	0.992491	138	118	-0.000018
04/08	0.993147	172	155	04/08	0.993129	132	120	0.000018
05/08	1.004836	150	138	05/08	1.004892	136	116	-0.000056
06/08	0.993992	152	136	06/08	0.994063	142	123	-0.000071
07/08	0.992447	162	150	07/08	0.992498	136	125	-0.000051
08/08	0.992210	162	151	08/08	0.992270	130	115	-0.000060
09/08	1.002600	148	131	09/08	1.002603	136	121	-0.000003
10/08	0.994482	174	152	10/08	0.994493	128	117	-0.000011
11/08	0.992010	136	125	11/08	0.992017	118	104	-0.000007
01/09	0.994184	174	152	01/09	0.994216	148	131	-0.000031
02/09	0.992266	118	101	02/09	0.992251	104	88	0.000015
03/09	0.991886	164	135	03/09	0.991875	126	108	0.000011
04/09	0.993632	180	150	04/09	0.993654	134	114	-0.000022
05/09	1.011061	116	100	05/09	1.011035	128	104	0.000026
06/09	0.992063	114	105	06/09	0.992104	116	106	-0.000041
07/09	0.992708	172	155	07/09	0.992720	144	140	-0.000012
08/09	0.993064	136	127	08/09	0.993139	110	102	-0.000075
09/09	1.005285	118	110	09/09	1.005276	112	108	0.000009
10/09	0.992905	150	132	10/09	0.992912	122	111	-0.000008
11/09	0.994016	142	127	11/09	0.994031	128	118	-0.000015
01/10	0.994734	170	152	01/10	0.994752	144	136	-0.000018
02/10	0.993177	120	110	02/10	0.993181	108	98	-0.000005
03/10	0.992799	148	136	03/10	0.992665	140	127	0.000134
04/10	0.995420	172	157	04/10	0.995502	142	128	-0.000082
05/10	1.002963	120	108	05/10	1.002851	130	119	0.000112
06/10	0.992546	120	113	06/10	0.992607	106	99	-0.000061
07/10	0.992831	174	152	07/10	0.992871	160	150	-0.000040
08/10	0.993184	144	130	08/10	0.993235	104	93	-0.000052
09/10	1.012293	114	103	09/10	1.012328	112	105	-0.000035
10/10	0.992289	154	136	10/10	0.992308	128	115	-0.000019

11/10	0.994649	130	112	11/10	0.994683	120	108	-0.000035
						average	Δ(BH-DE)	-0.0000162
						standard deviation	Δ(BH-DE)	0.0000447

Table 4: Precision parameters

	Hydrostatic balance (BH)	*Electronic densimeter (ED)*
No. of selected values	4347	3800
min	0.99189 g/cm^3	0.99187 g/cm^3
max	1.01229 g/cm^3	1.01233 g/cm^3
R	0.00067 g/cm^3	0.00025 g/cm^3
s_R	0.00024 g/cm^3	0.000091 g/cm^3
R%	0.067%	0.025%
r	0.00025 g/cm^3	0.00011 g/cm^3
s_r	0.000090 g/cm^3	0.000038 g/cm^3
r%	0.025%	0.011%

Key:
n: *number of selected values*
min: lower limit of the measurement range
max: upper limit of the measurement range
r: repeatability
s_r: repeatability standard deviation
r%: relative repeatability (r x 100 / average value)
R: reproducibility
s_R: reproducibility standard deviation
R%: relative reproducibility (R x 100 / average value)

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Evaluation of sugar by refractometry

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Saccharose % (m/m)	Refractive Index at 20 °C	Mass Density at 20 °C	Sugars in g/l	Sugars in g/kg	ABV % vol at 20 °C

55.0	1.43079	1.2635	707.8	560.2	42.06

55.1	1.43102	1.2639	709.4	561.3	42.16

55.2	1.43124	1.2645	711.0	562.3	42.25

55.3	1.43146	1.2652	712.7	563.3	42.36

55.4	1.43168	1.2659	714.4	564.3	42.46

55.5	1.43189	1.2665	716.1	565.4	42.56

55.6	1.43211	1.2672	717.8	566.4	42.66

55.7	1.43233	1.2679	719.5	567.5	42.76

55.8	1.43255	1.2685	721.1	568.5	42.85

55.9	1.43277	1.2692	722.8	569.5	42.96

56.0	1.43298	1.2699	724.5	570.5	43.06

Evaluation of sugar by refractometry

56.2	1.43343	1.2708	727.7	572.6	43.25

56.3	1.43365	1.2713	729.3	573.7	43.34

56.4	1.43387	1.2718	730.9	574.7	43.44

56.5	1.43409	1.2724	732.6	575.8	43.54

56.6	1.43431	1.2731	734.3	576.8	43.64

56.7	1.43454	1.2738	736.0	577.8	43.74

56.8	1.43476	1.2744	737.6	578.8	43.84

56.9	1.43498	1.2751	739.4	579.9	43.94

57.0	1.43519	1.2758	741.1	580.9	44.04

57.1	1.43542	1.2763	742.8	582.0	44.14

57.2	1.43564	1.2768	744.4	583.0	44.24

57.3	1.43586	1.2773	745.9	584.0	44.33

57.4	1.43609	1.2778	747.6	585.1	44.43

57.5	1.43631	1.2784	749.3	586.1	44.53

57.6	1.43653	1.2791	751.0	587.1	44.63

57.7	1.43675	1.2798	752.7	588.1	44.73

57.8	1.43698	1.2804	754.4	589.2	44.83

57.9	1.43720	1.2810	756.1	590.2	44.94

58.0	1.43741	1.2818	757.8	591.2	45.04

58.3	1.43909	1.2832	762.6	594.3	45.32

58.4	1.43832	1.2837	764.3	595.4	45.42

58.5	1.43854	1.2843	766.0	596.4	45.52

58.6	1.43877	1.2850	767.8	597.5	45.63

58.7	1.43899	1.2857	769.5	598.5	45.73

58.8	1.43922	1.2863	771.1	599.5	45.83

58.9	1.43944	1.2869	772.9	600.6	45.93

59.0	1.43966	1.2876	774.6	601.6	46.03

59.1	1.43988	1.2882	776.3	602.6	46.14

59.2	1.44011	1.2889	778.1	603.7	46.24

59.3	1.44034	1.2896	779.8	604.7	46.34

59.4	1.44057	1.2902	781.6	605.8	46.45

59.5	1.44079	1.2909	783.3	606.8	46.55

59.6	1.44102	1.2916	785.2	607.9	46.66

59.7	1.44124	1.2921	786.8	608.9	46.76

59.8	1.44147	1.2926	788.4	609.9	46.85

59.9	1.44169	1.2931	790.0	610.9	46.95

Evaluation of sugar by refractometry

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Evaluation of sugar by refractometry

Method OIV-MA-AS2-03A Type I method

Total dry matter

OIV/OENO 377/2009;
OIV/OENO 387/2009)
OIV-OENO 465-2012

1 Definition

The total dry extract or the total dry matter includes all matter that is non-volatile under specified physical conditions. These physical conditions must be such that the matter forming the extract undergoes as little alteration as possible while the test is being carried out.

The sugar-free extract is the difference between the total dry extract and the total sugars. The reduced extract is the difference between the total dry extract and the total sugars in excess of 1 g/L, potassium sulfate in excess of 1 g/L, any mannitol present and any other chemical substances which may have been added to the wine.

The residual extract is the sugar-free extract less the fixed acidity expressed as tartaric acid.

2 Principle

The weight of residue obtained when a sample of wine, previously absorbed onto filter paper, is dried in a current of air, at a pressure of 20 - 25 mm Hg at 70°C.

3 Method

3.1 *Apparatus*

3.1.1 Oven:

Cylindrical basin (internal diameter: 27 cm, height: 6 cm) made of aluminum with an aluminum lid, heated to 70°C and regulated to 1°C.

A tube (internal diameter: 25 mm) connecting the oven to a vacuum pump providing a flow rate of 50 L/h. The air, previously dried by bubbling through concentrated sulfuric acid, is circulated in the oven by a fan in order to achieve quick homogenous reheating. The rate of airflow is regulated by a tap and is to be 30-40 L per hour and the pressure in the oven is 25 mm of mercury.

The oven can then be used providing it is calibrated as in 3.1.3.

3.1.2 Dishes:

Stainless steel dishes (60 mm internal diameter, 25 mm in height) provided with fitting lids. Each dish contains 4-4.5 g of filter paper, cut into fluted strips 22 mm in length.

The filter paper is first washed with hydrochloric acid, 2 g/L, for 8 h, rinsed five times with water and then dried in air.

3.1.3 Calibration of apparatus and method

a) Checking the seal of the dish lids. A dish, containing dried filter paper, with the lid on, after first being cooled in a dessicator containing sulfuric acid, should not gain more than 1 mg/h when left in the laboratory.

b) Checking the degree of drying. A pure solution of sucrose, 100 g/L, should give a dry extract of 100 g ± 1 g/L.

c) A pure solution of lactic acid, 10 g/L, should give a dry extract of at least 9.5 g/L.

If necessary, the drying time in the oven can be increased or decreased by changing the rate of airflow to the oven or by changing the pressure in order that these conditions should be met.

NOTE - The lactic acid solution can be prepared as follows: 10 mL of lactic acid is diluted to approximately 100 mL with water. This solution is placed in a dish and heated on a boiling water bath for 4 h, distilled water is added if the volume decreases to less than 50 mL (approx). Make up the solution to 1 liter and titrate 10 mL of this solution with alkali, 0.1 M. Adjust the lactic acid solution to 10 g/L.

3.2 *Procedure*

3.2.1 Weighing the dish

Place the dish containing filter paper in the oven for 1 h. Stop the vacuum pump and immediately place the lid on the dish on opening the oven. Cool in a dessicator and weigh to the nearest 0.1 mg: the mass of the dish and lid is p_o g.

3.2.2 Weighing the sample

Place 10 mL of must or wine into the weigh dish. Allow the sample to be completely absorbed onto the filter paper. Place the dish in the oven for 2 h (or for the time used in the calibration of the standard in 3.1.3). Weigh the dish following the procedure 3.2.1 beginning "Stop the vacuum ..." The mass is p g.

Note: The sample weight should be taken when analyzing very sweet wines or musts.

3.3 *Calculation*

The total dry extract is given by:

$$(p - p_o) \times 100$$

For very sweet wines or musts the total dry extract is given by:

$$(p - p_o) \times \frac{\rho_{20}}{P} \times 1000$$

P = mass of sample in grams

ρ_{20} = density of wine or must in g/mL.

3.4 *Expression of results*

The total dry extract is expressed in g/L to one decimal place.

Note:

Calculate total dry extract by separately taking into account quantities of glucose and fructose (reducing sugars) and the quantity of saccharose, as follows:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) – saccharose

In the case that the method of analysis allows for sugar inversion, use the following formula for the calculation:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) - [(Sugars after inversion – Sugars before inversion) x 0,95]

Inversion refers to the process that leads to the conversion of a stereoisomer into compounds with reverse stereoisomerism. In particular, the process based on splitting sucrose into fructose and glucose, carried out by keeping acidified solutions containing sugars (100 ml solution containing sugars + 5 ml concentrated hydrochloric acid) for at least 15 min at 50°C or above in a water-bath (the water-bath is maintained at 60°C until the temperature of the solution reaches 50°C), is called *sugar inversion*. The final solution is laevo-rotatory due to the presence of fructose, while the initial solution is dextro-rotatory due to the presence of sucrose.

TABLE I

For the calculation of the total dry extract content (g/L)

Density to 2 decimal places	3rd decimal place									
	0	1	2	3	4	5	6	7	8	9
	Extract g/L									
1.00	0	2.6	5.1	7.7	10.3	12.9	15.4	18.0	20.6	23.2
1.01	25.8	28.4	31.0	33.6	36.2	38.8	41.3	43.9	46.5	49.1
1.02	51.7	54.3	56.9	59.5	62.1	64.7	67.3	69.9	72.5	75.1
1.03	77.7	80.3	82.9	85.5	88.1	90.7	93.3	95.9	98.5	101.1
1.04	103.7	106.3	109.0	111.6	114.2	116.8	119.4	122.0	124.6	127.2
1.05	129.8	132.4	135.0	137.6	140.3	142.9	145.5	148.1	150.7	153.3
1.06	155.9	158.6	161.2	163.8	166.4	169.0	171.6	174.3	176.9	179.5
1.07	182.1	184.8	.187.4	190.0	192.6	195.2	197.8	200.5	203.1	205.8
1.08	208.4	211.0	213.6	216.2	218.9	221.5	224.1	226.8	229.4	232.0
1.09	234.7	237.3	239.9	242.5	245.2	247.8	250.4	253.1	255.7	258.4
1.10	261.0	263.6	266.3	268.9	271.5	274.2	276.8	279.5	282.1	284.8
1.11	287.4	290.0	292.7	295.3	298.0	300.6	303.3	305.9	308.6	311.2
1.12	313.9	316.5	319.2	321.8	324.5	327.1	329.8	332.4	335.1	337.8
1.13	340.4	343.0	345.7	348.3	351.0	353.7	356.3	359.0	361.6	364.3
1.14	366.9	369.6	372.3	375.0	377.6	380.3	382.9	385.6	388.3	390.9
1.15	393.6	396.2	398.9	401.6	404.3	406.9	409.6	412.3	415.0	417.6
1.16	420.3	423.0	425.7	428.3	431.0	433.7	436.4	439.0	441.7	444.4
1.17	447.1	449.8	452.4	455.2	457.8	460.5	463.2	465.9	468.6	471.3
1.18	473.9	476.6	479.3	482.0	484.7	487.4	490.1	492.8	495.5	498.2
1.19	500.9	503.5	506.2	508.9	511.6	514.3	517.0	519.7	522.4	525.1
1.20	527.8	-	-	-	-	-	-	-	-	-

INTERPOLATION TABLE

4th decimal place	Extract g/L	4th decimal place	Extract g/L	4th decimal place	Extract g/L
1	0.3	4	1.0	7	1.8
2	0.5	5	1.3	8	2.1
3	0.8	6	1.6	9	2.3

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Method OIV-MA-AS2-03B Type IV method

Total dry matter

OIV/OENO 377/2009
OIV/OENO 387/2009)
OIV-OENO 465-2012

1 Definition

The total dry extract or the total dry matter includes all matter that is non-volatile under specified physical conditions. These physical conditions must be such that the matter forming the extract undergoes as little alteration as possible while the test is being carried out.

The sugar-free extract is the difference between the total dry extract and the total sugars. The reduced extract is the difference between the total dry extract and the total sugars in excess of 1 g/L, potassium sulfate in excess of 1 g/L, any mannitol present and any other chemical substances which may have been added to the wine.

The residual extract is the sugar-free extract less the fixed acidity expressed as tartaric acid.

2 Principle

The total dry extract is calculated indirectly from the specific gravity of the must and, for wine, from the specific gravity of the alcohol-free wine.

This dry extract is expressed in terms of the quantity of sucrose which, when dissolved in water and made up to a volume of one liter, gives a solution of the same gravity as the must or the alcohol-free wine.

3 Method

3.1 *Procedure*

Determine the specific gravity of a must or wine.
In the case of wine, calculate the specific gravity of the "alcohol free wine" using the following formula:

$$d_r = d_v - d_a + 1.000$$

where:
d_v = specific gravity of the wine at 20°C (corrected for volatile acidity [1])
d_a = specific gravity at 20°C of a water-alcohol mixture of the same alcoholic strength as the wine obtained using the formula:

$$d_r = 1.00180^{**} (r_v - r_a) + 1.000$$

where :
r_v = density of the wine at 20°C (corrected for volatile acidity [1])
r_a = density at 20°C of the water alcohol mixture of the same alcoholic strength as the wine obtained from Table 1 of chapter *Alcoholic strength by volume* for a temperature of 20°C.

3.2 *Calculation*
Use the value for specific gravity of the alcohol free wine to obtain the total dry extract (g/L) from table I

3.3 Expression of results
The total dry extract is reported in g/L to one decimal place.

Note:
Calculate total dry extract by separately taking into account quantities of glucose and fructose (reducing sugars) and the quantity of saccharose, as follows:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) – saccharose

[1] NOTE: Before carrying out this calculation, the specific gravity (or the density) of the wine measured as specified above should be corrected for the effect of the volatile acidity using the formula:

$$d_v = d\frac{20}{20} - 0.0000086\,a \quad \text{or} \quad \rho_v = \rho_{20} - 0.0000086\,a$$

where a is the volatile acidity expressed in milli-equivalents per liter.

** The coefficient 1.0018 approximates to 1 when r_v is below 1.05 which is often the case.

In the case that the method of analysis allows for sugar inversion, use the following formula for the calculation:
Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) - [(Sugars after inversion – Sugars before inversion) x 0,95]

Inversion refers to the process that leads to the conversion of a stereoisomer into compounds with reverse stereoisomerism. In particular, the process based on splitting sucrose into fructose and glucose, carried out by keeping acidified solutions containing sugars (100 ml solution containing sugars + 5 ml concentrated hydrochloric acid) for at least 15 min at 50°C or above in a water-bath (the water-bath is maintained at 60°C until the temperature of the solution reaches 50°C), is called *sugar inversion*. The final solution is laevo-rotatory due to the presence of fructose, while the initial solution is dextro-rotatory due to the presence of sucrose.

TABLE I
For the calculation of the total dry extract content (g/L)

Density to 2 decimal places	3rd decimal place									
	0	1	2	3	4	5	6	7	8	9
	Extract g/L									
1.00	0	2.6	5.1	7.7	10.3	12.9	15.4	18.0	20.6	23.2
1.01	25.8	28.4	31.0	33.6	36.2	38.8	41.3	43.9	46.5	49.1
1.02	51.7	54.3	56.9	59.5	62.1	64.7	67.3	69.9	72.5	75.1
1.03	77.7	80.3	82.9	85.5	88.1	90.7	93.3	95.9	98.5	101.1
1.04	103.7	106.3	109.0	111.6	114.2	116.8	119.4	122.0	124.6	127.2
1.05	129.8	132.4	135.0	137.6	140.3	142.9	145.5	148.1	150.7	153.3
1.06	155.9	158.6	161.2	163.8	166.4	169.0	171.6	174.3	176.9	179.5
1.07	182.1	184.8	.187.4	190.0	192.6	195.2	197.8	200.5	203.1	205.8
1.08	208.4	211.0	213.6	216.2	218.9	221.5	224.1	226.8	229.4	232.0
1.09	234.7	237.3	239.9	242.5	245.2	247.8	250.4	253.1	255.7	258.4
1.10	261.0	263.6	266.3	268.9	271.5	274.2	276.8	279.5	282.1	284.8
1.11	287.4	290.0	292.7	295.3	298.0	300.6	303.3	305.9	308.6	311.2
1.12	313.9	316.5	319.2	321.8	324.5	327.1	329.8	332.4	335.1	337.8
1.13	340.4	343.0	345.7	348.3	351.0	353.7	356.3	359.0	361.6	364.3
1.14	366.9	369.6	372.3	375.0	377.6	380.3	382.9	385.6	388.3	390.9
1.15	393.6	396.2	398.9	401.6	404.3	406.9	409.6	412.3	415.0	417.6
1.16	420.3	423.0	425.7	428.3	431.0	433.7	436.4	439.0	441.7	444.4
1.17	447.1	449.8	452.4	455.2	457.8	460.5	463.2	465.9	468.6	471.3
1.18	473.9	476.6	479.3	482.0	484.7	487.4	490.1	492.8	495.5	498.2
1.19	500.9	503.5	506.2	508.9	511.6	514.3	517.0	519.7	522.4	525.1
1.20	527.8	-	-	-	-	-	-	-	-	-

INTERPOLATION TABLE

4th decimal place	Extract g/L	4th decimal place	Extract g/L	4th decimal place	Extract g/L
1	0.3	4	1.0	7	1.8
2	0.5	5	1.3	8	2.1
3	0.8	6	1.6	9	2.3

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Method OIV-MA-AS2-04 Type I method

Ash
(A6 revised by OIV/OENO 377/2009)

1. Definition
The ash content is defined to be all those products remaining after igniting the residue left after the evaporation of the wine. The ignition is carried out in such a way that all the cations (excluding the ammonium cation) are converted into carbonates or other anhydrous inorganic salts.

2. Principle
The wine extract is ignited at a temperature between 500 and 550°C until complete combustion (oxidation) of organic material has been achieved.

3 Apparatus
3.1 boiling water-bath at 100°C;
3.2 balance sensitive to 0.1 mg;
3.3 hot-plate or infra-red evaporator;
3.4 temperature-controlled electric muffle furnace;
3.5 dessicator;
3.6 flat-bottomed platinum dish 70 mm in diameter and 25 mm in height.

4. Procedure
Pipette 20 mL of wine into the previously tared platinum dish (original weight p_0 g).

Evaporate on the boiling water-bath, and heat the residue on the hot-plate at 200°C or under the infra-red evaporator until carbonization begins. When no more fumes are produced, place the dish in the electric muffle furnace maintained at 525 ± 25°C. After 15 min or carbonization, remove the dish from the furnace, add 5 mL of distilled water, evaporate on the water-bath or under the infra-red evaporator, and again heat the residue to 525°C for 10 min.

If combustion (oxidation) of the carbonized particles is not complete, the following operations are repeated: washing the carbonized particles, evaporation of water, and ignition. For wines with a high sugar content, it is advantageous to add a few drops of pure vegetable oil to the extract before the first ashing to prevent excessive foaming. After cooling in the desiccator, the dish is weighed (p_1 g).

The weight of the ash in the sample (20 mL) is then calculated as $p = (p_1 — p_0)$ g.

5. Expression of results
The weight P of the ash in grams per liter is given to two decimal places by the expression: $P = 50\ p$.

Method OIV-MA-AS2-05 Type IV method

Alkalinity of Ash

(A7 revised by OIV/OENO 377/2009)

1. Definition

The alkalinity of the ash is defined as the sum of cations, other than the ammonium ion, combined with the organic acids in the wine.

2. Principle

The ash is dissolved in a known (excess) amount of a hot standardized acid solution; the excess is determined by titration using methyl orange as an indicator.

3. Reagents and apparatus

3.1. Sulfuric acid solution, 0.05 M H_2SO_4

3.2. Sodium hydroxide solution, 0.1 M NaOH

3.3. Methyl orange, 0.1% solution in distilled water

3.4. Boiling water-bath

4. Procedure

Add 10 mL 0.05 M sulfuric acid solution (3.1) to the ash from 20 mL of wine contained in the platinum dish. Place the dish on the boiling water-bath for about 15 min, breaking up and agitating the residue with a glass rod to speed up the dissolution. Add two drops of methyl orange solution and titrate the excess sulfuric acid against 0.1 M sodium hydroxide (3.2) until the color of the indicator changes to yellow.

5. Expression of results

5.1. *Method of calculation*

The alkalinity of ash, expressed in milliequivalents per liter to one decimal place, is given by:

$$A = 5\ (10 - n)$$

where n mL is the volume of sodium hydroxide, 0.1 M, used.

5.2. *Alternative expression*

The alkalinity of ash, expressed in grams per liter of potassium carbonate, to two decimal places, is given by:

$$A = 0.345\ (10 - n)$$

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Method OIV-MA-AS2-06 Type IV method

Measurement of the oxidation-reduction potential in wines

(Resolution OENO 3/2000)

1. PURPOSE AND SCOPE OF APPLICATION:

The oxidation-reduction potential (EH) is a measure of the oxidation or reduction state of a medium. In the field of enology, oxygen and the oxidation-reduction potential are two important factors in the pre-fermentation processing of the grape harvest, the winemaking process, growing, and wine storage.

Proposals are hereby submitted for equipment designed to measure the Oxidation-reduction Potential in Wines and a working method for taking measurements under normal conditions. This method has not undergone any joint analysis, given the highly variable nature of the oxidation-reduction state of a particular wine, a situation which makes this step in the validation process difficult to implement. As a result, this is a class 4 method[1] intended basically for production.

2. UNDERLYING PRINCIPLE

The oxidation-reduction potential of a medium is defined as the difference in potential between a corrosion-proof electrode immersed in this medium and a standard hydrogen electrode linked to the medium. Indeed, only the difference in oxidation-reductions potentials of two linked systems can be measured. Consequently, the oxidation-reduction potential of the hydrogen electrode is considered to be zero, and all oxidation-reduction potentials are compared to it. The oxidation-reduction potential is a measurement value permitting expression of the instantaneous physico-chemical state of a solution. Only potentiometric volumetric analysis of the total oxidation-reduction pairs and an estimate of the oxidizing agent/reducing agent ratio can yield a true quantitative measurement. Oxidation-reduction potential is measured using combined electrodes, whether in wine or in another solution. This system usually involves the use of a platinum electrode (measuring electrode) and a silver or mercurous chloride electrode (reference electrode).

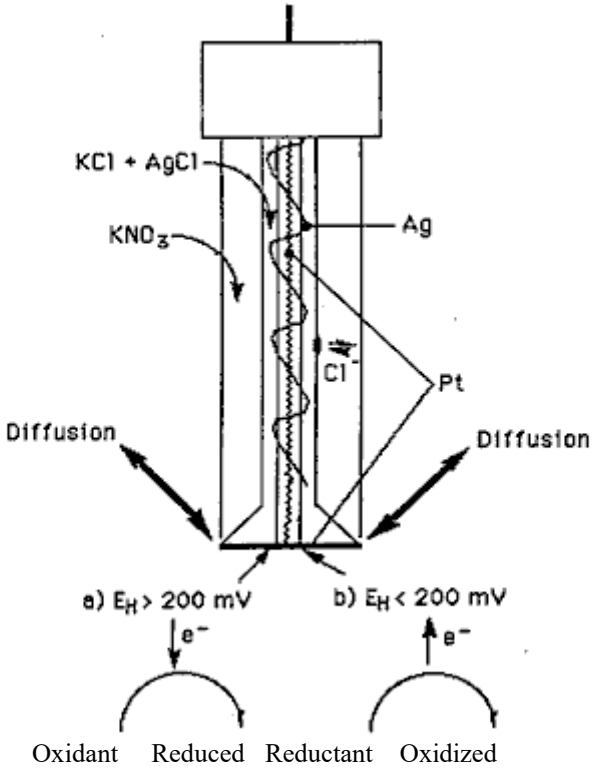
3. EQUIPMENT

Although several types of electrodes exist, it is recommended that an electrode adapted for measuring the EH in wine be used. It is recommended that use be made of a double-jacket combined electrode linked to a reference electrode (see figure). This system incorporates a measuring electrode, and a double-jacket reference electrode, both of which are linked to an ion meter. The inner jacket of the reference

[1] In conformity with the classification detailed in the Codex Alimentarius.

electrode is filled with a solution of 17.1% KNO_3; trace amounts of AgCl; trace amounts of Triton X-100; 5% KCL; 77.9% de-ionized water; and for the measuring electrode, the solution is made up of <1% AgCL; 29.8% KCL; and 70% de-ionized water.

Modified Combined Electrode



4. CLEANING AND CALIBRATION OF THE ELECTRODES

4.1. *Calibration*

The electrodes are calibrated using solutions with known, constant oxidation-reduction potentials. An equimolar solution (10 mM/l) of ferricyanide and potassium ferrous cyanide is used. Its composition is: 0.329g of $K_3Fe(CN)_6$; 0.422g of $K_4Fe(CN)_6$; 0.149g of KCl and up to 1000ml of water. At 20 °C this solution has an oxidation-reduction potential of 406 mV (±5 mV), but this potential changes over time, thus requiring that the solution not be stored for more than two weeks in the dark.

4.2. *Cleaning the Platinum in the Electrode*

The electrode platinum should be cleaned by immersing it in a solution of 30% hydrogen peroxide by volume for one hour, then washing it with water. Complete cleaning in water is required after each series of measurement. The system is normally cleaned after each week of use.

5. WORKING METHOD

5.1. *Filling the Inner Jacket*

The composition of the double jacket varies depending on the type of medium for which the EH is being measured (Table below).

Table
Composition of the Filler Solution in the Double Jacket of the Electrode as a Function of the Medium Measured

Medium to be measured	Solution Composition of the jacket
1 Dry wines	Ethanol 12% by vol., 5g tartaric acid, NaOH N up to pH 3.5, distilled water up to 1000 ml
2 Sweet wines	Solution 1 plus 20 g/l sucrose
3 Special sweet wines	Solution 2 plus 100 mg/l of SO_2 ($KHSO_3$)
4 Brandies	Ethanol 50% by vol., acetic acid up to pH 5, distilled water up to 1000 ml.

5.2. Balancing the Electrode with the Medium to Be Measured

Before taking any measurements, the electrodes must be calibrated in Michaelis solution, then stabilized for 15 minutes in a wine, if the measurement s are to be taken in wines. Next, for measurements taken on site, measurements are read after the electrodes have been immersed in the medium for 5 minutes. For laboratory measurements, the stability index is the ΔEH(mV) / T (minutes) ratio ; when this latter is ≤ 0.2, the potential can be read.

5.3. *Measurements Under Practical Conditions*

Measurements are systematically taken on site without any handling that could change the oxidation-reduction potential values. When taking measurements in storehouses, casks, vats, etc. care should be taken to record temperature, pH and dissolved oxygen content (method under preparation) at the same time as the EH measurement is taken, as these measurements will subsequently be used to interpret results. For wines in bottles, the measurement is taken in the wine after letting it sit

in a room whose temperature is 20 °C, immediately after the container is opened, under a constant flow of nitrogen, and after immersing the entire electrode unit in the bottle.

5.4. *Expression of Results*
Findings are recorded in mV as compared with the standard hydrogen electrode.

Method OIV-MA-AS2-07A

Chromatic Characteristics

2. Principle of the methods

A spectrophotometric method which makes it possible to determine the tristimulus values and the three chromaticity coefficients required to specify the color as described by the CIE (*Commission internationale de l'Éclairage*).

WITHDRAWN
(replaced by OIV-MA-AS2-11)

Method OIV-MA-AS2-07B Type IV method

Chromatic Characteristic

(Method A0 modified by OENO 1/2006
Modified by OIV-OENO 667-2022)

1. Definitions

The "chromatic characteristics" of a wine are its luminosity and chromaticity. Luminosity depends on transmittance and varies inversely with the intensity of color of the wine. Chromaticity depends on dominant wavelength (distinguishing the shade) and purity.
Conventionally, and for the sake of convenience, the chromatic characteristics of red and rosé wines are described by the intensity of color and shade, in keeping with the procedure adopted as the working method.

2. Principle of the methods

(applicable to red and rosé wines)

A spectrophotometric method whereby chromatic characteristics are expressed conventionally, as given below:

- The intensity of color is given by the sum of absorbencies (or optical densities) using a 1 cm optical path and radiations of wavelengths 420, 520 and 620 nm.
- The shade is expressed as the ratio of absorbance at 420 nm to absorbance at 520 nm.

3. Method

3.1. *Apparatus*

3.1.1 Spectrophotometer enabling measurements to be made between 300 and 700 nm.

3.1.2 Glass cells (matched pairs) with optical path *b* equal to 0.1, 0.2, 0.5, 1 and 2 cm.

3.2. *Preparation of the sample*

If the wine is cloudy, clarify it by centrifugation; young or sparkling wines must have the bulk of their carbon dioxide removed by agitation under vacuum.

3.3. *Method*

The optical path *b* of the glass cell used must be chosen so that the measured absorbance A, falls between 0.3 and 0.7.

Take the spectrophotometric measurements using distilled water as the reference liquid, in a cell of the same optical path *b*, in order to set the zero on the absorbance scale of the apparatus at the wavelengths of 420, 520 and 620 nm.
Using the appropriate optical path *b*, read off the absorbencies at each of these three wavelengths for the wine.

3.4. *Calculations*

Calculate the absorbencies for a 1 cm optical path for the three wavelengths by dividing the absorbencies found (A_{420}, A_{520} and A_{620}) by *b*, in cm.

3.5. *Expression of Results*

The color intensity I is conventionally given by:

$$I = A_{420} + A_{520} + A_{620}$$

and is expressed to three decimal places.
The shade N is conventionally given by:

$$N = \frac{A_{420}}{A_{520}}$$

and is expressed to three decimal places.

TABLE 1

Converting absorbance into transmittance (T%)

Method: find the first decimal figure of the absorbance value in the left-hand column (0-9) and the second decimal figure in the top row (0-9).

Take the figure at the intersection of column and row: to find the transmittance, divide the figure by 10 if absorbance is less than 1, by 100 if between 1 and 2 and by 1000 if between 2 and 3.

Note: The figure in the top right hand corner of each box enables the third decimal figure of the absorbance to be determined by interpolation.

	0	1	2	3	4	5	6	7	8	9
0	23 1000	22 977	22 955	21 933	21 912	20 891	20 871	19 851	19 932	19 813
1	18 794	18 776	17 759	17 741	16 724	16 708	16 692	15 676	15 661	15 646
2	14 631	14 617	14 603	14 589	13 575	13 562	13 549	12 537	12 525	12 513
3	11 501	11 490	11 479	11 468	10 457	9 447	9 436	10 427	10 417	9 407
4	9 398	9 389	9 380	8 371	8 363	8 355	8 347	 339	7 331	8 324
5	7 316	 309 71	7 302	7 295	6 288	7 282	6 275	6 269	6 263	6 257
6	6 251	5 245	6 240	5 234	5 229	5 224	5 219	5 214	5 209	5 204
7	4 199	5 195	4 190	4 186	4 182	4 178	4 174	4 170	4 166	4 162
8	3 158	4 155	3 151	4 148	4 144	3 141	3 138	3 135	3 132	3 129
9	3 126	3 123	3 120	2 117	3 115	2 112	3 110	2 107	3 105	2 102

Example:

Absorbance	0.47	1.47	2.47	3.47
T% 33.9%	3.4%	0.3%	0%	

Transmittance (T%) is expressed to the nearest 0.1%.

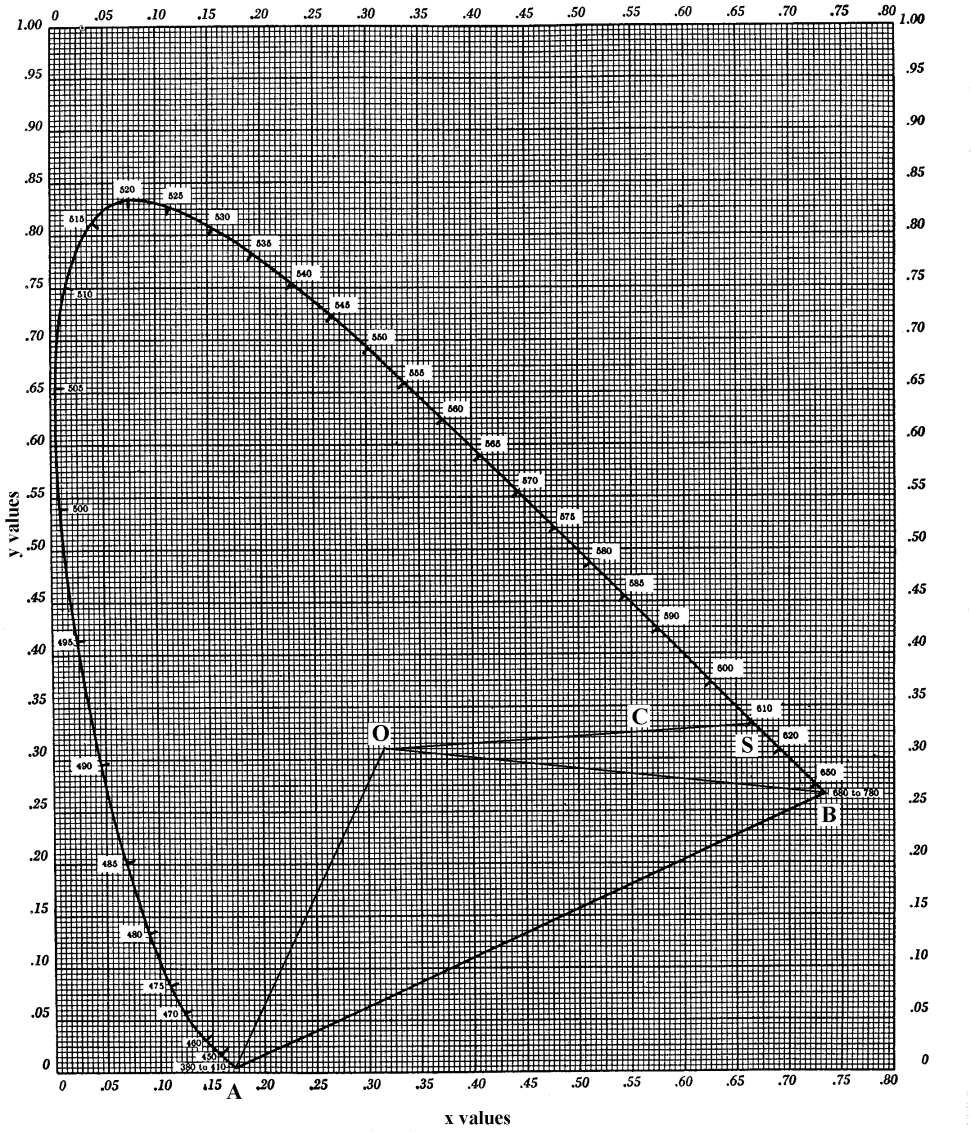


FIGURE 1

Chromaticity diagram, showing the locus of all colors of the spectrum

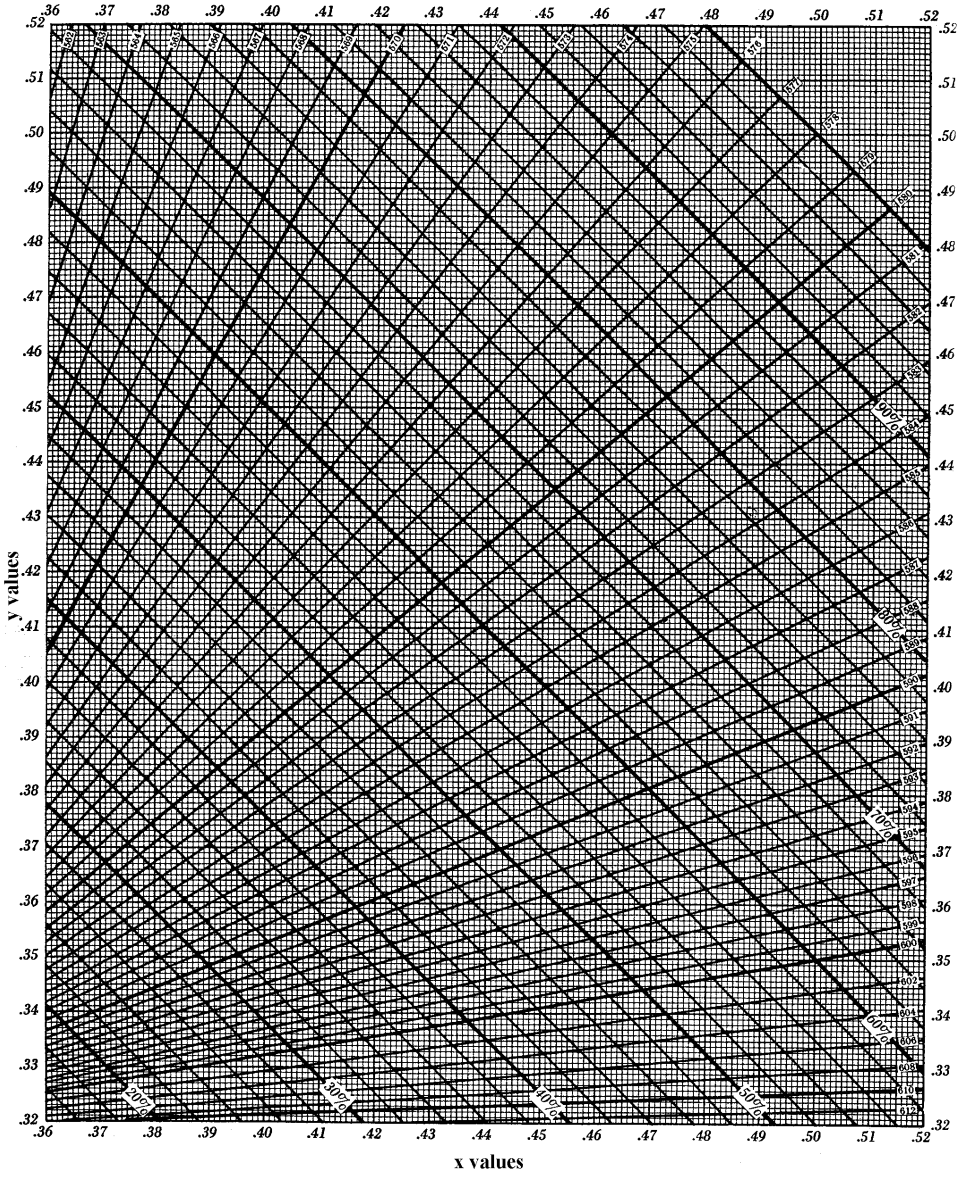


FIGURE 2
Chromaticity diagram for pure red wines and brick red wines

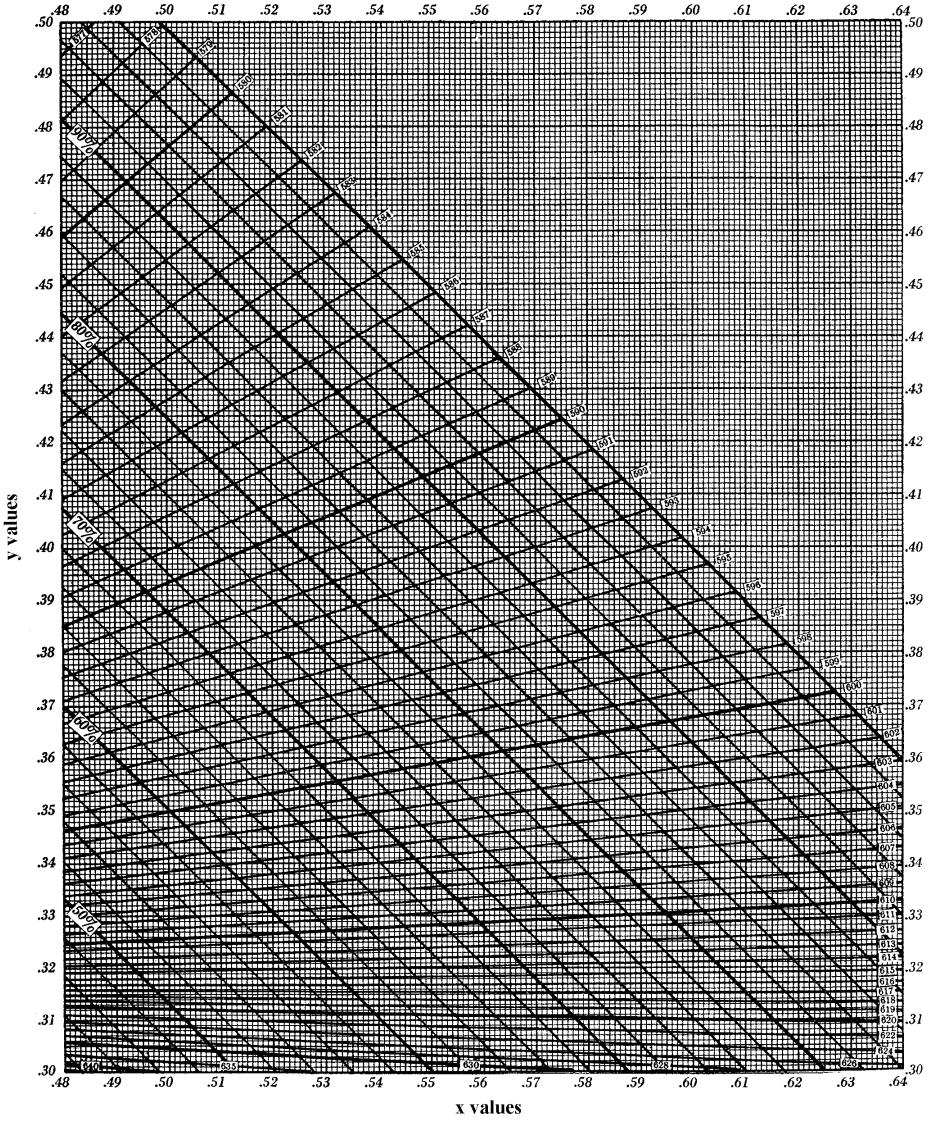


FIGURE 3

Chromaticity diagram for pure red wines and brick red wines

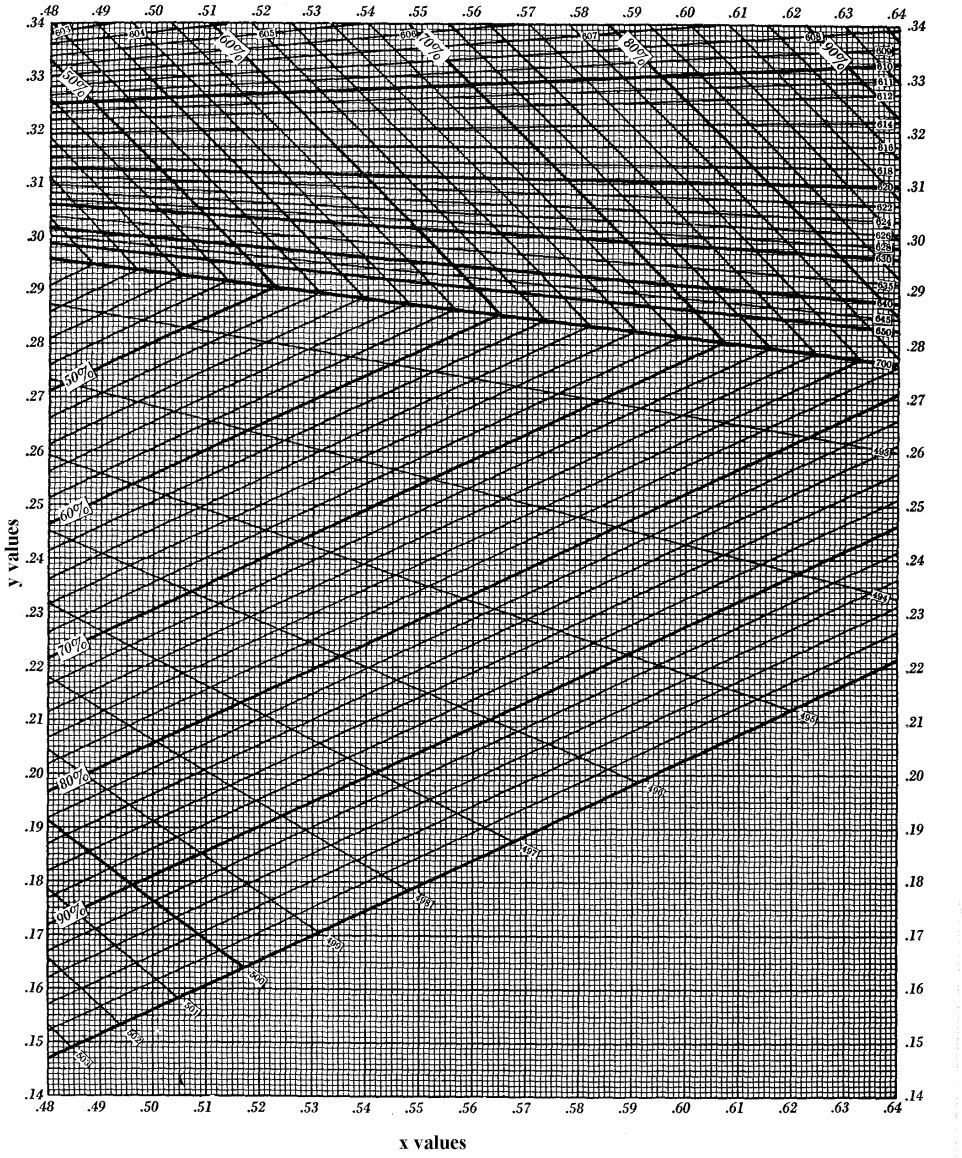


FIGURE 4

Chromaticity diagram for pure red wines and purple wines

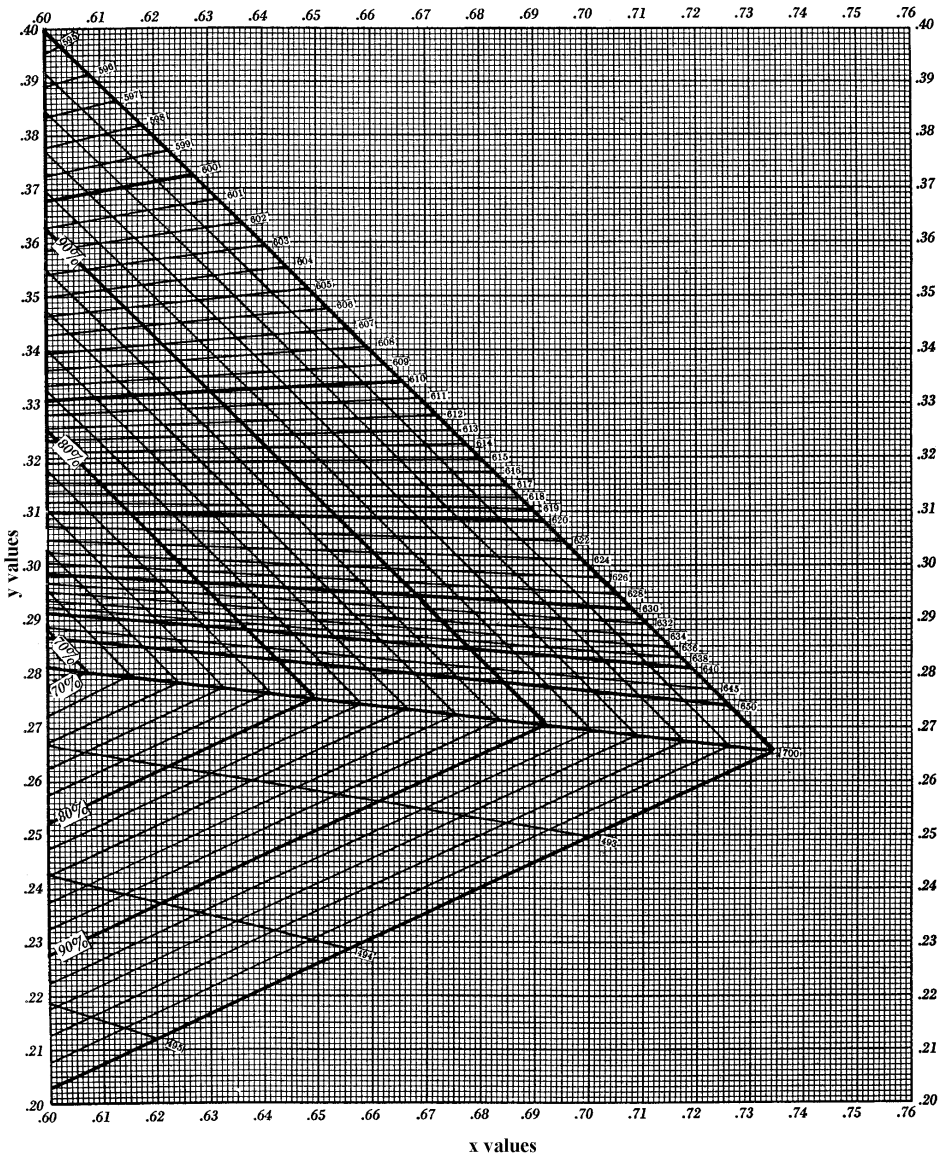


FIGURE 5

Chromaticity diagram for pure red wines and purple red wines

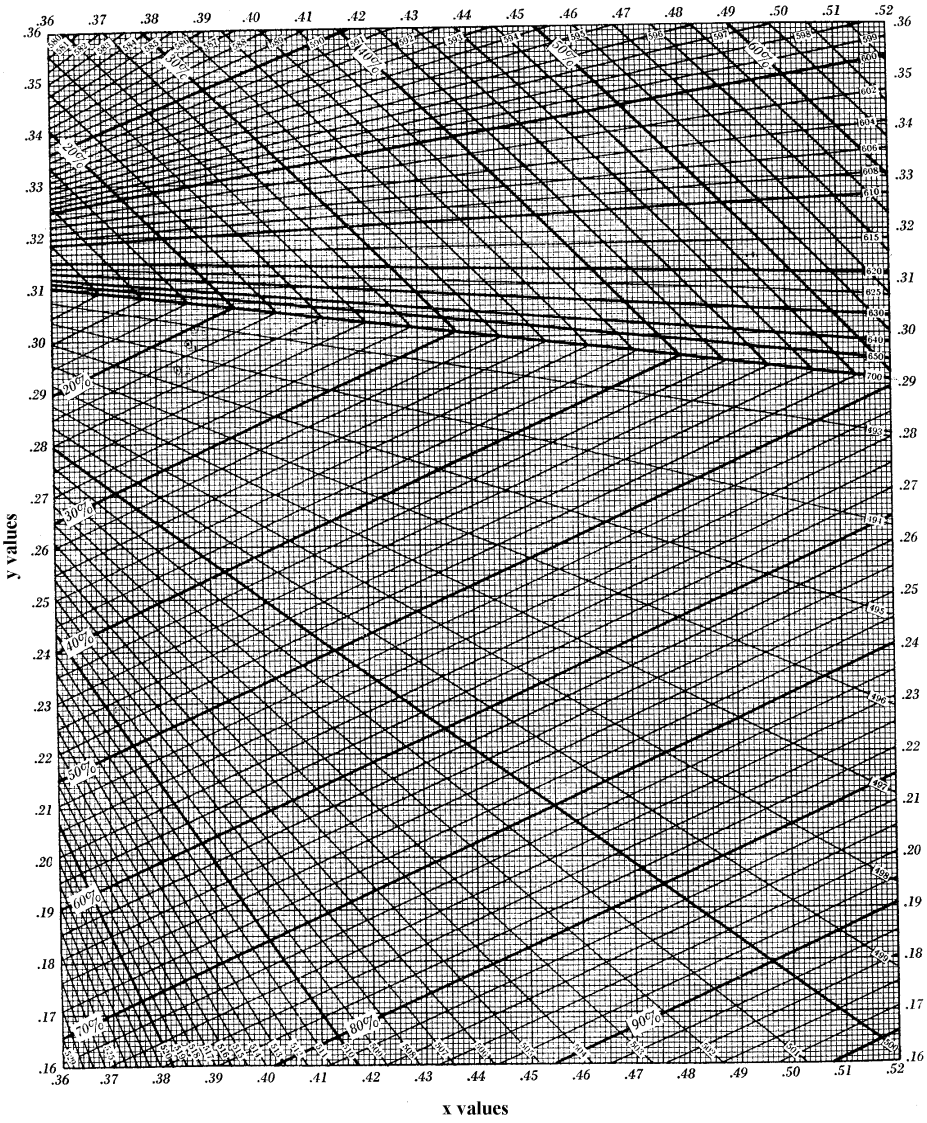


FIGURE 6

Chromaticity diagram for brick red wines and purple red wines

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Annex: Operative instructions for the determination of Chromatic Characteristics of wines and/or musts obtained by grape varieties characterized by high concentrations of colouring pigments and/or high sulfur dioxide levels

1. Principle of the method

1.1. Field of application

Applicable to red wine with high concentrations of colouring pigments, must, and must with high sulphur dioxide levels.

A spectrophotometric method whereby chromatic characteristics are expressed, conventionally, as given below:

- The intensity of colour is given by the sum of absorbencies (or O.D. = Optical Densities) using a 1 cm optical path and radiation of wavelengths 420, 520 and 620 nm.
- The shade is expressed as the ratio of absorbance at 420 nm to absorbance at 520 nm.

For grape varieties characterized by high concentrations of colouring pigments, given the nature of the chemical structure of these substances, the determination of the chromatic characteristics requires the dilution of the sample with a buffered solvent at pH 3.2. The use of a buffered solvent compared to dilution with water reduces the effect of the matrix and normalizes the O.D as the dilution increases.

2. Method

2.1. Apparatus

2.1.1. Spectrophotometer enabling measurements to be made between 300 and 700 nm.

2.1.2. Glass cuvettes or single use plastic cuvettes with optical path equal to 1 cm.

2.1.3. Volumetric glassware with variable volume according to needs

2.1.4. Syringe filter 0.45 μm

2.2. Reagents

2.2.1. Type II water for analytical use, ISO 3696 standard, or of equivalent purity
2.2.2. Tartaric acid 99.5% (CAS 87-69-4)
2.2.3. Sodium hydroxide NaOH 1 N (CAS 1310-73-2)
2.2.4. Hydrogen peroxide 30% w/w (CAS 7722-84-1)

2.3. Working solutions

2.3.1. *Buffer at pH 3.20*

Daily preparation: weigh 7 0.1 g of tartaric acid (2.2.2) in a 1000 mL volumetric flask, add 35 ml of NaOH 1 N (2.2.3) and make up to 1000 mL with water (2.2.1). Check the pH with a pH meter and verify that pH is 3.20±0.05. The solution should be checked and filtered (2.1.4) at the time of use.

2.3.2. Hydrogen peroxide 3% (v/v)

Dilute 1.0 mL of hydrogen peroxide (2.2.4) to 10 ml. The solution should be prepared at the time of use.

2.4. Preparation of the sample

If the sample is cloudy, clarify it by centrifugation (10 min, 1146 rcf); If there is carbon dioxide remove it by agitation under vacuum (or similar systems).

In the case of grape must whose alcoholic fermentation is inhibited by adding sulphur dioxide, add 0.1 mL of 3% hydrogen peroxide solution (2.3.2) per mL of sample used and make up to volume, depending on the dilution chosen, with the buffer solution at pH 3.2 (2.3.1). Wait 20 minutes, then proceed with spectrophotometric reading.

2.5. Spectrophotometric reading for wine and must with high colour intensity or high sulphur dioxide levels

Take the spectrophotometric measurements of the samples: the absorbance (A) will fall between 0.3 and 1.0 (the absorbance acceptability range can be extended if instrumental technology allows it) If the A-value is above the maximum limit, make an appropriate number of dilutions (*d*) of the sample using the buffer solution (2.3.1) to meet the acceptability criteria.

Take the spectrophotometric measurements using the buffer solution as the reference liquid to set the absorbance scale of the apparatus to zero at the wavelengths of 420, 520 and 620 nm.

2.6. Calculations

Calculate the optical densities (O.D.) for each of the three wavelengths by multiplying the detected absorbances (A_{420}, A_{520} and A_{620}) by the number of dilutions made (d):

2.7. Expression of results

The colour intensity (I) is conventionally given by:

and is expressed to three decimal places.

The shade (N) is conventionally given by:

and is expressed to three decimal places.

Method OIV-MA-AS2-08 Type IV method

Wine turbidity

(Resolution OENO 4/2000, revised by OIV/OENO 377/2009)

Determination by Nephelometric Analysis

1. Warning

Measurements of turbidity are largely dependent on the design of the equipment used. Therefore, comparative measurements from one instrument to another are not possible unless the same measuring principle is used.

The primary known sources of errors, which are linked to the type of turbidimeter employed, are:

- effect of stray light,
- effect of product color, especially in cases with low cloudiness values,
- electronic shifting due to aging electronic components,
- type of light source, photo detector and the dimensions and type of measurement the cell.

The present method uses a nephelometer incorporating a **double beam with optical compensation** design.

This category of instrument makes it possible to compensate for: electronic shift, fluctuations of mains voltage, and, in part, wine color. Furthermore, calibration is highly stable.

It should be noted that this method does not lend itself to a collation of data from various sources, given the impossibility of conducting an analysis in collaboration with others.

2. Purpose

The purpose of this document is to describe an optical method capable of measuring the turbidity (or diffusion) index of wine.

3. Scope of application

This method is used in the absence of instruments allowing a completely faithful duplication of measurements from one device to another, as well as full compensation for wine color. Therefore, findings are given for informational purposes only, and must be considered with caution.

Above all, this technique is intended for use in production, where it is the most objective criterion of the measurement of clarity.

This method, which cannot be validated accordingly to internationally recognized criteria, will be classified as class **4**[1].

4. General principle

Turbidity is an optical effect.

The diffusion index is an intrinsic property of liquids that makes it possible to describe their optical appearance. This optical effect is produced by the presence of extremely fine particles scattered in a liquid dispersion medium. The refraction index of these particles differs from that of the dispersion medium.

If a light is shown through a quantity of optically clean water placed in a container of known volume and the luminous flux diffused with respect to an incident beam is measured, the recorded value of this diffused flux will allow description of the molecular diffusion in the water.

If the value obtained for the water thus analyzed is greater than that of the molecular diffusion, which remains constant for a given wavelength, the same incident flux at the same angle measurement, in a tank of the same shape and at a given temperature, the difference can be attributed to the light diffused by solid, liquid or gaseous particles suspended in the water.

The measurement (taken as described) of the diffused luminous flux constitutes a nephelometric measurement.

5. Definitions

5.1. *Turbidity*

Reduction of the transparency of a liquid due to the presence of undissolved substances.

5.2. *Units of Measurement of the Turbidity Index*

The unit of turbidity used is: NTU - NEPHELOMETRIC TURBIDITY UNIT, which is the value corresponding to the measurement of the light diffused by a standard formazine suspension prepared as described under point 6.2.2, at a 90° angle to the direction of the incident beam.

6. Preparing the reference Formazine suspension ([2])

6.1. *Reagents*

All reagents must be of recognized analytical quality.
They must be stored in glass flasks.

6.1.1 Water for Preparing Control Solutions.

Soak a filter membrane with a pore size of 0.1µm (like those used in bacteriology) for one hour in 100 ml of distilled water. Filter 250 ml distilled water twice through this membrane, and retain this water for preparation of standard solutions.

[2(?)] Care must be given to the precautions for handling, since Formazine is somewhat toxic.

6.1.2. Formazine ($C_2H_4N_2$) Solutions

The compound known as formazine, whose formula is $C_2H_4N_2$, is not commercially available. It can be produced using the following solutions:

Solution A: Dissolve 10.0 g hexamethylene-tetramine $(CH_2)_6N_4$ in distilled water prepared according to the instructions in 6.1.1. Then fill to a volume of 100 ml using distilled water.

Solution B: Dissolve 1.0 g of hydrazinium sulfate, $N_2H_6SO_4$, in distilled water prepared according to the instruction in 6.1.1. Then fill to a volume of 100 ml using distilled water prepared according to 6.1.1.

WARNING: Hydrazinium sulfate is poisonous and may be carcinogenic.

6.2 Working Method

Mix 5 ml of Solution A and 5 ml of Solution B. Dilute the solution to a volume of 100 ml with water after 24 hours at 25 °C ± 3 °C (6.1.1).

The turbidity of this standard solution is 400 NTU.

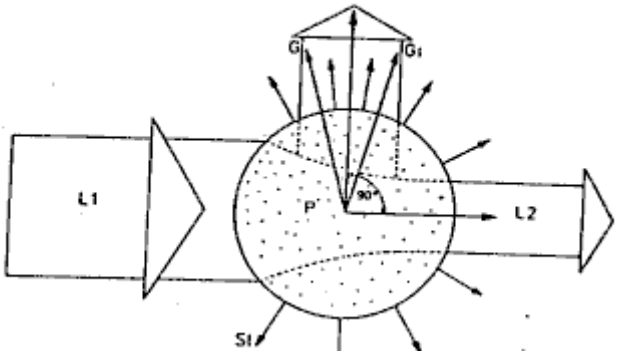
This standard suspension will keep for approximately 4 weeks at room temperature in the dark.

By diluting to 1/400 with recently prepared distilled water, a turbidity of 1 NTU will be obtained.

This solution remains stable for one week only.

N.B.: Standard formazine solutions have been compared to standard polymer-based solutions. The differences observed may be considered negligible. Nonetheless, polymer-based standard solutions have the following drawbacks: they are very expensive and they have a limited useful life. They must be handled with care to avoid breaking the polymer particles, as breakage would alter the turbidity value. Polymer use is suggested as an alternative to formazine.

7. Optical Measurement Principle



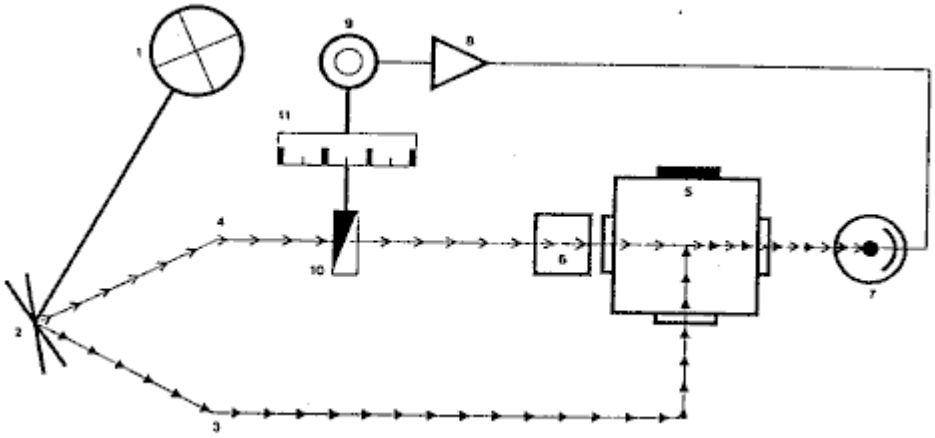
S1

Measurement principle:
L1 = Incident light beam
L2 = Beam after passing through sample
P = Sample
St = diffused light
G/G1 = Limiting rays from the diffused light beam used for measurement

The diffused light should be observed at an angle of 90° to the direction of propagation of the incident beam.

8. Instrumentation

8.1. *Optical principle of the dual-beam and optical compensation nephelometer*



A light source (1) powered by the electricity network projects a beam of light onto an oscillating mirror (2) which alternately reflects a measuring beam (3) and a comparison beam (4) at a rate of approximately 600 times per second.

The measuring beam (3) propagates through the fluid to be measured (5) while the comparison beam (4) propagates through an optically stable turbidity-comparison standard fluid (6).

The light diffused by the particles producing turbidity in the fluid (5) and the light diffused by the standard comparison solution (6) are alternately received by a photoelectric cell (7).

Accordingly, this cell receives a measuring beam (3) and a comparison (4) having the same frequency, but different whose luminous intensities.

The photoelectric cell (7) transforms these unequal luminous intensities into electric current which are in turn amplified (8) and fed to a synchronous motor (9) functioning as a servo-motor.

This motor uses a mechanical measuring diaphragm (10) to vary the intensity of the control beam, until the two beams strike the photoelectric cell with equal luminous intensity.
This equilibrium state allows the solid particle content of the fluid to be determined. The absolute value of the measurement depends on the dimensions of the standard comparison beam and on the position of the diaphragm.

8.2. *Characteristics*

Note: In order to take these measurements, regardless of the color of the wine, the nephelometer must be equipped with an additional interferential filter allowing measurement at a wavelength of 620 nm. However, the interferential filter is not needed if the light source is an infrared one.

8.2.1 The width of the spectral band of the incident radiation should be less than or equal to 60 nm.

8.2.2 There should be no divergence in the parallelism of the incident radiation, and convergence must not exceed 1.5°.

8.2.3 The angle of measurement between the optical axis of the incident radiation and that of the diffused radiation should be 90° ± 2.5°.

8.2.4 The apparatus must not cause error due to stray light greater than:

- 0.01 NTU of random light error
within a range of:
- 0 to 0.1 NTU.

9. Operating Method for measurement

9.1. *Checking the Apparatus*

Before taking any measurement or series of measurements, check to ensure the proper electrical and mechanical operation of the apparatus in accordance with the recommendations of the manufacturer.

9.2. *Check Measurement Scale Adjustment*

Before taking any measurement or series of measurements, use a previously calibrated instrument to check its measurement scale adjustment consistent with the principle underlying its design.

9.3 *Cleaning the Measuring Unit*

With the greatest care, clean the measuring tank before all analyses. Take all necessary precautions to avoid getting dust in the apparatus and especially in the measuring unit, before and during determination of the turbidity index.

9.4. *Taking Measurements*

- The operating temperature should be between 15° and 25 °C (Take the temperature of the wine to be measured into consideration to ensure proper comparison). Prior to taking the measurement, carefully homogenize the product and, without making any abrupt movement that could create an emulsion, the flask holding the product to be analyze.
- Carefully wash the measuring tank twice with a small amount of the product to be analyzed.

- Carefully pour the product to be analyzed into the measuring tank, taking care to avoid any turbulence in the flow of the liquid, since this would lead to the formation of air bubbles. Carry out the test measurements.
- Wait one minute if the index value is stable.
- Record the resulting turbidity index.

10. Expressing the results

The turbidity index of the wine undergoing analysis is recorded and expressed in:
*** NTU**

* if turbidity is less than 1 NTU, round off to 0.01 NTU
* if turbidity is between 1 NTU and 10 NTU, round off to 0.1 NTU
* if turbidity is between 10 NTU and 100 NTU, round off to 1 NTU

11. Test report

The test should contain the following information:

a) reference to this method
b) the results, expressed as indicated in 10
c) any detail or occurrence that may have affected the findings.

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SIGRIST PHOTOMETER SA, CH 6373 Ennetburgen
"Excerpts from technical instructions for nephelometers"

Method OIV-MA-AS2-09

Method for isotopic ratio $^{18}O/^{16}O$ of water content in wines

(Resolution OENO 2/96)

WITHDRAWN
(replaced by OIV-MA-AS2-12)

Method OIV-MA-AS2-10 Type IV method

Folin-Ciocalteu Index

(Recueil OIV ed.1990 revised by OIV/OENO 377/2009)

1. Definition

The Folin-Ciocalteu index is the result obtained by applying the method described below.

2. Principle

All phenolic compounds contained in wine are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten, W_8O_{23}, and molybdenum, Mo_8O_{23}. The blue coloration produced has a maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present.

3. Apparatus

Normal laboratory apparatus, in particular:

3.1 100 mL volumetric flasks.

3.2 Spectrophotometer capable of operating at 750 nm.

4. Reagents

4.1 Folin-Ciocalteu reagent

This reagent is available commercially in a form ready for use.

Alternatively it may be prepared as follows: dissolve 100 g of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 mL of distilled water. Add 50 mL phosphoric acid 85% (ρ_{20} = 1.71 g/mL), and 100 mL of concentrated hydrochloric acid (ρ_{20} = 1.19 g/mL). Bring to the boil and reflux for 10 hours. Then add 150 g of lithium sulfate, $Li_2SO_4.H_2O$, and a few drops of bromine and boil for 15 minutes. Allow to cool and make up to one liter with distilled water.

4.2 Anhydrous sodium carbonate, Na_2CO_3, made up into a 20% (*m/v*) solution.

5. Procedure

5.1 *Red wine*

Introduce the following into a 100 mL volumetric flask (3.1) strictly in the following order:

1 mL of the wine, previously diluted 1/5,
50 mL of distilled water,
5 mL of Folin-Ciocalteu reagent (4.1),
20 mL of sodium carbonate solution (4.2).

Bring to 100 mL with distilled water.

Mix to dissolve. Leave for 30 minutes for the reaction to stabilize. Determine the absorbance at 750 nm through a path length of 1 cm with respect to a blank prepared with distilled water in place of the wine.

If the absorbance is not in the region of 0.3 appropriate dilution should be made.

5.2 *White wine*

Carry out the same procedure with 1 mL of undiluted wine.

6. Expression of results

6.1 *Calculation*

The result is expressed in the form of an index obtained by multiplying the absorbance by 100 for red wines diluted 1/5 (or by the corresponding factor for other dilutions) and by 20 for white wines.

6.2 *Precision*

The difference between the results of two determinations carried out simultaneously or very quickly one after the other by the same analyst must not be greater than 1. Good precision of results is aided by using scrupulously clean apparatus (volumetric flasks and spectrophotometer cells).

Method OIV-MA-AS2-11 Type I method

Determination of chromatic characteristics according to CIELab

(Resolution OENO 1/2006, OIV/OENO 377/2009)

1. Introduction

The colour of a wine is one of the most important visual features available to us, since it provides a considerable amount of highly relevant information.

Colour is a sensation that we perceive visually from the refraction or reflection of light on the surface of objects. Colour is light—as it is strictly related to it—and depending on the type of light (illuminating or luminous stimulus) we see one colour or another. Light is highly variable and so too is colour, to a certain extent.

Wine absorbs a part of the radiations of light that falls and reflects another, which reaches the eyes of the *observer*, making them experience the sensation of colour. For instance, the sensation of very dark red wines is almost entirely due to the fact that incident radiation is absorbed by the wine.

1.1. Scope

The purpose of this spectrophotometric method is to define the process of measuring and calculating the *chromatic characteristics* of wines and other beverages derived from *trichromatic components*: X, Y and Z, according to the *Commission Internationale de l'Eclairage* (CIE, 1976), by attempting to imitate real observers with regard to their sensations of colour.

1.2. Principle and definitions

The colour of a wine can be described using 3 attributes or specific qualities of visual sensation: tonality, luminosity and chromatism.

Tonality—colour itself—is the most characteristic: red, yellow, green or blue. *Luminosity* is the attribute of visual sensation according to which a

wine appears to be more or less luminous. However, *chromatism,* or the *level of colouring,* is related to a higher or lower intensity of colour. The combination of these three concepts enables us to define the multiple shades of colour that wines present.

The *chromatic characteristics* of a wine are defined by the *colorimetric* or *chromaticity coordinates* (Fig. 1): *clarity* (L^*), *red/green colour component* (a^*), and *blue/yellow colour component* (b^*); and by its *derived magnitudes*: *chroma* (C^*), *tone* (H^*) and *chromacity* [(a^*, b^*) or (C^*, H^*)]. In other words, this CIELab colour or space system is based on a sequential or continuous Cartesian representation of 3 orthogonal axes: L^*, a^* and b^* (Fig. 2 and 3). Coordinate L^* represents clarity ($L^* = 0$ black and $L^* = 100$ colourless), a^* green/red colour component ($a^*>0$ red, $a^*<0$ green) and b^* blue/yellow colour component ($b^*>0$ yellow, $b^*<0$ blue).

1.2.1. Clarity

Its symbol is L^* and it is defined according to the following mathematical function:

$$L^*=116(Y/Y_n)^{1/3}-16 \qquad \textbf{(I)}$$

Directly related to the visual sensation of luminosity.

1.2.2.Red/green colour component

Its symbol is a^* and it is defined according to the following mathematical function:

$$a^*=500[(X/X_n)-(Y/Y_n)] \quad \textbf{(I)}$$

1.2.3. Yellow/blue colour component

Its symbol is b^* and it is defined according to the following mathematical function:

$$b^*=200-[(Y/Y_n)^{1/3}-(Z/Z_n)^{1/3} \quad \textbf{(I)}$$

1.2.4. Chroma

The chroma symbol is C^* and it is defined according to the following mathematical function:

$$C^* = \sqrt{\left(a^{*2} + b^{*2}\right)}$$

1.2.5. Tone

The tone symbol is H^*, its unit is the sexagesimal degree (º), and it is defined according to the following mathematical function:

$$H^* = tg^{-1}\,(b^*/a^*)$$

1.2.6 Difference of tone between two wines

The symbol is ΔH^* and it is defined according to the following mathematical function:

$$\Delta H^* = \sqrt{(\Delta E^*)^2 - (\Delta L^*)^2 - (\Delta C^*)^2}$$

(I) See explanation Annex I

1.2.7. Overall colorimetric difference between two wines

The symbol is ΔE^* and it is defined according to the following mathematical functions:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} = \sqrt{(\Delta L^*)^2 + (\Delta C^*)^2 + (\Delta H^*)^2}$$

1.3. Reagents and products

Distilled water.

1.4. Apparatus and equipment

Customary laboratory apparatus and, in particular, the following:

1.4.1. Spectrophotometer to carry out transmittance measurements at a wavelength of between 300 and 800 nm, with illuminant D65 and observer placed at 10º. Use apparatus with a resolution equal to or higher than 5 nm and, where possible, with scan.

1.4.2. Computer equipment and suitable programme which, when connected to the spectrophotometer, will facilitate calculating colorimetric coordinates (L^*, a^* and b^*) and their derived magnitudes (C^* and H^*).

1.4.3. Glass cuvettes, available in pairs, optical thickness 1, 2 and 10 mm.

1.4.4. Micropipettes for volumes between 0.020 and 2 ml.

1.5. Sampling and sample preparation

Sample taking must particularly respect all concepts of homogeneity and representativity.

If the wine is dull, it must be clarified by centrifugation. For young or sparkling wines, as much carbon dioxide as possible must be eliminated by vacuum stirring or using a sonicator.

1.6. Procedure

— Select the pair of cuvettes for the spectrophotometric reading, ensuring that the upper measurement limit within the linear range of the spectrophotometer is not exceeded. By way of indication, for white and rosé wines it is recommended to use cuvettes with 10 mm of optical thickness, and for red wines, cuvettes with 1 mm optical thickness.

- After obtaining and preparing the sample, measure its transmittance from 380 to 780 nm every 5 nm, using distilled water as a reference in a cuvette with the same optical thickness, in order to establish the base line or the white line. Choose illuminant D65 and observer 10º.

– If the optical thickness of the reading cuvette is under 10 mm, the transmittance must be transformed to 10 mm before calculating: L^*, a^*, b^*, C^* and H^*.
Summary:

Spectral measurements in transmittance from 780 to 380 nm
Interval: 5 nm
Cuvettes: use appropriately according to wine intensity: 1 cm (white and rosé wines) and 0.1 cm (red wines)
Illuminant D65
Observer reference pattern 10º

1.7. Calculations

The spectrophotometer must be connected to a computer programme to facilitate the calculation of the colorimetric coordinates (L^*, a^* and b^*) and their derived magnitudes (C^* and H^*), using the appropriate mathematical algorithms.
In the event of a computer programme not being available, see Annex I on how to proceed.

1.8. Expression of results

The colorimetric coordinates of wine will be expressed according to the recommendations in the following table.

Colorimetric coordinates	Symbol	Unit	Interval	Decimals
Clarity	L^*		0-100 0 black 100 colourless	1
Red/green colour component	a^*		>0 red <0 green	2
Yellow/blue colour component	b^*		>0 yellow <0 blue	2
Chroma	C^*			2
Tone	H^*	º	0-360º	2

1.9. Numerical Example

Figure 4 shows the values of the colorimetric coordinates and the chromaticity diagram of a young red wine for the following values:
X = 12.31; Y = 60.03 and Z = 10.24
L* = 29.2
a* = 55.08

b* = 36.10
C* = 66.00
H* = 33.26º

2. Accuracy

The above data were obtained from two interlaboratory tests of 8 samples of wine with blind duplicates of progressive chromatic characteristics, in accordance with the recommendations of the harmonized protocol for collaborative studies, with a view to validating the method of analysis.

2.1. Colorimetric coordinate L* (clarity, 0-100)

Sample Identification	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Year of interlaboratory test	2004	2002	2004	2004	2004	2004	2002	2004
No. of participating laboratories	18	21	18	18	17	18	23	18
No. of laboratories accepted after aberrant value elimination	14	16	16	16	14	17	21	16
Mean value ($\overline{X}$)	96.8	98.0	91.6	86.0	77.4	67.0	34.6	17.6
Repeatability standard deviation (s_r)	0.2	0.1	0.2	0.8	0.2	0.9	0.1	0.2
Relative repeatability standard deviation (RSD_r) (%)	0.2	0.1	0.3	1.0	0.3	1.3	0.2	1.2
Repeatability limit (r) (2.8 x s_r)	0.5	0.2	0.7	2.2	0.7	2.5	0.2	0.6
Reproducibility standard deviation (s_R)	0.6	0.1	1.2	2.0	0.8	4.1	1.0	1.0
Relative reproducibility standard deviation (RSD_R) (%)	0.6	0.1	1.3	2.3	1.0	6.1	2.9	5.6
Reproducibility limit (R) (2.8 x s_R)	1.7	0.4	3.3	5.5	2.2	11.5	2.8	2.8

2.2. Colorimetric coordinate a* (green/red)

Sample Identification	A	B	C	D	E	F	G	H
Year of interlaboratory	2004	2002	2004	2004	2004	2004	2002	2004
No. of participating laboratories	18	21	18	18	17	18	23	18
No. of laboratories accepted after aberrant value elimination	15	15	14	15	13	16	23	17
Mean value ($\overline{X}$)	-0.26	-0.86	2.99	11.11	20.51	29.29	52.13	47.55
Repeatability standard deviation (s_r)	0.17	0.01	0.04	0.22	0.25	0.26	0.10	0.53
Relative repeatability standard deviation (RSD_r) (%)	66.3	1.4	1.3	2.0	1.2	0.9	0.2	1.1
Repeatability limit (r) (2.8 x s_r)	0.49	0.03	0.11	0.61	0.71	0.72	0.29	1.49
Reproducibility standard deviation (s_R)	0.30	0.06	0.28	0.52	0.45	0.98	0.88	1.20
Relative reproducibility standard deviation (RSD_R) (%)	116.0	7.5	9.4	4.7	2.2	3.4	1.7	2.5
Reproducibility limit (R) (2.8 x s_R)	0.85	0.18	0.79	1.45	1.27	2.75	2.47	3.37

2.3. Colorimetric coordinate b* (blue/yellow)

Sample Identification	A	B	C	D	E	F	G	H
Year of interlaboratory	2004	2002	2004	2004	2004	2004	2002	2004
No. of participating laboratories	17	21	17	17	17	18	23	18
No. of laboratories accepted after aberrant value elimination	15	16	13	14	16	18	23	15
Mean value ($\overline{X}$)	10.95	9.04	17.75	17.10	19.68	26.51	45.82	30.07
Repeatability standard deviation (s_r)	0.25	0.03	0.08	1.08	0.76	0.65	0.15	0.36
Relative repeatability standard deviation (RSD_r) (%)	2.3	0.4	0.4	6.3	3.8	2.5	0.3	1.2
Repeatability limit (r) (2.8 x s_r)	0.71	0.09	0.21	3.02	2.12	1.83	0.42	1.01
Reproducibility standard deviation (s_R)	0.79	0.19	0.53	1.18	3.34	2.40	1.44	1.56
Relative reproducibility standard deviation (RSD_R) (%)	7.2	2.1	3.0	6.9	16.9	9.1	3.1	5.2
Reproducibility limit (R) (2.8 x s_R)	2.22	0.53	1.47	3.31	9.34	6.72	4.03	4.38

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APPENDIX 1

In formal terms, the trichromatic components X, Y, Z of a colour stimulus result

from the integration, throughout the visible range of the spectrum, of the functions obtained by multiplying the relative spectral curve of the colour stimulus by the colorimetric functions of the reference observer. These functions are always obtained by experiment. It is not possible, therefore to calculate the trichromatic components directly by integration. Consequently, the approximate values are determined by replacing these integrals by summations on finished wavelength intervals.

=

$T_{(\lambda)}$ is the measurement of the transmittance of the wine measured at the wavelength λ expressed at 1 cm from the optical thickness.

=

$\Delta_{(\lambda)}$ is the interval between the value of λ at which $T_{(\lambda)}$ is measured

=

$S_{(\lambda)}$: coefficients that are a function of λ and of the illuminant (Table 1).

=

: coefficients that are a function of λ and of the observer. (Table 1)

The values of Xn, Yn, and Zn represent the values of the perfect diffuser under an illuminant and a given reference observer. In this case, the illuminant is D65 and the observer is higher than 4 degrees.

$X_n = 94.825$; $Y_n = 100$; $Z_n = 107.381$

This roughly uniform space is derived from the space CIEYxy, in which the trichromatic components X, Y, Z are defined.

The coordinates L*, a* and b* are calculated based on the values of the trichromatic components X, Y, Z, using the following formulae.

L* = $116 (Y / Y_n)^{1/3} - 16$ where $Y/Yn > 0.008856$

L* = $903.3 (Y / Y_n)$ where $Y / Y_n <$ ó = 0.008856

a* = $500 [f(X / X_n) - f(Y / Y_n)]$

$\mathbf{b^*} = 200\ [f(Y / Y_n) - f(Z / Z_n)\ ]$

$f(X / X_n) = (X / X_n)^{1/3}$	where $(X / X_n) > 0.008856$
$f(X / X_n) = 7.787\ (X / X_n) + 16 / 166$	where $(X / X_n) < ó = 0.008856$
$f(Y / Y_n) = (Y / Y_n)^{1/3}$	where $(Y / Y_n) > 0.008856$
$f(Y / Y_n) = 7.787\ (Y / Y_n) + 16 / 116$	where $(Y / Y_n) < ó = 0.008856$
$f(Z / Z_n) = (Z / Z_n)^{1/3}$	where $(Z / Z_n) > 0.008856$
$f(Z / Z_n) = 7.787\ (Z / Z_n) + 16 / 116$	where $(Z / Z_n) < ó = 0.008856$

The total colorimetric difference between two colours is given by the CIELAB colour difference

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

In the CIELAB space it is possible to express not only overall variations in colour, but also in relation to one or more of the parameters L*, a* and b*. This can be used to define new parameters and to relate them to the attributes of the visual sensation.

Clarity, related to luminosity, is directly represented by the value of L*.

Chroma: $C^* = (a^{*\,2} + b^{*\,2})^{1/2}$ defines the chromaticness.

The angle of hue: $H^* = tg^{-1}\,(b^*/a^*)$ (expressed in degrees); related to hue.

The difference in hue: $\Delta H^* = [(\Delta E^*)^2 - (\Delta L^*)^2 - (\Delta C^*)^2]^{1/2}$

For two unspecified colours, ΔC^* represents their difference in chroma; ΔL^*, their difference in clarity, and ΔE^*, their overall variation in colour. We thus have:

$$\Delta E^* = [(\Delta L^*)2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} = [(\Delta L^*)^2 + (\Delta C^*)^2 + (\Delta H^*)^2]^{1/2}$$

Table 1.

Wavelength (λ) nm.	$S_{(\lambda)}$	$\bar{x}$	$\bar{y}$	$\bar{z}$
380	50.0	0.0002	0.0000	0.0007
385	52.3	0.0007	0.0001	0.0029
390	54.6	0.0024	0.0003	0.0105
395	68.7	0.0072	0.0008	0.0323
400	82.8	0.0191	0.0020	0.0860
405	87.1	0.0434	0.0045	0.1971
410	91.5	0.0847	0.0088	0.3894
415	92.5	0.1406	0.0145	0.6568
420	93.4	0.2045	0.0214	0.9725
425	90.1	0.2647	0.0295	1.2825
430	86.7	0.3147	0.0387	1.5535
435	95.8	0.3577	0.0496	1.7985
440	104.9	0.3837	0.0621	1.9673
445	110.9	0.3867	0.0747	2.0273
450	117.0	0.3707	0.0895	1.9948
455	117.4	0.3430	0.1063	1.9007
460	117.8	0.3023	0.1282	1.7454
465	116.3	0.2541	0.1528	1.5549
470	114.9	0.1956	0.1852	1.3176
475	115.4	0.1323	0.2199	1.0302
480	115.9	0.0805	0.2536	0.7721
485	112.4	0.0411	0.2977	0.5701
490	108.8	0.0162	0.3391	0.4153
495	109.1	0.0051	0.3954	0.3024
500	109.4	0.0038	0.4608	0.2185
505	108.6	0.0154	0.5314	0.1592
510	107.8	0.0375	0.6067	0.1120
515	106.3	0.0714	0.6857	0.0822
520	104.8	0.1177	0.7618	0.0607
525	106.2	0.1730	0.8233	0.0431
530	107.7	0.2365	0.8752	0.0305
535	106.0	0.3042	0.9238	0.0206
540	104.4	0.3768	0.9620	0.0137
545	104.2	0.4516	0.9822	0.0079
550	104.0	0.5298	0.9918	0.0040
555	102.0	0.6161	0.9991	0.0011
560	100.0	0.7052	0.9973	0.0000
565	98.2	0.7938	0.9824	0.0000

570	96.3	0.8787	0.9556	0.0000
575	96.1	0.9512	0.9152	0.0000
580	95.8	1.0142	0.8689	0.0000
585	92.2	1.0743	0.8256	0.0000
590	88.7	1.1185	0.7774	0.0000
595	89.3	1.1343	0.7204	0.0000
600	90.0	1.1240	0.6583	0.0000
605	89.8	1.0891	0.5939	0.0000
610	89.6	1.0305	0.5280	0.0000
615	88.6	0.9507	0.4618	0.0000
620	87.7	0.8563	0.3981	0.0000
625	85.5	0.7549	0.3396	0.0000
630	83.3	0.6475	0.2835	0.0000
635	83.5	0.5351	0.2283	0.0000
640	83.7	0.4316	0.1798	0.0000
645	81.9	0.3437	0.1402	0.0000
650	80.0	0.2683	0.1076	0.0000
655	80.1	0.2043	0.0812	0.0000
660	80.2	0.1526	0.0603	0.0000
665	81.2	0.1122	0.0441	0.0000
670	82.3	0.0813	0.0318	0.0000
675	80.3	0.0579	0.0226	0.0000
680	78.3	0.0409	0.0159	0.0000
685	74.0	0.0286	0.0111	0.0000
690	69.7	0.0199	0.0077	0.0000
695	70.7	0.0138	0.0054	0.0000
700	71.6	0.0096	0.0037	0.0000
705	73.0	0.0066	0.0026	0.0000
710	74.3	0.0046	0.0018	0.0000
715	68.0	0.0031	0.0012	0.0000
720	61.6	0.0022	0.0008	0.0000
725	65.7	0.0015	0.0006	0.0000
730	69.9	0.0010	0.0004	0.0000
735	72.5	0.0007	0.0003	0.0000
740	75.1	0.0005	0.0002	0.0000
745	69.3	0.0004	0.0001	0.0000
750	63.6	0.0003	0.0001	0.0000
755	55.0	0.0002	0.0001	0.0000
760	46.4	0.0001	0.0000	0.0000
765	56.6	0.0001	0.0000	0.0000
770	66.8	0.0001	0.0000	0.0000
775	65.1	0.0000	0.0000	0.0000

780	63.4	0.0000	0.0000	0.0000

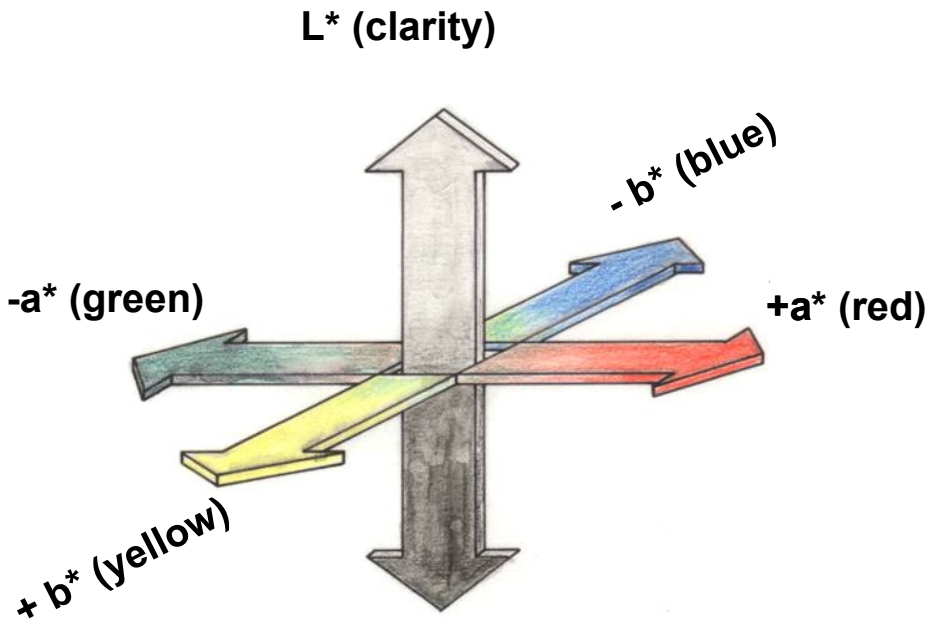


Figure 1. Diagram of colourimetric coordinates according to *Commission Internationale de l'Eclairage* (CIE, 1976)

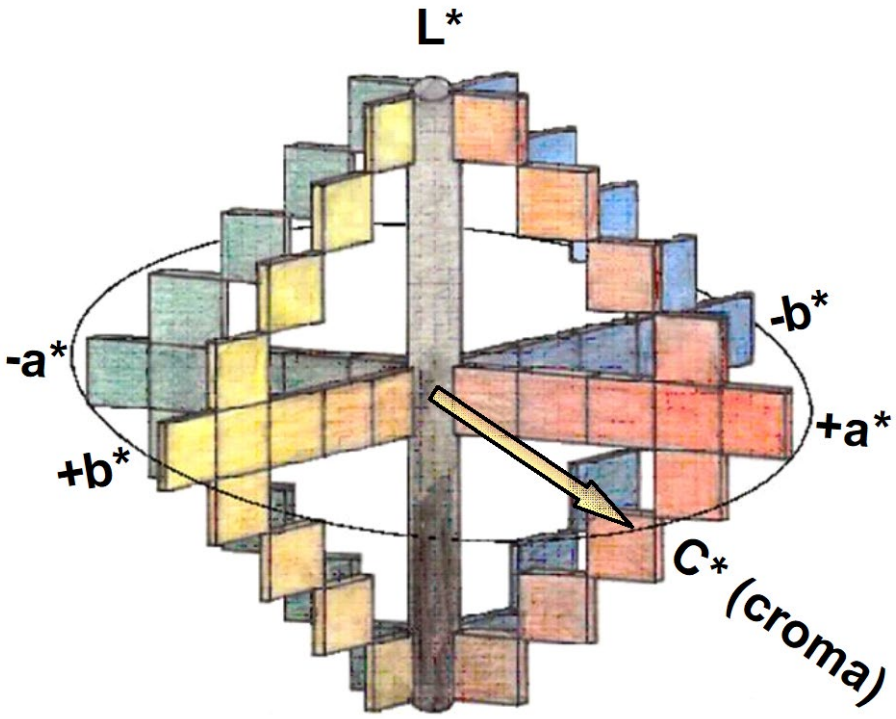


Figure 2. CIELab colourspace, based on a sequential or 3 orthogonal axis continual Cartesian representation L^*, a^* y b^*

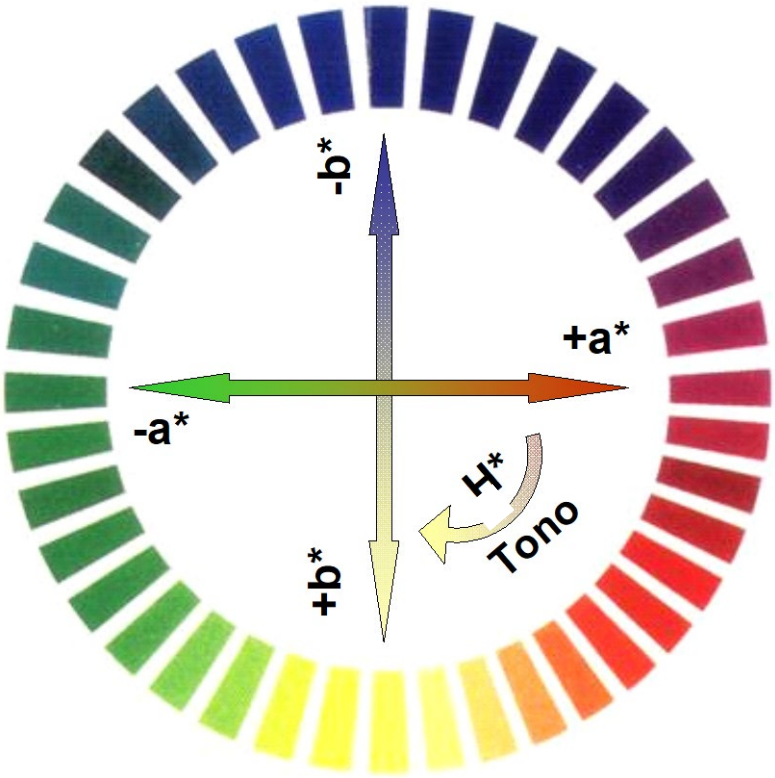
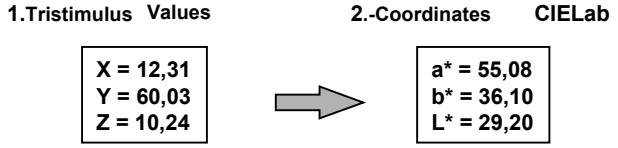


Figure 3. Sequential diagram and/or continuation of a and b colourimetric coordinates and derived magnitude, such as tone (H*)

Example: Young Red Wine

❒ **OBTENTION OF ANALYTICAL PARAMETERS:**



❒ **GRAPHIC REPRESENTATION AND ARTICULATION OF RESULTS:**

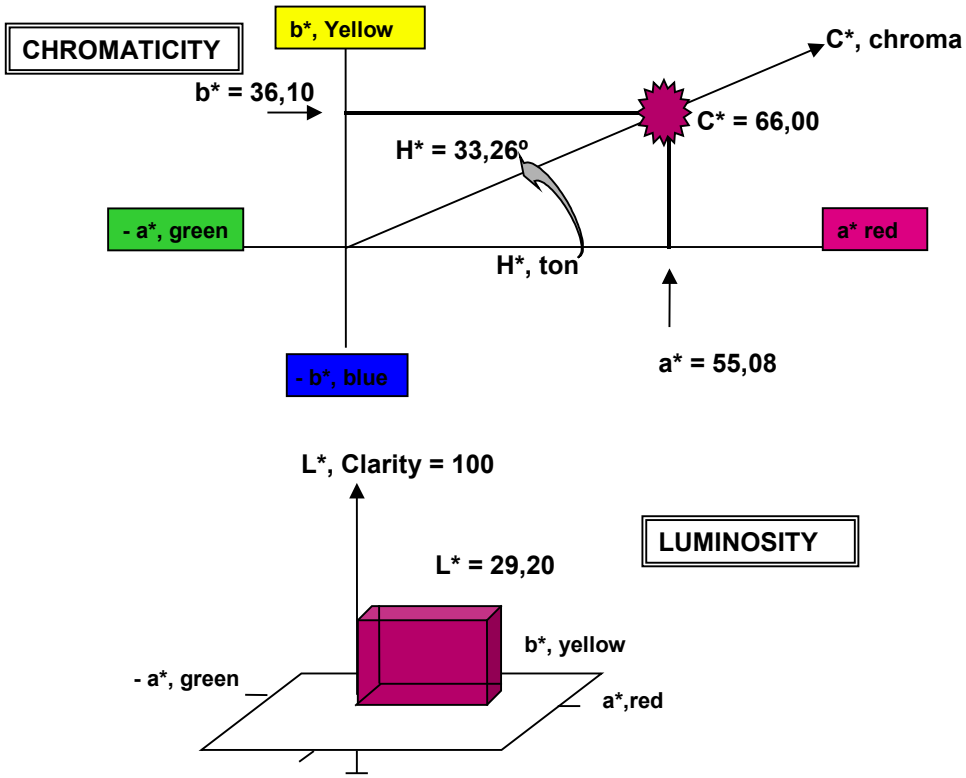


Figure 4. Representation of colour of young red wine used as an example in Chapter 1.8 shown in the CIELab three dimensional diagram.

Method OIV-MA-AS2-12 Type II method

Method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must

(Resolution OIV/OENO 353/2009)

1. SCOPE

The method describes the determination of the $^{18}O/^{16}O$ isotope ratio of water from wine and must after equilibration with CO_2, using the isotope ratio mass spectrometry (IRMS).

2. REFERENCE STANDARDS

ISO 5725:1994:	Accuracy (trueness and precision) of measurement methods and results: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
V-SMOW:	Vienna-Standard Mean Ocean Water ($^{18}O/^{16}O = R_{V\text{-}SMOW} = 0.0020052$)
GISP	Greenland Ice Sheet Precipitation
SLAP	Standard Light Antarctic Precipitation

3. DEFINITIONS

$^{18}O/^{16}O$	Isotope ratio of oxygen 18 to oxygen 16 for a given sample
$\delta^{18}O_{V\text{-}SMOW}$	Relative scale for the expression of the isotope ratio of oxygen 18 to oxygen 16 for a given sample. $\delta^{18}O_{V\text{-}SMOW}$ is calculated using the following equation:

$$\delta^{18}O_{V-SMOW} = \left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample} - \left(\frac{^{18}O}{^{16}O}\right)_{standard}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} \right] \times 1000 \quad [‰]$$

using the V-SMOW as standard and as reference point for the relative δ scale.

BCR	Community Bureau of Reference
IAEA	International Atomic Energy Agency (Vienna, Austria)
IRMM	Institute for Reference Materials and Measurements
IRMS	Isotope Ratio Mass Spectrometry
m/z	mass to charge ratio
NIST	National Institute of Standards & Technology
RM	Reference Material

4. PRINCIPLE

The technique described thereafter is based on the isotopic equilibration of water in samples of wine or must with a CO_2 standard gas according to the following isotopic exchange reaction:

$$C^{16}O_2 + H_2{}^{18}O \leftrightarrow C^{16}O^{18}O + H_2{}^{16}O$$

After equilibration the carbon dioxide in the gaseous phase is used for analysis by means of Isotopic Ratio Mass Spectrometry (IRMS) where the $^{18}O/^{16}O$ isotopic ratio is determined on the CO_2 resulting from the equilibration.

5. REAGENTS AND MATERIALS

The materials and consumables depend on the method used (see chapter 6). The systems generally used are based on the equilibration of water in wine or must with CO_2.
The following reference materials, working standards and consumables can be used:

5.1 Reference materials

Name	issued by	$\delta^{18}O$ versus V-SMOW
V-SMOW, RM 8535	IAEA / NIST	0 ‰
BCR-659	IRMM	-7.18 ‰
GISP, RM 8536	IAEA / NIST	-24.78 ‰
SLAP, RM 8537	IAEA / NIST	-55.5 ‰

5.2 Working Standards
5.2.1 Carbon dioxide as a secondary reference gas for measurement (CAS 00124-38-9).
5.2.2 Carbon dioxide used for equilibration (depending on the instrument this gas could be the same as 5.2.1 or in the case of continuous flow systems cylinders containing gas mixture helium-carbon dioxide can also be used)

5.2.3 Working Standards with calibrated $\delta^{18}O_{V\text{-}SMOW}$ values traceable to international reference materials.

5.3 Consumables
Helium for analysis (CAS 07440-59-7)

6. APPARATUS

6.1 Isotope ratio mass spectrometry (IRMS)
The Isotope ratio mass spectrometer (IRMS) enables the determination of the relative contents of ^{18}O of CO_2 gas naturally occurring with an internal accuracy of 0.05‰. Internal accuracy here is defined as the difference between 2 measurements of the same sample of CO_2.
The mass spectrometer used for the determination of the isotopic composition of CO_2 gas is generally equipped with a triple collector to simultaneously measure the following ion currents:

- m/z = 44 ($^{12}C^{16}O^{16}O$)
- m/z = 45 ($^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$)
- m/z = 46 ($^{12}C^{16}O^{18}O$, $^{12}C^{17}O^{17}O$ and $^{13}C^{17}O^{16}O$)

By measuring the corresponding intensities, the $^{18}O/^{16}O$ isotopic ratio is determined from the ratio of intensities of m/z = 46 and m/z = 44 after corrections for isobaric species ($^{12}C^{17}O^{17}O$ and $^{13}C^{17}O^{16}O$) whose contributions can be calculated from the actual intensity observed for m/z= 45 and the usual isotopic abundances for ^{13}C and ^{17}O in Nature.

The isotope ratio mass spectrometry must either be equipped with:
- a double introduction system (dual inlet system) to alternately measure the unknown sample and a reference standard.
- or a continuous flow system that transfers quantitatively the CO_2 from the sample vials after equilibration but also the CO_2 standard gas into the mass spectrometer.

6.2 Equipment and Materials
All equipments and materials used must meet stated requirements of the used method / apparatus (as specified by the manufacturer). However, all equipments and materials can be replaced by items with similar performance.
6.2.1 Vials with septa appropriate for the used system
6.2.2 Volumetric pipettes with appropriate tips
6.2.3 Temperature controlled system to carry out the equilibration at constant temperature, typically within ±1 °C
6.2.4 Vacuum pump (if needed for the used system)
6.2.5 Autosampler (if needed for the used system)
6.2.6 Syringes for sampling (if needed for the used system)
6.2.7 GC Column to separate CO_2 from other elementary gases (if needed for the used system)
6.2.8 Water removal device (e.g. cryo-trap, selective permeable membranes)

7. SAMPLING

Wine and must samples as well as reference materials are used for analysis without any pre-treatment. In the case of the possible fermentation of the sample, benzoic acid (or another anti-fermentation product) should be added or filtered with a with a 0,22 µm pore diameter filter.

Preferably, the reference materials used for calibration and drift-correction should be placed at the beginning and at the end of the sequence and inserted after every ten samples.

8. PROCEDURE

The descriptions that follow refer to procedures generally used for the determination of the $^{18}O/^{16}O$ isotopic ratios by means of equilibration of water with a CO_2 working standard and the subsequent measurement by IRMS. These procedures can be altered according to changes of equipment and instrumentation provided by the manufacturers as various kind of equilibration devices are available, implying various conditions of operation. Two main technical procedures can be used for introduction of CO_2 into the IRMS either through a dual inlet system or using a continuous flow system. The description of all these technical systems and of the corresponding conditions of operation is not possible. Note: all values given for volumes, temperatures, pressures and time periods are only indicative. Appropriate values must be obtained from specifications provided by the manufacturer and/or determined experimentally.

8.1 Manual equilibration

A defined volume of the sample/standard is transferred into a flask using a pipette. The flask is then attached tightly to the manifold.
Each manifold is cooled down to below – 80 °C to deep-freeze the samples (manifold equipped with capillary opening tubes do not require this freezing step). Subsequently, the whole system is evacuated. After reaching a stable vacuum the gaseous CO_2 working standard is allowed to expand into the various flasks. For the equilibration process each manifold is placed in a temperature controlled water-bath typically at 25°C (± 1 °C) for 12 hours (overnight). It is crucial that the temperature of the water-bath is kept constant and homogeneous.
After the equilibration process is completed, the resulting CO_2 is transferred from the flasks to the sample side bellow of the dual inlet system. The measurements are performed by comparing several times the ratios of the CO_2 contained in the sample side and the standard side (CO_2 reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.2 Use of an automatic equilibration apparatus

A defined volume of the sample/standard is transferred into a vial using a pipette. The sample vials are attached to the equilibration system and cooled down to below – 80 °C to deep-freeze the samples (systems equipped with capillary opening tubes do not require this freezing step). Subsequently, the whole system is evacuated. After reaching a stable vacuum the gaseous CO_2 working standard is expanded into the vials. Equilibrium is reached at a temperature of typically 22 ± 1 °C after a minimum period of 5 hours and with moderate agitation (if available). Since the equilibration duration depends on various parameters (e.g. the vial geometry, temperature, applied agitation ...), the minimum equilibrium time should be determined experimentally.
After the equilibration process is completed, the resulting CO_2 is transferred from the vials to the sample side bellow of the dual inlet system. The measurements are performed by comparing several times the ratios of the CO_2 contained in the sample side and the standard side (CO_2 reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.3 Manual preparation manual and automatic equilibration and analysis with a dual inlet IRMS

A defined volume of sample / standard (eg. 200 µL) is introduced into a vial using a pipette. The open vials are then placed in a closed chamber filled with the CO_2 used for equilibration (5.2.2). After several purges to eliminate any trace of air, the vials are closed and then placed on the thermostated plate of the sample changer. The equilibration is reached after at least 8 hours at 40 °C. Once the process of equilibration completed, the CO_2 obtained is dried and then transferred into the sample side of the dual inlet introduction system. The measurements are performed by comparing several times the ratios of the CO_2 contained in the sample side and the standard side (CO_2 reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.4 Use of an automatic equilibration apparatus coupled to a continuous flow system

A defined volume of the sample/standard is transferred into a vial using a pipette. The sample vials are placed into a temperature controlled tray.
Using a gas syringe the vials are flushed with mixture of He and CO_2. The CO_2 remains in the headspace of the vials for equilibration.
Equilibrium is reached at a temperature typically of 30 ± 1 °C after a minimum period of 18 hours.
After the equilibration process is completed the resulting CO_2 is transferred by means of the continuous flow system into the ion source of the mass spectrometer. CO_2 reference gas is also introduced into the IRMS by means of the continuous flow system. The measurement is carried out according to a specific protocol for each kind of equipment.

9. CALCULATION

The intensities for m/z = 44, 45, 46 are recorded for each sample and reference materials analysed in a batch of measurements. The $^{18}O/^{16}O$ isotope ratios are then calculated by the computer and the software of the IRMS instrument according to the principles explained in section 6.1. In practice the $^{18}O/^{16}O$ isotope ratios are measured against a working standard previously calibrated against the V-SMOW. Small variations may occur while measuring on line due to changes in the instrumental conditions. In such a case the $\delta\,^{18}O$ of the samples must be corrected according to the difference in the $\delta\,^{18}O$ value from the working standard and its assigned value, which was calibrated beforehand against V-SMOW. Between two

measurements of the working standard, the variation is the correction applied to the sample results that may be assumed to be linear. Indeed, the working standard must be measured at the beginning and at the end of all sample series. Therefore a correction can be calculated for each sample using linear interpolation between two values (the difference between the assigned value of the working standard and the measurements of the obtained values).
The final results are presented as relative $\delta^{18}O_{V\text{-}SMOW}$ values expressed in ‰.
$\delta^{18}O_{V\text{-}SMOW}$ values are calculated using the following equation:

$$\delta^{18}O_{V-SMOW} = \left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample} - \left(\frac{^{18}O}{^{16}O}\right)_{V-SMOW}}{\left(\frac{^{18}O}{^{16}O}\right)_{V-SMOW}} \right] \times 1000 \ [‰]$$

The $\delta^{18}O$ value normalized versus the V-SMOW/SLAP scale is calculated using the following equation:

$$\delta^{18}O_{V-SMOW/SLAP} = \left[\frac{\delta^{18}O_{sample} - \delta^{18}O_{V-SMOW}}{\delta^{18}O_{V-SMOW} - \delta^{18}O_{SLAP}} \right] \times 55.5 \ [‰]$$

The $\delta^{18}O_{V\text{-}SMOW}$ value accepted for SLAP is -55.5‰ (see also 5.1).

10. PRECISION

The repeatability (r) is equal to 0.24 ‰.
The reproducibility (R) is equal to 0.50 ‰.

Summary of statistical results

	General average (‰)		Standard deviation of repeatability (‰) s_r		Repeatability (‰) r		Standard deviation of reproducibility (‰) s_R		Reproducibility (‰) R
Water									
Sample 1	-8.20		0.068		0.19		0.171		0.48
Sample 2	-8.22		0.096		0.27		0.136		0.38
Wine N° 1									
Sample 5	6.87		0.098		0.27		0.220		0.62
Sample 8	6.02		0.074		0.21		0.167		0.47
Sample 9	5.19		0.094		0.26		0.194		0.54
Sample 4	3.59		0.106		0.30		0.205		0.57
Wine N° 2									
Sample 3	-1.54		0.065		0.18		0.165		0.46
Sample 6	-1.79		0.078		0.22		0.141		0.40
Sample 7	-2.04		0.089		0.25		0.173		0.49
Sample 10	-2.61		0.103		0.29		0.200		0.56

11. INTER-LABORATORIES STUDIES

Bulletin de l'O.I.V. janvier-février 1997, 791-792, p.53 - 65.

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Method OIV-MA-AS311-01A Type IV method

Reducing substances

(Resolution OIV/OENO 377/2009)

1. Definition

Reducing substances comprise all the sugars exhibiting ketonic and aldehydic functions and are determined by their reducing action on an alkaline solution of a copper salt.

2. Principle of the method

Clarification

The wine is treated with one of the following reagents:
- neutral lead acetate,
- zinc ferrocyanide (II).

3. Clarification

The sugar content of the liquid in which sugar is to be determined must lie between 0.5 and 5 g/L.
Dry wines should not be diluted during clarification; sweet wines should be diluted during clarification in order to bring the sugar level to within the limits prescribed in the following table.

Description	Sugar content (g/L)	Density	Dilution (%)
Musts and mistelles	> 125	> 1.038	1
Sweet wines, whether fortified or not	25 to 125	1.005 to 1.038	4
Semi-sweet wines	5 to 25	0.997 to 1.005	20
Dry wines	< 5	< 0.997	No dilution

3.1. Clarification by neutral lead acetate.

3.1.1. Reagents

- Neutral lead acetate solution (approximately saturated)
  Neutral lead acetate, $Pb(CH_3COO)_2 \cdot 3H_2O$ ..... 250 g
  Very hot water to ..... 500 mL
  Stir until dissolved.
- Sodium hydroxide solution, 1 M
- Calcium carbonate.

3.1.2 Procedure

- Dry wines.

Place 50 mL of the wine in a 100 mL volumetric flask; add 0.5 (n - 0.5) mL sodium hydroxide solution, 1 M, (where n is the volume of sodium hydroxide solution, 0.1 M, used to determine the total acidity in 10 mL of wine). Add, while stirring, 2.5 mL of saturated lead acetate solution and 0.5 g calcium carbonate. Shake several times and allow to stand for at least 15 minutes. Make up to the mark with water. Filter.
1 mL of the filtrate corresponds to 0.5 mL of the wine.

- Musts, mistelles, sweet and semi-sweet wines

Into a 100 mL volumetric flask, place the following volumes of wine (or must or mistelle), the dilutions being given for guidance:

Case 1 - Musts and mistelles: prepare a 10% (*v/v*) solution of the liquid to be analyzed and take 10 mL of the diluted sample.

Case 2 - Sweet wines, whether fortified or not, having a density between 1.005 and 1.038: prepare a 20% (*v/v*) solution of the liquid to be analyzed and take 20 mL of the diluted sample.

Case 3 - Semi-sweet wines having a density between 0.997 and 1.005: take 20 mL of the undiluted wine.
Add 0.5 g calcium carbonate, about 60 mL water and 0.5, 1 or 2 mL of saturated lead acetate solution. Stir and leave to stand for at least 15 minutes, stirring occasionally. Make up to the mark with water. Filter.

Note:
Case 1: 1 mL of filtrate contains 0.01 mL of must or mistelle.
Case 2: 1 mL of filtrate contains 0.04 mL of sweet wine.
Case 3: 1 mL of filtrate contains 0.20 mL of semi-sweet wine.

3.2. Clarification by zinc ferrocyanide (II)

This clarification process should be used only for white wines, lightly colored sweet wines and musts.

3.2.1 Reagents

Solution I: potassium ferrocyanide (II):

Potassium ferrocyanide (II), $K_4Fe(CN)_6 \cdot 3H_2O$	150 g
Water to	1000 mL

Solution II: zinc sulfate:

Zinc sulfate, $ZnSO_4 \cdot 7H_4O$	300 g
Water to	1000 mL

3.2.2 Procedure

Into a 100 mL volumetric flask, place the following volumes of wine (or must or mistelle), the dilutions being given for guidance:

Case 1 - Musts and mistelles. Prepare a 10% *(v/v)* solution of the liquid to be analyzed and take 10 mL of the diluted sample.

Case 2 - Sweet wines, whether fortified or not, having a density between 1.005 and 1.038: prepare a 20% *(v/v)* solution of the liquid to be analyzed and take 20 mL of the diluted sample.

Case 3 - Semi-sweet wines having a density at 20°C between 0.997 and 1.005: take 20 mL of the undiluted wine.

Case 4 - Dry wines: take 50 mL of undiluted wine.

Add 5 mL of solution I and 5 mL of solution II. Stir. Make up to the mark with water. Filter.

Note:

Case 1: 1 mL of filtrate contains 0.01 mL of must or mistelle.

Case 2: 1 mL of filtrate contains 0.04 mL of sweet wine.

Case 3: 1 mL of filtrate contains 0.20 mL of semi-sweet wine.

Case 4: 1 mL of filtrate contains 0.50 mL of dry wine.

4. Determination of sugars

4.1. *Reagents*

- Alkaline copper salt solution:
  - Copper sulfate, pure, $CuSO_4 \cdot 5H_2O$ ..... 25 g
  - Citric acid monohydrate ..... 50 g
  - Crystalline sodium carbonate, $Na_2CO_3 \cdot 10H_2O$ ..... 388 g
  - Water to ..... 1000 mL

  Dissolve the copper sulfate in 100 mL of water, the citric acid in 300 mL of water and the sodium carbonate in 300 to 400 mL of hot water. Mix the citric acid and sodium carbonate solutions. Add the copper sulfate solution and make up to one liter.
- Potassium iodide solution, 30% (*m/v*):
  - Potassium iodide, KI ..... 30 g
  - Water to ..... 100 mL

  Store in a colored glass bottle.
- Sulfuric acid, 25% (*m/v*):

Concentrated sulfuric acid, H_2SO_4, ρ_{20} = 1.84 g/Ml …….. 25 g
Water to ……. 100 mL

Add the acid slowly to the water, allow to cool and make up to 100 mL with water.

- Starch solution, 5 g/L:

  Mix 5 g of starch in with about 500 mL of water. Bring to boil, stirring all the time, and boil for 10 minutes. Add 200 g of sodium chloride, NaCl. Allow to cool and then make up to one liter with water.

- Sodium thiosulfate solution, 0.1 M.

- Invert sugar solution, 5 g/L, to be used for checking the method of determination.

  Place the following into a 200 mL volumetric flask:

  Pure dry sucrose ……. 4.75 g
  Water, approximately ……. 100 mL
  Conc. hydrochloric acid (ρ_{20}= 1.16 – 1.19 g/mL) …… 5 mL

  Heat the flask in a water-bath maintained at 60°C until the temperature of the solution reaches 50°C; then keep the flask and solution at 50°C for 15 minutes. Allow the flask to cool naturally for 30 minutes and then immerse it in a cold water-bath. Transfer the solution to a one-liter volumetric flask and make up to one liter. This solution keeps satisfactorily for a month. Immediately before use, neutralize the test sample (the solution being approximately 0.06 M acid) with sodium hydroxide solution.

4.2. *Procedure*

Mix 25 mL of the alkaline copper salt solution, 15 mL water and 10 mL of the clarified solution in a 300 mL conical flask. This volume of sugar solution must not contain more than 60 mg of invert sugar.

Add a few small pieces of pumice stone. Fit a reflux condenser to the flask and bring the mixture to the boil within two minutes. Keep the mixture boiling for exactly 10 minutes.

Cool the flask immediately in cold running water. When completely cool, add 10 mL potassium iodide solution, 30% (*m/v*); 25 mL sulfuric acid, 25% (*m/v*), and 2 mL starch solution.

Titrate with sodium thiosulfate solution, 0.1 M. Let *n* be the number of mL used. Also carry out a blank titration in which the 25 mL of sugar solution is replaced by 25 mL of distilled water. Let *n′* be the number of mL of sodium thiosulfate used.

4.3. *Expression of results*

4.3.1 Calculations

The quantity of sugar, expressed as invert sugar, contained in the test sample is given in the table below as a function of the number (n' - n) of mL of sodium thiosulfate used.

The sugar content of the wine is to be expressed in grams of invert sugar per liter to one decimal place, account being taken of the dilution made during clarification and of the volume of the test sample.

Table giving the relationship between the volume of sodium thiosulfate solution: (n'-n) mL, and the quantity of reducing sugar in mg.

$Na_2S_2O_3$ (ml 0.1 M)	Reducing sugars (mg)	Diff.	$Na_2S_2O_3$ (ml 0.1 M)	Reducing sugars (mg)	Diff.
1	2.4	2.4	13	33.0	2.7
2	4.8	2.4	14	35.7	2.8
3	7.2	2.5	15	38.5	2.8
4	9.7	2.5	16	41.3	2.9
5	12.2	2.5	17	44.2	2.9
6	14.7	2.6	18	47.2	2.9
7	17.2	2.6	19	50.0	3.0
8	19.8	2.6	20	53.0	3.0
9	22.4	2.6	21	56.0	3.1
10	25.0	2.6	22	59.1	3.1
11	27.6	2.7	23	62.2	
12	30.3	2.7			

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Method OIV-AS311-01B

Reducing sugars

(Resolution OIV/OENO 377/2009)

Principle of the method

Clarification

After neutralization and removal of alcohol, the wine is passed through an anion-exchange resin column in the acetate form, followed by clarification with neutral lead acetate.

WITHDRAWN

Method OIV-AS311-01C Type II method

Reducing sugars

(Resolution OIV/OENO 377/2009)

Principle of the method

Determination

Single method: the clarified wine or must is reacted with a specific quantity of an alkaline copper salt solution and the excess copper ions are then determined iodometrically.

WITHDRAWN

Method OIV-MA-AS311-02 Type II method

Glucose and fructose

(Resolution OIV/OENO 377/2009)

1. Definition

Glucose and fructose may be determined individually by an enzymatic method, with the sole aim of calculating the glucose/fructose ratio.

2. Principle

Glucose and fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalyzed by hexokinase (HK), to produce glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P):

$$\text{glucose} + \text{ATP} \xleftrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{fructose} + \text{ATP} \xleftrightarrow{\text{HK}} \text{F6P} + \text{ADP}$$

The glucose-6-phosphate is first oxidized to gluconate-6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced corresponds to that of glucose-6-phosphate and thus to that of glucose.

$$\text{G6P} + \text{NADP}^{+} \xleftrightarrow{\text{G6PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^{+}$$

The reduced nicotinamide adenine dinucleotide phosphate is determined from its absorption at 340 nm.

At the end of this reaction, the fructose-6-phosphate is converted into glucose-6-phosphate by the action of phosphoglucose isomerase (PGI):

$$\text{F6P} \xleftrightarrow{\text{PGI}} \text{G6P}$$

The glucose-6-phosphate again reacts with the nicotinamide adenine dinucleotide phosphate to give gluconate-6-phosphate and reduced nicotinamide adenine dinucleotide phosphate, and the latter is then determined.

3. Apparatus

- A spectrophotometer enabling measurements to be made at 340 nm, the wavelength at which absorption by NADPH is at a maximum. Absolute measurements are involved (i.e. calibration plots are not used but standardization is made using the extinction coefficient of NADPH), so that the wavelength scales of, and absorbance values obtained from, the apparatus must be checked.
  If not available, a spectrophotometer using a source with a discontinuous spectrum that enables measurements to be made at 334 nm or at 365 nm may be used.
- Glass cells with optical path lengths of 1 cm or single-use cells.
- Pipettes for use with enzymatic test solutions, 0.02, 0.05, 0.1, 0.2 mL.

4. Reagents

Solution 1: buffer solution (0.3 M triethanolamine, pH 7.6, 0.004 M Mg^{2+}): dissolve 11.2 g triethanolamine hydrochloride, $(CH_2CH_2OH)_3N.HCl$, and 0.2 g magnesium sulfate, $MgSO_4.7H_2O$, in 150 mL of double-distilled water, add about 4 mL 5 M sodium hydroxide solution to obtain a pH value of 7.6 and make up to 200 mL.
This buffer solution may be kept for four weeks at approx. + 4°C.

Solution 2: nicotinamide adenine dinucleotide phosphate solution (about 0.0115 M): dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 mL of double-distilled water.
This solution may be kept for four weeks at approx. +4°C.

Solution 3: adenosine-5'-triphosphate solution (approx. 0.081 M): dissolve 250 mg disodium adenosine-5'-triphosphate and 250 mg sodium hydrogen carbonate, NaHCO3, in 5 mL of double-distilled water.
This solution may be kept for four weeks at approx. +4°C.

Solution 4: hexokinase/glucose-6-phosphate-dehydrogenase: mix 0.5 mL hexo-kinase (2 mg of protein/mL or 280 U/mL with 0.5 mL glucose-6-phosphate-dehydrogenase (1 mg of protein/mL).
This mixture may be kept for a year at approx. +4°C.

Solution 5: phosphoglucose-isomerase (2 mg of protein/mL or 700 U/mL). The suspension is used undiluted.
This may be kept for a year at approx. +4°C.

Note: All solutions used above are available commercially.

5. Procedure

5.1. *Preparation of sample*

Depending on the estimated amount of glucose + fructose per liter (g/L) dilute the sample as follows:

Measurement at 340 and 344 nm (g/L)	Measurement at 365 nm (g/L)	Dilution with water	Dilution factor F
up to 0.4	0.8	-	-
up to 4.0	8.0	1 + 9	10
up to 10.0	20.0	1 + 24	25
up to 20.0	40.0	1 + 49	50
up to 40.0	80.0	1 + 99	100
above 40.0	80.0	1 + 999	1000

5.2. *Determination*

With the spectrophotometer adjusted to the 340 nm wavelength, make measurements using air (no cell in the optical path) or water as reference.

Temperature between 20 and 25°C.

Into two cells with 1 cm optical paths, place the following:

	Reference cell	Sample cell
Solution 1 (taken to 20°C) ..…… .....…..	2.50 mL	2.50 mL
Solution 2 .....…	0.10 mL	0.10 mL
Solution 3 .....…	0.10 mL	0.10 mL
Sample to be measured ....……		0.20 mL
Double -distilled water.....	0.20 mL	

Mix, and after three minutes read the absorbance of the solutions (A_1). Start the reaction by adding:

Solution 4	0.02 mL	0.02 mL

Mix, read the absorbance after 15 minutes and after two more minutes check that the reaction has stopped (A_2).

Add immediately:

Solution 5	0.02 mL	0.02 mL

Mix; read the absorbance after 10 minutes and after two more minutes check that the reaction has stopped (A_3).

Calculate the differences in the absorbance between the reference cell and sample cells.:

$A_2 - A_1$ corresponds to glucose,
$A_3 - A_2$ corresponds to fructose,

Calculate the differences in absorbance for the reference cells (ΔA_T) and the sample cell (ΔA_D) and then obtain:

for glucose: $\Delta A_G = \Delta A_D - \Delta A_T$
for fructose: $\Delta A_F = \Delta A_D - \Delta A_T$

Note: The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

5.3. *Expression of results*

5.3.1 Calculation

The general formula for calculating the concentrations is:

$$C = \frac{V \times MV}{\varepsilon \times d \times v \times 10000} \Delta A \text{ (g/L}$$

where:
V = volume of the test solution (mL)
v = volume of the sample (mL)
MW = molecular mass of the substance to be determined
d = optical path in the cell (cm)
ε = absorption coefficient of NADPH at 340 nm = 6.3 ($mmole^{-1} \times l \times cm^{-1}$)
V = 2.92 mL for the determination of glucose
V = 2.94 mL for the determination of fructose
v = 20 mL
PM = 180
d = 1

so that:
For glucose : $C(g/L) = 0.417 \times \Delta A_G$
For fructose: $C(g/L) = 0.420 \times \Delta A_F$

If the sample was diluted during its preparation, multiply the result by the dilution factor F.

Note: If the measurements are made at 334 or 365 nm, then the following expressions are obtained:

- measurement at 334 nm: $\varepsilon = 6.2$ (mmole $^{-1}$ × absorbance × cm $^{-1}$)
  for glucose : $C(g/L) = 0.425 \times \Delta A_G$
  for fructose: $C(g/L) = 0.428 \times \Delta A_F$

- measurement at 365 nm: $\varepsilon = 3.4$ (mmole $^{-1}$ × absorbance × cm $^{-1}$)
  for glucose: $C(g/L) = 0.773 \times \Delta A_G$
  for fructose: $C(g/L) = 0.778 \times \Delta A_F$

5.3.2 Repeatability (*r*):
$r = 0.056\, x_i$
x_i = the concentration of glucose or fructose in g/L

5.3.3 Reproducibility (R):
$R = 0.12 + 0.076\, x_i$
x_i = the concentration of glucose or fructose in g/L

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Method OIV-MA-AS311-03 Type II method[3]

Dosage of sugars in wine by HPLC

OENO 23/2003
OIV/OENO 377/2009
OIV-OENO 526-2016

1. SCOPE OF APPLICATION

This method is applicable to the direct quantification of sugars in musts and wines up to 20 g/L and, after dilution, beyond.
Glycerol (between 0.5 and 15 g/L) and sucrose (between 1 and 40 g/L) may also be quantified in the same way.

2. PRINCIPLE

Sugars and glycerol are separated by HPLC using an alkylamine column and detected by refractometer.

3. REAGENTS

3.1 - Demineralised Type I water (ISO 3696) or equivalent (HPLC grade);
3.2 - acetonitrile [75-05-8] (minimal transmission at 200 nm - purity ≥ 99%);
3.3 - fructose [57-48-7] (purity ≥ 99%);
3.4 - glucose [492-62-6] (purity ≥99%);
3.5 - sucrose [57-50-1] (purity ≥ 99%);
3.6 - glycerol [56-81-5] (purity ≥99%).

PREPARATION OF REAGENT SOLUTIONS

3.9 - Demineralised water (3.1): filtered through a 0.45 µm cellulose membrane;
3.10 - eluent: acetonitrile (3.2)/water (3.9) with a respective ratio of 80/20.
Note 2: the water/acetonitrile ratio may be adapted according to the objectives.

4. APPARATUS

4.1. - 0.45 µm Cellulose filtration membrane;
4.2. - silica-based, octadecyl-bonded filter cartridge (e.g. Sep-Pak C_{18});
4.3. - common apparatus for high-performance liquid chromatography;
4.4. - alkylamine column (5 µm, 250 x 4.6 mm);
Note 3: columns of different lengths, internal diameter and particle size may be used but the type II method refers to the dimensions provided.
4.5. - refractometric index detector (RID);

[3] Type II for glucose and fructose. Type IV for sucrose and glycerol.

4.6. - common laboratory apparatus.

5. SAMPLING

The samples are degassed beforehand if necessary (e.g. with nitrogen or helium, or in an ultrasonic bath).

6. PROCEDURE

6.1 - Preparation of the sample

6.1.1 - Dilution

Wines containing less than 20 g/L of (glucose + fructose) are analysed undiluted. Musts and wines containing more than 20 g/L have to be diluted to be within the range of calibration.

6.1.2 - Filtration

The samples must be filtered using a 0.45 µm membrane (4.1) before analysis.

6.1.3 - Elimination of phenolic compounds (if necessary)

For a must or wine, pass over a C_{18} cartridge (4.2).

6.2 - Analyses

6.2.1 - Analytical conditions

Note 4: The following instructions are mandatory for the type II method.

Note 5: Conditions may be adapted by the laboratory with the loss of the type II reference.

HPLC system (4.3) equipped with column (4.4) and RID (4.5).

Mobile phase: isocratic acetonitrile/water eluent (3.10).

Flow: 1 mL/min.

Injected volume: between 10 and 50 µL, to be adapted according to the material used.

Examples of chromatograms are shown in Annex B (Figures 1 and 2).

The fructose-glucose resolution is recommended to be ≥ 2.

6.2.2 - External calibration

The calibration solution that applies to all compounds described in this procedure may contain the following:

10 g/L glycerol (3.6) ± 0.01 g/L,

10 g/L fructose (3.3) ± 0.01 g/L,

10 g/L glucose (3.4) ± 0.01 g/L,

10 g/L sucrose (3.5) ± 0.01 g/L.

Note 6: if quantifying only one of these compounds, a solution that contains only the one required can be prepared.

6.3 - Calculation of response factors for external calibration used in routine analyses

$RF_i = area_i / C_i$

where

area i = peak area of the product in the calibration solution

and C_i = quantity of product present in the calibration solution.

It is also possible to use a calibration curve.

7. EXPRESSION OF RESULTS

7.1 - Calculation of concentrations

$C_e = area_e / RF_i$

where

$area_e$ = peak area of product present in the sample.

The results are expressed in g/L.

Note 7: the results are indicated to a maximum of one decimal place.

8. QUALITY ASSURANCE AND CONTROL

Traceable to the international references through mass, volume and temperature.

Synthetic mixtures or samples coming, for instance, from proficiency ring test are used as internal quality control. A control chart may be used

9. PERFORMANCE OF THE METHOD

No known compound co-elutes with fructose, glucose or sucrose.

Robustness: the analysis is sensitive to slight variations in temperature. Columns should be protected from temperature variations.

10. PRECISION

(See Annex B.3)

10.1 - Glucose (content ≥ 3 g/L)

Repeatability limit ≅ reproducibility limit = 13%

10.2 - Fructose (content ≥ 2 g/L)

Repeatability limit = 7%

Reproducibility limit = 10%

10.3 – Glucose + fructose (content ≥ 5 g/L)

Repeatability limit ≅ Reproducibility limit = 10%

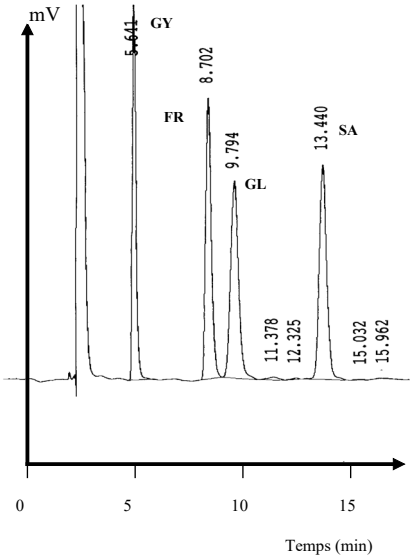


Figure 1
Chromatogram of a calibration solution (sugars and glycerol at 10 g/l.)

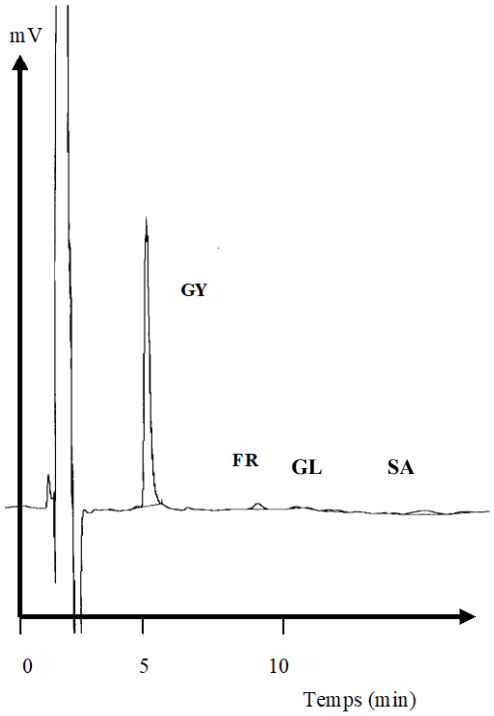
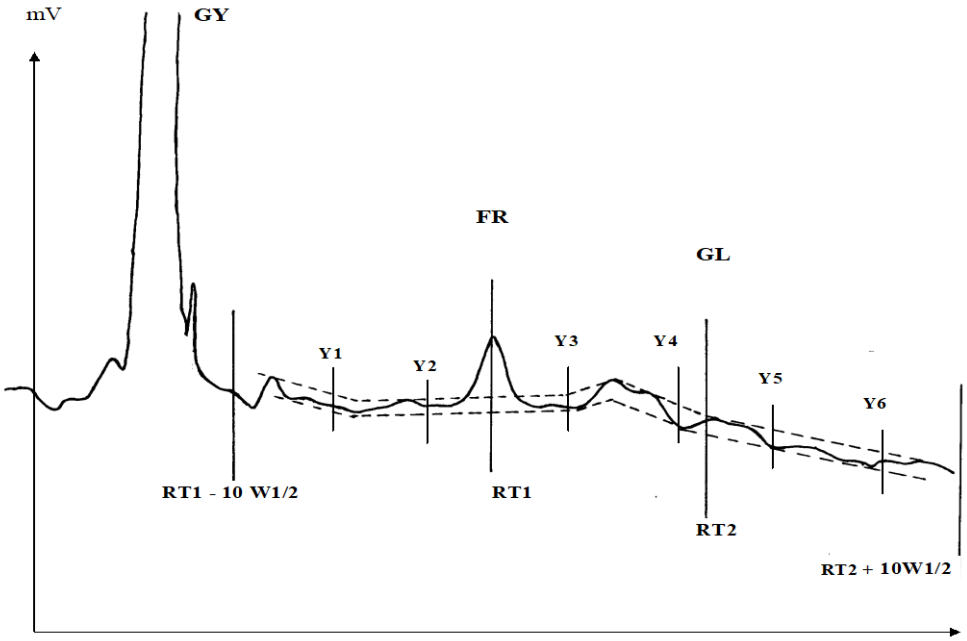


Figure 2
Chromatogram of a rosé wine

Glycerol (GY), fructose (FR), glucose (GL), saccharose (SA)



fructose (FR), glucose (GL), saccharose (SA) Glycerol (GY),

Figure 3 - Measure of pitches of noise after enlargement of chromatogram

RT1: retention time of fructose; RT2: retention time of glucose
W1/2: width of peak at mid-height; Yi: pitch of noise at point i

Annex B

(informative)

Precision data

B.1 - Samples in the interlaboratory test trial

This study was carried out by the Interregional Laboratory of the Répression de Fraudes in Bordeaux. The test trial involved 6 samples in blind duplicates (12 samples in total), identified as A to J (4 white wines and 4 red wines; 2 white Port wines and 2 red Port wines), containing glucose and fructose and whose content of each sugar was between 2 and 65 g/L. The wines from the region of Bordeaux were supplemented with glucose and fructose and stabilised with 100 mg/L of SO_2 (TRICARD and MEDINA, 2003).

B.2 - Chromatographic conditions

Considering the response factors of these two sugars and the scales of the chromatograms, the noise corresponds to a concentration of 0.04 g/L for fructose and of 0.06 g/L for glucose (see Figure A3).
The limits of detection (3 times the noise) and of quantification (10 times the noise) are then obtained:

$LD_{fructose} = 0.12$ g/L,
$LD_{glucose} = 0.18$ g/L,
$LQ_{fructose} = 0.4$ g/L,
$LQ_{glucose} = 0.6$ g/L.

These results are compliant with those determined by TUSSEAU and BOUNIOL (1986) and are repeatable on other chromatograms.

B.3 - Precision

Nine laboratories participated in the interlaboratory study:
Istituto Sperimentale per l'Enologia, Asti, Italy;
Laboratoire de la DGCCRF de Montpellier, France;
Laboratoire LARA, Toulouse, France;
Instituto do vinho do Porto, Porto, Portugal;
Instituto da Vinha e do Vinho, Unhos, Portugal;
Estación de Viticultura y Enología, Vilafranca del Penedés, Spain;
Comité Interprofessionnel du vin de Champagne, Epernay, France;
Station fédérale de Changins, Switzerland;

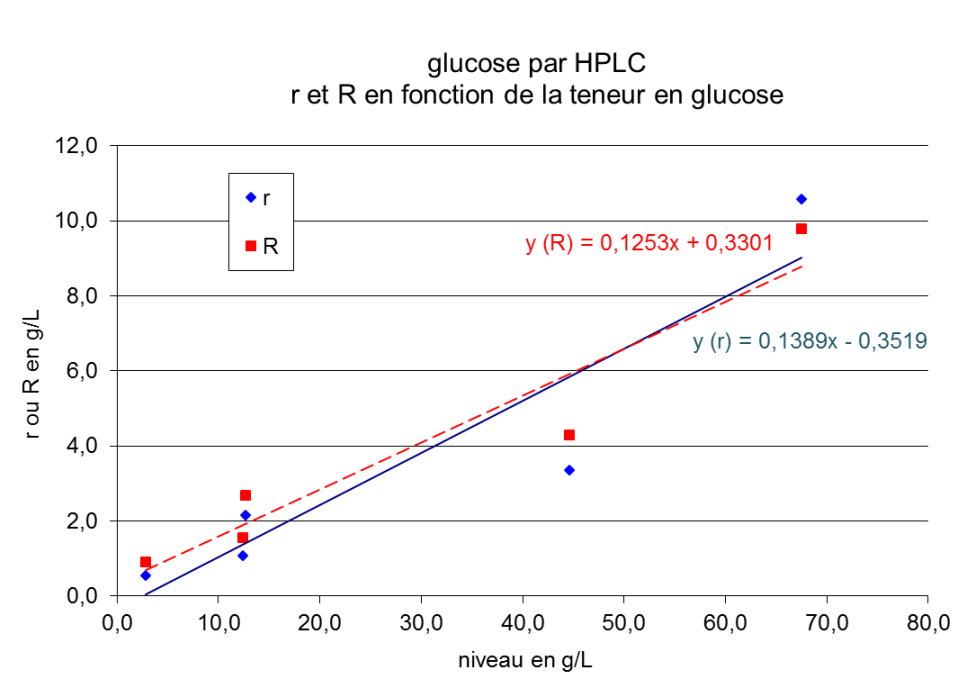
Laboratoire de la DGCCRF de Talence, France.

The analyses of 3 points of the set of calibration solutions and the 12 samples were carried out successively by applying the method of analysis given.
The results were analysed according to the OIV protocol (Validation protocol of methods of analysis – Resolution OENO 6/1999).
This protocol does not require the analyses to be repeated, whereas 4 laboratories gave results of analyses repeated 3 times. A single series was chosen (the first one) for the analysis of the results, in compliance with the OIV protocol.
The calculations of repeatability according to Youden, reproducibility and Cochran and Grubbs tests were performed.
Data on the repetitions made it possible to work out the standard deviations of repeatability in another way (according to ISO 5725).

B.3.1 – GLUCOSE

Glucose by HPLC (g/L)						
Number of laboratories	9	9	9	9	9	9
Number of samples	2	2	2	2	2	2
Average value	2.9	2.9	12.6	12.4	44.6	67.5
Repeatability standard deviation	0.44	0.17	0.67	0.34	1.05	3.31
Repeatability limit	1.42	0.55	2.15	1.07	3.35	10.58
Reproducibility standard deviation	0.78	0.30	0.90	0.52	1.43	3.28
Reproducibility limit	2.32	0.90	2.68	1.55	4.28	9.78
Horrat value	5.7*	2.1	1.84	1.08	1.01	1.62

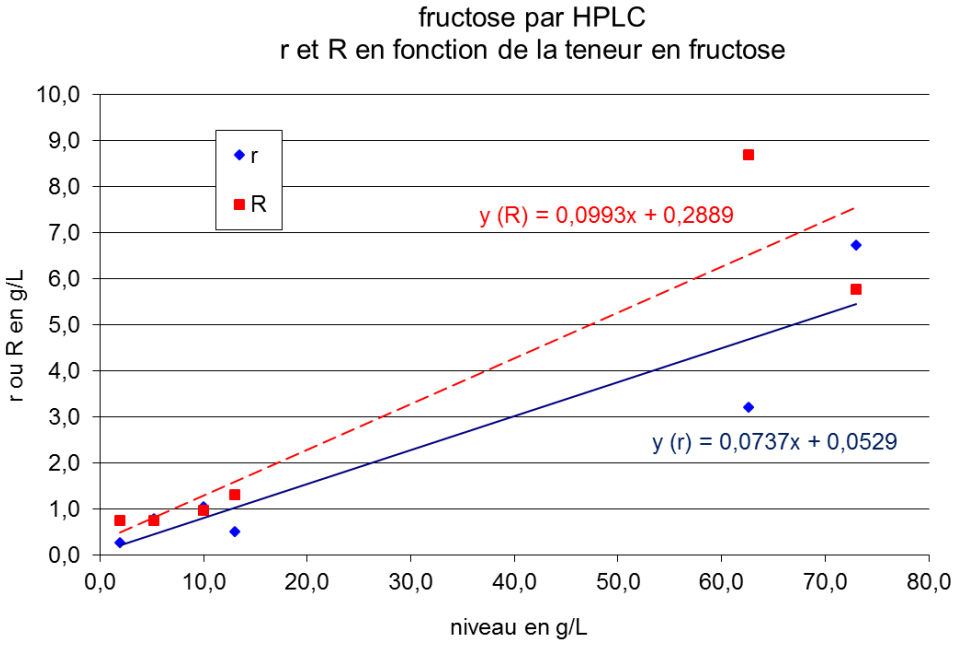
* not taken into account for the expression of precision



Correlation between r and R and the concentration for glucose (ISO 5725)

B.3.2 – FRUCTOSE

Fructose by HPLC (g/L)						
Number of laboratories	9	9	9	9	9	9
Number of samples	2	2	2	2	2	2
Average value	1.9	5.2	10.0	13.0	62.6	73.0
Repeatability standard deviation	0.09	0.24	0.32	0.16	3.20	2.10
Repeatability limit	0.27	0.79	1.03	0.51	3.20	6.72
Reproducibility standard deviation	0.25	0.25	0.32	0.43	2.91	1.93
Reproducibility limit	0.75	0.75	0.96	1.30	8.68	5.77
Horrat value	2.54	1.09	0.81	0.87	1.53	0.89

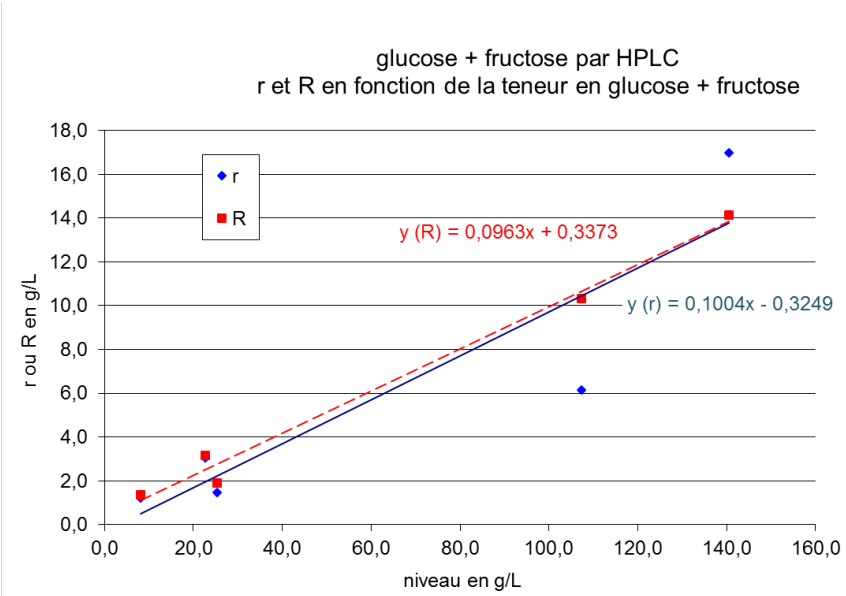


Correlation between r and R and the concentration for fructose (ISO 5725)

B.3.3 – GLUCOSE + FRUCTOSE

Glucose + fructose by HPLC (g/L)						
Number of laboratories	9	9	9	9	9	9
Number of samples	2	2	2	2	2	2
Average value	4.7	8.1	22.6	25.4	107.3	140.5
Repeatability standard deviation	0.48	0.38	1.06	0.46	1.92	5.30
Repeatability limit	1.52	1.21	3.07	1.48	6.13	17.0
Reproducibility standard deviation	0.89	0.46	1.06	0.64	3.47	4.74
Reproducibility limit	2.64	1.38	3.17	1.90	10.34	14.15
Horrat value	4.17*	1.39	1.33	0.72	1.15	1.26

* not taken into account for the expression of precision



Correlation between r and R and the concentration for glucose + fructose (ISO 5725)

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Method OIV-MA-AS311-04

Stabilization of Musts to Detect the Addition of Sucrose

1. Principle of the method

The sample is brought to pH 7 with a sodium hydroxide solution and an equal volume of acetone is added.

The acetone is removed by distillation prior to determination of sucrose by TLC (thin-layer chromatography) and HPLC (high-performance liquid chromatography) (see *Sucrose* Chapter).

2 Apparatus

Distillation apparatus, with a 100 mL round distillation flask.

3 Reagents

3.1 Sodium hydroxide solution, 20% (*m/v*)
3.2 Acetone (propanone).

4 Method

4.1 *Stabilizing the samples*

20 mL of must is placed in a 100 mL strong-walled flask and brought to pH 7 with the 20% sodium hydroxide solution (*m/V*) (six to twelve drops). 20 mL of acetone are added. Stopper and store at low temperature.

WARNING: **ACETONE HAS HIGH VAPOUR PRESSURE AND IS HIGHLY INFLAMMABLE.**

4.2 *Preparing the sample* to determine sucrose by TLC or HPLC.

Place the contents of the flask in the 100 mL round flask of the distillation apparatus. Distil and collect approximately 20 mL of distillate, which is discarded. Add 20 mL of water to the contents of the distilling flask and distil again, collecting about 25 mL of distillate, which is discarded.

Transfer the contents of the distillation flask to a graduated 20 mL volumetric flask and make up to the mark with the rinsing water from the round flask. Filter. Analyze the filtrate and (if detected) measure the sucrose using TLC or HPLC.

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Method OIV-MA-AS311-05 Type II method

Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance (SNIF-NMR/ RMN-FINS [4])

(OIV-OENO 426-2011)

1. Introduction

The deuterium contained in the sugars and the water in grape must is redistributed after fermentation in molecules I, II, III and IV of the wine:

$CH_2D\ CH_2\ OH$	$CH_3\ CHD\ OH$	$CH_3\ CH_2\ OD$	HOD
I	II	III	IV

2. Scope

The method enables measurement of the Deuterium isotope ratios (D/H) in wine ethanol and ethanol obtained by fermentation of products of the vine (musts, concentrated musts, rectified concentrated musts).

3. Definitions

$(D/H)_I$: Isotope ratio associated with molecule I
$(D/H)_{II}$: Isotope ratio associated with molecule II
$(D/H)^Q_W$: Isotope ratio of the water in the wine (or in fermented products)
$R = 2(D/H)_{II}/(D/H)_I$

[4] Fractionnement Isotopique Naturel Spécifique étudié par Résonance Magnétique Nucléaire (Site Specific Natural Isotope Fractionation studied by Nuclear Magnetic Resonance). Brevet: France, 8122710; Europe, 824022099; Etats Unis, 854550082; Japon 57123249.

R expresses the relative distribution of deuterium in molecules I and II; R is measured directly from the intensities h (peak heights) of the signals and then $R = 3h_{II}/h_I$.

4. Principle

The above defined parameters R, $(D/H)_I$ and $(D/H)_{II}$ are determined by nuclear magnetic resonance of the deuterium in the ethanol extracted from the wine or from the fermentation products of the must, the concentrated must or the grape sugar (rectified concentrated must) obtained under given conditions.

5. Reagents and materials

5.1 reagents:

5.1.1 reagents for the determination of water by the Karl Fischer method (when this method is used for the measurement of the alcohol grade of the distillate).

5.1.2 Hexafluorobenzene (C6F6) used as lock substance

5.1.3 Trifluoroacetic acid (TFA, CAS: 76-05-1) or alternatively trifluoroacetic anhydride (TFAA, CAS: 407-25-0)

5.2 Reference Materials (available from the Institute for Reference Materials and Measurements IRMM in Geel (B)):

5.2.1 Sealed NMR tubes CRM-123, used to check the calibration of the NMR instrumentation

5.2.2 Standard N,N-tetramethyl urea (TMU); standard TMU with a calibrated isotope ratio D/H.

5.2.3 Other CRMs available used to check the distillation and preparation steps:

CRM		Parameter	Certified value	Uncertainty
CRM-656	Ethanol from wine, 96% vol.			
		t^D (ethanol) in % w/w	94.61	0.05
		$\delta^{13}C$ (ethanol) in ‰ VPDB	-26.91	0.07
		$(D/H)_I$ (ethanol) in ppm	102.84	0.20
		$(D/H)_{II}$ (ethanol) in ppm	132.07	0.30
		R (ethanol)	2.570	0.005
CRM-660	hydro alcoholic solution, 12% vol.			
		t^Q (ethanol) in % vol.	11.96	0.06
		$\delta^{13}C$ (ethanol) in ‰ VPDB	-26.72	0.09
		$(D/H)_I$ (ethanol) in ppm	102.90	0.16
		$(D/H)_{II}$ (ethanol) in ppm	131.95	0.23
		R	2.567	0.005
		(D/H)w (water) in ppm	148.68	0.14

5.3 Apparatus

5.3.1 NMR spectrometer fitted with a specific 'deuterium' probe tuned to the characteristic frequency νo of the field Bo (e.g. for Bo = 7.05 T, νo = 46.05 MHz and for Bo = 9.4 T, νo = 61.4 MHz) having a proton decoupling channel (B2) and field-frequency stabilization channel (lock) at the fluorine frequency. The NMR instrument can possibly be equipped with an automatic sample changer and additional data-processing software for the evaluation of the spectra and computation of the results. The performance of the NMR spectrometer can be checked using the Certified Reference Materials (sealed tubes CRM 123).

5.3.2 10 mm NMR sample tubes

5.3.3 Distillation apparatus

Note: Any method for ethanol extraction can be used as long as the alcohol in the wine is recovered without isotopic fractionation.

The Cadiot column shown in figure 1 is an example of a manual distillation system that allows to extract 96 to 98.5% of the ethanol of a wine without isotopic fractionation and obtain a distillate with an alcohol grade of 92 to 93 in % w/w (95% vol.).

Such a system is composed of:

- Electric heating mantle with voltage regulator,
- One-liter round-bottom flask with ground glass neck joint,
- Cadiot column with rotating band (moving part in Teflon),
- conical flasks with ground glass neck joints, for collection of the distillate

Automatic distillation systems are also available.

The performance of the distillation system may be checked periodically for both the yield of extraction as well as for accuracy for the isotopic determination. This control can be done by distillation and measurement of CRM -660.

5.3.4 The following common laboratory equipment and consumables is needed:

-micropipette with appropriate tips,
-balance with 0.1 mg accuracy or better,
-balance with 0.1g accuracy or better
-single use syringe for transfer of liquids,

-precise graduated flasks (50ml, 100 ml, 250ml, …)
-flasks equipped with airtight closing systems and inert septa (for storage of aliquots of wines, distillates and residues until measurement)
-equipment and consumables as specified in the other methods referred to herein.
The laboratory equipment and consumables indicated in the above lists are examples and may be replaced by other equipment of equivalent performance.

6. Sampling (Preparation of the sample)

6.1 If not yet available, determine the alcoholic strength of the wine or of the fermented product (tv) to better than the nearest 0.05 % vol. (eg. using the OIV method MA-F-AS312-01-TALVOL).

6.2 Extraction of the ethanol
Using the appropriate graduated flask, introduce a homogeneous sample of a suitable volume V ml of the wine or the fermented product into the round-bottom flask of the distillation apparatus. Place a ground conical flask to receive the distillate. Heat the product to be distilled to obtain a constant reflux ratio at the level of the condenser. Start the collection of the distillate when a stable temperature of the vapours typical of the ethanol-water azeotrope (78 °C) is reached and stop the collection when the temperature increases. The collection of distillate should be continued until the ethanol-water azeotrope is completely recovered.

When using manually a Cadiot column (Figure 1) the following procedure can be applied:
-Collect the boiling liquid corresponding to the ethanol-water azeotrope, when the temperature increases, discontinue collection for five minutes. When the temperature returns to 78 °C, recommence collecting the distillate until the temperature of the vapours increases again. Repeat this operation until the temperature, after discontinuing collection, does not return to 78 °C. Alternatively, commercially available distillation systems can be used.

The weight m^D of distillate collected is weighed to better than 0.1g.
In order to avoid isotopic fractionation, the distillate should be kept in a tight vial preventing any evaporation until further use for determination of the alcoholic strength (6.3) and preparation of the NMR tube (7.1).

An aliquot of a few ml of the residues is kept. Its isotope ratio $(D/H)^Q_W$ may be determined if required.

6.3 Determination of the alcoholic strength of the distillate
The alcoholic strength (%w/w) of the distillate must be determined with a precision better than 0.1%.

The water content of the distillate (ρ' g) can be determined by the Karl Fischer method using a sample of about 0.5 ml of alcohol of exactly known mass ρ g .The alcohol strength by mass of the distillate is then given by:
t_m^D % w/w= 100 (1-ρ')/ ρ

Alternatively the alcoholic strength can be determined by densimetry for instance using a electronic densimeter.

6.4 Yield of distillation
The yield of distillation is estimated using the following formula:
Yield of dist.% = 100 t_m^D m^D /(V.tv)
Given the uncertainty on each term and especially on tv, the yield of distillation is estimated at ±0.5% (in the case of a wine of 10%v/v).
When using the Cadiot column, no significant isotope fractionation effect is expected for yield of extraction higher than 96%. In any case the operator may use a sufficient volume Vml of wine or fermented product for the distillation to ensure that the yield of extraction is sufficient. Typically aliquots of 750, 500, 400 and 300ml of wine sample should be sufficient to obtain a 96% yield when carrying out the above distillation procedure with the Cadiot column on wines or fermented products of respectively tv = 4, 6, 8 and 10% vol.

6.5 Fermentation of musts, concentrated musts and rectified concentrated musts

Prior to use, the yeast can be reactivated in a small volume of must. The fermentation vessel is equipped with a device to keep it airtight and to avoid loss of ethanol.

6.5.1 Musts
Place about one litre of must, whose concentration of fermentable sugars has been previously determined, in the fermentation vessel. Add about 1 g of dry yeast eventually reactivated beforehand. Insert device to keep it airtight. Allow fermentation to proceed until the sugar is used up. The fermented product can then be distilled following the procedure already described for wine in 6.1 to 6.4

Note: Musts preserved by addition of sulphur dioxide have to be de-sulphited by bubbling nitrogen through the must in a water bath at 70 to 80 °C under reflux in order to prevent isotope fractionation through evaporation of water. Alternatively, the sulphur dioxide can be removed by a small addition of a solution of hydrogen peroxide (H_2O_2).

6.5.2 Concentrated musts
Place V ml of concentrated must containing a known amount of sugar (approximately 170 g) into the fermentation vessel. Top up to one litre with (1000 - V) ml of water. Add dry yeasts (1 g) and 3 g of Bacto Yeast Nitrogen Base without amino acids. Homogenize and proceed as described in 6.5.1.

6.5.3 Grape sugar (Rectified concentrated musts)
Proceed as described in 6.5.2, topping up to one litre with (1000 - V) ml of water also containing 3 g of dissolved tartaric acid.

Note: Concentrated musts and rectified concentrated musts are diluted in local water having a (D/H) isotope concentration different of that of the original must. By convention, the $(D/H)_I$ and $(D/H)_{II}$ parameters measured on ethanol have to be normalised as if the must had fermented in water having the same deuterium concentration as V-SMOW (155.76 ppm).
This normalisation of the data is performed by using the following equations (Martin et al., 1996, J. AOAC, 79, 62-72):

$$\left(\frac{D}{H}\right)_I^{Norm.V-SMOW} = \left(\frac{D}{H}\right)_I - 0.19 \times \left[\left(\frac{D}{H}\right)_W^S - 155.76\right]$$

$$\left(\frac{D}{H}\right)_{II}^{Norm.V-SMOW} = \left(\frac{D}{H}\right)_{II} - 0.78 \times \left[\left(\frac{D}{H}\right)_W^S - 155.76\right]$$

where $\left(\frac{D}{H}\right)_W^S$ is the deuterium isotope ratio of the diluted must. This value can be computed using the equation of the Global Meteoric Water Line (Craig, 1961):

$$\left(\frac{D}{H}\right)_W^S = 155.76 \times \left[\frac{\left(8 \times \delta^{18}O + 10\right)}{1000} + 1\right]$$

Where $\delta^{18}O$ is measured on the diluted must by the method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must [OIV-MA-AS2-12].

Retain 50 ml of sample of must or sulphur dioxide treated must or concentrated must or rectified concentrated must with a view to the possible extraction of the water and the determination of its isotope ratio $(D/H)_W^Q$.

7. Procedure

7.1 Preparation of alcohol sample for NMR measurement

- 10 mm diameter NMR probe: in a previously weighed bottle, collect 3.2 ml of distillate as described in section 6.2 and weigh it to the nearest 0.1 mg (m_A); then take 1.3 ml sample of the internal standard TMU (5.2.2) and weigh to the nearest 0.1 mg (m_{ST}).

Depending on the type of spectrometer and probe used, add a sufficient quantity of hexafluorobenzene (5.1.2) as a field-frequency stabilization substance (lock):

Spectrometer	10 mm probe
7.05 T	150 µl
9.4 T	35 µl

These figures are indicative and the actual volume to be used should be adjusted to the sensitivity of the NMR instrument. While preparing the tube and until the NMR measurement, the operator should take care to avoid any evaporation of ethanol and TMU since this would cause isotopic fractionation, errors in the weights (m_A and m_{ST}) of the components and erroneous NMR results.
The correcteness of the procedure of measurement including this preparation step can be checked using the CRM 656.
Note: the hexafluorobenzene can be added with 10% (v/v) of trifluoroacetic acid (5.1.3) in order to catalyze the fast hydrogen exchange on hydroxyle bond resulting in a single NMR peak for both the hydroxyle and residual water signals.

7.2 Recording of ^2H NMR spectra of the alcohol
The homogeneity of the magnetic field B_0 in the sample is optimized through the "shimming" procedure maximizing the ^{19}F NMR lock signal observed the hexafluorobenzene. Modern NMR spectrometers can perform automatically and efficiently this "shimming" procedure provided that the initial settings are close enough to the optimal magnetic field homogeneity for a given sample as is generally the case for a batch of ethanol samples prepared as described in 7.1. The efficiency of this procedure can be checked through the resolution measured on the spectrum obtained without exponential multiplication (i.e. LB = 0) (Figure 2b) and expressed by the half-width of the methyl and methylene signals of ethanol and the methyl signal of TMU, which must be less than 0.5 Hz in the best conditions. The sensitivity, measured with an exponential multiplying factor LB equal to 2 (Figure 2a) must be greater than or equal to 150 for the methyl signal of ethanol of alcoholic strength 95 % vol (93.5 % mas).

7.2.2 Checking the instrumental settings

Carry out customary standardization for homogeneity and sensitivity according to the manufacturer's specifications.
Use the sealed tubes CRM123 (H: High , M: Medium, L: Low).
Following the procedure described below in 9.3, determine the isotope values of these alcohols, denoting them Hmeas, Mmeas, Lmeas .

Compare them with the given corresponding standard values, denoted by a superscript Hst, Mst, Lst.

Typically, as an indication the standard deviation obtained for 10 repetitions of each spectrum should be of the order of 0.01 for the ratio R and 0.5 ppm for $(D/H)_I$ and 1 ppm for $(D/H)_{II}$.

The average values obtained for the various isotopic parameters (R, $(D/H)_I$, $(D/H)_{II}$) must be within the corresponding standard deviation of repeatability given for those parameters for the CRM123. If they are not, carry out the checks again.

Once the settings have been optimized also other CRM materials can be used to monitor the quality of measurements in routine analysis.

7.3 Conditions for obtaining NMR spectra

Place a sample of alcohol prepared as in 7.1 in a 10 mm tube and introduce it into the probe.

Suggested conditions for obtaining NMR spectra are as follows:

- a constant probe temperature, set to better less than ±0.5°K variation in the range 302 K to 306 K depending on the heating power generated by the decoupling;
- acquisition time of at least 6.8 s for 1200 Hz spectral width (16K memory) (i.e. about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz);
- 90° pulse;
- parabolic detection: fix the offset 01 between the OD and CHD reference signals for ethanol and between the HOD and TMU reference signals for water;

- determine the value of the decoupling offset 02 from the proton spectrum measured by the decoupling coil on the same tube. Good decoupling is obtained when 02 is located in the middle of the frequency interval existing between the CH3- and CH2- groups. Use the wide band decoupling mode or composite pulse sequences (eg. WALTZ16) to ensure homogeneous decoupling on the whole spectrum.

For each spectrum, carry out a number of accumulations NS sufficient to obtain the signal-to-noise ratio indicated as sensitivity in 7.2 and repeat NE times this set of NS accumulations. The values of NS depend on the types of spectrometer and probe used. Examples of the possible choices are:

Spectrometer	10 mm probe
7.05 T	NS = 304
9.4 T	NS = 200

The number of repetitions NE should be statistically meaningful and sufficient to achieve the performance and precision of the method as reported below in §9.

In the case that two NMR sample tubes have been prepared following the procedure described in 7.1, five repetitions of NMR spectra (NE=5) can be recorded on each tube. The final result for each isotopic parameter corresponds to the mean value of the measurements obtained on the two NMR sample tubes. In that case, the acceptance criteria for validation of the results obtained with these two tubes are:

$|Mes1(D/H)_I - Mes2(D/H)_I| < 0.5ppm$, $|Mes1(D/H)_{II} - Mes2(D/H)_{II}| < 0.8ppm$

8. Expression of results

For each of the NE spectra (see NMR spectrum for ethanol, Figure 2a), determine:

$$R = 3 \cdot \frac{h_{II}}{h_I} = 3 \cdot \frac{\text{height of signal II } (CH_3\ CH_D\ OH)}{\text{height of signal I } (CH_2D\ CH_2\ OH)}$$

$$(D/H)_I = 1.5866 \cdot T_I \cdot \frac{m_{ST}}{m_A} \cdot \frac{(D/H)_{ST}}{t_m^D}$$

$$(D/H)_{II} = 2.3799 \cdot T_{II} \cdot \frac{m_{ST}}{m_A} \cdot \frac{(D/H)_{ST}}{t_m^D}$$

with

- $T_I = \frac{\text{height of signal I } (CH_2D\ CH_2\ OH)}{\text{height of signal of internal standard (TMU)}}$
- $T_{II} = \frac{\text{height of signal II } (CH_3\ CHD\ OH)}{\text{height of signal of internal standard (TMU)}}$

- m_{ST} and m_A, see 7.1
- t_m^D, see 6.3
- $(D/H)_{ST}$ = isotope ratio of internal standard (TMU) indicated on certificate delivered by IRMM.

The use of peak heights instead of peak area, which is less precise, supposes that peak width at half height is identical and is a reasonable approximation if applicable (Figure 2b).

For each of the isotope parameters, calculate the average and the confidence interval for the number of repeated spectra acquired on a given sample.

Optional softwares enable such calculations to be carried out on-line.

9. Precision

The repeatability and Reproducibility of the SNIF-NMR method has been studied through collaborative studies on fruit juices as reported in the bibliography here below. However these studies considered only the

parameter $(D/H)_I$. In the case of wine data from in-house studies carried out by several laboratories can be considered for establishing the standard deviation of repeatability and the limit of repeatability as presented in Annex I. The results of proficiency testing reported in Annex II provide data that can be used to compute the standard deviation of Reproducibility and the limit of Reproducibility for wines.

These figures can be summarised as follows:

	$(D/H)_I$	$(D/H)_{II}$	R
S_r	0.26	0.30	0.005
r	0.72	0.84	0.015
S_R	0.35	0.62	0.006
R	0.99	1.75	0.017

with

- S_r : standard deviation of repeatability
- r : limit of repeatability
- S_R: standard deviation of reproducibility
- R : limit of Reproducibility

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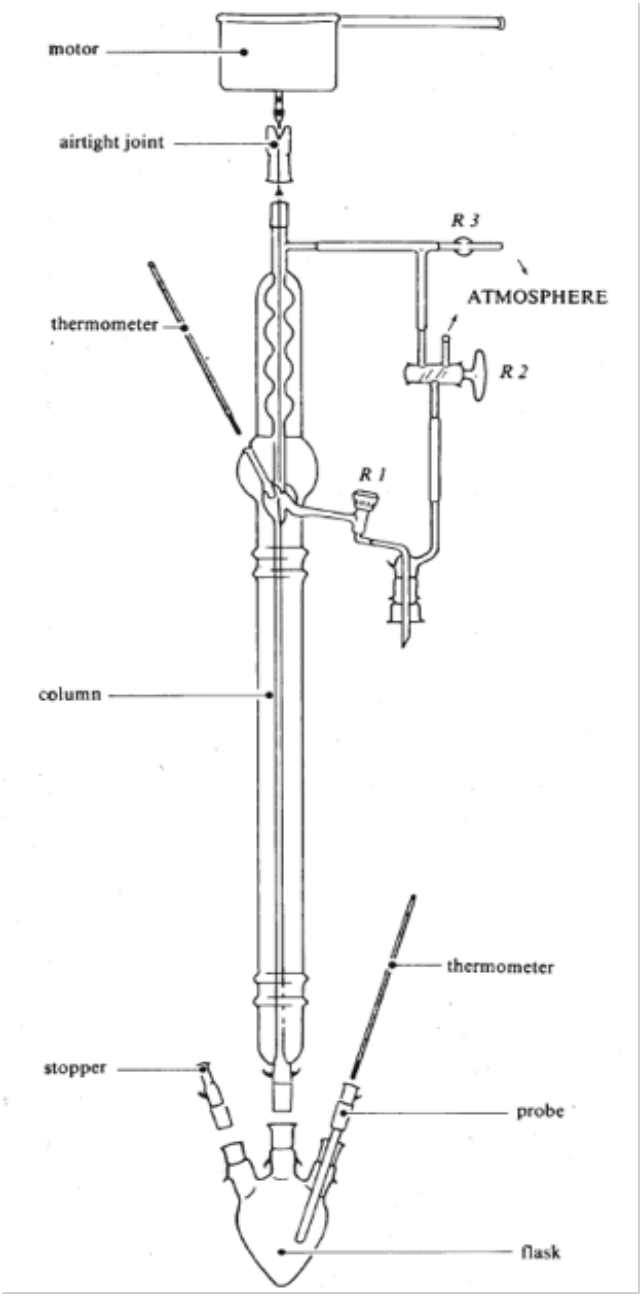


Figure 1 - Apparatus for extracting ethanol

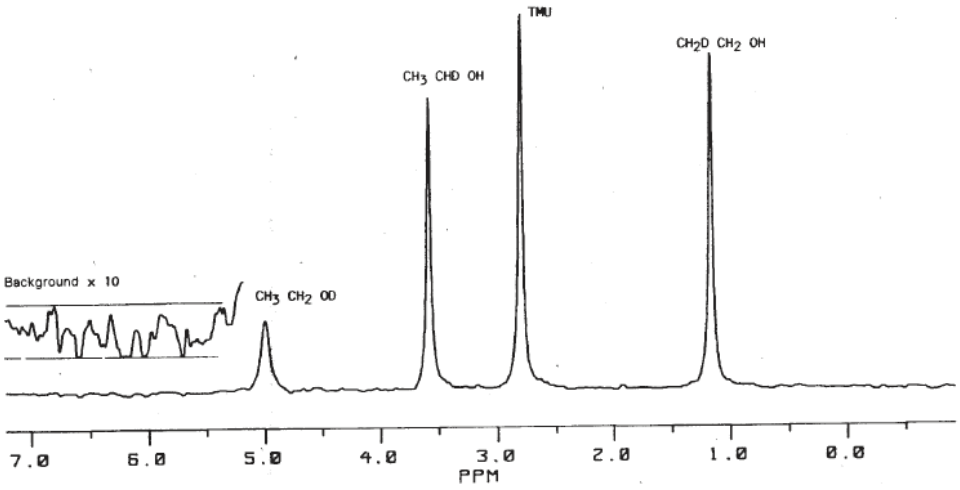


Figure 2a
^2H NMR spectrum of an ethanol from wine with an internal standard (TMU: N, N-tetramethylurea)

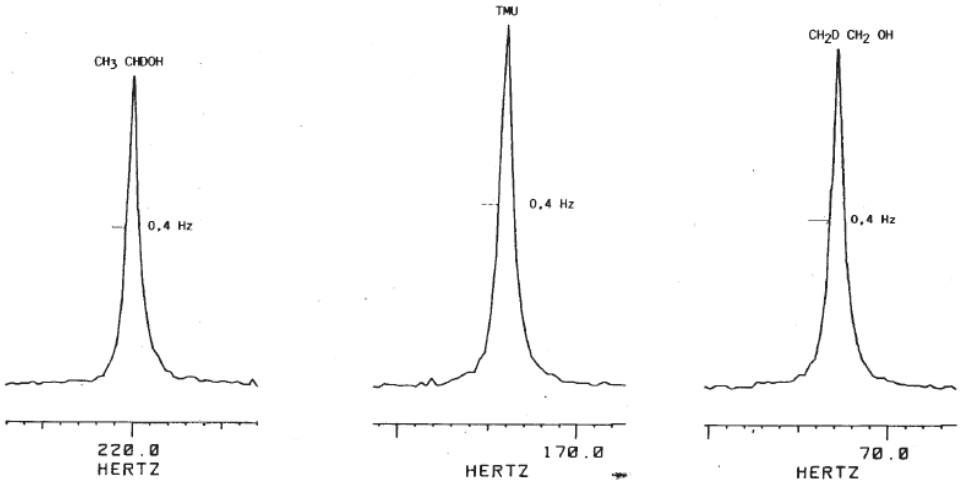


Figure 2b
^2H spectrum of ethanol taken under the same conditions as those of Figure 2a,

but without exponential multiplication (LB = 0)

Annex I: Estimation of the repeatability from in-house repeatability studies

The in-house repeatability studies performed in 4 laboratories provide data that allows the estimation of the repeatability of the SNIF-NMR method.
These in-house repeatability studies have been performed by duplicate distillations and measurements of 10, 9 or 15 different wine samples by the laboratories 1, 2 and 3.
Alternatively the laboratory 4 performed 16 distillations and measurements on the same wine in condition of repeatability on a short period of time.

Table I-1: **lab 1 : 10 wines analysed in duplicates**

				$(D/H)_I$ abs $(\Delta(D/H)_I)$	Squares	$(D/H)_{II}$ abs $(\Delta(D/H)_{II})$	Squares	R abs $(\Delta(R))$	Squares
Sample	$(D/H)_I$	$(D/H)_{II}$	R						
1	103.97	130.11	2.503	0.55	0.302	0.68	0.462	0.000	0.00000
	104.52	130.79	2.503						
2	103.53	130.89	2.529	0.41	0.168	0.32	0.102	0.016	0.00026
	103.94	130.57	2.513						
3	102.72	130.00	2.531	0.32	0.102	0.20	0.040	0.004	0.00002
	103.04	130.20	2.527						
4	105.38	132.39	2.513	0.14	0.020	0.20	0.040	0.000	0.00000
	105.52	132.59	2.513						
5	101.59	127.94	2.519	0.48	0.230	0.20	0.040	0.016	0.00026

	101.11	128.14	2.535						
6	103.23	132.14	2.560	0.30	0.090	0.36	0.130	0.001	0.00000
	102.93	131.78	2.561						
7	103.68	130.95	2.526	0.15	0.023	0.75	0.563	0.011	0.00012
	103.53	130.20	2.515						
8	101.76	128.86	2.533	0.24	0.058	0.42	0.176	0.003	0.00001
	101.52	128.44	2.530						
9	103.05	129.59	2.515	0.04	0.002	0.44	0.194	0.007	0.00005
	103.01	129.15	2.508						
10	101.47	132.63	2.614	0.50	0.250	0.18	0.032	0.010	0.00010
	100.97	132.45	2.624						
				Sum of squares:	1.245		1.779		0.00081
				Sr	**0.25**		**0.30**		**0.006**
				r	**0.71**		**0.84**		**0.018**

Table I-2: **lab 2 : 9 wines analysed in duplicates**

				(D/H)I abs (Δ(D/H)$_I$)	Squares	**(D/H)II** abs (Δ(D/H)$_{II}$)	Squares	**R** abs (Δ(R))	Squares
Sample	**(D/H)$_I$**	**(D/H)$_{II}$**	**R**						
1	105.02	133.78	2.548	0.26	0.068	0.10	0.010	0.008	0.00007
	104.76	133.88	2.556						
2	102.38	130.00	2.540	0.73	0.533	0.40	0.160	0.010	0.00011
	101.65	129.60	2.550						
3	100.26	126.08	2.515	0.84	0.706	0.64	0.410	0.008	0.00007
	99.42	125.44	2.523						
4	101.17	128.83	2.547	0.51	0.260	0.45	0.203	0.004	0.00002
	100.66	128.38	2.551						
5	101.47	128.78	2.538	0.00	0.000	0.26	0.068	0.005	0.00003
	101.47	128.52	2.533						
6	106.14	134.37	2.532	0.12	0.014	0.04	0.002	0.002	0.00000
	106.26	134.41	2.530						
7	103.62	130.55	2.520	0.05	0.003	0.11	0.012	0.003	0.00001
	103.57	130.66	2.523						
8	103.66	129.88	2.506	0.28	0.078	0.55	0.302	0.004	0.00001
9	103.38 103.50 103.93	129.33 129.66 129.44	2.502 2.506 2.491	0.43	0.185	0.22	0.048	0.015	0.00021
				Sum of squares:	1.846		1.214		0.00053
				Sr	**0.32**		**0.26**		**0.005**
				r	**0.91**		**0.74**		**0.015**

Table I-3: **lab 3 : 15 wines analysed in duplicates**

				(D/H)I		**(D/H)II**		**R**	
				abs ($\Delta(D/H)_I$)	Squares	abs ($\Delta(D/H)_{II}$)	Squares	abs ($\Delta(R)$)	Squares
Sample	**$(D/H)_I$**	**$(D/H)_{II}$**	**R**						
1	101.63	125.87	2.477	0.06	0.004	0.46	0.212	0.007	0.00005
	101.57	125.41	2.470						
2	99.24	124.41	2.507	0.05	0.002	0.04	0.002	0.001	0.00000
	99.19	124.37	2.508						
3	101.23	125.07	2.471	0.06	0.004	0.16	0.026	0.005	0.00002
	101.17	125.23	2.476						
4	100.71	125.29	2.488	0.07	0.005	1.16	1.346	0.024	0.00058
	100.78	124.13	2.464						
5	99.89	124.02	2.483	0.18	0.032	0.56	0.314	0.007	0.00005
	99.71	123.46	2.476						
6	100.60	124.14	2.468	0.19	0.036	0.66	0.436	0.018	0.00032
	100.41	124.80	2.486						
7	101.47	125.60	2.476	0.23	0.053	0.14	0.020	0.003	0.00001
	101.70	125.74	2.473						
8	102.02	124.00	2.431	0.13	0.017	0.07	0.005	0.005	0.00002
	102.15	123.93	2.426						
9	99.69	124.60	2.500	0.40	0.160	0.53	0.281	0.000	0.00000
	100.09	125.13	2.500						
10	99.17	123.71	2.495	0.30	0.090	0.19	0.036	0.004	0.00002
	99.47	123.90	2.491						
11	100.60	123.89	2.463	0.40	0.160	0.54	0.292	0.001	0.00000
	101.00	124.43	2.464						
12	99.38	124.88	2.513	0.33	0.109	0.55	0.302	0.002	0.00000
	99.05	124.33	2.511						
13	99.51	125.24	2.517	0.44	0.194	0.01	0.000	0.011	0.00012
	99.95	125.25	2.506						
15	101.34	124.68	2.460	0.43	0.185	0.41	0.168	0.002	0.00000
	101.77	125.09	2.458						
			Sum of squares:		1.050		3.437		0.00120

				Sr	**0.19**		**0.34**		**0.006**
				r	**0.53**		**0.96**		**0.018**

Table I-4:
lab 4 : one wine analysed 16 times

Repetition	$(D/H)_I$	$(D/H)_{II}$	R		$(D/H)_I$	$(D/H)_{II}$	R
1	101.38	126.87	2.503	Variance:	0.0703	0.0840	0.000013
2	101.30	126.22	2.492				
3	100.98	125.86	2.493	**Sr**	**0.27**	**0.29**	**0.004**
4	100.94	126.00	2.497				
5	100.71	125.79	2.498	**r**	**0.75**	**0.82**	**0.010**
6	100.95	126.05	2.497				
7	101.17	126.30	2.497				
8	101.22	126.22	2.494				
9	100.99	125.91	2.494				
10	101.29	126.24	2.493				
11	100.78	126.07	2.502				
12	100.65	125.65	2.497				
13	101.01	126.17	2.498				
14	100.89	126.05	2.499				
15	101.66	126.52	2.489				
16	100.98	126.11	2.498				

The pooled data for the standard deviation of repeatability and for the limit of repeatability can thus be estimated as:

	$(D/H)_I$	$(D/H)_{II}$	R
Sr	0.26	0.30	0.005
limit of repeatability r	0.72	0.84	0.015

Data of in-house repeatability studies were provided by (in alphabetic order):

-Bundesinstitut für Risikobewertung,
Thielallee 88-92 PF 330013 D-14195 BERLIN – GERMANY

-Fondazione E. Mach-Istituto Agrario di San Michele all'Adige,
Via E. Mach, 1 - 38010 San Michele all'Adige (TN), ITALY

-Joint Research Centre - Institute for Health and Consumer Protection,
I-21020 ISPRA (VA) – ITALY

-Laboratorio Arbitral Agroalimentario, Carretera de la Coruña, km 10,7
E-28023 MADRID –SPAIN

Annex II: Evaluation of the Reproducibility from proficiency testing data

Since December 1994 international proficiency testing exercises on the determination of isotopic parameters on wine and various other food matrices have been regularly organised. These proficiency testing exercises allow participating laboratories to assess their performance and the quality of their analyses. The statistical exploitation of these results obtained on a large number of samples over a long period of time allows the appreciation of the variability of the measurements under conditions of reproductibility. This enables a good estimation of the variance parameters and of the reproducibility limit of the method. The results of 40 rounds of proficiency testing since 1994 until 2010 for various type of wine (red, white, rosé, dry, sweet and sparkling) are summarised in the table II-1 here below.

For $(D/H)_I$ and $(D/H)_{II}$ the pooled S_R can thus be calculated using the following equation:

$$\sqrt{\frac{\sum_i^K (N_i - 1) S_{R,i}^2}{\sum_i^K (N_i - 1)}}$$

with N_i ,and $S_{R,i}$ the number of values and the standard deviation of reproducibility of the i^{th} round, and K the number of rounds.

Considering the definition of the intramolecular ratio R, and applying the standard error propagation rules assuming that $(D/H)_I$ and $(D/H)_{II}$ are uncorrelated (the covariance terms are then zero), one can also estimate the standard deviation of Reproducibility for this parameter.

The following figures can thus be calculated:

	$(D/H)_I$	$(D/H)_{II}$	R
S_R:	0.35	0.62	0.006
Limit of Reproducibility R	0.99	1.75	0.01

Table II-1 : FIT Proficiency Testing – Summary of statistical values observed on wine samples:

			$(D/H)_I$			$(D/H)_{II}$		
Sample	Year	Round	N	Mean	S_R	N	Mean	S_R
Red wine	1994	R1	10	102.50	0.362	10	130.72	0.33
Rosé wine	1995	R1	10	102.27	0.333	10	128.61	0.35
Red wine	1995	R2	11	101.45	0.389	11	127.00	0.55
Red wine	1996	R1	11	101.57	0.289	11	132.23	0.34
Rosé wine	1996	R2	12	102.81	0.322	12	128.20	0.60
White wine	1996	R3	15	103.42	0.362	15	127.97	0.51
Red wine	1996	R4	15	102.02	0.377	13	131.28	0.30
Rosé wine	1997	R1	16	103.36	0.247	16	126.33	0.44
White wine	1997	R2	16	103.42	0.444	15	127.96	0.53
Sweet White Wine	1997	R2	14	99.16	0.419	15	130.02	0.88
Wine	1997	R3	13	101.87	0.258	15	132.03	0.61
Sweet Wine	1997	R3	12	102.66	0.214	12	128.48	0.48

Rosé wine	1997	R4	16	102.29	0.324	16	129.29	0.63
Sweet Wine	1997	R4	15	102.04	0.269	13	131.27	0.30
White wine	1998	R1	16	105.15	0.302	16	127.59	0.59
Sweet Wine	1998	R3	16	102.17	0.326	16	129.60	0.56
Red wine	1998	R4	17	102.44	0.306	17	131.60	0.47
White wine	1999	R1	14	102.93	0.404	13	129.64	0.46
Sweet Wine	2000	R2	15	103.19	0.315	14	129.43	0.60
Wine	2001	R1	12	105.28	0.264	16	131.32	0.68
Sweet Wine	2001	R2	14	101.96	0.249	15	128.99	1.05
Wine	2002	R1	17	101.01	0.365	16	129.02	0.74
Wine	2002	R2	17	101.30	0.531	17	129.28	0.93
Wine	2003	R1	18	100.08	0.335	18	128.98	0.77
Sweet Wine	2003	R2	17	100.51	0.399	18	128.31	0.80
Wine	2004	R1	18	102.88	0.485	19	128.06	0.81
Sweet Wine	2004	R3	16	101.47	0.423	16	130.10	0.71
Wine	2005	R1	19	101.33	0.447	19	129.88	0.76
Sweet wine	2005	R2	15	102.53	0.395	15	131.36	0.38
Dry wine	2006	R1	18	101.55	0.348	18	131.30	0.51
Sweet wine	2006	R2	18	100.31	0.299	18	127.79	0.55
Wine	2007	R1	18	103.36	0.403	18	130.90	0.90
Sweet wine	2007	R2	19	102.78	0.437	19	130.72	0.55
Wine	2008	R1	24	103.20	0.261	23	131.29	0.59
Sweet wine	2008	R2	20	101.79	0.265	19	129.73	0.34
Dry wine	2009	R1	24	102.96	0.280	23	130.25	0.49
Sweet wine	2009	R2	21	101.31	0.310	21	127.07	0.50
Dry wine	2010	R1	21	101.80	0.350	20	129.65	0.40
Sparkling wine	2010	R1	11	101.51	0.310	11	129.09	0.68
Dry wine	2010	R2	20	104.05	0.290	19	133.31	0.58

Method OIV-MA-AS311-06 Type IV method

Determination of polyols derived from sugars and residual sugars found in dry wines by means of gas chromatography

(Resolution OENO 9/2006 ; OIV/OENO 377/2009)

1. Scope

Simultaneous determination of the erythritol, arabitol, mannitol, sorbitol and meso-inositol content of wines.
Because the determination of sugars by gas chromatography (GC) is long and complicated, it is reserved for the determination of traces of sugars and, especially, of sugars for which no other routine enzyme method exists – (Arabinose, Rhamnose, Mannose and Galactose) although it is also applicable to glucose and fructose, the advantage being that it is possible to simultaneously determine all sugar monomers, dimers and even trimers.

Comment 1 - It is not possible to determine sugars once they have been reduced to alditol form because of the presence of corresponding polyols.
Comment 2 - In the form of trimethylsilylated derivatives (TMS), sugars give 2 α and β forms and occasionally 3 or 4 (Gamma…) corresponding to the different anomers present in wines.
Comment 3 - Without prior dilution, it is difficult to determine glucose and fructose content using this method when it exceeds 5 g/l.

2. Principle

Residual sugars in dry wines can be determined by gas chromatography after the formation of their trimethylsilylated derivatives.

The internal standard is pentaerythritol.

3. Reagents

Silane mixture for example purposes:
3.1 Pure hexamethyldesilazane (HMDS)
3.2 Pure trifluoroacetic anhydride (TFA)
3.3 Pure pyridine
3.4 Pure pentaerythritol

3.5. Distilled water
3.6 10 g/l pentaerythritol (internal standard solution): dissolve 0.15 g of pentaerythritol (3.4) in 100 ml of water (3.5)
3.7 Pure products that may be used to prepare control solutions, notably glucose, fructose, arabinose, mannitol and sorbitol (non-exhaustive list)
3.8 Control solutions of pure products at 200 mg/l: dissolve 20 mg of each of the products to be determined (3.6) in 100 ml of water.
Comment – Sugar solutions should be prepared immediately prior to use.

4. Apparatus and Equipment

4.1 1-ml pipettes, with $1/10^{th}$ ml graduations
4.2 Propipette™ bulbs
4.3 100-µl syringe
4.4 5-ml tubes with screw stoppers fitted with a Teflon-coated sealing cap.
4.5 Rotary vacuum evaporator capable of housing screw-cap test tubes (4.4) in order to evaporate samples to dryness
4.6 Gas chromatograph fitted with a flame ionisation detector x g, and an injector operating in "split" mode - $1/30^{th}$ to $1/50^{th}$ division of the injected volume (1 µl)
4.7 Non-polar capillary column (SE-30, CPSil-5, HP-1, etc.) 50 m x 0.25 mm, 15 mµ stationary phase film thickness (as an example).
4.8 10-µl injection syringe
4.9 Data acquisition system
4.10 Ultra-sonic bath
4.11 Laboratory fume cupboard

5. Preparation of samples

5.1 Addition of the internal standard: 1 ml of wine (pipette, 4.1) or of 200 mg/l control solution (3.6) is placed in the screw-cap test tube (4.4)
Note: It is possible to operate with lower volumes of wine especially in high content sugar environments.

50 µl of the 10 g/l pentaerythritol solution (3.5) is added by means of the syringe (4.3)

5.2 Obtaining dry solid matter:
The screw-cap test tube is placed on the rotary evaporator, with a water bath kept below 40°C. Evaporation continues until all traces of liquid have disappeared.
5.3 Addition of reagents
5.3.1 Place the tubes containing the dry solid matter and reagents 3.1, 3.2 and 3.3 in the fume cupboard (4.11) and switch on the ventilation.

5.3.2 Using the pipettes (4.1) and Propipette™ bulbs (4.2), add 0.20 ml of pyridine (3.3), 0.7 ml of HMDS (3.1) and 0.1 ml of TFA (3.2) to the test tube one after the other.
5.3.3 Seal the test tube with its stopper.
5.3.4 Put the test tube in the ultra-sonic bath (4.10) for 5 minutes until the dry solid matter has completely dispersed.
5.3.5 Place the test tube in a laboratory kiln at 60°C for two hours in order to obtain the total substitution of the hydroxyl or acid hydrogen by the trimethylsilyl groups (TMS).

Comment: a single phase only should remain after heating (if not, water would be left in the test tube). Likewise, there should be no brownish deposit, which would indicate an excess of non-derived sugar.

6 Chromatographic assay

6.1 Place the cooled test tube in the ventilated fume cupboard (4.11), remove 1 µl with the syringe (4.8) and inject into the chromatograph in "split" mode (permanent split).
Treat the wine-derived and control sample in the same way.
6.2 Programme the kiln temperature, for example from 60°C to 240°C at a rate of 3°C per minute, such that the complete assay lasts, for example, one hour for complete mannitol and sorbitol separation (resolution higher than 1.5).

7. Calculations

Example: calculation of sorbitol concentration
If
s = the peak area of the sorbitol in the wine
S = the peak area of the sorbitol in the control solution
i = the peak area of the internal standard in the wine
I = the peak area of the internal standard in the control solution

The sorbitol content of the wine (ts) will be

$$ts = 200 \times \frac{s}{S} \times \frac{I}{i} \quad \text{in mg per litre}$$

The same logic makes it possible to calculate the glucose content (tg)

$$tg = 200 \times \frac{g}{G} \times \frac{I}{i} \quad \text{in mg per litre}$$

when g is the sum of the areas of the two peaks of glucose in the wine and G is the sum of the areas of the two peaks of glucose in the control solution.

8. Characteristics of the method

Detection threshold approximately 5 mg/l for a polyol (a single chromatographic peak). Average repeatability in the region of 10% for a sugar or polyol concentration in the region of 100 mg/l.

Table 1 Repeatability of the determination of a number of substances found in the dry solid matter of wine after TMS derivatization.

	Tartaric acid	Fructose	Glucose	Mannitol	Sorbitol	Dulcitol	Meso-inositol
Average (mg/l)	2013	1238	255	164	58	31	456
Typical variance(mg/l)	184	118	27	8	2	2	28
CV (%)	9	10	11	5	3	8	6

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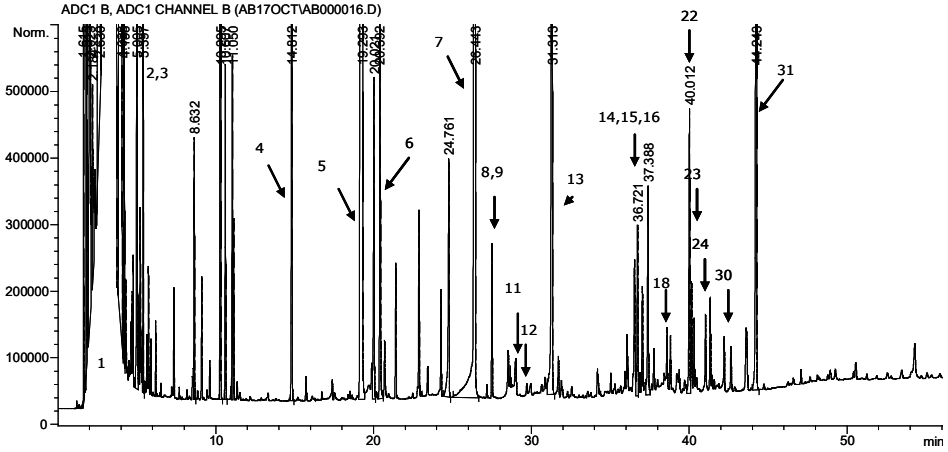
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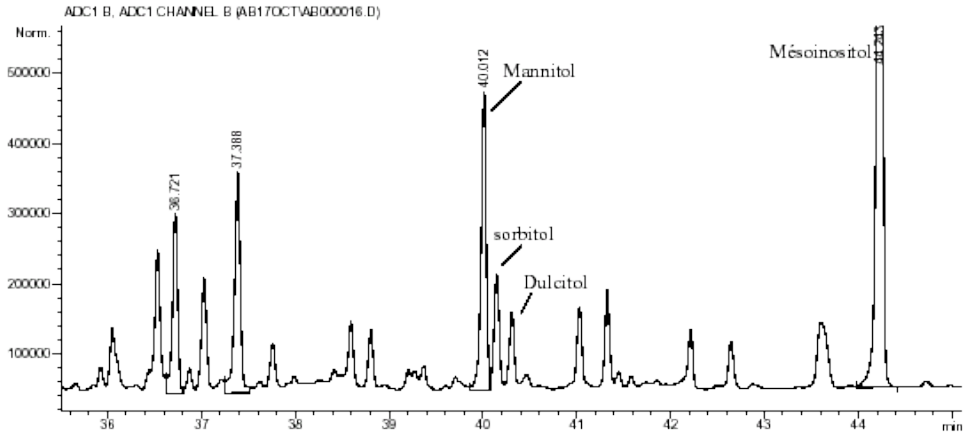
Figure 1

Chromatogram of a white wine following silylation. CPSil-5CB 50 m x 0.25 mm x 0.15 µm column. Split injection, 60°C, 3°C/min, 240°C. Magnification below.



Identification of peaks: 1 : reactive mixture; 2 and 3: unknown acids; 4: pentaerythriol; 5 and 6: unknown; 7: tartaric acid and arabinose; 8, 10 and 11: rhamnose; 9: arabinose; 12: xylitol; 13: arabitol; 14, 15 and 16: fructose; 17: galactose and unknown; 18: glucose α; 19: galactose and galacturonic acid; 20 and 21: unknown; 22: mannitol; 23: sorbitol; 24: glucose β; 25 and 27: unknown; 26: galacturonic acid; 28 and 30: galactonolactone; 29: mucic acid; 31: meso-inositol.

Chromatogram of a white wine following silylation. CPSil-5CB 50 m x 0.25 mm x 0.15 µm column. Split injection, 60°C, 3°C/min, 240°C. Magnification below.



Method OIV-MA-AS311-07 Type III method

Joint determination of the glucose and fructose content in wines by differential ph-metry

(Resolution OENO 10/2006 ; OIV/OENO 377/2009)

1. SCOPE

This method is applicable to the analysis of glucose and fructose in wines between 0 and 60 g/L (average level) or 50 and 270 g/L (high level).

2. PRINCIPLE

The joint determination of glucose and fructose content by differential pH-metry consists in the phosphorylation of the glucose and fructose by hexokinase. The H^+ ions generated stoechiometrically in relation to the quantities of glucose and fructose are then quantified.

3. REACTIONS

The glucose and fructose present are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} + H^+$$

$$\text{fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{fructose-6-phosphate} + \text{ADP} + H^+$$

4. REAGENTS

4.1 Demineralised Water (18 MΩ) or bi-distilled

4.2 2-Amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) purity ≥ 99%

4.3 Disodic adenosine triphosphate (ATP, 2Na) purity ≥ 99%

4.4 Trisodium phosphate with twelve water molecules (Na3PO4·12H2O) purity ≥ 99%

4.5 Sodium hydroxide (NaOH) purity ≥ 98%

4.6 Magnesium chloride with six water molecules ($MgCl_2 \cdot 6H_2O$) purity ≥ 99%

4.7 Triton X 100

4.8 Potassium chloride (KCl) purity ≥ 99%

4.9 2-Bromo-2-nitropropane-1,3-diol (Bronopol) ($C_3H_6BrNO_4$)

4.10 Hexokinase (EC. 2.7.1.1) 1 mg ≅145 U (e.g. Hofmann La Roche, Mannheim, Germany ref. Hexo-70-1351)

4.11 Glycerol purity ≥ 98%

4.12 Glucose purity ≥ 99%

4.13 Reaction buffer pH 8.0 commercial or prepared according to the following method:
In a graduated 100-ml flask (5.2) pour roughly 70 ml (5.3) of water (4.1), and continuously stir (5.5). Add 0.242 g ± 0.001 g (5.4) of TRIS (4.2), 0.787 g ± 0.001 g (5.4) of ATP (4.3), 0.494 g ± 0.001 g (5.4) of sodium phosphate (4.4), 0.009 mg ± 0.001 g (5.4) of sodium hydroxide (4.5), 0.203 g ± 0.001 g (5.4) of magnesium chloride (4.6), 2.000 ± 0.001 g (5.4) of Triton X 100 (4.7), 0.820 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 ± 0.001 g (4.9) of bronopol. Adjust to volume with water (4.1). The final pH must be 8.0 ± 0.1 (5.6), otherwise adjust it with sodium hydroxide or hydrochloric acid. The buffer thus prepared is stable for two months at 4°C.

4.14 Enzyme solution commercial or prepared according to the following method: Using a graduated pipette (5.7) place 5 ml of glycerol (4.11) into a graduated 10-ml flask, adjust to volume with water (4.1) and homogenize. Dissolve 20 mg ±1 mg (5.4) of hexokinase (4.10) and 5 mg of Bronopol (4.9) in 10 ml of the glycerol solution. The activity of the enzyme solution must be 300 U ± 50 U per ml for the hexokinase. The enzyme solution is stable for 6 months at 4°C.

4.15 Preparation of the calibration solution (average level) if the supposed content is less than 50 g/L of glucose + fructose)
Place 3.60 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 36 g/L of glucose. The solution is stable for 6 months at 4 °C.

4.16 Preparation of the calibration solution (high level) if the supposed content is above 50 g/L of glucose + fructose)

Place 18.0 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 180 g/L of glucose. The solution is stable for 6 months at 4°C.

5. APPARATUS

5.1 Differential pH-metry apparatus (EUROCHEM CL 10 plus, Microlab EFA or equivalent) see appendix A

5.2 Graduated 100-ml flask, class A

5.3 Graduated 100-ml test-tube with sole

5.4 Precision balance to weigh within 1 mg

5.5 Magnetic stirrer and magnetic Teflon bar

5.6 pH-meter

5.7 Graduated 3-mL, 5-mL pipettes, class A

5.8 Graduated 10-ml flask, class A

5.9 Automatic syringe pipettes, 25 and 50 µL

6. PREPARATION OF SAMPLES

The samples should not be too charged with suspended matter; in the contrary case, centrifuge or filter them. Sparkling wines must be degassed.

7. PROCEDURE

The operator must respect the instructions for use of the equipment (5.1). Before any use, the instrument must be stabilized in temperature. The circuits must be rinsed with the buffer solution (4.13) after cleaning, if required.

7.1 Determination of the blank (determination of the enzyme signal)
Fill the electrode compartments (EL_1 and EL_2) of the differential pH-meter (5.1) with the buffer solution (4.13); the potential difference between the two electrodes (D_1) must range between ± 150 mpH;
Add 24 µL of enzyme solution (4.14) to the reaction vessel (using the micropipette 5.9 or the preparer) and fill electrode EL_2;
Measure the potential difference (D_2) between the two electrodes;
Calculate the difference in pH, ΔpH_o for the blank using the following formula:

$$\Delta pH_o = D_2 - D_1$$

where
ΔpH_o = the difference in pH between two measurements for the blank;
D_1 = the value of the difference in pH between the two electrodes filled with the buffer solution;
D_2 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer solution and the other with the buffer solution and enzyme solution.
The value of ΔpH_o is used to check the state of the electrodes during titration as well as their possible drift over time; it must lie between –30 and 0 mpH and ≤ 1.5 mpH between two consecutive readings. If not, check the quality of the buffer pH and the cleanliness of the hydraulic system and electrodes, clean if necessary and then repeat the blank.

7.2 Calibration

7.2.1 Average level
Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.13);
Add 25 µL (with the micropipette 5.9 or the preparer) of the standard glucose solution (4.15) to the reaction vessel;
Fill the electrodes EL_1 and EL_2 with the buffer + standard solution;
Measure the potential difference (D_3) between the two electrodes;
Add 24 µL of enzyme solution (4.14) and fill electrode EL_2 with the buffer + standard solution + enzyme;
After the time necessary for the enzymatic reaction, measure the potential difference (D_4) between the two electrodes;
Calculate the difference in pH, ΔpH_c for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 - D_3) - \Delta pH_o$$

where
ΔpH_c = the difference between two measurements D_3 and D_4 for the calibration sample minus the difference obtained for the blank;
D_3 = the value of the difference in pH between the two electrodes filled with the reference buffer/solution mixture;
D_4 = the value of the difference in pH between the two electrodes, one of which is filled with the reference buffer/solution and the other with the buffer/ enzyme / reference solution.

Calculate the slope of the calibration line:

$$s = C_u / \Delta pH_c$$

where
C_u is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 25 µL of standard solution (ML) of glucose (4.15) according to the procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

7.2.2 High level

Fill the electrode compartments (EL_1 and EL_2) with the buffer (4.13);
Add 10 µL (with the micropipette 5.9 or the preparer) of standard solution (HL) of glucose (4.16) to the reaction vessel;
Fill the electrodes EL_1 and EL_2 with the buffer + standard solution mixture;
Measure the potential difference (D_3) between the two electrodes;
Add 24 µL of enzyme solution (4.14) and fill electrode EL_2 with the buffer + standard solution + enzyme mixture;
After the time required for the enzymatic reaction, measure the potential difference (D_4) between the two electrodes;
Calculate the difference in pH, ΔpH_c for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 - D_3) - \Delta pH_o$$

where
ΔpH_c = the difference in pH between two measurements D_3 and D_4 for the calibration sample minus the difference obtained for the blank;
D_3 = the value of the difference in pH between the two electrodes filled with the buffer/ reference solution mixture;
D_4 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/ reference solution and the other with the buffer/ reference solution /enzyme.

Calculate the slope of the calibration line:

$$s = C_u/\Delta pH_c$$

where
C_u is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution of glucose (4.16) in accordance with procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

7.3 Quantification

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.13)
Add 10 µL (high level) or 25 µL (mean level) (with the micropipette 5.9 or the preparer) of the sample solution to the reaction vessel;
Fill electrodes EL_1 and $EL_2$with the buffer + sample mixture;
Measure the potential difference (D_5) between the two electrodes;
Add 24 µL of the enzyme solution (4.14) and fill electrode EL_2 with the buffer mixture + sample + enzyme;
Measure the potential difference (D6) between the two electrodes;
Calculate the quantity of aqueous solution in the sample using the following formula:

$$w = s \times [(D_6 – D_5) - \Delta pH_o]$$

where
w = the quantity of aqueous solution in the sample (in g/L);
S is the slope determined by the calibration line;
ΔpH_o = the difference in pH between two measurements for the blank;

D_5 = the value of the difference in pH between the two electrodes filled with the sample/ reference solution;
D_6 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/sample and the other with the buffer/ sample /enzyme.

8 EXPRESSION OF RESULTS

The results are expressed in g/L of glucose + fructose with one significant figure after the decimal point.

9 PRECISION

The details of the interlaboratory test on the precision of the method are summarized in appendix B.

9.1 Repeatability

The absolute difference between two individual results obtained in an identical matter tested by an operator using the same apparatus, in the shortest interval of time possible, shall not exceed the repeatability value *r* in 95% of the cases.
The value is: $r = 0.021x + 0.289$ where x is the content in g/L of glucose + fructose

9.2 Reproducibility

The absolute difference between two individual results obtained with an identical matter tested in two different laboratories, shall not exceed the reproducibility value of R in 95% of the cases.
The value is: $R = 0.033x + 0.507$ where w is the content in g/L of glucose + fructose

10 OTHER CHARACTERISTICS OF THE ANALYSIS

10.1 Detection and quantification limits

10.1.1 Detection limit
The detection limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to three standard deviations. In this case, the method gave as a result a detection limit of 0.03 g/L. Tests by successive dilutions confirmed this value.

10.1.2 Quantification limit

The quantification limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to ten standard deviations. In this case, the method gave as a result a quantification limit of 0.10 g/L. Tests by successive dilutions confirmed this value. The quantifications of white and red wine carried out by the laboratories that took part in the interlaboratory analysis also confirm these figures.

10.2 Accuracy

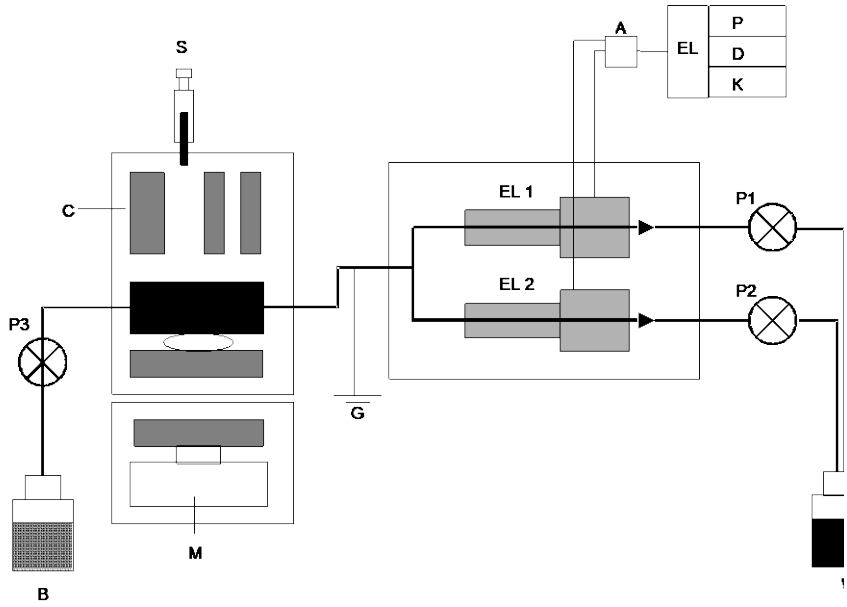
Accuracy is evaluated based on the average coverage rate calculated for the loaded wines analysed double-blind during the interlaboratory test (wines A, B, C, D, F and J). It is equal to 98.9% with a confidence interval of 0.22%.

11. QUALITY CONTROL

Quality controls can be carried out with certified reference materials, wines whose characteristics have been determined by consensus, or loaded wines regularly used in analytical series, and by following the related control charts.

Appendix A

Diagram of the differential pH-metry apparatus



A: differential amplifier; B: buffer solution; C: mixing chamber; D: indicator; EL_1 and EL_2 capillary electrodes; EL: electronics; G: ground; K: keyboard; M: magnetic stirrer; P: printer; P_1 to P_3: peristaltic pumps; S: injection syringe for the sample and enzyme; W: waste.

Appendix B

Statistical data obtained with the interlaboratory test results

In accordance with ISO 5725-2:1994, the following parameters were defined during an interlaboratory test. This test was carried out by the laboratory of the Inter-trade Committee for Champagne Wine in Epernay (France).
Year of the interlaboratory test: 2005
Number of laboratories: 13 double blind
Number of samples: 10

	Wine A	Wine B	Wine C	Wine D	Wine E	Wine F	Wine G	Wine H	Wine I	Wine J
Average in g/L	8.44	13.33	18.43	23.41	28.03	44.88	86.40	93.34	133.38	226.63
Number of laboratories	13	13	13	13	13	13	13	13	13	13
Number of laboratories after elimination of greatest dispersions	13	13	13	13	13	13	13	13	13	13
Standard deviation of repeatability	0.09	0.13	0.21	0.21	0.29	0.39	0.81	0.85	1.19	1.51
Repeatability limit	0.27	0.38	0.61	0.62	0.86	1.14	2.38	2.51	3.52	4.45
RSDr, 100%	1.08	0.97	1.13	0.91	1.04	0.86	0.94	0.91	0.89	0.67
HORRAT r	0.26	0.25	0.31	0.26	0.30	0.27	0.32	0.32	0.33	0.47
Standard deviation of reproducibility	0.17	0.27	0.37	0.59	0.55	0.45	1.27	1.43	1.74	2.69
Reproducibility limit	0.50	0.79	1.06	1.71	1.60	1.29	3.67	4.13	5.04	7.78
RSDR, 100%	2.05	2.05	1.99	2.54	1.97	1.00	1.47	1.53	1.31	1.19
HORRAT R	0.50	0.54	0.55	0.72	0.58	0.31	0.51	0.53	0.48	0.47

Types of samples:
Wine A: white wine naturally containing sugar, loaded with 2.50 g/L glucose and of 2.50 g/L of fructose;
Wine B: white wine naturally containing sugar (wine A), loaded with 5.00 g/L glucose and 50 g/L of fructose;
Wine C: white wine naturally containing sugar (wine A), loaded with 7.50 g/L glucose and 7,50 g/L of fructose;
Wine D: white wine naturally containing sugar (wine A), loaded with 10.0 g/L glucose and 10.0 g/L of fructose;
Wine E: aromatised wine;
Wine F: white wine naturally containing less than 0.4 g/L of sugar, loaded with 22.50 g/L glucose and 22.50 g/L of fructose;
Wine G: naturally sweet red wine;
Wine H: sweet white wine;
Wine I: basis wine;
Wine J: white wine naturally containing less than 0.4 g/L of sugar, loaded with 115.00 g/L glucose and 115.00 g/L of fructose;

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Method OIV-MA-AS311-08 Type IV method

Whole determination of glucose, fructose and saccharose content in wines by differential ph-metry

(Resolution OENO 11/2006 ; OIV/OENO 377/2009)

1. SCOPE

This method is applicable to the analysis of glucose and fructose in wines between 0 and 270 g/L.
This quantification is different from glucose and fructose quantification by its differential pH-metry which can not be substituted.

2. PRINCIPLE

The determination by differential pH-metry of glucose, fructose and saccharose content consists in the preliminary hydroloysis of saccharose by invertase, followed by phosphorylation of the glucose and fructose by hexokinase. The H^+ ions generated stoechiometrically in relation to the quantities of glucose and fructose are then quantified.

3. REACTIONS

Possible traces of saccharose are hydrolysed by invertase (EC 3.2.1.26)

$$\text{Saccharose} \xrightarrow{\text{invertase}} \text{glucose + fructose}$$

The glucose and fructose initially or consecutively present to invertase action are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)

$$\text{glucose + ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate + ADP + } H^+$$

$$\text{fructose + ATP} \xrightarrow{\text{HK}} \text{fructose-6-phosphate + ADP+ } H^+$$

4. REAGENTS

4.1 Demineralised Water (18 MΩ) or bi-distilled

4.2 2-Amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) purity ≥ 99%

4.3 Disodic adenosine triphosphate (ATP, 2Na) purity ≥ 99%

4.4 Trisodium phosphate with twelve water molecules ($Na_3PO_4.12H_2O$) purity ≥ 99%

4.5 Sodium hydroxide (NaOH) purity ≥ 98%

4.6 Magnesium chloride with six water molecules ($MgCl_2.6H_2O$) purity ≥ 99%

4.7 Triton X 100

4.8 Potassium chloride (KCl) purity ≥ 99%

4.9 2-Bromo-2-nitropropane-1,3-diol (Bronopol) ($C_3H_6BrNO_4$)

4.10 Invertase (EC 3.2.1.26) 1 mg ≅ 500 U (ex Sigma ref I-4504)

4.11 Hexokinase (EC. 2.7.1.1) 1 mg ≅145 U (e.g. Hofmann La Roche, Mannheim, Germany ref. Hexo-70-1351)

4.12 Glycerol purity ≥ 98%

4.13 Saccharose purity ≥ 99%

4.14 Reagent buffer pH 8.0 commercial (ex. DIFFCHAMB GEN 644) or prepared according to the following method:
In a graduated 100-ml flask (5.2) pour roughly 70 ml (5.3) of water (4.1), and continuously stir (5.5). Add 0.242 g ± 0.001 g (5.4) of TRIS (4.2), 0.787 g ± 0.001 g (5.4) of ATP (4.3), 0.494 g ± 0.001 g (5.4) of sodium phosphate (4.4), 0.009 mg ± 0.001

g (5.4) of sodium hydroxide (4.5), 0.203 g ± 0.001 g (5.4) of magnesium chloride (4.6), 2.000 ± 0.001 g (5.4) of Triton X 100 (4.7), 0.820 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 ± 0.001 g (4.9) of bronopol. Adjust to volume with water (4.1). The final pH must be 8.0 ± 0.1 (5.6), otherwise adjust it with sodium hydroxide or hydrochloric acid. The buffer thus prepared is stable for two months at 4°C.

4.15 Enzyme solution commercial or prepared according to the following method:
Using a graduated pipette (5.7) place 5 ml of glycerol (4.11) into a graduated 10-ml flask, adjust to volume with water (4.1) and homogenize. Dissolve 300 mg ±1 mg (5.4) of invertase (4.10) 10 mg ± 1 mg (5.4) of hexokinase (4.11) in 3 mL of glycerol solution. Enzyme solution activity must be 50 000 U ± 100 U per ml for intervase and 480 U ± 50 U for hexokinase. The enzyme solution is stable for 6 months at 4°C.

4.16 PREPARATION OF REFERENCE SOLUTION

Place 17,100 g ± 0.01 g (5.4) of saccharose (4.13) (desiccated 12 hours beforehand
at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g (5.4) of bronopol in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 171 g/L of saccharose. The solution is stable for 6 months at 4°C.

5. APPARATUS

5.1 Differential pH-metry apparatus (EUROCHEM CL 10 plus, Microlab EFA or equivalent) see appendix A

5.2 Graduated 100-ml flask, class A

5.3 Graduated 100-ml test-tube with foot

5.4 Precision balance to weigh within 1 mg

5.5 Magnetic stirrer and magnetic Teflon bar

5.6 pH-meter

5.7 Graduated 3-mL, 5-mL pipette, class A

5.8 Graduated 10-ml flask, class A

5.9 Automatic syringe pipettes, 25 and 50 µL

6. PREPARATION OF SAMPLES

Samples must not contain excessive suspended matter. If this occurs, the solution centrifuge and filter. Sparkling wines must be degassed

7. PROCEDURE

The operator must respect the instructions for use of the equipment (5.1). Before any use, the instrument must be stabilized in temperature. The circuits must be rinsed with the buffer solution (4.14) after cleaning, if required.

7.1 Determination of the blank (determination of the enzyme signal)
Fill the electrode compartments (EL_1 and EL_2) of the differential pH-meter (5.1) with the buffer solution (4.14); the potential difference between the two electrodes (D_1) must range between ± 150 mpH;
Add 32 µL of enzyme solution (4.15) to the reaction vessel (using the micropipette 5.9 or the preparer) and fill electrode EL_2;
Measure the potential difference (D_2) between the two electrodes;
Calculate the difference in pH, ΔpH_o for the blank using the following formula:

$$\Delta pH_o = D_2 - D_1$$

where
ΔpH_o = the difference in pH between two measurements for the blank;
D_1 = the value of the difference in pH between the two electrodes filled with the buffer solution;
D_2 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer solution and the other with the buffer solution and enzyme solution.
The value of ΔpH_o is used to check the state of the electrodes during titration as well as their possible drift over time; it must lie between –30 and 0 mpH and ≤ 1.5 mpH between two consecutive readings. If not, check the quality of the buffer pH and the cleanliness of the hydraulic system and electrodes, clean if necessary and then repeat the blank.

7.2 Calibration

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.14);
Add 10 µL (with the micropipette 5.9 or the preparer) of the standard saccharose solution (5) to the reaction vessel;
Fill the electrodes EL_1 and EL_2 with the buffer + standard solution;
Measure the potential difference (D_3) between the two electrodes;
Add 32 µL of enzyme solution (4.15) and fill electrode EL_2 with the buffer + standard solution + enzyme;
After the time necessary for the enzymatic reaction, measure the potential difference (D_4) between the two electrodes;

Calculate the difference in pH, ΔpH_c for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 – D_3) - \Delta pH_o$$

where
ΔpH_c = the difference between two measurements D_3 and D_4 for the calibration sample minus the difference obtained for the blank;

D_3 = the value of the difference in pH between the two electrodes filled with the reference buffer/solution mixture;
D_4 = the value of the difference in pH between the two electrodes, one of which is filled with the reference buffer/solution and the other with the buffer/ enzyme / reference solution.
Calculate the slope of the calibration line:

$$s = C_u / \Delta pH_c$$

where
C_u is the concentration of saccharose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution (ML) of saccharose (5) according to the procedure (8.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

7.3 Quantification

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.14)
Add 10 µL (with the micropipette 5.9 or the preparer) of the sample solution to the reaction vessel;
Fill electrodes EL_1 and $EL_2$with the buffer + sample mixture;
Measure the potential difference (D_5) between the two electrodes;
Add 32 µL of the enzyme solution (4.15) and fill electrode EL_2 with the buffer mixture + sample + enzyme;
Measure the potential difference (D6) between the two electrodes;
Calculate the quantity of aqueous solution in the sample using the following formula:

$$w = s \times [(D_6 – D_5) - \Delta pH_o]$$

where

w = the quantity of aqueous solution in the sample (in g/L);
S is the slope determined by the calibration line;
ΔpH_o = the difference in pH between two measurements for the blank;
D_5 = the value of the difference in pH between the two electrodes filled with the sample/ reference solution;
D_6 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/sample and the other with the buffer/ sample /enzyme.

8 EXPRESSION OF RESULTS

The results are expressed in g/L of glucose with one significant figure after the decimal point.

9 CHARACTERISTICS OF THE ANALYSIS

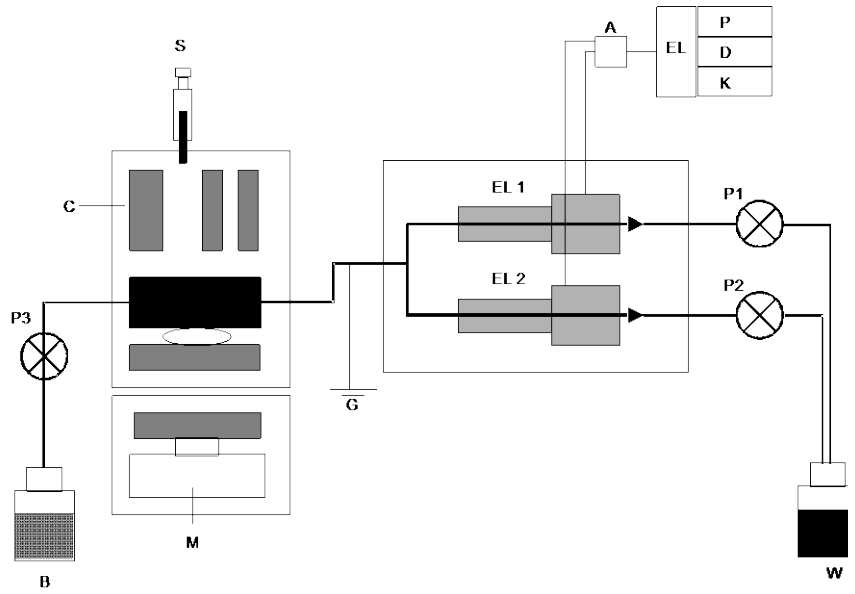
Due to the hydrolysis of saccharose in wines and musts, it is not possible to organise an inter-laboratory analysis according to the OIV protocol.
Inter-laboratory studies of this method demonstrate that for saccharose, the linearity between 0 and 250 g/l, a detection limit of 0.2 g/l, a quantification limit of 0.6 g/l, repeatability of 0.0837x -0.0249 g/l and reproducibility of 0.0935x - 0.073 g/l (saccharose content).

10 QUALITY CONTROL

Quality controls can be carried out with certified reference materials, wines whose characteristics have been determined by consensus, or loaded wines regularly used in analytical series, and by following the related control charts.

Appendix A

Diagram of the differential pH-metry apparatus



A: differential amplifier; B: buffer solution; C: mixing chamber; D: indicator; EL_1 and EL_2 capillary electrodes; EL: electronics; G: ground; K: keyboard; M: magnetic stirrer; P: printer; P_1 to P_3: peristaltic pumps; S: injection syringe for the sample and enzyme; W: waste.

Appendix B

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Method OIV-MA-AS311-09 Type II and III method

Determination of the $^{13}C/^{12}C$ isotope ratios of glucose, fructose, glycerol, ethanol in production of vitivinicultural origin by high-performance liquid chromatography coupled to isotope ratio mass spectrometry

(Resolution OIV-OENO 479-2017)

1. Scope of application

This method applies to products of vitivinicultural origin.
This method is:

- type II for glucose, fructose and glycerol,
- type III for ethanol.

2. Principle

The samples are injected into the HPLC instrument after any necessary dilution and filtration. After oxidation in a liquid interface, the $^{13}C/^{12}C$ isotope ratio of the compounds is determined using isotope ratio mass spectrometry. This liquid interface, symbolised by the acronym "*co"*, permits the chemical oxidation of the organic matter into CO2. HPLC-*co*-IRMS coupling can therefore be used to determine the isotope ratio of the following compounds simultaneously: glucose, fructose, glycerol and ethanol.

3. Reagents

3.1 Pure water - resistivity > 18 MΩ cm, HPLC quality
3.2 Ammonium persulfate – analytical purity – [CAS No.: 7727-54-0]
3.3 Orthophosphoric acid (concentration 85%) – analytical purity – [CAS No.: 7664-38-2]
3.4 Analytical-grade helium, used as a carrier gas [CAS No.: 07440-59-7]
3.5 Reference gas: analytical-grade CO_2 (carbon dioxide), used as a secondary reference gas [CAS No.: 00124-38-9]
3.6 International standards

4. Equipment

4.1 Everyday laboratory equipment
4.2 High-performance liquid chromatography instrument
4.3 Liquid interface for the oxidation of eluted compounds
4.4 Isotope ratio mass spectrometer

5. Analysis of the samples

5.1 Preparation of the samples

Depending on the sugar, glycerol and ethanol contents, the samples should be diluted with the water (3.1) beforehand in order to obtain a concentration which is observable under the experimental conditions. Depending on the concentrations of the compounds, two measurements are needed with different dilutions.

5.2 Example of analytical conditions

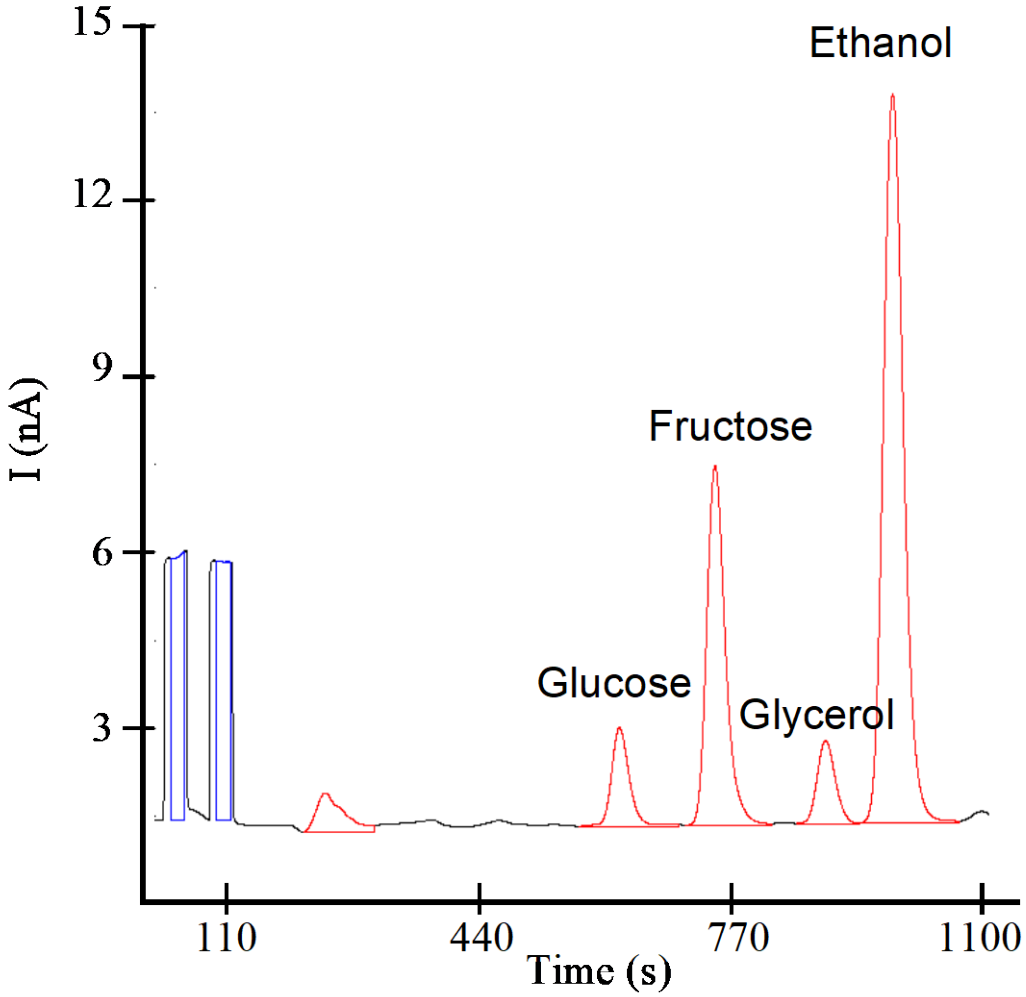
Total analysis duration: 20 minutes
As an indication, the dilution of grape juices and wines is around 1:200, while that of concentrated musts is approximately 1:500.
HPLC:
Column: carbohydrate-type column (e.g. 700-CH Carbohydrate column, HyperRez XP Carbohydrate H^+)
Injection volume: 25 µl
Mobile phase: water (3.1)
Flowrate: 0.4 mL/min
Column T°: 80 °C

Liquid Interface:
Solution of ammonium persulfate (3.2) (15% in mass) and orthophosphoric acid (2.5% in volume)
Peristaltic pump flow: 0.6 mL/min
Heater temperature: 93 °C
Flow of the helium carrier gas: 15 mL/min
Helium flow for drying: 50 mL/min

IRMS:
Trap current: 300 µA

5.3 Example chromatogram

Chromatogram of a sweet wine analysed using HPLC-*co*-IRMS



6. Determination of isotope ratios

The reference gas, CO_2, is calibrated from international commercial standards. The isotope ratios are expressed in δ ‰ in relation to the Pee Dee Belemnite (PDB) and are defined as:

$$\delta^{13}C_{Sam}\ (‰) = [(R_{Sam} / R_{St}) - 1\ ] * 10^3$$

Where: Sam = sample; St = standard; R = $^{13}C/^{12}C$ isotope ratio

7. Method characteristics

The characteristics of the method for the measurement of the $\delta^{13}C$ isotope ratios of glucose, fructose, glycerol and ethanol by HPLC-*co*-IRMS have been determined from the results obtained from an inter-laboratory analysis of four samples (dry wine, sweet wine, grape juice and rectified concentrated must). The results obtained for each compound analysed and each type of matrix are annexed.

8. Bibliography

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Annex

Statistical treatment of the HPLC-*co*-IRMS inter-laboratory analysis for the determination of the precision of the method (repeatability and reproducibility)

List of laboratories in alphabetical order of country of origin.

Country	Laboratory
Belgium	IRMM
China	CNRIFFI
Czech Republic	SZPI
France	SCL-33
Germany	INTERTEK
Germany	UNI DUE
Germany	ELEMENTAR
Germany	QSI
Germany	LVI
Italy	FLORAMO
Japan	AKITA Univ.
Spain	MAGRAMA

Responses:

12 laboratories / 14 responses

Treatment of the results of inter-laboratory analyses according to ISO 5725-2

Samples:

1 dry wine (Wine A)
1 sweet wine (Wine B)
1 rectified concentrated must (RCM)
1 grape juice

Analytical conditions:

Each sample was analysed in duplicate (repeatability) and double blind (reproducibility)

Expression of results in ‰ vs. PDB

Precision of the glucose measurement

Repeatability and reproducibility

	Wine B	RCM	Grape juice
Number of laboratories	12	12	12
Number of responses	14	13	14
Number of responses retained (elimination of outliers)	13	13	12
Minimum value	-26.33	-25.04	-25.78
Maximum value	-23.72	-23.74	-24.62
Mean value	-25.10	-24.24	-25.19
Repeatability variance	0.02	0.01	0.01
Repeatability standard deviation (S_r)	0.14	0.10	0.09
Repeatability limit (r ‰)	0.40	0.29	0.24
Reproducibility variance	0.39	0.14	0.11
Reproducibility standard deviation (S_R)	0.62	0.38	0.33
Reproducibility limit (R ‰)	1.77	1.06	0.94

Precision of the fructose measurement

Repeatability and reproducibility

	Wine B	RCM	Grape juice
Number of laboratories	12	11	12
Number of responses	14	13	14
Number of responses retained (elimination of outliers)	13	13	13
Minimum value	-25.56	-24.19	-25.33
Maximum value	-24.12	-23.19	-23.98
Mean value	-24.87	-23.65	-24.56
Repeatability variance	0.02	0.03	0.02
Repeatability standard deviation (S_r)	0.14	0.16	0.14
Repeatability limit (r ‰)	0.40	0.46	0.39
Reproducibility variance	0.15	0.10	0.18
Reproducibility standard deviation (S_R)	0.39	0.32	0.42
Reproducibility limit (R ‰)	1.10	0.90	1.19

Precision of the glycerol measurement

Repeatability and reproducibility

	Wine A	Wine B
Number of laboratories	12	12
Number of responses	12	12
Number of responses retained (elimination of outliers)	11	11
Minimum value	-32.91	-30.74
Maximum value	-30.17	-28.27
Mean value	-31.75	-29.54
Repeatability variance	0.13	0.04
Repeatability standard deviation (S_r)	0.36	0.19
Repeatability limit (r ‰)	1.03	0.55
Reproducibility variance	0.57	0.37
Reproducibility standard deviation (S_R)	0.76	0.61
Reproducibility limit (R ‰)	2.14	1.72

Precision of the ethanol measurement

Repeatability and reproducibility

	Wine A	Wine B
Number of laboratories	12	12
Number of responses	11	12
Number of responses retained (elimination of outliers)	10	12
Minimum value	-27.85	-27.60
Maximum value	-26.50	-26.06
Mean value	-27.21	-26.82
Repeatability variance	0.03	0.03
Repeatability standard deviation (S_r)	0.16	0.17
Repeatability limit (r ‰)	0.47	0.47
Reproducibility variance	0.16	0.23
Reproducibility standard deviation (S_R)	0.40	0.47
Reproducibility limit (R ‰)	1.14	1.34

Method OIV-MA-A311-10 Type III method

Determination of D-glucose and D-fructose in wines by automated enzymatic method

(Résolution OIV-OENO 600-2018)

1. Scope of application

This method makes it possible to determine the sum of D-glucose and D-fructose in wine by specific enzyme analysis using an automatic sequential analyser.
In this document a collaborative study is reported which demonstrates application of the method for measurement of D-glucose and D-fructose from 0.1 to 96.31 g/L, taking into account the introduction of a dilution of the sample above 5 g/L.

Note: Where necessary, each laboratory using this method may refine, and potentially widen, this range through a validation study.

2. Standard references

- OIV *Compendium of International Methods of Analysis*: Glucose and fructose – enzymatic method, OIV-MA-AS311-02,
- ISO 78-2: Chemistry – Layouts for standards.

3. Reaction principles

D-glucose and D-fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) to produce glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P).

$$D-glucose \; + \; ATP \overset{HK}{\leftrightarrow} G6P \; + \; ADP$$

$$D - fructose + ATP \overset{HK}{\leftrightarrow} F6P + ADP$$

Glucose-6-phosphate is first oxidised to gluconate-6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) is directly correlated with that of glucose-6-phosphate and thus with that of D-glucose.

$$G6P + NADP^{+} \overset{G6PDH}{\longleftrightarrow} gluconate - 6 - phosphate + NADPH + H^{+}$$

Fructose-6-phosphate (F6P) is converted into glucose-6-phosphate (G6P) in the presence of phosphoglucose isomerase (PGI):

$$F6P \overset{PGI}{\leftrightarrow} G6P$$

The glucose-6-phosphate thus formed reacts as shown in the above formula.
The reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced is measured based on its absorption at 340 nm.

4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical usage (ISO 3696 standard)

4.1.2. Triethanolamine hydrochloride (CAS no. 637-39-8)

4.1.3. NADP (nicotinamide adenine dinucleotide phosphate) (CAS no. 24292-60-2)

4.1.4. ATP (adenosine-5'-triphosphate) (CAS no. 34369-07-8)

4.1.5. MgSO4 (anhydrous magnesium sulphate) (CAS no. 7487-88-9)

4.1.6. Sodium hydroxide (CAS no. 1310-73-2)

4.1.7. Hexokinase (HK) (CAS no. 9001-51-8)

4.1.8. Glucose-6-phosphate dehydrogenase (G6PDH) (CAS no. 9001-40-5)

4.1.9. Phosphoglucose isomerase (PGI): lyophilised powder, 400-600 units/mg protein (CAS no. 9001-41-6)

Note: One unit ensures the conversion of 1.0 µmole of D-fructose-6-phosphate into D-glucose-6-phosphate per minute at pH 7.4 and 25 °C

4.1.10. Polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8)

4.1.11. D-glucose: purity ≥ 99.5% (CAS no. 50-99-7)

4.1.12. D-fructose: purity ≥ 99% (CAS no. 57-48-7)

Note 1: There are commercial kits for the determination of D-glucose and D-fructose. The user needs to check the composition to ensure it contains the above-indicated reagents.

Note 2: The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme protein molecules. This is the case particularly in red wines. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

4.2. Working solutions

4.2.1. Triethanolamine hydrochloride buffer and magnesium sulphate adjusted to pH 7.6. The preparation may be as follows:

- triethanolamine hydrochloride (4.1.2): 11.2 g,
- magnesium sulphate (4.1.5): 0.2 g,
- PVP (4.1.10): 2 g,

- water for analytical usage (4.1.1): 150 mL.

The mixture is adjusted to pH 7.6 using a 5 M sodium hydroxide solution, then made up to 200 mL with water for analytical usage. The solution is stable for at least 4 weeks at 2-8 °C.

4.2.2. R1 working solution (example):
- triethanolamine buffer (4.2.1): 50 mL,
- NADP (4.1.3): 117 mg,
- ATP (4.1.4): 150 mg.

4.2.3. R2 working solution (example):
- triethanolamine buffer (4.2.1): 2 mL,
- HK (4.1.7): 270 U,
- G6PDH (4.1.8): 340 U,
- PGI (4.1.9): 640 U.

Note: Commercial preparations of a HK/6GPDH mixture may be used.

Note: When preparing these solutions, they should be mixed gently to prevent foam from forming. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

4.3. Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of D-glucose and D-fructose prepared by weighing and covering the measurement range.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with UV detector. The reaction temperature should be stable (around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software ensuring its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a

minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature is 37 °C. Certain pieces of apparatus may use slightly different values.

5.1.5. Wavelength

The wavelength of maximum absorption of the NADPH formed by the reaction is 340 nm. This wavelength will be selected for the spectrophotometers commonly used. Some analysers are equipped with photometers that use a mercury-vapor lamp. In this case, a wavelength with a reading of 365 or 334 nm is to be selected.

5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

5.3. pH meter

5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

6. Sampling

6.1 Preparation of samples of musts and wines

The majority of wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

filtration should be used for highly turbid samples,
sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values exceeding the measurement range. By way of example, factors of

10x, 20x or 40x are used for musts. Given their impact on the uncertainty budget, these dilutions should be conducted with the utmost care.

6.2 Preparation of samples of wines containing CO_2

Wine samples containing CO_2 may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly observed. This also applies to the different enzymatic kits available on the market.

The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction takes place.

By way of example, the quantities of different elements may be as follows:

- sample: 2.0 µL,
- R1: 40 µL,
- R2: 40 µL.

The equipment takes regular measurements (every 12 seconds, for example) that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

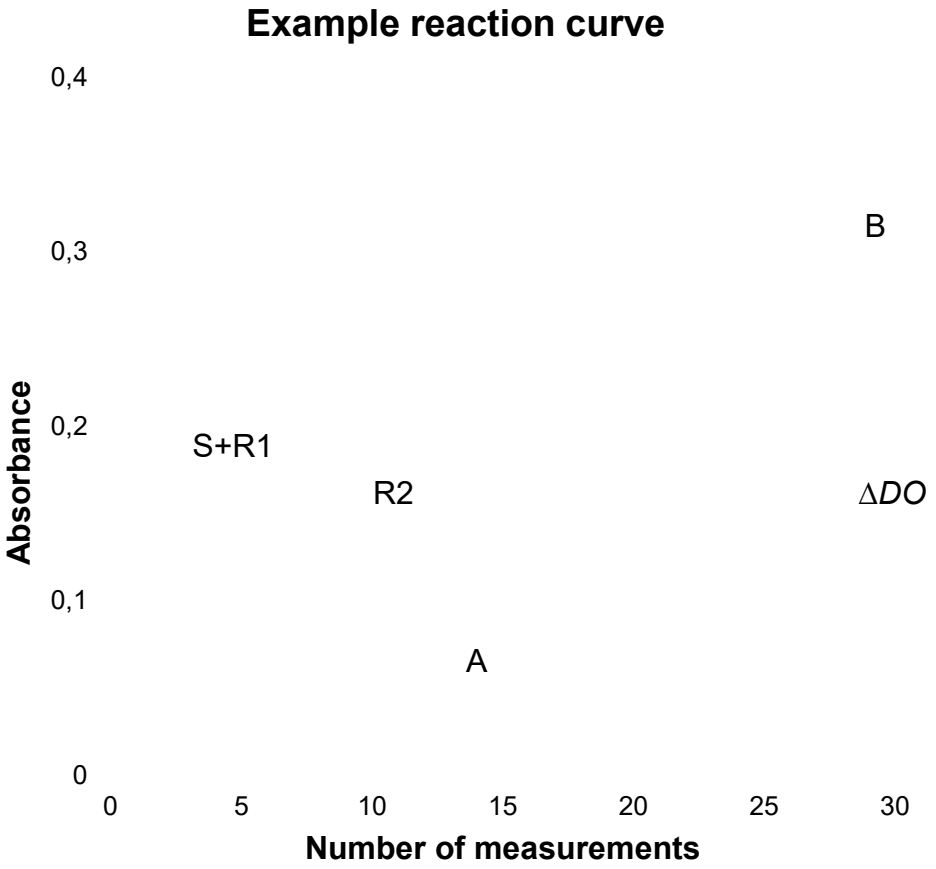


Figure 1: Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

8. Calculation of results

The measurement used for the determination of the result is as follows:

$$\Delta DO = (\text{Absorbance } B - \text{Absorbance } A)$$

In order to correlate this ΔDO value with the desired concentration of D-glucose and D-fructose, calibration of the equipment is carried out using the calibration solutions at a minimum of 3 points (§4.3) covering the measurement range. In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

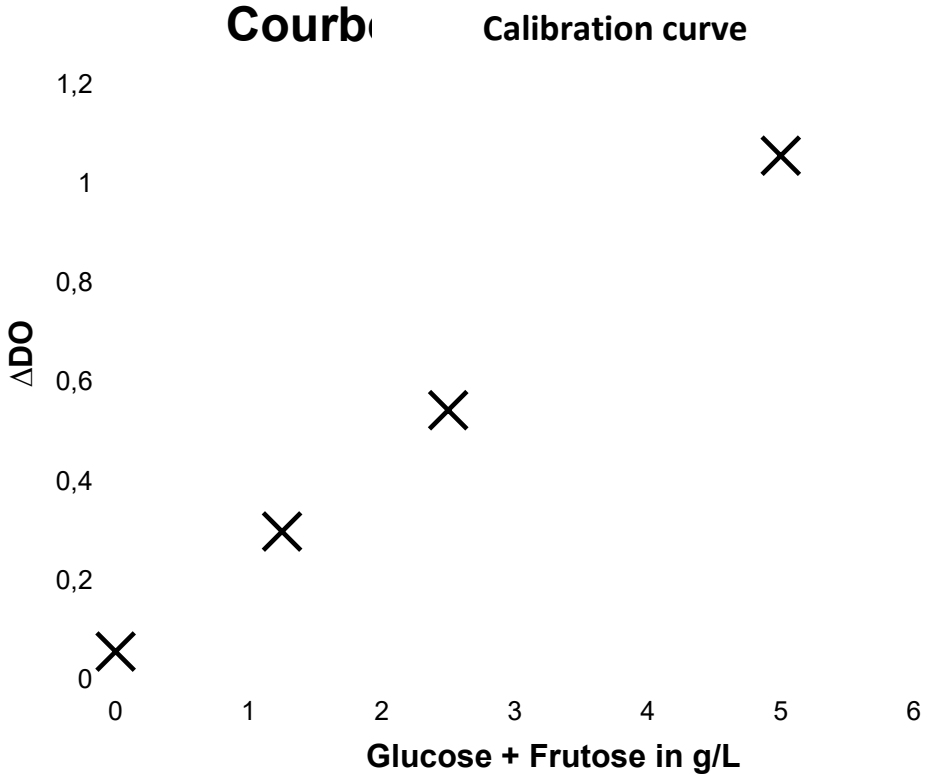


Figure 2: Calibration curve

The calibration curve can be order 1 (*Concentration* = $a.\Delta DO + b$) or even order 2 (*Concentration* = $a.\Delta DO^2 + b.\Delta DO + c$). If using a calibration curve of order 2, the laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve).

The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The D-glucose + D-fructose results are expressed in g/L to 2 d.p.

10. Precision

Interlaboratory reproducibility

RSD_R = 5% (from 1 g/L)
CV_R% (k=2) = 2·RSD_R= 10%, (from 1 g/L)

Repeatability

RSD_r= 1.5% (from 1 g/L)
CV_r% (k=2) = 2·RSD_r = 3% (from 1 g/L)

Limit of quantification

Validated LOQ = 0, 10 g/L
(Concentration where CV_R% (k=2) = 60%)

ANNEX
Results of the interlaboratory tests

Collaborative study
A total of 17 laboratories from different countries participated in the collaborative study, organised in 2016.

Labo	Country
Miguel Torres S.A.- Finca Mas La Plana	SPAIN
Estación Enológica de Castilla y León	SPAIN

INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	SPAIN
Estación Enológica de Haro	SPAIN
Instituto dos Vinhos do Douro e do Porto, IP	PORTUGAL
Comissão de Viticultura da Região dos Vinhos Verdes	PORTUGAL
Laboratoires Dubernet	FRANCE
Laboratoire Dicœnos Rhône	FRANCE
Laboratoire Natoli	FRANCE
SCL Montpellier	FRANCE
Agricultural institute of Slovenia	SLOVENIA
Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	GERMANY
HBLAuBA Wein - und Obstbau	AUSTRIA
Landesuntersuchungsamt Mainz	GERMANY
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	GERMANY
Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach	ITALY
Unione Italiana Vini soc. Coop.	ITALY

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur wines.

Sample		A		B		C		D		E		F		G		H		I		J	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Labo3	rep#1	94.00	96.00	3.40	3.50	**0.40**	**0.40**	0.90	1.10	**2.10**	**2.50**	0.10	0.10	1.40	1.40	5.60	5.90	**4.70**	**4.20**	17.50	17.00
	rep#2	96.00	98.00	3.50	3.60	**0.40**	**0.30**	1.00	1.10	**2.20**	**2.40**	0.10	0.10	1.40	1.40	5.70	6.00	**4.30**	**4.50**	17.50	17.00
Labo6	rep#1	97.50	95.00	3.42	3.25	**0.35**	**0.48**	1.05	0.98	**3.24**	**2.65**	0.08	0.05	1.42	1.40	5.49	5.57	4.04	4.11	13.63	19.00
	rep#2	97.00	94.50	3.39	3.29	**0.37**	**0.57**	1.08	1.01	**3.34**	**2.66**	0.08	0.08	1.52	1.45	5.42	5.52	3.95	4.13	13.70	20.50
Labo7	rep#1	99.22	99.53	3.46	3.56	0.31	0.34	1.00	0.98	2.50	2.58	0.04	0.04	1.49	1.39	5.77	5.75	4.26	4.35	17.66	17.35
	rep#2	100.30	98.90	3.53	3.53	0.31	0.32	1.02	1.02	2.48	2.50	0.04	0.02	1.48	1.34	5.89	5.79	4.23	4.40	17.21	17.94
Labo9	rep#1	92.00	94.20	3.05	3.03	0.29	0.30	0.93	0.97	2.30	2.16	0.04	0.04	1.25	1.25	5.02	5.01	3.98	3.76	15.60	15.76
	rep#2	95.00	97.25	3.03	3.23	0.32	0.31	0.94	0.90	2.20	2.29	0.03	0.04	1.27	1.25	5.14	5.39	3.80	4.06	16.64	16.40
Labo10	rep#1	90.79	92.31	3.27	3.36	0.34	0.34	0.97	1.01	2.28	2.30	0.09	0.07	1.28	1.26	5.46	5.42	3.27	3.36	17.92	17.99

	rep#2	92.13	91.65	3.34	3.24	0.32	0.35	0.97	1.04	2.28	2.33	0.08	0.08	1.32	1.28	5.18	5.37	3.34	3.24	17.58	17.68
Labo11	rep#1	91.40	91.28	3.06	3.12	0.57	0.30	0.95	0.93	2.15	2.18	0.07	0.05	1.16	1.22	5.19	5.34	3.70	3.86	16.22	16.47
	rep#2	90.13	89.94	3.10	3.14	0.56	0.30	0.93	0.93	2.14	2.18	0.07	0.06	1.16	1.20	5.28	5.18	3.76	3.86	16.13	16.33
Labo12	rep#1	100.00	100.00	3.25	3.27	0.34	0.33	1.03	1.10	**2.35**	**2.75**	0.08	0.10	1.30	1.39	5.66	5.64	4.07	4.13	17.30	17.44
	rep#2	101.00	97.00	3.22	3.25	0.34	0.33	1.03	1.11	**2.36**	**2.75**	0.08	0.10	1.30	1.39	5.62	5.68	4.07	4.15	17.50	17.80
Labo13	rep#1	96.60	96.00	3.04	3.07	0.34	0.31	0.97	0.94	2.26	2.50	0.05	0.04	1.25	1.25	5.21	5.29	3.84	3.99	16.08	16.03
	rep#2	96.00	95.10	3.07	3.12	0.32	0.32	0.97	1.04	2.25	2.25	0.04	0.04	1.25	1.28	5.24	5.31	3.90	3.97	15.95	16.18
Labo14	rep#1	**104.00**	**98.00**	3.19	3.16	0.33	0.33	0.97	0.96	2.47	2.44	0.05	0.05	1.34	1.32	5.77	5.81	4.20	4.21	17.76	17.04
	rep#2	**103.00**	**96.00**	3.18	3.17	0.33	0.33	0.97	0.97	2.48	2.44	0.05	0.05	1.34	1.32	5.77	5.78	4.20	4.14	17.44	17.24
Labo15	rep#1	**110.03**	**99.25**	3.63	3.60	0.20	0.19	0.94	0.97	2.54	2.36			1.30	1.20	**5.65**	**6.14**	4.56	4.43	**17.16**	**19.33**
	rep#2	**104.39**	**99.34**	3.59	3.72	0.20	0.20	0.94	0.95	2.52	2.32			1.32	1.20	**5.62**	**6.19**	4.39	4.54	**17.41**	**19.29**
Labo16	rep#1	95.20	94.08	3.20	3.22	0.32	0.32	0.96	0.96	2.24	2.26	0.06	0.06	1.23	1.23	5.19	5.19	3.89	3.84	17.82	17.38
	rep#2	96.00	94.41	3.17	3.18	0.31	0.33	0.95	0.94	2.25	2.22	0.06	0.06	1.24	1.22	5.13	5.15	3.85	3.86	17.84	17.24

Labo17	rep#1	96.68	97.10	3.28	3.38	0.47	0.43	1.03	1.03	2.41	2.46	**0.10**	**0.20**	1.36	1.36	5.52	5.53	4.09	4.00	16.42	17.30
	rep#2	97.08	99.40	3.24	3.33	0.39	0.38	0.95	0.96	2.30	2.36	**0.20**	**0.15**	1.32	1.24	5.38	5.40	3.95	4.10	16.50	16.60
Labo18	rep#1	90.23	91.39	3.14	3.26	0.46	0.47	1.12	1.10	2.30	2.44	**0.23**	**0.24**	1.38	1.30	5.19	5.49	3.91	4.10	14.83	14.89
	rep#2	90.02	91.74	3.18	3.31	0.47	0.47	1.07	1.07	2.31	2.40	**0.23**	**0.24**	1.38	1.32	5.23	5.45	3.94	4.04	14.82	14.85
Labo19	rep#1	99.63	103.55	3.34	3.41	0.32	0.32	0.98	0.97	2.38	2.41	0.04	0.05	1.29	1.30	5.68	5.56	4.10	4.11	17.61	17.49
	rep#2	100.57	103.28	3.36	3.42	0.32	0.32	0.98	0.97	2.36	2.42	0.05	0.05	1.29	1.31	5.61	5.59	4.10	4.11	17.53	17.51
Labo20	rep#1	96.41	96.18	3.20	3.23	0.32	0.32	0.96	0.95	2.26	2.32	0.07	0.08	1.24	1.24	5.35	5.40	3.92	4.03	16.36	16.51
	rep#2	96.32	95.89	3.18	3.23	0.32	0.32	0.96	0.95	2.26	2.32	0.07	0.08	1.24	1.24	5.35	5.38	3.92	4.03	16.38	16.49
Labo21	rep#1	103.60	102.02	3.37	3.60	0.23	0.25	0.95	0.98	2.41	2.49	0.05	0.05	1.27	1.33	5.95	6.12	**4.02**	**4.53**	**18.41**	**19.70**
	rep#2	102.50	103.02	3.34	3.51	0.23	0.26	0.92	0.98	2.45	2.45	0.03	0.05	1.26	1.27	6.02	5.99	**4.09**	**4.42**	**18.96**	**19.90**
Labo22	rep#1	96.73	96.59	3.25	3.28	0.28	0.28	0.92	0.93	2.25	2.31	0.06	0.05	1.23	1.28	5.51	5.47	4.02	3.98	17.09	17.10
	rep#2	97.06	96.34	3.24	3.21	0.30	0.30	0.93	0.93	2.26	2.30	0.04	0.05	1.21	1.24	5.40	5.39	4.03	4.04	17.05	17.01

Table of the data obtained. The values in bold correspond with the values rejected in accordance with the Cochran (variance

outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

Sample	A	B	C	D	E	F	G	H	I	J
No. of laboratories selected	15	17	14	17	14	14	17	16	15	14
No. of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	90.69	3.08	0.20	0.93	2.16	0.04	1.19	5.14	3.80	14.85
Max.	102.79	3.64	0.47	1.09	2.52	0.10	1.45	6.02	4.48	17.79
Overall average	96.31	3.29	0.32	0.98	2.34	0.06	1.30	5.50	4.05	16.86
Repeatability variance	1.449	0.004	0.000	0.001	0.004	0.000	0.001	0.009	0.005	0.065
Inter-laboratory stand. dev.	3.60	0.16	0.06	0.05	0.10	0.02	0.07	0.26	0.17	0.83
Reproducibility variance	14.037	0.029	0.004	0.003	0.013	0.000	0.006	0.073	0.034	0.739
Repeatability variance	1.20	0.06	0.01	0.04	0.06	0.01	0.04	0.09	0.07	0.26
r limit	3.40	0.17	0.04	0.10	0.17	0.02	0.11	0.26	0.21	0.72

Repeatability RSD_r	1.2%	1.8%	4.4%	3.6%	2.5%	13.2%	2.9%	1.7%	1.8%	1.5%
Reproducibility stand. dev.	3.75	0.17	0.07	0.06	0.11	0.02	0.08	0.27	0.19	0.86
R limit	10.60	0.48	0.19	0.16	0.32	0.06	0.22	0.76	0.52	2.43
Reproducibility RSD_R	3.9%	5.1%	20.4%	5.7%	4.8%	35.3%	6.1%	4.9%	4.6%	5.1%
Horwitz RSD_r	1.877	3.120	4.425	3.742	3.284	5.694	3.588	2.889	3.025	2.440
$Horrat_r$	0.666	0.587	1.001	0.952	0.773	2.315	0.804	0.585	0.593	0.621
Horwitz RSD_R	2.84	4.73	6.70	5.67	4.98	8.63	5.44	4.38	4.58	3.70
$Horrat_R$	1.368	1.086	3.036	0.997	0.965	4.087	1.123	1.122	1.000	1.378

Table of the results obtained

Note: The results from sample F should be taken with caution due to the very low concentration levels, which are below to the laboratories' limit of quantification.

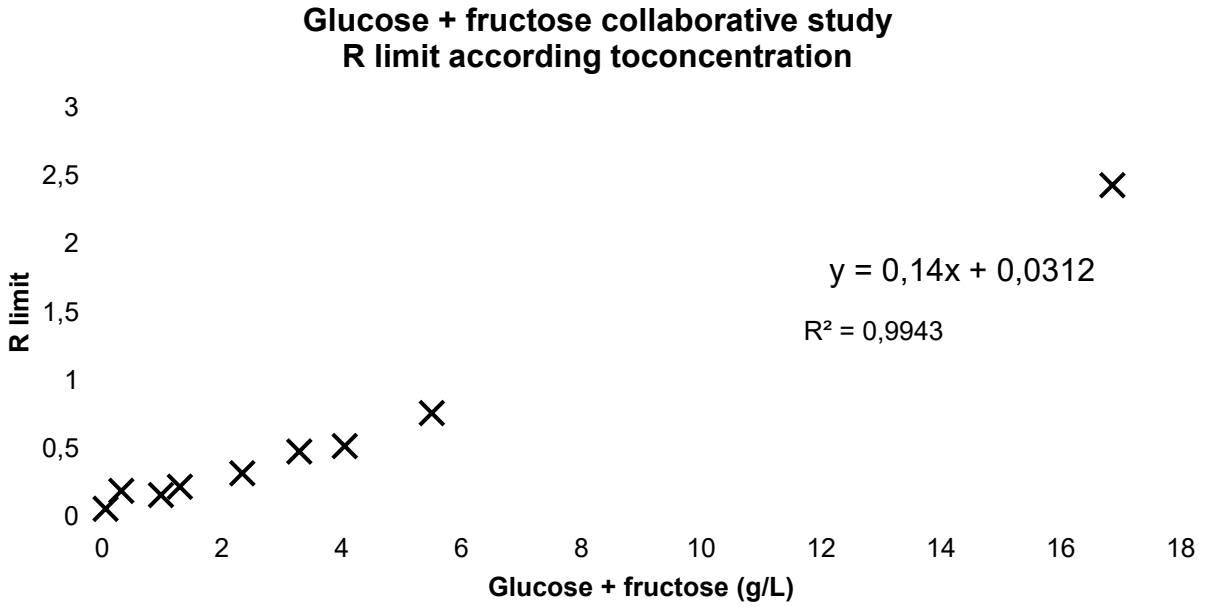

Glucose + fructose collaborative study
R limit according toconcentration
R limit
Glucose + fructose (g/L)
y = 0,14x + 0,0312
R² = 0,9943
3
2,5
2
1,5
1
0,5
0
0
2
4
6
8
10
12
14
16
18

Figure 3: R limit according to concentration

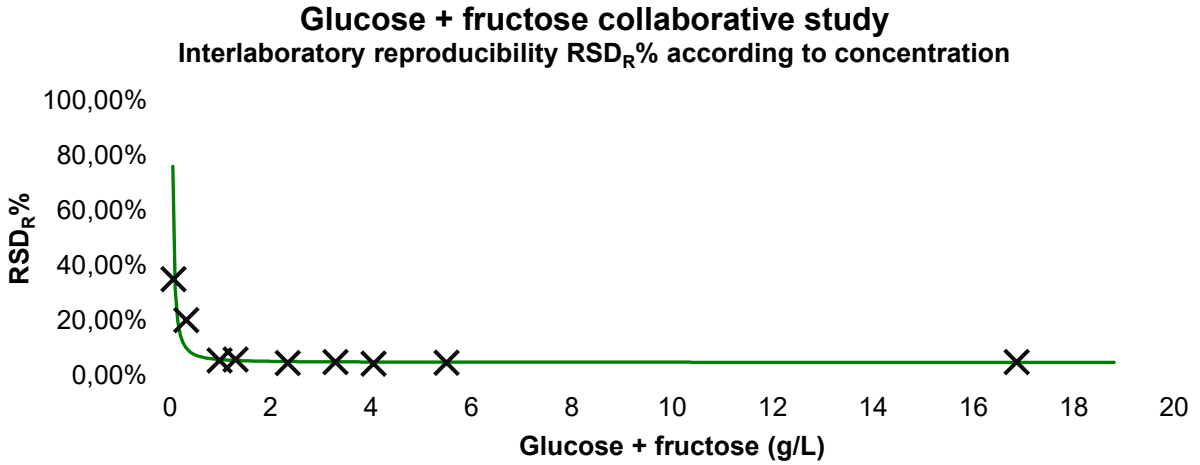


Figure 4: Interlaboratory RSD_R % according to concentration.
Modelling: $\mathbf{RSD_R\% = 1 \cdot C^{(-1.424)} + 5}$

Method OIV-MA-AS312-01 Type I and Type IV Methods

Alcoholic strength by volume

(A2; OENO 8/2000
OENO 24/2003
OIV/OENO 377/2009
OIV-OENO 601B - 2021

1 Introduction

This resolution is made up of one part on distillate preparation, followed by 4 methods (A, B, C and D) to determine the alcoholic strength by volume of this distillate.

2 Scope of application

This resolution is applicable for determining the alcoholic strength by volume at 20 °C of vitivinicultural beverages, using any of the following:

- Method A: Pycnometry — Type I Method,
- Method B: Electronic densimetry using a frequency oscillator — Type I Method,
- Method C: Hydrostatic balance — Type I Method,
- Method D: Hydrometry and refractometry — Common Type IV Method.

3 Definition

The alcoholic strength by volume (ABV) of a beverage is the number of litres of ethanol contained in 100 litres of hydroalcoholic solution with the same density as the beverage distillate; both volumes being determined at a temperature of 20 °C. It is expressed by the symbol '% vol.'.

4 Principle and methods

4.1 Principle

The principle of the method consists firstly of distilling the beverage by volume to volume after alkalinisation by a suspension of calcium hydroxide, which prevents the entrainment of volatile acids. This distillation enables the elimination of non-volatile substances. The homologues of ethanol, in addition to ethanol and its homologues in esters are included in the ABV since they are present in the distillate.

Secondly, the density of the distillate is measured. The density of a liquid at a given temperature is equal to the quotient of its mass over its volume:

$\rho = m / V$, and for a vitivinicultural beverage, it is expressed as g/cm^3.

For hydro-alcoholic solutions such as distillates, when the temperature is known, the tables can be used to match the density up to the ABV (OIV, MA-AS312-02: R2009 Table 1). This ABV corresponds to that of the beverage (distillation by volume to volume).

4.2 Methods of determination of ABV

The principle and procedure for each method are detailed in the following parts:

Part A: Determination of the alcoholic strength by volume of a beverage by measuring of the density of the distillate using a pycnometer;

Part B: Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate by electronic densimetry using a frequency oscillator;

Part C: Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate by densimetry using a hydrostatic balance;

Part D: Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate by hydrometry or by refractometry.

The test temperature is set at 20 °C.

4.3 Safety precautions

Respect the safety guidelines for the usage of distillation apparatus, and for the handling of hydro-alcoholic and cleaning solutions.

5 Obtaining the distillate

5.1 *Reagents*

5.1.1 Type II water for analytical usage (ISO 3696 standard), or of equivalent purity

5.1.2 Suspension of calcium hydroxide, 12% m/v
Obtain by carefully pouring 1 L of water at 60-70 °C onto 120 g of quicklime (CaO).

5.1.3 Anti-foaming agent

5.2 *Apparatus*

Any type of distillation or steam distillation apparatus may be used provided that it satisfies the following test:
Distil a hydro-alcoholic mixture with an alcoholic strength of 10% vol. five times in succession. The distillate should have an alcoholic strength of at least 9.9% vol. after the fifth distillation, i.e. the loss of alcohol during each distillation should not be more than 0.02% vol.

By way of example, use one of the following two sets of apparatus.

5.2.1 Distillation apparatus, consisting of:
- a round bottomed 1-L flask with a ground-glass standard joint,
- a rectifying column of about 20 cm in height or any system designed to prevent priming,
- a source of heat (any pyrolysis of extracted matter should be prevented by a suitable arrangement),
- a condenser terminated by a tapered tube taking the distillate to the bottom of a graduated receiver flask containing several mL of water.

5.2.2 Steam distillation apparatus consisting of:
- a steam generator,

- a bubbler,
- a rectifying column,
- a condenser.

5.3 *Preparation of the sample*

Remove the bulk of any carbon dioxide from samples with bubbles (e.g. by stirring 250 to 300 mL of the wine in a 500-mL flask).

5.4 *Procedure*

5.4.1 Procedure for beverages with an ABV greater than or equal to 1.5% vol.

Take a sample of a 200-mL volume of beverage using a calibrated flask. Note the temperature of the sample.

Pour it into the flask of the distillation apparatus or into the bubbler of the steam distillation apparatus. Rinse the calibrated flask four times with approx. 5 mL of water and add this to the apparatus' flask or bubbler.

Add approx. 10 mL 2 M calcium hydroxide ((5.1.2). If necessary, several fragments of inert porous material (e.g. pumice, etc.) and/or several drops of anti-foaming agent (5.1.3) may also be added to facilitate distillation.

Collect the distillate in the 200-mL calibrated flask used to measure the beverage. Collect a volume of about three-quarters of the initial volume if distillation is used or 198-199 mL of distillate if steam distillation is used.

Make up to 200 mL with distilled water, keeping the distillate at within ± 2 °C of the initial temperature.

Carefully mix using a circular motion.

Note: In the case of wines containing particularly large concentrations of ammonium ions, the distillate may be redistilled under the conditions described above, but replacing the suspension of calcium hydroxide with 1 mL sulphuric acid diluted to 10% (v/v).

5.4.2 Procedure for beverages with an ABV less than 1.5% vol.

Take a sample of a 200-mL volume of beverage using a calibrated flask. Note the temperature of the beverage. Pour it into the flask of the distillation apparatus or into the bubbler of the steam distillation apparatus. Rinse the calibrated flask four times with approx. 5 mL of water and add this to the apparatus' flask or bubbler. Add approx. 10 mL 2 M calcium hydroxide

(5.1.2) and, in the case of distillation, if necessary, a boiling regulating agent (e.g. pumice, etc.). In a 100-mL calibrated flask, collect a volume of distillate of about 75 mL if distillation is used or 98-99 mL of distillate if steam distillation is used.
Make up to 100 mL with distilled water, keeping the distillate at within ± 2 °C of the initial temperature. Carefully mix using a circular motion.

Part A:
Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate using a pycnometer
(Type I Method)

(Method A2/1978 – Resolution 377/2009)

A.1 Principle

The density of the distillate is determined, which is matched to the ABV using the Tables.

The density is measured for a specific temperature using a glass pycnometer. This comprises a flask of known capacity, onto which a hollow ground-glass stopper is fitted equipped with a capillary tube. When the flask is closed, the overflow rises in the capillary. The volumes of the flask and the capillary being known, the density is determined by weighing using precision balances before and after filling of the pycnometer.

A.2 Reagents and products

A.2.1 Type II water for analytical usage (ISO 3696 standard), or of equivalent purity

A.2.2 Sodium chloride solution (2% m/v)

To prepare 1 litre, weigh out 20 g sodium chloride and dissolve to volume with water.

A.3 Apparatus and materials

Common laboratory apparatus, including the following:

A.3.1 Pyrex-glass pycnometer of around 100 mL capacity with a removable thermometer, with ground-glass joint and 10^{th}-of-a-degree graduations, from 10 °C to 30 °C. This thermometer should be calibrated (Fig. 1).

Any pycnometer of equivalent characteristics may be used.

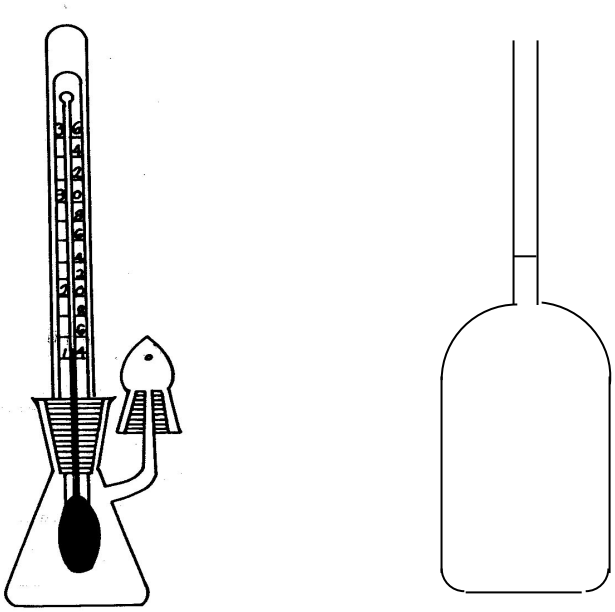


FIGURE 1: Pycnometer and its tare bottle

This pycnometer includes a side tube of 25 mm in length and an inside diameter of at most 1 mm, terminated by a ground-glass conical joint. This side tube may be capped by a 'reservoir stopper' composed of a ground-glass conical tube, terminated by a tapered joint. This stopper serves as an expansion chamber.

The two joints of the apparatus should be prepared with great care.

A.3.2 Tare bottle of the same external volume (to within 1 mL) as the pycnometer and with a mass equal to the mass of the pycnometer filled with a liquid of a density of 1.01 (2% m/v sodium chloride solution).

A.3.3 Thermally-insulated jacket that fits the body of the pycnometer exactly.

A.3.4 Twin-pan balance accurate to the nearest 0.1 mg
or
single-plate balance accurate to the nearest 0.1 mg.

A.3.5 Masses calibrated by an accredited body.

A.4 Procedure

A.4.1 Pycnometer calibration

The calibration of the pycnometer comprises the determination of the following characteristics:

- tare weight,
- volume at 20 °C,
- water mass at 20 °C.

A.4.1.1 Using a twin-pan balance

Place the tare bottle on the left-hand pan and the clean, dry pycnometer with its 'reservoir stopper' on the right-hand pan. Balance them by placing weights of known mass on the pycnometer side: *p* grams.

Fill the pycnometer carefully with water (A.2.1) at room temperature and fit the thermometer.

Carefully wipe the pycnometer dry and place it in the thermally-insulated jacket.

Shake by inverting the container until the thermometer's temperature reading is constant. Accurately adjust the level to the upper rim of the side tube. Wipe the side tube clean and fit the reservoir stopper.

Read the temperature, *t* °C, carefully and if necessary correct for any inaccuracies in the temperature scale.

Weigh the water-filled pycnometer, with the weight in grams, *p*, making up the equilibrium.

Calculations

- Tare of the empty pycnometer:

Tare weight = $p + m$ where m = mass of the air contained in the pycnometer, in g

$$m\ (\text{g}) = 0.0012\ (p - p')$$

- Volume at 20 °C in mL:

$V_{20\,°C}$ (mL) = $(p + m - p') \times F_t$

F_t = factor for temperature, *t* °C, taken from Table I

$V_{20\,°C}$ should be known to ± 0.001 mL

- Water mass at 20 °C:
  $M_{20\,°C} = V_{20\,°C}$ x 0.998203, in g

0.998203 = water density at 20 °C, in g/cm^3

A.4.1.2 Using a single-pan balance

Determine:

- the mass of the clean, dry pycnometer: P,
- the mass of the water-filled pycnometer at *t* °C: P_1 following the instructions outlined in A.4.1.1,
- the mass of the tare bottle, T_0.

- Calculations

Tare of the empty pycnometer:

Tare weight: P – *m* where *m* (g) = mass of the air contained in the pycnometer, in g

$$m\ (g) = 0.0012\ (P_1 - P)$$

- Volume at 20 °C in mL:
  $V_{20\,°C}$ (mL) = [P1 - (P - *m*)] x F_t

F_t = factor for temperature, t °C, taken from Table I

V_{20} °C should be known to ± 0.001 mL.

- Water mass at 20 °C:

$M_{20\,°C}$ (g) = V_{20} °C x 0.998203

0.998203 = water density at 20 °C, in g/cm^3

A.4.2. Determination of the density of the distillate

Measure the apparent density of the distillate at t °C using a twin-pan or single-pan balance:

A.4.2.1 Using a twin-pan balance

Weigh the pycnometer filled with the test sample following the instructions outlined in A.4.1.1.
Where *p*" represents the mass in grams that makes up the equilibrium at *t* °C, taking into account that the liquid mass contained in the pycnometer = $p + m - p''$,
the apparent density at *t* °C, in g/cm^3, is given by the following equation:

$$\rho_{t°C} = \frac{p + m - p''}{V_{20°C}}$$

A.4.2.2 Using a single-pan balance

Weigh the tare bottle, where T_1 is its mass in g.
Calculate $dT = T_1 - T_o$
Mass of the empty pycnometer at the time of measurement = $P - m + dT$, in g
Weigh the pycnometer filled with the test sample following the instructions outlined in A.4.1.1.
Where P_2 represents its mass at *t* °C,
the liquid mass contained in the pycnometer at t °C = P_2 ($P - m + dT$), in g
and the apparent density at t °C, in g/cm^3, is as follows:

$$\rho_{t\,°C} = \frac{P_2 - (P - m + dT)}{V_{20°C}}$$

A.5 Expression of results and precision parameters

A.5.1 Method of calculation

A.5.1.1 Beverages with an ABV greater than or equal to 1.5% vol.

Find the alcoholic strength at 20 °C in % vol. to 2 d.p. using Table I of Method OIV-MA-AS312-02A. Please note, this table uses the unit kg/m^3 and not g/cm^3. The relationship is as follows: 1 g/cm^3 = 1000 kg/m^3.

In the horizontal line of this table corresponding to the temperature, T, (expressed as a whole number) immediately below t °C, find the smallest density greater than ρ_t. Use the tabular difference just below this density to calculate the density ρ at this temperature, T.

On the line of the temperature, T, find the density ρ' immediately above ρ and calculate the difference between the densities ρ and ρ'. Divide this difference by the tabular difference just to the right of the density ρ'. The quotient gives the decimal part of the alcoholic strength, while the whole number part of this strength is shown at the head of the column in which the density ρ' is located.
An example of calculation of the alcoholic strength is given in Annex 1 to this Chapter.

Note: This temperature correction has been incorporated into a computer program and might possibly be carried out automatically.

A.5.1.2 Beverages with an ABV less than 1.5% vol.

Identical to A.5.1.1, dividing the alcoholic strength by volume of the distillate (ABVD) by 2.
ABV = ABVD/2, % vol. to 2 d.p.

A.6 Precision

Repeatability (r): $r = 0.10$ % vol.

Reproducibility (R) *R = 0.19 % vol.*

The validation parameters for beverages with a low alcohol content are given in Annex II.

A.7 Example of the calculation of the alcoholic strength of a wine

A.7.1 Measurement by pycnometer on a twin-pan balance

The constants of the pycnometer have been determined and calculated as described in the method **OIV-MA-AS2-01**, 'Density and specific gravity', paragraph A.7.

Numerical example

1. Weighing of the distillate-filled pycnometer*:*

Tare = pycnometer + distillate at *t* °C + *p"*

- *t* °C = 18.90 °C
- *corrected t* °C = 18.70 °C
- *p"* = 2.8074 g

p + *m* – *p"* = mass of the distillate at *t* °C

105.0698 – 2.8074 = 102.2624 g

Apparent density at *t* °C:

$$\rho_t = \frac{\mathrm{p + m - p"}}{\text{volume of the pycnometer at 20 °C}}$$

$$\rho_{18.7°C} = \frac{102.2624}{104.0299} = 0.983076\ \mathrm{g/cm^3}$$

2. Calculation of the alcoholic strength:

Consult the table of apparent densities of hydro-alcoholic mixtures at different temperatures, as indicated above.

On the line 18 °C of the table of apparent densities, the smallest density greater than the observed density of 0.983076 is 0.98398, in the column 11%.

The density at 18 °C is:

$(98307.6 + 0.7 \times 22)\ 10^{-5} = 0.98323$

$0.98398 - 0.98323 = 0.00075$

The decimal portion of the alcoholic strength is: 75 / 114 = 0.65

The alcoholic strength is: 11.65% vol.

A.7.2 Measurement by pycnometer on a single-pan balance

The constants of the pycnometer have been determined and calculated as described in the method **OIV-MA-AS2-01**, 'Density and specific gravity', paragraph A.7.

Numerical example

1. Weighing of the pycnometer filled with distillate:

Weight of tare bottle at the time of measurement	: T1 = 171.9178 g
Pycnometer filled with distillate at 20.50 °C	: P2 = 167.8438 g
Variation in the buoyancy of air	: dT = 171.9178 – 171.9160 = + 0.0018
Mass of the distillate at 20.5 °C	: Lt = 167.8438 – (67.6695 + 0.0018) = 100.1725 g
Apparent density of the distillate 100.1725/101.8194 =	: $\rho_{20.5\,°C}$ = 0.983825 g/cm^3

2. Calculation of alcoholic strength:

Refer to the table of apparent densities of hydro-alcoholic mixtures at different temperatures, as indicated above.

On the line 20 °C of the table of apparent densities, the smallest density greater than the observed density of 0.983825 is 0.98471, in the column 10% vol.
The density at 20 °C is:
$(98382.5 + 0.5 \times 24) \times 10^{-5} = 0.983945$
$0.98471 - 0.983945 = 0.000765$

The decimal portion of the alcoholic strength is: 76.5 / 119 = 0.64

The alcoholic strength is: 10.64% vol.

2. Calculation of alcoholic strength:

Refer to the table of apparent densities of hydro-alcoholic mixtures at different temperatures, as indicated above.

Part B
Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate by electronic densimetry using a frequency oscillator

(Type I method)

(Resolution OENO 8/2000 – 377/2009)

B.1 Principle

In the present method the distillate density is measured by electronic densimetry using a frequency oscillator. The principle consists of measuring the period of oscillation of a tube containing the sample undergoing electromagnetic stimulation. The density is thus calculated and is linked to the period of oscillation by the following formula:

$$\rho = T^2 \times \left(\frac{C}{4\pi^2 V} \right) - \left(\frac{M}{V} \right) \qquad (1).$$

ρ = density of the sample
T = period of induced vibration
M = mass of empty tube
C = spring constant
V = volume of vibrating sample

This relationship is in the form $\rho = A\ T^2 - B$ (2), so there is a linear relationship between the density and the period squared. The constants A and B are specific to each oscillator are estimated by measuring the period of fluids of known density.

B.2 Reagents and products

B.2.1 Reference fluids

Two reference fluids are used to adjust the densimeter. The densities of the reference fluids should encompass the densities of the distillates to be analysed. A spread of

greater than 0.01000 g/cm^3 between the densities of the reference fluids is recommended.

The reference fluids for the determination of the ABV of vitivinicultural beverages by electronic densimetry are as follows:

- dry air (unpolluted),
- Type II water for analytical usage (ISO 3696 standard), or of equivalent purity,
- hydro-alcoholic solutions of densities determined by another reference method, for which the uncertainty does not exceed 0.00005 g/cm^3 at the temperature of 20.00 ± 0.05 °C,
- solutions calibrated with traceability to the International System of Units (SI), with viscosities of less than 2 mm^2/s, for which the uncertainty does not exceed 0.00005 g/cm^3 at the temperature of 20.00 ± 0.05 °C.

B.2.2 Cleaning and drying products

Use products that ensure the perfectly clean and dried state of the measuring cell, according to the manufacturer's indications. For example:

- detergents, acids, etc.,
- organic solvents: 96% vol. ethanol, pure acetone, etc.

B.3 Apparatus and equipment

B.3.1 Electronic densimeter with frequency oscillator

The electronic densimeter consists of the following elements:

- a measuring cell consisting of a measuring tube and a temperature-controlled enclosure,
- a system for setting up an oscillation tube and measuring the period of oscillation,
- a digital display and possibly a calculator.

The densimeter is placed on a perfectly stable support isolated from all vibrations.

B.3.2 Temperature control of the measuring cell

Locate the measuring tube in the temperature-controlled enclosure. Temperature stability should be better than ± 0.02 °C.

It is necessary to control the temperature of the measuring cell when the densimeter makes this possible because this strongly influences the determination results. The density of a hydro-alcoholic solution with an ABV of 10% vol. is 0.98471 g/cm^3 at 20 °C and 0.98447 g/cm^3 at 21 °C, equating to a spread of 0.00024 g/cm^3.

The test temperature is set at 20 °C. The temperature is taken at the cell level, and done with a thermometer that has a resolution accurate to 0.01 °C and is calibrated to national standards. This should enable a temperature measurement with an uncertainty of better than ± 0.07 °C.

B.3.3 Calibration of the apparatus

The apparatus should be calibrated before using it for the first time, then periodically or if the verification is not satisfactory. The objective is to use two reference fluids to calculate the constants A and B [see formula (2), B.1). To carry out the calibration in practice, refer to the user manual of the apparatus. In principle, this calibration is carried out with dry air (taking into account the atmospheric pressure) and very pure water (B.2.1).

B.3.4 Calibration verification

In order to verify the calibration, measure the density of the reference fluids.

- Every day of use, a density check of the air is carried out. A difference between the theoretical density and observed density of more than 0.00008 g/cm^3 may indicate that the tube is clogged. In that case, it should be cleaned. After cleaning, verify the air density again. If the verification is not conclusive, adjust the apparatus.
- Check the density of the water; if the difference between the theoretical density and the density observed is greater than 0.00008 g/cm^3, adjust the apparatus.
- If verification of the cell temperature is difficult, it is possible to directly check the density of a hydro-alcoholic solution of comparable ABV to those of the distillates analysed.

-

B.3.5 Checks

When the difference between the theoretical density of the reference solution (known with an uncertainty of ± 0.00005 g/cm^3) and the measured density is above 0.00008 g/cm^3, the calibration of the apparatus should be checked.

B.4 Procedure

After obtaining a distillate, measure the density by densimetry and match to the ABV using the Tables.

Ensure the stability of the temperature of the measuring cell. The distillate in the densimeter cell should not contain air bubbles and should be homogeneous. If there is a lighting system available that makes it possible to verify the absence of bubbles, turn it off quickly after checking because the heat generated by the lamp can influence the measuring temperature.

For apparatus with a permanent lighting system, this statement is not applicable.

If the apparatus only provides the period, the density can be calculated from the A and B constants (see Annex I). If the apparatus does not provide the ABV directly, by knowing the density, obtain the ABV using the tables (Table I, OIV-MA-312-02).

B.5 Expression of results

B.5.1 Expression of results

B.5.1.1 Beverages with an ABV greater than or equal to 1.5% vol.

The alcoholic strength by volume of the beverage is obtained from the distillate. This is expressed as '% vol'.

If the temperature conditions are not respected, a correction should be made to express the temperature at 20 °C. The result is given to two decimal places.

B.5.1.2 Beverages with an ABV less than 1.5% vol.

Identical to B.5.1.1, dividing the alcoholic strength of the distillate (ABVD) by 2.

ABV = ABVD/2, % vol. to 2 d.p.

The validation parameters for beverages with a low alcohol content are given in Annex II.

B.5.2 Comments

The volume introduced into the cell should be sufficient enough to avoid possible contamination caused from the previous sample. It is thus necessary to carry out at least two tests. If these do not provide results included in the repeatability limits, a third test is necessary. In general, the results from the last two tests are homogeneous and the first value can then be eliminated.

B.6 Precision

For samples with an ABV of greater than 4% vol., the validation data and precision results are given in Annex III.

Repeatability (r) = 0.067 (% vol.)
Reproducibility (R) = 0.0454 + 0.0105 x ABV (% vol.)

For samples with an ABV of less than 4% vol. the validation data and precision results are given in Annex II.

Part C:
Determination of the alcoholic strength by volume of a beverage by measuring the density of the distillate by densimetry using a hydrostatic balance (Type I Method)

(Resolution Oeno 24/2003 – 377/2009)

C.1 Principle

The alcoholic strength by volume can be determined by densimetry using a hydrostatic balance following the Archimedes principle, by which any body immersed in a fluid experiences an upward force equal to the weight of the displaced fluid.

C.2 Reagents and products

C.2.1 Type II water for analytical usage (ISO 3696 standard), or of equivalent purity,

C.2.2 Floater-washing solution (sodium hydroxide, 30% m/v).

To prepare a 100 mL solution, weigh out 30 g of sodium hydroxide and fill to volume using 96% vol. ethanol.

C.3 Apparatus and materials

Common laboratory apparatus, including the following:

C.3.1 Single-pan hydrostatic balance with 1 mg precision.

C.3.2 Floater with at least 20 mL volume, specifically adapted for the balance, suspended by a thread with a diameter of less than or equal to 0.1 mm.

C.3.3 Cylindrical test tube with level indicator.

The floater should be able to fit entirely within the test tube volume below the level indicator; only the hanging thread should break the surface of the liquid. The cylindrical test tube should have an inside diameter at least 6 mm greater than that of the floater.

C.3.4 Thermometer (or temperature-measurement probe) with degree and 10^{th}-of-a-degree graduations, from 10 °C to 40 °C, calibrated to ± 0.05 °C.

C.3.5 Masses calibrated by an accredited body.

C.4 Procedure

After each measurement, the floater and the test tube should be cleaned with distilled water, wiped with soft laboratory paper that does not lose its fibres and rinsed with solution whose density is to be determined. These measurements should be carried out once the apparatus has reached a stable level in order to limit alcohol loss through evaporation.

C.4.1 Apparatus calibration

C.4.1.1 Balance calibration

While balances usually have internal calibration systems, hydrostatic balances should be calibrated with weights with traceability to the International System of Units (SI).

C.4.1.2 Floater calibration

Fill the cylindrical test tube up to the level indicator with water (C.2.1) whose temperature is between 15 °C and 25 °C, but preferably at 20 °C.

Plunge the floater and the thermometer into the liquid, shake, note down the density on the apparatus and, if necessary, adjust the reading in order for it to be equal to that of the water at the measurement temperature.

C.4.1.3 Control using a hydro-alcoholic solution

Fill the cylindrical test tube up to the level indicator with a known titre of hydro-alcoholic solution at a temperature of between 15 °C and 25 °C, preferably at 20 °C.

Plunge the floater and the thermometer into the liquid, shake and note down the density on the apparatus (or the alcoholic strength if possible). The established alcoholic strength should be equal to the previously determined alcoholic strength.

Note: This solution of known alcoholic strength can also replace water for floater calibration.

C.4.2 Measurement of the density of the distillate (or alcoholic strength if possible)

Pour the test sample into the cylindrical test tube up to the level indicator.
Plunge the floater and the thermometer into the liquid, shake and note down the density on the apparatus (or the alcoholic strength if possible).
Note the temperature if the density is measured at *t* °C (ρ_t).
Correct ρ_t using a ρ_t density table of hydro-alcoholic mixtures (Table II in Annex I of the method OIV-MA-AS312-02 in the OIV *Compendium of International Methods of Analysis*).

C.4.3 Cleaning of the floater and cylindrical test tube

Plunge the floater into the washing solution in the test tube.
Allow to soak for one hour while turning the floater regularly.
Rinse with tap water, then with distilled water.
Wipe with soft laboratory paper that does not lose its fibres.
Carry out these operations when the floater is used for the first time and then on a regular basis as necessary.

C.5 Expression of results

C.5.1 Beverages with an ABV greater than or equal to 1.5% vol.

Using the density ρ_{20}, calculate the real alcoholic strength using the table indicating the alcoholic strength by volume (% vol.) at 20°C according to the density at 20°C of the hydro-alcoholic mixtures. This is the international table adopted by the International Organization of Legal Metrology in its Recommendation No. 22 (1973).
The values are expressed in % vol. to 2 d.p.

C.5.2 Beverages with an ABV less than or equal to 1.5% vol.

Identical to C.5.1, dividing the alcoholic strength of the distillate (ABVD) by 2.

ABV = ABVD/2, % vol. to 2 d.p.

The validation parameters for beverages with a low alcohol content are given in Annex II.

C.6 Precision

Repeatability (r)= 0.074 (% vol.)

Reproducibility (R)= 0.229 (% vol.)

Part D:
Determination of the alcoholic strength by volume of a wine by measuring the density of the distillate by hydrometry or refractometry (Type IV Method)

D.1 Principle

The alcoholic strength may be determined by densimetry using an alcoholometer following the Archimedes principle. A weighted cylinder equipped with a graduated stem is more or less immersed into the distillate whose density is to be determined. The density of the liquid is read directly on the graduation of the stem at the level of the meniscus.

D.2 Hydrometry

D.2.1 Apparatus and materials

D.2.1.1 Alcoholometer

The alcoholometer should meet the specifications for Class I or Class II alcoholometers as defined in OIML (International Organization of Legal Metrology) International Recommendation 44 "Alcoholometers and alcohol hydrometers for use in alcoholometry".

This apparatus should be calibrated with traceability to the International System of Units (SI).

D.2.1.2 Thermometer calibrated with traceability to the International System of Units (SI) with degree and 10^{th}-of-a-degree graduations, from 0 °C to 40 °C, calibrated to ± 0.05 °C.

D.2.1.3 Measuring cylinder with dimensions that allow for the immersion of the thermometer and the alcoholometer without contact with the sides, held vertically.

D.2.2 Procedure

Pour the distillate into the measuring cylinder, ensure that the cylinder is kept vertical, and insert the thermometer and alcoholometer. Stir and wait 1 minute to allow temperature equilibration of the measuring cylinder, the thermometer, the alcoholometer and the distillate before reading the thermometer. Remove the thermometer and, after 1 minute of rest, read the apparent alcoholic strength.

Take at least three readings from the bottom of the meniscus using a magnifying glass. Correct the apparent strength measured at *t* °C to account for the effect of the temperature using the Tables. The temperature of the liquid must differ very little from the room temperature (at most, by 5 °C).

D.3 Refractometry

D.3.1 Apparatus

Refractometer enabling the refractive indices in the range 1.330 to 1.346 to be measured.

Depending on the type of apparatus, measurements are taken:

- either at 20 °C, with a suitable instrument,
- or at room temperature, *t* °C, with a thermometer enabling the temperature to be determined to within at least 0.05 °C (a temperature correction table will be provided with the apparatus).

D.3.2 Procedure

The refractive index of the wine distillate (5) is measured by following the procedure prescribed for the type of instrument used.

D.3.3 Expression of results

Table IV in Chapter OIV-MA-AS312-02 is used to find the alcoholic strength corresponding to the refractive index at 20 °C.

Note: Table IV gives the alcoholic strengths corresponding to the refractive indices for both pure hydro-alcoholic mixtures and for wine distillates. In the case of wine distillates, it takes into account the presence of impurities in the distillate (mainly higher alcohols). The presence of methanol lowers the refractive index and thus the alcoholic strength.

Note: To obtain the alcoholic strength from the density of the distillate, use Tables I, II and III in Chapter OIV-MA-AS312-02. These have been calculated from the international alcoholometric tables published in 1972 by the

International Organization of Legal Metrology in its Recommendation No. 22 and adopted by the OIV.

7. Annexes

ANNEX I

Formula for the calculation of alcoholic strength tables for mixtures of ethanol and water.

The density, ρ, expressed in kilograms per cubic metre (kg/m³) of a mixture of ethanol and water at the temperature t, expressed in degrees Celsius, is given by the following formula, according to the following:

- the mass concentration p, expressed by a decimal number (*),
- the temperature t, expressed in degrees Celsius (IPTS 68),
- the numerical coefficients in the tables below.

The formula is valid for temperatures of between –20 °C and +40 °C.

$$\rho = A_1 + \sum_{k=2}^{12} A_k \, \rho^{k-1} + \sum_{k=1}^{6} B_k (t - 20°C)^k + \sum_{i=1}^{n} \sum_{k=1}^{m} C_{i,k} \, \rho^k (t - 20°C)^i$$

$$\begin{aligned} n &= 5 \\ m_1 &= 11 \\ m_2 &= 10 \\ m_3 &= 9 \\ m_4 &= 4 \\ m_5 &= 2 \end{aligned}$$

(*) E.g. For a mass concentration of 12%, $p = 0.12$.

Numeric coefficients for the formula

k	A_k kg/m^3	B_k
1	$9{,}982\,012\,300 \cdot 10^{2}$	$-2{,}061\,851\,3 \cdot 10^{-1}$ kg/(m^3 · °C)
2	$-1{,}929\,769\,495 \cdot 10^{2}$	$-5{,}268\,254\,2 \cdot 10^{-3}$ kg/(m^3 · °C^2)
3	$3{,}891\,238\,958 \cdot 10^{2}$	$3{,}613\,001\,3 \cdot 10^{-5}$ kg/(m^3 · °C^3)
4	$-1{,}668\,103\,923 \cdot 10^{3}$	$-3{,}895\,770\,2 \cdot 10^{-7}$ kg/(m^3 · °C^4)
5	$1{,}352\,215\,441 \cdot 10^{4}$	$7{,}169\,354\,0 \cdot 10^{-9}$ kg/(m^3 · °C^5)
6	$-8{,}829\,278\,388 \cdot 10^{4}$	$-9{,}973\,923\,1 \cdot 10^{-11}$ kg/(m^3 · °C^6)
7	$3{,}062\,874\,042 \cdot 10^{5}$	
8	$-6{,}138\,381\,234 \cdot 10^{5}$	
9	$7{,}470\,172\,998 \cdot 10^{5}$	
10	$-5{,}478\,461\,354 \cdot 10^{5}$	
11	$2{,}234\,460\,334 \cdot 10^{5}$	
12	$-3{,}903\,285\,426 \cdot 10^{4}$	

k	$C_{1,k}$ kg/(m^3 · °C)	$C_{2,k}$ k g/(m^3 · °C2)
1	$1{,}693\,443\,461530\,087 \cdot 10^{-1}$	$-1{,}193\,013\,005\,057\,010 \cdot 10^{-}$
2	$-1{,}046\,914\,743\,455\,169 \cdot 10^{1}$	$2{,}517\,399\,633\,803\,46\,1 \cdot 10^{-1}$
3	$7{,}196\,353\,469\,546\,523 \cdot 10^{1}$	$-2{,}170\,575\,700\,536\,993$
4	$-7{,}047\,478\,054\,272\,792 \cdot 10^{2}$	$1{,}353\,034\,988\,843\,029 \cdot 10^{1}$
5	$3{,}924\,090\,430\,035\,045 \cdot 10^{3}$	$-5{,}029\,988\,758\,547\,014 \cdot$
6	$-1{,}210\,164\,659\,068\,747 \cdot 10^{4}$	$1{,}096\,355\,666\,577\,570 \cdot 10^{2}$
7	$2{,}248\,646\,550\,400\,788 \cdot 10^{4}$	$-1{,}422\,753\,946\,421\,155 \cdot$
8	$-2{,}605\,562\,982\,188\,164 \cdot 10^{4}$	$1{,}080\,435\,942\,856\,230 \cdot 10^{2}$
9	$1{,}852\,373\,922\,069\,467 \cdot 10^{4}$	$-4{,}414\,153\,236\,817\,392 \cdot$
10	$-7{,}420\,201433\,430\,137 \cdot 10^{3}$	$7{,}442\,971\,530\,188\,783$
11	$1{,}285\,617\,841\,998\,974 \cdot 10^{3}$	

k	$C_{3,k}$ kg/(m^3 · °C3)	$C_{4,k}$ kg/(m^3 · °C4)	$C_{5,k}$ kg/(m^3 · °C5)
1	$-6{,}802\,995\,733\,503\,803 \cdot 10^{-4}$	$-4{,}075\,376\,675\,622\,027 \cdot 10^{-}$	$-2{,}788\,074\,354\,782\,409 \cdot 10^{-8}$
2	$-1{,}876\,837\,790\,289\,664 \cdot 10^{-2}$	$-8{,}763\,058\,573\,471\,110 \cdot 10^{-}$	$1{,}345\,612\,883493\,354 \cdot 10^{-}$
3	$-2{,}002\,561\ 813734\,156 \cdot 10^{-1}$	$6{,}515\,031\,360\,099\,368 \cdot 10^{-8}$	
4	$-1{,}022\,992\,966\,719\,220$	$-1{,}515\,784\,836\,987\,210 \cdot 10^{-}$	
5	$-2{,}895\,696\,483\,903\,638$		
6	$-4{,}810\,060\,584\,300\,675$		
7	$-4{,}672\,147\,440\,794\,683$		
8	$-2{,}458\,043\,105\,903\,461$		
9	$-5{,}411\,227\,621\,436\,812 \cdot 10^{-1}$		

ANNEX II
Validation parameters relating to the measurement of the ABV of beverages with a low alcohol content

This document presents the results of the validation study for the method for beverages with a low alcohol content (update).

The study was carried out in accordance with documents OIV MA-F-AS1-08-FIDMET and MA-F-AS1-09-PROPER.

1. Sample

Sample no.	1	2	3	4	5	6
Nature	Grape juice	Beverage obtained by dealcoholisation of wine	Beverage obtained by partial dealcoholisation of wine	Partially fermented grape juice	Cider	Wine-based beverage
Approximate ABV in %vol.	< 0.5	0.5	1.5	2.5	4.5	6.5

Table 1: Samples analysed for the validation

2. Analyses
   Each of the 12 samples received by the laboratories were analysed by simple distillation or by steam distillation according to the following two procedures:

- OIV reference method with use of 200 mL and recovery of 200 mL of distillate,
- Alternative method with use of 200 mL and recovery of 100 mL of distillate.

3. Participating laboratories

19 laboratories from different countries took part:

Laboratório CVRVV	4050-501 Porto	Portugal
Laboratório de Análises da CVRA	7006-806 Évora	Portugal
Testing Laboratory CAFIA	603 00 BRNO	Czech Republic
Laboratório ASAE - LBPV	1649-038 Lisboa	Portugal
Agroscope - Site de Changins	1260 Nyon 1	Switzerland
Labo SCL de Bordeaux	33608 Pessac	France
Labo SCL de Montpellier	34196 Montpelllier	France
Laboratorio Arbitral Agroalimentario	28023 Madrid	Spain
Estación Enológica de Haro	26200 Haro La Rioja	Spain
Instituto dos Vinho do Douro do Porto	Porto 4050-253	Portugal
IVICAM	13700 Tomelloso, Ciudad Real	Spain
INCAVI	08720 Vilafranca del Penedès	Spain
ICQRF Laboratorio di Conegliano/Susegana	31058 SUSEGANA (TV)	Italy
ICQRF Laboratorio di Catania	95122 CATANIA	Italy
ICQRF Laboratorio di Modena	41100 Modena	Italy
ICQRF laboratorio di Perugia	06128 Perugia	Italy
ICQRF laboratorio di Salerno	84098 Salerno	Italy
ICQRF Laboratorio centrale di Roma	00149 Rome	Italy
Laboratoires DUBERNET	11100 Narbonne	France

Table 2: Laboratories that took part in the validation

4. Results

	Sample No. 1		Sample No. 2		Sample No.°3		Sample No. 4		Sample No. 5		Sample No. 6	
	POSITION :											
LAB	2	7	4	11	6	12	5	8	9	10	1	3
A	0.21	0.21	0.55	0.55	1.34	1.34	2.58	2.58	4.59	4.60	6.54	6.50
B	0.11	0.14	0.49	0.50	1.32	1.38	2.60	2.57	4.68	4.72	6.52	6.55
C	0.33	0.28	0.68	0.61	1.43	1.35	2.63	2.60	4.63	4.66	6.58	6.51
D			0.62	0.62	1.38	1.36	2.68	2.67	4.69	4.73	6.62	6.64
E	0.20	0.21	0.55	0.56	1.36	1.40	2.61	2.62	4.67	4.68	6.56	6.55
F	0.18	0.12	0.52	0.51	1.31	1.30	2.56	2.56	4.70	4.66	6.51	6.54
G	0.22	0.22	0.55	0.56	1.37	1.37	2.62	2.62	4.68	4.68	6.58	6.57
H			0.41	0.42	1.25	1.27	2.46	2.49	4.57	4.56	6.39	6.40
I	0.20	0.13	0.54	0.48	1.32	1.28	2.60	2.58	4.62	4.62	6.57	6.55
J	0.24	0.24	0.58	0.60	1.41	1.37	2.63	2.63	4.69	4.67	6.55	6.55
K	0.22	0.22	0.56	0.55	1.35	1.35	2.63	2.63	4.67	4.68	6.59	6.58
L	0.22	0.23	0.56	0.57	1.38	1.36	2.63	2.61	4.66	4.67	6.56	6.57
M	0.18	0.18	0.53	0.53	1.33	1.29			4.66	4.65	6.53	6.52
N	0.22	0.23	0.56	0.57	1.38	1.41	2.26	2.61	4.67	4.67	6.51	6.57
O	0.12	0.19	0.53	0.52	1.33	1.33	2.64	2.62	4.67	4.67	6.51	6.55
P	0.25	0.25	0.57	0.58	1.39	1.41	2.66	2.65	4.70	4.68	6.62	6.62
Q	0.22	0.20	0.55	0.59	1.34	1.33	2.61	2.63	4.65	4.63	6.52	6.54
R	0.21	0.21	0.55	0.52	1.29	1.28	2.52	2.55	4.62	4.56	6.50	6.53
S	0.18	0.17	0.41	0.42	1.38	1.37	2.61	2.58	4.63	4.58	6.51	6.48

Table 3: Results obtained for a 200 mL distillation with recovery volume of 200 mL

Results not presented were rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test) and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

	Sample No. 1		Sample No. 2		Sample No.°3		Sample No. 4		Sample No. 5		Sample No. 6	
	POSITION :											
LAB	2	7	4	11	6	12	5	8	9	10	1	3
A												
B	0.17	0.18	0.52	0.53	1.34	1.36	2.62	2.62	4.62	4.60	6.48	6.52
C	0.25	0.25	0.56	0.62	1.35	1.36	2.50	2.46	4.48	4.44	6.12	6.19
D	0.29	0.29	0.63	0.63	1.43	1.42	2.66	2.65	4.68	4.69	6.58	6.59
E	0.24	0.24	0.58	0.58	1.39	1.39	2.64	2.64	4.66	4.67	6.55	6.57
F	0.21	0.18	0.53	0.53	1.31	1.27	2.41	2.48	4.30	4.31	6.22	5.89
G	0.24	0.24	0.56	0.57	1.35	1.36	2.58	2.57	4.57	4.56	6.46	6.43
H	0.19	0.18	0.48	0.55	1.33	1.32	2.51	2.55	4.59	4.54	6.38	6.42
I	0.25	0.18	0.56	0.53	1.34	1.33	2.62	2.61	4.64	4.64	6.25	6.28
J	0.24	0.24	0.55	0.56	1.31	1.32	2.49	2.53	4.37	4.34	6.14	6.12
K	0.25	0.25	0.57	0.57	1.37	1.38	2.60	2.61	4.60	4.61	6.48	6.38
L	0.24	0.24	0.55	0.55	1.35	1.31	2.52	2.47	4.38	4.31	6.09	6.06
M	0.19	0.20	0.55	0.55	1.34	1.31			4.68	4.67	6.52	6.54
N	0.28	0.26	0.58	0.59	1.28	1.28	2.52	2.47	4.44	4.32	6.01	6.15
O	0.19	0.25	0.57	0.57	1.39	1.39	2.63	2.64	4.66	4.66	6.57	6.57
P	0.25	0.26	0.57	0.57	1.36	1.36	2.58	2.56	4.54	4.53	6.34	6.38
Q	0.24	0.24	0.57	0.57	1.38	1.38	2.63	2.62	4.66	4.67	6.56	6.56
R	0.23	0.23	0.54	0.55	1.32	1.30	2.54	2.56	4.56	4.52	6.40	6.35
S	0.27	0.26	0.55	0.57	1.34	1.34	2.46	2.43	4.53	4.51	6.36	6.36

Table 4: Results obtained for a 200 mL distillation with recovery volume of 100 mL

Results not presented were rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test) and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
No. of laboratories selected	17	19	19	17	19	18
No. of repetitions	2	2	2	2	2	2
Minimum	0.11	0.41	1.25	2.46	4.56	6.48
Maximum	0.33	0.68	1.43	2.68	4.73	6.64
Overall average	0.20	0.54	1.35	2.60	4.65	6.55
Repeatability variance	0.00052	0.00033	0.00050	0.00019	0.00036	0.00047
Reproducibility variance	0.00211	0.00345	0.00190	0.00229	0.00181	0.00147
Inter-laboratory standard deviation	0.043	0.057	0.041	0.047	0.040	0.035
Repeatability standard deviation	0.02	0.02	0.02	0.01	0.02	0.02
r limit	0.06	0.05	0.06	0.04	0.05	0.061
Repeatability CV	11.1	3.3	1.7	0.5	0.4	0.3
Reproducibility standard deviation	0.046	0.059	0.044	0.048	0.043	0.038
R limit	0.130	0.166	0.123	0.135	0.120	0.109
Reproducibility CV	22.5	10.9	3.2	1.8	0.9	0.6
Horwitz RSDr	3.36	2.90	2.52	2.29	2.09	1.99
$Horrat_r$	3.3	1.1	0.7	0.2	0.2	0.2
Horwitz RSD_R	5.10	4.39	3.82	3.46	3.17	3.01
$Horrat_R$	4.4	2.5	0.8	0.5	0.3	0.2

Table 5: Data obtained for a 200 mL distillate from a 200 mL sample

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
No. of laboratories selected	16	15	18	17	17	17
No. of repetitions	2	2	2	2	2	2
Minimum	0.17	0.52	1.27	2.41	4.30	6.01
Maximum	0.29	0.63	1.43	2.66	4.69	6.59
Overall average	0.24	0.56	1.35	2.56	4.55	6.38
Repeatability variance	0.00006	0.00003	0.00016	0.00050	0.00039	0.00135
Inter-laboratory standard deviation	0.03209	0.02496	0.03752	0.07013	0.12167	0.17621
Reproducibility variance	0.001	0.001	0.001	0.005	0.015	0.031
Repeatability standard deviation	0.01	0.01	0.01	0.02	0.02	0.04
r limit	0.02	0.02	0.04	0.06	0.06	0.104
Repeatability CV	3.2	1.0	0.9	0.9	0.4	0.6
Reproducibility standard deviation	0.033	0.025	0.039	0.072	0.122	0.178
R limit	0.092	0.071	0.109	0.203	0.347	0.504
Reproducibility CV	13.8	4.5	2.9	2.8	2.7	2.8
Horwitz RSDr	3.27	2.88	2.52	2.29	2.10	2.00
$Horrat_r$	1.0	0.4	0.4	0.4	0.2	0.3
Horwitz RSD_R	4.96	4.36	3.82	3.47	3.18	3.03
$Horrat_R$	2.8	1.0	0.8	0.8	0.9	0.9

Table 6: Data obtained for a 100 mL distillate from a 200 mL sample

Annex III
Validation parameters relating to the measurement of the ABV by electronic densimetry (Part B)

1. Inter-laboratory tests: precision and accuracy on additions

1.1 Samples
The samples used for this joint study are described in Table 7.

No.	Nature	Approximate ABV (% vol.)
C0	Cider (filtered through a membrane to remove CO_2)	~5
V0	Filtered wine	~10
V1	Filtered wine then doped	~11
V2	Filtered wine then doped	~12
V3	Filtered wine then doped	~13
P0	Liqueur wine	~16

Table 7: Samples for the joint study

All samples were homogenised before filling the bottles to be sent to the participants. For wine, 40 litres were homogenised before sending and carrying out the additions.

For the additions, absolute ethanol was poured into a 5-L volumetric flask, then filled up to the line with filtered wine. This was repeated two times. The volumes of ethanol were 50, 100 and 150 mL respectively for the V1, V2 and V3 samples.

1.2 Participating laboratories

The participating laboratories in the joint study are outlined in Table 8.

Laboratory	Postcode	City
ALKO Group LTD	FIN-00101	Helsinki
Bénédictine	76400	Fécamp
Casanis	18881	Gemenos
CIVC	51200	Epernay
Cointreau	49181	St Barthélémy d'Anjou
Courvoisier	16200	Jarnac
Hennessy	16100	Cognac
IDAC	44120	Vertou
Laboratoire Gendrot	33000	Bordeaux
Martell	16100	Cognac
Ricard	94320	Thiais
SOEC Martin Vialatte	51319	Epernay

Table 8: List of laboratories participating in the joint study

In order to not introduce a methodological bias, the results of the Station Viticole du Bureau National Interprofessionnel du Cognac (the joint-study organiser) are not taken into account.

1.2.1 Analyses

The C0 and P0 products were distilled independently two times, and the V0, V1, V2 and V3 products three times. Three ABV tests were done for each distillate. The results are displayed in the results table.

1.2.2 Results

The second test (out of the three carried out) was kept for the precision study (Table 9).

Laboratory	C0	V0	V1	V2	V3	P0
	6.020	9.500	10.390	11.290	12.100	17.080
1	5.970	9.470	10.380	11.260	12.150	17.080
		9.450	10.340	11.260	12.150	
	6.040	9.500	10.990	11.270	12.210	17.050
2	6.040	9.500	10.390	11.280	12.210	17.050
		9.510	10.400	11.290	12.200	
	5.960	9.460	10.350	11.280	12.170	17.190
3	5.910	9.460	10.360	11.280	12.150	17.200
		9.450	10.340	11.260	12.170	
	6.020	9.470	10.310	11.250	12.160	16.940
4	6.020	9.450	10.350	11.250	12.120	17.070
		9.450	10.330	11.210	12.130	
	5.950	9.350	10.250	11.300	12.050	17.000
5	5.950	9.430	10.250	11.300	12.050	17.000
		9.430	10.250	11.300	12.050	
	6.016	9.513	10.370	11.275	12.222	17.120
6	6.031	9.513	10.336	11.266	12.222	17.194
		9.505	10.386	11.275	12.220	
	5.730	9.350	10.230	11.440	12.080	17.010
7	5.730	9.430	10.220	11.090	12.030	16.920
		9.460	10.220	11.080	11.930	
	5.990	9.400	10.340	11.160	12.110	17.080
8	6.000	9.440	10.320	11.150	12.090	17.110
		9.440	10.360	11.210	12.090	
	6.031	9.508	10.428	11.289	12.180	17.089
9	6.019	9.478	10.406	11.293	12.215	17.084
		9.509	10.411	11.297	12.215	
	6.030	9.500	10.380	11.250	12.150	17.130

10	6.020	9.510	10.380	11.250	12.150	17.100
		9.510	10.380	11.250	12.160	
	6.020	9.480	10.400	11.260	12.150	17.040
11	6.000	9.470	10.390	11.260	12.140	17.000
		9.490	10.370	11.240	12.160	

Table 9: Results (second test per distillate) (% vol.)

1.2.3 Repeatability and reproducibility calculations

The repeatability and reproducibility calculations were carried out in compliance with the standard NF X 06-041, September 1983, ISO 5725. Table 10 presents the standard deviation per cell (laboratory x sample).

Laboratory	C0	V0	V1	V2	V3	P0
1	0.0354	0.0252	0.0265	0.0173	0.0289	0.0000
2	0.0000	0.0058	0.3436	0.0100	0.0058	0.0000
3	0.0354	0.0058	0.0100	0.0115	0.0115	0.0071
4	0.0000	0.0115	0.0200	0.0231	0.0208	0.0919
5	0.0000	0.0462	0.0000	0.0000	0.0000	0.0000
6	0.0106	0.0046	0.0255	0.0052	0.0012	0.0523
7	0.0000	0.0569	0.0058	0.2050	0.0764	0.0636
8	0.0071	0.0231	0.0200	0.0321	0.0115	0.0212
9	0.0085	0.0176	0.0115	0.0040	0.0202	0.0035
10	0.0071	0.0058	0.0000	0.0000	0.0058	0.0212
11	0.0141	0.0100	0.0153	0.0115	0.0100	0.0283

Table 10: Dispersion table (standard deviation in % vol.)

Three cells presented strong dispersions (probability with Cochran test under 1%). These cells are represented in grey (Table 10). For laboratory 7 and the

V3 product, the standard deviation of 0.0764 was maintained despite the Cochran test because it is on the same high level as that observed at the same laboratory for the V0 product.

An examination of the figures for each distillate (Table 9) led to the elimination of the following:

- laboratory 2, product V1, value 10.990,
- laboratory 7, product V2, value 11.440.

After eliminating these two values, the cell averages were calculated (laboratory x sample). The results are presented in Table 11.

Laboratory	C0	V0	V1	V2	V3	P0
1	5.9950	9.4733	10.3700	11.2700	12.1333	17.0800
2	6.0400	9.5033	10.3950	11.2800	12.2067	17.0500
3	5.9350	9.4567	10.3500	11.2733	12.1633	17.1950
4	6.0200	9.4567	10.3300	11.2367	12.1367	17.0050
5	5.9500	9.4033	10.2500	11.3000	12.0500	17.0000
6	6.0235	9.5103	10.3640	11.2720	12.2213	17.1570
7	5.7300	9.4133	10.2233	11.0850	12.0133	16.9650
8	5.9950	9.4267	10.3400	11.1733	12.0967	17.0950
9	6.0250	9.4983	10.4150	11.2930	12.2033	17.0865
10	6.0250	9.5067	10.3800	11.2500	12.1533	17.1150
11	6.0100	9.4800	10.3867	11.2533	12.1500	17.0200

Table 11: Table of averages (means in % vol.)

The figures given by laboratory 7 are generally low (Table 11). In the case of cider, the average for this laboratory is very far from the figures of the other laboratories (probability with the Dixon test under 1%). This laboratory's results for this product were eliminated.

Table 12 presents the calculated repeatability and reproducibility.

Sample	P	n	ABV	S^2r	S^2L	r	R
l_{C0}	10	20	6.002	0.000298	0.001033	0.049	0.103
V0	11	33	9.466	0.000654	0.001255	0.072	0.124
V1	11	32	10.344	0.000255	0.003485	0.045	0.173
V2	11	32	11.249	0.000219	0.003113	0.042	0.163
V3	11	33	12.139	0.000722	0.003955	0.076	0.194
P0	11	22	17.070	0.001545	0.004154	0.111	0.214

Table 12: Repeatability and reproducibility calculations

Key:
p: *number of laboratories retained*
n: *number of values retained*
ABV: *mean ABV (% vol.)*
S^2r: *repeatability variance (% vol.)^2*
S^2L: *interlaboratory variance (% vol.)^2*
r: *repeatability (% vol.)*
R: *reproducibility (% vol.)*

Reproducibility increases with the sample's ABV (Figure 2). The increase in repeatability according to ABV is less noticeable and the overall repeatability was calculated according to the mean repeatability variance. As such, for the samples with an ABV of between 4 and 18% vol.,

Repeatability (r) = 0.067 (% vol.),
Reproducibility (R) = 0.0454 + 0.0105 x ABV.

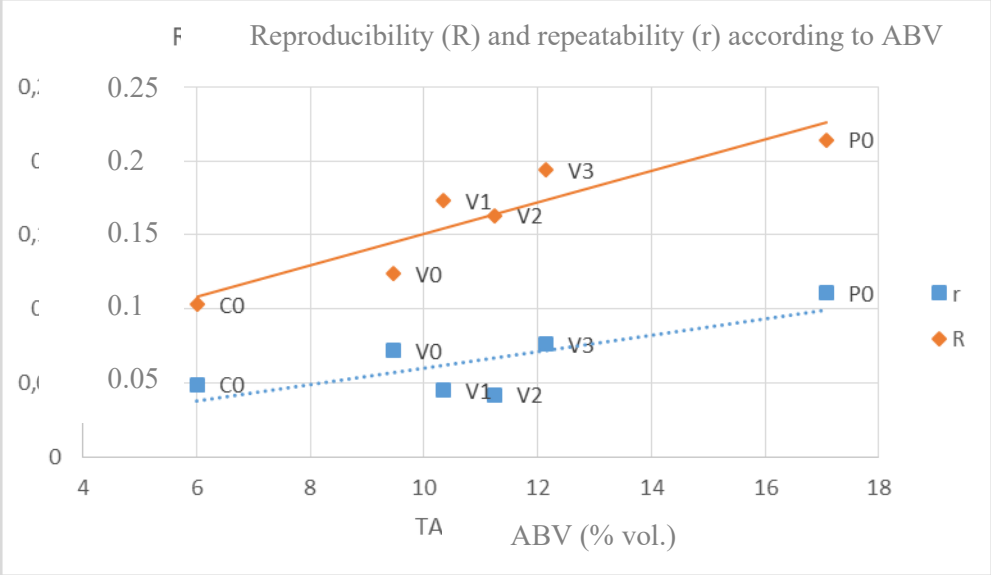


Figure 2: Repeatability and reproducibility according to ABV

1.2.4 Accuracy with regard to additions carried out on wine

The regression line of alcoholic strength after addition according to the volume of ethanol added provides, for a volume of 0 mL, an estimation of the initial alcoholic strength of the product (Figure 3). This regression is carried out with mean values for each laboratory (Table 11).

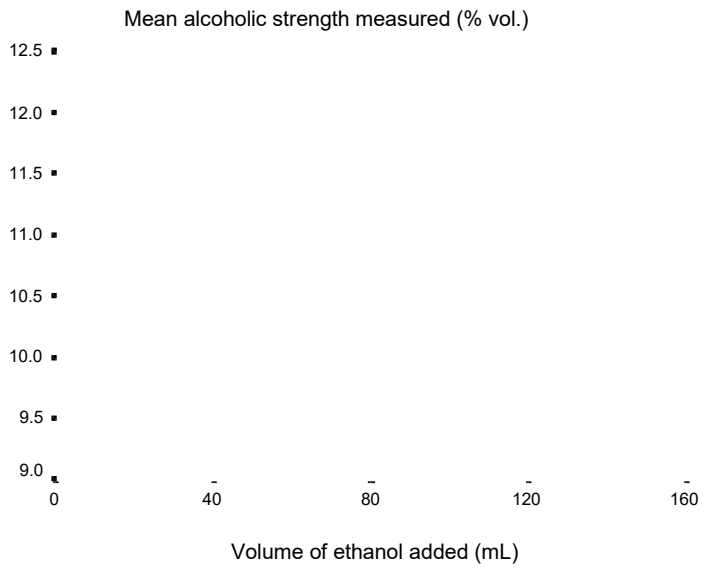


Figure 3: Regression of ABV measured by volume of ethanol added

Measurements carried out on initial products are not included in this estimation. This estimation was compared with the mean of the measurements taken on this product before additions; the intervals of relative confidence on these two estimations were calculated (Table 13).

LB	mean of measurements	UB	LB	estimation with measurements of products + additions	UB
9.440	9.466	9.492	9.392	9.450	9.508

Table 13: Additions to products

Key:

LB: lower bound of confidence interval at 95%

UB: upper bound of confidence level at 95%

The two confidence intervals cover a large overlapping centre. Thanks to the measurements on 'doped' samples, the alcoholic strength by volume of the initial product could be found.

1.2.5 Conclusion of inter-laboratory tests

The repeatability and reproducibility indications by inter-laboratory tests provide the following equations, for products with ABVs of between 4% and 18% vol.:

Repeatability (r) = 0.067 (% vol.),
Reproducibility (R) = 0.0454 + 0.0105 x ABV (% vol.).

The Horwitz indicators, Hor and HoR, are low (Table 14). This therefore indicates good precision of the method in relation to the analyte measured.

Sample	C0	V0	V1	V2	V3	P0
n	20	33	32	32	33	22
p	10	11	11	11	11	11
ABV	6.0019	9.4662	10.3443	11.2492	12.1389	17.0699
r	0.0489	0.0724	0.0452	0.0419	0.0760	0.1113
sr	0.0173	0.0256	0.0160	0.0148	0.0269	0.0393
RSDr	0.2878	0.2702	0.1543	0.1316	0.2214	0.2303
RSDrH	2.0159	1.8822	1.8573	1.8340	1.8131	1.7224
Hor	0.1428	0.1436	0.0831	0.0718	0.1221	0.1337
R	0.1033	0.1237	0.1731	0.1634	0.1935	0.2136
sR	0.0365	0.0437	0.0612	0.0577	0.0684	0.0755
RSDR	0.6080	0.4616	0.5912	0.5131	0.5634	0.4423
RSDRH	3.0543	2.8519	2.8141	2.7788	2.7471	2.6097
HoR	0.1991	0.1619	0.2101	0.1847	0.2051	0.1695

Table 14: Summary table of method precision

Key:

n:	*number of values retained*
p:	*number of laboratories retained*
ABV:	*mean ABV (% vol.)*
r:	*repeatability (% vol.)*
sr:	*repeatability standard deviation (% vol.)*
RSDr:	repeatability coefficient of variation (sr x 100 / ABV) (%)
RSDrH:	*Horwitz repeatability coefficient of variation (0.66 x RSDRH) (%)*
Hor:	*Horrat repeatability value (RSDr/RSDrH)*
R:	*reproducibility (% vol.)*
sR:	*reproducibility standard deviation (% vol.)*
RSDR:	*reproducibility coefficient of variation (sR x 100 / ABV) (%)*
RSDRH:	*Horwitz reproducibility coefficient of variation ($2^{(1-0.5\log(ABV))}$) (%)*
HoR:	*Horrat reproducibility value (RSDR/RSDRH)*

The measurements carried out during inter-laboratory tests on wine with additions made it possible to find the value obtained before the addition. The values 9.45% and 9.47% vol. were found respectively.

Annex IV

Comparison of measurements carried out using a hydrostatic balance (Method C) with those obtained by electronic densimetry (Méthode B)

Using samples with alcoholic strengths between 4% vol. and 18% vol., the repeatability and reproducibility were measured using an inter-laboratory test. The alcoholic strength of the different samples as measured using a hydrostatic balance and using electronic densimetry were compared, including the repeatability and reproducibility values derived from the multi-year inter-comparison tests performed on a large scale.

1. *Samples:* Wines with different densities and alcoholic strengths prepared monthly on an industrial scale, taken from a stock of bottles stored under normal conditions, and supplied anonymously to the laboratories.

2. *Laboratories:* Laboratories participating in the monthly tests organised by *Unione Italiana Vini* (Verona, Italy) according to ISO 5725 (UNI 9225) regulations and the International Harmonized Protocol for the Proficiency Testing of Analytical Chemical Laboratories produced by the AOAC, ISO and IUPAC (J. AOAC Intern., 1993, *74/4*), and ISO 43 and ILAC G13 guidelines. An annual report is provided by the above-mentioned organisation to all participants.

3. *Apparatus*:
   - An electronic hydrostatic balance (with precision to 5 decimal places), equipped if possible with a data-processing device.
   - An electronic densimeter, equipped if possible with an autosampler.

4. *Analyses*
   The measurement of the distillate was repeated twice.

5. *Results*
   Table 15 shows the results of the measurements obtained by the laboratories using a hydrostatic balance.

Table 16 shows the results obtained by the laboratories using an electronic densimeter.

6. *Evaluation of results*

Interlaboratory reproducibility

A Horrat value of 1 usually indicates satisfactory interlaboratory reproducibility, whereas a value of more than 2 normally indicates unsatisfactory reproducibility, i.e. one that is too variable for analytical purposes or where the variation obtained is greater than that expected for the type of method employed. Hor is also calculated and used to assess interlaboratory reproducibility, using the following approximation:

RSDr (Horwitz) = 0.66 RSDR (Horwitz) (this assumes the approximation that r = 0.66 R).

Table 17 shows the differences between the measurements obtained by laboratories using an electronic densimeter and those using a hydrostatic balance. Excluding the sample 2000/3, which has a very low alcohol strength and for which both techniques show poor reproducibility, good concordance is generally observed for the other samples.

7. *Precision parameters*

Table 18 shows the overall averages of the precision parameters calculated from all monthly tests carried out between January 1999 and May 2001.

In particular:

Repeatability (r) = 0.074 (% vol.) for the hydrostatic balance and 0.061 (% vol.) for electronic densimetry,

Reproducibility (R) = 0.229 (% vol.) for the hydrostatic balance and 0.174 (% vol.) for electronic densimetry.

Table 15: Hydrostatic balance (HB)

	mean	n	outliers	n1	r	sr	RSDr	Hor	R	sR	RSDR	HoR	no. of replicates	difference
1999/1	11.043	17	1	16	0.0571	0.0204	0.1846	0.1004	0.1579	0.0564	0.5107	0.18	2	0.108
1999/2	11.247	14	1	13	0.0584	0.0208	0.1854	0.1011	0.1803	0.0644	0.5727	0.21	2	0.1241
1999/3	11.946	16	0	16	0.0405	0.0145	0.1211	0.0666	0.1593	0.0569	0.4764	0.17	2	0.1108
1999/4	7.653	17	1	16	0.0502	0.0179	0.2344	0.1206	0.1537	0.0549	0.7172	0.24	2	0.1057
1999/5	11.188	17	0	17	0.0871	0.0311	0.278	0.1515	0.2701	0.0965	0.8622	0.31	2	0.186
1999/6	11.276	19	0	19	0.0846	0.0302	0.268	0.1462	0.2957	0.1056	0.9365	0.34	2	0.2047
1999/7	8.018	17	0	17	0.089	0.0318	0.3964	0.2054	0.2573	0.0919	1.1462	0.39	2	0.1764
1999/9	11.226	17	0	17	0.058	0.0207	0.1846	0.1423	0.2796	0.0999	0.8896	0.45	2	0.1956
1999/10	11.026	17	0	17	0.0606	0.0216	0.1961	0.1066	0.2651	0.0947	0.8588	0.31	2	0.185
1999/11	7.701	16	1	15	0.0643	0.0229	0.298	0.1535	0.233	0.0832	1.0805	0.37	2	0.1616
1999/12	10.987	17	2	15	0.0655	0.0234	0.2128	0.1156	0.1258	0.0449	0.4089	0.15	2	0.0827
2000/1	11.313	16	0	16	0.0986	0.0352	0.3113	0.1699	0.2577	0.092	0.8135	0.29	2	0.1754
2000/2	11.232	17	0	17	0.0859	0.0307	0.2731	0.1489	0.2535	0.0905	0.806	0.29	2	0.174
2000/3	0.679	10	0	10	0.068	0.0243	3.5773	1.2783	0.6529	0.2332	34.3395	8.1	2	0.4604
2000/4	11.223	18	0	18	0.0709	0.0253	0.2257	0.123	0.2184	0.078	0.6951	0.25	2	0.1503
2000/5	7.439	19	1	18	0.063	0.0225	0.3023	0.1549	0.1522	0.0544	0.7307	0.25	2	0.1029
2000/6	11.181	19	0	19	0.0536	0.0191	0.171	0.0932	0.2783	0.0994	0.889	0.32	2	0.195
2000/7	10.858	16	0	16	0.0526	0.0188	0.1731	0.0939	0.1827	0.0653	0.6011	0.22	2	0.1265
2000/9	12.031	17	1	16	0.0602	0.0215	0.1787	0.0985	0.2447	0.0874	0.7263	0.26	2	0.1704
2000/10	11.374	18	0	18	0.0814	0.0291	0.2555	0.1395	0.2701	0.0965	0.8482	0.31	2	0.1866
2000/11	7.644	18	0	18	0.0827	0.0295	0.3863	0.1988	0.2289	0.0817	1.0694	0.36	2	0.1565
2000/12	11.314	19	1	18	0.0775	0.0277	0.2447	0.1336	0.2421	0.0864	0.7641	0.28	2	0.1667
2001/1	11.415	19	0	19	0.095	0.0339	0.2971	0.1623	0.241	0.0861	0.7539	0.27	2	0.1636
2001/2	11.347	19	0	19	0.0792	0.0283	0.2493	0.1361	0.1944	0.0694	0.6119	0.22	2	0.1316
2001/3	11.818	16	0	16	0.0659	0.0235	0.199	0.1093	0.2636	0.0941	0.7965	0.29	2	0.1834
2001/4	11.331	17	0	17	0.1067	0.0381	0.3364	0.1836	0.1895	0.0677	0.5971	0.22	2	0.1229
2001/5	8.063	19	1	18	0.0782	0.0279	0.3465	0.1797	0.1906	0.0681	0.8442	0.29	2	0.129

Table 16: Electronic densimetry (ED)

	MEAN no.1	n	outliers	n1	r	Sr	RSDr	Hor	R	sR	RSDR	HoR	no of replicates	difference
D1999/1	11.019	18	1	17	0.0677	0.0242	0.2196	0.1193	0.1996	0.0713	0.6470	0.23	2	0.1370
D1999/2	11.245	19	2	17	0.0448	0.0160	0.1423	0.0776	0.1311	0.0468	0.4165	0.15	2	0.0900
D1999/3	11.967	21	0	21	0.0701	0.0250	0.2091	0.1151	0.1552	0.0554	0.4631	0.17	2	0.1040
D1999/4	7.643	19	1	18	0.0610	0.0218	0.2852	0.1467	0.1340	0.0479	0.6262	0.21	2	0.0897
D1999/5	11.188	21	3	18	0.0260	0.0093	0.0829	0.0452	0.2047	0.0731	0.6536	0.24	2	0.1442
D1999/6	11.303	21	0	21	0.0652	0.0233	0.2061	0.1125	0.1466	0.0523	0.4631	0.17	2	0.0984
D1999/7	8.026	21	0	21	0.0884	0.0316	0.3935	0.2039	0.1708	0.0610	0.7600	0.26	2	0.1124
D1999/9	11.225	17	0	17	0.0372	0.0133	0.1183	0.0645	0.1686	0.0602	0.5366	0.19	2	0.1178
D1999/10	11.011	19	0	19	0.0915	0.0327	0.2969	0.1613	0.1723	0.0615	0.5588	0.20	2	0.1129
D1999/11	7.648	21	1	20	0.0615	0.0220	0.2872	0.1478	0.1538	0.0549	0.7183	0.24	2	0.1043
D1999/12	10.999	16	1	15	0.0428	0.0153	0.1389	0.0755	0.2015	0.0720	0.6541	0.23	2	0.1408
D2000/1	11.248	22	1	21	0.0697	0.0249	0.2212	0.1206	0.1422	0.0508	0.4516	0.16	2	0.0944
D2000/2	11.240	19	3	16	0.0448	0.0160	0.1424	0.0776	0.1619	0.0578	0.5145	0.19	2	0.1123
D2000/3	0.526	12	1	11	0.0327	0.0117	2.2185	0.7630	0.9344	0.3337	63.4009	14.39	2	0.6605
D2000/4	11.225	19	1	18	0.0476	0.0170	0.1514	0.0825	0.1350	0.0482	0.4295	0.15	2	0.0924
D2000/5	7.423	21	0	21	0.0628	0.0224	0.3019	0.1547	0.2635	0.0941	1.2677	0.43	2	0.1836
D2000/6	11.175	23	2	21	0.0606	0.0217	0.1938	0.1056	0.1697	0.0606	0.5424	0.20	2	0.1161
D2000/7	10.845	21	5	16	0.0440	0.0157	0.1449	0.0786	0.1447	0.0517	0.4766	0.17	2	0.0999
D2000/9	11.983	22	1	21	0.0841	0.0300	0.2507	0.1380	0.2410	0.0861	0.7183	0.26	2	0.1651
D2000/10	11.356	22	1	21	0.0635	0.0227	0.1997	0.1090	0.1865	0.0666	0.5866	0.21	2	0.1280
D2000/11	7.601	27	0	27	0.0521	0.0186	0.2448	0.1258	0.1685	0.0602	0.7916	0.27	2	0.1162
D2000/12	11.322	25	1	24	0.0476	0.0170	0.1503	0.0820	0.1594	0.0569	0.5028	0.18	2	0.1102
D2001/1	11.427	29	0	29	0.0706	0.0252	0.2207	0.1206	0.1526	0.0545	0.4771	0.17	2	0.1020
D2001/2	11.320	29	1	28	0.0675	0.0241	0.2128	0.1161	0.1570	0.0561	0.4952	0.18	2	0.1057
D2001/3	11.826	34	1	33	0.0489	0.0175	0.1476	0.0811	0.1762	0.0629	0.5322	0.19	2	0.1222
D2001/4	11.339	31	2	29	0.0639	0.0228	0.2012	0.1099	0.1520	0.0543	0.4788	0.17	2	0.1026
D2001/5	8.058	28	0	28	0.0473	0.0169	0.2098	0.1088	0.2025	0.0723	0.8976	0.31	2	0.1412

Table 17: Comparison of results from a hydrostatic balance (HB) and from electronic densimetry (ED)

	Mean (HB)	n	Values	n1		Mean (ED)	n	Values	n1		%ABV (HB-ED)
1999/1	11.043	17	1	16	D1999/1	11.019	18	1	17		0.024
1999/2	11.247	14	1	13	D1999/2	11.245	19	2	17		0.002
1999/3	11.946	16	0	16	D1999/3	11.967	21	0	21		-0.021
1999/4	7.653	17	1	16	D1999/4	7.643	19	1	18		0.010
1999/5	11.188	17	0	17	D1999/5	11.188	21	3	18		0.000
1999/6	11.276	19	0	19	D1999/6	11.303	21	0	21		-0.028
1999/7	8.018	17	0	17	D1999/7	8.026	21	0	21		-0.008
1999/9	11.226	17	0	17	D1999/9	11.225	17	0	17		0.002
1999/10	11.026	17	0	17	D1999/10	11.011	19	0	19		0.015
1999/11	7.701	16	1	15	D1999/11	7.648	21	1	20		0.052
1999/12	10.987	17	2	15	D1999/12	10.999	16	1	15		-0.013
2000/1	11.313	16	0	16	D2000/1	11.248	22	1	21		0.065
2000/2	11.232	17	0	17	D2000/2	11.240	19	3	16		-0.008
2000/3	0.679	10	0	10	D2000/3	0.526	12	1	11	•	0.153
2000/4	11.223	18	0	18	D2000/4	11.225	19	1	18		-0.002
2000/5	7.439	19	1	18	D2000/5	7.423	21	0	21		0.016
2000/6	11.181	19	0	19	D2000/6	11.175	23	2	21		0.006
2000/7	10.858	16	0	16	D2000/7	10.845	21	5	16		0.013
2000/9	12.031	17	1	16	D2000/9	11.983	22	1	21		0.049
2000/10	11.374	18	0	18	D2000/10	11.356	22	1	21		0.018
2000/11	7.644	18	0	18	D2000/11	7.601	27	0	27		0.043
2000/12	11.314	19	1	18	D2000/12	11.322	25	1	24		-0.008
2001/1	11.415	19	0	19	D2001/1	11.427	29	0	29		-0.012
2001/2	11.347	19	0	19	D2001/2	11.320	29	1	28		0.027
2001/3	11.818	16	0	16	D2001/3	11.826	34	1	33		-0.008
2001/4	11.331	17	0	17	D2001/4	11.339	31	2	29		-0.008
2001/5	8.063	19	1	18	D2001/5	8.058	28	0	28		0.004
					Overall difference / %ABV (HB-ED)						0.014
					Standard deviation/difference						0.036
				*	Test 2000/3 was not taken into account						

Table 18: Precision parameters

MEAN	Hydrostatic balance	Electronic densimetry
n1	441	557
relative repeatability variance	0.309	0.267
r	0.074	0.061
sr	0.026	0.022
relative reproducibility variance	2.948	2.150
R	0.229	0.174
sR	0.082	0.062

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TABLE I International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																							
t°	**0**		**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**	
0°	999.64	1.50	998.14	1.44	996.70	1.40	995.30	1.35	993.95	1.30	992.65	1.24	991.41	1.19	990.22	1.14	989.08	1.10	987.98	1.05	986.93	1.00	985.93	0.95
	-0.07		-0.06		-0.06		-0.06		-0.06		-0.06		-0.06		-0.05		-0.04		-0.03		-0.02		-0.01	
1°	999.71	1.51	998.20	1.44	996.76	1.40	995.36	1.35	994.01	1.30	992.71	1.24	991.47	1.20	990.27	1.15	989:12	1.11	988.01	1.06	986.95	1.01	985.94	0.97
	-0.05		-0.05		-0.04		-0.04		-0.04		-0.04		-0.03		-0.03		-0.02		-0.02		-0.01		0.00	
2°	999.76	1.51	998.25	1.45	996.80	1.40	995.40	1.35	994.05	1.30	992.75	1.25	99150	1.20	990.30	1.16	989.14	1.11	988.03	1.07	986.96	1.02	985.94	0.98
	-0.03		-0.03		4.03		-0.02		-0.02		-0.02		-0.02		-0.01		-0.01		0.00		0.01		0.02	
3°	999.79	1.51	998.28	1.45	996.83	1.41	995.42	1.35	994.07	1.30	992.77	1.25	991.52	1.21	990.31	1.16	989.15	1.12	988.03	1.08	986.95	1.03	985.92	1.00
	-0.02		-0.02		-0.01		-0.02		-0.01		-0.01		0.00		0.00		0.01		0.02		0.03		0.04	
4°	999.81	1.51	998.30	1.46	996.84	1.40	995.44	1.36	994.08	1.30	992.78	1.26	991.52	1.21	990.31	1.17	989.14	1.13	988:01	1.09	986.92	1.04	985.88	1.00
	0.00		0.00		0.00		0.00		0.01		0.02		0.02		0.02		0.02		0.03		0.04		0.05	
5°	999.81	1.51	998.30	1.46	996.84	1.40	995.44	1.37	994.07	1.31	992.76	1.26	991.50	1.21	990.29	1.17	989.12	1.14	987.98	1.10	986.88	1.05	985.83	1.01
	0.01		0.01		0.01		0.02		0.01		0.02		0.03		0.04		0.05		0.05		0.05		0.06	
6°	999.80	1.51	998.29	1.46	996.83	1.41	995.42	1.36	994.06	1.32	992.74	1.27	991.47	1.22	990.25	1.18	989:07	1.14	987.93	1.10	986.83	1.06	985.77	1.02
	0.03		0.03		0.03		0.03		0.04		0.04		0.04		0.05		0.06		0.07		0.08		0.09	
7°	999.77	1.51	998.26	1.46	996.80	1.41	995.39	1.37	994.02	1.32	99270	1.27	99143	1.23	99020	1.19	989.01	1.15	987:86	1.11	986.75	1.07	985.68	1.03
	0.05		0.04		0.04		0.05		0.05		0.05		0.05		0.06		0.06		0.07		0.08		0.09	
8°	999.72	1.50	998.22	1.46	996.76	1.42	995.34	1.37	993.97	1.32	992.65	1.27	991.38	1.24	990.14	1.19	988.95	1.16	987.79	1.12	986.67	1.08	985.59	1.05
	0.05		0.06		0.06		0.06		0.06		0.06		0.07		0.07		0.08		0.09		0.10		0.11	
9°	999.67	1.51	998.16	1.46	996.70	1.42	995.28	1.37	993.91	1.32	992:59	1.28	99131	1.24	990.07	1.20	988.87	1.17	987.70	1.13	986.57	1.09	985.48	1.06
	0.07		0.07		0.07		0.07		0.07		0.08		0.08		0.09		0.09		0.10		0.11		0.12	
10°	999.60	1.51	998.09	1.46	996.63	1.42	995.21	1.37	993.84	1.33	992.51	1.28	991.23	1.25	989.98	1.20	988.78	1.17	987.60	1.14	986.46	1.10	985.36	1.06
	0.09		0.09		0.09		0.08		0.09		0.09		0.10		0.10		0.11		0.11		0.12		0.13	
11°	999.51	1.51	998.00	1.46	996.54	1.41	995.13	1.38	993.75	1.33	992.42	1.29	991.13	1.25	989.88	1.21	988.67	1.18	987.49	1.15	986.34	1.11	985.23	1.07
	0.10		0.09		0.09		0.10		0.10		0.11		0.11		0.11		0.12		0.13		0.13		0.14	
12°	999.41	1.50	997.91	1.46	996.45	1.42	995.03	1.38	993.65	1.34	992.31	1.29	991.02	1.25	989.77	1.22	988.55	1.19	987.36	1.15	986.21	1.12	98.509	1.09
	0.11		0.11		0.11		0.11		0.11		0.11		0.12		0.12		0.13		0.14		0.15		0.16	
13°	999.30	1.50	997.80	1.46	996.34	1.42	994.92	1.38	993.54	1.34	992.20	1.30	990.90	1.25	989.65	1.23	988.42	1.20	987.22	1.16	986.06	1.13	984.93	1.09
	0.12		0.12		0.13		0.13		0.13		0.13		0.14		0.14		0.15		0.16		0.16		0.16	
14°	999.18	1.50	997.68	1.46	996.22	1.43	994.79	1.38	993.41	1.34	992.07	1.30	990.77	1.26	989.51	1.23	988.28	1.21	987.07	1.17	985.90	1.13	984.77	1.11
	0.14		0.14		0.13		0.13		0.14		0.14		0.15		0.16		0.16		0.17		0.18		0.18	
15°	999.05	1.51	997.54	1.46	996.08	1.42	994.66	1.38	993.28	1.35	991.93	1.30	990.63	1.27	989.36	1.24	988.12	1.21	986.91	1.18	985.73	1.14	984.59	1.12
	0.14		0.14		0.15		0.15		0.15		0.16		0.16		0.17		0.17		0.18		0.19		0.19	
16°	998.90	1.50	997.40	1.46	995.94	1.43	994.51	1.38	993.13	1.35	991.78	1.31	990.47	1.27	989.20	1.25	987.95	1.21	986.74	1.19	985.55	1.15	984.40	1.13
	0.16		0.16		0.16		0.16		0.17		017		0.18		0.18		0.19		0.19		0.20		0.20	
17°	998.74	1.50	997.24	1.46	995.78	1.43	994.35	1.38	992.97	1.36	991.61	1.31	990.30	1.28	989.02	1.25	987.17	1.22	986.55	1.19	985.36	1.16	984.20	1.14
	0.17		0.17		0.16		0.17		0.17		0.18		0.18		0.19		0.20		0.21		0.22		0.22	
18°	998.57	1.50	997.07	1.46	995.61	1.42	994.19	1.39	992.80	1.36	991.44	1.32	990.12	1.28	988.84	1.26	987.58	1.23	986.35	1.20	985.15	1.17	983.98	1.14
	0.18		0.18		0.19		0.19		0.19		0.19		0.20		0.20		0.20		0.21		0.21		0.22	
19°	998.39	1.50	996.89	1.46	995.43	1.43	994.00	1.39	992.61	1.36	991.25	1.32	989.93	1.29	988.64	1.26	987.38	1.23	986.15	1.21	984.94	1.10	983.76	1.16
	0.19		0.19		0.19		0.19		0.19		0.20		0.20		0.21		0.22		0.23		0.24		0.24	
20°	998.20	1.50	996.70	1.46	995.24	1.43	993.81	1.39	992.42	1.36	991.06	1.33	989.73	1.29	988.44	1.27	987.17	1.24	985.93	1.22	984.71	1.19	983.52	1.16

TABLE I (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																							
t°	**0**		**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**	
20°	998.20	1.50	996.70	1.46	995.24	1.43	993.81	1.39	992.42	1.36	991.06	1.33	989.73	1.29	988.44	1.27	987.17	1.24	985.93	1.22	984.71	1.19	983.52	1.16
	0.20		0.20		0.20		0.20		0.21		0.21		0.21		0.22		0.22		0.23		0.24		0.24	
21°	998.00	1.50	996.50	1.46	995.04	1.43	993.61	1.40	992.21	1.36	990.85	1.33	989.52	1.30	988.22	1.27	986.95	1.25	985.70	1.23	984.47	1.19	983.28	1.18
	0.21		0.21		0.21		0.21		0.21		0.22		0.22		0.23		0.24		0.24		0.24		0.26	
22°	997.79	1.50	996.29	1.46	994.83	1.43	993.40	1.40	992.00	1.37	990.63	1.33	989.30	1.31	987.99	1.28	986.71	1.25	985.46	1.23	984.23	1.21	983.02	1.18
	0.22		0.22		023		0.23		0.23		0.23		0.24		0.24		0.24		0.25		0.26		0.25	
23°	997.57	1.50	996.07	1.47	994.60	1.43	993.17	1.40	991.77	1.37	990.40	1.34	989.06	1.31	987.75	1.28	986.47	1.26	985.21	1.24	983.97	1.20	982.77	1.20
	0.24		0.23		0.23		0.23		0.24		0.24		0.24		0.25		0.26		0.26		0.27		0.29	
24°	997.33	1.49	995.94	1.47	994.37	1.43	992.94	1.41	991.53	1.37	990.16	1.34	988.82	1.32.	987.50	1.29	986.21	1.26	984.95	1.25	983.70	1.22	982.48	1.20
	0.24		0.25		0.24		0.25		0.24		0.25		0.26		0.26		0.26		0.27		0.28		0.28	
25°	997.09	1.50	995.59	1.46	994.13	1.44	992.69	1.40	991.29	1.38	989.91	1.35	988.56	1.32	987.24	1.29	985.95	1.27	984.68	1.26	983.42	1.22	982.20	1.21
	0.25		0.25		0.26		0.25		0.26		0.26		0.26		0.26		0.28		0.28		0.28		0.30	
26°	996.84	1.50	995.34	1.47	993.87	1.43	992.44	1.41	991.03	1.38	989.65	1.35	988.30	1.32	986.98	1.31	985.67	1.27	984.40	1.26	983.14	1.24	981.90	1.22
	0.26		0.26		0.26		0.27		0.27		0.27		0.27		0.28		0.28		0.29		0.30		0.30	
27°	996.58	1.50	995.68	1.47	993.61	1.44	992.17	1.41	990.76	1.38	989.38	1. 35	988.03	1.33	986.70	1.31	985.39	1.28	984.11	1.27	982.84	1.24	981.60	1.23
	0.27		0.27		0.27		0.27		0.28		0.28		0.29		0.29		0.29		0.30		0.31		0.32	
28°	996.31	1.50	994.81	1.47	993.34	1.44	991.90	1.42	990.48	1.38	989.10	1.36	987.74	1.33	986.41	1.31	985.10	1.29	983.81	1.28	982.53	1.25	981.28	1.23
	0.28		0.28		0.28		0.29		0.28		0.29		0.29		0.30		0.31		0.31		0.31		0.32	
29°	996.03	1.50	994.53	1.47	993.06	1.45	991.61	1.41	990.20	1.39	988.81	1.36	987.45	1.34	986.11	1.32	984.79	1.29	983.50	1.28	982.22	1.26	980.96	1.24
	0.28		0.29		0.29		0.29		0.30		0.30		0.31		0.31		0.31		0.32		0.32		0.33	
30°	995.75	1.51	994.24	1.47	992.77	1.45	991.32	1.42	989.90	1.39	988.51	1.37	987.14	1.34	985.80	1.32	984.48	1.30	983.18	1.28	981.90	1.27	980.63	1.25
	0.30		0.30		0.30		0.30		0.31		0.31		0.31		0.31		0.32		0.33		0.34		0.34	
31°	995.45	1.51	993.94	1.47	992.47	1.45	991.02	1.43	989.59	1.39	988.20	1.37	986.83	1.34	985.49	1.33	984.16	1.31	982.85	1.29	981.56	1.27	980.29	1.26
	0.31		0.31		0.31		0.32		0.31		0.32		0.32		0.33		0.33		0.34		0.35		0.36	etc
30°	995.14	1.51	993.63	1.47	992.16	1.46	990.70	1.42	989.28	1.40	987.88	1.37	986.51	1.35	985.16	1.33	983.83	1.32	982.51	1.30	981.21	1.28	979.93	1.26
	0.31		0.31		0.32		0.32		0.32		0.33		0.33		0.34		0.35		0.35		0.35		0.35	
33°	994.93	1.51	993.32	1.48	991.84	1.46	990.38	1.42	988.96	1.41	987.55	1.37	986.18	1.36	984.82	1.34	983.48	1.32	982.16	1.30	980.86	1.28	979.58	1.28
	0.32		0.33		0.33		0.33		0.35		0.34		0.35		0.35		0.34		0.35		0.36		0.37	
34°	994.51	1.52	992.99	1.48	991.51	1.46	990.05	1.44	988.61	1.40	987.21	1.38	985.83	1.36	984.47	1.33	983.14	1.33	981.81	1.31	980.50	1.29	979.21	1.28
	0.33		0.33		0.34		0.35		0.34		0.35		0.35		0.35		0.36		0.36		0.36		0.37	
35°	994.18	1.52	992.66	1.49	991.17	1.47	989.70	1.43	988.27	1.41	986.86	1.38	985.48	1.36	984.12	1.34	982.78	1.33	981.45	1.31	980.14	1.30	978.84	1.29
	0.34		0.35		0.35		0.35		0.35		0.35		0.35		0.36		0.36		0.37		0.37		0.38	
36°	993.84	1.53	992.31	1.49	990.82	1.47	989.35	1.43	987.92	1.41	986.51	1.38	985.13	1.37	983.76	1.34	982.42	1.34	981.08	1.31	979.77	1.31	978.46	1.29
	0.35		0.35		0.36		0.35		0.36		0.36		0.37		0.37		0.38		0.37		0.39		0.39	
37°	993.49	1.53	991.96	1.50	990.46	1.46	989.00	1.44	987.56	1.41	986.15	1.39	984.76	1.37	983.39	1.35	982.04	1.33	980.71	1.33	97938	1.31	978.07	1.30
	0.36		0.36		0.36		0.37		0.37		0.37		0.37		0.37		0.38		0.39		0.38		0.39	
38°	993.13	1.53	991.60	1.50	990.10	1.47	988.63	1.44	987.19	1.41	985.78	1.39	984.39	1.37	983.02	1.36	981.66	1.34	980.32	1.32	979.00	1.32	977.68	1.31
	0.36		0.37		0.37		0.37		0.38		0.38		0.38		0.39		0.38		0.39		0.40		0.40	
39°	992.77	1.54	991.23	1.50	989.73	1.47	988.26	1.45	986.81	1.41	985.40	1.39	994.01	1.38	982.63	1.35	981.28	1. 35	979.93	1.33	978.60	1.32	977.28	1.32
	0.37		0.37		0.38		0.39		0.38		0.39		0.39		0.39		0.40		0.39		0.40		0.41	
40⁰	992.40	1.54	990.86	1.51	989.35	1.48	987.87	1.44	986.43	1.42	985.01	1.39	983.62	1.38	982.24	1.36	980.88	1.34	979.54	1.34	978.20	1.33	976.87	1.32

TABLE I (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																					
t°	10		11		12		13		14		15		16		17		18		19		20		21	
0	986.93	1.00	985.93	0.95	984.98	0.92	984.06	0.88	983.1	0.84	982.34	0.80	981.54	0.78	980.76	0.75	980.01	0.73	979.28	0.72	978.56	0.70	977.86	0.70
	-0.02		-0.01		0.01		0.01		0.03		0.04		0.07		0.08		0.10		0.12		0.14		0.17	
1	986.95	1.01	995.94	0.97	984.97	0.92	984.05	0.90	983.1	0.85	982.30	0.83	981.47	0.79	980.68	0.77	979.91	0.75	979.16	0.74	978.42	0.73	977.69	0.72
	-0.01		0.00		0.01		0.03		0.04		0.07		0.08		0.10		0.12		0.14		0.16		0.18	
2	986.96	1.02	985.94	0.98	984.96	0.94	984.02	0.91	983.1	0.98	982.23	0.84	981.39	0.81	980.58	0.79	979.79	0.77	979.02	0.76	978.26	0.75	977.51	0.74.
	0.01		0.02		0.04		0.05		0.06		0.07		0.09		0.11		0.13		0.15		0.17		0.19	
3	986 95	1.03	985.92	1.00	984.92	0.95	983.97	0.92	983.0	0.89	982.16	0.86	981.30	0.83	980.47	0.81	979.66	0.79	978.87	0.78	978.09	0.77	977.32	0.77
	0.03		0.04		0.04		0.06		0.07		0.09		0.10		0.12		0.14		0.16		0.18		0.20	
4	986 92	1.04	985.88	1.00	984.88	0.97	983.91	0.93	982.9	0.91	982.07	0.87	981.20	0.85	980.35	0.83	979.52	0.81	978.71	0.80	977.91	0.79	977.12	0.79
	0.04		0.05		0.06		0.07		0.09		0.10		0.12		0.14		0.15		0.17		0.19		0.22	
5	986.88	1.05	985.83.	1.01	984.82	0.98	983.84	0.95	982.8	0.92	981.97	0.89	981.08	0.87	980.21	0.84	979.37	0.83	978.54	0.82	977.72	0.82	976 90	0.80
	0.05		0.06		0.08		0.09		0.10		0.12		0.13		0.14		0.17		0.19		0.21		0.22	
6	986.93	1.06	985.77	1.03	984.74	0.99	983.75	0.96	982.7	0.94	981.85	0.90	980.95	0.88	980.07	0.87	979.20	0.85	978.35	0.84	977.51	0.83	976.68	0.83
	0.08		0.09		0.09		0.10		0.12		0.13		0.15		0.16		0.18		0.19		0.21		0.23	
7	986.75	1.07	995.68	1.03	984.65	1.00	983.65	0.98	982.6	0.95	981.72	0.92	980.80	0.89	979.91	0.89	979.02	0.86	978.16	0.86	977.30	0.85	976.45	0.85
	0.08		0.09		0.11		0.13		0.13		0.14		0.15		0.18		0.19		0.21		0.23		0.25	
8	986.67	1.08	985.59	1.05	984.54	1.02	983.52	0.98	982.5	0.96	981.58	0.93	980.65	0.92	979.73	0.90	978.83	0.88	977.95	0.88	977.07	0.87	976.20	0.87
	0.10		0 11		0.12		0.12		0.14		0.16		0.18		0.19		0.21		0.22		0.24		0.26	
9	986.57	1.09	985.48	1.06	984.42	1.02	983.40	1.00	982.4	0.98	981.42	0.95	980.47	0.93	979.54	0.92	978.62	0.89	977.73	0.90	976.83	0.89	975.94	0.89
	0.11		0.12		0.12		0.14		0.16		0.17		0.18		0.20		0.20		0.23		0.24		0.26	
10	986.46	1.10	985.36	1.06	984.30	1.04	983.26	1.02	982.2	0.99	981.25	0.96	980.29	0.95	979.34	0.92	978.42	.0.92	977.50	0.91	976.59	0.91	975.68	0.91
	0.12		0.13		0.14		0.16		0.16		0.17		0.19		0.20		0.23		0.25		0.27		0.29	
11	986.34	1.11	985.23	1.07	984.16	1.06	983.10	1.02	982.0	1.00	981.08	0.98	980.10	0.96	979.14	0.95	978.19	0.94	977.25	0.93	976.32	0.93	975.39	0.92
	0.13		0.14		0.16		0.16	1	0.18		0.19		0.21		0.22		0 24		0.25		0.27		0.28	
12	986.21	1.12	985.09	1.09	984.00	1.06	982.94	1.04	981.9	1.01	980.89	1.00	979.89	0.97	978.92	0.97	977.95	0.95	977.00	0.95	976.05	0.94	975.11	0.95
	0.15		0.16		0.16		0.18		0.19		0.20		0.21		0.23		0 24		0.26		0.28		0.30	
13	986.06	1.13	984.93	1.09	983.84	1.08	982.76	1.05	981.71	1.02	980.69	1.01	979.68	0.99	978.69	0.98	977.71	0.97	976.74	0.97	975.77	0.96	974.81	096
	0.16		0.16		0.18		0.18		0.20		0.22		0.23		0.24		0.26		0.27		0.28		0.30	
14	985.90	1.13	994.77	1.11	983.66	1.08	982.58	1.07	981.51	1.04	980.47	1.02	979.45	1.00	978.45	1.00	977.45	0.98	976.47	0.98	975.49	0.98	975.51	0.98
	0.17		0.18		0.19		0.20		0.21		0.22		0.24		0.25		0.26		0.28		0.30		0.32	
15	985.73	1.14	994.59	1.12	983.47	1.09	982.38	1.08	981.30	1.05	960.25	1.04	979.21	1.01	978.20	1.01	977.19	1.00	976.19	1.00	975.19	1.00	974.19	1.00
	0.18		0.19		0.20		0.22		0.22		0.24		0.24		0.27		0.28		0.30		0.31		0.32	
16	985.55	1.15	984.40	1.13	983.27	1.11	982.16	1.08	981.08	1.07	980.01	1.04	978.97	1.04	977.93	1.02	976.91	1.02	975.89	1.01	974.88	1.01	973.87	1.02
	0.19		0.20		0.21		0.22		0.23		0.24		0.26		0.27		0.29		0.30		0.32		0.33	
17	985.13	1.16	984.20	1.14	983.06	1.12	981.94	1.09	980.85	1.08	979.77	1.06	978.71	1.05	977.66	1.04	976.62	1.03	975.59	1.03	974.56	1.02	973.54	1.04
	0.21		0.22		0.22		0 23		0.25		0.26		0.27		0.28		0.29		0.31		0.32		0.35	
18	985.15	1.17	983.76	1.14	982.84	1.13	981.71	1.11	980.60	1.09	979.51	1.07	978.44	1.06	977.38	1.05	976.33	1.05	975.28	1.04	974.24	1.05	973.19	1.05
	0.21		0.22		0.24		0.24		0.25		0.26		0.28		0.29		0.31		0.32		0.34		0.35	
19	984.94	1.18	983.76	1.16	982.60	1.13	981.47	1.12	980.35	1.10	979.25	1.09	978.16	1.07	977.09	1.07	976.02	1.06	974.96	1.06	973.90	1.06	972.84	1.06
	0.23		0.24		0.24		0.26		0.27		0.28		0.29		0.30		0.31		0.33		0.34		0.36	
20	984.71	1.19	983.52	1.16	982.36	1.15	981.21	1.13	980.08	1.11	978.97	1.10	977.87	1.08	1976.7	1.08	1975.7	1.08	974.63	1.07	973.56	1.08	972.48	1.08

TABLE I (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																					
t°	10		11		12		13		14		15		16		17		18		19		20		21	
20°	984.71	1.19	983.52	1.16	982.36	1.15	981.21	1.13	980.08	1.11	978.97	1.10	977.87	1.08	976.79	1.08	975.71	1.08	974.63	1.07	973.56	1.08	972.48	1.08
	0.24		0.24		0.26		0.26		0.27		0.28		0.29		0.31		0.33		0.34		0.36		0.37	
21°	994.47	1.19	983.28	1.18	982.10	1.15	980.95	1.14	978.81	1.12	978.69	1.11	977.58	1.10	976.48	1.10	975.38	1.09	974.29	1.09	973.20	1.09	972.11	1.09
	0.24		0.26		0.28		0.29		0.30		0.31		0.33		0.33		0.35		0.35		0.36		0.37	
22°	984.23	1.21	983.02	1.18	981.84	1.17	980.67	1.15	979.52	1.13	978.39	1.12	977.27	1.12	976.15	1.10	975.05	1.11	973.94	1.10	972.84	1.10	971.74	1.12
	0.26		0.26		0.27		0.28		0.29		0.31		0.32		0.33		0.35		0.35		0.37		0.39	
23°	983.97	1.20	982.77	1.20	981.57	1.18	980.39	1.16	979.23	1.15	978.08	1.13	976.95	1.13	975.82	1.12	974.70	1.11	973.59	1.12	972.47	1.12	971.47	1.12
	0.27		0.29		0.29		0.29		0.30		0.31		0.33		0.33		0.35		0.37		0.38		0.40	
24°	983.70	1.22	982.48	1.20	981.28	1.18	980.10	1.17	978.93	1.16	977.77	1.15	976.62	1.13	975.49	1.14	974.35	1.13	973.22	1.13	972.09	1.14	970.95	1.14
	0.28		0.28		0.29		0.31		0.32		0.33		0.33		0.35		0.36		0.37		0.39		0.40	
25°	983.42	1.22	982.20	1.21	980.99	1.20	979.79	1.18	978.61	1.17	977.44	1.15	976.29	1.15	975.14	1.15	973.99	1.14	972.85	1.15	971.70	1.15	970.55	1.16
	0.28		0.30		0.31		0.31		0.32		0.33		0.35		0.36		0.37		0.39		0.40		0.41	
26°	983.14	1.24	981.90	1.22	980.68	1.20	979.48	1.19	978.29	1.18	977.11	1.17	975.94	1.16	974.78	1.16	973.62	1.16	972.46	1.16	971.30	1.16	970.14	1.17
	0.30		0.30		0.31		0.32		0.33		0.34		0.35		0.36		0.38		0.39		0.40		0.42	
27°	982.84	1.24	981.60	1.23	980.37	1.21	979.16	1.20	977.96	1.19	976.77	1.18	975.59	1.17	974.42	1.18	973.24	1.17	972.07	1.17	970.90	1.18	969.72	1.18
	0.31		0.32		0.32		0.33		0.34		0.35		0.36		0.38		0.38		0.40		0.41		0.43	
28°	982.53	1.25	981.28	1.23	980.05	1.22	978.83	1.21	977.62	1.20	976.42	1.19	975.23	1.19	974.04	1.18	972.86	1.19	971.67	1.18	970.49	1.20	969.29	1.20
	0.31		0.32		0.33		0.34		0.35		0.36		0.37		0.38		0.40		0.40		0.42		0.43	
29°	982.22	1.26	980.96	1.24	979.72	1.23	978.49	1.22	977.27	1.21	976.06	1.20	974.86	1.20	973.66	1.20	972.46	1.19	971.27	1.20	970.07	1.21	968.86	1.22
	0.32		0.33		0.34		0.35		0.36		0.37		0.38		0.40		0.41		0.43		0.44		0.45	
30°	981.90	1.27	980.63	1.25	979.38	1.24	978.14	1.23	976.91	1.22	975.69	1.21	974.48	1.22	973.26	1.21	972.05	1.21	970.84	1.21	969.63	1.22	968.41	1.23
	0.34		0.34		0.35		0.36		0.37		0.38		0.40		0.40		0.41		0.42		0.44		0.45	
31°	981.56	1.27	980.29	1.26	979.03	1.25	977.78	1.24	976.54	1.23	975.31	1.23	974.08	1.22	972.86	1.22	971.64	1.22	970.42	1.23	969.19	1.23	967.96	1.24
	0.35		0.36		0.36		0.37		0.38		0.39		0.39		0.40		0.42		0.43		0.44		0.46	
32°	981.21	1.28	979.93	1.26	978.67	1.26	977.41	1.25	976.16	1.24	974.92	1.23	973.69	1.23	972.46	1.24	971.22	1.23	969.99	1.24	968.75	1.25	967.50	1.25
	0.35		0.35		0.37		0.37		0.38		0.39		0.40		0.42		0.42		0.44		0.45		0.46	
33°	980.86	1.28	979.58	1.28	978.30	1.26	977.04	1.26	975.78	1.25	974.53	1.24	973.29	1.25	972.04	1.24	970.80	1.25	969.55	1.25	968.30	1.26	967.04	1.27
	0.36		0.37		0.37		0.38		0.39		0.40		0.41		0.42		0.43		0.44		0.46		0.47	
34°	980.50	1.29	979.21	1.28	977.93	1.27	976.66	1.27	975.39	1.26	974.13	1.25	972.88	1.26	971.62	1.25	970.37	1.26	969.11	1.27	967.84	1.27	966.57	1.29
	0.36		0.37		0.38		0.39		0.39		0.40		0.42	-	0.42		0.44		0.46		0.46		0.48	
35°	980.14	1.30	978.94	1.29	977.55	1.28	976.27	1.27	975.00	1.27	973.73	1.27	972.46	1.26	971.20	1.27	969.93	1.28	968.65	127	967.38	1.29	966.09	1.30
	0.37		0.38		0.38		0.39		0.40		0.41		0.42		0.44		0.45		0.45		0.47		0.48	
36°	979.77	1.31	978.46	1.29	977.17	1.29	975.88	1.28	974.60	1.28	973.32	1.28	972.04	1.28	970.76	1.28	969.48	1.28	968.20	1.29	966.91	1.30	965.61	1.32
	0.39		0.39		0.40		0.40		0.41		0.42		0.43		0.44		0.45		0.47		0.48		0.49	
37°	978.38	1.31	978.07	1.30	976.77	1.29	975.48	1.29	974.19	1.29	972.90	1.29	971.61	1.29	970.32	1.29	969.03	1.30	967.73	1.30	966.43	1.31	965.12	1.33
	0.38		0.39		0.40		0.41		0.42		0.43		0.44		0.45		0.46		0.47		0.49		0.50	
38°	979.00	1.32	977.68	1.31	976.37	1.30	975.07	1.30	973.77	1.30	972.47	1.30	971.17	1.30	969.87	1.30	968.57	1.31	967.26	1.32	965.94	1.32	964.62	1.34
	0.40		0.40		0.41		0.42		0.42		0.43		0.44		0.45		0.47		0.48		0.49		0.50	
39°	978.60	1.32	977.28	1.32	975.96	1.31	974.65	1.30	973.35	1.31	972.04	1.31	970.73	1.31	969.42	1.32	968.10	1.32	966.78	1.33	965.45	1.33	964.12	1.36
	0.40		0.41		0.41		0.42		0.43		0.44		0.45		0.46		0.47		0.48		0.49		0.51	
40°	978.20	1.33	976.87	1.32	975.55	1.32	974.23	1.31	972.92	1.32	971.60	1.52	970.28	1.32	968.96	1.33	967.63	1.33	966.30	1.34	964.96	1.35	963.61	1.37

TABLE I (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - <u>Pyrex pycnometer</u> Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																													
t°	20		21		22		23		24		25		26		27		28		29		30		31							
0	978.56	0.70	977.86	0.70	977.16	0.69	976.47	0.71	975.76	0.71	975.05	0.72	974.33	0.75	973.58	0.77	972.81	0.80	972.01	0.83	971.18	0.87	970.31	0.90						
	0.14		0.17		0.19		0.22		0.24		0.26		0.29		0.31		0.34		0.36		0.39		0.41							
1	978.42	0.73	977.69	0.72	976.97	0.72	976.25	0.73	975.52	0.73	974.79	0.75	974.04	0.77	973.27	0.80	972.47	0182	971.65	0.86	970.79	0.89	969.90	0.92						
	0.16		0.18		0.20		0.23		0.25		0.28		0.30		0.32		0.34		0.37		0.39		0.41							
2	978.26	0.75	977.51	0.74	976.77	0.75	976.02	0.75	975.27	0.76	974.51	0.77	973.74	0.79	972.95	0.82	972.13	0.85	971.28	0.88	970.40	0.91	969.49	0.95						
	0.17		0.19		0.22		0.23		0.26		0.28		0.31		0.33		0.36		0.38		0.40		0.42							
3	978.09	0.77	977.32	0.77	976.55	0.76	975.79	0.78	975.01	0.78	974.23	0.80	973.43	0.81	972.62	0.85	971.77	0.87	970.90	0.90	970.00	0.93	969.07	0.98						
	0.18		0.20		0.22		0.25		0.27		0.29		0.31		0.34		0.36		0.38		0.40		0.43							
4	977.91	0.79	977.12	0.79	976.33	0.79	975.54	0.80	974.94	0.80	973.94	0.82	973.12	0.84	972.28	0.87	971.41	0.89	970.52	0.92	969.60	0.96	968.64	1.00						
	0.19		0.22		0.23		0.26		0.27		0.30		0.33		0.35		0.37		0.39		0.42		0.44							
5	977.72	0.82	976.90	0.80	976.10	0.82	975.28	0.81	974.47	0.83	973.64	0.85	972.79	0.86	971.93	0.89	971.04	0.91	970.13	0.95	969.18	0.98	968.20	1.01						
	0.21		0.22		0.25		0.26		0.29		0.31		0.33		0.35		0.37		0.40		0.42		0.44							
6	977.51	0.83	976.68	0.83	975.85	0.83	975.02	0.84	974.18	0.85	973.33	0.87	972.46	0.86	971.58	0.91	970.67	0.94	969.73	0.97	968.76	1.00	967.76	1.03						
	0.21		0.23		0.25		0.28		0.30		0.32		0.34		0.36		0.36		0.40		0.42		0.44							
7	977.30	0.85	976.45	0.85	975.60	0.86	974.74	0.86	973.88	0.87	973.01	0.89	972.12	0.90	971.22	0.93	970.20	0.96	969.33	0.99	968.34	1.02	967.32	1.06						
	0.23		0.25		0.27		0.28		0.31		0.33		0.35		0.37		0.40		0.42		0.43		0.46							
8	977.07	0.87	976.20	0.87	975.33	0.87	974.46	0.89	973.57	0.89	972.68	0.91	971.77	0.92	970.85	0.96	969.89	0.98	968.91	1.00	967.91	1.05	966.86	1.07						
	0.24		0.26		0.28		0.30		0.31		0.34		0.35		0.38		0.39		0.41		0.44		0.46							
9	976.83	0.89	97.594	0.89	97.505	0.89	974.16	0.90	973.26	0.92	972.34	0.92	971.42	0.95	970.47	0.97	969.50	1.00	968.50	1.03	967.47	1.07	966.40	1.09						
	0.24		0.26		0.28		0.30		0.33		0.34		0.37		0.39		0.41		0.43		0.45		0.46							
10	976.59	0.91	975.68	0.91	974.77	0.91	973.86	0.93	972.93	0.93	972.00	0.95	971.05	0.97	970.08	0.99	969.09	1.02	968.07	1.05	967.02	1.08	965.94	1.12						
	0.27		0.29		0.30		0.33		0.34		0.36		0.38		0.40		0.42		0.44		0.46		0.47							
11	976.32	0.93	975.39	0.92	974.47	0.94	973.53	0.94	972.59	0.95	971.64	0.97	970.67	0.99	969.68	1.01	968.67	1.04	967.63	1.07	966.56	1.09	965.47	1.13						
	0.27		0.28		0.31		0.32		0.34		0.36		0.38		0.40		0.42		0.44		0.45		0.48							
12	976.05	0.94	975.11	0.95	974.16	0.95	973.21	0.96	972.25	0.97	971.28	0.99	970.29	1.01	969.28	1.03	968.25	1.06	967.19	1.08	966.11	1.12	964.99	1.15						
	0.28		030		0.31		0.33		0.35		0.37		0.39		0.41		0.43		0.45		0.47		0.49							
13	975.77	0.96	974.81	0.96	973.85	0.97	972.88	0.98	971.90	0.99	970.91	1.01	969.90	1.03	968.87	1.05	967.82	1.08	966.74	1.10	965.64	1.14	964.50	1.17						
	0.28		0.30		0.32		0.34		0.36		0.38		0.40		0.41		0.43		0.45		0.47		0.49							
14	975.49	0.98	974.51	0.98	973.53	0.99	972.54	1.00	971.54	1.01	970.53	1.03	969.50	1.04	968.46	1.07	967.39	1.10	966.29	1.12	965.17	1.16	964.01	1.19						
	0.30		0.32		0.34		0.35		0.37		0.39		0.40		0.42		0.44		0.46		0.48		0.49							
15	975.19	1.00	974.19	1.00	973.19	1.00	972.19	1.02	971.17	1.03	970.14	1.04	969.10	1.06	968.04	1.09	966.95	1.12	965.83	1.14	964.69	1.17	963.52	1.21						
	0.31		0.32		0.34		0.36		0.37		0.39		0.41		0.43		0.45		0.46		0.48		0.51							
16	974.88	1.01	973.87	1.02	972.85	1.02	971.83	1.03	970.80	1.05	969.75	1.06	968.69	1.08	967.61	1.11	966.50	1.13	965.37	1.16	964.21	1.20	963.01	1.22						
	0.32		0.33		0.35		0.37		0.39		0.40		0.42		0.44		0.45		0.48		0.50		0.50							
17	974.56	1.02	973.54	1.04	972.50	1.04	971.46	1.05	970.41	1.06	969.35	1.08	968.27	1.10	967.17	1.12	966.05	1.16	964.89	1.18	963.71	1.20	962.51	1.24						
	0.32		0.35		0.36		0.37		0.39		0.41		0.43		0.45		0.47		0.48		0.49		0.52							
18	974.24	1.05	973.19	1.05	972.14	1.05	971.09	1.07	970.02	1.08	968.94	1.10	967.84	1.12	966.72	1.14	965.58	1.17	964.41	1.19	963.22	1.23	961.99	1.25						
	0.34		0.35		0.36		0.39		0.40		0.42		0.43		0.45		0.47		0.48		0.50		0.52							
19	973.90	1.06	972.84	1.06	971.78	1.08	970.70	1.08	969.62	1.10	968.52	1.11	967.41	1.14	966.27	1.16	965.11	1.18	963.93	1.21	962.72	1.25	961.47	1.27						
	0.34		0.36		0.38		0.39		0.41		0.42		0.45		0.46		0.47		0.49		0.51		0.52							
20	973.56	1.08	972.48	1.08	971.40	1.09	970.31	1.10	969.21	1.11	968.10	1.14	966.96	1.15	965.81	1.17	964.64	1.20	963.44	1.23	962.21	1.26	960.95	1.29						

TABLE I (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures - <u>Pyrex pycnometer</u> Densities at *t*°C. corrected for air buoyancy

	Alcohol % by volume																							
t °	20		21		22		23		24		25		26		27		28		29		30		31	
20	973.56	1.08	972.48	1.08	971.40	1.09	970.31	1.10	969.21	1.11	968.10	1.14	966.96	1.15	965.81	1.17	964.64	1.20	963.44	1.23	962.21	1.26	960.95	1.29
	0.36		0.37		0.38		0.40		0.42		0.44		0.45		0.46		0.49		0.50		0.52		0.53	
21	973.20	1.09	972.11	1.09	971.02	1.11	969.91	1.12	968.79	1.13	967.66	1.15	966.51	1.16	965.35	1.20	964.15	1.21	962.94	1.25	961.69	1.27	960.42	1.31
	0.36		0.37		0.40		0.41		0.42		0.44		0.45		0.48		0.49		0.51		0.52		0.54	
22	972.84	1.10	971.74	1.12	970.62	1.12	969.50	1.13	968.37	1.15	967.22	1.16	966.06	1.19	964.87	1.21	963.66	1.23	962.43	1.26	961.17	1.29	959.88	1.32
	0.37		0.39		0.40		0.42		0.43		0.45		0.47		0.48		0.49		0.51		0.53		0.55	
23	972.47	1.12	971.35	1.13	970.22	1.14	969.08	1.14	967.94	1.17	966.77	1.18	965.59	1.20	964.39	1.22	963.17	1.25	961.92	1.28	960.64	1.31	959.33	1.33
	0.38		0.40		0.41		0.42		0.44		0.45		0.47		0.49		0.51		0.52		0.54		0.55	
24	972.09	1.14	970.95	1.14	969.81	1.15	968.66	1.16	967.50	1.18	966.32	1.20	965.12	1.22	963.90	1.24	962.66	1.26	961.40	1.30	960.10	1.32	958.78	1.35
	0.39		0.40		0.42		0.43		0.45		0.47		0.48		0.49		0.51		0.53		0.54		0.55	
25	971.70	1.15	970.55	1.16	969.39	1.16	968.23	1.18	967.05	1.20	965.85	1.21	964.64	1.23	963.41	1.26	962.15	1.28	960.87	1.31	959.56	1.33	958.23	1.37
	0.40		0.41		0.42		0.44		0.46		0.47		0.49		0.50		0.51		0.53		0.54		0.57	
26	971.30	1.16	970.14	1.17	968.97	1.18	967.79	1.20	966.59	1.21	965.38	1.23	964.15	1.24	962.91	1.27	961.64	1.30	960.34	1.32	959.02	1.36	957.66	1.38
	0.40		0.42		0.43		0.45		0.46		0.48		0.49		0.51		0.53		0.54		0.56		0.56	
27	970.90	1.18	969.72	1.18	968.54	1.20	967.34	1.21	966.13	1.23	964.90	1.24	963.66	1.26	962.40	1.29	961.11	1.31	959.80	1.34	958.46	1.36	957.10	1.40
	0.41		0.43		0.45		0.46		0.47		0.48		0.50		0.52		0.54		0.56		0.57		0.59	
28	970.49	1.20	969.29	1.20	968.09	1.21	966.88	1.22	965.66	1.24	964.42	1.26	963.16	1.28	961.88	1.31	960.57	1.33	959.24	1.35	957.89	1.38	956.51	1.41
	0.42		0.43		0.45		0.47		0.49		0.50		0.52		0.53		0.53		0.55		0.56		0.58	
29	970.07	1.21	968.86	1.22	967.64	1.23	966.41	1.24	965.17	1.25	963.92	1.28	962.64	1.29	961.35	1.31	960.04	1.35	958.69	1.36	957.33	1.40	955.93	1.42
	0.44		0.45		0.46		0.47		0.49		0.50		0.51		0.53		0.55		0.55		0.58		0.58	
30	969.63	1.22	968.41	1.23	967.18	1.24	965.94	1.26	964.68	1.26	963.42	1.29	962.13	1.31	960.82	1.33	959.49	1.35	958.14	1.39	956.75	1.40	955.35	1.44
	0.44		0.45		0.46		0.48		0.49		0.51		0.52		0.53		0.55		0.57		0.58		0.60	
31	969.19	1.23	967.96	1.24	966.72	1.26	965.46	1.27	964.19	1.28	962.91	1.30	961.61	1.32	960.29	1.35	958.94	1.37	957.57	1.40	956.17	1.42	954.75	1.44
	0.44		0.46		0.47		0.48		0.50		0.51		0.53		0.54		0.55		0.57		0.58		0.59	
32	968.75	1.25	967.50	1.25	966.25	1.27	964.98	1.29	963.69	1.29	962.40	1.32	961.08	1.33	959.75	1.36	958.39	1.39	957.00	1.41	955.59	1.43	954.16	1.46
	0.45		0.46		0.48		0.49		0.50		0.52		0.53		0.55		0.57		0.57		0.59		0.61	
33	968.30	1.26	967.04	1.27	965.77	1.28	964.49	1.30	963.19	1.31	961.88	1.33	960.55	1.35	959.20	1.38	957.82	1.39	956.43	1.43	955.00	1.45	953.55	1.47
	0.46		0.47		0.49		0.50		0.51		0.53		0.54		0.56		0.56		0.59		0.59		0.60	
34	967.84	1.27	966.57	1.29	965.28	1.29	963.99	1.31	962.68	1.33	961.35	1.34	960.01	1.37	958.64	1.38	957.26	1.42	95584	1.43	954.41	1.46	952.95	1.49
	0.46		0.48		0.49		0.51		0.52		0.53		0.55		0.56		0.58		0.58		0.60		0.62	
35	967.38	1.29	966.09	1.30	964.79	1.31	963.48	1.32	962.16	1.34	960.82	1.36	959.46	1.38	958.08	1.40	956.68	1.42	955.26	1.45	953.81	1.48	952.33	1 50
	0.47		0.48		0.50		0.51		0.53		0.54		0.55		0.57		0.58		0.60		0.61		0.62	
36	966.91	1.30	965.61	1.32	964.29	1.32	962.97	1.34	961.63	1.35	960.28	1.37	958.91	1.40	957.51	1.41	956.10	1.44	954.66	1.46	953.20	1.49	951.71	1.51
	0.48		0.49		0.50		0.52		0.53		0.55		0.56		0.57		0.59		0.60		0.61		0.62	
37	966.43	1.31	965.12	1.33	963.79	1.34	962.45	1.35	961.10	1.37	959.73	1.38	958.35	1.41	956.94	1.43	955.51	1.45	954.06	1.47	952.59	1.50	951.09	1.53
	0.49		0.50		0.51		0.52		0.54		0.55		0.57		0.58		0.59		0.60		0.62		0.63	
38	965.94	1.32	964.62	1.34	963.28	1.35	961.93	1.37	960.56	1.38	959.18	1.40	957.78	1.42	956.36	1.44	954.92	1.46	953.46	1.49	951.97	1.51	950.4	1.54
	0.49		0.50		0.52		0.53		0.54		0.56		0.57		0.58		0.60		0.61		0.62		0.64	
39	965.45	1.33	964.12	1.36	962.76	1.36	961.40	1.38	960.02	1.40	958.62	1.41	957.21	1.43	955.78	1.46	954.32	1.47	952.85	1.50	951.35	1.53	949.82	1.55
	0.49		0.51		0.52		0.54		0.55		0.56		0.58		0.59		0.60		0.62		0.63		0.64	
40	964.96	1.35	963.61	1.37	962.24	1.38	960.86	1.39	959.47	1.41	958.06	1.43	956.63	1.44	955.19	1.47	953.72	1.49	952.23	1.51	950.72	1.54	949.18	1.57

TABLE II International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature

Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

Temperatures	To add	Apparent alcoholic strength at *t*°C																
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0		0.76	0.77	0.82	0.87	0.95	1.04	1.16	1.31	1.49	1.70	1.95	2.26	2.62	3.03	3.49	4.02	4.56
1°		0.81	0.83	0.87	0.92	1.00	1.09	1.20	1.35	1.52	1.73	1.97	2.26	2.59	2.97	3.40	3.87	4.36
2°		0.85	0.87	0.92	0.97	1.04	1.13	1.24	1.38	1.54	1.74	1.97	2.24	2.54	2.89	3.29	3.72	4.17
3°		0.88	0.91	0.95	1.00	1.07	1.15	1.26	1.39	1.55	1.73	1.95	2.20	2.48	2.80	3.16	3.55	3.95
4°		0.90	0.92	0.97	1.02	1.09	1.17	1.27	1.40	1.55	1.72	1.92	2.15	2.41	2.71	3.03	3.38	3.75
5°		0.91	0.93	0.98	1.03	1.10	1.17	1.27	1.39	1.53	1.69	1.87	2.08	2.33	2.60	2.89	3.21	3.54
6°		0.92	0.94	0.98	1.02	1.09	1.16	1.25	1.37	1.50	1.65	1.82	2.01	2.23	2.47	2.74	3.02	3.32
7°		0.91	0.93	0.97	1.01	1.07	1.14	1.23	1.33	1.45	1.59	1.75	1.92	2.12	2.34	2.58	2.83	3.10
8°		0.89	0.91	0.94	0.98	1.04	1.11	1.19	1.28	1.39	1.52	1.66	1.82	2.00	2.20	2.42	2.65	2.88
9°		0.86	0.88	0.91	0.95	1.01	1.07	1.14	1.23	1.33	1.44	1.57	1.71	1.97	2.05	2.24	2.44	2.65
10°		0.82	0.84	0.87	0.91	0.96	1.01	1.08	1.16	1.25	1.35	1.47	1.60	1.74	1.89	2.06	2.24	2.43
11°		0.78	0.79	0.82	0.86	0.90	0.95	1.01	1.08	1.16	1.25	1 36	1.47	1.60	173	1.88	2.03	2.20
12°		0.72	0.74	0.76	0.79	0.83	0.88	0.93	0.99	1.07	1.15	1.24	1.34	1.44	1.56	1.69	1.82	1.96
13°		0.66	0.67	0.69	0.72	0.76	0.80	0.84	0.90	0.96	1.03	1.11	1.19	1.28	1.38	1.49	1.61	1.73
14°		0.59	0.60	0.62	0.64	0.67	0.71	0.74	0.79	0.85	0.91	0.97	1.04	1.12	1.20	1.29	1.39	1.49
15°		0.51	0.52	0.53	0.55	0.58	0.61	0.64	0.68	0.73	0.77	0.83	0.89	0995	1.02	1.09	1.16	1.24
16°		0.42	0.43	0.44	0.46	0.48	0.50	0.53	0956	0.60	0963	0.67	0.72	0.77	0.82	0.88	0.94	1.00
17°		0.33	0.33	0.34	0.35	0.37	0.39	0.41	0.43	0.46	0.48	0.51	0.55	0.59	0.62	0.67	0.71	0.75
18°		0.23	0.23	0.23	0.24	0.25	0.26	0.27	0.29	0.31	0.33	0.35	0.37	0.40	0.42	0.45	0.48	0.51
19°		0.12	0.12	0.12	0.12	0.13	0.13	0.14	0.15	0.16	0.17	0.18	0.19	0.20	0.21	0.23	0.24	0.25

TABLE II (continued)
International alcoholic strength at 20°C
Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature
Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

Temperatures		To subtract	Apparent alcoholic strength at *t*°C																
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	21°			0.13	0.13	0.13	0.14	0.14	0.15	0.16	0.17	0.18	0.19	0.19	0.20	0.22	0.23	0.25	0.26
	22°			0.26	0.27	0.28	0.29	0.30	0.31	0.32	0.34	0.36	0.37	0.39	0.41	0.44	0.47	0.49	0.52
	23°			0.40	0.41	0.42	0.44	0.45	0.47	0.49	0.51	0.54	0.57	0.60	0.63	0.66	0.70	0.74	0.78
	24°			0.55	0.56	0.58	0.60	0.62	0.64	0.67	0.70	0.73	0.77	0.81	0.85	0.89	0.94	0.99	1.04
	25°			0.69	0.71	0.73	0.76	0.79	0.82	0.85	0.89	0.93	0.97	1.02	1.07	1.13	1.19	1.25	1.31
	26°			0.85	0.87	0.90	0.93	0.96	1.00	1.04	1.08	1.13	1.18	1.24	1.30	1.36	1.43	1.50	1.57
	27°				1.03	1.07	1.11	1.15	1.19	1.23	1.28	1.34	1.40	1.46	1.53	1.60	1.68	1.76	1.84
	28°				1.21	1.25	1.29	1.33	1.38	1.43	1.49	1.55	1.62	1.69	1.77	1.85	1.93	2.02	2.11
	29°				1.39	1.43	1.47	1.52	1.58	1.63	1.70	1.76	1.84	1.92	2.01	2.10	2.19	2.29	2.39
	30°				1.57	1.61	1.66	1.72	1.78	1.84	1.91	1.98	2.07	2.15	2.25	2.35	2.45	2.56	2.67
	31°				1.75	1.80	1.86	1.92	1.98	2.05	2.13	2.21	2.30	2.39	2.49	2.60	2.71	2.83	2.94
	32°				1.94	2.00	2.06	2.13	2.20	2.27	2.35	2.44	2.53	2.63	2.74	2.86	2.97	3.09	3.22
	33°					2.20	2.27	2.34	2.42	2.50	2.58	2.67	2.77	2.88	2.99	3.12	3.24	3.37	3.51
	34°					2.41	2.48	2.56	2.64	2.72	2.81	2.91	3.02	3.13	3.25	3.38	3.51	3.65	3.79
	35°					2.62	2.70	2.78	2.86	2.95	3.05	3.16	3.27	3.39	3.51	3.64	3.78	3.93	4.08
	36°					2.83	2.91	3.00	3.09	3.19	3.29	3.41	3.53	3.65	3.78	3.91	4.05	4.21	4.37
	37°						3.13	3.23	3.33	3.43	3.54	3.65	3.78	3.91	4.04	4.18	4.33	4.49	4.65
	38°						3.36	3.47	3.57	3.68	3.79	3.91	4.03	4.17	4.31	4.46	4.61	4.77	4.94
	39°						3.59	3.70	3.81	3.93	4.05	4.17	4.44	4.58	4.74	4.90	5.06	5.06	5.23

	40°					3.82	3.94	4.06	4.18	4.31	4.44	4.57	4.71	4.86	5.02	5.19	5.36	5.53

TABLE II (continued)
International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature
Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

Temperatures	To add	Apparent alcoholic strength at *t*°C																
		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	0°	3.49	4.02	4.56	5.11	5.65	6.16	6.63	7.05	7.39	7.67	7.91	8.07	8.20	8.30	8.36	8.39	8.40
	1°	3.40	3.87	4.36	4.86	5.35	5.82	6.26	6.64	6.96	7.23	7.45	7.62	7.75	7.85	7.91	7.95	7.96
	2°	3.29	3.72	4.17	4.61	5.05	5.49	5.89	6.25	6.55	6.81	7.02	7.18	7.31	7.40	7.47	7.51	7.53
	3°	3.16	3.55	3.95	4.36	4.77	5.17	5.53	5.85	6.14	6.39	6.59	6.74	6.86	6.97	7.03	7.07	7.09
	4°	3.03	3.38	3.75	4.11	4.48	4.84	5.17	5.48	5.74	5.97	6.16	6.31	6.43	6.53	6.59	6.63	6.66
	5°	2.89	3.21	3.54	3.86	4.20	4.52	4.83	5.11	5.35	5.56	5.74	5.89	6.00	6.10	6.16	6.20	6.23
	6°	2.74	3.02	3.32	3.61	3.91	4.21	4.49	4.74	4.96	5.16	5.33	5.47	5.58	5.67	5.73	5.77	5.80
	7°	2.58	2.83	3.10	3.36	3.63	3.90	4.15	4.38	4.58	4.77	4.92	5.05	5.15	5.24	5.30	5.34	5.37
	8°	2.42	2.65	2.88	3.11	3.35	3.59	3.81	4.02	4.21	4.38	4.52	4.64	4.74	4.81	4.87	4.92	4.95
	9°	2.24	2.44	2.65	2.86	3.07	3.28	3.48	3.67	3.84	3.99	4.12	4.23	4.32	4.39	4.45	4.50	4.53
	10°	2.06	2.24	2.43	2.61	2.80	2.98	3.16	3.33	3.48	3.61	3.73	3.83	3.91	3.98	4.03	4.08	4.11
	11°	1.88	2.03	2.20	2.36	2.52	2.68	2.83	2.98	3.12	3.24	3.34	3.43	3.50	3.57	3.62	3.66	3.69
	12°	1.69	1.82	1.96	2.10	2.24	2.38	2.51	2.64	2.76	2.87	2.96	3.04	3.10	3.16	3.21	3.25	3.27
	13°	1.49	1.61	1.73	1.84	1.96	2.08	2.20	2.31	2.41	2.50	2.58	2.65	2.71	2.76	2.80	2.83	2.85
	14°	1.29	1.39	1.49	1.58	1.68	1.78	1.88	1.97	2.06	2.13	2.20	2.26	2.31	2.36	2.39	2.42	2.44
	15°	1.09	1.16	1.24	1.32	1.40	1.48	1.56	1.64	1.71	1.77	1.83	1.88	1.92	1.96	1.98	2.01	2.03
	16°	0.88	0.94	1.00	1.06	1.12	1.19	1.25	1.31	1.36	1.41	1.46	1.50	1.53	1.56	1.58	1.60	1.62
	17°	0.67	0.71	0.75	0.80	0.84	0.89	0.94	0.98	1.02	1.05	1.09	1.12	1.14	1.17	1.18	1.20	1.21
	18°	0.45	0.48	0.51	0.53	0.56	0.59	0.62	0.65	0.68	0.70	0.72	0.74	0.76	0.78	0.79	0.80	0.81
	19°	0.23	0.24	0.25	0.27	0.28	0.30	0.31	0.33	0.34	0.35	0.36	0.37	0.38	0.39	0.40	0.41	0.41

TABLE II (continued)
International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature
Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

Temperatures	To subtract	Apparent alcoholic strength at *t*°C																		
		14			15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	21°	0.23	0.25	0.26	0.28	0.29	0.30	0.31	0.33	0.34	0.35	0.35	0.37	0.38	0.38	0.39	0.39	0.40		
	22°	0.47	0.49	0.52	0.55	0.57	0.60	0.62	0.65	0.67	0.70	0.72	0.74	0.75	0.76	0.78	0.79	0.80		
	23°	0.70	0.74	0.78	0.82	0.86	0.90	0.93	0.97	1.01	1.04	1.07	1.10	1.12	1.15	1.17	1.18	1.19		
	24°	0.94	0.99	1.04	1.10	1.15	1.20	1.25	1.29	1.34	1.39	1.43	1.46	1.50	1.53	1.55	1.57	1.59		
	25°	1.19	1.25	1.31	1.37	1.43	1.49	1.56	1.62	1.68	1.73	1.78	1.83	1.87	1.90	1.94	1.97	1.99		
	26°	1.43	1.50	1.57	1.65	1.73	1.80	1.87	1.94	2.01	2.07	2.13	2.19	2.24	2.28	2.32	2.35	2.38		
	27°	1.68	1.76	1.84	1.93	2.01	2.10	2.18	2.26	2.34	2.41	2.48	2.55	2.61	2.66	2.70	2.74	2.77		
	28°	1.93	2.02	2.11	2.21	2.31	2.40	2.49	2.58	2.67	2.76	2.83	2.90	2.98	3.03	3.08	3.13	3.17		
	29o	2.19	2.29	2.39	2.50	2.60	2 70	2.81	2.91	3.00	3.09	3.18	3.26	3.34	3.40	3.46	3.51	3.55		
	30o	2.45	2.56	2.67	2.78	2.90	3.01	3.12	3.23	3.34	3.44	3.53	3.62	3.70	3.77	3.84	3.90	3.95		
	31°	2.71	2.83	2.94	3.07	3.19	3.31	3.43	3.55	3.67	3.78	3.88	3.98	4.07	4.15	4.22	4.28	4.33		
	32o	2.97	3.09	3.22	3.36	3.49	3.62	3.74	3.87	4.00	4.11	4.22	4.33	4.43	4.51	4.59	4.66	4.72		
	33o	3.24	3.37	3.51	3.65	3.79	3.92	4.06	4.20	4.33	4.45	4.57	4.68	4.79	4.88	4.97	5.04	5.10		
	34°	3.51	3.65	3.79	3.94	4.09	4.23	4.37	4.52	4.66	4.79	4.91	5.03	5.15	5.25	5.34	5.42	5.49		
	35°	3.78	3.93	4.08	4.23	4.38	4.53	4.69	4.84	4.98	5.12	5.26	5.38	5.50	5.61	5.71	5.80	5.87		
	36°	4.05	4.21	4.37	4.52	4.68	4.84	5.00	5.16	5.31	5.46	5.60	5.73	5.86	5.97	6.08	6.17	6.25		
	37°	4.33	4.49	4.65	4.82	4.98	5.15	5.31	5.48	5.64	5.80	5.95	6.09	6.22	6.33	6.44	6.54	6.63		
	38°	4.61	4.77	4.94	5.12	5.29	5.46	5.63	5.80	5.97	6.13	6.29	6.43	6.57	6.69	6.81	6.92	7.01		
	39°	4.90	5.06	5.23	5.41	5.59	5.77	5.94	6.12	6.30	6.47	6.63	6.78	6.93	7.06	7.18	7.29	7.39		
	40°	5.19	5.36	5.53	5.71	5.90	6.08	6.26	6.44	6.62	6.80	6.97	7.13	7.28	7.41	7.54	7.66	7.76		

TABLE III International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength in %																							
t°	**0**		**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**	
0	999.34	1.52	997.82	1.45	996.37	1.39	994.98	1.35	993.63	1.29	992.34	1.24	991.10	1.18	989.92	1.15	988.77	1.09	987.68	1.05	986.63	1.00	985.63	0.96
	--0.09		-0.09		-0.09		-0.08		-0.08		-0.08		-0.07		-0.05		-0.05		-0.04		-0.03		-0.02	
1	999.43	1.52	997.91	1.45	996.46	1.40	995.06	1.35	993.71	1.29	992.42	1.25	991.17	1.20	989.97	1.15	988.82	1.10	987.72	1.06	986.66	1.01	985.65	0.97
	--0.06		-0.06		-0.06		-0.06		-0.06		-0.05		-0.05		-0.04		-0.03		-0.02		0.02		-0.01	
2	999.49	1.52	997.97	1.40	996.52	1.40	995.12	1.35	993.77	1.30	992.47	1.25	991.22	1.21	990.01	1.16	988.85	1.11	987.74	1.06	986.68	1.02	985.66	0.98
	-0.05		-0.05		-0.04		-0.04		-0.04		-0.04		-0.03		-0.03		-0.03		-0.02		0.00		0.01	
3	999.54	1.52	998.02	1.46	996.56	1.40	995.16	1.35	993.81	1.30	992.51	1.26	991.25	1.21	990.04	1.16	988.88	1.12	987.76	1.08	986.68	1.03	985.65	0.99
	-0.03		-0.03		-0.03		-0.03		-0.02		-0.02		-0.02		-0.01		0.00		0.01		0.01		0.02	
4	999.57	1.52	998.05	1.46	996.59	1.40	995.19	1.36	993.83	1.30	992.53	1.26	991.27	1.22	990.05	1.17	98888	1.13	987.75	1.08	986.67	1.04	985.63	1.00
	-0.02		-0.02		-0.02		-0.02		-0.02		-0.01		0.00		0.00		0.00		0.01		0.02		0.03	
5	999.59	1.52	998.07	1.46	996.61	1.40	995.21	1.36	993.85	1.31	992.54	1.27	991.27	1.22	990.05	1.17	988.88	1.14	987.74	1.09	986.65	1.05	985.60	1.01
	0.00		0.00		0.00		0.01		0.01		0.01		0.01		0.02		0.03		0.03		0.04		0.06	
6	999.59	1.52	998.07	1.46	996.61	1.41	995.20	1.36	993.84	1.31	992.53	1.27	991.26	1.23	990.03	1.18	988.85	1.14	987.71	1.10	986.61	1.07	985.54	1.03
	0.01		0.01		0.01		0.01		0.01		0.02		0.02		0.02		0.03		0.04		0.05		0.06	
7	999.58	1.52	998.06	1.46	996.60	1.41	995.19	1.36	99383	1.32	992.51	1.27	991.24	1.23	990.01	1.19	988.82	1.15	987.67	1.11	986.56	1.08	985.48	1.04
	0.03		0.03		0.03		0.03		0.04		0.04		0.05		0.05		0.06		0.07		0.07		0.08	
8	999.55	1.52	998.03	1.46	996.57	1.41	995.16	1.37	993.79	1.32	992.47	1.28	991.19	1.23	989.96	1.20	988.76	1.16	987.60	1.11	986.49	1.09	985.40	1.05
	0.04		0.04		0.04		0.04		0.04		0.04		0.05		0.06		0.06		0.06		0.08		0.08	
9	99951	1.52	997.99	1.46	996.53	1.41	995.12	1.37	993.75	1.32	992.43	1.29	991.14	1.24	989.90	1.20	988.70	1.16	987.54	1.13	986.41	1.09	985 32	1.05
	0.06		0.06		0.06		0.06		0.06		0.07		0.07		0.07		0.08		0.09		0.10		0.11	
10	999.45	1.52	997.93	1.46	996.47	1.41	995.06	1.37	993.69	1.33	992.36	1.29	991.07	1.24	989.83	1.21	988.62	1.17	987.45	1.14	986.31	1.10	985.21	1.06
	0.07		0.06		0.06		0.07		0.07		0.07		0.07		0.08		0.09		0.10		0.10		0.11	
11	999.38	1.51	997.87	1.46	996.41	1.42	994.99	1.37	993.62	1.33	992.29	1.29	991.00	1.25	989.75	1.22	988.53	1.18	987.35	1.14	986.21	1.11	985.10	1.07
	0.09		0.09		0.09		0.09		0.09		0.09		0.10		0.11		0.11		0.11		0.1 2		0.13	
12	999.29	1.51	997.78	1.46	996.32	1.42	994.90	1.37	993.53	1.33	992.20	1.30	990.90	1.26	989.64	1.22	988.42	1.18	987.24	1.15	986.109	1.12	984.97	1.08
	0.09		0.09		0.09		0.09		0.10		0.10		0.10		0.10		0.11		0.12		0.13		0.14	
13	999.20	1.51	997.69	1.46	996.23	1.42	994.81	1.38	993.43	1.33	992.10	1.30	990.80	1.26	989.54	1.23	988.31	1.19	987.12	1.16	985.96	1.13	984.83	1.09
	0.11		0.11		0.11		0.11		0.11		0.12		0.12		0.13		0.13		0 14		0.15		0.16	
14	999.09	1.51	997.58	1.46	996.12	1.42	994.70	1.38	993.32	1.34	991.98	1.30	990.68	1.27	989.41	1.23	988.18	1.20	986.98	1.17	985.81	1.14	984.67	1.10
	0.12		0.12		0.12		0.12		0.12		0.12		0.13		0.13		0.14		0.14		0.15		0.16	
15	998.97	1.51	997.46	1.46	996.00	1.42	994.58	1.38	993.20	1.34	991.86	1.31	990.55	1.27	989.28	1.24	988.04	1.20	986.94	1.18	985.66	1.15	984.51	1.11
	0.13		0.13		0.13		0.13		0.14		0.14		0.14		0.15		0.15		0.17		0.17		0.18	
16	998.84	1.51	997.33	1.46	995.87	1.42	994.45	1.39	993.06	1.34	991.72	1.31	990.41	1.28	989.13	1.24	987.89	1.22	986.67	1.18	985.49	1.16	984.33	1.13
	0.14		0.14		0.14		0.14		0.14		0.15		0.15		0.15		0.16		0.17		0.17		0.18	
17	998.70	1.51	997.19	1.46	995.73	1.42	994.31	1.39	992.92	1.35	991.57	1.31	990.26	1.28	988.98	1.25	987.73	1.22	986.50	1.18	985.32	1.17	984.15	1.14
	0.15		0.15		0.16		0.16		0.16		0.16		0.17		0.17		0.18		0.18		0.19		0.19	
18	998.55	1.51	997.04	1.47	995.57	1.42	994.15	1.39	992.76	1.35	991.41	1.32	990.09	1.28	988.81	1.26	987.55	1.23	986.32	1.19	985.13	1.17	983.96	1.15
	0.17		0.16		0.16		0.16		0.16		0.16		0.17		0.18		0.18		0.19		0.20		0.21	
19	998.38	1.50	996.88	1.47	995.41	1.42	993.99	1.39	992.60	1.35	991.25	1.33	989.92	1.29	988.63	1.26	987.37	1.24	986 13	1.20	98493	1.18	983.75	1.16
	0.18		0.18		0.18		0.18		0.19		0.19		0.19		0.20		0.21		0.22		0.22		0.23	
20	998.20	1.50	996.70	1.47	995.23	1.42	993.81	1.40	992.41	1.35	991.06	1.33	989.73	1.30	988.43	1.27	987.16	1.24	985 92	1.21	984.71	1.19	983 52	1.17

TABLE III (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength in %																							
t°	0		1		2		3		4		5		6		7		8		9		10		11	
20	998.20	1.50	996.70	1.47	995.23	1.42	993.81	1.40	992.41	1.35	991.06	1.33	989.73	1.30	988.43	1.27	987.16	1.24	985.92	1.21	984.71	1.19	983.52	1.17
	0.19		0.19		0.19		0.19		0.19		0.20		0.20		0.21		0.21		0.22		0.23		0.23	
21	998.01	1.50	996.51	1.47	995.04	1.42	993.62	1.40	992.22	136	990.86	1.33	989.53	1.31	988.22	1.27	986.95	1.25	985.70	1.22	984.48	1.19	983.29	1.17
	0.20		0.20		0.19		0.20		0.20		0.20		0.21		0.21		0.22		0.22		0.23		0.24	
22	987.81	1.50	996.31	1.46	994.85	1.43	993.42	1.40	992.02	1.36	990.66	1.34	989.32	1.31	988.01	1.28	986.73	1.25	985.48	1.23	984.25	1.20	983.05	1.18
	0.21		0.21		0.21		0.21		0.21		0.22		0.22		0.22		0.23		0.24		0.24		0.25	
23	997.60	1.50	996.10	1.46	994.64	1.43	993.21	1.40	991.81	1.37	990.44	1.34	989.10	1.31	987.79	1.29	986.50	1.26	985.24	1.23	984.01	1.21	982.80	1.19
	0.21		0.21		0.22		0.22		0.22		0.22		0.23		0.23		0.23		0.24		0.25		0.26	
24	997.39	1.50	995.89	1.47	994.42	1.43	992.99	1.40	991.59	1.37	990.22	1.35	988.87	1.31	987.56	1.29	986.27	1.27	985.00	1.24	98376	1.22	982.54	1.20
	0.23		0.23		0.23		0.23		0.24		0.24		0.24		0.25		0.25		0.25		0.26		0.27	
25	997.16	1.50	995.66	1.47	994.19	1.43	992.76	1.41	991 35	1.37	989.98	1.35	988.63	1.32	987.31	1.29	986.02	1.27	984.75	1.25	983.50	1.23	982.27	1.21
	0.23		0.23		0.23		0.24		0.24		0.24		0.24		0.25		0.26		0.27		0.27		0.28	
26	996.93	1.50	995.43	1.47	993.96	1.44	992.52	1.41	991.11	1.37	989.74	1.35	988.39	1.33	987.06	1.30	985.76	1.28	984.48	1.25	983.23	1.24	981.99	1.21
	0.25		0.25		0.25		0.25		0.25		0.26		0.26		0.26		0.27		0.28		0.29		0.29	
27	996.68	1.50	995.18	1.47	993.71	1.44	992.27	1.41	990.86	1.38	989.48	1.35	988.13	1.33	986.80	1.31	985.49	1.29	994.20	1.26	982.94	1.24	981.70	1.22
	0.25		0.25		0.26		0.26		0.26		0.26		0.27		0.28		0.28		0.28		0.29		0.30	
28	996.43	1.50	994.93	1.48	993.45	1.44	992.01	1.41	990.60	1.38	989.22	1.36	987.86	1.34	986.52	1.31	985.21	1.29	983.92	1.27	982.65	1.25	981.40	1.22
	0.26		0.27		0.27		0.27		027		0.28		0.28		0.28		0.29		0.29		0.30		0.31	
29	996 17	1.51	994.66	1.48	993 18	1.44	991.74	1.41	990.33	1.39	988.94	1.36	98758	1.34	98624	1.32	984.92	1.29	983.63	1.28	98235	1.26	98109	1.23
	0.27		0.27		0.27		0.28		0.28		0.28		0.28		0.29		0.29		0.30		0.31		0.32	
30	995.90	1.51	994.39	1.48	992.91	1.45	991.46	1.41	990.05	1.39	988.66	1.37	987.29	1.34	985.95	1.32	984.63	1.30	983.33	1.29	982.04	1.27	980.77	1.24
	0.29		0.29		0.29		0.29		0.30		0.30		0.30		0.31		0.31		0.32		0.32		0.32	
31	995.61	151	994.10	1.48	992.62	1.45	991.17	1.42	989.75	1.39	988.36	1.37	986.99	1.35	985.64	1.33	984.31	1.30	983.01	1.29	981.72	1.27	980.45	1.24
	0.29		0.29		0.29		0.29		0.30		0.31		0.31		0.31		0.31		0.32		0.33		0.34	
32	995.32	1.51	993.81	1.48	992.33	1.45	990.88	1.42	989.45	1.40	988.05	1.37	986.68	1.35	985.33	1.33	984.00	1.31	982.69	1.30	981.39	1.28	980.11	1.26
	0.30		0.31		0.31		0.31		0.31		0.31		0.31		0.32		0.33		0.33		0.34		0.34	
33	995.02	1.52	993.50	1.48	992.02	1.45	990.57	1.43	989.14	1.40	987.74	1.37	986.37	1.36	985.01	1.34	983.67	1.31	982.36	1.31	981.05	1.28	979.77	1.27
	0.30		0.31		0.31		0.31		0.31		0.32		0.33		0.33		0.33		0.34		0.34		0.35	
34	994.72	1.53	993.19	1.48	991.71	1.45	990.26	1.43	988.83	1.41	987.42	1.38	98604	1.36	984.68	1.34	983.34	1.32	982.02	1.31	980.71	1.29	979.42	1.28
	0.32		0.32		0.32		0.33		0.33		0.33		0.33		0.33		0.33		0.34		0.34		0.35	
35	994.40	1.53	992.87	1.48	991.39	1.46	989.93	1.43	988.50	1.41	987.09	1.38	985.71	1.36	984.35	1.34	983.01	1.33	981.68	1.31	980.37	1.30	979.07	1.29
	0.32		0.32		0.33		0.33		0.33		0.33		0. 34		0.34		0.35		0.35		0.36		0.37	
36	994.08	1.53	992.55	1.49	991.06	1.46	989.60	1.43	988.17	1.41	986.76	1.39	985.37	1.36	984.01	1.35	982.66	1.33	981.33	1.32	980.01	1.31	978.70	1.29
	0.33		0.34		0.34		0.34		0.35		0.35		0.35		0.35		0.36		0.36		0.36		0.37	
37	993.75	1.54	992.21	1.49	990.72	1.46	989.26	1.44	987.82	1.41	986.41	1.39	985.02	1.37	983.65	1.35	982.30	1.33	980.97	1.32	979.65	1.32	978.33	1.30
	0.34		0.34		0.35		0.36		0.36		0.36		0.36		0.36		0.37		0.38		0.38		0.38	
38	993.41	1.54	991.87	1.50	990.37	1.47	988.90	1.44	87.46	1.41	986.05	1.39	984.66	1.37	983.29	1.36	981.93	1.34	980.59	1.32	979.27	1.32	977.95	1.31
	0.35		0.35		0.36		0.36		0.36		0.37		0.37		0.37		0.37		0.38		0.38		0.39	
39	993.06	1.54	991.52	1.51	990.01	1.47	98854	1.44	987.10	1.41	98568	1.39	984.29	1.37	982.92	1.36	981.56	1.34	980.22	1.33	978.89	1.33	977.56	1.31
	0.35		0.36		0.36		0.37		0.38		0.38		0.38		0.38		0.38		0.39		0.39		0.39	
40	992.71	1.55	991.16	1.51	989.65	1.48	988.17	1.45	986.72	1.42	985.30	1.39	983.91	1.37	982.54	1.36	981.18	1.35	979.83	1.33	978.50	1.33	977.17	1.32

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength in %																					
t°	10		11		12		13		14		15		16		17		18		19		20		21	
0	986.63	1.00	985.63	0.96	984.67	0.92	983.75	0.87	982.88	0.84	982.04	0.81	981.23	0.77	980.46	0.75	979.71	0.73	978.98	0.72	978.26	0.70	977.56	0.70
	-0.03		-0.02		-0.01		0.00		0.02		0.04		0.05		0.07		0.09		0.11		0.13		0.15	
1	986.66	1.01	985.65	0.97	984.68	0.93	983.75	0.89	982.86	0.86	982.00	0.82	981.18	0.79	98039	0.77	979.62	0.75	978.87	0.74	978.13	0.72	977.41	0.72
	-0.02		-0.01		0.00		0.01		0.03		0.04		0.06		0.08		0.10		0.12		0.14		0.17	
2	986.68	1.02	985.66	0.98	984.68	0.94	983.74	0.91	982.83	0.87	981.96	0.84	981.12	0.81	980.31	0.79	979.52	0.77	978.75	0.76	977.99	0.75	977.24	0.74
	0.00		0.01		0.02		0.04		0.05		0.06		0.08		0.10		0.12		0.14		0.16		0.18	
3	986.68	1.03	985.65	0.99	984.66	0.96	983.70	0.92	982.78	0.88	981.90	0.86	981.04	0.83	980.21	0.81	979.40	0.79	978.61	0.78	977.83	0.77	977.06	0.76
	0.01		0.02		0.03		0.04		0.05		0.07		0.08		0.10		0.12		0.14		0.16		0.18	
4	986.67	1.04	985.63	1.00	984.63	0.97	983.66	0.93	982.73	0.90	981.83	0.87	980.96	0.85	980.11	0.83	979.28	0.81	978.47	0.80	977.67	0.79	976.88	0.79
	0.02		0.03		0.05		0.06		0.08		0.09		0.11		0.13		0.14		0.16		0.18		0.20	
5	986.65	1.05	985.60	1.02	984.58	0.98	983.60	0.95	982.65	0.91	981.74	0.89	980.85	0.87	979.98	0.84	979.11	0.83	978.31	0.82	977.49	0.81	976.68	0.81
	0.04		0.06		0.06		0.07		0.08		0.10		0.11		0.13		0.15		0.17		0.19		0.21	
6	986.61	1.07	985.54	1.02	984.52	0.99	983.53	0.96	982.57	0.93	981.64	0.90	980.74	0.89	979.85	0.86	978.99	0.85	978.14	0.84	977.30	0.83	976.47	0.83
	0.05		0.06		0.08		0.09		0.10		0.12		0.14		0.15		0.17		0.19		0.20		0.22	
7	986.56	1.08	985.48	1.04	994.44	1.00	983.44	0.97	982.47	0.95	981.52	0.92	980.60	0.90	979.70	0.88	978.82	0.87	977.95	0.85	977.10	0.85	976.25	0.85
	0.07		0.08		0.09		0.10		0.11		0.12		0.14		0.16		0.18		0.19		0.21		0.23	
8	986.49	1.09	985.40	1.05	984.35	1.01	983.34	0.98	982.36	0.96	981.40	0.94	980.46	0.92	979.54	0.90	978.64	0.88	977.76	0.87	976.89	0.87	976.02	0.87
	0.08		0.08		0.09		0.11		0.13		0.14		0.15		0.16		0.18		0.20		0.22		0.24	
9	986.41	1.09	985.32	1.06	984.26	1.03	983.23	1.00	982.23	0.97	981.26	0.95	980.31	0.93	979.38	0.92	978.48	0.90	977.56	0.89	976.67	0.89	975.78	0.89
	0.10		0.11		0.12		0.13		0.14		0.16		0.17		0.18		0.19		0.21		0.23		0.25	
10	986.31	1.10	985.21	1.07	984.14	1.04	983.10	1.01	982.09	0.99	981.10	0.96	980.14	0.94	979.20	0.93	918.27	0.92	977.35	0.91	976.44	0.91	975.53	0.91
	0.10		0.11		0.12		0.13		0.15		0.16		0.17		0.19		0.21		0.23		0.25		0.27	
11	986.21	1.11	985.10	1.08	984.02	1.05	982.97	1.03	981.94	1.00	980.94	0.97	979.97	0.96	979.01	0.95	978.06	0.94	977.12	0.93	976.19	0.93	975.26	0.92
	0.12		0.13		0.14		0.15		0.16		0.17		0.19		0.21		0.22		0.24		0.26		0.27	
12	986.09	1.12	984.97	1.09	983.88	1.06	982.82	1.04	981.78	1.01	980.77	0.99	979.78	0.98	978.80	0.96	977.84	0.96	976.88	0.95	975.93	0.94	974.99	0.94
	0.13		0.14		0.15		0.16		0.17		0.19		0.20		0.21		0.23		0.24		0.26		0.28	
13	985.96	1.13	984.83	1.10	983.73	1.07	982.66	1.05	981.61	1.03	980.58	1.00	979.58	0.99	978.59	0.98	977.61	0.97	976.64	0.97	975.67	0.96	974.71	0.96
	0.15		0.16		0.17		0.18		0.19		0.20		0.22		0.23		0.24		0.26		0.27		0.29	
14	985.81	1.14	984.67	1.11	983.56	1.08	982.48	1.06	981.42	1.04	980.38	1.02	979.36	1.00	978.36	0.99	977.37	0.99	976.38	0.98	975.40	0.98	974.42	0.98
	0.15		0.16		0.17		0.18		0.19		0.20		0.22		0.24		0.26		0.27		0.28		0.30	
15	985.66	1.15	984.51	1.12	983.39	1.09	982.30	1.07	981.23	1.05	980.18	1.04	979.14	1.02	978.12	1.01	977.11	1.00	976.11	0.99	975.12	1.00	974.12	1.00
	0.17		0.18		0.19		0.20		0.21		0.22		0.23		0.25		0.26		0.28		0.30		0.31	
16	985.49	1.16	984.33	1.13	983.20	1.10	982.10	1.08	981.02	1.06	979.96	1.05	978.91	1.04	977.87	1.02	976.85	1.02	975.83	1.01	974.82	1.01	973.81	1.02
	0.17		0.18		0.19		0.20		0.21		0.23		0.24		0.25		0.27		0.29		0.30		0.31	
17	985.32	1.17	984.15	1.14	98.301	1.11	981.90	1.09	980.81	1.08	979.73	1.06	978.67	1.05	977.62	1.04	976.58	1.04	975.54	1.02	974.52	1.02	973.95	1.04
	0.19		0.19		0.20		0.22		0.24		0.25		0.26		0.27		0.28		0.29		0.31		0.33	
18	985.13	1.17	983.96	1.15	982.81	1.13	981.68	1.11	980.57	1.09	979.48	1.07	978.41	1.06	977.35	1.05	976.30	1.05	975.25	1.04	974.21	1.04	973.17	1.05
	0.20		0.21		0.22		0.23		0.24		0.25		0.26		0.27		0.29		0.30		0.32		0.34	
19	984.93	1.18	983.75	1.16	982.59	1.14	981.45	1.12	980.33	1.10	979.23	1.08	978 15	107	977.08	1.07	976.01	1.06	974.94	1.05	973.89	1.06	972.83	1.06
	0.22		0.23		0.24		0.24		0.25		0.26		0.28		0.29		0.30		0.31		0.33		0.35	
20	984.71	1.19	983.52	1.17	982.35	1.14	981.21	1.13	980.08	1.11	978.97	1.10	977.87	1.08	976.79	1.08	975.71	1.08	974.63	1.07	973.56	1.08	972.48	1.08

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength in %																							
t°	**10**		**11**		**12**		**13**		**14**		**15**		**16**		**17**		**18**		**19**		**20**		**21**	
20	984.71	1.19	983.52	1.17	982.35	1.14	981.21	1.13	980.08	1.11	978.97	1.10	977.87	1.08	976.79	1.08	975.71	1.08	974.63	1.07	973.56	1.08	972.48	1.08
	0.23		0.23		0.23		0.25		0.26		0.28		0.29		0.31		0.32		0.33		0.35		0.36	
21	984.48	1.19	983.29	1.17	982.12	1.16	980.96	1.14	979.82	1.13	978.69	1.11	97758	1.10	976.48	1.09	975.39	1.09	974.30	1.09	973.21	1.09	972.12	1.09
	0.23		0.24		0.25		0.26		0.27		0.28		0.29		0.31		0.32		0.33		0.35		0.36	
22	984.25	1.20	983.05	1.18	981.97	1.17	980.70	1.15	979.55	1.14	978.41	1.12	977.29	1.12	976.17	1.10	975.07	1.10	973.97	1.10	972.86	1.10	971.76	1.11
	0.24		0.25		0.26		0.27		0.28		0.29		0.30		0.31		0.33		0.34		0.35		0.37	
23	984.01	1.21	982.80	1.19	981.61	1.18	980.43	1.16	979.27	1.15	978.12	1.13	976.99	1.13	975.86	1.12	974.74	1.11	973.63	1.12	972.51	1.12	971.39	1.13
	0.25		0.26		0.27		0.28		0.29		0.30		0.31		0.32		0.33		0.35		0.36		0.38	
24	983.76	1.22	982.54	1.20	981.34	1.19	980.15	1.17	978.98	1.16	977.82	1.14	976.68	1.14	97554	1.13	974.41	1.13	97328	1.13	972.15	1.14	971.01	1.14
	0.26		0.27		0.28		0.29		0.30		0.31		0.32		0.33		0.35		0.36		0.38		0.39	
25	983.50	1.23	982.27	1.21	981.06	1.20	979.86	1.18	978.68	1.17	977.51	1.16	976.36	1.15	975.21	1.15	974.06	1.14	972.92	1.15	971.77	1.15	970.62	1.16
	0.27		0.28		0.29		0.29		0.30		0.31		0.33		0.34		0.35		0.37		0.38		0.39	
26	983.23	1.24	981.99	1.22	980.77	1.20	979.57	1.19	978.38	1.18	977.20	1.17	976.03	1.16	974.87	1.16	973.71	1.16	972.55	1.16	971.39	1.16	970.23	1.17
	0.29		0.29		0.30		0.31		0.32		0.33		0.34		0.36		0.37		0.38		0.39		0.41	
27	982.94	1.24	981.70	1.23	980.47	1.21	979.26	1.20	978.06	1.19	976.87	1.18	975.69	1.18	974.51	1.17	973.34	1.17	972.17	1.17	971.00	1.18	969.82	1.18
	0.29		0.30		0.30		0.31		0.32		0.33		0.35		0.36		0.38		0.39		0.40		0.41	
28	982.65	1.25	981.40	1.23	980.17	1.22	978.95	1.21	977.74	1.20	976.54	1.20	975.34	1.19	974.15	1.19	972.96	1.18	971.78	1.18	970.60	1.19	969.41	1.20
	0.30		0.31		0.32		033		0.34		0.35		0.36		0.37		0.38		0.39		0.40		0.42	
29	982.35	1.26	981.09	1.24	979.85	1.23	978.62	1.22	977.40	1.21	976.19	1.21	974.98	1.20	973.78	1.20	972.58	1.19	971.39	1.19	970.20	1.21	968.99	1.20
	0.31		0.32		0.33		0.34		0.35		0.36		0.37		0.38		0.38		0.40		0.42		0.43	
30	982.04	1.27	980.77	1.25	979.52	1.24	978.28	1.23	977.05	1.22	975.83	1.21	974.62	1.21	973.41	1.21	972.20	1.21	970.99	1.21	969.78	1.22	968.56	1.22
	0.32		0.32		0.33		0.34		0.35		0.36		0.37		0.38		0.39		0.40		0.42		0.43	
31	981.72	1.27	980.45	1.26.	979.19	1.25	977.94	1.24	976.70	1.23	975.47	1.22	974.25	1.22	973.03	1.22	971.81	1.22	970.59	1.23	969.36	1.23	968.13	1.23
	0.33		0.34		0.34		0.35		0.36		0.37		0.38		0.39		0.40		0.42		0.43		0.45	
32	981.39	1.28	980.11	1.26	978.95	1.26	977.59	1.25	976.34	1.24	975.10	1.23	973.87	1.23	972.64	1.23	971.41	1.24	970.17	1.24	968.93	1.25	967.68	1.25
	0.34		0.34		0.35		0.35		0.36		0.37		0.39		0.40		0.41		0.42		0.43		0.45	
33	981.05	1.28	979.77	1.27	978.50	1.26	977.24	1.26	975.78	1.25	974.73	1.25	973.48	1.24	972.24	1.24	971.00	1.25	969.75	1.25	968.50	1.27	967.23	1.27
	0.34		0.35		0.36		0.37		0.38		0.39		0.40		0.41		0.42		0.43		0.45		0.45	
34	98071	1.29	979.42	1.28	978.14	1.27	976.97	1.27	975. 60	1.26	974.34	1.26	973.08	1.25	971.83	1.25	970.58	1.26	969.32	1.27	968.05	1.27	966.78	1.28
	0.34		0.35		0.36		0.37		0.38		0.39		0.40		0.41		0.43		0.44		0.45		0.47	
35	980.37	1.30	979.07	1.29	977.78	1.28	976.50	1.28	975.22	1.27	973.95	1.27	972.68	1.26	971.42	1.27	970.15	1.27	968.88	1.28	967.60	1.29	966.31	1.30
	0.36		0.37		0.37		0.38		0.38		0.39		0.40		0.42		0.43		0.44		0.45		0.47	
36	980.01	1.31	978.70	1.29	977.41	1.29	976.12	1.28	974.84	1.28	973.56	1.28	972.28	1.28	971.00	1.28	969.72	1.28	968.44	1.29	967.15	1.31	965.84	1.31
	0.36		0.37		0.38		0.39		0.40		0.41		0.42		0.43		0.44		0.45		0.46		0.47	
37	979.65	1.32	978.33	1.30	977.03	1.30	975.73	1.29	974.44	1.29	973.15	1.29	971.86	1.29	970.57	1.29	969.28	1.29	967.99	1.30	966.69	1.32	965.37	1.32
	0.38		0.38		0.39		0.39		0.40		0.41		0.42		0.43		0.44		0.46		0.47		0.48	
38	979.27	1.32	977.95	1.31	976.64	1.30	975.34	1.30	974.04	1.30	972.74	1.30	971.44	1.30	970.14	1.30	968.84	1.31	967.53	1.31	966.22	1.33	964.89	1.34
	0.38		0.39		0.39		0.40		0.41		0.42		0.43		0.44		0.45		0.46		0.48		0.49	
39	978.89	1.33	977.56	1.31	976.25	1.31	974.94	1.31	973.63	1.31	972.32	1.31	971.01	1.31	969.70	1.31	968.39	1.32	967.07	1.33	965.74	1.34	964.40	1.36
	0.39		0.39		0.40		0.41		0.42		0.42		0.43		0.45		0.47		0.48		0.49		0.50	
40	978.50	1.33	977.17	1.32	975.85	1.32	974.53	1.32	973.21	1.31	971.90	1.32	970.58	1.33	969.25	1.33	967.92	1.33	966.59	1.34	565.25	1.35	963.90	1.37

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength in %																					
t°	**20**		**21**		**22**		**23**		**24**		**25**		**26**		**27**		**28**		**29**		**30**		**31**	
0	978.26	0.70	977.56	0.70	976.86	0.69	976.17	0.70	975.47	0.72	974.75	0.72	974.03	0.74	973.29	0.77	972.52	0.80	971.72	0.83	970.89	0.87	970.02	0.90
	0.13		0.15		0.17		0.20		0.22		0.24		0.27		0.30		0.32		0.35		0.37		0.39	
1	978.13	0.72	977.41	0.72	976.69	0.72	975.97	0.72	975.25	0.74	974.51	0.75	973.76	0.77	972.99	0.79	972.20	0.83	971.37	0.85	970.52	0.89	969.63	0.93
	0.14		0.17		0.19		0.21		0.24		0.26		0.29		0.31		0.34		0.36		0.38		0.41	
2	977.99	0.75	977.24	0.74	976.50	0.74	975.76	0.75	975.01	0.76	974.25	0.78	973.47	0.79	972.68	0.82	971.86	0.85	971.01	0.87	970.14	0.92	960.22	0.96
	0.16		0.18		0.20		0.23		0.25		0.27		0.29		0.32		0.34		0.36		0.38		0.40	
3	977.83	0.77	977.06	0.76	976.30	0.77	975.53	0.77	974.76	0.78	973.98	0.80	973.18	0.82	972.36	0.84	971.52	0.87	970.65	0.89	969.76	0.94	968.82	0.98
	0.16		0.18		0.21		0.23		0.25		0.28		0.30		0.32		0.34		0.36		039		0.42	
4	977.67	0.79	976.98	0.79	976.09	0.79	975.30	0.79	974.51	0.81	973.70	0.82	972.88	0.84	972.04	0.86	971.18	0.89	970.29	0.92	969.37	0.96	968.40	1.00
	0.18		0.20		0.22		0.24		0.26		0.28		0.30		0.33		0.35		0.38		0.40		0.41	
5	977.49	0.81	976.68	0.81	975.87	0.81	975.06	0.81	974.25	0.83	973.42	0.84	972.58	0.86	971.71	0.88	970.83	0.92	969.91	0.94	968.97	0.98	967.99	1.01
	0.19		0.21		0.23		0.25		0.27		0.30		0.33		0.34		0.37		0.39		0.41		0.43	
6	977.30	0.83	976.47	0.83	975.64	0.83	974.81	0.84	973.97	0.85	973.12	0.87	972.25	0.88	971.37	0.91	970.46	0.94	969.52	0.96	968.56	1.00	967.56	1.03
	0.20		0.22		0.24		0.26		0.28		0.30		0.32		0.35		0.37		0.39		0.41		0.43	
7	976.10	0.85	976.25	0.85	975.40	0.85	974.55	0.96	973.69	0.87	972.82	0.89	971.93	0.91	971.02	0.93	970.09	0.96	969.13	0.98	968.15	1.02	967.13	1.05
	0.21		0.23		0.25		0.27		0.29		0.31		0.33		0.35		0.37		0.39		0.42		0.44	
8	976.89	0.87	976.02	0.87	975.15	0.87	974.28	0.88	973.40	0.89	972.51	0.91	971.60	0.93	970.67	0.95	969.72	0.98	968.74	1.01	967.73	1.04	966.69	1.08
	0.22		0.24		0.26		0.28		0.30		0.32		0.34		0.36		0.39		0.41		0.43		0.45	
9	976.67	0.89	975.78	0.89	974.89	0.89	974.00	0.90	973.10	0.91	972.19	0.93	971.26	0.95	970.31	0.98	969.33	1.00	968.33	1.03	967.30	1.06	966.24	1.10
	0.23		0.25		0.27		0.29		0.31		0.33		0.35		0.37		0.39		0.41		0.43		0.45	
10	976.44	0.91	975.53	0.91	974.62	0.91	973.71	0.92	972.79	0.93	971.86	0.95	970.91	0.97	969.94	1.00	968.94	1.02	967.92	1.05	966.87	1.08	965.79	1.11
	0.25		0.27		0.28		0.30		0.32		0.34		0.36		0.38		0.40		0.42		0.44		0.45	
11	976.11	0.93	975.26	0.92	97434	0.93	973.41	0.94	972.47	0.95	971.52	0.97	970.55	0.99	969.56	1.02	968.54	1.04	967.50	1.07	966.43	1.09	965.34	1.12
	0.26		0.27		0.29		0.31		0.33		0.35		0.37		0.39		0.40		0.42		0.44		0.46	
12	975.93	0.94	974.99	0.94	974.05	0.95	973.10	0.96	972.14	0.97	971.17	0.99	970.18	1.01	969.17	1.03	968.14	1.06	967.08	1.09	965.99	1.11	964.88	1.14
	0.26		0.28		0.30		0.32		0.34		0.36		0.38		0.39		0.41		0.43		0.45		0.47	
13	975.67	0.96	974.71	0.96	973.75	0.97	972.78	0.98	971.80	0.99	970.81	1.01	969.80	1.02	968.78	1.05	967.73	1.08	966.65	1.11	965.54	1.13	964.41	1.17
	0.27		0.29		0.31		0.33		0.35		0.37		0.38		0.40		0.42		0.44		0.45		0.47	
14	975.40	0.98	974.42	0.98	973.44	0.99	972.45	1.00	971.45	1.01	970.44	1.02	969.42	1.04	968.38	1.07	967.31	1.10	966.21	1.12	965.09	1.15	963.94	1.18
	0.28		0.30		0.32		0.33		0.35		0.37		0.39		0.41		0.43		0.45		0.47		0.49	
15	975.12	1.00	974.12	1.00	973.12	1.00	972.12	1.02	971.10	1.03	970.07	1.04	969.03	1.06	967.97	1.09	966.88	1.12	965.76	1.14	964.62	1.17	963.45	1.20
	0.30		0.31		0.33		0.35		0.36		0.38		0.40		0.42		0.44		0.45		0.47		0.49	
16	974.82	1.01	973.81	1.02	972.79	1.02	971.77	1.03	970.74	1.05	969.69	1.06	968.63	1.08	967.55	1.11	966.44	1.13	965.31	1.16	964.15	1.19	962.96	1.22
	0.30		0.31		0.33		0.35		0.37		0.38		0.40		0.42		0.43		0.45		0.47		0.49	
17	974.52	1.02	973.50	1.04	972.46	1.04	971.42	1.05	970.37	1.06	969.31	1.08	968.23	1.10	967.13	1.12	966.01	1.15	964.86	1.18	963.68	1.21	962.47	1.24
	0.31		0.33		0.34		0.36		0.38		0.40		0.42		0.43		0.45		0.47		0.48		0.50	
18	974.21	1.04	973.17	1.05	972.12	1.06	971.06	1.07	969.99	1.08	968.91	1.10	967. 81	1.11	966.70	1.14	965.56	1.17	964.39	1.19	963.20	1.23	961.97	1.26
	0.32		0.34		0.35		0.36		0.38		0.40		0.42		0.44		0.46		0.47		0.49		0.50	
19	973.89	1.06	972.83	1.06	971.77	1.07	970.70	1.09	969.61	1.10	968.51	1.11	967.39	1.13	966.26	1.16	965.10	1.18	963.92	1.21	962.71	1.24	961.47	1.28
	0.33		0.35		0.37		0.39		0.40		0.41		0.42		0.45		0.46		0.48		0.51		052	
20	973.56	1.08	972.48	1.08	971.40	1.09	979.31	1.10	969.21	1.11	968.10	1.13	966.97	1.14	965.81	1.17	964.64	1.20	963.44	1.23	962.21	1.26	960.95	1.29

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

	Alcoholic strength at %																							
	20		21		22		23		24		25		26		27		28		29		30		31	
0	973.56	1.08	972.48	1.08	971.40	1.09	970.31	1.10	969.21	1.11	968.10	1.13	966.97	1.16	965.81	1.17	964.64	1.20	963.44	1.23	962.21	1.26	960.95	1.2
	0.35		0.36		0.37		0.39		0.40		0.42		0.44		0.45		0.47		0.49		0.50		0.52	
1	973.21	1.09	972.12	1.09	971.03	1.11	969.92	1.11	968.81	1.13	967.68	1.15	966.53	1.17	965.36	1.19	964.17	1.22	962.95	1.24	961.71	1.28	960.43	1.3
	0.35		0.36		0.38		0.39		0.41		0.43		0.44		0.46		0.48		0.49		0.51		0.52	
2	972.86	1.10	971.76	1.11	970.65	1.12	969.53	1.13	968.40	1.15	967.25	1.16	966.09	1.19	964.90	1.21	963.69	1.23	962.46	1.26	961.20	1.29	959.91	1.3
	0.35		0.37		0.39		0.40		0.42		0.43		0.45		0.46		0.48		0.50		0.52		0.53	
3	972.51	1.12	971.39	1.13	970.26	1.13	969.13	1.15	967.98	1.16	966.82	1.18	965.64	1.20	964.44	1.23	963.21	1.25	961.96	1.28	960.68	1.30	959.38	1.3
	0.36		0.38		0.39		0.41		0.42		0.44		0.46		0.48		0.49		0.51		0.53		0.54	
4	972.15	1.14	971.01	1.14	969.87	1.15	968.72	1.16	967.56	1.18	966.38	1.20	965.18	1.22	963.96	1.24	962.72	1.27	961 45	1.29	960.16	1.32	958.84	1.3
	0.38		0.39		0.40		0.42		0.44		0.45		0.46		0.48		0.50		0.51		0.53		0.54	
5	971.77	1.15	970.62	1.15	969.47	1.17	968.30	1. 18	96	1.19	965.93	1.21	964.72	1.24	963.48	1.26	962.22	1.28	960.94	1.31	959.63	1.33	958.30	1.3
	0.38		0.39		0.41		0.42		0.44		0.46		0.48		0.49		0.50		0.52		0.53		0.55	
6	971.39	1.16	970.23	1.17	969.06	1.18	967.88	1.20	966.68	1.21	965.47	1.23	964.24	1.25	962.99	1.27	961.72	1.30	960.42	1.32	959.10	1.35	957.75	1.3
	0.39		0.41		0.42		0.44		0.45		0.46		0.48		0.50		0.51		0.52		0.53		0.55	
7	971.00	1.18	969.82	1.18	968.64	1.20	967.44	1.21	966.23	1.22	965.01	1.25	963.76	1.27	962.49	1.28	961.21	1.31	959.90	1.33	958.57	1.37	957.20	1.3
	0.40		0.41		0.43		0.44		0.46		0.48		0.49		0.50		0.52		0.53		0.55		0.56	
8	970.60	1.19	969.41	1.20	968.21	1.21	967.00	1.23	965.77	1.24	964.53	1.26	963.27	1.28	961.99	1.30	960.69	1.32	959.37	1.35	958.02	1.38	956.64	1.3
	0.40		0.42		0.43		0.45		0.46		0.48		0.49		0.50		0.52		0.54		0.55		0.56	
9	970.20	1.21	968.99	1.21	96778	1.23	966.55	1.24	965.31	1.26	964.05	1.27	962.78	1.29	961.49	1.32	960.17	1.34	958.83	1.36	957.47	1.39	956.08	1.3
	0.42		0.43		0.45		0.46		0.47		0.48		0.50		0.52		0.53		0.54		0.56		0.58	
30	969.78	1.22	968.56	1.23	967.33	1.24	966.09	1.25	964.84	1.27	963.57	1.29	962.28	1.31	960.97	1.33	959.64	1.35	958.29	1.38	956.91	1.41	955.50	1.4
	0.42		0.43		0.44		0.45		0.47		0.49		0.51		0.52		0.53		0.55		0.56		0.58	
31	969.36	1.23	968.13	1.24	966.89	1.25	965.64	1.27	964.37	1.29	963.08	1.31	961.77	1.32	960.45	1.34	959.11	1.37	957.74	1.39	956.35	1.43	954.92	1.4
	0.43		0.45		0.46		0.48		0.49		0.50		0.51		0.52		0.54		0.56		0.57		0.58	
32	968.93	1.25	967.68	1.25	966.43	1.27	965.16	1.28	963.88	1.30	962.58	1.32	961.26	1.33	959.93	1.36	958.57	1.39	957.18	1.40	955.78	1.44	954.34	1.4
	0.43		0.45		0.47		0.48		0.50		0.51		0.52		0.54		0.55		0.56		0.58		0.59	
33	968.50	1.27	967.23	1.27	965.96	1.28	964.68	1.30	963.38	1.31	962.07	1.33	960.74	1.35	959.39	1.37	958.02	1.40	956.62	1.42	955.20	1.45	953.75	1.4
	0.45		0.45		0.47		0.49		0.50		0.51		0.52		0.54		0.55		0.56		0.58		0.60	
34	968.05	1.27	966.78	1.29	965.49	1.30	964 19	1.31	962.88	1.32	961.56	1.34	960.22	1.37	958.85	1.38	957.47	1.41	956.06	1.44	954.62	1.47	953.15	1.4
	0.45		0.47		0.48		0.49		0.50		0.52		0.54		0.55		0.57		0.58		0.59		0.60	
35	967.60	1.29	996.31	1.30	965.01	1.31	963.70	1.32	962.38	1.34	961.04	1.36	959.68	1.38	958. 0	1.40	956.90	1.42	955.48	1.45	954.03	1.48	952.55	1.5
	0.45		0.47		0.48		0.49		0.51		0.53		0.54				0.57		0.59		0.60		061	
36	967.15	1.31	965.84	1.31	964.53	1.32	963.21	1.34	961.87	1.36	960.51	1.37	959.14	1.39	957.75	1.42	956.33	1.44	954.89	1.46	953.43	1.49	951.94	1.5
	0.46		0.47		0.48		0.50		0.52		0.53		0.55		0.56		0.57		0.58		0.60		0.61	
37	966.69	1.32	965.37	1.32	964.05	1.34	962.71	1.36	961.35	1.37	959.98	1.39	958.59	1.40	957.19	1.43	955.76	1.45	954.31	1.48	952.83	1.50	951.33	1.5
	0.47		0.48		0.50		0.51		0.52		0.54		0.55		0.57		0.58		0.59		0.60		0.61	
38	966.22	1.33	964.89	1.34	963.55	1.35	962.20	1.37	960.83	1.39	959.44	1.40	958.04	1.42	956.62	1.44	955.18	1.46	953.72	1.49	952.23	1.51	950.72	1.5
	0.48		0.49		0.51		0.52		0.53		0.54		0.56		0.57		0.58		0.60		0.61		0.62	
39	965.74	1.34	964.40	1.36	963.04	1.36	961.68	1.38	960.30	1.40	958.90	1.42	957.48	1.43	956.05	1.45	954.60	1.48	953 12	1.50	951.62	1.52	950.10	1.5
	0.49		0.50		0.51		0.53		0.54		055		0.56		058		0.60		0.61		0.62		0.64	
40	965.25	1.35	963.90	1.37	962.53	1.38	961.15	1.39	959.76	1.41	958.35	1.43	956.92	1.45	955.47	1.47	954.00	1.49	952.51	1.51	951.00	1.54	949.49	1.5

TABLE IV
Table giving the refractive indices of pure ethanol-water mixtures
and distillates at 20°C
and the corresponding alcoholic strengths at 20°C

Refractive index at 20°C	Alcoholic strength at 20°C				Refractive index at 20°C	Alcoholic strength at 20°C			
	Water-ethanol mixtures		Distillates			Water-ethanol mixtures		Distillates	
1.33628	6.54	0.25	6.48	0.26	1.34222	16.76	0.23	16.65	0.23
1.33642	6.79	0.26	6.74	0.26	1.34236	16.99	0.23	16.88	0.24
1.33656	7.05	0.25	7.00	0.27	1.34250	17.22	0.22	17.12	0.22
1.33670	7.30	0.28	7.27	0.27	1.34264	17.44	0.24	17.34	0.22
1.33685	7.58	0.25	7.54	0.25	1.34278	17.68	0.21	17.56	0.22
1.33699	7.83	0.26	7.79	0.26	1.34291	17.89	0.23	17.78	0.23
1.33713	8.09	0.25	8.05	0.25	1.34305	18.12	0.24	18.01	0.22
1.33727	8.34	0.28	8.30	0.26	1.34319	18.36	0.23	18.23	0.23
1.33742	8.62	0.25	8.56	0.25	1.34333	18.59	0.23	18.46	0.24
1.33756	8.87	0.25	8.81	0.25	1.34347	18.82	0.23	18.70	0.22
1.33770	9.12	0.24	9.06	0.24	1.34361	19.05	0.23	18.92	0.25
1.33784	9.36	0.27	9.30	0.25	1.34375	19.28	0.23	19.17	0.23
1.33799	9.63	0.24	9.55	0.26	1.34389	19.51	0.24	19.40	0.22
1.33813	9.87	0.25	9.81	0.24	1.34403	19.75	0.23	19.62	0.24
1.33827	10.12	0.23	10.05	0.24	1.34417	19.98	0.24	19.86	0.23
1.33841	10.35	0.26	10.29	0.25	1.34431	20.22	0.22	20.09	0.24
1.33856	10.61	0.25	10.54	0.24	1.34445	20.44	0.21	20.33	0.21
1.33870	10.86	0.24	10.78	0.24	1.34458	20.65	0.24	20.54	0.22
1.33884	11.10	0.23	11.02	0.24	1.34472	20.89	0.22	20.76	0.23
1.33898	11.33	0.24	11.26	0.24	1.34486	21.11	0.23	20.99	0.22
1.33912	11.47	0.24	11.50	0.24	1.34500	21.34	0.21	21.21	0.23
1.33926	11.81	0.24	11.74	0.24	1.34513	21.55	0.23	21.44	0.21
1.33940	12.05	0.25	11.98	0.24	1.34527	21.78	0.22	21.65	0.22
1.33955	12.30	0.23	12.22	0.24	1.34541	22.00	0.23	21.87	0.23
1.33969	12.53	0.23	12.46	0.23	1.34555	22.23	0.21	22.10	0.21
1.33983	12.76	0.24	12.69	0.23	1.34568	22.44	0.23	22.31	0.23
1.33997	13.00	0.23	12.92	0.23	1.34582	22.67	0.23	22.54	0.21
1.34011	13.23	0.24	13.15	0.25	1.34596	22.90	0.23	22.75	0.21
1.34025	13.47	0.23	13.40	0.22	1.34610	23.13	0.20	22.96	0.21
1.34039	13.70	0.23	13.62	0.24	1.34623	23.33	0.24	23.17	0.23
1.34053	13.93	0.23	13.86	0.23	1.34637	23.57	0.24	23.40	0.21
1.34067	14.16	0.25	14.09	0.23	1.34651	23.81	0.23	23.61	0.24
1.34081	14.41	0.25	14.32	0.25	1.34665	24.04	0.22	23.85	0.24
1.34096	14.66	0.23	14.57	0.24	1.34678	24.26	0.22	24.09	0.22
1.34110	14.89	0.24	14.81	0.25	1.34692	24.48	0.24	24.31	0.25
1.34124	15.13	0.23	15.06	0.22	1.34706	24.72	0.23	24.56	0.22
1.34138	15.36	0.23	15.28	0.22	1.34720	24.95	0.21	24.78	0.22
1.34152	15.59	0.24	15.50	0.24	1.34733	25.16	0.24	25.00	0.23
1.34166	15.83	0.23	15.74	0.22	1.34747	25.40	0.22	25.23	0.22
1.34180	16.06	0.23	15.96	0.23	1.34760	25.62	0.24	25.45	0.25
1.34194	16.29	0.23	16.19	0.22	1.34774	25.86	0.24	25.70	0.23
1.34208	16.52	0.24	16.41	0.24	1.34788	26.10	0.22	25.93	0.22

Method OIV-MA-AS312-03A Type II method

Methanol

A41 revised by OIV/OENO 377/2009
OIV-OENO 480-2014)

1. Scope of application

This method is applicable to the determination of methanol in wine for concentrations between 50 and 500 mg/L.

2. Principle

Methanol is determined in the distillate, to which an internal standard is added, using gas chromatography with a flame ionisation detector (FID).

3. Reagents and materials

3.1. Type II water, according to ISO standard 3696

3.2. Ethanol: purity ≥ 96 % (CAS no. 64-17-5)

3.3. Hydrogen: minimum specifications: 99.999% purity (CAS no. 1333-74-0)

3.4. Helium: minimum specifications: 99.999% purity (CAS no. 7440-59-7)

3.5. Methanol: purity ≥ 99 % (CAS no. 67-56-1)

3.6. 4-Methyl-2-pentanol (internal standard): purity ≥ 98 % (CAS no. 108-11-2). Internal standard used in the validation.

Note 1: Other internal standards can be used, such as:

- 3-pentanol: purity ≥ 98% (CAS no. 584-02-1)
- 4-methyl-1-pentanol: purity ≥ 98% (CAS no. 626-89-1)
- Methyl nonanoate: purity ≥ 98% (CAS no. 1731-84-6)

3.7. Reference materials: these may be, for example, wines from laboratory proficiency tests.

3.8. Preparation of working solutions (by way of example):

3.8.1. Approximately 10% v/v aqueous-alcoholic mixture

This mixture should be as close as possible to the alcohol content of the wine to be analysed. Pour 100 mL of ethanol (3.2) into a 1 L calibrated flask (4.2), make up to volume with demineralised water (3.1) and mix.

3.8.2. 10 g/L Internal standard solution

Using an analytical balance (4.1), weigh approximately 1 g of internal standard (3.6) into a 100 mL calibrated flask (4.3) that contains around 60 mL of 10% ethanol solution (3.8.1), so as to minimise evaporation of the internal standard. Make up to volume with the ethanol solution (3.8.1) and mix.

3.8.3. 1 g/L Internal standard solution

Add 10 mL of the 10 g/L internal standard solution (3.8.2) using a pipette (4.8) and make up to 100 mL (4.3) using the 10% v/v hydroalcoholic mixture (3.8.1).

3.8.4. 5 g/L Methanol stock solution

Using an analytical balance (4.1), weigh approximately 500 mg of methanol (3.5) into a 100 mL calibrated flask (4.3) that contains about 60 mL of 10% ethanol solution (3.8.1), so as to minimise evaporation of the methanol. Make up to volume with the ethanol solution (3.8.1) and mix.

3.8.5. Working calibration solutions

By way of example, a method for plotting a calibration curve is outlined below.

Each solution should be prepared with the 10% aqueous-alcoholic mixture (3.8.1).

3.8.5.1. 500 mg/L Methanol standard solution

Add 10 mL of the 5 g/L stock solution (3.8.4) to a 100 mL calibrated flask (4.3) using a pipette (4.8) and make up to volume with the 10% v/v ethanol solution (3.8.1).

3.8.5.1.1. 250 mg/L Methanol standard solution

Add 10 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.8) and make up to volume with the 10% v/v ethanol solution (3.8.1).

3.8.5.1.2. 200 mg/L Methanol standard solution

Add 20 mL of the 500 mg/L methanol solution (3.8.5.1) to a 50 mL calibrated flask (4.4) using a pipette (4.7) and make up to volume with the 10% v/v ethanol solution (3.8.1).

3.8.5.1.3. 150 mg/L Methanol standard solution

Add 6 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.9) and make up to volume with the 10% v/v ethanol solution (3.8.1).

3.8.5.1.4. 100 mg/L Methanol standard solution

Add 4 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.10) and make up to volume with the 10% v/v ethanol solution (3.8.1).

3.8.5.1.5. 50 mg/L Methanol standard solution

Add 2 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.11) and make up to volume with the 10% v/v ethanol solution (3.8.1).

4. Apparatus

4.1. Analytical balance (1 mg precision)

4.2. 1 L Class A calibrated flasks

4.3. 100 mL Class A calibrated flasks

4.4. 50 mL Class A calibrated flasks

4.5. 20 mL Class A calibrated flasks

4.6. 10 mL Class A calibrated flasks

4.7. 20 mL Class A pipettes with two marks

4.8. 10 mL Class A pipettes with two marks

4.9. 6 mL Class A pipettes with two marks

4.10. 4 mL Class A pipettes with two marks

4.11. 2 mL Class A pipettes with two marks

4.12. 1 mL Class A pipettes with two marks or 1 mL micropipettes

4.13. Temperature-programmable gas chromatograph with a flame ionisation detector and a data processing system capable of calculating areas or measuring peak heights

4.14. Fused silica capillary column coated with a Carbowax 20M-type polar stationary phase (for example):

- Chrompack CP-wax 57 CB, 50 m x 0.32 mm x 0.45 µm
- DB-WAX 52, 30 m x 25 mm x 0.2 µm

5. Sample preparation

Sparkling and/or young wines must be pre-degassed, for example, by mixing 200 mL of wine in a 1 L flask. Subsequently, the samples are distilled according to the method for determining alcoholic strength by volume (OIV-MA-AS312-01). The distillation can be carried out without adding calcium hydroxide in this case.

5.1. Addition of internal standard (by way of example)

Pour 10 mL of distillate into a 10 mL calibrated flask (4.6), add 1 mL (4.12) of internal standard solution (3.8.3) and mix.

6. Procedure

The calibration curve standards are treated in the same way as the samples (point 5.1).

It is recommended that the aqueous-alcoholic mixture (3.8.1) is injected at the start of the sequence in order to verify that it does not contain methanol.

6.1. Operating conditions (as a guide):

Carrier gas: helium or hydrogen

Carrier gas flow: 7 mL/min

Injection: split (ratio: 7:50)

Injection volume: 1 or 2 µL

Injector temperature: 200-260 ºC

Detector temperature: 220-300 ºC

Temperature programme: from 35 ºC (for 2 minutes) to 170 ºC, at 7.5 ºC/min

7. Calculations

Calculate the concentration of methanol (C_i), using the following equation:

$$C_i = \frac{C_p}{m}\left(\frac{A_i}{A_p} - b\right)$$

A_i – Peak area of methanol

A_p – Peak area of internal standard

C_p – Concentration of internal standard

m - Slope of the calibration curve

b - Y-intercept of the calibration curve

8. Expression of the results

The concentration of methanol may be expressed in mg/L or in mg/100 mL absolute alcohol; in the latter case, the alcohol content by volume of the wine should be determined.

Note 2: mg/100 mL absolute alcohol = mg/L x 10/alcohol content by volume

9. Precision

The data from the international interlaboratory test is outlined in Annex A.

10. Quality control

Internal quality control may be carried out using certified reference materials or wines whose characteristics have been determined from a consensus (3.7). These should be prepared as for the samples (point 5). Participation in proficiency tests is recommended.

11. Report of the results

The results are expressed to the nearest whole number (in accordance with the uncertainty).

12. Bibliography

Compendium of international methods of wine and must analysis. Method OIV-MA-AS312-01 (Alcoholic strength).

Annex A
Statistical results of the interlaboratory test

Design of validation study

The validation study was conducted with 10 samples: 2 white wines, one dry and one sweet, 2 red wines, one of which was oaked, and 1 fortified wine (Port), including blind duplicates, according to OIV recommendations. The approximate concentration of methanol is shown in the following table.

Sample	White wine Dry	White wine Sweet	Red wine	Red wine oaked	Fortified wine port
Methanol (mg/L)	50	150*	270	400*	120

(*) In this particular indicated case, methanol was added to the wine to cover a greater range of concentrations. The wine was then mixed, stabilised and bottled.

Participating laboratories:

Samples were sent to 17 laboratories in 9 different countries.

Laboratorios Agroalimentarios, Madrid (Spain)
Estación de Viticultura y Enología de Galicia, EVEGA (Spain)
Estació de Viticultura i Enologia de Vilafranca del Penedès, (Spain)
Estación Enológica de Haro, La Rioja (Spain)
Estación de Viticultura y Enología de Galicia (Spain)
Lab. Bordeaux, Service Commun des Lab., Pessac (France)
Laboratoire d'Ile-de-France, Paris (France)
Laboratoires Inter Rhône (France)
Comité Interprof. du Vin de Champagne (CIVC) (France)
Bfr-Bundesinst. f. Risikobewertung (Germany)
Landesuntersuchungsamt Mainz (Germany)
Instituto Nacional de Vitivinicultura, Mendoza (Argentina)

ALKO Inc., Alcohol Control Lab. (ACL) (Finland)

Instituto dos Vinhos do Douro e do Porto (Portugal)

Czech Agriculture and Food Inspection Authority (CAFIA), Brno (Czech Republic) CZ

National Food Safety Office, Directorate of Oenology and Alcoholic Beverages (NÉBIH BAII), Budapest (Hungary)

Lehr- und Forschungszentrum, Klosterneuburg (Austria)

	Dry white		Collaborative study on methanol				Oaked red		Port	
Laboratory code	A	G	B	H	C	I	D	J	E	K
A	39.99	38.13	127.42	136.25	144.80	145.71	496.53	513.00	192.13	219.39
B	41.20	40.90	157.60	160.50	150.40	146.90	484.90	477.80	222.40	219.60
C	36.80	35.60	133.50	129.20	119.10	134.10	454.10	478.40	197.00	174.80
D	36.00	39.60	**177.40**	**145.50**	160.80	138.00	**302.00**	**494.50**	216.10	248.50
E	**68.00**	**70.00**	163.00	169.00	178.00	177.00	503.00	495.50	225.00	227.00
F	37.00	37.10	148.30	148.20	143.40	142.40	484.10	474.00	206.30	206.90
G	41.40	42.30	152.60	152.40	149.70	150.50	489.60	491.10	216.60	217.20
H	36.80	32.40	140.80	129.10	128.00	137.70	440.60	429.30	187.50	192.80
I	42.90	43.30	153.50	155.50	139.70	147.40	468.30	456.10	225.30	225.60
J	40.90	40.60	155.50	154.60	148.50	149.10	496.40	499.80	217.10	217.00
K	39.30	36.20	**103.10**	**143.10**	131.90	115.90	**437.90**	**334.00**	156.10	172.60
L	35.00	39.00	164.00	167.00	157.00	160.00	492.00	508.00	249.00	220.00
M	43.60	43.40	157.30	154.90	155.50	158.90	506.80	496.10	217.70	219.50
N	34.20	33.60	126.50	125.70	125.90	133.60	429.10	429.00	192.10	188.90
O	34.00	35.70	149.00	154.80	144.20	141.80	482.80	473.60	210.40	218.10
P	44.70	43.70	151.60	146.90	140.70	147.60	451.20	472.80	205.40	205.80
Q	40.70	38.80	153.00	149.80	158.20	153.40	498.20	497.50	225.50	217.20

Note: The values in bold correspond to values rejected according to the Cochran (variance outliers) and Grubbs (mean outliers) tests.

Indicators	Dry white	Sweet white	Red	Oaked red	Port
Number of accepted laboratories	16	15	17	15	17
Number of repetitions	2	2	2	2	2
Minimum	32.40	125.70	115.90	429.00	156.10
Maximum	44.70	169.00	178.00	513.00	249.00
Repeatability variance s_r^2	2.2466	12.1330	39.0164	76.3567	105.3390
Intergroup variance s_r^2	9.61893	146.39249	151.90249	535.61827	292.14282
Reproducibility variance s_r^2	11.8655	158.5254	190.9189	611.9750	397.4819
Overall mean	38.90	148.92	145.76	478.97	210.37
Repeatability standard deviation	1.50	3.48	6.25	8.74	10.26
r Limit	4.242	9.858	17.677	24.729	29.046
Repeatability CV	3.9	2.3	4.3	1.8	4.9
Reproducibility standard deviation	3.44	12.59	13.82	24.74	19.94
R Limit	9.748	35.632	39.103	70.009	56.422
Reproducibility CV	8.9	8.5	9.5	5.2	9.5
Horwitz RSD	6.09	4.97	4.99	4.17	4.72
Horrat r	0.6	0.5	0.9	0.4	1.0
Horwitz RSD	9.22	7.53	7.56	6.32	7.15
Horrat R	1.0	**1.1**	**1.3**	0.8	**1.3**

According to the Horrat values, the repeatability and reproducibility of the method are acceptable

Z-scores obtained by the participants: of the 85 Z-scores, 3 are unsatisfactory and 4 are questionable

Laboratory code	Z-score Dry white wine	Z-score Sweet white wine	Z-score Red wine	Z-score Oaked red wine	Z-score Port
A	0.05	-1.36	-0.04	1.04	-0.23
B	0.62	0.80	0.21	0.10	0.53
C	-0.78	-1.40	-1.39	-0.51	-1.23
D	-0.32	1.00	0.26	**-3.26**	1.10
E	**8.74**	1.36	**2.30**	0.81	0.78
F	-0.54	-0.05	-0.21	0.00	-0.19
G	0.86	0.28	0.31	0.46	0.33
H	-1.25	-1.11	-0.93	-1.78	-1.01
I	1.22	0.44	-0.16	-0.68	0.76
J	0.54	0.49	0.22	0.77	0.34
K	-0.33	**-2.05**	-1.58	**-3.76**	**-2.31**
L	-0.55	1.32	0.92	0.85	1.21
M	1.34	0.57	0.83	0.91	0.41
N	-1.45	-1.81	-1.16	**-2.02**	-1.00
O	-1.18	0.24	-0.20	-0.03	0.19
P	1.54	0.03	-0.12	-0.69	-0.24
Q	0.25	0.20	0.73	0.76	0.55

Method OIV-MA-AS312-03B Type IV method

Methanol

(A41 revised by OIV/OENO 377/2009)

1. Principle

The wine distillate is diluted to an ethanol content of 5% (*v/v*). Methanol is oxidized to formaldehyde (methanol) by potassium permanganate (acidified by phosphoric acid). The amount of formaldehyde is determined by the violet color formed by the reaction of chromotropic acid in a sulfuric medium. The intensity of the color is determined by spectrophotometry at 575 nm.

2.. Method

2.1 *Reagents*

2.1.1 Chromotropic Acid

4,5–Dihydroxy–2,7–naphthalenedisulfonic acid, ($C_{10}H_8O_8S_2 \cdot 2H_2O$), (MW 356.34 g)

White or light brown powder, soluble in water. The *di*-sodium salt of this acid that forms a yellow or light brown substance, and is very soluble in water can also be used.

Purification - The chromotropic acid must be pure and give a negligible color in the blank tests of reagents prepared with it. If this is not the case, proceed with purification using the following procedure:

Dissolve 10 g of chromotropic acid or its salt in 25 mL of distilled water. If the salt has been used, add 2 mL of concentrated sulfuric acid (ρ_{20} = 1.84 g/mL) to release the acid. Add 50 mL of methanol, heat to boiling and filter. Add 100 mL of *iso*-propanol to precipitate the pure crystals of chromotropic acid, allow the crystals formed to drain and cold dry.

Reaction - The addition of ferric chloride (1 drop) to 10 mL of a 0.1 g/L solution should give a green color.

Sensitivity test - Dilute 0.5 mL of analytical grade formaldehyde to 1 L with water. To 5 mL of 0.05%chromotropic acid solution in sulfuric acid, 75% (*v/v*), add 0.1 mL of formaldehyde solution and heat to 70°C for 20 min. A violet color should be produced.

2.1.2 Chromotropic acid solution, 0.05%, in sulfuric acid solution, 75% (*v/v*).

Dissolve 50 mg chromotropic acid (2.1.1) or its sodium salt in 35 mL of distilled water. Cool this solution with iced water and add carefully 75 mL of concentrated sulfuric acid (= 1.84 g/mL) in small portions, while mixing.

This solution must be prepared just before use.

2.1.3 Methanol, 5 g/L, standard solution in alcohol 5%, (*v/v*)

Pure methanol (E_{760} = 64.7 ± 0.2)	0.5 g
Absolute alcohol (without methanol)	50 mL
Distilled water to	1 liter

2.1.4 Dilution solution

Absolute alcohol (without methanol)	50 mL
Distilled water to	1 liter

2.1.5 Phosphoric acid solution, 50% (*m/v*)

2.1.6 Potassium permanganate solution, 5% (*m/v*)

2.1.7 Neutral sodium sulfite solution, 2% (*m/v*)

Solution rapidly oxidizes in air. Determine the exact strength by iodometric titration.

2.2 *Procedure*

Dilute the wine distillate (see chapter *Alcoholic strength*) to reduce the alcoholic strength to 5% vol.

Into a ground-glass stopper test tube place 0.5 mL of the diluted distillate, add 1 drop of phosphoric acid, 50%, 2 drops of potassium permanganate solution, 5%, shake and allow to stand for 10 minutes.

Decolorize the permanganate by adding a few drops, usually 4, of neutralized 2% sodium sulfite solution, (avoid any excess). Add 5 mL 0.05% chromotropic acid. Place in a water bath at 70°C for 20 min. Allow to cool.

Determine the absorbance A_S, at 570 nm, the zero of the absorbance being adjusted on the control sample prepared with 0.5 mL of the dilution solution.

Calibration curve

In a series of 50 mL volumetric spherical flasks, place 2.5, 5, 10, 15, 20, 25 mL respectively of the methanol, 0.5 g/L, solution in ethanol 5%. Make up to volume with a 5% ethanol solution. These solutions contain 25, 50, 100, 150, 200 and 250 mg of methanol per liter.

Treat simultaneously 0.5 mL of the dilution solution and 0.5 mL of each of the standard solutions, with the same technique as used to bring the wine distillate to an ethanol concentration of 5%.

Determine the absorbance of these solutions at 570 nm, in the conditions described above.

The graph of absorbance of the standard solutions as a function of concentration should be a straight line.

2.3 *Calculations*

Determine the methanol concentration, expressed in mg/L of the wine distillate brought to an alcoholic strength of 5% vol., and plotted as A_S on the calibration line.

Express the concentration in wine in mg/L taking into account the dilution performed to bring the strength to 5% vol.

Method OIV-MA-AS312-04 Type IV method

Glycerol and 2,3-Butanediol

(Resolution OIV/OENO 377/2009)

1. Principle

Glycerol and 2,3-butanediol are oxidized by periodic acid after treatment through an anion exchange resin column to fix the sugars and a large proportion of mannitol and sorbitol. The product obtained by the action of phloroglucinol on formaldehyde (by glycerol oxidation) is determined colorimetrically at 480 nm. The product formed by the action of piperidine solution and sodium nitroferricyanide solution with the ethanol (by oxidation of 2,3-butanediol) is determined colorimetrically at 570 nm.

2 . Apparatus

2.1 Glass column 300 mm long and approximately 10-11 mm internal diameter fitted with a stopcock.

2.2 Spectrophotometer allowing measurement to be made between 300 and 700 nm and glass cells with optical path length of 1 cm.

3. Reagents

3.1 Glycerol, $C_3H_8O_3$

3.2 2,3-Butanediol, $C_4H_{10}O_2$

3.3 A strongly basic anion exchange resin e.g. anion exchange resin of Merck strength III or Amberlite IRA 400.

3.4 Polyvinylpolypyrrolidone (PVPP) (see *International Oenological Codex*).

3.5 Periodic acid, 0.1 M, in sulfuric acid, 0.05 M.

Weigh 10.696 g of sodium periodate, $NaIO_4$, place into a 500 mL volumetric flask, dissolve with 50 mL of sulfuric acid, 0.5 M, and make up to 500 mL with distilled water.

3.6 Periodic acid, 0.05 M, in sulfuric acid, 0.025 M.

The above solution (3.5) is diluted 1 : 1 with distilled water.

3.7 Sulfuric acid, 0.5 M.

3.8 Sodium hydroxide solution, 1 M.

3.9 Sodium hydroxide solution, 5% (*m/v*).

3.10 Ethanol, 96% (*v/v*).

3.11 Phloroglucinol solution, 2% (*m/v*), to be prepared fresh daily.

3.12 Sodium acetate solution, 27% (*m/v*), prepared from anhydrous sodium acetate, CH_3COONa.

3.13 Sodium nitroferricyanide solution, $Na_2Fe(CN)_5NO.2H_2O$, 2% (*m/v*), to be prepared fresh daily

3.14 Piperidine solution, $C_5H_{11}N$ 25% (*v/v*), to be prepared fresh daily.

3.15 Standard glycerol solution

Prepare a solution containing 250 g glycerol in 100 mL and determine the glycerol content by the enzymatic or periodimetric method (see section 6).

Prepare the standard glycerol solution as follows: weigh in a 100 mL volumetric flask a mass "*m*" corresponding to 250 mg of pure glycerol, make up to 100 mL with water.

3.16 Standard 2,3-butanediol solution

Prepare a solution containing 250 mg of 2,3-butanediol sample in 100 mL and determine the 2,3-butanediol content by the periodimetric method (see section 6).

Prepare the standard solution of 2,3-butanediol by weighing in a 100 mL volumetric flask a mass "*m*" corresponding to 250 mg of pure 2,3-butanediol; make up to 100 mL with water.

3.17 Alkaline copper solution:

Copper Solution A

Copper sulfate, $CuSO_4.5H_20$	40 g
Sulfuric acid (r=1.84 g/mL)	2 mL

Make up to 1000 mL with water

Alkaline tartaric solution B

Potassium sodium tartrate tetrahydrate

$KNaC_4H_4O_6.4H_2O$	200 g
Sodium hydroxide	150 g

Make up to 1000 mL with water

The copper alkaline solution is obtained by mixing solution A and B in equal quantities at the time of use.

4. Procedure

4.1 *Preparation of an anion exchange column*

The anion exchange resin (Cl^-) must be kept in a flask filled with decarbonated distilled water.

Put 30 mL of anion exchange resin (3.3) in the column (2.1), place a wool plug on the top of the column to stop air contact with the resin. Pass 150 mL of 5% sodium hydroxide (3.9) through the column at a flow rate of 3.5 to 5 mL per minute followed by a similar quantity of decarbonated distilled water at the same

flow rate until the eluent is neutral or slightly alkaline to phenolphthalein. The resin is then ready for use.

The anion exchange resin can only be used once. It can be regenerated by treating with 5% hydrochloric acid for a few hours and then rinsed with water until free of chloride. (Check for absence of chloride).

4.2 *Preparation of sample*

The wine sample is diluted 10/50.

In case of strongly colored wines, first decolorize with PVPP (3.4): place 10 mL wine in a 50 mL volumetric flask, dilute with water (20 mL) and add 300 mg of PVPP (3.4). Leave for 20 min. stirring occasionally, make to the mark and filter through fluted filter paper. Take 10 mL of diluted wine (treated or untreated with PVPP) and place on the anion exchange column. Allow to drain, drop by drop, at flow rate not exceeding 2 mL per minute. When the level of diluted wine reaches 5-10 mm above the glass wool plug, add decarbonated distilled water to bring the volume of the eluent to 100 mL at a flow rate 2-3 mL per minute. The eluate must be free of sugars. To ensure this, boil rapidly 5 mL of eluate with 5 mL of alkaline copper solution (3.17). There should not be any discoloration or precipitation.

4.3 *Determination of glycerol*

4.3.1 Photometric determination

Place into a 100 mL conical ground necked vessel:

10 mL eluate and add successively

10 mL distilled water and

10 mL periodic acid solution, 0.05 M (3.6).

Stir carefully; leave exactly 5 min. for the oxidation to take place. Add 10 mL sodium hydroxide solution, (3.8), and 5 mL 96% ethanol (*v/v*) (3.10).

Stir after each addition, then add 10 mL phloroglucinol solution (3.11)

Mix rapidly and transfer the solution into a 1 cm cell. The purple coloration is obtained very quickly. Its intensity reaches a maximum after 50 to 60 seconds, then decreases. Note the maximal absorbance. The measurement is carried out at 480 nm using air as a reference.

4.3.2 Preparation of the calibration curve

Pipette into 100 mL volumetric flasks:

3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mL glycerol standard solution (3.15) and make up to volume with distilled water.

These solutions correspond, according to the conditions in 4.2, to the following concentrations:

3.75, 5.00, 6.25, 7.50, 8.75 and 10.00 g/L of glycerol.

Proceed with the determination as described in 4.3.1, replacing the eluate by the same volume of each of the standard solutions.

4.4 *Determination of 2,3-butanediol*

4.4.1 Photometric determination

Place into a conical 100 mL ground stoppered vessel:
20 mL eluate and add successively
5 mL sodium acetate solution (3.12) and
5 mL 0.1 M periodic acid solution (3.5).
Stir to mix, leave for 2 min exactly for oxidation to take place

Add:
5 mL sodium nitroferricyanide solution (3.13) and
5 mL piperidine solution (3.14).

Transfer the solution into a 1 cm cell. The purple color is obtained very rapidly; its intensity reaches a maximum after 30-40 sec then diminishes. Note the maximal absorbance. The measurement is carried out at 570 nm using air as a reference.

4.4.2 Preparation of the calibration curve

Put 10.0 mL of 2,3-butanediol standard solution (3.16) in a 100 mL volumetric flask and make up with distilled water. From this solution prepare standard solutions by pipetting respectively into 100 mL volumetric flasks:

2.0, 4.0, 6.0, 8.0 and 10.0 mL, make up with distilled water

These solutions correspond, according to the conditions described in 4.2 to the following concentrations: 0.25, 0.50, 0.74, 1.00 and 1.25 g/L of 2,3-butanediol.

Proceed with the determination as described in 4.4.1, replacing the eluate by the same volume of each of the standard solutions. The straight line of the calibration graph should pass through the origin.

5. Calculation and expression of results

5.1 *Glycerol*

5.1.1 Method of calculation

Read the glycerol content from the calibration curve. The result is expressed in g/L to one decimal place.

5.1.2 Repeatability

5.1.3 Reproducibility

5.2 *2,3-Butanediol*

5.2.1 Method of calculation

Read the 2,3-butanediol content on the calibration. The result is expressed in g/L to two decimal places.

5.2.2 Repeatability

5.2.3 Reproducibility

6. Glycerol and 2,3-butanediol by periodimetric titration

6.1 *Reagents*

6.1.1 Sodium hydroxide solution, 1 M.

6.1.2 Sulfuric acid solution, 0.5 M.

6.1.3 Periodic acid solution, 0.025 M.

6.1.4 Sodium bicarbonate solution, $NaHCO_3$, 8% (*m/v*).

6.1.5 Sodium arsenate solution, 0.025 M.

In a 1000 mL volumetric flask, dissolve 2.473 g of arsenic III oxide, As_2O_3, with 30 mL 1 M sodium hydroxide, (6.1.1) add 35 mL 0.5 M sulfuric acid (6.1.2), and make up to the mark with distilled water.

6.1.6 Iodine solution, 0.025 M.

6.1.7 Potassium iodide, 10% (*m/v*).

6.1.8 Starch paste, 2% (*m/v*).

6.2 *Procedure*

In a 300 mL conical flask add:
5 mL glycerol sample solution (3.15)
45 mL distilled water

or

25 mL 2,3-butanediol sample solution (3.16)
25 mL distilled water

Add:
20 mL periodic acid, 0.025 M (6.1.3), leave for 15 min, shaking from time to time
10-20 mL sodium bicarbonate solution (6.1.4)
20 mL sodium arsenate solution (6.1.5)

Leave for 15 min shaking from time to time and add:
5 mL potassium iodide solution (6.1.7)
2 mL starch paste (6.1.8)

Titrate the excess sodium arsenate with 0.025 M iodine solution (6.1.6).

Prepare at the same time a blank test using 50 mL distilled water and the same quantity of reagents.

6.3 *Method of calculation*

6.3.1 Glycerol

1 mL periodic acid, 0.025 M, oxidizes 1.151 mg glycerol.

The glycerol content in g/L of the glycerol standard solution (3.15) is:

$$G = \frac{(X - B) \times 1{,}151}{\alpha}$$

The percentage of glycerol used in the standard glycerol solution (3.15) is:

$$\frac{G}{2{,}5} \times 100$$

X = mL of the iodine solution, 0.025 M, used up by the standard solution (3.15)

B = mL of the iodine solution, 0.025 M, in the blank test

a = mL of the solution test (3.15) (equal to 5 mL)

6.3.2 2,3-Butanediol

1 mL periodic acid, 0.025 M, oxidizes 2.253 mg of 2,3-butanediol.

The 2,3-butanediol content in g/L of the 2,3-butanediol standard solution (3.16) is:

$$BD = \frac{(X' - B') \times 2{,}253}{b}$$

The percentage of 2,3-butanediol used in the 2,3-butanediol standard solution (3.2) is:

$$\frac{BD}{2{,}5} \times 100$$

X' = mL of iodine solution, 0.025 M, used up by the standard solution (3.16)

B' = mL of iodine solution, 0.025 M, used in blank test

b = mL of the solution test (3.16) (equal to 25 mL)

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Method OIV-MA-AS312-05 Type IV method

Glycerol

(Recueil OIV ed. 1990 revised by OIV/OENO 377/2009)

1 Principle

The glycerokinase (GK) catalyses the phosphorylation of glycerol to glycerol-3-phosphate by adenosine-5'-triphosphate (ATP) (1):

(1) $$\text{Glycerol + ATP} \xleftrightarrow{\text{GK}} \text{glycerol-3-phosphate + ADP}$$

The adenosine-5'-diphosphate (ADP) is then converted into ATP by phosphoenol-pyruvate (PEP) in presence of pyruvate-kinase (PK) with pyruvate (2) formation:

(2) $$\text{ADP + PEP} \xleftrightarrow{\text{PK}} \text{ATP + pyruvate}$$

Pyruvate is converted into lactate by reduced nicotinamide-adenine dinucleotide NADH) in presence of lactate-dehydrogenase (LDH) (3):

(3) $$\text{pyruvate + NADH + H}^+ \xleftrightarrow{\text{LDH}} \text{lactate + NAD}^+$$

The quantity of NAD^+ formed during the reaction is proportional to the quantity of glycerol. The NADH oxidization is measured by the decrease of its extinction at wavelengths of 334 nm, 340 nm or 365 nm.

2. Apparatus

2.1 Spectrophotometer enabling measurements to be made at 340 nm, at which absorption by NADH is at a maximum.

If not available, a photometer using a source with a discontinuous spectrum enabling measurements to be made at 334 nm or at 365 nm, may be used.

2.2 Glass cells of 1 cm optical path length or single-use cells.

2.3 Micropipettes enabling the selection of volumes from 0.02 to 2 mL.

3. Reagents

3.1 Buffer solution (0.75 M glycylglycine, Mg^{2+} 10^{-3} M, pH = 7.4)

Dissolve 10.0 g of glycylglycine and 0.25 g of magnesium sulfate ($MgSO_4 \cdot 7 H_2O$) in about 80 mL of double distilled water, add about 2.4 mL of 5 M sodium hydroxide solution to obtain a pH of 7.4 and make up to 100 mL. This buffer solution may be kept for 3 months at + 4°C.

3.2 (NADH $8.2.10^{-3}$ M, ATP 33.10^{-3} M, PEP 46.10^{-3} M)

Dissolve:

42 mg of nicotinamide-adenine-dinucleotide reduced - Na_2
120 mg of adenosine-5'-triphosphate, Na_2H_2
60 mg of phosphoenol pyruvate, Na and
300 mg of sodium bicarbonate ($NaHCO_3$)
in 6 mL of double distilled water.

This may be kept for 2-3 days at + 4°C.

3.3 Pyruvate-kinase/lactate-dehydrogenase (PK/LDH)

(PK 3 mg /mL, LDH 1 mg /mL)
Use the suspension without diluting it.

This may be kept for a year at about + 4°C.

3.4 Glycerokinase (GK 1 mg/mL)

The suspension may be kept for a year at about + 4°C.

Note: All reagents needed for the above are available commercially.

4. Preparation of sample

The determination of glycerol is generally made directly on the wine, which is diluted with double distilled water so that the resulting glycerol concentration is between 30 and 500 mg/L. Wine diluted 2 /100 is usually sufficient.

5. Procedure

With spectrophotometer adjusted to 340 nm wavelength the absorbance measurements are made in the glass cells with optical path length of 1 cm, with air as a reference.

Into cells with 1 cm optical paths place the following:

	Reference cell	Sample cell
Solution 3.1	1.00 Ml	1.00 mL
Solution 3.2	0.10 mL	0.10 mL
Sample to be measured	- 0.10 mL	
Water	2.00 mL	1.90 mL
Suspension 3.3	0.01 mL	0.01 mL

Mix, and after about 5 min, read the absorbances (A_1). Start the reaction by adding:

Suspension 3.4	0.01 mL	0.01 mL

Mix, wait until the end of the reaction (5 to 10 min), read the absorbance of the solutions (A_2). Read the absorbance after 10 min and check every 2 min until the absorbance is constant for 2 min.
Calculate the differences in the absorbance:

$$A_2 - A_1$$

for the reference and sample cells.

Calculate the differences in absorbance between the reference cell (ΔA_T) and the sample cell (ΔA_D) using the equation:

$$\Delta A = \Delta A_D - \Delta A_T$$

6. Expression of results

6.1 *Calculation*

The general formula for calculating the concentration is:

$$C = \frac{V \times PM}{\varepsilon \times d \times v \times 1000} X \Delta A$$

V = volume of the test in mL (3.12 mL)
v = volume of the sample mL (0.1 mL)
PM = molecular weight of the substance to be determined (glycerol = 92.1)
d = optical path length of the cell (1 cm)
ε = absorption coefficient of NADH at 340 nm

$$\varepsilon = 6.3 \ (mmol^{-1} \times l \times cm^{-1})$$

When using the amounts given in brackets this reduces to:

$$C = 0.456 \times \Delta A \times F$$

F = dilution factor

Note:
— Measurement at 334 nm, $\varepsilon = 6.2 \ (mmol^{-1} \times l \times cm^{-1})$

$$C = 0.463 \times \Delta A \times F$$

— Measurement at 365 nm, $\varepsilon = 3.4 \ (mmol^{-1} \times l \times cm^{-1})$

$$C = 0.845 \times \Delta A \times F$$

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Method OIV-MA-AS312-06 Type II method

Determination by isotope ratio mass spectometry $^{13}C/^{12}C$ of wine ethanol or that obtained through the fermentation of musts, concentrated musts or grape sugar.

(Resolution OENO 17/2001)

1. FIELD OF APPLICATION

The method enables the measuring of isotope ratio $^{13}C/^{12}C$ of ethanol in wine and ethanol obtained after fermentation of products derived from the vine (musts, concentrated musts, grape sugar).

2. REFERENCE STANDARDS

ISO 5725 :1994 «Accuracy (trueness and precision) of measurement methods and results: Basic method for the determination of repeatability and reproducibility of a standard measurement method»

V-PDB : Vienna-Pee-Dee Belemnite (R_{PDB} = 0.0112372).

Method OIV «Detection of enriching musts, concentrated musts, grape sugar and wine by application of nuclear magnetic deuterium resonance (RMN-FINS): »

3. TERMS AND DEFINITIONS

$^{13}C/^{12}C$: Carbon 13 and carbon 12 isotope ratio for a given sample

$\delta^{13}C$: Carbon 13 contents (^{13}C) expressed in parts per 1000 (‰)

RMN-FINS : Fractioning the specific natural isotope studied by nuclear magnetic resonance

V-PDB : Vienna-Pee-Dee Belemnite. or PDB, is the main reference for measuring natural variations of carbon 13 isotopic contents. Calcium carbonate comes from a Cretaceous belemnite from the Pee Dee formation in South Carolina (USA). Its isotopic ratio $^{13}C/^{12}C$ or R_{PDB}

is R_{PDB} = 0.0112372. PDB reserves have been exhausted for a long time, but it has natural variations of Carbon 13 isotopic contents. Reference material is calibrated based on this content and is available at the International Agency of Atomic Energy (IAEA) in Vienna (Austria). The isotopic indications of naturally occurring carbon 13 are expressed by V-PDB, as is the custom.

m/z: Mass to charge ratio

4. PRINCIPLE

During photosynthesis, the assimilation of carbonic gas by plants occurs according to 2 principle types of metabolism that are: metabolism C_3 (Calvin cycle) and C_4 (Hatch and Slack). These two means of photosynthesis present a different type of isotope fractionation. Products, such as sugars and alcohol, derived from C_4 plants and fermentation, have higher levels of Carbon 13 than from C_3 .plants. Most plants, such as vines and sugar beets belong to the C_3.group. Sugar cane and corn belong to the group C_4. Measuring the carbon 13 content enables the detection and the quantification of C_4 (sugar cane or corn isoglucose) origin sugars which are added to products derived from grapes (grape musts, wines). The combined information on carbon 13 content and information obtained from RMN-FINS enable the quantification of mixed sugars added or alcohol of plant origin C_3 and C_4.

The carbon 13 content is determined by carbonic gas resulting from the complete combustion of the sample. The abundance of main mass isotopomers 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$ et $^{12}C^{17}O^{16}O$) and 46 ($^{12}C^{16}O^{18}O$), resulting from different possible combinations of isotopes ^{18}O, ^{17}O, ^{16}O, ^{13}C et ^{12}C, are determined from ionic currents measured by three different collectors of mass isotopic spectrometers. The contributions of isotopomers $^{13}C^{17}O^{16}O$ et $^{12}C^{17}O_2$ are sometimes neglected because of their small presence. The ionic current for $m/z = 45$ is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is calculated according to the current intensity measured for $m/z = 46$ while considering the relative abundance of ^{18}O et ^{17}O (Craig adjustment). The comparison with the calibrated reference and the international reference V-PDB enable the calculation of carbon 13 content on a relative scale of $\delta^{13}C$.

5. REAGENTS

The material and the consumables depend on the apparatus (6) used by the laboratory. The systems generally used are based on elementary analysers. These systems can be equipped to introduce the samples placed in sealed metal capsules or for the injection of liquid samples through a septum using a syringe.

Depending on the type of instrument used, the reference material, reagents, and consumables can be used:

- Reference material

available from the IAEA:

Name	Material	$\delta^{13}C$ versus V-PDB (9)
- IAEA-CH-6	saccharose	-10.4 ‰
-IAEA-CH-7	polyethylene	-31.8 ‰
- NBS22	oil	-29.7 ‰
- USGS24	graphite	-16.1 ‰

available from the IRMM de Geel (B) (Institut des Matériaux et Mesures de Référence) :

Name	Material	$\delta^{13}C$ versus V-PDB (9)
- CRM 656	Wine alcohol	-26.93 ‰
- CRM 657	glucose	-10.75 ‰
- CRM 660	hydroalcoholic solution (TAV 12%)	-26.72 ‰

Standard work sample with a known calibrated $^{13}C/^{12}C$ ratio with international reference materials.

A standard list of consumables established for continuous flow systems follows here under :

- Helium for analysis (CAS 07440-59-7)
- Oxygen for analysis (CAS 07782-44-7)
- Carbon dioxide for analysis, used as a secondary reference gas for the content of carbon 13 (CAS 00124-38-9)
- Oxidation reagent for the oven and the combustion system as follows: copper oxide (II) for elementary analyzed (CAS 1317-38-0)
- Drying agent to eliminate water produced by combustion. For example: anhydrone for elementary analysis (magnesium perchlorate) (CAS 10034-81-8).

This is not necessary for apparatuses equipped with a water elimination system by cryo-trapping or through selective permeable capillaries.

6. APPARATUS AND MATERIAL

6.*1.. Isotope ratio mass spectometry (IRMS)*

Isotope ratio mass spectometry (IRMS) enables the determination the relative contents of ^{13}C of CO_2 gas naturally occurring with an internal accuracy of 0.05‰ or expressed in relative value (9). Internal accuracy here is defined as the difference between 2 measurements of the same sample of CO_2. the mass spectrometer used to measure isotope ratios is generally equipped with a triple collector to simultaneously measure m/z = 44, 45 and 46 intensities. The isotope ratio mass spectrometry must either be equipped with a double introduction system to alternately measure the unknown sample and a reference sample, or use an integrated system that carries out quantitative combustion on samples and separates the carbon dioxide from the other combustion products before measuring the mass spectrum.

6.2. Combustion apparatus

Combustion apparatus able to quantitively convert ethanol in carbon dioxide and able of eliminating all other combustion products including water, without any isotopic fractioning. The apparatus can be either an integrated continual flow system integrated with mass spectometry (6.2.1), of an autonomous combustion system (6.2.2). The apparatus must be as precise as indicated in (11).

6.2.1. Continual flow system

These are made up by an elementary analyser, either by chromatography in gaseous state equipped with an online combustion system.

The following laboratory material is used for systems equipped for the introduction of samples contained in metallic capsules :

- volumetric micropipette with appropriate cones
- scale with μg accuracy or better
- pliers for encapsulation
- tin capsules for liquid samples
- tin capsules for solid samples

The following laboratory material is needed when using an elementary analyser equipped with a liquid injector or in the case of a preparation system for combustion chromatography:

- syringe for liquids
- flasks equipped with sealed closing system and inert septa

The laboratory material indicated in the lists are examples that are susceptible of being replaced by other equivalent performance material depending on the type of combustion apparatus and of mass spectometry used by the laboratory.

6.2.2 Autonomous preparation system

The samples of carbon dioxide resulting from the combustion of samples to be analyzed and the reference sample are collected in bulbs which are then put in a double entry spectometry system to carry out isotopic analyses. Several combustion apparatuses described in writings can be used:

- Closed combustion system filled with oxygen gas circulating
- Elementary analyser with helium and oxygen flow
- Bulb sealed in glass filled with copper oxide (II) used as an oxidation agent

7. PREPARATION OF SAMPLES FOR TRIALS

Ethanol must be extracted from wine before isotopic testing. This is carried out by distilling wine as described in §3.1 using the RMN-FINS method.

Sugars must be fermented in ethanol first as described in the RMN-FINS method in the case of grape musts, concentrated rectified grape musts (grape sugar).

8. PROCEDURE

All preparation steps must be carried out without any significant ethanol loss through evaporation, which would change the isotopic composition of the sample.

The description that follows makes reference to the procedure generally used for ethanol sample combustion using commercial automatic combustion systems. All other methods, ensuring that ethanol samples are converted by quantity in carbon dioxide without the evaporation of ethanol, can use the preparation of carbon dioxide for isotopic analyses. An experimental procedure based on the usage of an elementary analyser:

a) Placing the samples in capsules

- use capsules, a tweezers and a clean preparation tray
- take an appropriate sized capsule using a tweezers
- introduce an appropriate amount of liquid into the capsule using a micropipette

Note: 3.84 mg of absolute ethanol or 4.17 mg of distillate with an alcohol content of 92% m/m are necessary to obtain 2 mg of carbon. The appropriate quantity of distillate must be calculated in the same way according to the quantity of carbon necessary based on the mass spectometry instruments' sensitivity.

- close the capsule with the tweezers.
- each capsule must be completely sealed. If not, it must be discarded and the capsule must be repaired.
- two capsules must be prepared for every sample
- place the capsules in an appropriate place on the tray elementary analyser sample. Every capsule must be carefully identified in order by number .
- systematically place capsules containing work references at the beginning and the end of the sample series
- regularly insert a check sample in the sample series.

b) check and adjust the elementary analysis and mass spectometry instruments

- adjust the temperature of the elementary analyzer ovens and the helium and oxygen gas flow for an optimal combustion of the sample;
- check the elementary analysis system and the mass spectometry system for leaks (for example by checking the ionic current where m/z = 28 corresponding to N_2.);
- adjust the mass spectrometer to measure the intensities of ionic current where m/z = 44, 45 and 46;
- check the system using known reference samples before starting to measure the samples.

c) To carry out a series of measurements

The samples that are placed under the elementary or chromatography are introduced successively. The carbon dioxide for each sample combustion is eluted towards the mass spectrometer which measures the ionic current. The interface computer records the ionic current intensities and calculates the values δ for each sample (9).

9. CALCULATION

The objective of the method is to measure the isotopic ratio $^{13}C/^{12}C$ ethanol extract from wine or from products derived from grapes following fermentation. The isotopic ratio $^{13}C/^{12}C$ can be expressed by its deviation compared to the reference work. Carbon 13 ($\delta\,^{13}C$)'s isotopic ratio is calculated on a delta scale per thousand

by comparing the results obtained for the sample to be measured to the reference work calibrated before based on the primary international reference (V-PDB). The values $\delta\ ^{13}C$ are expressed compared to reference work:

$$\delta^{13}C_{ech/ref}‰ = 1000 \times (R_{ech}-R_{ref})/R_{ref}$$

where R_{ech} and R_{ref} are respectively the isotopic ratio $^{13}C/^{12}C$ of the sample and the work reference.

The values $\delta\ ^{13}C$ are thus expressed using V-PDB:

$$\delta^{13}C_{ech/V\text{-}PDB}‰ = \delta^{13}C_{ech/ref} + \delta^{13}C_{ref/V\text{-}PDB} + (\delta^{13}C_{ech/ref} \times \delta^{13}C_{ref/V\text{-}PDB})/1000$$

where $\delta^{13}C_{ref/V\text{-}PDB}$ is the isotopic deviation determined beforehand for the work reference to V-PDB.

Small variations may occur while measuring on line due to changes in the instrumental conditions. In this case the $\delta\ ^{13}C$ samples must be corrected according to the difference in the value $\delta^{13}C$ from the work reference and the real value, which was calibrated beforehand against V-PDB by comparison with one of the international reference materials. Between two measurements of the reference work, the variation is the correction applied to the sample results that may be assumed to be linear. The reference work must be measured at the beginning and at the end of all sample series. A correction can be calculated for each sample using linear interpolation between two values (the difference between the assigned value of the reference work and the measurements of obtained values).

10. QUALITY INSURANCE AND CONTROL

Check that the value ^{13}C for the reference work does not differ by more than 0.5‰ of the admitted value. If not, the spectrometer instrument adjustments must be checked and possibly readjusted.

For each sample, verify that the difference in result between the 2 capsules measured successively is under 0.3‰. The final result for a given sample is the average value between the 2 capsules. If the deviation is higher than 0.3‰ the measurement should be repeated.

Measurement condition monitoring can be based on the ionic current intensity where m/z = 44 and is proportional to the quantity of carbon injected in the elementary analyzer. Under standard conditions, the ionic current intensity should be almost constant for the samples analysed. A significant deviation could be indicative of ethanol evaporation (an imperfect seal on a capsule), an instability of the elementary analyser or the mass spectrometer.

11. METHOD PERFORMANCE TRAITS (Accuracy)

One joint analysis (11.1) was carried out on distillates containing alcohol of vinous origin and cane and beet alcohol, in addition to different mixtures of these three origins. This study did not take into account the distillation step, further information from other joint laboratory studies on wine (11.2) and namely circuits of aptitude tests (11.3) for isotopic measurements were also considered. The results show that different distillation systems under satisfactory conditions, and in particular those used to measure RMN-FINS, do not have significant varieties for determining $\delta^{13}C$ of ethanol in wine. The precision parameters observed for wine are almost identical to those obtained in the joint study on distillates (11.1) sur les distillats.

11.1. Joint study on distillates

Year of joint laboratory study: 1996

Number of laboratories: 20

Number of samples: 6 samples in double-blind comparison

Analysis: $\delta\ ^{13}C$ ethanol

Sample code	Vinous origin alcohol	Beet alcohol	Sugar cane alcohol
A & G	80%	10%	10%
B & C	90%	10%	0%
D & F	0%	100%	0%
E & I	90%	0%	10%
H & K	100%	0%	0%
J & L	0%	0%	100%

Samples	A / G	B / C	D / F	E / I	H / K	J / L
Number of laboratories retained after eliminating aberrant results	19	18	17	19	19	19
Number of results accepted	38	36	34	38	38	38
Average value ($\delta\ ^{13}C$) ‰	-25.32	-26.75	-27.79	-25.26	-26.63	-12.54
Sr^2	0.0064	0.0077	0.0031	0.0127	0.0069	0.0041
Repeatability standard deviation (Sr) ‰	0.08	0.09	0.06	0.11	0.08	0.06
Repeatability limit r ($2,8 \times S_r$) ‰	0.22	0.25	0.16	0.32	0.23	0.18
S_R^2	0.0389	0.0309	0.0382	0.0459	0.0316	0.0584
Reproductability standard deviation (S_R) ‰	0.20	0.18	0.20	0.21	0.18	0.24
Reproductability limit R ($2,8 \times S_R$) ‰	0.55	0.9	0.55	0.60	0.50	0.68

11.2. Joint laboratory study on two wines and one alcohol

Year of joint laboratory trial: 1996

Number of laboratories: 14 for distillation of wine and 7 for also measuring δ ^{13}C of ethanol in wine

8 for measuring δ ^{13}C in alcohol sample

Number of samples 3 (White wine TAV 9.3% vol., White wine TAV 9.6% Alcohol strength 93% m/m)

Analysis: δ ^{13}C of ethanol

Samples	Red wine	White wine	Alcohol
Number of laboratories	7	7	8
Number of accepted results	7	7	8
Average value (δ ^{13}C) ‰	-26.20	-26.20	-25.08
Reproductability variance S_R^2	0.0525	0.0740	0.0962
Reproductability standard deviation (S_R) ‰	0.23	0.27	0.31
Reproductability limit R (2,8× S_R) ‰	0.64	0.76	0.87

Different distillation systems were used by the participating laboratories. The isotopic indications δ ^{13}C carried out in one laboratory on the whole number of distillates returned by the participants, does not reveal any absurd values or significant distinct average values. The variation in results ($S^2 = 0.0059$) is comparable to repeatability variances Sr^2 from the joint study on distillates (11.1).

11.3. Results from the exercises of aptitude circuits to isotopic trials

Since December 1994 international aptitude exercises to determine the isotopic measurements for wine and alcohol (TAV distillates 96% vol.) have been regularly organized. The results enable participating laboratories to check the quality of their

analyses. Statistical results enable the appreciation of the variety of derterminants under the reproductability conditions. This enables the estimating the variance parametres and the reproductability limit. The results obtained for the wine and distillate ethanol $\delta\ ^{13}C$ determants are summarized in the table below:

	Wine				Distillates			
Date	N	S_R	S^2_R	R	N	S_R	S^2_R	R
Dec. 1994	6	0.210	0.044	0.59	6	0.151	0.023	0.42
June 1995	8	0.133	0.018	0.37	8	0.147	0.021	0.41
Dec. 1995	7	0.075	0.006	0.21	8	0.115	0.013	0.32
March 1996	9	0.249	0.062	0.70	11	0.278	0.077	0.78
June 1996	8	0.127	0.016	0.36	8	0.189	0.036	0.53
Sept. 1996	10	0.147	0.022	0.41	11	0.224	0.050	0.63
Dec. 1996	10	0.330	0.109	0.92	9	0.057	0.003	0.16
March 1997	10	0.069	0.005	0.19	8	0.059	0.003	0.16
June 1997	11	0.280	0.079	0.78	11	0.175	0.031	0.49
Sept 1997	12	0.237	0.056	0.66	11	0.203	0.041	0.57
Dec. 1997	11	0.127	0.016	0.36	12	0.156	0.024	0.44
March 1998	12	0.285	0.081	0.80	13	0.245	0.060	0.69
June 1998	12	0.182	0.033	0.51	12	0.263	0.069	0.74
Sept 1998	11	0.264	0.070	0.74	12	0.327	0.107	0.91
Weighted average		0.215	0.046	0.60		0.209	0.044	0.59

N : number of participating laboratories

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Method OIV-AS312-07 Type of the method **IV**

Method for the determination of the $^{13}C/^{12}C$ isotope ratio of glycerol in wines by Gas Chromatography Combustion or High performance Liquid Chromatography coupled to Isotopic Ratio Mass Spectrometry (GC-C-IRMS or HPLC-IRMS)

(OIV/OENO 343/2010)

1. SCOPE

The present methods, based on gas chromatography [1] or liquid chromatography [2] coupled to an isotope ratio mass spectrometer (GC-C-IRMS or HPLC-IRMS), permit measurements of the $^{13}C/^{12}C$ ratio of glycerol. If its quantification is required simultaneously with the $^{13}C/^{12}C$ isotope ratio, GC-IRMS may be used.
The use of 1,5-pentanediol, as internal standard, also allows the determination of the glycerol concentration during the same analysis of the 13C/12C ratio.

2. DEFINITIONS

- $^{13}C/^{12}C$: ratio of carbon-13 (^{13}C) to carbon-12 (^{12}C) isotopes for a given sample.
- $\delta^{13}C$: carbon-13 content (^{13}C) expressed in parts per 1000 (‰, per mil).
- GC-C-IRMS: hyphenated technique of gas chromatography coupled to a combustion interface and isotope ratio mass spectrometry.
- V-PDB: Vienna-Pee-Dee-Belemnite. PDB is the primary reference material for measuring natural variations of carbon-13 isotope content, consisting of calcium carbonate from a Cretaceous belemnite rostrum from the Pee Dee Formation in South Carolina (USA). Its $^{13}C/^{12}C$ isotope ratio or R_{PDB} is 0.0112372. PDB reserves have been exhausted for a long time, but it has remained the primary reference for expressing natural variations of carbon-13 isotope content and against which the reference material available at the IAEA (*International Atomic Energy Agency*) in Vienna (Austria) is calibrated. Isotopic indications of naturally occurring carbon-13 are conventionally expressed in relation to V-PDB.

3. PRINCIPLE

A significant difference exists between the carbon-13 content of sugars from plants following the different photosynthetic C_3 (Calvin cycle) and C_4 (Hatch-Slack) cycles. Most plants, such as the vine and beet, belong to the C_3 group, whilst maize and cane belong to the C_4 group. The carbon-13 contents of the sugar and of the corresponding metabolites obtained by fermentation (ethanol, glycerol) are correlated.
The measurement of the carbon-13 content of glycerol may enable possible detection of addition of glycerol from maize (C_4 plant) or from synthesis (fossil sources) to wines or to spirit drinks.
The separation of glycerol from the wine matrix is achieved using gas or liquid chromatography.
In GC-C-IRMS, after the chromatographic separation the effluent undergoes a combustion and a reduction step, passing through the oxidation and the reduction ovens of a combustion interface. Components other than the glycerol, namely the solvent, are vented with a back-flush valve during the run, to avoid oven soiling and interferences in chromatograms. The carbon-13 content is determined on the carbon dioxide gas resulting from the oxidation of the glycerol contained in the sample. Once the glycerol is oxidized, CO_2 and H_2O are produced. Water produced during the combustion is eliminated by a water-removing trap, consisting of a Nafion® membrane. The carbon dioxide is eluted by a helium stream to the IRMS source for $^{13}C/^{12}C$ analysis.
In HPLC-IRMS, after the chromatographic separation the sample is oxidized while still in the mobile phase at the interface. The CO_2 formed is removed on-line from the solvent stream through a gas-exchange membrane into a stream of He. This He stream passes through a water trap consisting of a Nafion® membrane, and is then admitted to the ion source of the IRMS via an open split.
The various possible combinations of the ^{18}O, ^{17}O, ^{16}O and ^{13}C, ^{12}C, isotopes lead to the mass 44 corresponding to the $^{12}C^{16}O_2$ isotopomer, the mass 45 corresponding to $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ species and the mass 46 to the $^{12}C^{16}O^{18}O$ isotopomer ($^{13}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can be neglected due to their very low abundance). The corresponding ion currents are determined on three different collectors. The ion current m/z 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the current intensity measured for m/z 46 by considering the relative abundance of ^{18}O and ^{17}O (Craig correction). The comparison with a reference calibrated against the international standard V-PDB permits the calculation of the carbon-13 content on the $\delta^{13}C$ ‰ relative scale.

4. REAGENTS

The following reagents and working standards should be used:

4.1 Anhydrous ethanol (CAS number 64-17-5).
4.2 Pure glycerol ≥ 99 % (CAS 56-81-5).
4.3 1,5-pentanediol (CAS 111-29-5).
4.4 Bulk solution of 1,5-pentanediol (4.3) in ethanol (4.1). This solution prepared at a precisely known concentration, in the range of 0.5 to 1.0 g L^{-1} is used to dilute wine samples.
4.5 Orthophosphoric acid
4.6 Sodium peroxodisulfate, used as oxidation reagent
4.7 Helium for analysis, used as carrier gas (CAS 07440-59
4.8 Oxygen for analysis, used as regenerating gas for the combustion reactor (CAS 07782-44-7).
4.9 Cylinder of carbon dioxide for analysis, used as a secondary reference gas for the carbon-13 content (CAS 00124-38-9).
4.10 Working standard samples of glycerol with a known $^{13}C/^{12}C$ ratio calibrated against international reference materials.
4.11 Working standard samples of 1,5-pentanediol with a known $^{13}C/^{12}C$ ratio calibrated against international reference materials.

5. APPARATUS AND EQUIPMENT

5.1. Isotope ratio mass spectrometer

Isotope ratio mass spectrometer (IRMS) capable of determining the relative ^{13}C content of naturally-occurring CO_2 gas with an internal accuracy of 0.05 ‰ or better expressed as a relative value (point 8. Calculation). Internal accuracy here is defined as the difference between two measurements of the same sample of CO_2. The mass spectrometer used to measure isotope ratios is equipped with a triple collector to simultaneously measure intensities for m/z = 44, 45 and 46. The IRMS is equipped with software for running the analysis, acquisition of data and processing of analytical results for computation of isotope ratios.

5.2. Gas chromatograph

Gas chromatograph (GC) coupled through a combustion interface to an isotope ratio mass spectrometer (5.1).

The gas chromatograph must be equipped with a polar capillary column enabling the chromatographic separation of glycerol from the other wine components (e.g. Chrompack WCOT fused silica capillary column filled with bonded polyethylene glycol CP-Wax-57 CB, 25 m, 0.25 mm id, 0.20 µm film thickness).

Combustion interface generally made up of an oxidation reactor (a ceramic tube containing nickel, platinum and copper wires) and of a reduction reactor (ceramic tube containing copper wires).

5.3. Liquid chromatograph

Liquid chromatograph (LC) coupled through a LC Isolink interface to an isotope ratio mass spectrometer (5.1).

The liquid chromatograph must be equipped with a column enabling the chromatographic separation of glycerol from the other wine components without using organic solvents or additives (e.g. HyperREZ Carbohydrate H^+, 30 cm, 8 mm).

Isolink interface made up of a capillary oxidation reactor and a membrane exchanger (three membranes).

5.4. Equipment

Usual laboratory equipment and in particular the following:

- Sample injection syringes or autosampler

- Volumetric flasks, 0.2 µm filters, chromatographic vials and 10 µL syringe for liquids.

The laboratory equipment indicated in the above list is an example and may be replaced by other equipment of equivalent performance.

6. PREPARATION OF TEST SAMPLES

6.1. $^{13}C/^{12}C$ determination of glycerol by GC-C-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted (in the ratio 1:4) with ethanol. Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis.

6.2. $^{13}C/^{12}C$ ratio of glycerol and its quantification by GC-C-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted (in the ratio 1:4) with the bulk solution of 1,5-pentanediol (4.4). Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis.

6.3. $^{13}C/^{12}C$ determination of glycerol by HPLC-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted with water. Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis

7. PROCEDURE

7.1. GC-C-IRMS

The following description refers to the procedures generally used for glycerol $^{13}C/^{12}C$ isotope-ratio determination using commercial automated GC-C-IRMS systems.

Procedures may be adapted according to changes introduced by the manufacturers. Note: volumes, temperature, flows and times are indicative. The correct values should be optimized according to the manufacturer's instructions.

7.1.1 Working conditions

Using the column and combustion interface described as an example in 5.2 the following parameters can be applied:
A. The injector temperature is set to 270 °C.
B. The temperature program is set as follows: initial column temperature of 120 °C; a holding time of 2 min; then a temperature increase at a rate of 10 °C min^{-1}, up to the final value of 220 °C, with a final holding time of 2 min.
Each run takes 14 min, not taking into account the time needed for cooling.
C. He is used as the carrier gas.
D. The temperatures of the combustion and reduction reactors of the GC combustion interface are set to 960 and 640°C respectively.
E. In each injection 0.3 µL of sample solution is introduced into the column using a high-split mode (split flow 120 mL min^{-1}).
At regular intervals (e.g. once a week) re-oxidation of the oxidation reactor with O_2 is required (the intervals depend on the total amount of substances that has passed through the reactor).

7.1.2. $^{13}C/^{12}C$ ratio of glycerol

During each $^{13}C/^{12}C$ analysis, at least two pulses of reference CO_2 gas (4.9) from the cylinder are introduced. This CO_2 is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference CO_2 gas may also be calibrated against in-house standards. Each wine sample (6.1) is injected 3 times. Suitable control references must be included in each batch.
A typical batch is as follows:
• Control Sample
• Control Sample
• Sample 1
• Sample 1
• Sample 1
• Sample 2
Each sample is measured 3 times
• …..
• Sample 6
• Sample 6
• Sample 6
• Control sample
• Control sample

The control sample is an ethanol solution of glycerol with a known accurately-measured $\delta^{13}C$ value (by an elemental analyser-IRMS for instance) and enables possible drift during the sequence of measurements to be checked and the correction of results.

7.1.3 $^{13}C/^{12}C$ ratio of glycerol and its quantification

If quantification of glycerol is required at the same time as $^{13}C/^{12}C$ isotope ratio measurement, the previous procedure (7.1.2) is applied to the samples prepared as described in 6.2.
The 1,5-pentanediol (4.3) permits the determination of the concentration of glycerol. Furthermore, $\delta^{13}C$ values for the internal reference can be used to assess the correctness of the injections and the quality control of the isotopic determinations and of the combustion reaction step.
The concentration of glycerol in wine samples is determined using the internal-standard method. To do this, a calibration curve must be produced, using a constant known concentration for the internal standard, 1,5-pentanediol, and five glycerol solutions at different known concentrations, from 0.50 to 10 g L^{-1}. These solutions are prepared by weighing and dissolving glycerol (4.2) and 1,5-pentanediol in ethanol (4.1), using volumetric flasks. Ensure that the response is linear by successively analysing in triplicate each of the linearity standard solutions containing the internal standard.

7.2. HPLC-IRMS

The following description refers to the procedures generally used for glycerol $^{13}C/^{12}C$ isotope ratio determination using commercial automated HPLC-IRMS systems.

Procedures may be adapted according to changes introduced by the manufacturers. Note: volumes, temperature, flows and times are indicative. The correct values should be optimized according to the manufacturer's instructions.

7.2.1 Working conditions

Using the column and interface described as an example in 5.3 the following parameters can be applied:

A. Flow rate of the eluent is set at 400 μL min^{-1}
B. Flow rate of the acid and oxidant reagents in the LC interface is set at 40 and 30 μL min^{-1}, respectively
C. The temperatures of the interface reactor and the column are set at 99.9 and 65 °C, respectively
D. Helium flow rate of the separation unit is set at 1 μL min^{-1}

The reagent bottles are degassed with helium during the complete chromatographic run.

7.2.2. $^{13}C/^{12}C$ ratio of glycerol

During each $^{13}C/^{12}C$ analysis, at least two pulses of reference CO_2 gas (4.9) from the cylinder are introduced (see example of chromatogram in 11.2). This CO_2 is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference CO_2 gas may also be calibrated against in-house standards.
Each wine sample (6.3) is injected 3 times. Suitable control references must be included in each batch.
A typical batch is as follows:
• Control sample
• Control sample
• Sample 1
• Sample 1
• Sample 1
• Sample 2
Each sample is measured 3 times
• …..
• Sample 6
• Sample 6
• Sample 6
• Control sample
• Control sample
The control sample is a solution of glycerol with a known accurately measured $\delta^{13}C$ value (by an elemental analyser-IRMS for instance) and enables possible drift during the sequence of measurements to be checked and the correction of results.

8. CALCULATION

8.1. $^{13}C/^{12}C$ ratio

The $^{13}C/^{12}C$ isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of carbon-13 ($\delta^{13}C$) is then calculated on a delta scale per thousand ($\delta/1000$ or δ ‰) by comparing the results obtained for the sample to be measured with those for a working reference, previously calibrated on the basis of the primary international reference (V-PDB). During $^{13}C/^{12}C$ analyses, a reference CO_2 gas is introduced, which is calibrated against other PDB-calibrated international standards.
The $\delta\ ^{13}C$ values are expressed in relation to the working reference as follows:

$$\delta^{13}C_{\text{sample/ref}}‰ = (R_{\text{sample}}/R_{\text{ref}} - 1) \times 1000$$

where R_{sample} and R_{ref} are respectively the $^{13}C/^{12}C$ isotope ratios of the sample and of the carbon dioxide used as the reference gas (4.9).
The $\delta\ ^{13}C$ values are expressed in relation to V-PDB as follows:

$$\delta^{13}C_{\text{sample/V-PDB}}‰ = \delta^{13}C_{\text{sample/ref}} + \delta^{13}C_{\text{ref/V-PDB}} + (\delta^{13}C_{\text{sample/ref}} \times \delta^{13}C_{\text{ref/V-PDB}})/1000$$

where $\delta^{13}C_{\text{ref/V-PDB}}$ is the previously determined isotopic deviation of the working reference from V-PDB

Small variations may occur while measuring on-line due to changes in the instrumental conditions. In this case the $\delta\ ^{13}C$ values of the samples must be corrected according to the difference between the measured $\delta^{13}C$ value of the standard working sample and its true value, previously calibrated against V-PDB by comparison with one of the international reference materials. Between two measurements of the standard working sample, the variation, and therefore the correction to be applied to the results obtained from the samples, may be assumed to be linear. The standard working sample must be measured at the beginning and at the end of all sample series. A correction can then be calculated for each sample using linear interpolation.

8.2. Glycerol concentration by GC-IRMS

When producing the calibration curve, for each injection, the measured parameter which is taken into account is the area S (in V*s) given by the spectrometer.

Calculate the ratio R as expressed in equation 1 below, and plot a graph of R versus the concentration ratio of glycerol to the internal standard (IS), C.
A linear plot should be obtained, with a correlation coefficient of at least 0.99.

Equation 1 $$R=\frac{\text{Peak area glycerol}}{\text{Peak area of IS}}$$

Using the analytical conditions described (7.1.1), 1,5-pentanediol being less polar than glycerol shows a retention time of around 310 sec, while that of glycerol is 460 sec ((see example of a chromatogram in 11.1).
The concentration of glycerol in each injection is calculated using the following equation:

Equation 2 $$C_{glyc_{Sample}} = K \cdot C_{1,5PD_{Sample}} \cdot \frac{S_{glyc_{Sample}}}{S_{1,5PD_{Sample}}} \times \text{dilution factor}$$

Where:

Cx_{Sample} is the concentration in g L^{-1} of the species in the sample;

SX_{sample} is the area of the peaks produced;

K (the response factor) is calculated as follows:

$$K = \frac{C_{glyc_{St}}}{C_{1,5PD_{St}}} \cdot \frac{S_{1,5PD_{St}}}{S_{glyc_{St}}}$$ Equation 3 (see 8.2)

The St suffix indicates the concentrations and the areas of 1,5-pentandiol and glycerol in the five standard solutions prepared for the calibration (7.1.3);

Dilution factor: considering the sampling conditions described above (7), the dilution factor is 4.

The concentration value in g L^{-1} of each sample is the mean of the three injections

9. QUALITY ASSURANCE AND CONTROL

9.1. GC-C-IRMS

For each sample, check that the standard deviation (SD) in three vials measured successively is less than 0.6 ‰. The final result for a given sample is the average value for the three measurements. If the deviation is greater than 06 ‰, the measurement must be repeated.

Checks on correct measurement can be based on the ion current of m/z = 44, which is proportional to the quantity of carbon injected into the system. Under standard conditions, the ion current should be almost constant for the samples analysed. A significant deviation could be indicative of imperfect separation and oxidation of glycerol or instability of the mass spectrometer.

9.2. HPLC-IRMS

Check that the ^{13}C value for the working reference does not differ by more than 0.5 ‰ from the admissible value. If not, the spectrometer settings should be checked and, if necessary, adjusted.

For each sample, check that the standard deviation (SD) in three vials measured successively is less than 0.6 ‰. The final result for a given sample is the average value for the three measurements. If the deviation is greater than 0.6 ‰, the measurement must be repeated.

Checks on correct measurement can be based on the ion current of m/z = 44, which is proportional to the quantity of carbon injected into the system. Under standard conditions, the ion current should be almost constant for the samples analysed. A significant deviation could be indicative of imperfect separation and oxidation of glycerol or instability of the mass spectrometer.

10. PERFORMANCE CHARACTERISTICS OF THE METHOD

10.1. GC-C-IRMS

10.1.1 Precision

Preliminary studies have been performed on 4 synthetic wine solutions (water-ethanol-glycerol), prepared using glycerol samples of different origins and with a $\delta^{13}C$ value already determined by EA-IRMS. For the 3 repetitions, n=3, using the GC-C-IRMS technique a standard deviation SD ≤ 0.6 ‰ was considered acceptable. Precision can be affected by overlapping between 1,5-PD and other wine components or by-products when measuring sweet wines.

10.1.2. Determination of the concentration of glycerol

For the validation of this method, 2 glycerol solutions were used. Assuming that the typical concentration of glycerol is 4 to 10 g L^{-1} in dry wine, the 2 solutions represent this range. The first solution was 4.0 g L^{-1} and gave an experimental concentration of 3.6 g L^{-1} (SD=0.2, n=8). The second solution, 8.0 g L^{-1}, gave a value of 7.9 g L^{-1} (SD=0.3, n=8).
Furthermore, 5 wine samples (A-E) already analysed for their glycerol concentration using other methods* through the BIPEA proficiency–testing scheme were injected to test the method.

Table 1: Comparison with the concentration of 5 dosed wines.

Sample	A	B	C	D	E
Type	White	Rosé	White	Red	White
Given range	6.2 - 8.4	4.8 - 6.6	5.7 - 7.7	6.3 - 8.5	4.6 - 6.2
Mean value	7.3	5.4	6.7	7.4	5.4
by GC-C-IRMS	6.4	5.4	6.7	7.8	5.4

* BIPEA determinations were performed by HPLC and/or enzymatic analysis.
Concentrations are given in g L^{-1}. n>3 and SD < 0.6.

obtained using other analytical techniques such as enzymatic determination or HPLC.

10.2. HPLC-IRMS

Internal validation of HPLC-IRMS method

For the validation of the HPLC-IRMS method, the following samples have been used: a glycerol standard, three synthetic wines (glycerol concentrations ranged within typical concentration found in wines) and a wine.
The precision of the measurement for glycerol was determined by repeating the analysis ten times on each sample, under repeatable conditions, and by performing ten independent analyses on the same sample on three different days, under reproducible conditions (Table 2).

Table 2. Accuracy and precision of δ13C values of glycerol obtained by HPLC-IRMSa

		HPLC-IRMS							
		Day 1		Day 2		Day 3		Precision	
Sample	Repetitions per sample	Mean $\delta^{13}C$ (‰)	SD (‰)	Mean $\delta^{13}C$ (‰)	SD (‰)	Mean $\delta^{13}C$ (‰)	SD (‰)	r (‰)	R (‰)
Glycerol (standard)[b]	10	-27.99	0.05	-27.94	0.04	-27.95	0.08	0.17	0.18
Synthetic wine (6 g/l)	10	-28.06	0.13	-28.14	0.12	-28.14	0.11	0.34	0.35
Synthetic wine (8 g/l)	10	-28.11	0.12	-28.18	0.07	-28.21	0.07	0.25	0.28
Synthetic wine (10 g/l)	10	-28.06	0.06	-28.06	0.09	-28.05	0.09	0.23	0.24
Wine	10	-28.88	0.10	-28.85	0.27	-28.72	0.23	0.60	0.62

[a]Values of $\delta^{13}C$ are expressed in ‰ vs V-PDB

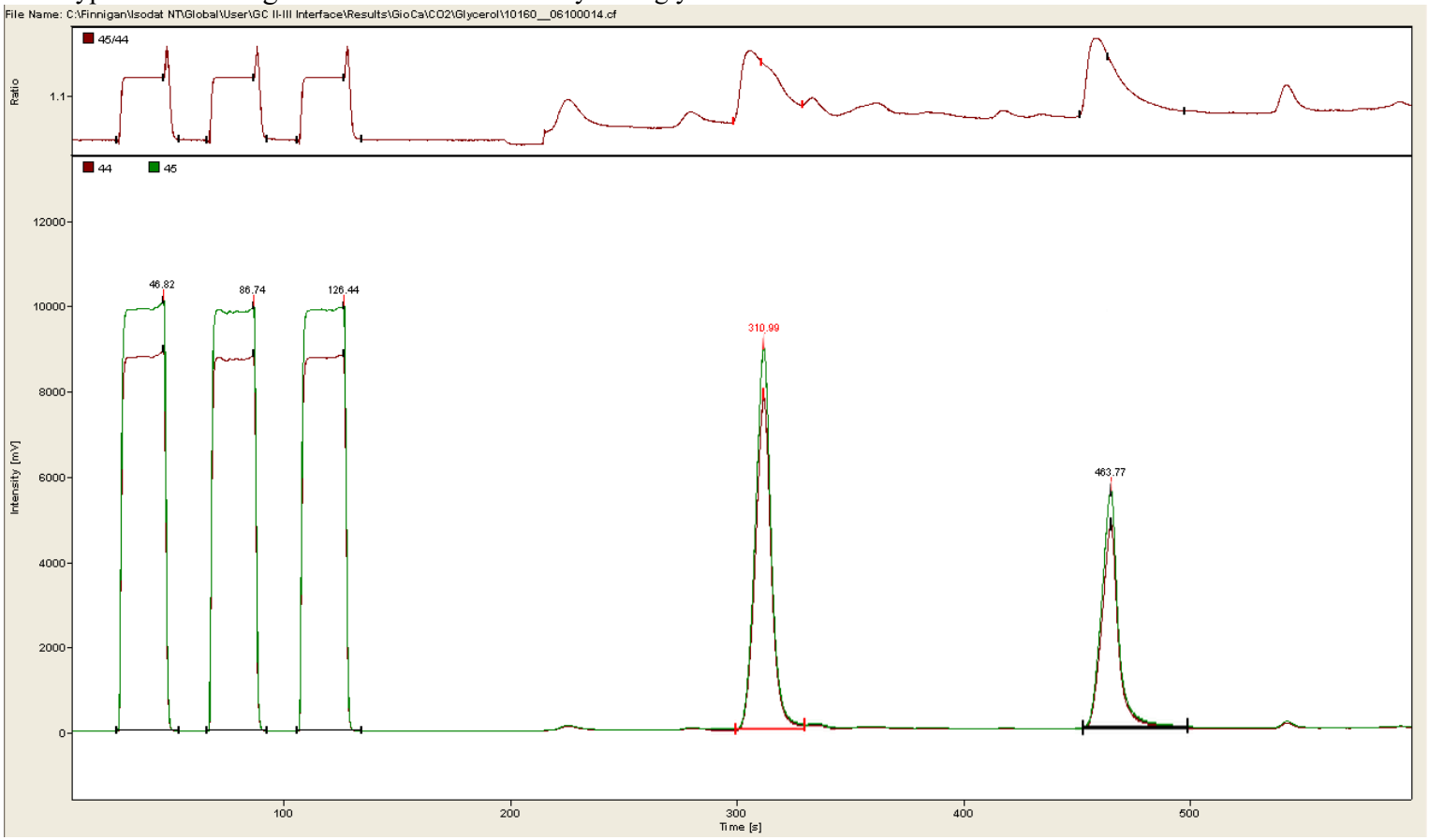
[b]EA-IRMS glycerol (standard) result: -28.02 ± 0.09 ‰

The following performance parameters for determining the $\delta^{13}C$ of glycerol were obtained from a wine sample:

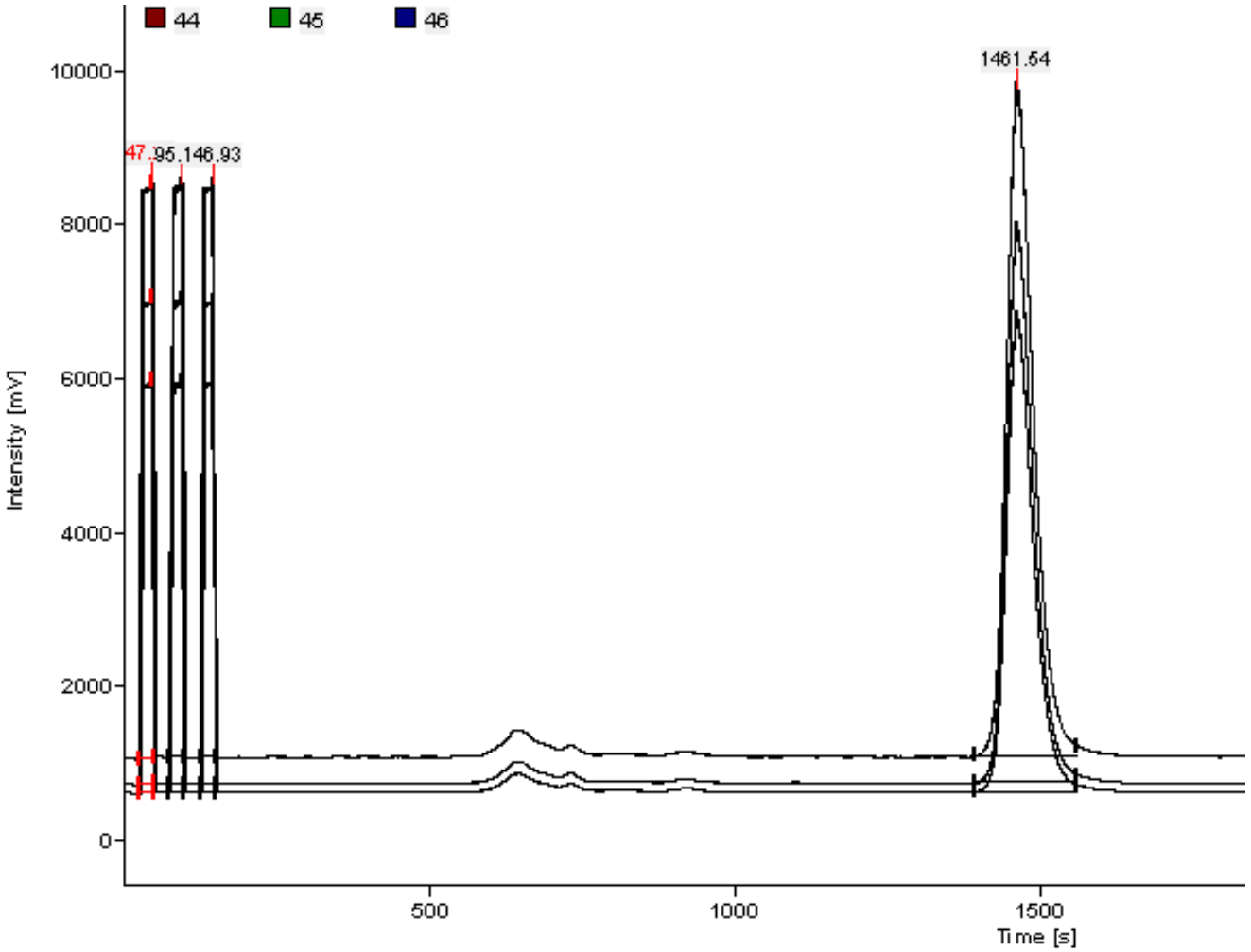
- Repeatability **r: 0,60 ‰**
- Reproducibility **R: 0,62 ‰**

11. ANNEX

11.1 Typical chromatogram of a GC-C-IRMS analysis of glycerol in wine



11.2 Typical chromatogram of a HPLC-IRMS analysis of glycerol



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Method OIV-MA-AS313-01 Type I method

Total acidity
(OIV-OENO 551-2015)

1. Definition

The total acidity of the wine is the sum of its titratable acidities when it is titrated to pH 7 against a standard alkaline solution. Carbon dioxide is not included in the total acidity.

2. Principle

Potentiometric titration or titration with bromothymol blue as indicator and comparison with an end-point color standard.

3. Apparatus

3.1 *Water vacuum pump*.

3.2 *Vacuum flask, 500 mL*.

3.3 *Potentiometer* with scale graduated in pH values, and electrodes. The glass electrode must be kept in distilled water. The calomel/saturated potassium chloride electrode must be kept in a saturated potassium chloride solution.

3.4 *Beakers of 12 cm diameter*.

4. Reagents

4.1 *Buffer solution pH 7.0*:

potassium *di*-hydrogen phosphate, KH_2PO_4 ..... 107.3 g
sodium hydroxide solution, NaOH, 1 mol/L ..... 500 mL
water to ..... 1000 mL

Alternatively, ready-made buffer solutions are available commercially.

4.2 Sodium hydroxide solution, NaOH, 0.1 mol/L.

4.3 Bromothymol blue indicator solution, 4 g/L.

bromothymol blue ..... 4 g
neutral ethanol, 96% (*v/v*) ..... 200 mL

Dissolve and add:

water free of CO_2 ..... 200 mL

sodium hydroxide solution, 1 mol/L, sufficient to produce

blue green color (pH 7) ..... ~ 7.5 mL

water to ..... 1000 mL

5. Procedure

5.1 *Preparation of sample:* elimination of carbon dioxide.

Place approximately 50 mL of wine in a vacuum flask; apply vacuum to the flask using a water pump for one to two min, while shaking continuously.

5.2 *Potentiometric titration*

5.2.1 Calibration of pH meter

The pH meter is calibrated for use at 20°C, according to the manufacturer's instructions, with the pH 7 buffer solution at 20°C.

5.2.2 Method of measurement

Into a beaker, introduce a volume of the sample, prepared as described in 5.1, equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add about 10 mL of distilled water and then add sodium hydroxide solution, 0.1 mol/L, from a burette until the pH is equal to 7 at 20°C. The sodium hydroxide must be added slowly and the solution stirred continuously. Let *n* mL be the volume of sodium hydroxide, 0.1 mol/L, added.

5.3 *Titration with indicator (bromothymol blue)*

5.3.1 Preliminary test: end-point color determination.

Into a beaker (3.4) place 25 mL of boiled distilled water, 1 mL of bromothymol blue solution and a volume prepared as in 5.1 equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the color changes to blue-green. Then add 5 mL of the pH 7 buffer solution.

5.3.2 Measurement

Into a beaker (3.4) place 30 mL of boiled distilled water, 1 mL of bromothymol blue solution and a volume of the sample, prepared as described in 5.1, equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the same color is obtained as in the preliminary test above (5.3.1). Let *n* mL be the volume of sodium hydroxide solution, 0.1 mol/L, added.

6. Expression of results

6.1 *Method of calculation*

- The total acidity expressed in milliequivalents per liter is given by:

$$A = 10\,n.$$

It is recorded to one decimal place.

- The total acidity expressed in grams of tartaric acid per liter is given by:

$$A' = 0.075 \times A$$

The result is quoted to two decimal places.

- The total acidity expressed in grams of sulfuric acid per liter is given by:

$$A' = 0.049 \times A$$

The result is quoted to two decimal places.

6.2 *Repeatability (r)* for titration with the indicator:(5.3):

r = 0.9 meq/L
r = 0.04 g sulfuric acid/L
r = 0.07 g tartaric acid/L

6.3 *Reproducibility (*R*)* for titration with the indicator (5.3):

For white and rosé wines:

R = 3.6 meq/L
R = 0.2 g sulfuric acid/L
R = 0.3 g tartaric acid/L

For red wines:

R = 5.1 meq/L
R = 0.3 g sulfuric acid/L
R = 0.4 g tartaric acid/L

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Method OIV-MA-AS313-02 Type I method

Volatile Acidity
(OIV-OENO 549-2015)

1. Definition

The volatile acidity is derived from the acids of the acetic series present in wine in the free state and combined as salts.

2. Principle

Carbon dioxide is first removed from the wine. Volatile acids are separated from the wine by steam distillation and titrated using standard sodium hydroxide.

The acidity of free and combined sulfur dioxide distilled under these conditions should be subtracted from the acidity of the distillate.

The acidity of any sorbic acid, which may have been added to the wine, must also be subtracted.

Note: Part of the salicylic acid used in some countries to stabilize the wines before analysis is present in the distillate. This must be determined and subtracted from the acidity. The method of determination is given in the Annex of this Chapter.

3 . Apparatus

3.1 Steam distillation apparatus consisting of:

- a steam generator; the steam must be free of carbon dioxide;
- a flask with steam pipe;
- a distillation column;
- a condenser.

This equipment must pass the following three tests:

(a) Place 20 mL of boiled water in the flask. Collect 250 mL of the distillate and add to it 0.1 mL sodium hydroxide solution, 0.1 M, and two drops of phenolphthalein solution. The pink coloration must be stable for at least 10 sec (i.e. steam to be free of carbon dioxide).

(b) Place 20 mL acetic acid solution, 0.1 M, in the flask. Collect 250 mL of the distillate. Titrate with the sodium hydroxide solution, 0.1 M: the volume of the titer must be at least 19.9 mL (i.e. at least 99.5% of the acetic acid entrained with the steam).

(c) Place 20 mL lactic acid solution, 1 M, in the flask. Collect 250 mL of the distillate and titrate the acid with the sodium hydroxide solution, 0.1 M.
The volume of sodium hydroxide solution added must be less than or equal to 1.0 mL (i.e. not more than 0.5% of lactic acid is distilled).

Any apparatus or procedure which passes these tests satisfactorily fulfils the requirements of official international apparatus or procedures.

3.2 Water aspirator vacuum pump.

3.3 Vacuum flask.

4. Reagents

4.1 Tartaric acid, crystalline.

4.2 Sodium hydroxide solution, 0.1 M.

4.3 Phenolphthalein solution, 1%, in neutral alcohol, 96% (*m/v*).

4.4 Hydrochloric acid (ρ_{20} = 1.18 to 1.19 g/mL) diluted 1/4 with distilled water.

4.5 Iodine solution, 0.005 M.

4.6 Potassium iodide, crystalline.

4.7 Starch solution, 5 g/L.

Mix 5 g of starch with about 500 mL of water. Bring to the boil, stirring continuously and boil for 10 min. Add 200 g sodium chloride. When cool, make up to one liter.

4.8 Saturated solution of sodium tetraborate, $Na_2B_4O_7.10H_2O$, about 55 g/L at 20°C.

4.9 Acetic acid, 0.1 M.

4.10 Lactic acid solution, 0.1 M

100 mL of lactic acid is diluted in 400 mL of water. This solution is heated in an evaporating dish over a boiling water bath for four hours, topping up the volume occasionally with distilled water. After cooling, make up to a liter. Titrate the lactic acid in 10 mL of this solution with 1 M sodium hydroxide solution. Adjust the solution to 1 M lactic acid (90 g/L).

5. Procedure

5.1 *Preparation of sample*: elimination of carbon dioxide. Place about 50 mL of wine in a vacuum flask; apply vacuum to the flask with the water pump for one to two min while shaking continuously. Other CO_2 elimination systems may be used if the CO_2 elimination is guaranteed.

5.2 *Steam distillation*

Place 20 mL of wine, freed from carbon dioxide as in 5.1, into the flask. Add about 0.5 g of tartaric acid. Collect at least 250 mL of the distillate.

5.3 *Titration*

Titrate with the sodium hydroxide solution, (4.2), using two drops of phenolphthalein (4.3) as indicator. Let *n* mL be the volume of sodium hydroxide used.

Add four drops of the dilute hydrochloric acid (4.4), 2 mL starch solution (4.7) and a few crystals of potassium iodide (4.6). Titrate the free sulfur dioxide with the iodine solution, 0.005 M (4.5). Let n' mL be the volume used.

Add the saturated sodium tetraborate solution (4.8) until the pink coloration reappears. Titrate the combined sulfur dioxide with the iodine solution, 0.005 M (4.5). Let n'' mL be the volume used.

6. Expression of results

6.1 *Method of calculation*

The volatile acidity, expressed in milliequivalents per liter to one decimal place, is given by:

$$5\ (n - 0.1\ n' - 0.05\ n'').$$

The volatile acidity, expressed in grams of sulfuric acid per liter to two decimal places, is given by:

$$0.245\ (n - 0.1\ n' - 0.05\ n'').$$

The volatile acidity, expressed in grams of acetic acid per liter to two decimal places, is given by:

$$0.300\ (n - 0.1\ n' - 0.05\ n'').$$

6.2 *Repeatability (r)* $r = 0.7$ meq/L
$r = 0.03$ g sulfuric acid/L
$r = 0.04$ g acetic acid/L.

6.3 *Reproducibility* (R) R = 1.3 meq/L
R = 0.06 g sulfuric acid/L
R = 0.08 g acetic acid/L.

6.4 *Wine with sorbic acid present*

Since 96% of sorbic acid is steam distilled with a distillate volume of 250 mL, its acidity must be subtracted from the volatile acidity, knowing that 100 mg of sorbic acid corresponds to an acidity of 0.89 milliequivalents or 0.053 g of acetic acid and knowing the concentration of sorbic acid in mg/L as determined by other methods.

ANNEX

Determination of Salicylic Acid entrained in the distillate from the volatile acidity

1. *Principle*

After the determination of the volatile acidity and the correction for sulfur dioxide, the presence of salicylic acid is indicated, after acidification, by the violet coloration that appears when an iron (III) salt is added.

The determination of the salicylic acid entrained in the distillate with the volatile acidity is carried out on a second distillate having the same volume as that on which the determination of volatile acidity was carried out. In this distillate, the salicylic acid is determined by a comparative colorimetric method. It is subtracted from the acidity of the volatile acidity distillate.

2 *Reagents*

- Hydrochloric acid, HCl, (ρ_{20} = 1.18 to 1.19 g/L).
- Sodium thiosulfate solution, $Na_2S_2O_3.5H_2O$, 0.1 M.
- Iron (III) ammonium sulfate solution, $Fe_2(SO_4)_3(NH_4)_2SO_4.24H_2O$, 10% (*m/v*)
- Sodium salicylate solution, 0.01 M: containing 1.60 g/L sodium salicylate, $NaC_7H_5O_3$.

3. *Procedure*

3.1 *Identification of salicylic acid in the volatile acidity distillate*

Immediately after the determination of the volatile acidity and the correction for free and combined sulfur dioxide, introduce into a conical flask 0.5 mL hydrochloric acid, 3 mL of the sodium thiosulfate solution, 0.1 M, and 1 mL of the iron (III) ammonium sulfate solution. If salicylic acid is present, a violet coloration appears.

3.2 *Determination of salicylic acid*

On the above conical flask, indicate the volume of the distillate by a reference mark. Empty and rinse the flask. Subject a new test sample of 20 mL wine to steam distillation and collect the distillate in the conical flask up to the reference mark. Add 0.3 mL concentrated hydrochloric acid, and 1 mL of the iron (III) ammonium sulfate solution. The contents of the conical flask turn violet.

Into a conical flask identical to that carrying the reference mark, introduce distilled water up to the same level as that of the distillate. Add 0.3 mL concentrated hydrochloric acid and 1 mL of the iron (III) ammonium sulfate solution. From the burette run in the sodium salicylate solution, 0.01 M, until the violet coloration obtained has the same intensity as that of the conical flask containing the wine distillate.

Let n''' mL be the volume of solution added from the burette.

4. *Correction to the volatile acidity*

Subtract the volume 0.1 x n'''' mL from the volume n mL of sodium hydroxide solution, 0.1 M, used to titrate the acidity of the distillate during the determination of volatile acidity.

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Method OIV-MA-AS313-03 Type I method

Fixed acidity

(A11, revised by OIV/OENO 377/2009)

1. Principle

The fixed acidity is calculated from the difference between total acidity and volatile acidity.

2. Expression of results

The fixed acidity is expressed in:

— milliequivalents per liter.
— grams of sulfuric acid per liter.
— grams of tartaric acid per liter.

Method OIV-MA-AS313-04 Type IV method

Organic acids

(Recueil OIV ed. 1990, revised by OIV/OENO 377/2009)

Wine organic acids may be separated and simultaneously determined by *high performance liquid chromatography* (HPLC).

1. Principle of method

Wine organic acids may be separated using two stationary phases: octyl-bonded silica and ion exchange resin columns. The acids are detected by spectrophotometric absorbance in ultraviolet.

For the determination of malic and tartaric acids, it is advisable to use an octyl-bonded silica column and for citric and lactic acids, an ion exchange resin column. The determination of these acids is performed with reference to an external standard analyzed under similar conditions.

This method is also able to give an evaluation of contents of shikimic, acetic, succinic and fumaric acids.

Note: other types of columns may also give a good separation. The columns and operating conditions given below are given as examples.

2. Apparatus

2.1. *Cellulose membrane filtration apparatus* (*diameter of pores: 0.45 µm*)

2.2. *Octadecyl-bonded silica fitted cartridges* (*e.g. Sep Pak - Waters Assoc.*)

2.3. *High Performance Liquid Chromatograph equipped with:*

— a 10 µL loop injector,
— a temperature control apparatus,
— spectrophotometer detector capable of making absorbance measurements at 210 nm,
— a chart recorder, or integrator.

Operating conditions

2.3.1 In the case of citric, lactic and acetic acid separation:

— a column containing a strong cation (H^+) exchange resin (300 mm length, 7.8 mm internal diameter, 9 µm particle size) (e.g. HPX-87 H BIO-RAD),
— mobile phase: sulfuric acid solution, 0.0125 mol/L,
— flow rate: 0.6 mL/min,
— temperature: 60 - 65°C. (Depending on the type of resin).

2.3.2 In the case of fumaric, succinic, shikimic, lactic, malic and tartaric acid separation.

— two columns (250 mm length, 4 mm internal diameter) placed in series, fitted with octyl-bonded silica, spherical particles of 5 µm diameter,
— mobile phase: potassium *di*-hydrogen phosphate solution, 70 g/L, ammonium sulfate, 14 g/L, and adjusted to pH 2.1 by adding phosphoric acid,
— flow rate: 0.8 mL/min,
— temperature: 20°C.

3. Reagents

3.1. Distilled water of HPLC quality

3.2. Distilled methanol

3.3. Tartaric acid

3.4. Malic acid

3.5. Sodium lactate

3.6. Shikimic acid

3.7. Sodium acetate

3.8. Succinic acid

3.9. Citric acid

3.10. Fumaric acid

3.11. Sulfuric acid (ρ_{20} = 1.84 g/mL)

3.12. Sulfuric acid solution, 0.0125 mol/L

3.13. Potassium *di*-hydrogen *ortho*-phosphate, KH_2PO_4

3.14. Ammonium sulfate, $(NH_4)_2SO_4$

3.15. O*rtho*-phosphoric acid, 85% (ρ_{20} = 1.71 g/mL)

3.16. Reference solution made of: tartaric acid, 5 g/L, malic acid, 5 g/L, sodium lactate, 6.22 g/L, shikimic acid, 0.05 g/L, sodium acetate, 6.83 g/L, succinic acid, 5 g/L, fumaric acid, 0.01 g/L and citric acid, 5 g/L.

4. Procedure

4.1. *Preparation of sample*

First wash cartridge (2.2) with 10 mL methanol (3.2) then with 10 mL water (3.1).

Remove gas from wine or must sample. Filter through membrane (0.45 µm) (2.1). Put 8 mL of filtered sample into a syringe already rinsed with the sample; pass through the cartridge. Disregard the first 3 mL and collect the following 5 mL (prevent the cartridge from becoming dry).

4.2. *Chromatography*

Inject successively into the chromatograph 10 µL reference solution and 10 µL sample solution prepared according to 4.1. Repeat these injections three times in the same order.

5. Calculation

5.1. *Qualitative analysis*

Determine the respective times of retention for each of the eluates.

The organic acids of the reference solution are divided in order of elution as follows:

— citric, tartaric, malic, succinic + shikimic, lactic, fumaric and acetic acids in the technique 2.3.1.

— tartaric, malic, shikimic, lactic, acetic, citric, succinic and fumaric acids in the technique 2.3.2.

5.2. *Quantitative analysis*

Measure the area of each of the peaks and determine the average of the three answers for the reference and sample solutions to be analyzed. Deduct the sample concentration from the organic acids.

6. Expression of results

The concentrations are expressed as follows:

— grams per liter to one decimal place for the tartaric, malic, lactic and succinic acids

— milligrams per liter for the citric, acetic and fumaric acids.

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Method OIV-MA-AS313-05A Type IV method

Tartaric acid

(A12 revised by OIV/OENO 377/2009)

1. Principle

Tartaric acid is precipitated in the form of calcium (±)tartrate and determined gravimetrically. This determination may be completed using a volumetric procedure for comparison. The conditions for precipitation (pH, total volume used, concentrations of precipitating ions) are such that precipitation of the calcium (±)tartrate is complete whereas the calcium D(–) tartrate remains in solution.

When *meta*-tartaric acid has been added to the wine, which causes the precipitation of the calcium (±)tartrate to be incomplete, it must first be hydrolyzed.

2. Method

2.1. *Gravimetric method*

2.1.1. Reagents

- Calcium acetate solution containing 10 g of calcium per liter:

Calcium carbonate, $CaCO_3$	25 g
Acetic acid, glacial, CH_3COOH (ρ_{20}= 1.05 g/mL)	40 mL
Water to	1000 mL

- Calcium (±)tartrate, crystallized: $CaC_4O_6H_4 \cdot 4H_2O$.

Place 20 mL of L(+) tartaric acid solution, 5 g/L, into a 400 mL beaker.

Add 20 mL of ammonium D(–) tartrate solution, 6.126 g/L, and 6 mL of calcium acetate solution containing 10 g of calcium per liter.

Allow to stand for two hours to precipitate. Collect the precipitate in a sintered glass crucible of porosity No 4, and wash it three times with about 30 mL of distilled water. Dry to constant weight in the oven at 70°C. Using the quantities of reagent indicated above, about 340 mg of crystallized calcium (±) tartrate is obtained. Store in a stoppered bottle.

- Precipitation solution (pH 4.75):

D(–) ammonium tartrate	150 mg
Calcium acetate solution, 10 g calcium/L	8.8 mL
Water to	1000 mL

Dissolve the D(-) ammonium tartrate in 900 mL water; add 8.8 mL calcium acetate solution and make up to 1000 mL. Since calcium (±)tartrate is slightly soluble in this solution, add 5 mg of calcium (±)tartrate per liter, stir for 12 hours and filter.

Note: The precipitation solution may also be prepared from D(-) tartaric acid.

D(–) tartaric acid ..... 122 mg
Ammonium hydroxide solution (ρ_{20}= 0.97 g/mL), 25 % (*v/v*) ..... 0.3 mL

Dissolve the D(–) tartaric acid, add the ammonium hydroxide solution and make up to about 900 mL; add 8.8 mL of calcium acetate solution, make up to a liter and adjust the pH to 4.75 with acetic acid. Since calcium (±)tartrate is slightly soluble in this solution, add 5 mg of calcium (±)tartrate per liter, stir for 12 hours and filter.

2.1.2. Procedure

— Wines with no added *meta*-tartaric acid

Place 500 mL of precipitation solution and 10 mL of wine into a 600 mL beaker. Mix and initiate precipitation by rubbing the sides of the vessel with the tip of a glass rod. Leave to precipitate for 12 hours (overnight).

Filter the liquid and precipitate through a weighed sintered glass crucible of porosity No. 4 fitted on a clean vacuum flask. Rinse the vessel in which precipitation took place with the filtrate to ensure that all precipitate is transferred.

Dry to constant weight in an oven at 70°C. Weigh. Let *p* be the weight of crystallized calcium (±)tartrate, $CaC_4O_6H_4 \cdot 4H_2O$, obtained.

— Wines to which *meta*-tartaric acid has been added.

When analyzing wines to which *meta*-tartaric acid has been or is suspected of having been added, proceed by first hydrolyzing this acid as follows:

Place 10 mL of wine and 0.4 mL of glacial acetic acid, CH_3COOH, (ρ_{20}= 1.05 g/mL) into a 50 mL conical flask. Place a reflux condenser on top of the flask and boil for 30 min. Allow to cool and then transfer the solution in the conical flask to a 600 mL beaker. Rinse the flask twice using 5 mL of water each time and then continue as described above.

Meta-Tartaric acid is calculated and included as tartaric acid in the final result.

2.1.3. Expression of results

One molecule of calcium (±)tartrate corresponds to half a molecule of L(+) tartaric acid in the wine.

- The quantity of tartaric acid per liter of wine, expressed in milliequivalents, is equal to:

384.5 *p*.

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of tartaric acid, is equal to:

28.84 *p*.

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of potassium tartrate, is equal to:

36.15 *p*.

It is quoted to one decimal place.

2.2. *Comparative volumetric analysis*

2.2.1. Reagents

- Hydrochloric acid (ρ_{20} = 1.18 to 1.19 g/mL) diluted 1:5 with distilled water
- EDTA solution, 0.05 M:

EDTA (ethylenediaminetetraacetic acid disodium salt).....	18.61 g
Water to	1000 mL

- Sodium hydroxide solution, 40% (*m/v*):

Sodium hydroxide, NaOH	40 g
Water to	100 mL

- Complexometric indicator: 1% (*m/m*)

2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)

-3-naphthoic acid	1 g
Sodium sulfate, Na_2SO_4 (anhydrous)	100 g

2.2.2. Procedure

After weighing, replace the sintered glass crucible containing the precipitate of calcium (±)tartrate on the vacuum flask and dissolve the precipitate with 10 mL of dilute hydrochloric acid. Wash the sintered glass crucible with 50 mL of distilled water.

Add 5 mL 40% sodium hydroxide solution and about 30 mg of indicator. Titrate with EDTA solution, 0.05 M. Let the number of mL used be *n*.

2.2.3. Expression of results

- The quantity of tartaric acid per liter of wine, expressed in milliequivalents, is equal to:

5 *n*.

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of tartaric acid, is equal to:

$$0.375\ n.$$

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of potassium acid tartrate, is equal to:

$$0.470\ n.$$

It is quoted to one decimal place.

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Method OIV-MA-AS313-05B

Tartaric acid

1. Principle

The tartaric acid, separated using an ion exchange column, is determined colorimetrically in the eluate by measurement of the red color produced on reaction with vanadic acid. The eluate also contains lactic and malic acids that do not interfere.

WITHDRAWN
(resolution OIV/OENO 377/2009)

Method OIV-MA-AS313-06

Lactic acid

1. Principle

The lactic acid, separated by passage through an ion exchange resin column, is oxidized to acetaldehyde (ethanol) and determined by colorimetry after reacting with sodium nitroprusside and piperidine.

WITHDRAWN
(Resolution OIV/OENO 377/2009)

Method OIV-MA-AS313-07 Type II method

Lactic acid

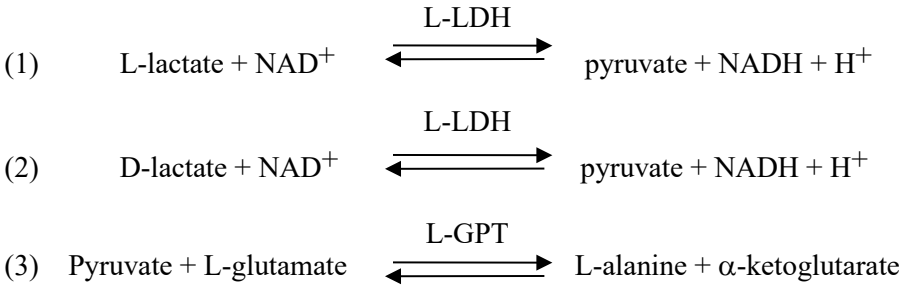
Enzymatic method
(Recueil OIV ed. 1990; revised by OIV/OENO 377/2009)

1. Principle

Total lactic acid (L-lactate and D-lactate) is oxidized by nicotinamide adenine dinucleotide (NAD) to pyruvate in a reaction catalyzed by L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH).

The equilibrium of the reaction normally lies more strongly in favor of the lactate. Removal of the pyruvate from the reaction mixture displaces the equilibrium towards the formation of pyruvate.

In the presence of L-glutamate, the pyruvate is transformed into L-alanine in a reaction catalyzed by glutamate pyruvate transaminase (GPT):



The amount of NADH formed, measured by the increase in absorbance at the wavelength of 340 nm, is proportional to the quantity of lactate originally present.

Note: L-lactic acid may be determined independently by using reactions (1) and (3), while D-lactic acid may be similarly determined by using reactions (2) and (3).

2. Apparatus

2.1. A spectrophotometer permitting measurements to be made at 340 nm, the wavelength at which the absorbance of NADH is a maximum.
Failing that, a spectrophotometer with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm may be used.

2.2. Glass cells with optical path lengths of 1 cm or single-use-cells.

2.3. Micropipettes for pipetting sample volumes in the range 0.02 to 2 mL.

3. Reagents

Double-distilled water

3.1. Buffer solution, pH 10 (glycylglycine, 0.6 M; L-glutamate, 0.1 M):

Dissolve 4.75 g of glycylglycine and 0.88 g of L-glutamic acid in approximately 50 mL of double distilled water; adjust the pH to 10 with a few milliliters sodium hydroxide, 10 M, and make up to 60 mL with double distilled water.
This solution will remain stable for at least 12 weeks at 4°C.

3.2. Nicotinamide adenine dinucleotide (NAD) solution, approximately 40 x 10^{-3} M: dissolve 900 mg of NAD in 30 mL of double distilled water. This solution will remain stable for at least four weeks at 4°C.

3.3. Glutamate pyruvate transaminase (GPT) suspension, 20 mg/mL.
The suspension remains stable for at least a year at 4°C.

3.4. L-lactate dehydrogenase (L-LDH) suspension, 5 mg/mL.
This suspension remains stable for at least a year at 4°C.

3.5. D-lactate dehydrogenase (D-LDH) suspension, 5 mg/mL.
This suspension remains stable for at least a year at 4°C.
It is recommended that, prior to the determination, the enzyme activity should be checked.

Note: All the reagents are available commercially.

4. Preparation of the sample

Lactate determination is normally carried out directly on the wine, without prior removal of pigmentation (coloration) and without dilution provided that the lactic acid concentration is less than 100 mg/L. However, if the lactic acid concentration lies between:

100 mg/L and 1 g/L, dilute 1/10 with double distilled water, 1 g/L and 2.5 g/L, dilute 1/25 with double distilled water, 2.5 g/L and 5 g/L, dilute 1/50 with double distilled water.

5. Procedure

Preliminary note:

No part of the glassware that comes into contact with the reaction mixture should be touched with the fingers, since this could introduce L-lactic acid and thus give erroneous results.

The buffer solution must be at a temperature between 20 and 25°C before proceeding to the measurement.

5.1. *Determination of total lactic acid*

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using 1 cm cells, with air as the zero absorbance (reference) standard; (no cell in the optical path) or with water as the standard.
Place the following in the 1 cm cells:

	Reference cell (mL)	Sample cell (mL)
Solution 3.1.	1.00	1.00
Solution 3.2.	0.20	0.20
Double distilled water	1.00	0.80

Suspension 3.3.	0.02	0.02
Sample to be measured	—	0.20

Mix using a glass stirrer or a rod of synthetic material with a flattened end; after about five min, measure the absorbencies of the solutions in the reference and sample cells (A_1).

Add 0.02 mL of solution 3.4 and 0.05 mL of solution 3.5, homogenize, wait for the reaction to be completed (about 30 min) and measure the absorbencies of the solutions in the reference and sample cells (A_2).

Calculate the differences ($A_2 - A_1$) in the absorbencies of the solutions in the reference and sample cells, ΔA_S and ΔA_R.

Finally, calculate the difference between those differences:

$$\Delta A = \Delta A_S - \Delta A_R$$

5.2. *Determination of L-lactic acid and D-lactic acid*

Determination of the L-lactic acid or D-lactic acid can be carried out independently by applying the procedure for total lactic acid up to the determination of A_1 and then continuing as follows:

Add 0.02 mL of solution 3.4, homogenize, wait until the reaction is complete (about 20 min) and measure the absorbencies of the solutions in the reference and sample cells (A_2).

Add 0.05 mL of solution 3.5, homogenize, wait until the reaction is complete (about 30 min) and measure the absorbencies of the solutions in the reference and sample cells (A_3).

Calculate the differences ($A_2 - A_1$) for L-lactic acid and ($A_3 - A_2$) for D-lactic acid between the absorbencies of the solutions in the reference and sample cells, ΔA_S and ΔA_R.

Finally, calculate the difference between those differences:

$$\Delta A = A_S - \Delta A_R.$$

Note: The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch. When determining the L-lactic acid alone, the incubation time may be reduced to 10 min.

6. Expression of results

Lactic acid concentration is given in grams per liter (g/L) to one decimal place.

6.1. *Method of calculation*

The general formula for calculating the concentration in g/L is:

$$C = \frac{V \times M}{\varepsilon \times \delta \times \nu \times 1000} \times \Delta A$$

where

V = volume of test solution in mL (V = 2.24 mL for L-lactic acid, V = 2.29 mL for D-lactic acid and total lactic acid)

v = volume of the sample in mL (0.2 mL)
M = molecular mass of the substance to be determined (for DL-lactic acid, M = 90.08)
δ = optical path in the cell in cm (1 cm)
ε = absorption coefficient of NADH, at 340 nm

(ε = 6.3 $mmol^{-1}$ x l x cm^{-1}).

6.1.1 Total lactic acid and D-lactic acid

$$C = 0.164 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note:

- Measurement at 334 nm: $C = 0.167 \times \Delta A$, ($\varepsilon$ = 6.2 $mmol^{-1}$ x 1 x cm^{-1}).
- Measurement at 365 nm: $C = 0.303 \times \Delta A$, ($\varepsilon$ = 3.4 $mmol^{-1}$ x 1 x cm^{-1}).

6.1.2 L-lactic acid

$$C = 0.160 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note:

- Measurement at 334 nm: $C = 0.163 \times \Delta A$, ($\varepsilon$ = 6.2 $mmol^{-1}$ x 1 x cm^{-1}).
- Measurement at 365 nm: $C = 0.297 \times \Delta A$, ($\varepsilon$ = 3.4 $mmol^{-1}$ x 1 x cm^{-1}).

6.2 *Repeatability (r)*

$$r = 0.02 + 0.07\, x_i$$

x_i is the lactic acid concentration in the sample in g/L.

6.3. *Reproducibility (R)*

$$R = 0.05 + 0.125\, x_i$$

x_i is the lactic acid concentration in the sample in g/L.

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Method OIV-MA-AS313-08 Type IV method

Citric acid

Chemical method

1. Principle

Citric acid is fixed with other wine acids onto an anion exchange column. The citramalic acid is obtained by fractionating the elute.

The citric acid is oxidized to acetone, which is separated by distillation. The acetaldehyde (ethanol) is oxidized to acetic acid and acetone is determined by iodometry.

2. Apparatus

2.1. *Anion exchange column*

In a 25 mL burette with tap, place a glass wool plug and pour 20 mL of Dowex resin 1 x 2.

Initially the resin goes through two complete regeneration cycles with alternate passages of hydrochloric acid solution, 1 M, and sodium hydroxide solution, 1 M. Rinse with 50 mL distilled water [(1)]. Saturate the resin with acetate ions by adding 250 mL acetic acid solution, 4 M. Wash with 100 mL distilled water.

[(1)] The passage of the sodium hydroxide causes a contraction that, followed by a swelling during washings, stops the flow. It is recommended to stir the resin as soon as the first few mL of water pass through the column to stop the resin from sticking to the bottom of the burette.

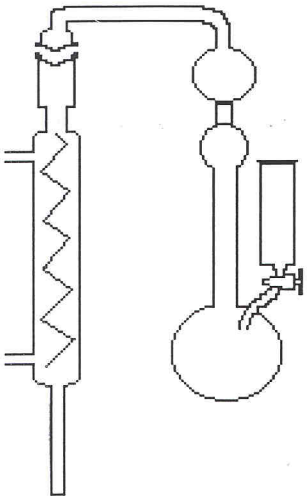


Fig: 1 The oxidation and distillation

The sample is passed through a column conforming to the description below. After the elution of the acids, rinse with 50 mL of distilled water and proceed once more to saturate the resin with acetic acid solution, 4 M. Rinse with 100 mL water. The resin is then ready for re-use.

2.2 *Oxidation apparatus*

The use of a distillation apparatus with oxidation round bottom flask, see drawing Fig. 1 facilitates the introduction of potassium permanganate, with a very regular flow.

If unavailable, use a 500 mL round bottom flask and a funnel fitted with a tap and a tapered end, to ensure that there is as regular flow of potassium permanganate as possible.

3. Reagents

- Dowex resin 1 x 2 (50 - 100 mesh)
- Acetic acid solution, 4 M
- Acetic acid solution, 2.5 M
- Sodium hydroxide solution, 2 M
- Sulfuric acid (r_{20} = 1.84 g/mL) diluted $^1/_5$ (*v*/*v*)
- Buffer solution of pH 3.2 - 3.4

Potassium *di*-hydrogen phosphate KH_2PO_4 ..... 150 g
Concentrated phosphoric acid (p_{20} = 1.70 g/mL) ..... 5 mL
Water to: ..... 1000 mL

- Manganese sulfate solution, $MnSO_4.H_2O$, 50 g/L
- Pumice stone
- Potassium permanganate solution, 0.01 M
- Sulfuric acid (r_{20} = 1.84 g/mL) diluted $^1/_3$ (*v*/*v*)
- Potassium permanganate solution, 0.4 M
- Iron (II) sulfate, $FeSO_4.7H_2O$, 40% (*m*/*v*)
- Sodium hydroxide solution, 5 M
- Iodine solution, 0.01 M
- Sodium thiosulfate solution, 0.02 M
- Thiodene or starch

4. Method

4.1 *Separation of citramalic and citric acids*

Pass 25 mL wine through the ion exchange Dowex 1 x 2 resin column (in an acetate form) at a flow rate of 3 mL every 2 minutes. Rinse the column three times with 20 mL distilled water. Elute the acids with 200 mL acetic acid solution, 2.5 M, at the same flow rate. This eluate fraction contains succinic, lactic, galacturonic, citramalic acids and nearly all of the malic acid.

Proceed with the elution of citric and tartaric acids by passing 100 mL sodium hydroxide solution, 2 M, through the column. Collect the eluate in the oxidation flask.

4.2. *Oxidation*

In the flask containing this second eluate, add sulfuric acid diluted 1/5 (about 20 mL) to bring the pH to between 3.2 and 3.8. Add 25 mL of pH 3.2-3.4 buffer solution, 1 mL of manganese sulfate solution and few grains of pumice stone.

Bring to the boil and distil over 50 mL, which is discarded.

Put the potassium permanganate solution, 0.01 M, in the funnel and introduce at 1 drop per second into the boiling eluate. The distillate is collected in a 500 mL ground glass stoppered flask containing few millimeters of water. The oxidation is carried on until a brown coloration of the liquid appears indicating an excess of permanganate.

4.3. *Separation of the acetone*

If the volume of the distillate is less than 90 mL, make up with distilled water, add 4.5 mL of sulfuric acid diluted $^1/_3$, and 5 mL potassium permanganate solution, 4.4 M. If the collected distillate largely exceeds this volume, complete to 180 mL and double the amounts of the reagents.

Under those conditions (i.e. sulfuric acid, 0.25 M, and potassium permanganate, 0.02 M), acetaldehyde (ethan0l) is oxidized into acetic acid while acetone remains intact.

The stoppered flask is left to rest for 45 minutes at room temperature. After which the excess of permanganate is destroyed by addition of iron (II) sulfate solution.

Distillate and collect about 50 mL of distillate in a ground glass stoppered flask containing 5 mL sodium hydroxide solution, 5 M.

4.4. *Determination of acetone*

Add 25 mL iodine solution, 0.01 M, to the flask [*]. Leave for 20 minutes. Add 8 mL of sulfuric acid diluted 1/5. Titrate the excess of iodine by sodium thiosulfate, 0.02 M, in the presence of thiodene or starch, *n* mL.

[*] This amount is suitable for citric acid contents not exceeding 0.5 to 0.6 g/L. For higher contents the volume of the iodine solution prescribed is not sufficient and the solution does not take a yellow color which is typical of an iodine excess. In this case double or triple the quantity of iodine until the solution becomes really yellow. However, in exceptional cases where the amount of citric acid in wine exceeds 1.5 g/L, it is recommended to restart the analysis on 10 mL of wine.

Under the same conditions make a blank determination replacing 50 mL of distillate by 50 mL of distilled water.

n' mL of thiosulfate used.

5. Calculations

1 mL iodine, 0.01 M, corresponds to 0.64 mg of citric acid.

Under the same given conditions, the quantity of citric acid in milligrams per liter corresponds to:

$$(n' - n) \times 25.6$$

6. Expression of results

The concentration of citric acid is expressed in milligrams per liter.

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Method OIV-MA-AS313-09 Type II method

Citric acid

Enzymatic method
(Recueil OIV ed. 1990, revised by OIV/OENO 377/2009

1. Principle

Citric acid is converted into oxaloacetate and acetate in a reaction catalyzed by citratelyase (CL):

$$\text{Citrate} \xrightleftharpoons{\text{CL}} \text{oxaloacetate} + \text{acetate}$$

In the presence of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), the oxaloacetate and its decarboxylation derivative, pyruvate, are reduced to L-malate and L-lactate by reduced nicotinamide adenine dinucelotide (NADH):

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

The amount of NADH oxidized to NAD+ in these reactions is proportional to the amount of citrate present. The oxidation of NADH is measured by the resultant decrease in absorbance at a wavelength of 340 nm.

2. Apparatus

2.1 A spectrophotometer permitting measurement to be made at 340 nm, the wavelength at which absorbance of NADH is a maximum.
Alternatively, a spectrophotometer, with a discontinuous spectrum source permitting measurements to be made at 334 nm or 365 nm, may be used.
Since absolute absorbance measurements are involved (i.e. calibration curves are not used but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

2.2 Glass cells with optical path lengths of 1 cm or single-use cells.

2.3 Micropipettes for pipetting volumes in the range 0.02 to 2 mL.

3. Reagents

3.1 Buffer solution pH 7.8 (glycylglycine, 0.51 M; pH 7.8; Zn^{+}(0.6 x 10^{-3} M): dissolve 7.13 g of glycylglycine in approximately 70 mL of double distilled water. Adjust the pH to 7.8 with approximately 13 mL sodium hydroxide solution, 5 M, add 10 mL of zinc chloride, $ZnCl_2$, (80 mg in 100 mL double distilled water) solution and make up to 100 mL with double distilled water.

3.2 Reduced nicotinamide adenine dinucleotide, NADH, solution (approximately 6 x 10^{-3}M): dissolve 30 mg NADH and 60 mg $NaHCO_3$ in 6 mL of double distilled water.

3.3 Malate dehydrogenase/lactate dehydrogenase solution (MDH/LDH) (0.5 mg MDH/mL; 2.5 mg LDH/mL): mix together 0.1 mL MDH (5 mg MDH/mL), 0.4 mL ammonium sulfate solution, 3.2 M, and 0.5 mL LDH (5 mg/mL).
This suspension remains stable for at least a year at 4°C.

3.4 Citrate-lyase (CL, 5 mg protein/mL): dissolve 168 mg lyophilisate in 1 mL ice-cold water. This solution remains stable for at least a week at 4°C and for at least four weeks if frozen.
It is recommended that, prior to the determination, the enzyme activity should be checked.

3.5 Polyvinylpolypyrrolidone (PVPP).

Note: All the reagents above are available commercially.

4. Preparation of the sample

Citrate determination is normally carried out directly on wine, without preliminary removal of pigmentation (coloration) and without dilution, provided that the citric acid content is less than 400 mg/L. If not, dilute the wine until the citrate concentration lies between 20 and 400 mg/L (i.e. between 5 and 80 µg of citrate in the test sample).

With red wines that are rich in phenolic compounds, preliminary treatment with PVPP is recommended:

Form a suspension of about 0.2 g of PVPP in water and allow to stand for 15 min. Filter using a fluted filter.

Place 10 mL of wine in a 50 mL conical flask, add the moist PVPP removed from the filter with a spatula. Shake for two to three minutes. Filter.

5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using the 1 cm cells, with air as the zero absorbance (reference) standard (no cell in the optical path). Place the following in the 1 cm cells:

	Reference cell (mL)	Sample cell (mL)
Solution 3.1	1.00	1.00
Solution 3.2	0.10	0.10
Sample to be measured	-	0.20
Double distilled water	2.00	1.80
Solution 3.3	0.02	0.02

Mix, and after about five min read the absorbance of the solutions in the reference and sample cells (A_1).

Add:

Solution 3.4	0.02 mL	0.02 mL

Mix; wait until the reaction is completed (about five min) and read the absorbance of the solutions in the reference and sample cells (A_2).

Calculate the absorbance difference (A_1- A_2) for the reference and sample cells, ΔA_S and ΔA_R.

Finally, calculate the difference between those differences:

$$\Delta A = \Delta A_S - \Delta A_R.$$

Note: The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

6. Expression of results

Citric acid concentration is given in milligrams per liter to the nearest whole number.

6.1 *Method of calculation*

The general formula for calculating the concentration in mg/L is:

$$C=\frac{VxM}{exdxv}x\Delta A$$

where:

V = volume of test solution in mL (3.14 mL)

v = volume of the sample in mL (0.2 mL)

M = molecular mass of the substance to be determined (for anhydrous citric acid, M = 192.1)

d = optical path in the cell in cm (1 cm)

ε = absorption coefficient of NADH, (at 340 nm, ε = 6.3 $mmol^{-1}$ x l x cm^{-1}).

so that:

$$C = 479 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note:

- At 334 nm: C = 488 x Δ*A* (ε= 6.3 $mmol^{-1}$ x l x cm^{-1}).
- At 365 nm: C = 887 x Δ*A* (ε= 3.4 $mmol^{-1}$ x l x cm^{-1}).

6.2 *Repeatability (r)*

Citric acid concentration less than 400 mg/L: *r* = 14 mg/L.

Citric acid concentration greater than 400 mg/L: *r* = 28 mg/L.

6.3 *Reproducibility (R)*

Citric acid concentration less than 400 mg/L: *R* = 39 mg/L.

Citric acid concentration greater than 400 mg/L: *R* = 65 mg/L.

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Method OIV-MA-AS313-10 Type IV method

Total Malic Acid

(A33 revised by OIV/OENO 377/2009)

1. Principle

Malic acid, separated by means of an anion exchange column, is determined colorimetrically in the eluent by measuring the yellow coloration it forms with chromotropic acid in the presence of concentrated sulfuric acid. A correction for interfering substances is made by subtracting the absorbance, obtained using 86% sulfuric and chromotropic acid respectively (malic acid does not react at these acid concentrations), from the absorbance obtained from using 96% strength acids.

2. Apparatus

2.1 Glass column 250 mm approximately in length and 35 mm internal diameter, fitted with drain tap.

2.2 Glass column approximately 300 mm in length and 10 to 11 mm internal diameter, fitted with drain tap.

2.3 Thermostatically controlled water bath at 100°C.

2.4 Spectrophotometer set to measure absorbance at 420 nm using cells of 1 cm optical path.

3. Reagents

3.1 A strongly basic anion exchanger (e.g. Merck III)

3.2 Sodium hydroxide, 5% (*m/v*).

3.3 Acetic acid, 30% (*m/v*).

3.4 Acetic acid, 0.5% (*m/v*).

3.5 Sodium sulfate solution, 10% (*m/v*).

3.6 Concentrated sulfuric acid, 95-97% (*m/m*).

3.7 Sulfuric acid, 86% (*m/m*).

3.8 Chromotropic acid, 5% (*m/v*).

Prepare fresh solution before each determination by dissolving 500 mg sodium chromotropate, $C_{10}H_6Na_2O_8S_2.2H_2O$, in 10 mL distilled water

3.9 0.5 g DL-malic acid per liter solution

Dissolve 250 g malic acid ($C_4H_6O_5$) in sodium sulfate solution, 10%, to obtain 500 mL.

4. Procedure

4.1 *Preparation of ion exchanger*

Place a plug of cotton impregnated with distilled water in a 35 x 250 mm glass column. Pour a suspension of the anion exchange resin into the glass column. The level of the liquid should be 50 mm above the top of the resin. Rinse with 1000 mL of distilled water. Wash the column with sodium hydroxide solution, 5%, allow to drain to approximately 2 to 3 mm of the top of the resin and repeat with two further washings of sodium hydroxide, 5%, and leave for one hour. Wash the column with 1000 mL of distilled water. Refill the column with acetic acid, 30%, allow to drain to approximately 2 to 3 mm above the top of the resin and repeat with two further washings of acetic acid, 30%. Leave for at least 24 hours before use. Keep the ion exchange resin in acetic acid, 30%, for the subsequent analysis.

4.2 *Preparation of ion exchange column.*

Place a plug of cotton wool at the bottom of the column measuring 11 x 300 mm above the tap. Pour in the ion exchanger prepared as described above in 4.1 to a height of 10 cm. Open the tap and allow the acetic acid solution, 30%, to drain to approximately 2 to 3 mm above the surface of the exchanger. Wash the exchanger with a 50 mL acetic acid solution, 0.5%.

4.3 *Separation of DL-Malic acid*

Pour onto the column (4.2) 10 mL of wine or must. Allow to drain drop by drop (average rate of one drop per second) and stop the flow 2 to 3 mm from the top of the resin. Wash the column with 50 mL acetic acid, 0.5% (*m/v*), then with 50 mL of distilled water and allow to drain at the same rate as previously, stopping the flow 2 to 3 mm from the top of the resin.

Elute the acids absorbed on the exchange resin with sodium sulfate solution, 10%, at the same rate as in the previous steps (1 drop/sec). Collect the eluate in a 100 mL volumetric flask. The ion exchange column can be regenerated using the procedure described in 4.1

4.4 *Determination of malic acid*

Take two wide necked 30 mL tubes fitted with ground glass stoppers, A and B. In each tube add 1.0 mL of the eluate and 1.0 mL chromotropic acid solution, 5%. Add to tube A 10.0 mL sulfuric acid, 86% (*m/m*), (reference) and to the tube B 10.0 mL sulfuric acid, 96% (*m/m*), (sample). Stopper and shake to homogenize carefully, without wetting the glass stopper. Immerse the tubes in a boiling water bath for exactly 10 min. Cool the tubes in darkness at 20 C for exactly 90 min. Immediately measure the absorbance of tube B relative to the sample tube A at 420 nm in 1 cm cells.

4.5 *Plotting the calibration curve*

Pipette 5, 10, 15 and 20 mL of the DL-malic acid solution (0.5g/L) into separate 50 mL volumetric flasks. Make up to the mark with sodium sulfate solution, 10%.

These solutions correspond to eluates obtained from wines containing 0.5, 1.0, 1.5 and 2.0 g DL-malic acid per liter.

Continue as indicated in 4.4. The graph of the absorbencies of these solutions verses their malic acid concentration should appear as a straight line passing through the origin.

The intensity of the coloration depends to a large extent on the strength of the sulfuric acid used. It is necessary to check the calibration curve to see if the concentration of the sulfuric acid has changed.

5. Expression of results

Plot the absorbance on calibration graph to obtain the content of DL-malic acid in grams per liter. This content is expressed with 1 decimal.

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Method OIV-MA-AS313-11 Type II method

L-Malic acid

(Recueil OIV ed. 1990 REVISED BY OIV/OENO 377/2009)

1. Principle of the method

L-malic acid (L-malate) is oxidized by nicotinamide adenine dinucleotide (NAD) to oxaloacetate in a reaction catalysed by L-malate dehydrogenase (L-MDH). The equilibrium of the reaction normally lies more strongly in favour of the malate. Removal of the oxaloacetate from the reaction mixture displaces the equilibrium towards the formation of oxaloacetate. In the presence of L-glutamate, the oxaloacetate is transformed into L-aspartate in a reaction catalysed by glutamate oxaloacetate transaminase (GOT):

(1) L-malate + NAD^+ $\overset{\text{L-MDH}}{\rightleftharpoons}$ oxaloacetate + NADH + H^+

(2) Oxaloacetate + L-glutamate $\overset{\text{GOT}}{\rightleftharpoons}$ L-aspartate + α-ketoglutarate

The amount of NADH formed, measured by the increase in absorbance at the wavelength of 340 nm, is proportional to the quantity of L-malate originally present.

2. Apparatus

2.1. A spectrophotometer permitting measurement to be made at 340 nm, the wavelength at which absorption by NADH is at a maximum. Failing that, a spectrophotometer, with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm, may be used.

Since absolute measurements of absorbance are involved (i.e. calibration curves are not used, but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

2.2. Glass cells with optical path lengths of 1 cm or single-use cells.

2.3. Micropipettes for pipetting sample volumes in the range 0,01 to 2 ml.

3. Reagents

Doubly distilled water

3.1. *Buffer solution, pH 10*

(glycylglycine 0,6 M; L-glutamate 0,1 M):
dissolve 4,75 g of glycylglycine and 0,88 g of L-glutamic acid in approximately 50 ml of doubly distilled water; adjust the pH to 10 with about 4,6 ml of 10 M

sodium hydroxide and make up to 60 ml with doubly distilled water. This solution will remain stable for at least 12 weeks at 4 °C.

3.2. Nicotinamide adenine dinucleotide (NAD) solution, approximately 47 × 10 3 M: dissolve 420 mg of NAD in 12 ml of doubly distilled water. This solution will remain stable for at least four weeks at 4 °C.

3.3. Glutamate oxaloacetate transaminase (GOT) suspension, 2 mg/ml. The suspension remains stable for at least a year at 4 °C.

3.4. L-malate dehydrogenase (L-MDH) solution, 5 mg/ml. This solution remains stable for at least a year at 4 °C.

Note: All the reagents above are available commercially.

4. Preparation of the sample

L-malate determination is normally carried out directly on the wine, without prior removal of pigmentation (colouration) and without dilution provided that the L–malic acid concentration is less than 350 mg/l (measured at 365 mg/l). If this is not so, dilute the wine with doubly distilled water until the L-malate concentration lies between 30 and 350 mg/l (i.e. amount of L-malate in the test sample lies between 3 and 35 µg).

If the malate concentration in the wine is less than 30 mg/l, the volume of the test sample may be increased up to 1 ml. In this case, the volume of water to be added is reduced in such a way that the total volumes in the two cells are equal.

5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using the cells having optical paths of 1 cm, with air as the zero absorbance (reference) standard (no cell in the optical path) or with water as the standard.

Place the following in the cells having 1 cm optical paths:

	Reference cell (ml)	Sample cell (ml)
Solution 3.1	1,00	1,00
Solution 3.2	0,20	0,20
Doubly distilled water	1,00	0,90
Suspension 3.3	0,01	0,01
Sample to be measured	-	0,10

Mix; after about three minutes, measure the absorbances of the solutions in the reference and sample cells (A1).

Add:

Solution 3.4	0,01 ml	0,01 ml

Mix; wait for the reaction to be completed (about 5 to 10 minutes) and measure the absorbances of the solutions in the reference and sample cells (A_2).

Calculate the differences ($A_2 - A_1$) in the absorbances of the solutions in the reference and sample cells, ΔA_R and ΔA_S.

Finally, calculate the difference between those differences: $\Delta A = \Delta A_S - \Delta A_R$

Note: The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

6. Expression of results

L-malic acid concentration is given in grams per litre to one decimal place.

6.1. *Method of calculation*

The general formula for calculating the concentration in g/l is:

$$C = \frac{V \times PM}{\varepsilon \times \delta \times 1000} \times \Delta A$$

where:

V = volume of test solution in ml (here 2,22 ml)

v = volume of the sample in ml (here 0,1 ml)

M = molecular mass of the substance to be determined (here, for L-malic acid, M=134,09)

δ = optical path in the cell in cm (here, 1 cm)

ε = absorption coefficient of NADH, (at 340 nm

ε = 6,3 m mol 1 × l × cm 1),

so that for L-malate:

$$C = 0{,}473 \times \Delta A \text{ g/l}$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note:

- Measurement at 334 nm, ε = 6,2 ($mmole^{-11} \times 1 \times cm^2$)
  $C = 0{,}482 \times \Delta A$

- Measurement at 365 nm, ε = 6,2 ($mmole^{-1\text{-}1} \times 1 \times cm^2$)
  $C = 0{,}876 \times \Delta A$

6.2. *Repeatability (r)*

$r = 0{,}03 + 0{,}034\ x_i$

x_i is the malic acid concentration in the sample in g/l.

6.3. *Reproducibility (R)*

$R = 0{,}05 + 0{,}071\ x_i$

x_i is the malic acid concentration in the sample in g/l.

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Method OIV-MA-AS313-12A Type II method

D-Malic acid

Enzymatic method
(OENO 6/98, revised by OIV/OENO 377/2009)

1. Principle

In the presence of D-malate-dehydrogenase (D-MDH), D-malic acid (D-malate) is oxidized to oxalo-acetate by nicotinamide-adenine-dinucleotide (NAD). The formed oxalo-acetate is transformed into pyruvate and carbon dioxide.

(1) D-malate + NAD $\overset{\text{+ D-MDH}}{\rightleftharpoons}$ pyruvate + CO_2 + NADH + H^+

The formation of NADH, measured by the increase of absorbance for 334, 340 or 365 nm wave lengths, is proportional to the quantity of D-malate present.

2. Reagents

Reagents that allow 30 determinations to be made are marketed in a set which includes:

1/ Flask 1 containing about 30 ml of solution of Hepes buffer acid [N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic] pH = 9.0 and stabilizers;
2/ Flask 2 containing about 210 mg of NAD lyophilizate;
3/ Flask 3 (three flasks), containing D-MDH lyophilizate, with a titer of about 8 units.

Preparation of the solutions

1/ Use the content of flask 1 without dilution. Bring the solution to a temperature of 20-25°C before using it.
2/ Dissolve the content of flask 2 in 4 ml of double-distilled water.
3/ Dissolve the content of one the flasks 3 in 0,6 ml of double-distilled water. Bring the solution to a temperature of 20-25 °C before using it.

Stability of the solutions

The contents of flask 1 can be kept for at least one year at + 4°C; solution 2 can be kept about 3 weeks at + 4 °C and 2 months at - 20 °C; solution 3 can be kept 5 days at + 4 °C.

3. Apparatus

3.1. Spectrophotometer which is able to measure at the NADH absorption maximum of 340 nm. If this is not available, a spectrophotometer with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm may be used. Since absolute absorbance measurements are involved (i.e. calibration curves are not used, but standardization is made by consideration of the

extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

3.2. Cells with a 1 cm path of glass or single-use cells.

3.3. Micropipettes capable of pipetting volumes between 0.01 and 2 ml.

4. Preparation of the sample

The analysis of D-malate is generally carried out directly on the wine without preliminary decoloration.

The quantity of D-malate in the cell must be between 2 µg and 50 µg; wine should be diluted so the malate concentration will be between 0.02 and 0.5 g/L or 0.02 and 0.3 g/L depending on the apparatus used.

Dilution table:

Estimated quantity of D-malate/liter		Dilution with water	Dilution factor F
Measured at: 340 or 334 nm	365 nm		
< 0.3 g	< 0.5 g	-	1
0.3-3.0 g	0.5-5.0 g	1 + 9	10

5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using 1 cm cells, with air as the zero absorbance (reference) standard (no cell in the optical path) or with water as the standard.

Place the following in the 1 cm cells:

	Reference cell (mL)	Sample cell (mL)
Solution 1	1.00 mL	1.00 mL
Solution 2	0.10 mL	0.10 mL
Double-distilled Water	1.80 mL	1.70 mL
Sample	-	0.10 mL

Mix: after approximately 6 minutes, measure the absorbance of the reference and sample solutions (A_1).

Add

	Reference	Sample
Solution 3	0.05 mL	0.05 mL

Mix: wait for the end of the reaction (about 20 min.) and measure the absorbance of the reference and sample solutions (A_2).

Determine the absorbance differences (A_2 - A_1) of the control (ΔA_T) and trial (ΔA_D).

Deduct the control absorbance difference from the trial absorbance difference:

$$\Delta A = \Delta A_D - \Delta A_T$$

Comment: the time required for the enzymes' action can vary from one batch to the other. It is given here only as an indication. It is recommended it be determined for each batch.
D-malic acid reacts rapidly. An additional activity of the enzyme also transforms L-tartaric acid even though it is not as rapid. This is the reason why there is a small side reaction which may be corrected by means of extrapolation (see annex 1).

6. Expression of the results

The concentration in milligrams per liter is calculated with the general formula:

$$C = \frac{V \times PM}{\varepsilon \times d \times \nu} \times \Delta A$$

V = volume of the test in ml (here 2.95 mL)
ν = volume of the sample in ml (here 0.1 mL)
PM = molecular mass of the substance to be measured
(here, D-malic acid = 134.09)
d = cell path length in cm (here 1 cm)
ε = absorption coefficient of NADH:
at 340 nm = 6.3 ($l\ mmol^{-1}\ cm^{-1}$)
at 365 nm = 3.4 ($l\ mmol^{-1}\ cm^{-1}$)
at 334 nm = 6.18($l\ mmol^{-1}\ cm^{-1}$).

If a dilution was made during the preparation of the sample, multiply the result by the dilution factor. The concentration in D-malic acid is given in milligrams per liter (mg/L) without decimal.

7. Accuracy

The details of the interlaboratory trial on the accuracy of the method are summarized in annex 2. The derived values of the interlaboratory study may not be applicable to ranges of concentration of the analyte and to other matrices other than those given in annex 2.

7.1. *Repeatability*

The absolute difference between individual results obtained on an identical matter submitted to a trial by an operator using the same apparatus, within the shortest time interval, will not exceed the value of repeatability r in more than 5% of the cases. The value is: r = 11 mg/L.

7.2. *Reproducibility*

The absolute difference between individual results obtained on an identical material submitted to a test in two laboratories will not exceed the value of reproducibility R in more than 5% of the cases. The value is: R = 20 mg/L.

8. Comments

Taking into account the method's accuracy, the values of D-malic acid less than 50 mg/L must be confirmed by another analytical method using another measuring principle such as that of PRZYBORSKI et al, (1993). Values of D-malic acid less than 100 mg/L must not be interpreted as an addition of D, L-malic acid to wine.

The wine content in the cuvette must not exceed 0.1mL to avoid a possible inhibition of enzymatic activity by polyphenols.

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ANNEX 1
How to treat side reactions

Side reactions are generally due to secondary reactions of the enzyme, in the presence of other enzymes in the sample's matrix, or the interaction of one or several elements of the matrix with a co-factor of the enzymatic reaction.

With a normal reaction, absorbance reaches a constant value after a certain time, generally between 10 min and 20 min, according to the speed of the specific enzymatic reaction. However, when secondary reactions occur, the absorbance does not reach a constant value, but increases regularly with time; this type of process is commonly called a « side reaction ».

When this problem arises, one should measure the solution's absorbance at regular intervals (2 min to 5 min), after the required time for the standard solution to reach its final absorbance. When the absorbance increases regularly, carry out 5 or 6 measurements, than establish a graphic or calculated extrapolation, in order to obtain what the solution's absorbance would have been when the final enzyme was added (T0). The difference in extrapolated absorbance at this time (Af-Ai) is used for the calculation of the substrate concentration.

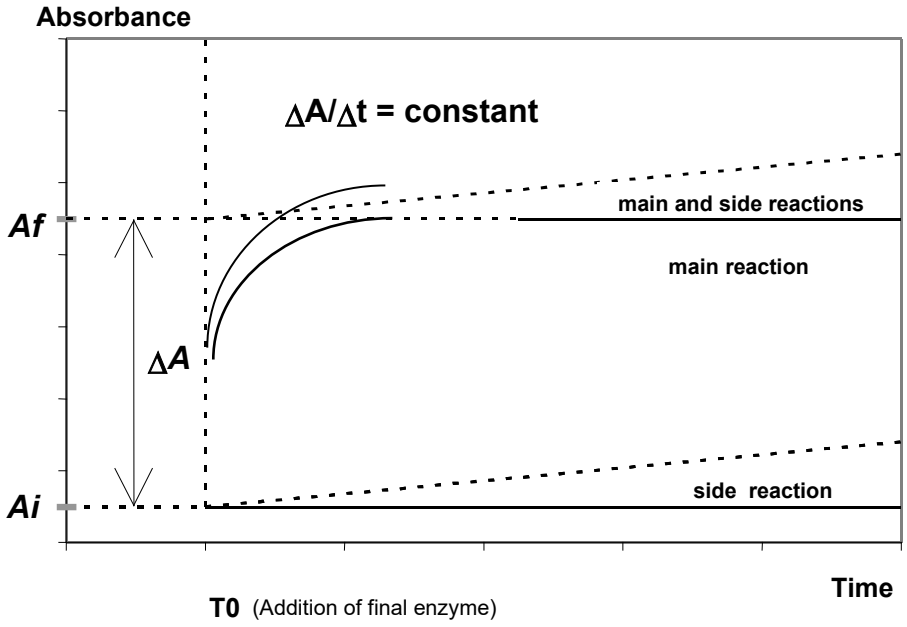


Figure 1: Side reaction

ANNEX 2

Interlaboratory trials statistical results

Year of the interlaboratory trial 1995
Number of laboratories 8
Number of samples 5 with addition of D-malic acid

Sample	A	B	C	D	E
Number of laboratories retained after elimination of laboratories presenting aberrant results	7	8	7	8	7
Number of laboratories presenting aberrant results	1	-	1	-	1
Number of accepted results	35	41	35	41	36
Average value(⊠) (mg/L)	161.7	65.9	33.1	106.9	111.0
Standard deviation of repeatability (s_r) (mg/L)	4.53	4.24	1.93	4.36	4.47
Relative standard deviation of repeatability (RSD_r) (%)	2.8	6.4	5.8	4.1	4.00
Limit of repeatability (r) (mg/L)	12.7	11.9	5.4	12.2	12.5
Standard deviation of reproducibility (s_R) (mg/L)	9.26	7.24	5.89	6.36	6.08
Relative standard deviation of reproducibility (RSD_R) (%)	5.7	11	17.8	5.9	5.5
Limit of reproducibility (R) (mg/L)	25.9	20.3	16.5	17.8	17.0

Types of samples:

A	red wine	C	white wine
B	red wine	D	white wine
		E	white wine

Method OIV-MA-AS313-12B Type IV method

Determination of d-malic acid in wines at low concentrations using the enzymatic method

(Resolution OENO 16/2002, revised by OIV/OENO 377/2009)

1. FIELD OF APPLICATION

The method described is applied to dosage, by the enzymatic means, of malic acid D of wines with contents under 50 mg/l.

2. PRINCIPLE

The principle of the method is based on malic acid D(+) oxidation (D-malate) by nicotinamide-adenine-dinucleotide (NAD) in oxaloacetate that is transformed into pyruvate and carbon dioxide; the formation of NADH, measured by the increase of absorbance in wave length at 340 nm, is proportional to the quantity of D-malate present (principle of the method described for malic acid D determination for concentrations above 50 mg/l), after introducing a quantity of malic acid D of 50 mg/l in a cuvette.

3. REAGENTS

Malic acid D solution of 0.199 g/l, above reagents indicated in the methods described for contents above 50 mg/l.

4. APPARATUS

Apparatus indicated in the method described for concentration above 50 mg/l.

5. SAMPLE PREPARATION

Sample preparation is indicated in the method described for concentrations above 50 mg/l.

6. PROCEDURE

The procedure is indicated in the method described for concentrations above 50 mg/l. (Resolution Oeno 6/98), but with the introduction in the tank of a quantity of malic acid D equivalent to 50 mg/l. (Introduction of 0.025 mL of malic acid D at 0.199 g/l, substituting the equivalent volume of water); the values obtained are decreased by 50 mg/l.

7. INTERNAL VALIDATION

Summary of the internal validation file on the dosage of malic acid D(+)- after the addition of 50 mg/l of this isomer

Work level	0 mg of 70 mg of malic acid D(+)-per liter. Within these limits, the method is linear with a correlation coeffiency between 0.990 and 0.994
Setting limit	24.4 mg/l
Detection limit	8.3 mg/l
Sensitivity	0.0015 abs / mg/l
Recovery percent range	87.5 to 115.0% for white wines and 75 to 105% for red wines
Repeatability	=12.4 mg/l for white wines (according to the OIV method =12,5 mg/l) =12.6 mg/l for red wines (according to OIV method=12,7 mg/l)
Percentage standard deviation	4.2% to 7.6% (white wines and red wines)
Intralaboratory variability	CV=7.4% (s=4.4mg/l; X average=59.3 mg/l)

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Method OIV-MA-AS313-13A Type IV method

L-Ascorbic acid

(A28, revised by OIV/OENO 377/2009)

1. Principle

The following methods enable the presence of L-ascorbic acid and dehydroascorbic acid in wines or musts to be determined.

Ascorbic acid is converted on activated carbon to dehydroascorbic acid. The latter forms a fluorescent compound on reaction with orthophenylenediamine (OPDA). A control prepared in the presence of boric acid enables spurious fluorescence to be determined (by the formation of a boric acid/dehydroascorbic acid complex). The sample and the control are analyzed fluorometrically and the concentration of dehydroascorbic acid calculated.

2. Method (fluorimetric method)

2.1 *Apparatus*

2.1.1 Fluorometer.

A spectrofluorometer equipped with a lamp giving a continuous spectrum and using it at minimum power.

The optimum excitation and emission wavelengths for the test are to be determined beforehand and depend on the equipment used. As a guide, the excitation wavelength will be approximately 350 nm and the emission wavelength approximately 430 nm. Cells of 1 cm path length.

2.1.2 Sintered glass filter of porosity 3.

2.1.3 Test tubes (diameter approximately 10 mm).

2.1.4 Stirring rods for test tubes.

2.2 *Reagents*

2.2.1 Orthophenylenediamine dihydrochloride solution ($C_6H_{10}Cl_2N_2$), 0.02 % (*m/v*), prepared just before use.

2.2.2 Sodium acetate trihydrate solution ($CH_3COONa \cdot 3H_2O$), 500 g/L.

2.2.3 Mixed solution of boric acid and sodium acetate:

Dissolve 3 g of boric acid, (H_3BO_3) in 100 mL of a 500 g/L sodium acetate solution. This solution must be prepared just before use.

2.2.4 Acetic acid solution (CH_3COOH) 56%: glacial acetic acid (r_{20}= 1.05 g/mL), diluted to 56% (*v/v*), pH approximately 1.2.

2.2.5 L-Ascorbic acid standard solution, 1 g/L.

Just before use, dissolve 50 mg of L-ascorbic acid previously dehydrated in a desiccator and protected against light, in 50 mL of acetic acid solution (2.2.4).

2.2.6 Very pure analytical grade activated carbon.

Place 100 g of activated carbon into a 2-liter conical flask and add 500 mL aqueous hydrochloric acid solution, 10% (*v/v*), (r_{20}= 1.19 g/mL). Bring to a boil, and filter through a sintered glass filter of porosity 3. Collect the carbon treated in this way in a 2-liter conical flask. Add 1 liter of water, shake and filter using a sintered glass filter of porosity 3. Repeat this operation two more times. Place the residue in an oven controlled to 115ºC ± 5 °C for 12 hours (or overnight).

2.3 *Procedure*

2.3.1 Preparation of the sample of wine or must

Take a volume of the wine or must and dilute to 100 mL in a graduated flask with the acetic acid solution, 56% (2.2.4), in order to obtain a solution with an ascorbic acid concentration between 0 and 60 mg/L. Thoroughly mix the contents of the flask by shaking. Add 2 g of activated carbon and allow to stand for 15 minutes, shaking occasionally. Filter using ordinary filter paper, discarding the first few milliliters of filtrate.

Pipette 5 mL of the filtrate into two 100 mL graduated flasks. Add to the first 5 mL of the mixed solution of boric acid and sodium acetate solution (2.2.3) (sample blank) and to the second 5 mL of the sodium acetate solution (2.2.2) (sample). Allow to stand for 15 minutes, stirring occasionally. Make to 100 mL with distilled water. Pipette 2 mL from the contents of each flask into a test tube and add 5 mL of orthophenylenediamine solution. Stir with the stirring rod and allow the reaction proceed for 30 minutes in the dark and then make the spectrofluorometric measurements.

2.3.2.Preparation of the calibration curve.

Into three 100 mL graduated flasks pipette 2, 4, and 6 mL respectively of the standard ascorbic acid solution (2.2.5), make to 100 mL with acetic acid solution and thoroughly mix by stirring. The standard solutions prepared in this way contain 2, 4 and 6 mg per 100 mL of L-ascorbic acid respectively.

Add 2 g of activated carbon to each of the flasks and allow to stand for 15 minutes, stirring occasionally. Filter through ordinary filter paper, discarding the first few milliliters. Pipette 5 mL of each filtrate into three 100 mL graduated flasks (first series). Repeat the operation and obtain a second series of three graduated flasks. To each of the flasks in the first series (corresponding to the blank test) add 5 mL of the mixed solution of boric acid and sodium acetate (2.2.3), and to each of the flasks in the second series add 5 mL of the sodium acetate solution (2.2.2). Let stand for 15 minutes, stirring occasionally. Make up to 100 mL with distilled water. Take 2 mL of the contents of each flask; add

5 mL of orthophenylenediamine solution. Stir and allow the reaction to proceed for 30 minutes in the dark and then make the spectrofluorometric measurements.

2.3.3 Fluorometric determination

Set the zero on the scale of measurement using the corresponding control test sample for each solution. Measure the intensity of the fluorescence for each solution over the calibration range and for the solution to be determined. Plot the calibration curve, which should be a straight line passing through the origin. From the graph determine the concentration C of ascorbic acid and dehydroascorbic acid in the solution analyzed.

2.4 *Expression of results*

The concentration of L-ascorbic acid and the dehydroascorbic acid in the wine in milligrams per liter is given by:

$$C \times F$$

where F is the dilution factor.

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Method OIV-AS313-13B Type IV method

L-Ascorbic acid

(Resolution OIV/OENO 377/2009)

WITHDRAWN

Method OIV-MA-AS313-14A Type IV method

Sorbic acid

(A30, revised by OIV/OENO 377/2009)

1. Principle of Method

Determination using ultraviolet absorption spectrophotometry

Sorbic acid (*trans, trans*, 2,4-hexadienoic acid) extracted by steam distillation is determined in wine distillate by ultraviolet absorption spectrophotometry. Substances that interfere with the measure of absorption in ultraviolet are removed by evaporation to dryness using a slightly alkaline calcium hydroxide solution. Samples with less than 20 mg/L are confirmed using thin layer chromatography (sensitivity: 1 mg/L).

2. Determination by ultraviolet absorption spectrophotometry

2.1 *Apparatus*

2.1.1 Steam distillation apparatus (see chapter "Volatile Acidity")

2.1.2 Water bath 100 °C

2.1.3 Spectrophotometer allowing absorbance measurements to be made at a wavelength of 256 nm and having a quartz cell with a 1 cm optical path

2.2 *Reagents*

2.2.1 Crystalline tartaric acid

2.2.2 Calcium hydroxide solution, approx. 0.02 M

2.2.3 Sorbic acid standard solution, 20 mg/L:

Dissolve 20 mg sorbic acid in approximately 2 mL 0.1 M sodium hydroxide solution. Pour into a 1 L volumetric flask, and make up to volume with water. Alternatively dissolve 26.8 mg of potassium sorbate, $C_6H_7KO_2$, in water and make up to 1 L with water.

2.3 *Procedure*

2.3.1 Distillation

Place 10 mL of wine in the bubbler of the steam distillation apparatus and add about 1 g tartaric acid. Collect 250 mL of distillate.

2.3.2 Preparation of the calibration curve

Prepare, by dilution of the standard solution (2.2.3) with water, four dilute standard solutions containing 0.5, 1.0, 2.5 and 5 mg of sorbic acid per liter.

Measure their absorbance with the spectrophotometer at 256 nm using distilled water as a blank. Plot a curve showing the variation of absorbance as a function of concentration. The relationship is linear.

2.3.3 Determination

Place 5 mL of the distillate in an evaporating dish of 55 mm diameter, add 1 mL of calcium hydroxide solution (2.2.2). Evaporate to dryness on a boiling water bath. Dissolve the residue in several mL of distilled water, transfer completely to a 20 mL volumetric flask and bring to volume with the rinsing water. Measure the absorbance at 256 nm using a solution obtained by diluting 1 mL of calcium hydroxide solution to 20 mL with water as the blank. Plot the value of the absorbance on the calibration curve and from this interpolate the concentration *C* of sorbic acid in the solution.

Note: In this method the loss due to evaporation is negligible and the absorbance is measured on the treated distillate diluted 1/4 with distilled water.

2.4 *Expression of results*

2.4.1 Calculation

The sorbic acid concentration in the wine expressed in mg/L is given by:

$$100 \times C$$

C = concentration of sorbic acid in the solution obtained in 2.3.3 expressed in mg/L.

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Method OIV-MA-AS313-14B Type IV method

Sorbic acid

(A30, revised by OIV/OENO 377/2009)

1. Principle of Methods

Determination by gas chromatography

Sorbic acid extracted in diethyl ether is determined by gas chromatography using an internal standard.

2. Determination by gas chromatography

2.1 *Apparatus*

2.1.1. Gas chromatograph fitted with a flame ionization detector and a stainless steel column (4 m x 1/8 inch) previously treated with dimethyldichlorosilane and packed with a stationary phase consisting of a mixture of diethyleneglycol succinate, 5%, and phosphoric acid, 1%, (DEGS - H_3PO_4), or of a mixture of diethyleneglycol adipate, 7%, and phosphoric acid, 1%, (DEGA - H_3PO_4) bonded on Gaschrom Q 80 - 100 mesh.

Treatment of column with dimethyldichlorosilane (DMDCS): pass a solution containing 2 to 3 g of (DMDCS) in toluene through the column.

Immediately wash with methanol, followed by nitrogen and then wash with hexane followed by more nitrogen. The column is now ready to be packed.

Operating conditions:

- Oven temperature: 175 °C
- Temperature of the injector and detector: 230 °C.
- Carrier gas: nitrogen (flow rate = 200 mL/min)

Note: Other types of columns can also give a good separation, particularly capillary columns (e.g. FFAP). The working method described below is given as an example.

2.1.2 Microsyringe, 10 µL capacity graduated in 0.1 µL.

2.2 *Reagents*

2.2.1 Diethyl ether distilled just before use

2.2.2 Internal standard: solution of undecanoic acid, $C_{11}H_{22}O_2$, 1 g/L in ethanol, 95% (*v/v*)

2.2.3. Aqueous solution of sulfuric acid, H_2SO_4, (ρ_{20} = 1.84 g/mL) diluted 1/3 (*v/v*)

2.3 *Procedure*

2.3.1 Preparation of sample to be analyzed

Into a glass test tube of approximately 40 mL capacity and fitted with a ground glass stopper, place 20 mL of wine, 2 mL of the internal standard (2.2.2) and 1 mL of dilute sulfuric acid.

After mixing the solution by repeatedly turning the tube over, add 10 mL of diethyl ether (2.2.1). Extract the sorbic acid into the organic phase by shaking the tube for five minutes. Allow to settle.

2.3.2 Preparation of the spiked sample

Select a wine for which the chromatogram of the ether extract shows no peak corresponding to the elution of sorbic acid. Fortify this wine with sorbic acid at a concentration of 100 mg/L. Treat 20 mL of the sample prepared in this way according to the procedure described in 2.3.1.

2.3.3. Chromatography

Inject 2 µL of the ether-extract phase obtained in 2.3.2, into the chromatograph using a microsyringe, followed by 2 µL of the ether-extracted phase obtained in 2.3.1.

Record the respective chromatograms: check the identity of the respective retention times of the sorbic acid and the internal standard. Measure the height (or area) of each of the recorded peaks.

2.4 *Expression of results*

2.4.1 Calculation

The concentration of sorbic acid in the analyzed wine, expressed in mg/L, is given by:

$$100 \times \frac{h}{H} \times \frac{I}{i}$$

where

H = height of the sorbic acid peak in the spiked solution

h = height of the sorbic acid peak in the sample for analysis

I = height of the internal standard peak in the spiked solution

i = height of the internal standard peak in the sample for analysis

Note: The sorbic acid concentration may be determined in the same way from measurements of the respective peak areas.

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Method OIV-MA-AS313-14C Type IV method

Sorbic acid

(A30, revised by OIV/OENO 377/2009)

1. Principle of Methods

Identification of traces by thin-layer chromatography

Sorbic acid extracted in ethyl ether is separated by thin layer chromatography and its concentration is evaluated semi-quantitatively.

2. Identification of traces of sorbic acid by thin layer chromatography

2.1 *Apparatus*

2.1.1 Precoated 20 x 20 cm plates for thin layer chromatography coated with polyamide gel (0.15 mm thick) with the addition of a fluorescence indicator

2.1.2 Chamber for thin layer chromatography

2.1.3 Micropipette or microsyringe for delivering volumes of 5 µL to within ± 0.1 µL

2.1.4 Ultraviolet lamp (254 nm)

2.2. *Reagents*

2.2.1 Diethyl ether, $(C_2H_5)_2$ O

2.2.2 Aqueous sulfuric acid solution: sulfuric acid (ρ_{20}= 1.84 g/mL), diluted 1/3 (*v*/*v*)

2.2.3 Standard solution of sorbic acid, approximately 20 mg/L, in a 10% (*v*/*v*) ethanol/water mixture.

4.2.4 Mobile phase: hexane + pentane + acetic acid (20:20:3).

4.2.5

2.3 *Procedure*

2.3.1 Preparation of sample to be analyzed

Into a glass test tube of approximately 25 mL capacity and fitted with a ground glass stopper, place 10 mL of wine; add 1 mL of dilute sulfuric acid (2.2.2) and 5 mL of diethyl ether (2.2.1). Mix by repeatedly inverting the tube. Allow to settle.

2.3.2 Preparation of dilute standard solutions

Prepare five dilute standard solutions from the solution in 2.2.3. containing 2, 4, 6, 8 and 10 mg sorbic acid per liter.

2.3.3 Chromatography

Using a microsyringe or micropipette, deposit 5 µL of the ether-extracted phase obtained in 2.3.1 and 5 µL each of the dilute standard solutions (2.3.2) at points 2 cm from the lower edge of the plate and 2 cm apart from each other.

Place the mobile phase in the chromatograph tank to a height of about 0.5 cm and allow the atmosphere in the tank to become saturated with solvent vapor. Place the plate in the tank. Allow the chromatogram to develop over 12 to 15 cm (development time approximately 30 minutes). Dry the plate in a current of cool air. Examine the chromatogram under a 254 nm ultraviolet lamp.

The spots indicating the presence of sorbic acid will appear dark violet against the yellow fluorescent background of the plate.

2.4 *Expression of the results*

A comparison of the intensities of the spots produced by the test sample and by the standard solutions will enable a semi-quantitative assessment of a sorbic acid concentration between 2 and 10 mg/L. A concentration equal to 1 mg/L may be determined by using a 10 µL sample size.

Concentrations above 10 mg/L may be determined using a sample volume of less than 5 µL (measured out using a microsyringe).

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Method OIV-MA-AS313-15 Type I method

pH

(A31, OIV-OENO 438-2011)

1. Principle

The difference in potential between two electrodes immersed in the liquid under test is measured. One of these two electrodes has a potential that is a function of the pH of the liquid, while the other has a fixed and known potential and constitutes the reference electrode.

2. Apparatus

2.1 pH meter with a scale calibrated in pH units and enabling measurements to be made to at least ±0.01 pH units.

2.2 Electrodes:

- glass electrode, kept in distilled water;
- calomel-saturated potassium chloride reference electrode, kept in a saturated solution of potassium chloride; or,
- a combined electrode, kept in distilled water.

3. Reagents

– Buffer solutions:

- Saturated potassium hydrogen tartrate solution, containing 5.7 g/L potassium hydrogen tartrate ($CO_2HC_2H_4O_2CO_2K$) at 20°C. (This solution may be kept for up to two months by adding 0.1 g of thymol per 200 mL.)

$$\text{pH} \begin{cases} 3.57 \text{ at } 20\ ^{o}\text{C} \\ 3.56 \text{ at } 25\ ^{o}\text{C} \\ 3.55 \text{ at } 30\ ^{o}\text{C} \end{cases}$$

- Potassium hydrogen phthalate solution, 0.05 M, containing 10.211 g/L potassium hydrogen phthalate, $CO_2HC_6H_4CO_2K$, at 20°C. (This solution may be kept for up to two months.)

$$\text{pH} \begin{cases} 3.999 \text{ at } 15\ ^{o}\text{C} \\ 4.003 \text{ at } 20\ ^{o}\text{C} \\ 4.008 \text{ at } 25\ ^{o}\text{C} \\ 4.015 \text{ at } 30\ ^{o}\text{C} \end{cases}$$

Solution containing:
potassium *di*-hydrogen phosphate, KH_2PO_4 ...……..... 3.402 g
di-potassium hydrogen phosphate, K_2HPO_4 ...……..... 4.354 g
water to .....… …./....……. 1 litre
(This solution may be kept for up to two months)

pH { 6.90 at 15 °C
6.88 at 20 °C
6.86 at 25 °C
6.85 at 30 °C

Note: commercial reference buffer solutions traceable to the SI may be used.

For example: pH 1.679 ±0.01 at 25°C

pH 4.005 ±0.01 at 25°C

pH 7.000 ±0.01 at 25°C

4. Procedure

4.1 *Zeroing of the apparatus*

Zeroing is carried out before any measurement is made, according to the instructions provided with the apparatus used.

4.2 *Calibration of the pH meter*

The pH meter must be calibrated at 20°C using standard buffer solutions connected to the SI. The pH values selected must encompass the range of values that may be encountered in musts and wines. If the pH meter used is not compatible with calibration at sufficiently low values, a verification using a standard buffer solution linked to the SI and which has a pH value close to the values encountered in the musts and wines may be used.

4.3 *Determination*

Dip the electrode into the sample to be analyzed, the temperature of which should be between 20 and 25°C and as close as possible to 20°C. Read the pH value directly off the scale.

Carry out at least two determinations on the same sample.

The final result is taken to be the arithmetic mean of two determinations.

5. Expression of results

The pH of the must or the wine is reported to two decimal places.

Method OIV-MA-AS313-16 Type IV method

Determination of organic acids and mineral anions in wines by ionic chromatography

(OENO 23/2004; OIV/OENO 377/2009)

Preamble

The development of high performance ionic chromatography in laboratories has enabled the study the determination of organic acids and mineral anions in alcoholic and non alcoholic beverages by this technique.
Particularly concerning the analysis of wines, the results of intercomparison test trials and the measurements of recovery rates have enabled us to validate an analytical methodology.
The major interest of this method is that the ion exchange columns allow the separation of most organic acids and anions, and the detection by conductimetry frees the analysis from interferences due to the presence of phenolic compounds. This type of interference is very notable in chromatographic methods that include detection in ultra-violet radiation at 210 nm.

1 - OBJECT AND FIELD OF APPLICATION

This method for mineral anions and organic acids by ionic chromatography is applicable to alcoholic beverages (wines, wine spirits and liqueurs). It enables the determination of organic acids in the ranges of concentration listed in table 1; these concentrations are obtained by diluting samples.

Table 1: range of concentration of anions for their analysis by ionic chromatography

Sulfate	0.1 to 10	mg/l
Ortho-phosphate	0.2 to 10	mg/l
Malic acid	1 to 20	mg/l
Tartaric acid	1 to 20	mg/l
Citric acid	1 to 20	mg/l
Isocitric acid	0.5 to 5	mg/l

The ranges of the above-mentioned work are given as an example. They include the methods of calibration commonly practiced and are therefore adaptable according to

the type of apparatus used (nature of column, sensitivity of the detector, etc.) and procedure (volume of sample injected, dilution, etc.).

2- PRINCIPLE

Separation of mineral and organic anions on an ion exchanger resin.
Detection by conductimetry.
Identification after the retention time and quantification using the calibration curve.

3 - REAGENTS

All the reagents used during the analysis must be of analytical quality. The water used for the preparation of solutions must be distilled or deionised water of a conductivity lower than 0.06 µS, free from anions determined at thresholds compatible with the detection limits of the apparatus used.

3.1 Eluant

The composition of the eluant depends on the nature of the separation column and the nature of the sample to be analysed. Nevertheless it is always prepared using aqueous solutions of sodium hydroxide.
The performances of the chromatographic analysis are alternated by carbonation of the sodium hydroxide solution; consequently, the mobile phase flasks are swept with helium before adding sodium hydroxide and all precautions should be taken in order to avoid contaminating them with room air.
Lastly, commercial concentrated sodium hydroxide solutions will be used.

Remark

The table in chapter 9 mentions the main interferents susceptible of being present in the samples.
It is therefore necessary to know beforehand if they coelute with the ions to be determined and if they are present at such a concentration that the analysis is disrupted.

Fermented drinks contain succinic acid which can interfere with the malic acid determination. To this effect, it is necessary to add methanol to the eluant in order to improve the resolution of the column for these two substances (20% of methanol).

3.2 Calibration reference solutions

Prepare calibration reference solutions of precise concentrations close to those indicated in the following table. Dissolve in water, quantities of salts or corresponding acids in 1000 ml volumetric flasks. (Table 2)

Table 2: Concentration of anions determined in calibration reference solutions

Anions and acids	Compounds weighed	Concentration final (mg/l)	Quantity weighed (mg)
Sulphate	Na2SO4	500	739.5
Orthophosphate	KH2PO4	700	1003.1
Malic acid	Malic acid	1000	1000.0
Tartaric acid	Tartaric acid	1000	1000.0
Citric acid	Citric acid, H_2O	1000	1093.8
Isocitric acid	Isocitrate 3Na, $2H_2O$	400	612.4

Remark

The laboratory must take the necessary precautions regarding the hygroscopic character of certain salts.

3.3 Calibration solutions

The calibration solutions are obtained by diluting the reference solutions of each ion or acid in water.
These solutions should contain all the ions or acids determined in a range of concentrations covering those corresponding to the samples to be analysed. They must be prepared the day of their use.

At least two calibration solutions and a blank must be analysed so as to establish, for each substance, the calibration curves using three points (0, maximum semi-concentration, maximum concentration).

Remark

Table 1 gives indications on the maximum concentrations of anions and acids in calibration solutions but the performances of the chromatographic columns are better with very diluted solutions.
So the best adequation possible between the performances of the column and the level of dilution of the samples should be looked for.

In general, the sample is diluted between 50 and 200 times maximum except for particular cases.
For prolonging the life span of the dilution solutions, it is preferable to prepare them in a water/methanol solution (80/20).

4 - APPARATUS

4.1 Instrument system for ionic chromatography including:

4.1.1 Eluant reservoir(s)

4.1.2 Constant-stroke pump, without pulsing action

4.1.3 Injector, either manual or automatic with a loop sampling valve (for example 25 or 50 µl).

4.1.4 Separation columns

System made up of an anion exchanger column of controlled performance, possibly a precolumn of the same type as the main column. For example, it is possible to use the AS11 columns and DIONEX® AG11 precolumn.

4.1.5 Detection system

Circulation conductivity cell of very low volume connected to a conductivity meter with several ranges of sensitivity.
In order to lower the conductivity of the eluant, a chemical suppression mechanism, a cation exchanger is installed in front of the conductivity cell.

4.1.6 Recorder, integrator or other device for the treatment of signals.

4.2 Precise balance to 1 mg

4.3 Volumetric flasks from 10 to 1000 ml

4.4 Calibrated pipettes from 1 to 50 ml

4.5 Filtrating membranes with an average pore diameter of 0.45 µm.

5 - SAMPLING

The samples are diluted while taking into account the mineral anions and organic acids that are to be determined.
If their concentration is very variable in the sample, two levels of dilution will be necessary in order to respect the ranges of concentration covered by the calibration solutions.

6 - PROCEDURE

Turn on the apparatus by following the manufacturer's instructions.
Adjust the pumping (eluant flux) and detection conditions so as to obtain good separations of the peaks in the range of concentrations of ions to be analysed.
Allow the system to balance until a stable base line is obtained.

6.1 Calibration
Prepare the calibration solutions as indicated in 3.3.
Inject the calibration solutions so that the volume injected is at least 5 times that of the sampling loop to allow the rinsing of the system.
Trace the calibration curves for each ion. These must normally be straight.

6.2 Blank trial
Inject the water used for the preparation of the calibration solutions and samples.
Control the absence of parasite peaks and quantify the mineral anions present (chloride, sulphate, etc.).

6.3 Analysis
Dilute the sample possibly at two different levels as indicated in 5, so that the anions and acids to be determined are present in the range of concentrations of the calibration solutions.
Filter the diluted sample on a filtrating membrane (4.5) before injection.
Then proceed as for the calibration (6.1).

7 - REPEATABILITY, REPRODUCIBILITY

An interlaboratory circuit tested this method, but this does not constitute a formal validation according to The OIV protocol (Oeno 6/99).
A repeatability limit and a reproducibility limit for the determination of each ion in wine were calculated according to the ISO 5725 standard.
Each analysis was repeated 3 times.

Number of participating laboratories: 11; the results were as follows:

White wine

	No labs	**Average** (mg/l)	**Repeatability** (mg/l)	**Reproducibility** (mg/l)
Malic acid	11/11	2745	110	559
Citric acid	9/11	124	13	37
Tartaric acid	10/11	2001	96	527
Sulphate	10/11	253	15	43
O.phosphate	9/11	57	5	18

Red wine

	No labs	Average (mg/l)	Repeatability (mg/l)	Reproducibility (mg/l)
Malic acid	8/11	128	16	99
Citric acid	8/10	117	8	44
Tartaric acid	9/11	2154	48	393
Sulphate	10/11	324	17	85
O.phosphate	10/11	269	38	46

8 – CALCULATION OF RECOVERY RATE

The supplemented sample is a white wine.

Determination	No labs	Concentration initial (mg/l)	Real addition (mg/l)	Measured addition (mg/l)	Recovery rate (%)
Citric acid	11/11	122	25.8	24.2	93.8
Malic acid	11/11	2746	600	577	96.2
Tartaric acid	11/11	2018	401	366	91.3

9 - RISKS OF INTERFERENCES

Any substance whose retention time coincides with that of one of the ions analysed can constitute an interference.
The most common interference include the following:

Anions or	Interferents acids
Nitrate	bromide
Sulphate	oxalate, maleate
Orthophosphate	phtalate
Malic acid	Succinic acid, Citramalic acid
Tartric acid	Malonic acid
Citric acid	-
Isocitric acid	-

Remark: The addition of methanol in the mobile phase can resolve certain analytical problems.

Method OIV-MA-AS313-17 Type II method

Determination of shikimic acid in wine by HPLC and UV-detection

(OENO 33/2004; OIV/OENO 377/2009)

1. INTRODUCTION

Shikimic acid (3,4,5-Trihydroxy-1-cyclohexene-1-carboxylic acid) is biosynthetically synthesized from chinic acid by dehydration and plays a major role as a precursor of phenylanaline, tyrosine, tryptophan and plant alkaloids [1]. As a minor carboxylic acid shikimic acid is naturally found in a wide range of fruits [2].

Member states are encouraged to continue research in this area to avoid any non scientific evaluation of the results.

This method has been validated in an international collaborative study via the analyses of wine samples with naturally occurring amounts of shikimic acid ranging from about 10 to 150 mg/l. The trueness has been proved by an interlaboratory comparison using HPLC and GC/FID and GC/MS respectively [3].

2. SCOPE

This paper specifies an isocratic routine method for the quantitative determination of shikimic acid in red, rosé and white wine (included sparkling and special wines) at concentration levels ranging from 1 mg/l up to 300 mg/l by high performance liquid chromatography. When the method is applied to sparkling wine the samples must be previously degassed (for instance by sonication).

3. PRINCIPLE

Shikimic acid is determined directly without previous sample preparation by high performance liquid chromatography using a coupled column system. In a first step the organic acids in wine are pre-separated with a C_{18} reversed phase column followed by a cation exchange column at 65 °C performing the final separation. By using slightly acidified water as elution solvent a baseline resolution of shikimic acid is achieved without any interferences from the wine matrix . Due to the double bond within the cyclohexene ring system shikimic

acid has a strong absorption and can therefore be detected easily with an UV-detector at its absorption maximum at 210 nm.

4. REAGENTS AND MATERIALS

4.1 Shikimic acid (CAS 138-59-0) , at least 98 % pure

4.2 Sulfuric acid 0,5 M

4.3 Bidestilled water

4.4 Preparation of the elution solvent (0,01 M H_2SO_4)
Pipette 20 ml of the 1 N sulfuric acid (4.2) to a 1000 ml volumetric flask, fill up with bidestilled water (4.3) to about 900 ml, shake and adjust to 1000 ml. Filter the elution solvent with a filter of a pore size less than or equal to 0,45 µm and degas.

4.5 Preparation of stock standard solution (500 mg/l shikimic acid)
Weigh exactly 50 mg shikimic acid (4.1), transfer them without loss to a 100 ml volumetric flask, fill up with bidestilled water (4.3) to about 90 ml, shake and adjust to 100 ml. At –18 °C the stock standard solution can be stored for months.

4.6 Preparation of working standard solutions (5 , 25 , 50 , 100, 150 mg/l shikimic acid) Dilute stock solution 500 mg/l (4.5) appropriately with bidestilled water (4.3) to give five working standards of 5 , 25 , 50 , 100, 150 mg/l shikimic acid. Prepare working standard solutions daily.

5. APPARATUS

Usual laboratory equipment, in particular, the following:

5.1 HPLC system capable of achieving baseline resolution of shikimic acid

5.1.1 High-performance liquid chromatograph with a six-way injection valve fitted with a 5 µl loop or any other device, either automatic or manual, for a reliable injection of microvolumes

5.1.2 Isocratic pumping system enabling one to achieve and maintain a constant or programmed rate of flow with great precision.

5.1.3 Column heater enabling one to heat a 300 mm column to 65 °C

5.1.4 UV-VIS detector with a flow cell and wavelength set of 210 nm

5.1.5 Computational integrator or other data collection system

5.2 HPLC column system of stainless steel

5.2.1 Guard column

It is recommended that a suitable pre-column is attached in front of the analytical column system.

5.2.2 Analytical column system

1. Reversed Phase Column (ambient)
   Material: stainless steel
   Internal diameter: 4 - 4,6 mm
   Length : 200 - 250 mm
   Stationary phase: spherical C_{18} reversed phase material, particles 5µ in diameter*)
   coupled with

2. Cation exchange column (heated up to 65 ° C)
   Material: stainless steel
   Internal diameter: 4 - 7,8 mm
   Length : 300 mm
   Stationary phase: Sulfonated sterene-divinylbenzene gel type resin (S-DVB),containing a hydrogen packing, cross linked 8 %**)

6. SAMPLING

Clear samples are filled directly into sample vials and supplied to chromatography without any sample preparation. Cloudy wine samples are filtered through a 0,45 µm membrane filter before injection, while the first fractions of filtrates are rejected.

7. PROCEDURE

7.1 Operating conditions of HPLC analysis

Inject 5 µL of wine into the chromatographic apparatus by full loop injection system.
Flow rate: 0,4 ml/min (if internal diameter of the cation exchange column is 4 mm)
0,6 ml/min (if internal diameter of the cation exchange column is 7,8 mm)
Mobile Phase: 0,01 M H_2SO_4
Column heater for cation exchange column: 65 °
Run time: 40 min

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*) Lichrospher™ 100 RP-18 , Hypersil™-ODS or Omnichrom™ YMC-ODS-A are examples of suitable columns available commercially

* *) Aminex™ HPX 87-H or Rezex™ ROA-Organic Acid are examples of suitable columns available commercially

Equilibration time: 20 min (to ensure that all substances from the wine matrix are completely eluted)

Detection wavelength: 210 nm

Injection volume: 5 µL

Note: *Due to the different separation properties of various columns and different dead volumes of various HPLC-equipments the absolute retention time (min) for the shikimic acid peak may vary more or less significantly. Even though shikimic acid can be identified easily by calculating the a relative retention (r) related to a reference peak, here tartaric acid, a major organic acid naturally occurring in wine and the first and dominant peak in the chromatogram . By trying different* C_{18} reversed phase columns and various cation exchange *columns a relative retention (r) of 1*.33 (± 0.2) *has been calculated.*

7.2. Detection limit
The detection limit of this method calculated according to the OIV protocol was estimated to 1 mg/l.

8. CALCULATION

Prepare a 5-point calibration curve from the working standard solutions (4.6).

Following the method of external standard the quantification of shikimic acid is performed by measuring the peak areas at shikimic acid retention time and comparing them with the relevant calibration curve. The results are expressed in mg/l shikimic acid at 1 decimal place.

9. PRECISION

The method was tested in a collaborative study with 19 international laboratories participating. Design and assessment followed O.I.V. Resolution Oeno 8/2000 "Validation Protocol of Analytical Methods". The study included 5 different samples of red and white wines. The samples covered concentration levels from 10 to 120 mg/l (see Annex 3).

The Standard Deviations of Repeatability and Reproducibility correlated with the shikimic acid concentration (see Annex 2). The actual performance parameters can be calculated by

$s_r = 0{,}0146 \cdot x + 0{,}2716$
$s_R = 0{,}0286 \cdot x + 1{,}4883$

x: shikimic acid concentration (mg/l)

Example:

shikimic acid: 50 mg/l
s_r = ±1,0 mg/l
s_R = ±2,92 mg/l

10. ANNEX

A typical separation of shikimic acid from other organic acids in wine is given in the Annex 1.

The correlationship of shikimic acid concentration and the standard deviation of repeatability and reproducibility is given in Annex 2.

The statistical data derivated from the results of the interlaboratory study is given in Annex 3.

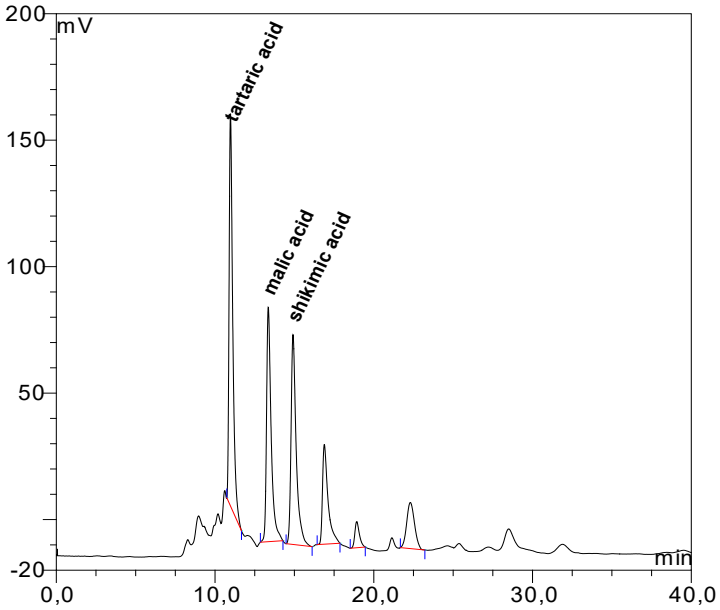
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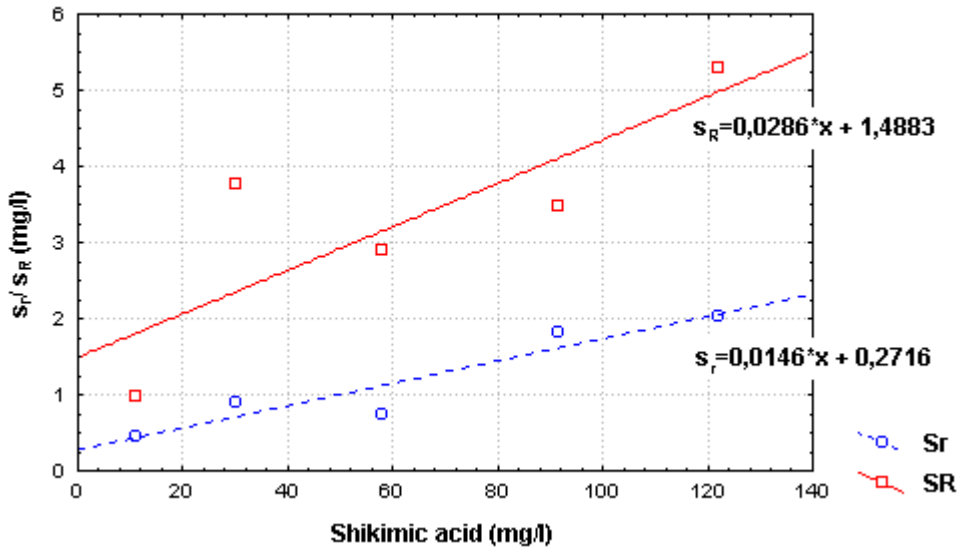
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Annex 1: Chromatogram of organic acids in wine



Annex 2: Correlationship of shikimic acid concentration and standard deviation of repeatability and reproducibility respectively



Annex 3: Table of method performance parameters

sample identification	**A**	**B**	**C**	**D**	**E**
Number of participating laboratories	19	19	19	19	19
Number of accepted laboratories	17	18	17	18	18
mean	58.15	30.05	11.17	122.17	91.20
s_r^2	0.54588	0.84694	0.19353	4.32417	2.67306
s_r	0.73884	0.92030	0.43992	2.07946	1.63495
RSD_r (%)	1.27	3.06	3.93	1.70	1.79
r	2.07	2.58	1.23	5.82	4.58
s_L^2	8.45221	13.27078	0.73013	24.62737	8.55508
s_R^2	8.99809	14.11773	0.92366	28.95154	11.22814
s_R	2.99968	3.75736	0.96107	5.38066	3.35084
RSD_R (%)	5.16	12.50	8.60	4.40	3.67
R	8.40	10.52	2.69	15.07	9.38

s_r^2	variance of repeatability
s_r	standard deviation of repeatability
RSD_r (%)	relative standard deviation of repeatability
r	repeatability
s_L^2	variance between laboratory
s_R^2	variance of reproducibility
s_R	variance of reproducibility
RSD_R (%)	relative standard deviation of reproducibility
R	reproducibility

Method OIV-MA-AS313-18 Type IV method

Determination of sorbic acid in wines by capillary electrophoresis

OENO 4/2006; OIV/OENO 377/2009

1 Scope

The present method is used to determine the sorbic acid in wines in a range from 0 to 300 mg/l.

2 Principle

The negatively charged sorbate ion naturally enables easy separation by capillary electrophoresis. At the capillary outlet, detection is carried out in the ultraviolet spectrum at 254 Nm.

3 Reagents and products

3.1 Reagents

3.1.1 Sodium dihydrogenophosphate [10049-21-5] purity > 96%
3.1.2 Sodium hydrogenophosphate [10028-24-7] purity > 99%
3.1.3 Sodium hydroxide [1310-73-2] purity > 97%
3.1.4 Hippuric sodium [532-94-5] purity > 99%
3.1.5 Demineralised water (< 15 MOHMS) or double-distilled

3.2 Migration buffer solution

The migration buffer is made up in the following way:

Sodium dihydrogenophosphate (3.1.1):	5 mM
Sodium hydrogenophosphate(3.1.2)	5 mM

3.3 Internal standard

Hippuric spdium (3.1.4) in an aqueous solution 0.5 g.L-1

3.4 Rinse solutions

3.4.1 Sodium hydroxide (3.1.3) N/10
3.4.2 Sodium hydroxide (3.1.3) N

4 Sample preparation

The wine samples are prepared as follows, which involves a 1/20 dilution:

Wine to be analyzed: 0.5 ml
Sodium hydroxide (3.1.3): 0.5 ml
Internal standard (3.1.4) with 0.5 g. L^{-1}: 0.5 ml
Qsp 10 ml with demineralized water (3.1.5)

5 Operating conditions

5.1 Conditioning the capillary

Before its first use, and as soon as the migration times increase, the capillary must be conditioned according to the following process:

5.1.1 Rinse with sodium hydroxide solution 1N (3.4.2) at 20 psi (140 kPA) for 8 min.
5.1.2 Rinse with sodium hydroxide solution (3.4.1) 0.1 N at 20 psi (140 kPA) for 12 min.
5.1.3 Rinse with water (3.1.5) at 20 psi (140 kPA) for 10 min.
5.1.4 Rinse with the migration buffer (3.2) at 20 psi (140 kPA) for 30 min.

5.2 Migration conditions

These conditions may be slightly changed depending on the equipment used.

5.2.1 The molten silica capillary is 31 cm long, with a diameter of 50 microns.
5.2.2 Migration temperature: 25°C
5.2.3 Reading wavelength: 254 nm.
5.2.4 Reading of the signal in direct mode (sorbic acid absorbs in the UV spectrum).
5.2.5 First Pre-rinse under pressure 30 psi (210 kPA) with sodium hydroxide solution 0.1 N (3.4.1) for 30 seconds
5.2.6 Second Pre-rinse under pressure 30 psi (210 kPA) with the migration buffer (3.2) for 30 seconds.
5.2.7 The injection is done under a pressure of 0.3 psi (2.1 kPA) for 10 seconds.
5.2.8 The migration lasts approximately 1.5 to 2 minutes under a potential difference of + 25 kV, in normal polarity (cathod at the exit).
5.2.9 Certain capillary electrophoresis apparatus propose large-capacity vials for migration buffer solutions. This is preferable when several analyses are carried out in series, because the electrolytic properties are maintained longer.

5.3 Reading the results

The absorption peaks for the internal standard and the sorbic acid are obtained on average 1 to 1.5 minutes after the start of the migration phase live. Migration time is fairly constant, but can slightly vary according to the state of the capillary. If the migration time degrades, reconditioning of the capillary is necessary, and if the nominal conditions are not restored, the capillary must be replaced.

6 Characteristics of the method

The different validation steps described were carried out according to the OIV resolution OENO 10/2005.

6.1 Intralaboratory repeatability

Standard repeatability deviation **Sr**	**1.6 mg/** L^{-1}
Repeatability **r**	**4.6 mg/** L^{-1}

6.2 Linearity

Regression line	Y = **0,99491 X + 2,52727**
Correlation coefficient **r**	**0,9997**
Residual standard deviation **Sxy**	**1,6 mg.**L^{-1}
Standard deviation slope **Sb**	**0,008 mg.**L^{-1}

6.3 Intralaboratory reproducibility

Standard reproductibility deviation **Sr**	**2.1 mg/** L^{-1}
Reproductibility **R**	**5.8 mg/** L^{-1}

6.4 Detection and quantification limits

Detection limit **Ld**	**1.8 mg/** L^{-1}
Quantification limit **Lq**	**4.8 mg/** L^{-1}

6.5 Robustness

6.5.1 Determination

Since the method is relative, any slight variations in the analysis conditions will have no effect on the final result, but will primarily influence the migration time.

6.6 Method specificity

Possible influence of principle oenological additives were tested. None of them modify the results obtained.

6.7 Correlating the method with the OIV reference method

The OIV reference method is determination by ultraviolet absorption spectrometry. The sorbic acid, extracted by steam distillation, is determined in the wine distillate by ultraviolet absorption spectrometry at 256 Nm.

6.7.1 Comparison of repeatabilities

	Capillary electrophoresis	OIV reference method
Standard deviation of repeatability $\mathbf{S_r}$	**1.6 mg/l**	**2.5 mg/** L^{-1}
Repeatability **r**	**4.6 mg/l**	**7.0 mg/** L^{-1}

6.7.2 Accuracy of the usual method in relation to the reference method

Coefficient of correlation **r**	**0.999**
Average bias **Md**	**0.03 mg** L^{-1}
Average bias standard deviation **Sd**	**3.1 mg** L^{-1}
Z-score (Md/Sd)	**0.01**

Method OIV-MA-AS313-19 Method Type: II for organic acids
III for sulphate

Determination of the principal organic acids of wines and sulphates by capillary electrophoresis

OENO 5/2006
OIV-OENO 407-2011

1. Introduction

Tartaric, malic and lactic acids and sulphates are separated and assayed by capillary electrophoresis after simple dilution and addition of an internal standard.

2. Title

Determination of the principal organic acids of wines and sulphates by capillary electrophoresis

3. Scope

Capillary electrophoresis can be used to assay the tartaric and malic acid in musts, as well as the tartaric, malic and lactic acids and sulphates in wines that have been diluted, degassed and filtered beforehand if need be.

4. Définitions

4.1 Capillary electrophoresis

Capillary electrophoresis: all the techniques that use a capillary tube of very small diameter with an appropriate buffer solution to effectively separate small and large electrically charged molecules in the presence a high-voltage electric current.

4.2 Buffer for electrophoresis

Solution containing one or more solvents and aqueous solutions with suitable electrophoretic mobilities to buffer the pH of the solution.

4.3 Electrophoretic mobility

Aptitude of an ion to move quickly under the effect of an electric field.

4.4 Electroosmotic flow

Flow of solvent in the buffer solution along the internal wall of the capillary tube due to displacement of the solvated ions under the effects of the field and the electric charges of the silica.

5. Principle

Separations of the aqueous solutions of a mixture by capillary electrophoresis are obtained by differential migrations in a buffered electrolyte referred to as a buffer. The electrophoresis takes place in a silica tube with an inside diameter ranging between 25 and 75 µm. The aqueous solutions to be separated are simultaneously driven by 2 forces that can act in the same direction or in the opposite direction. These two forces are caused by the **electric field** and the **electroosmotic flow**.

The electric field is represented by the voltage in volts applied between the electrodes brought to within one centimetre of the capillary tube, and is expressed in $V.cm^{-1}$. Mobility is a characteristic of ions. The smaller the molecules, the greater their electrophoretic mobility.

If the internal wall of the capillary tube is not coated, the negative electric charges of the silica fix part of the cations of the buffer. The solvation and displacement towards the cathode of part of the cations of the buffer create the electroosmotic flow. The pH of the buffer and additives can be chosen in order to control the direction and the intensity of the electroosmotic flow.

The addition of a chromophoric ion in the buffer can be used to obtain negative peaks that quantitatively represent the solutions to be separated which do not absorb at the used wavelength.

6. Reagents and products

6.1 Chemically pure grade products for analysis at least at 99%

6.1.1 Sodium sulphate or Potassium sulphate

6.1.2 L-tartaric acid

6.1.3 D,L- malic acid

6.1.4 Monohydrated citric acid

6.1.5 Succinic acid

6.1.6 D,L Lactic acid

6.1.7 Sodium dihydrogenophosphate

6.1.8 Sodium gluconate

6.1.9 Sodium chlorate

6.1.10 Dipicolinic acid

6.1.11 Cethyltrimethyl ammonium bromure

6.1.12 Acetonitrile for HPLC
6.1.13 Deionized ultra filtered pure water
6.1.14 Sodium hydroxide

6.2 Solutions

6.2.1 Calibration stock solution

- Solution in pure water (6.1.13) of different acids and sulphates to be measured (6.1.1 to 6.1.6) at exact known concentrations ranging between 800 and 1200 mg l^{-1}
- Solution to be kept at +5° C for a maximum of 1 month

6.2.2 Internal standard solution

- Solution of sodium chlorate (6.1.9) at approximately 2 g l^{-1} in pure water (6.1.13)
- Solution to be kept at +5° C for a maximum of 1 month

6.2.3 Calibration solution to be injected

In a graduated 50-ml class "A" flask using class "A" pipettes, deposit:

- 2 ml of calibration solution (6.2.1)
- 1 ml of internal standard solution (6.2.2)
- Adjust solution to 50 ml with pure water (6.1.13)

Homogenize by agitation
Solution to be prepared each day

6.2.4 Sodium hydroxide solutions

6.2.4.1 Sodium hydroxide solution M
In a 100-ml flask place 4g of sodium hydroxide (6.1.14)
Adjust with pure water (6.1.13)
Shake until completely dissolved.

6.2.4.2 sodium hydroxide solution 0.1M
In a 100 ml flask place 10 ml of sodium hydroxide M (6.2.4.1)
Adjust with pure water (6.1.13)
Homogenise.

6.2.5 Electrophoretic buffer solution

In a graduated 200-ml class "A" flask, place:

- 0.668 g of dipicolinic acid (6.1.10)
- 0.364 g of cethyltrimethyl-ammonium bromide. (6.1.11)

- 20 ml of acetonitrile (6.1.12)
- Approximately 160 ml of pure water (6.1.13)
- Shake until complete dissolution (if need be, place in ultrasound bath to eliminate any aggregated material)
- Bring M sodium hydroxide solution M (6.2.4.1) to pH 5.64 and then 0.1M sodium hydroxide (6.2.4.2)
- Make up to 200 ml with pure water (6.1.13)
- Homogenize by agitation
- Solution to be prepared each month.
- Store at laboratory temperature.

This buffer can be replaced by equivalent commercial product.

7. Apparatus

The capillary electrophoresis apparatus required for these determinations basically comprises:

- A sample changer
- Two bottles (phials) containing the buffer
- A non-coated silica capillary tube, internal diameter 50 µm, length 60 cm, between the inlet of the capillary tube and the detection cell. Depending on the apparatus, an additional 7 to 15 cm are required so that the outlet of the capillary tube is immersed in the centre of another bottle
- A high voltage DC power supply capable of outputting voltages of -30 to + 30 kV. The electrodes immersed in the two bottles where the outlets of the capillary tube emerge are connected to the terminals of the generator
- A pressurization system capable of circulating the buffer in the capillary tube and enabling the injection of the test specimen
- A UV detector
- A data acquisition system

8. Preparation of samples for tests

8.1 Degassing and filtration

The samples rich in carbon dioxide are degassed for 2 min with ultra-sound. Turbid samples are filtered on a membrane with an average pore diameter of 0.45 µm.

8.2 Dilution and addition of internal standard

Place 2 ml of sample in a graduated flask of 50 ml. Add 1 ml of internal standard solution (6.2.2). Adjust to 50 ml with pure water (6.1.13)

Homogenize.

9. Procedure

9.1 Conditioning of a new capillary tube (for example)

- Circulate pure water (6.1.13) in the opposite direction (from the outlet of the capillary tube towards the inlet flask) for 5 min at a pressure of approximately 40 psi (2.76 bar or 276 kPa)
- Circulate 0.1M sodium hydroxide (6.2.4.2) in the opposite direction for 5 min at the same pressure
- Circulate pure water (6.1.13) in the opposite direction (from the outlet of the capillary tube towards the inlet flask) for 5 min at the same pressure
- Repeat the cycle of circulating pure water, 0.1M sodium hydroxide , pure water
- Circulate electrophoretic buffer (6.2.5) in the opposite direction for 10 min

9.2. Reconditioning a capillary tube in the course of use (optional)

When the quality of the separations becomes insufficient, new conditioning of the capillary tube is essential. If the results obtained are still not satisfactory, change capillary tube and condition it.

9.3. Checking the quality of the capillary tube (optional)

Analyse 5 times the calibration solution under the recommended analysis conditions.

9.4. Separation and detection conditions (for example)

- Light the detector lamp 1 hour before the start of the analyses

- Rinse the capillary tube by circulating the buffer for 3 min in the opposite direction at a pressure of 40 psi
- Pressure inject the samples (prepared at 8.1) at 0.5 psi for 6 to 15 seconds
- The polarity is regulated such that the anode is on the detector side
- Apply a voltage from 0 to 16 kV in 1 min then 16 kV for approximately 18 min (the duration of separation can slightly vary depending on the quality of the capillary tube)
- Maintain the temperature at + 25 C°
- Detection in the ultraviolet is at 254 Nm
- Rinse the capillary tube by circulating the electrophoretic buffer (6.2.5) for 2 min in the opposite direction at a pressure of 40 psi
- Change the electrophoretic buffer (6.2.5) contained in the inlet and outlet flasks at least every 6 injections

9.5 Order that the analyses are to be carried out (for example)
Change the electrophoretic buffer (6.2.5) for every new series of analyses

- The sequence of analysis in order contains:Analysis of reference material (external concentration sample known for different acids to be measured)
- Analysis of samples prepared in 8.2,chromatograms should look like those presented in appendix A
- At the end of analysis, rinse with pure water (6.1.13) 10 mm in opposite direction (outlet of capillary tube toward the inlet)
- Switch off detector lamp

10. Calculation of results

The calculations are based on the surface areas of the peaks obtained after integration.
The surface areas of the peaks of the aqueous solutions of the calibration solution (6.2.3) are corrected by taking into account the variations in the surface areas of the peaks of the internal standard. The response factor for each acid is calculated.

The surface areas of the peaks of the internal standard and the peaks of the aqueous solutions are read off for each sample. The surface areas of the aqueous solutions to be assayed are recalculated by taking into account variations in the surface areas of the peaks of the internal standard a second time in order to obtain "corrected" surface areas.
The corrected surface areas are then multiplied by the value of the corresponding response factor.
It is possible to use an automatic data management system, so that they can be controlled in accordance with the principles described above as well as with the best practices (calculation of response factor and / or establishment of a calibration curve).

CALCULATION FORMULA

The abbreviations used to calculate the concentration in an acid are given in the following table:
Surfaces are expressed by the whole numbers of integration units.
The concentrations are given in g/L (only indicate to two decimal places).

ABBREVIATIONS		
	REFERENCE SOLUTION	**SAMPLE**
SURFACE AREAS OF TITRATED PEAKS	S_{AR}	S_{AE}
INTERNAL STANDARD PEAKS	S_{EIR}	S_{EIE}
CONCENTRATION	C_{AR}	C_E

The calculation formula is:

$$C_E = \frac{C_{AR} \times S_{AE} \times S_{EIR}}{S_{AR} \times S_{EIE}}$$

Whenever possible, a duplicate analysis is used to highlight a possible error in the recognition of the peaks or inaccuracy of integration. The sample changer makes it possible to carry out the analyses in automatic mode day and night.

11. Precision

11.1 Organization of the tests

Interlaboratory trials and correspondent results are described in appendix B1 and B2

11.2 Measurement of precision

ASSESSEMENT OF PRECISION BY INTERLABORATORY TRIALS

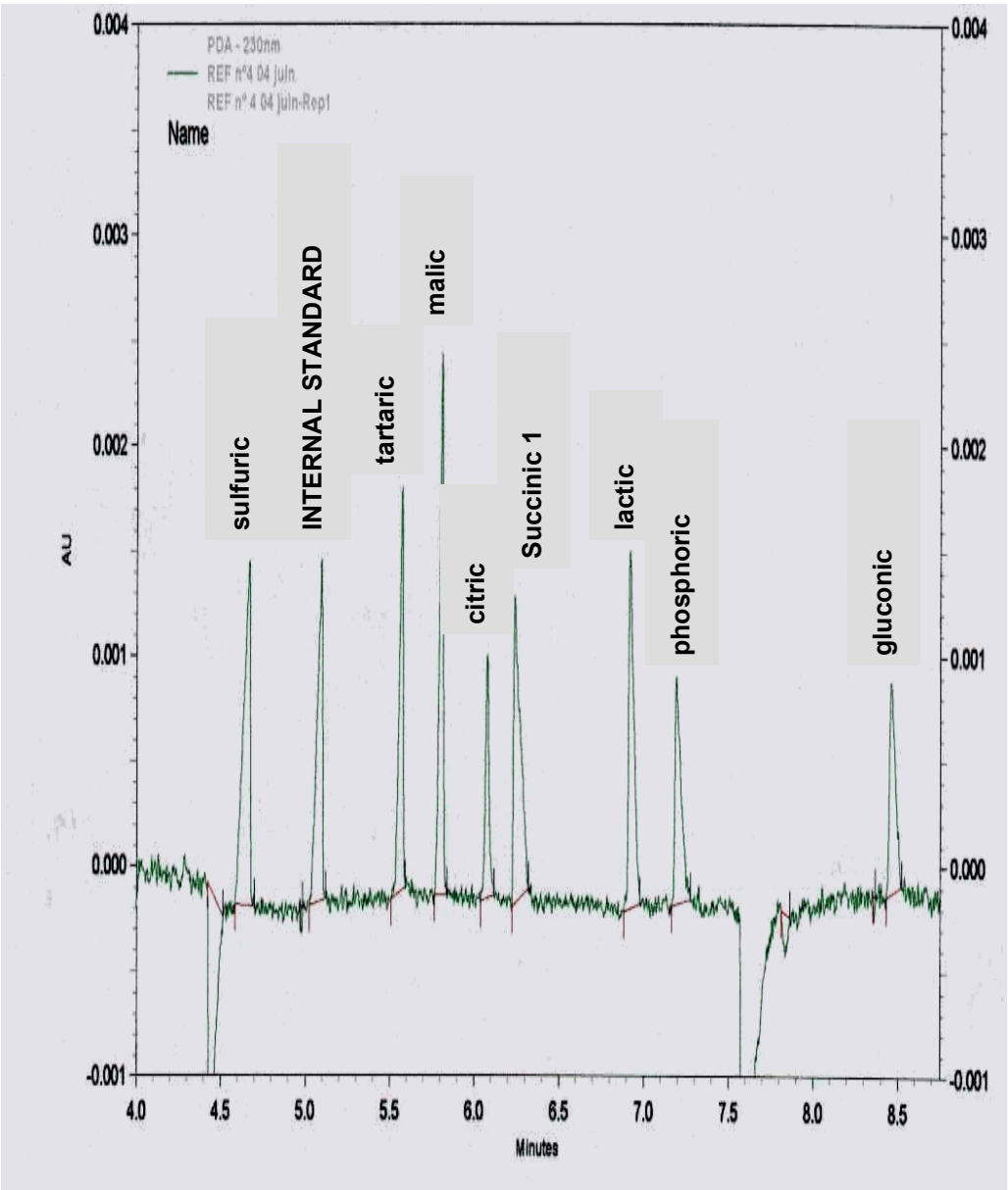
Number of laboratories involved: 5

Results expressed in mg / L

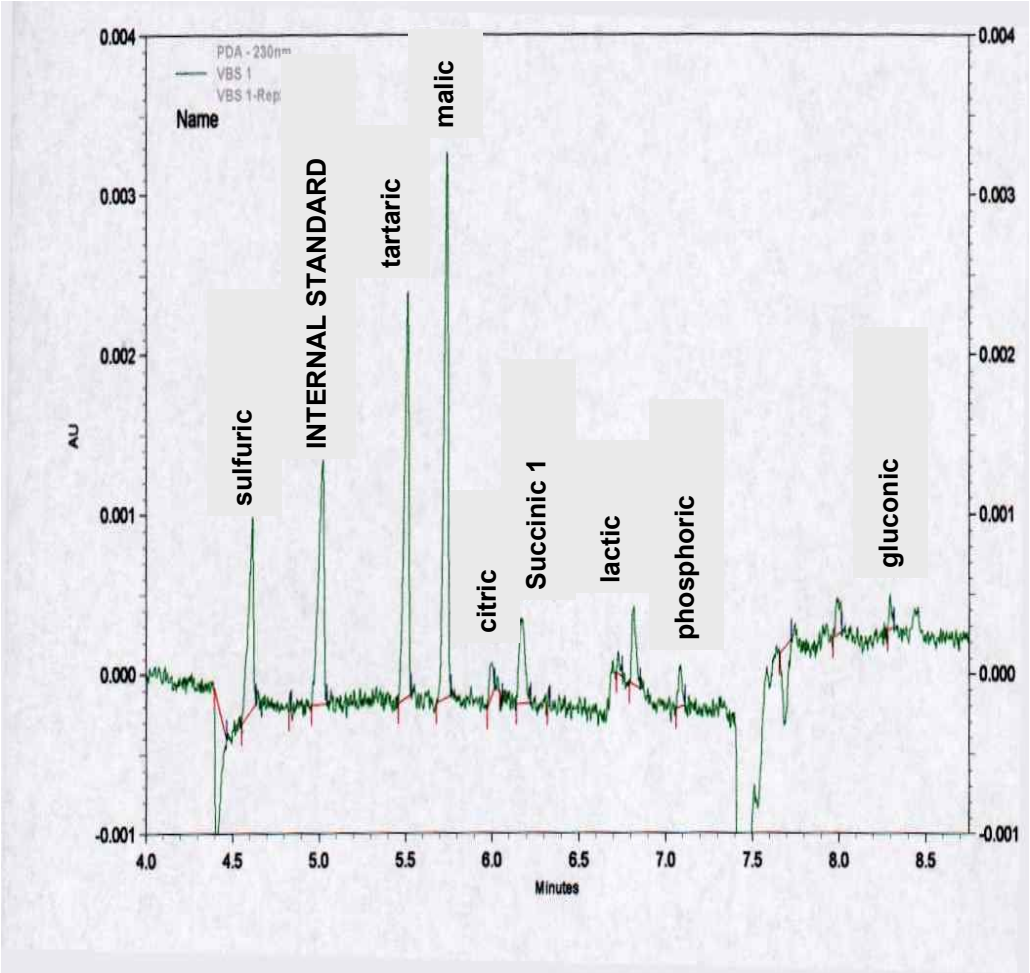
	TARTRIC ACID	**MALIC ACID**	**LACTIC ACID**
Average values of concentrations	1395	1884	1013
Average values of standard deviations in repeatability	38	54	42
Average values of standard deviations in reproducibility	87	113	42

12. APPENDICES

APPENDIX A
ELECTROPHOREGRAM OF A STANDARD SOLUTION OF ACI



ELECTROPHEROGRAM OF A WINE



APPENDIX B1

Statistic data obtained from the results of the interlaboratory trials (2006)

According to ISO 5725-2:1994, the following parameters have been defined during an interlaboratory trial. This trial has been conducted by the laboratory « Direction Générale de la Consommation et de la Répression des Fraudes de Bordeaux (France). »

Year of interlaboratory trial: 2006
Number of laboratories: 5
Number of samples: 8 double-blind (2 dry white wines, 2 sweet white wines, 2 rosé wines and 2 red wines)

INTERLABORATORY TESTS

Determination of TARTRIC ACID by capillary electrophoresis

Identification of the sample	dry white wines		liquorous white wines		rosé wines		red wines	
	A + D	**B + C**	**E + F**	**G + H**	**I + J**	**K + L**	**M + N**	**O + P**
Number of laboratories taking part	5	5	5	5	5	5	5	5
Number of results accepted	5	5	4	5	5	5	4	4 5
Average value in mg/l	1943	2563	1440	255	553	1885	1373	1148
Accepted value in mg/l	1943	2563	1387	2217	1877	1593	1370	1830
Standard deviation of repeatability (Sr)	27	25	106	23	40	31	25	24
Repeatability coefficient of variation	1.4	1,0	7.7	1,0	2.2	1.9	1.8	1.3
Limit of repeatability (r)	77	70	298	65	113	86	70	66
Standard deviation of reproducibility (SR)	96	128	174	80	57	55	52	53
Reproducibility coefficient of variation in %	4.9	5	12.6	3.6	3	3.5	3.8	2.9
Reproducibility limit (R)	268	359	488	223	160	154	145	148

INTERLABORATORY TESTS

Determination of MALIC ACID by capillary electrophoresis

Identification of the sample	dry white wines		liquorous white wines		rosé wines		red wines	
	A + D	**B + C**	**E + F**	**G + H**	**I + J**	**K + L**	**M + N**	**O + P**
Number of laboratories taking part	5	5	5	5	5	5	5	5
Number of results accepted	5	5	5	5	5	5	4	4 5
Average value in mg/l	2571	1602	1680	2539	3524	2109	173	869
Accepted value in mg/l	2571	1602	1680	2539	3524	2109	177	869
Standard deviation of repeatability (Sr)	54	19	113	35	61	109	7	32
Repeatability coefficient of variation	2.1	1.2	6.7	1.4	1.7	5.2	4.1	3.7
Repeatability limit (r)	151	54	315	99	170	305	20	89

INTERLABORATORY TESTS

Determination of LACTIC ACID by capillary electrophoresis
results in mg/l

Sample identification	dry white wines		liquorous white wines		rosé wines		red wines	
	A + D	**B + C**	**E + F**	**G + H**	**I + J**	**K + L**	**M + N**	**O + P**
Number of laboratories taking part	5	5	5	5	5	5	5	5
Number of results accepted	4	5	5	5	5	5	4	4 5
Average value in mg/l	659	1324	258	255	553	1885	2066	1148
Accepted value in mg/l	650	1324	258	255	553	1885	2036	1148
Standard deviation of repeatability (Sr)	20	42	20	39	27	99	75	16
Repeatability coefficient of variation	3.1	3.2	7.8	15.1	4.8	5.3	3.7	16,0

APPENDIX B2

Statistic data obtained from the results of the interlaboratory trials (sulphates 2010)

According to ISO 5725-2:1994, the following parameters have been defined during an interlaboratory trial. This trial has been conducted by the laboratory "Instituto dos Vinhos do Douro e do Porto (Portugal)"

Year of interlaboratory trial: 2010-2011
Number of laboratories: 7 (one laboratorysent two sets of results obtained by means of two different instruments)
Number of samples: 6 double-blind

Indicators	White wine (A/G)	Rosé (B/F)	Rosé (C/O)	Red wine (D/M)	Liquor wine (E/N)	Liquor wine (I/K)	White wine (H/Q)	Red wine (J/P)	Liquor wine (L)
Number of groups	7	7	6	7	8	7	7	7	8
Number of repetitions	2	2	2	2	2	2	2	2	2
Minimum (g/L K_2SO_4)	0,71	0,34	0,40	0,62	1,79	1,06	1,38	1,96	2,17
Maximum (g/L K_2SO_4)	0,88	0,54	0,52	0,75	2,40	1,35	1,70	2,30	2,85
Repeatability variation s_r^2	0,0012	0,0011	0,0001	0,0016	0,0063	0,0013	0,0036	0,0015	0,0053
Intergroup variation s_L^2	0,00148	0,00230	0,00163	0,00055	0,01952	0,01082	0,00668	0,01744	0,03552
Reproducibility variation s_R^2	0,0027	0,0034	0,0018	0,0022	0,0258	0,0122	0,0103	0,0189	0,0408
Mean (g/L K_2SO_4)	**0,78**	**0,43**	**0,44**	**0,69**	**2,01**	**1,19**	**1,49**	**2,15**	**2,41**
Standard deviation of Repeatability (g/LK_2SO_4)	**0,04**	**0,03**	**0,01**	**0,04**	**0,08**	**0,04**	**0,06**	**0,04**	**0,07**
Limit r (g/L K_2SO_4)	0,100	0,093	0,031	0,115	0,224	0,103	0,170	0,109	0,206
Repeatability CV	5%	8%	3%	6%	4%	3%	4%	2%	3%
Standard deviation of Reproducibility (g/L K_2SO_4)	**0,05**	**0,06**	**0,04**	**0,05**	**0,16**	**0,11**	**0,10**	**0,14**	**0,20**
Limit R (g/L K_2SO_4)	0,148	0,165	0,118	0,132	0,454	0,312	0,287	0,389	0,572
Reproducibility CV	7%	14%	10%	7%	8%	9%	7%	6%	8%
HORRAT	1,1	2,1	1,5	1,1	1,6	1,7	1,3	1,3	1,7

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KLAMPF C.F. (1999): Analysis of organic acids and inorganic anions in different types of beer using capillary zone electrophoresis. J. Agric. Food Chem., 47, 987-990.

Method OIV-MA-AS313-20 Type II / IV method

Determination of sorbic, benzoic and salicylic acid content in wine by the use of high-performance liquid chromatography

(Resolution OENO 6/2006; OIV/OENO 377/2009; OIV-OENO 687-2023)
(Type II for sorbic acid; Type IV for benzoic and salicylic acids)

1. Introduction

Sorbic acid and its potassium salt constitute an antiseptic that can be used in wine-making, although some countries will not tolerate even traces of it, the main reason being the smell of geraniums that develops when sorbic acid is broken down by lactic acid bacteria. Benzoic acid and salicylic acid are still prohibited in wine, but are used in other beverages.

2. Scope

All wines and grape musts, especially those likely to contain only traces of sorbic, benzoic or salicylic acid.

3. Principle

The antiseptics are determined using HPLC by direct injection of the sample into a column functioning by isocratic reversed-phase partition chromatography with ultraviolet detection[5].

4. Products

4.1 Micro-filtered fresh water (e.g. resistivity greater than 18.2 MΩ)
4.2 Pure tetrahydrofuran
4.3 Pure methanol
4.4 0.1 M hydrochloric acid (prepared by means of dilution funnels)
4.5 Water with a pH of 2: adjust the pH of 650 ml of water (4.1) to pH2 using a pH meter (5.5) and by adding 0.1 M hydrochloric acid drop by drop without stirring (4.4)
4.6 Elution solution: mix 650 ml of water at pH2 (4.5) with 280 ml of methanol (4.3) and 7 ml of tetrahydrofuran (4.2)

[5] For sorbic acid analysis the column and the wavelength are given as an example.

Note: it is likewise possible to use other elution solvents, for example: 80% ammonium acetate 0.005M (0.38 g/l) adjusted to pH 4 with pure acetic acid + 20% acetonitrile.

4.7 Pure sorbic acid

4.8 Pure benzoic acid

4.9 Pure salicylic acid

4.10 Absolute alcohol

4.11 50% vol. hydro-alcohol solution or other suitable concentration: put 500 ml of absolute alcohol (4.10) into a 1-litre flask and dilute to volume with distilled water (4.1)

4.12 Stock solution of sorbic acids at 500 mg/l: dissolve 50 mg of sorbic acids (4.7), benzoic (4.8) and salicylic (4.9) acids in 100 ml of the 50% vol. hydro-alcohol solution (4.11)

4.13 Sorbic, benzoic and salicylic acid surrogate solutions: dilute the stock solution (4.12) in the hydro-alcohol solution (4.11) in such a way as to obtain the final concentrations required. For example, for a solution of

- 200 mg/l: put 20 ml of stock solution (4.12) into a 50-ml flask and top up to the filling mark with 4.11.
- 1 mg/l: put 2 ml of stock solution (4.12) into a 50-ml flask and top up to the filling mark with 4.11.

Intermediate solutions may be produced in the same way to satisfy calibration requirements.

5. Apparatus

5.1 Laboratory glassware, especially pipette and volumetric flasks

5.2 Ultrasonic bath

5.3 Vacuum filtration device for large volumes (1 litre) using membrane filters with a pore diameter of under 1 µm (generally 0.45 µm)

5.4 Mini-filter for samples (1 to 2 ml) using membrane filters with a pore diameter of under 1 µm (generally 0.45 µm)

5.5 pH meter

5.6 Isocratic-mode liquid phase chromatograph equipped with an injection system for small volumes (for example), 10 or 20-µl loop valve.

5.7 Detector capable of functioning at an ultraviolet rating of 235 nm (see footnote 1) and fitted with a circulating tank for HPLC (for example, 8 µl for 1 cm of optical thickness)

5.8 A 5-µm stationary phase HPLC column of the silica-type with immobilisation by octadecyl groups (C18), length 20 cm, inside diameter 4 mm (see footnote 1)

5.9 Data acquisition system

6. Preparation of samples and the elution solvent

6.1 Filter the samples to be analysed using the mini-filter (5.4)
6.2 Degas the elution solvent (4.6) for 5 minutes using the ultrasonic bath (5.2) or any other suitable way
6.3 Filter the solvent using the device in (5.3)

7. Procedure

7.1 Column conditioning. Prior to injection, start the pump and rinse the column with the solvent for at least 30 minutes.
7.2 Inject one of the surrogate solutions (4.13) to check system sensitivity and ensure the resolution of the peaks of the substances to be analysed is satisfactory.
7.3 Inject the sample to be analysed. It is possible to analyse an identical sample, to which the acids sought have been added (adapt the amount added to the quantity observed during the previous analysis - for 1 mg present, add 1 mg, and so on).

Check the resolution of the peaks of the acids sought with the peaks of the wines (normally, there are none in this zone)

8. Calculation

Having located the peaks of the acids to be determined in the sample, compare the peak area with those of the acids of a surrogate solution (4.13) with a known concentration C.
For example, let s be the peak area of the acid to be determined, and S is the peak area of the solution (4.13) with concentration C

$$X_{\text{in the sample}} = C \times \frac{s}{S} \qquad \text{in mg/l}$$

It is also possible to use a calibration curve and determine the concentration by interpolation.

9. Characteristics of the method

9.1 For sorbic acid

Interlaboratory trial and correspondent results are described in Annex A.

- r = 0.0148 x + 0.5498
- R = 0.0936 x + 1.5542
- x: sorbic acid concentration (mg/l)

9.2 For benzoic and salicylic acids

	Benzoic acid	Salicylic acid
Linearity range	0 to 200 mg/l	0 to 200 mg/l
Accuracy (rate of recuperation)	> 90 %	> 90 %
Répétabilité : r*	3%	8%
Reproducibility: R*	9%	12%
Detection limit	3 mg/l	3 mg/l
Quantification limit	6 mg/l	7 mg/l
Uncertainty	12%	13%

Bibliography

- Dosage de l'acide sorbic dans les vins par chromatographie en phase gazeuse. 1978. BERTRAND A. et SARRE Ch., *Feuillets Verts O.I.V.,* 654-681.

- Dosage de l'acide salicylic dans les vins par chromatographie en phase gazeuse. 1978. BERTRAND A. et SARRE Ch., *Feuillets Verts O.l.V.,* 655-682.

- Dosage de l'acide benzoic, dans les sodas et autres produits alimentaires liquides, par chromatographie en phase gazeuse. 1978. BERTRAND A. et SARRE Ch. *Ann. Fals. Exp. Chim. 71, 761,* 35-39.

ANNEX A - Statistic data obtained from the results of the interlaboratory trials

The following parameters have been defined during an interlaboratory trial. This trial has been conducted by the laboratory " Instituto dos Vinhos do Douro e do Porto (Portugal). "

Year of interlaboratory trial: 2020

1. Laboratories: 23 laboratories from 14 different countries

HBLA und Bundesamt für Wein- und Obstbau Klosterneuburg	Austria
Department of Agriculture	Cyprus
Czech Agriculture and Food Inspection Authority	Czech Republic
Comité Interprofessionnel du Vin de Champagne	France
Laboratoires Dubernet	France
Service Commun des Laboratoires-Laboratoire de Montpellier	France
Service Commun des Laboratoires-Laboratoire de Bordeaux	France
Landesuntersuchungsamt, Institut für Lebensmittelchemie	Germany
Lebensmittel- u. Veterinärinstitut Braunschweig/Hannover	Germany
National Food Chain Safety Office	Hungary
Unione Italiana Vini Servizi	Italy
Vassanelli Lab s.r.l.	Italy
AsureQuality Ltd Laboratory Services	New Zealand
Arcus Norway AS	Norway
ASAE-Autoridade de Segurança Alimentar e Económica	Portugal
Instituto dos Vinhos do Douro e do Porto	Portugal
RUDN University	Russian Federation
National Laboratory of Health, Environment and Food (Novo Gorica)	Slovenia
National Institute of Chemistry	Slovenia
National Laboratory of Health, Environment and Food (Novo Mesto)	Slovenia

Laboratorio Arbitral Agroalimentario.	Spain
Estación Enológica de Haro- La Rioja	Spain
Cukurova University Food Engineering Dept.	Turkey

2. Number of samples: 6 samples in duplicate

- 2 white wines: A/G, C/K
- 1 white wine - late harvest: I/O
- 1 red wine: D/M
- 1 rosé wine: B/F
- 1 red fortified wine (alcoholic strength - 19 % vol.): E/N

3. Analytical conditions

The analytical conditions are summarized in Table 1.

4. Individual results

The individual results for Sorbic Acid (mg/L) are summarized in Table 2.

5. Data Analysis

- Statistical analysis was performed according to the recommendations of the OIV to obtain precision data
- The values below the quantification limit were treated as normal values.
- Determination of outliers was assessed by Cochran and Grubbs test
- The results are summarized in tables 3 and 4.
- Repetibility and reproducibility limit according to sorbic acid concentration are represented in figure 1.
- The Z-score for each laboratory was evaluated against the global values for the different samples.The results are in figure 2.

TABLE 1 – ANALYTICAL CONDITIONS

Lab. Code	*Calibration Curve*	*Column*	*Detection*	*Mobile phase*	*Flow rate (mL/min)*	*Column temperature (°C)*	*Injection volume (µL)*
1	yes	Altima C18LL 5 µm 250 x 4.6 mm	λ = 235 nm	Water pH=2.0 / MeOH/ THF (650/280/70)	1	ambient	20
2	yes	C18 250 x 4.6 Luna Phenomenex	λ = 235 nm	0.389 Ammonium acetate in 1L water + 250 mL ACN (pH=4 adjusted w/ CH_3COOH)	1.25	35	20
3	yes	Poreshell 120 EC-C18.150 x 4.4 mm; 2.7 µm	λ = 235 nm	A: H2O 0.7 % THF (pH=2) /B: MeOH (80 % A e 20 % B)	1.5	40	5
4	yes	RP-18 (250-4)	λ = 254 nm	Ammonium acetate buffer (pH 4.4): MeOH = 70:30	1	40	20

6	yes	PerfectSil Target ODS-3 HD. 5 µm. 250 x 4.6 mm	λ = 235 nm	ACN:Buffer solution (35:65); Buffer solution = Ammonium acetate (1.8 g/l) + Acetic acid. pH=4.3	1	40	2
7	yes	PoroShell C18 4.6 x 75 mm; 2.7 µm	λ = 235 nm	Isocratic: 80 % H_2O acid. with sulfuric acid; 20 % ACN	1.5	25	20
8	yes	Rezex-ROA- Organic acid H+ (8 %) -300 x 7.8 mm	λ = 254 nm	Elution solvent Sulfuric acid = 0.025M	1	90	50
9	yes	HICROM Ultrasphere 5 µm ODS column. 250 x 4.6 mm	λ = 235 nm	650 mL H_2O pH 2 with 280 mL MeOH + 7 mL THF	0.6	25	20
10	yes	Teknokroma Trace Excel 120 ODSB 5 µm. 20 x 0.4 cm	λ = 235 nm	650 mL H_2O pH 2 (HCl 0.1M) + 280 mL MeOH + 7 mL THF	1	24	20
11	yes	C18 5 µm. 200x4 mm	λ = 235 nm				

12	yes	RP18 Chromolith 100 x 4.6 mm; 5 µm	λ = 235 nm	770 Water pH=2.3 (0.8 mL H_3PO_4): isopropanol 200 mL. ACN 30 mL	1	25	10
13	yes	Phenomenex Gemini 5 µm. C18; 150 x 2 mm	λ = 240 nm	0.925 g ammonium acetate + 1.2 L H_2O + 0.8 L MeOH (pH 5.5 acetic acid)	0.4	40	10
15	yes	C18 5 µm. 200 x4 mm	λ = 235 nm	90 % v/v Water 15mL Acetic acid+ 15 g ammonium acetate 10 % v/v MeOH	1	40	10
16	yes	Prodigy 5 µm ODS-3 100. 150 x 4.6 mm w/ C18 guard column	λ = 261 nm	80 % v/v H_2O pH=4 (pure acetic acid) + 0.005M ammonium acetate 20 % v/v ACN	1.2	40	50
17		Kinetex; RP-C18 150x4.6 mm; 5 µm	λ = 230 nm	11/89. MeOH: Buffer (Buffer = 3.0 g KH2PO4 +3.0 g K2HPO4+ 100 mL water)	0.6	40	6

18	yes	C18 Luna. Phenomenex	λ = 224 nm	95 % phosphate buffer: 5 % ACN	1.2	ambient	20
19	yes	Phenomenex Aqua C18 125A 250 x 4.6 mm; 5 µm	λ = 235 nm	0.005M ammonium acetate (pH 4): ACN = 8:2	1	25	2
20	yes	Zorbax Eclipse XDB-C18 4.6 x 150 mm. 5 µm	λ = 235 nm	650 mL H_2O pH 2 (HCl 0.1M) + 280 mL MeOH + 7 mL THF	1	ambient	5
21	yes	RP-18 Nucloedur 100-C18. 3 µm. 50 mm	λ = 262 nm	A:B = 80:20; A: ammonium acetate. 0.002 mol/l. pH= 4.1 (adjusted by conc. Acetic acid); B: MeOH/ACN 2:1	1	60	6
22	yes	HYPERSIL ODS 250X4.6 mm. 5 µm	λ = 235 nm		1.8	40	20

23	yes	RP-C18. 150 x 4.6 mm; 5 µm	λ = 235 nm		1	25	20
24	yes	RP-C18 Purospher.125 x 4 mm; 5 µm	λ = 235 nm	MeOH / 0.02M sodium acetate buffer pH 4.4 = (30/70 v/v)	0.8	40	5
26	yes	Kinetex XB-C18 100 x 4.6 mm; 2.6 µm	λ = 254 nm	Water 0.1 % TFA. ACN 0.1 % TFA	1.5	30	10

TABLE 2 - INDIVIDUAL RESULTS FOR SORBIC ACID (mg/L)

	White wine (A)		Rosé (B)		White Wine (C)		Red wine (D)		Fortified wine (E)		Rosé (F)		White wine (G)		White wine (I)		White wine (K)		Red wine (M)		Fortified wine (N)		White wine(O)	
Laboratory code	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	36.3	35.5	226.0	221.0	88.0	86.0	5.2	5.3	1.7	1.8	226,0	224.0	36.1	35.9	125.0	124.0	90.0	87.0	4.7	4.6	1.8	2.0	126.0	126.0
2	33.1	33.2	210.7	208.3	80.9	81.4	4.5	4.4	1.7	2.4	210.4	209.5	33.0	32.5	116.0	114.9	81.2	80.6	4.3	5.7	2.0	2.4	116.3	116.1
3	34.1	34.5	216.8	215.5	84.7	84.5	5.3	5.2	2.5	2.3	216.3	217.2	34.5	34.3	119.8	120.1	84.5	84.3	5.7	5.7	2.4	2.4	120.5	120.2
4	34.7	35.0	223.1	224.7	89.3	88.6	5.1	5.1	2.1	2.2	220.0	221.6	34.5	34.8	123.1	123.2	86.7	86.4	5.2	5.2	2.1	2.1	123.1	123.5
6	34.3	34.6	214.2	215.2	83.9	84.0	5.5	5.6	1.9	2.0	215.0	215.6	34.6	34.7	120.6	120.7	83.6	83.9	6.2	6.5	2.0	2.1	120.1	121.1

7	34,0	35.0	217.0	218.0	84.0	85.0	5.0	6.0	3.0	3.0	218.0	218.0	35.0	35.0	122.0	123.0	86.0	84.0	5.0	5.0	2.0	2.0	122.0	122.0
8	33.9	33.4	216.8	218.5	86.2	83.8	4.5	4.5	2.0	2.0	218.8	215.5	35.2	33.9	122.0	118.9	84.2	84.3	4.3	4.1	2.0	2.0	124.5	123.3
9	32.5	32.5	197.4	198.2	81.0	80.7	3.4	3.4	1.4	1.4	197.0	195.8	32.1	32.2	112.5	112.2	79.3	79.4	3.3	3.3	1.3	1.2	113.5	113.7
10	34.1	34.1	216.0	217.0	83.7	83.6	5.7	5.6	2.2	2.1	216.0	216.0	34.2	34.0	118.0	119.0	83.4	83.4	5.6	5.8	2.1	2.1	120.0	119.0
11	36.8	37.0	227.2	229.9	90.2	90.4	4.6	4.8	2.1	1.9	224.2	225.6	37.1	36.8	127.4	127.0	88.4	89.0	4.0	4.3	2.1	1.9	128.0	128.5
12	31.0	31.0	207.0	202.0	78.0	78.0	4.0	3.0	2.0	2.0	209.0	209.0	32.0	32.0	111.0	111.0	79.0	78.0	4.0	5.0	2.0	2.0	109.0	109.0
13	35.0	35.0	219.0	220.0	86.0	87.0	6.0	6.0	3.0	3.0	220.0	220.0	35.0	36.0	124.0	124.0	85.0	87.0	7.0	7.0	3.0	3.0	123.0	123.0
15	32.0	32.0	200.0	199.0	79.0	78.0	4.0	4.0	1.7	1.6	200.0	199.0	32.0	32.0	111.0	111.0	79.0	78.0	4.0	4.0	1.7	1.6	111.0	111.0
16	31.3	29.1	213.1	214.2	84.7	84.2	4.3	4.3	1.9	1.9	217.3	216,0	30.3	31.0	120.1	119.6	83.5	84.2	4.2	4.6	2.0	2.0	119.3	120.1
17	35.6	35.4	217.8	221.4	87.0	86.0	5.2	5.4	2.3	2.4	215.5	219.5	35.6	35.3	122.2	123.4	85.9	86.5	5.4	5.5	2.7	2.5	122.3	123.9
18	35.0	37.0	235.0	246.0	88.0	85,0	6.1	5.2	7.5	7.7	220.0	209.0	36.0	37.0	124.0	125.0	94.0	88.0	6.5	6.6	7.8	7.4	127.0	128.0
19	31.0	30.0	183.0	187.0	71.0	72.0	8.0	8.0	-2.0	0.0	177.0	179.0	30.0	27.0	105.0	106.0	67.0	71.0	4.0	5.0	-1.0	-1.0	116.0	115.0
20	36.2	34.8	217.4	217.0	84.9	84.4	7.1	8.9	1.9	2.7	219.0	217.1	35.1	34.4	121.2	119.0	85.8	84.3	5.6	5.9	2.4	2.3	121.9	119.2
21	32.2	32.2	204.1	204.2	79.3	79.1	4.3	4.4	1.9	2.0	204.9	204.4	32.6	32.3	111.9	112.4	78.6	78.7	4.3	4.4	1.9	2.0	115.0	114.2
22	34.0	34.0	216.0	216.0	83.0	83.0	6.0	6.0	3.0	3.0	216.0	217.0	34.0	34.0	121.0	121.0	83.0	83.0	6.0	6.0	3.0	3.0	121.0	121.0
23	35.3	35.0	219.3	217.6	87.3	86.1	4.1	4.5	1.6	1.7	219.3	217.9	35.4	35.9	124.3	123.0	87.0	86.0	3.9	4.1	1.6	1.8	124.6	123.5
24	34.2	34.0	214.0	216.0	81.8	82.1	4.1	4.1	2.7	2.8	215.0	215.0	33.7	33.8	117.0	118.0	83.7	83.4	4.1	4.1	2.8	2.7	119.0	119.0
26	35.1	35.2	214.4	214.5	86.2	86.2	5.0	5.1	2.2	2.2	214.9	214.2	35.0	35.1	122.3	122.5	86.0	86.3	5.1	5.1	2.2	2.2	122.2	122.5

TABLE 3- RESULTS FOR SORBIC ACID (mg/L)

	White wine		Rosé		White wine		Red wine		Fortified wine		White wine	
Laboratory code	A	G	B	F	C	K	D	M	E	N	I	O
1	35.90	36.00	223.50	225.00	87.00	88.50	5.25	4.65	1.75	1.90	124.50	126.00
2	33.15	32.25	209.50	209.95	81.15	80.80	4.45	5.00	2.05	2.20	115.45	116.20
3	34.30	34.40	216.15	216.75	84.60	84.40	5.25	5.70	2.40[e)]	2.40[e)]	119.95	120.35
4	34.85	34.65	223.90	221.00	88.95	86.55	5.10	5.20	2.15	2.10	123.15	123.30
6	34.45	34.65	214.70	215.30	83.95	83.75	5.58[e)]	6.34	1.95[e)]	2.08[e)]	120.65	120.60
7	34.50	35.00	217.50	218.00	84.50	85.00	5.50	5.00	*3.00[b)]*	*2.00[b)]*	122.50	122.00
8	33.65	34.55	217.65	217.15	85.00	84.25	4.50	4.20	2.00	2.00	120.45	123.90

9	32.49	32.16	197.83	196.38	80.81	79.40	3.42	3.32	1.41	1.25	112.38	113.56
10*	34.10	34.10	216.50	216.00	83.65	83.40	5.66	5.68	2.15[c)]	2.13[c)]	118.50	119.50
11*	36.90	36.95	228.55	224.90	90.30	88.70	4.70	4.15	2.00[c)]	2.00[c)]	127.20	128.25
12	31.00	32.00	204.50	209.00	78.00	78.50	3.50	4.50	2.00	2.00	111.00	109.00
13	35.00	35.50	219.50	220.00	86.50	86.00	6.00	7.00	3.00	3.00	124.00	123.00
15*	32.00	32.00	199.50	199.50	78.50	78.50	4.00	4.00	1.65	1.65	111.00	111.00
16	30.20	30.65	213.65	216.65	84.45	83.85	4.28	4.38	1.94	1.96	119.85	119.70
17	35.53	35.44	219.61	217.53	86.51	86.19	5.30	5.47	2.38	2.61	122.82	123.12
18	36.00	36.50	240.50[b)]	214.50[b)]	86.50[b)]	91.00[b)]	5.65	6.55	*7.60[a)]*	*7.60[a)]*	124.50	127.50
19	30.50[b)]	28.50[b)]	185.00[a)]	98.00[a)]	71.50[a)]	69.00[a)]	8.00[b)]	4.50[c)b)]	-1.00[c)a)]	-1.00[c)a)]	105.50[b)]	115.50[b)]
20	35.50	34.75	217.20	218.05	84.65	85.05	8.00[b)]	5.75[b)]	2.30[c)]	2.35[c)]	120.10	120.55
21	32.20	32.45	204.15	204.65	79.20	78.65	4.36	4.36	1.94	1.94	112.15	114.60
22	34.00	34.00	216.00	216.50	83.00	83.00	6.00	6.00	3.00	3.00	121.00	121.00
23	35.15	35.65	218.45	218.60	86.70	86.50	4.30	4.00	1.65[c)]	1.70[c)]	123.65	124.05
24	34.10	33.75	215.00	215.00	81.95	83.55	4.10 [c)]	4.10[c)]	2.75[c)]	2.75 [c)]	117.50	119.00
26	35.13	35.06	214.45	214.58	86.21	86.15	5.07	5.07	2.17	2.17	122.37	122.35

a) Values rejected by Grubbs test on mean outliers

b) Values rejected by Cochran test variance outliers

c) Value identified by the laboratory below Quantification Limit

TABLE 4 - PRECISION DATA

Indicators	White wine (A/G)	Rosé (B/F)	White wine (C/K)	Red wine (D/M)	Fortified wine (E/N)	White wine (I/O)
Number of laboratories	22	21	21	21	20	22
Number of repetitions	2	2	2	2	2	2
Minimum (mg/L)	30.20	196.38	78.00	3.32	1.25	109.00

Maximum (mg/L)	36.95	228.55	90.30	7.00	3.00	128.25
Repeatability variance s_r^2	0.0942	1.5249	0.4191	0.1201	0.0037	0.9397
Intergroup variance s_L^2	2.6370	55.1140	9.7049	0.6418	0.1793	22.4914
Reproducibility variance s_R^2	2.7312	56.6389	10.1240	0.7619	0.1830	23.4311
Average (mg/L)	**34.16**	**214.72**	**83.96**	**4.92**	**2.15**	**120.07**
Repeatability standard deviation (mg/L)	0.31	1.23	0.65	0.35	0.06	0.97
Repeatability limit (mg/L)	0.869	3.495	1.832	0.981	0.172	2.743
Repeatability RSD %	0.9	0.6	0.8	7.0	2.8	0.8
Reproducibility standard deviation (mg/L)	1.65	7.53	3.18	0.87	0.43	4.84
Reproducibility limit (mg/L)	4.677	21.298	9.005	2.470	1.211	13.699
Reproducibility RSD %	4.8	3.5	3.8	17.7	19.9	4.0
HORRAT	**0.5**	**0.5**	**0.5**	**1.4**	**1.4**	**0.5**

FIGURE 1 – CORRELATION BETWEEN SORBIC ACID CONCENTRATION AND PRECISION

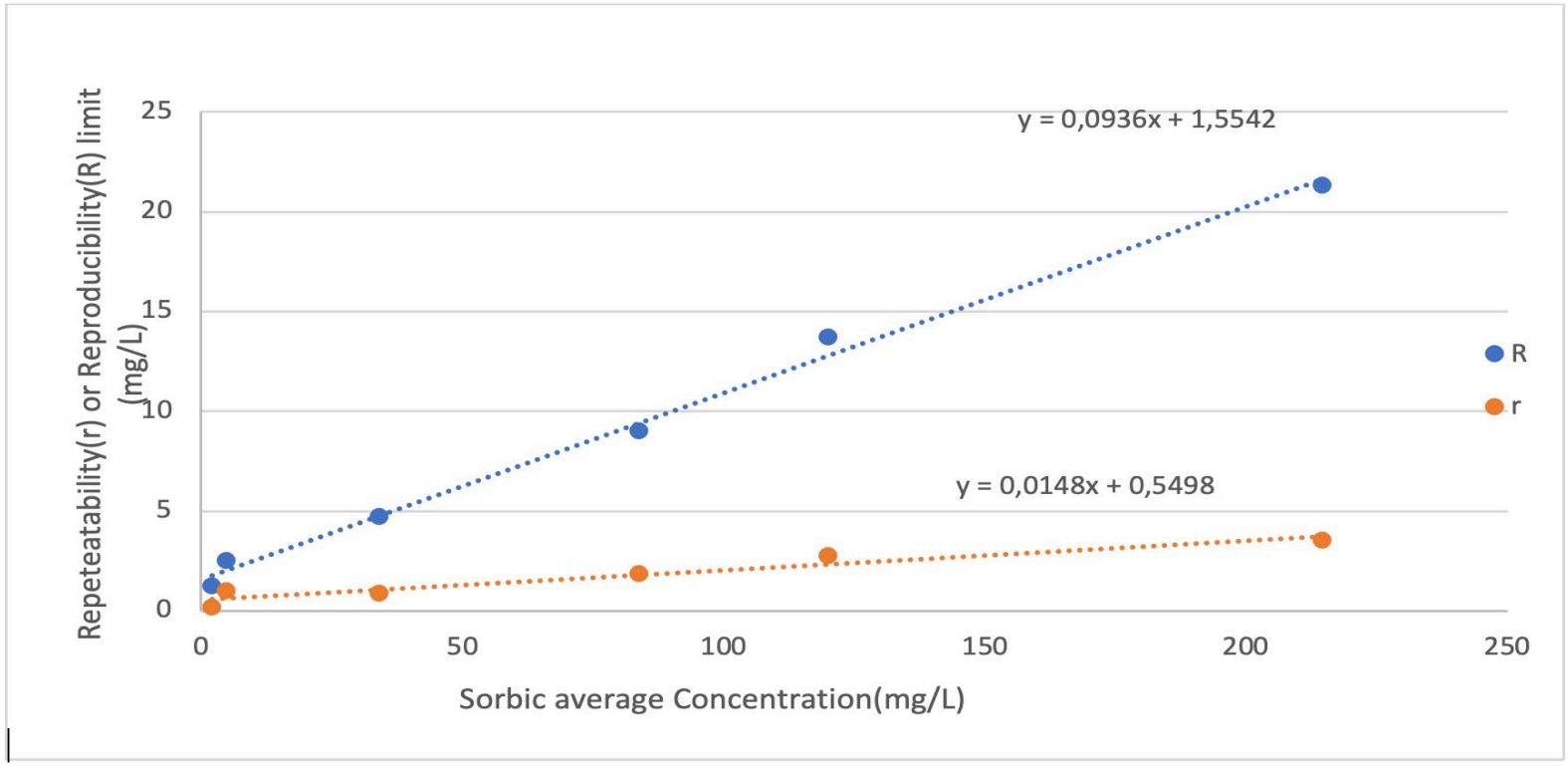
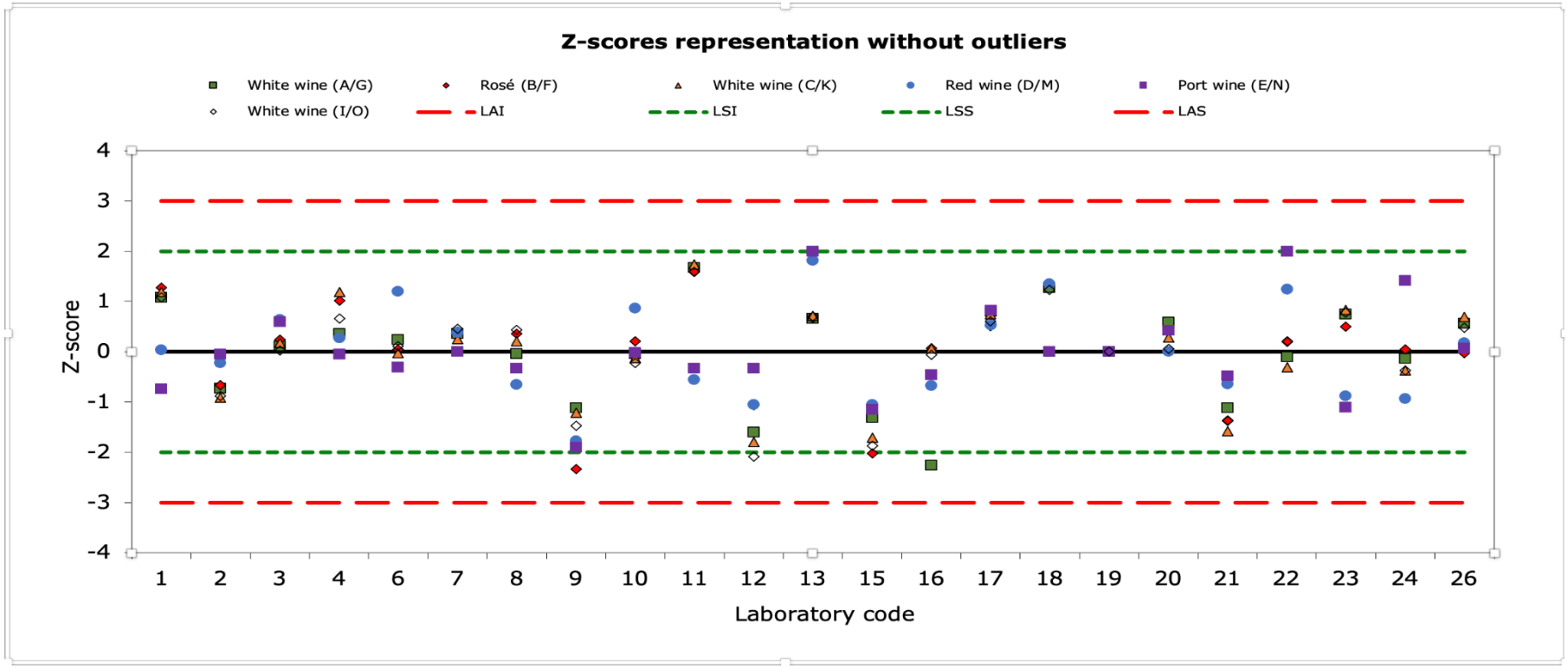


FIGURE 2 - Z-SCORES REPRESENTATION WITHOUT OUTLIERS



**LAI -lower action limit; LAS - upper action limit; LSI - lower warning limit; LSS - upper warning*

Method OIV-MA-AS313-21 Type IV method

Determination of the presence of metatartaric acid

(Resolution OENO 10/2007; OIV/OENO 377/2009)

1. Introduction

Metatartaric acid added to the wine to avoid tartaric precipitation is traditionally proportioned by the difference between the total tartaric acid following hot hydrolysis of metatartaric acid and natural tartaric acid preceding hydrolysis. However, taking into account the precision of the determination of tartaric acid, traces of metatartaric acid are not detectable by this method, and the additive, which is not accepted in certain countries, must therefore be characterised using a more specific method.

2. Scope

Wines likely to contain traces of metatartaric acid.

3. Principle

In relatively acid mediums, metatartaric acid forms an insoluble precipitate with cadmium acetate; it is the only one of all the elements present in must and wine to give such a precipitate .

Note: Tartaric acid is also precipitated with cadmium acetate, but only in the presence of an alcohol content greater than 25% vol. The precipitate redissolves in water, unlike the precipitate obtained with metatartaric acid.

The cadmium precipitate of metatartaric acid breaks down by heating with sodium hydroxide and releases tartaric acid. The latter produces a specific orange colour with ammonium metavanadate.

4. Reagents

4.1 Cadmium acetate solution at 5 p.100
- 4.1.1 Dihydrated cadmium acetate at 98%
- 4.1.2 Pure acetic acid
- 4.1.3 Distilled or demineralized water

4.1.4 Cadmium acetate solution: dissolve 5 g of cadmium acetate (4.1.1) in 99 mL of water (4.1.3) add 1 mL of pure acetic acid (4.1.2)

4.2 Sodium hydroxide 1M

4.3 Sulfuric acid 1M

4.4 Solution of ammonium metavanadate 2% w/v

4.4.1 Ammonium metavanadate

4.4.2 Trihydrated sodium acetate at 99%

4.4.3 Sodium acetate solution at 27 p. 100: dissolve 478 g of sodium acetate (4.4.2) in 1 liter of water (4.1.3)

4.4.4 Solution of ammonium metavanadate: dissolve 10 g of ammonium metavanadate (4.4.1) in 150 mL of sodium hydroxide 1 M (4.2) add 200 of the sodium acetate solution at 27 p. 100 (4.4.3) and fill to 500 mL with water (4.1.3)

4.5 Ethanol at 96% vol.

5. Apparatus

5.1 Centrifuge with a rotor capable of housing 50-mL bottles

5.2 Spectrometer capable of operating in the visible spectrum and of housing cuvets with an optical thickness of 1 cm.

6. Operating method

6.1 Centrifuge 50 mL of wine for 10 minutes at 11000 rpm

6.2 Take 40 mL of limpid wine using a test-tube and place the sample in a centrifuge flask

6.3 Add 5 mL of ethanol at 96% vol (4.5)

6.4 Add 5 mL of the cadmium acetate solution (4.1.4)

6.5 Mix and leave to rest for 10 minutes

6.6 Centrifuge for 10 minutes at 11000 rpm

6.7 Decant by completely reversing the flask (once) and throw away the supernatant.

In the presence of metatartaric acid, a lamellate precipitate is formed at the bottom of the tube.

In the absence of any precipitate, the sample will be regarded as free from metatartaric acid. In the contrary case, or if the presence of a light precipitate is to be established with certainty, proceed as follows:

6.8 Wash the precipitate once with 10 mL of water (4.1.3) in the form of an energetic jet towards the bottom of the tube in order to detach the precipitate from the bottom
6.9 Add 2 mL of cadmium acetate solution (4.1.4)
6.10 Centrifuge at 11000 rpm for 10 minutes then throw away the supernatant by completely reversing the tube (once)
6.11 After adding one mL of sodium hydroxide 1M (4.2), plunge the tube to be centrifuged for 5 minutes in a water bath at 100° C
6.12 After cooling, add 1 mL of sulfuric acid 1M (4.3) and 1 mL of ammonium metavanadate solution (4.4.4)
6.13 Wait 15 minutes
6.14 Centrifuge for 10 minutes at 11000 rpm
6.15 Pour the supernatant into a spectrophotometer tank and measure the absorbance at 530 nm, after determining the zero point with water (4.1.3)

i.e. Abs_E

Standard. In parallel, produce a standard comprising the same wine as that analyzed but heated beforehand for 2.5 minutes using a microwave generator set to maximum power or with a water bath at 100° C for 5 minutes.

i.e. Abs_T

7. Calculation

The presence of metatartaric acid in the wine is established when, at 530 nm:

$$Abs_E - Abs_T > 0.050$$

Method OIV-MA-AS313-22 Type II method

Simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by HPLC and UV-detection

(Resolution OENO 11/2008)

1. Introduction

Ascorbic acid is an antioxidant that is naturally occurring in a wide range of foods. The natural amount of ascorbic acid in grapes decreases during must and wine production, but it can be added to musts and to wines within certain limits.
The method described has been validated in a collaborative study by the analyses of wine samples with spiked amounts of 30 mg/L to 150 mg/l for L-ascorbic acid and 10 mg/L to 100 mg/l for D-isoascorbic acid respectively.

2. Scope

This method is suitable for the simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by high performance liquid chromatography and UV-detection in a range of 3 mg/L to 150 mg/l.
For contents above 150 mg/l, sample dilution is necessary.

3. Principle

The samples are directly injected into the HPLC system after membrane filtration. The analytes are separated on a reversed phase column and UV-detection at 266 nm. The quantification of L-ascorbic acid and D-iso-ascorbic acid is done with reference to an external standard.

Note: The columns and operating conditions are given as example. Other types of columns may also give a good separation.

4. Reagents and Material

4.1 Reagents

4.1.1. N-octylamine, puriss. ≥ 99.0 %
4.1.2. Sodium acetate, 3 H_2O, puriss ≥ 99.0 %
4.1.3. Pure acetic acid, 100 %
4.1.4. Phosphoric acid, approx. 25%
4.1.5. Oxalic acid, puriss. ≥ 99.0 %

4.1.6. Ascorbate oxidase
4.1.7. L-ascorbic acid, ultra ≥ 99.5 %
4.1.8. D-iso-ascorbic acid, puriss. ≥ 99.0 %
4.1.9. Bi-distilled water
4.1.10. Methanol, p.A. 99.8 %

4.2 Preparation of the mobile phase

4.2.1 Solutions for the mobile phase

For the mobile phase prepare the following solutions:

4.2.1.1 12.93 g n-octylamine in 100 ml methanol
4.2.1.2 68.05 g sodium acetate, 3 H_2O in 500 ml bi-distilled water
4.2.1.3 12.01 g pure acetic acid in 200 ml bi-distilled water
4.2.1.4 Buffer solution (pH 5.4) : 430 ml sodium acetate solution (4.2.1.2) and 70 ml acetic acid solution (4.2.1.3)

4.2.2 Preparation of the mobile phase

Add 5 ml of n-octylamine solution (4.2.1.1) to approximately 400 ml bi-distilled water in a beaker. Adjust this solution to a pH of 5.4 to 5.6 by adding 25% phosphoric acid (4.1.4) drop by drop. Add 50 ml of the buffer solution (4.2.1.4), transfer the composite mix to a 1000 ml volumetric flask and fill up with bi-distilled water. Before use, the mobile phase has to be filtered through a membrane (regenerated cellulose, 0.2 µm) and if possible degassed with helium (approximately 10 minutes) depending on the needs of the HPLC system used.

4.3 Preparation of the standard solution

Note: All standard solutions (stock solution 4.3.1. and working solutions 4.3.2) have to be prepared daily and preferably stored cold in a refrigerator prior to injection.

4.3.1 Preparation of the stock solution (1 mg/ml)

Prepare a 2% aqueous oxalic acid solution and eliminate dissolved oxygen by blowing through nitrogen.

Weigh exactly 100 mg each of L-ascorbic acid and D-iso-ascorbic acid in a 100 ml volumetric flask and make to the mark with the 2% aqueous oxalic acid solution.

4.3.2 Preparation of the working solutions

For the working solutions dilute the stock solution (4.3.1) to the desired concentrations with the 2% oxalic acid solution. Concentrations between 10 mg/l and 120 mg/l are recommended, e.g. 100 µl, 200 µl, 400 µl, 800 µl, 1200µl to 10 ml, corresponding to 10, 20, 40, 80 and 120 mg/l.

5. Apparatus

Usual laboratory equipment, in particular the following:

5.1 HPLC-pump
5.2 Loop injector, 20 µl
5.3 UV-detector

6. Sampling
Wine samples are fltered through a membrane with pore size 0.2 µm before injection. For contents above 150 mg/L, it is necessary to dilute the sample.

7. Procedure
7.1 Operating conditions for HPLC
Inject 20 µl of the membrane-filtered sample into the chromatographic apparatus.

Precolumn:	e.g. Nucleosil 120 C18 (4cm x 4 mm x 7 µm)
Column:	e.g. Nucleosil 120 C18 (25 cm x 4 mm x 7 µm)
Injection Volume:	20 µl
Mobile Phase:	see 4.2.2, isocratic
Flow rate:	1ml/min
UV-detection:	266 nm
Rinse cycle:	at least 30ml bi-distilled water followed by 30ml methanol and 30ml acetonitrile

7.2 Identification/Confirmation
Identification of peaks is done by the comparison of retention times between standards and samples. With the chromatographic system described as an example, the retention times are: for L-ascorbic acid 7.7 min. and for D-iso-ascorbic 8.3 min. respectively. (See figure 1, chromatogram A).
For further confirmation of positive findings these samples should be treated with a spatula of ascorbate oxidase and measured again (see figure 1, chromatogram B).
As a result of the degradation of L-ascorbic acid and D-iso-ascorbic acid caused by the ascorbate oxidase, no signal should be found at the retention time of L-ascorbic acid and D-iso-ascorbic acid. If interfering peaks are detected, their peak area should be taken into account for the calculation of the concentration of the analytes.

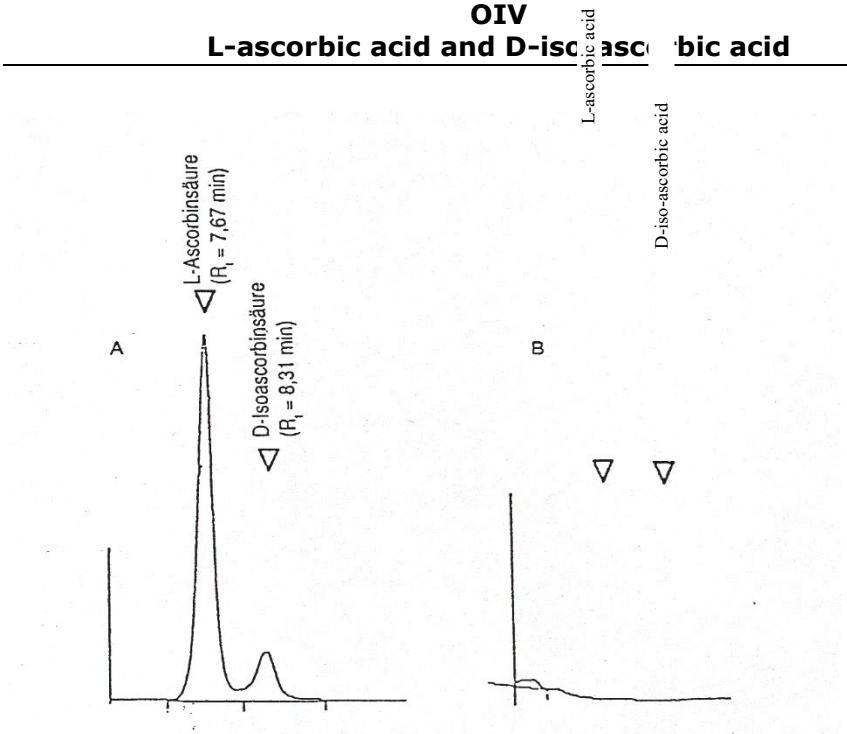


Figure 1: Example of a chromatogram of white wine: A: prior to treatment with ascorbate oxidase; B: after treatment

Note: It is recommended to analyse the ascorbate oxidase treated samples at the end of a sequence, followed by the rinse cycle for removing remaining ascorbate oxidase from the column. Otherwise the L-ascorbic acid and the D-iso-ascorbic acid may be converted by the remaining ascorbate oxidase during the HPLC-measurement and the result may be altered.

8 Calculation

Prepare a calibration curve from the working solutions (4.3.2). Following the method of external standard the quantification of L-ascorbic acid and D-isoascorbic acid is performed by measuring the peak areas and comparing them with the relevant concentration in the calibration curve.

Expression of results

The results are expressed in mg/l L-ascorbic acid and D-isoascorbic acid respectively with one decimal (e.g. 51,3 mg/l).
For contents above 150 mg/L, take into account the dilution.

9. Precision

The method was tested in a collaborative study with 27 laboratories participating, organised by the former Bundesgesundheitsamt (Germany) in 1994. The design of the
collaborative trial followed the § 35 of the German Food Law that has been accepted by the O.I.V until the new protocol (OENO 6/2000) was introduced.
The study included four different samples of wine - two white wines and two red wines - of which five repetitions of each were requested. Due to the fact that it was not possible to prepare samples with a sufficient stability of the analytes (different degradation rates) it was decided to send defined amounts of pure standard substances together with the wine samples to the participants. The laboratories were advised to transfer the standards quantitatively to the wine samples and to analyse them immediately. Amounts of 30 to 150 mg/l for L-ascorbic acid and 10 to 100 mg/l for D-iso-ascorbic acid were analysed. In the Annex the detailed study results are presented. Evaluation was done following the DIN/ISO 5725 (Version 1988) standard.

The standard deviations of repeatability (s_r) and reproducibility (s_R) were coherent with the L-ascorbic acid and D-iso-ascorbic acid concentrations. The actual precision parameters can be calculated by the following equations:

L-ascorbic acid

$s_r = 0.011\ x + 0.31$
$s_R = 0.064\ x + 1.39$
x: L-ascorbic acid concentration (mg/l)

D-iso-ascorbic acid

$s_r = 0.014\ x + 0.31$
$s_R = 0.079\ x + 1.29$
x: D-iso-ascorbic acid concentration (mg/l)

Example:
D-iso-ascorbic acid 50 mg/l $s_r = 1.0$ mg/l
$s_R = 5.2$ mg/l

10. Other characteristics of the analysis

10.1 Limit of detection

The limit of detection of this method was estimated at 3mg/l for L-ascorbic acid and D-iso-ascorbic acid

10.2. Trueness

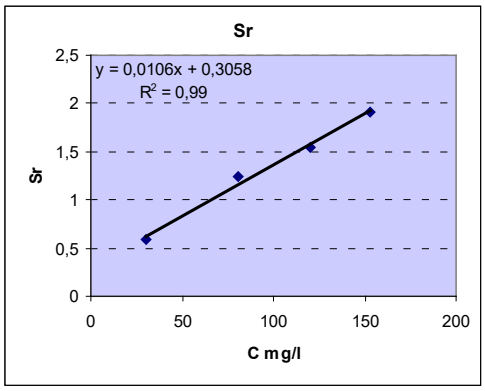
The mean recovery calculated from the collaborative trial over four samples (see Annex) was:

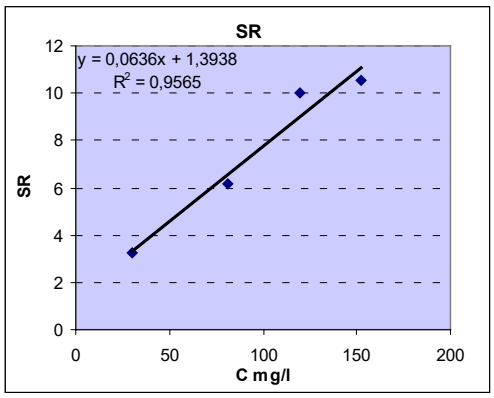
100.6 % for L-ascorbic acid
103.3 % for D-iso-ascorbic acid

11. ANNEX: Collaborative Trial

L-Ascorbic Acid

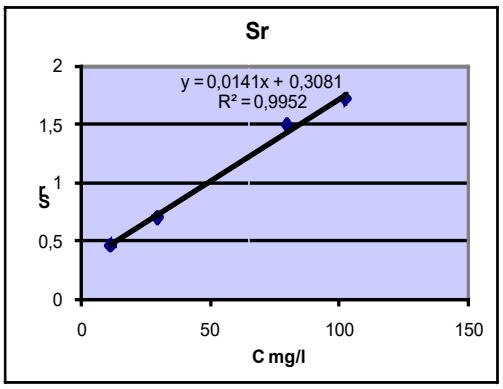
		Red Wine I	White Wine II	Red Wine III	White Wine IV
X	mg/l	**152.7**	**119.8**	**81.0**	**29.9**
Amount spiked	mg/l	150	120	80	30
Recovery	%	101.8	99.8	101.3	99.7
n		25	23	25	23
Outliers		1	3	1	3
Repeatability s_r	mg/l	1.92	1.55	1.25	0.58
RSD_r	%	1.3	1.3	1.5	1.9
HorRat		**0.17**	**0.17**	**0.19**	**0.20**
r	mg/l	5.4	4.3	3.5	1.6
Reproducibility S_R	mg/l	10.52	10.03	6.14	3.26
RSD_R	%	6.9	8.4	7.6	10.9
Horwitz RSD_R	%	7.5	7.8	8.3	9.6
HorRat		**0.92**	**1.08**	**0.92**	**1.14**
R	mg/l	29.5	28.1	17.2	9.1

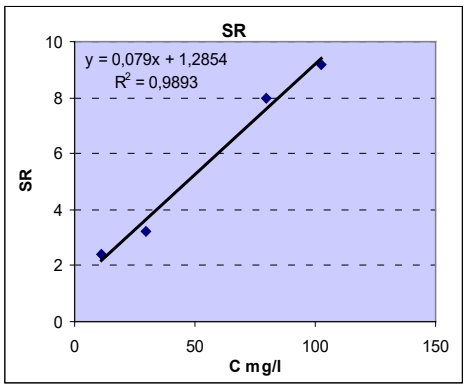




D-Isoascorbic Acid

		Red Wine I	White Wine II	Red Wine III	White Wine IV
X	mg/l	**102.4**	**79.8**	**11.3**	**29.4**
Amount Spiked	mg/l	100	80	10	30
Recovery	%	102.4	99.8	113.0	98.0
n		25	23	24	22
Outliers		1	3	2	4
Repeatability s_r	mg/l	1.71	1.49	0.47	0.70
RSD_r	%	1.7	1.9	4.1	2.4
HorRat		**0.21**	**0.23**	**0.37**	**0.25**
r	mg/l	4.8	4.2	1.3	2.0
Reproducibility S_R	mg/l	9.18	7.96	2.394	3.23
RSD_R	%	9.0	10.0	21.2	11.0
Horwitz RSD_R	%	8.0	8.3	11.1	9.6
HorRat		**1.12**	**1.21**	**1.91**	**1.14**
R	mg/l	25.7	22.3	6.7	9.0





12. Bibliography

B. Seiffert, H. Swaczyna, I. Schaefer (1992): Deutsche Lebensmittelrundschau, 88 (2) p. 38-40

C. Fauhl: Simultaneous determination of L -ascorbic acid and D –iso-ascorbic acid (erythorbic acid) in wine by HPLC and UV-detection – OIV FV 1228, 2006

Method OIV-MA-AS313-23 Type IV method

Identification of L- tartaric acid as being of plant or fossil origin by measuring its ^{14}C activity

(Resolution OENO 12/2008)

1. PURPOSE AND SCOPE

The method can be used to identify tartaric acid as being of plant or fossil origin, and in cases of a mixture of the two, to determine the respective proportions of the two types. In these situations, the method enables the detection of fossil-derived L(+)-tartaric acid quantities below 10%.

2. PRINCIPLE

In the majority of cases, commercially available tartaric acid of plant origin is a product of winemaking. The potassium hydrogénotartrate present in the lees is extracted and marketed in the form of L-tartaric acid. The ^{14}C concentration in the acid is therefore related, as with ethanol from wine, to the ^{14}C concentration in the carbon dioxide in wines from the same year of production. This concentration is relatively high as a result of the human activity involved.
Synthetic tartaric acid on the other hand, derived from fossil fuel by-products, has a much lower or even negligible concentration of ^{14}C.
Measuring the ^{14}C activity in DPM/gram of carbon (Disintegrations Per Minute) using liquid scintillation therefore allows the origin to be determined as well as any combination of the types.

3. REAGENTS AND PRODUCTS

3.1 Reagents

3.1.1 Scintillation fluid such as Instagel Plus
3.1.2 ^{14}C toluene reference with activity certified by laboratory for callibration, for calculating the sensitivity and efficiency of the machine by the definition of a quench curve
3.1.3 ^{14}C and ^{3}H standards and ^{12}C toluene for the background noise, for calibrating the scintillation counter
3.1.4 Nitromethane 99%

3.1.5 Ultrapure water (>18 MΩ)
3.1.6 ^{14}C toluene solution with activity of approx. 430 DPM/ml obtained by diluting stock ^{14}C reference solution in ^{12}C toluene.

3.2 Standards

3.2.1 Defining the quench curve

Once the scintillator has been calibrated using the three certified ^{14}C, ^{3}H and ^{12}C toluene standards, plot a quench curve using the following procedure.

Prepare a dozen vials with 10 ml of a solution of 500 g/l of fossil-derived L-tartaric acid in water, then add the quantity of toluene ^{14}C standard needed for approx. 400-1000 DPM in total per vial (if necessary, make up an intermediate solution of standard solution in toluene), then add increasing quantities of nitromethane, e.g. for 12 vials: 0, 0, 0, 5, 10, 15, 20, 35, 50, 100, 200 and 400 µL followed by 10 m of scintillation fluid. There must be at least 3 samples containing no nitromethane.

Define a quench curve once a year, analysing the vials in increasing order of nitromethane content.

The quench curve can then be used to determine the sensitivity or mean efficiency.

3.2.2 Determination of background noise (test blank)

Using fossil-derived L-tartaric acid, such as that used for calculating the efficiency, determine the background noise, or test blank value. This test should be performed immediately after defining the quench curve, then roughly every three months.

3.2.3 Defining the calibration curve

The purity of the plant and fossil-derived L-tartaric acids must be checked using HPLC before the scintillation test is done.

Calibration using a mixture of tartaric acid (which is known with certainty to be of plant origin) containing between 0% and 100 % of this type in combination with the fossil-derived type.

Preparation of 500 g/l solutions			
	Blank or background noise	Standards	Internal standard
Weighing	respectively in 50 ml volumetric flasks		
	25 g fossil-derived L-tartaric acid	25 g known combinations of fossil and plant L-tartaric acid	Use the blank
Dissolution	Seal		
	Homogenise the mixture well by shaking and/or tumbling		
Preparation of scintillation mixtures			
	In plastic vials, add respectively		
Sample taken from the 500 g/l solution	10 ml using volumetric pipettes		
Added concentration	//////////////////////////	//////////////////////////	100 µL
Added scintillation fluid	10 ml using an automatic burette		
	Screw the cap on		
	Wait 5 min. then analyse for 500 min.		

3.3. Internal control

3.3.1 Nature of product used for internal control

A 500 g/l solution of fossil-derived L-tartaric acid is enriched with a quantity of toluene ^{14}C (DPM<100)
The background noise should be determined using the same fossil-derived L-tartaric acid solution.

3.3.2 Nature of internal control

Measurement of the added concentration provides verification that there is no spectral interference in the medium being studied.

3.3.3 Internal control limits

The control limits depend on the equipment used: a 5% value is acceptable.

3.3.4 Inspection frequency and procedure

Once a month during frequent use, or at each analysis sequence, an internal control is performed on the scintillator. The same check is also carried out at every change of scintillation fluid batch or after a new quench curve has been defined.

3.3.5 Decision rules to be taken depending on the results of the internal control

If the results fall outside the internal control limits, calibrate the scintillator after checking the protocol, then repeat the internal control.
If the calibration is accurate but the new internal control measurement is not, make a new quench curve and carry out a new control.

4. APPARATUS

4.1 Liquid scintillation spectrometre with computer and printer previously callibrated with quenching curve established with nitromethane

4.2 Low content potassium identical bottles (40K) with screw top stopper, and low background noise

4.3 10 mL 2 graduations pipettes

4.4 Automatic distribution burette adapted to screw top for liquid scintillating bottle

4.5 Glass laboratory

1) 5. SAMPLES

The purity of the samples can be checked using HPLC if required, before running the scintillation analysis.
Make up a 500 g/solution of the sample to be analysed in ultra-pure water.

Preparation of 500 g/l solutions				
	Test blank or background noise	Standards	Internal standard	Sample
Weighing	respectively in 50 ml volumetric flasks			
	25 g fossil-derived L-tartaric acid	25 g known combinations of fossil-derived and plant L-tartaric acid	Use the blank	25 g
Dissolution	Seal			
	Homogenise the mixture well by shaking and/or tumbling			
Preparation of scintillation mixtures				
	In plastic vials, add respectively			
Sample taken from the 500 g/l solutions	10 ml using volumetric pipettes			
Added concentration	//////////////////////	/////////////////	100 µL	///////////////
Added scintillation flui	10 ml using an automatic burette			
	Screw the cap on and shake hard			
	Wait 5 min. then analyse for 500 min.			
Notes	Every 5 to 10 test samples, run a sample with 0 % plant tartaric acid, i.e. 10 ml fossil tartaric acid and 10 ml scintillation fluid.			
	Measure the background noise at the end of each analysis sequence			

6. CALCULATION

Measurements are given directly in Counts Per Minute CPM, but these must be converted to DPM/gram of carbon.

6.1 Results:

Calculation of the specific ^{14}C radioactivity of the sample in DPM/gram of carbon:

$$\frac{(X - X') \text{ x } 100 \text{ x } 3.125_{(1)}}{Rm \text{ x } m}$$

- **A**: radioactivity in disintegrations/minute and per gram of carbon
- **X**: CPM of the sample
- **X':** CPM for the fossil L-tartaric acid used for the background noise
- **m:** mass of the tartaric acid in the 10 ml sample from the 500 g/l solution, i.e. in 5 g of acid
- **Rm:** the mean efficiency expressed as a percentage

(1) There are 3.125 grams of tartaric acid to each gram of carbon (ratio of the molar mass of the acid (150 g/mol) to the total mass of carbon (or 4 *x 12 = 48 g/mol)

The result is expressed to one decimal place.

6.2 Verification of the results using internal controls:

The check should be carried out by comparing the value obtained at § 3.5.1 with the result given by the added concentration method. If the difference is significant (> 5 %), recalculate the DPM value from the CPM value as below:

$$\text{Recalculated DPM} = \frac{CPM}{Rm}$$

with the mean efficiency being obtained from the quench curve.

The two results must not differ by more than 5% from their mean value. If they do, repeat the analysis on the sample, doubling the quantity of the internal standard. Compare the 2 results obtained with the standards: if they do not differ by more than 5 % from the mean of the 2, give the mean result.

Note: in this case, that would mean that the quenching of the sample is so great that direct analysis cannot be used.

6.3 Uncertainty

The uncertainty value obtained under standard test conditions is +/- 0.7 DPM/gram of carbon.

7. VALIDATION BY COMPARISON WITH A REFERENCE METHOD

7.1 Principle

Tartaric acid is converted to CO_2 by combustion then converted to benzene; Measurement is then carried out using liquid scintillation.

After undergoing a pre-treatment designed to eliminate any contamination, the CO_2 from the sample is converted to benzene following the reaction chain below:

$C + O_2 \rightarrow CO_2$ (1)

$CaCO_3 + 2\ HCl \rightarrow CO_2 + H_2O + Ca^{2+} + 2Cl^-$ (2)

$$2\ CO_2 + 10\ Li \xrightarrow{800°C} Li_2C_2 + 4\ Li_2O \quad (3)$$

$Li_2C_2 + 2\ H_2O \rightarrow C_2H_2 + 2\ LiOH$ (4)

$$3\ C_2H_2 \xrightarrow{Al_2O_3\ avec\ Cr^{3+}\ et\ V} C_6H_6 \quad (5)$$

(1) Organic sample: the carbon flushed with oxygen plus a heat source (or by combustion in the presence of pressurised oxygen) produces carbon dioxide from the sample (CO_2).

(2) Mineral sample (marine or continental carbonates, water, etc.): The carbonate is attacked by pure hydrochloric acid (HC) to produce the carbon dioxide (CO_2) from the sample plus water and ionised calcium.

(3) The action of the CO_2 on lithium metal heated to between +600°C and +800°C produces lithium carbide and lithium oxide (-Li_2 O).

(4) The action of water (hydrolysis) on the lithium carbide produces acetylene (C_2H_2), lithium hydroxide,. Non-tritiated, radon-free water must be used.

(5) Trimerisation of the acetylene over a chrome-plated aluminium-based catalyst support at approx. 185 °C produces benzene (C_6H_6).

7.2 Procedure:

The carbon dioxide (CO_2) from a sample, obtained either by burning, combustion or acid attack, is preserved in a storage cylinder. The necessary quantity of lithium (lithium = catalyst for a chemical transformation) is placed in a nickel capsule, which is then placed at the bottom of a heat reaction chamber. A vacuum is created inside the chamber and its lower part is heated while its upper part is cooled at the sides with the help of a water circulation partition.

7.2.1 Carburisation.

After approximately one hour of heating, the temperature reaches 650°C. The CO_2 can then be brought into contact with melted lithium. The quantity of lithium is always higher in relation to the quantity of carbon in the sample. The excess amount of lithium to use in relation to the **stoechiometric** conditions varies from 20% to 100% according to different sources.

The chemical reaction (carburisation or "pickup") is almost instantaneous and the first few minutes of pickup are the most crucial in the carburisation process.

The reaction is **exothermic** (an increase of 200°C). Carburisation is quite rapid and is considered to be at the carburised stage after the first 20 minutes, but heating continues for 45 to 50 minutes in order to any eliminate traces of **radon** (a by-product of uranium), which could be mixed in with the carbon dioxide.

7.2.2 Cooling

Once the treatment period (heating) is complete, the reaction chambers are allowed to cool until they reach room temperature (25-30°C).

7.2.3 Hydrolysis of Lithium Carbide

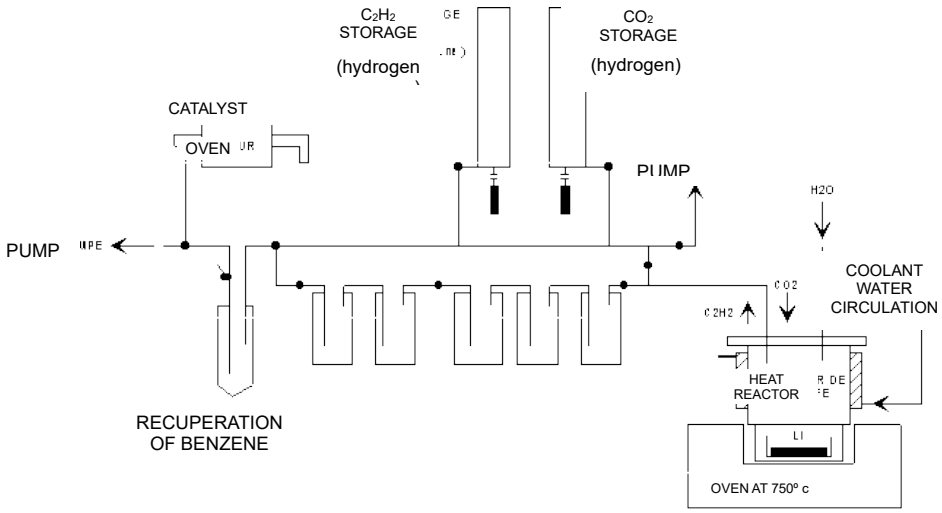
Water is introduced into the reaction chambers, in a much higher quantity than that required by the reaction (1.5 L). The chemical reaction is instantaneous and the acetylene is released at the same time. This reaction is also exothermic (temperature increase between +80°C and +100°C).

The acetylene produced is then brought to a vapour state (sublimation) and trapped over the chrome-plated (Cr3+) aluminium catalyst support. This is previously air dried for a minimum of three hours, then vacuum dried for two hours under heat at +380°C. Drying is vital in order to eliminate any water remaining in the catalyst support balls.

7.2.4 Trimerisation - Polymerisation of acetylene to benzene by catalysis

Before trimerisation, the temperature of the catalyst support must have dropped to between +60°C and +70°C, and since this reaction is also exothermic, automatic temperature maintenance is needed. The catalyst support is then reheated to +180°C for 1½ hours and the vaporised **benzene** is desorbed then trapped in a trap tube surrounded by liquid nitrogen. Desorption takes place under dynamic vacuum. At the end of the experiment, the crystallised benzene is left to reheat to room temperature so that it regains its liquid state before being used for the counting.

7.3 Benchtop arrangement for the synthesis of Benzene



7.4 Reference Chemical solution for the Counting

A **solution volume set at 4 ml** is used as the reference for the liquid scintillation counting.

The solution comprises a target base of 3.52g benzene from the sample (solvent) + the scintillation fluid (solute) made up of 2 scintillation fluids, one main and one secondary.

Since the mass per volume of benzene is 0.88 g/litre, **0.88 x 4ml = 3.52 g**.

Main scintillation fluid	**Buthyl-PBD**
Chemical composition	**(2-(4-Biphenylyl)-5-(4-tert-buthyl-phenyl)-1,3,4-oxadiazole)**
Maximum wavelength fluorescence	**367 nanometers**
Secondary scintillation fluid	**bis-MSB**
Chemical composition	**1,4-Di-(2-Methylstyryl)-Benzene**
Maximum wavelength fluorescence	**415 nanometers**

Optical absorption and coupling emission of the two fluids:	
Maximum absorption wavelength	**409 nanometers**
Maximum absorption wavelength	**412 nanometers**

7.5 Delta ^{13}C correction for Isotope Fractionation

The measurement involves a correction for isotope fractionation using the standardisation procedure with a stand PDB ^{13}C **with a value of - 25 o/oo**.

8. CHARACTERISTICS OF THE METHOD

8.1 Procedure

One sample of wine-derived tartaric acid and one sample of synthetic acetic acid were used to prepare test tartaric acid solutions at 500 g/l.

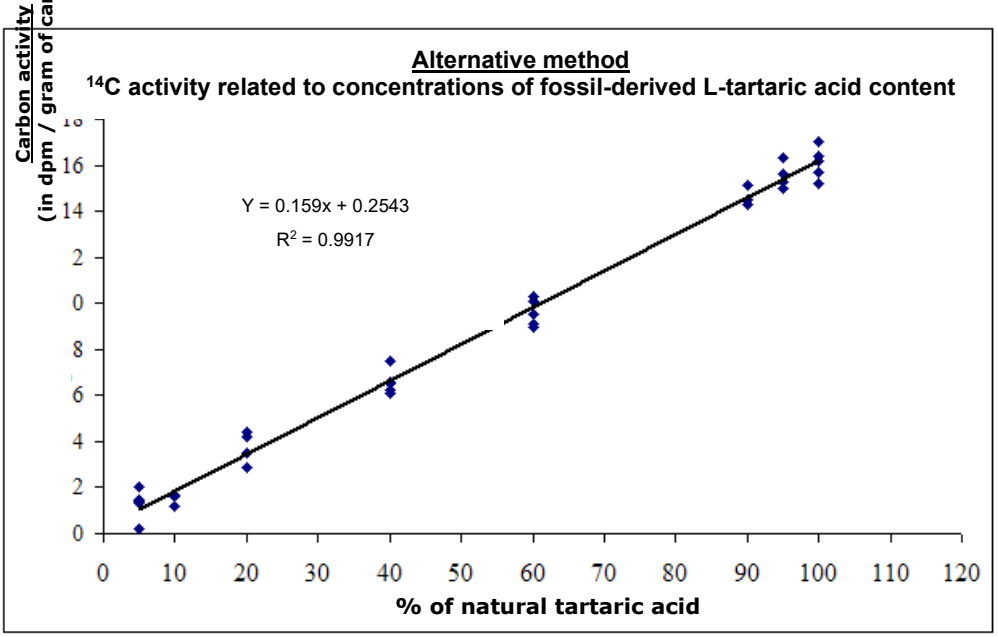
The concentrations of the wine-derived tartaric acid in the solutions varied between 0°C and 100%.

The origin and purity of the two starter samples had been previously checked using the reference method.

8.2 Results:

The results are given in the table and diagram below:

% OF WINE-DERIVED TARTARIC ACID		
ACTUAL CONCENTRATIONS	RESULTS FROM THE ALTERNATIVE METHOD	RESULTS FROM THE REFERENCE METHOD
0	0 and 0	0
10	3.5 and 6.0	12
20	11.4 and 12	22
30	24.6 and 25.4	31
40	34.7 and 38	40
50	41.4 and 50.6	50
60	57.8 and 58.8	63
70	60 and 63.3	70
80	81	81
85	84	86
90	88	91
95	94	96
100	100	100



8.3 Accuracy,trueness:

Accuracy is 6.9%.
The standard deviation of repeatability for the alternative method is: 2.86 % of plant tartaric acid.

9. BIBLIOGRAPHY

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S. COHEN, B. CRESTO, S. NACHAMPASSAK, T. PAYOT, B. MEDINA, S. CHAUVET, Détermination de l'origine de l'acide tartrique L(+): naturelle ou fossile par la détermination de son activité C^{14} - Document OIV FV 1238, 2006

Method OIV-MA-A313-24 Type IV method

Determination of total ethanol in wine by high-performance liquid chromatography

(Resolution OIV-OENO 595-2018)

SCOPE OF APPLICATION

The method described is suitable for the determination of total (free and sulphur-dioxide-bound) ethanal in wine for concentrations between 0.2 and 80 mg/L.

1. PRINCIPLE

The analyte is quantified after derivatisation of the molecule with 2,4-Dinitrophenylhydrazine (DNPH) followed by elution using HPLC. Detection is based on the retention time at the wavelength of 365 nm.

2. REAGENTS AND PRODUCTS

2.1. 2,4-Dinitrophenylhydrazine (DNPH), CAS no. 119-26-6, purity ≥ 99.0% (HPLC)
2.2. Sulphur dioxide (SO_2), as $K_2S_2O_5$, CAS no. 16731-55-8, purity ≥ 98%
2.3. Sulphuric acid (H_2S0_4), CAS no. 7664-93-9, purity 95.0-98.0%
2.4. Formic acid (CH_20_2), CAS no. 64-18-6, purity ≈ 98%
2.5. Acetonitrile (C_2H_3N), CAS no. 75-05-8, purity ≥ 99.9%
2.6. Ethanal (CH_3CHO), CAS no. 75-07-0, purity ≥ 99.5%
2.7. Ultra-pure, HPLC-grade type I water compliant with standards ASTM D1193 and ISO 3696, CAS no. 7732-18-5
2.8. Perchloric acid ($HClO_4$), CAS no. 7601-90-3, purity 70 %

Preparation of reagent solutions

2.9. Freshly-prepared sulphur dioxide solution at a concentration of 1120 mg/L SO_2, obtained by preparing a 2 g/L solution of $K_2S_2O_5$ (2.2) with ultra-pure, HPLC-grade water

2.10. 25% v/v sulphuric acid solution prepared by dilution of concentrated sulphuric acid (2.3) with ultra-pure, HPLC-grade water

2.11. 2.8% acetonitrile solution acidified with perchloric acid, obtained through the dilution of perchloric acid (2.8) in acetonitrile (2.5)

2.12. Freshly-prepared solution of 2,4-dinitrophenylhydrazine (2.1) in acidified acetonitrile (2.11) at a concentration of 2 g/L DNPH

2.13. Preparation of calibration solutions

The stock solution is prepared by dilution of an appropriate quantity of ethanal (density = 0.785 g/mL) in ultra-pure, HPLC-grade water in order to obtain a concentration of between 300 and 400 mg/L. Given that pure ethanal is highly volatile, the stock solution should be prepared by sampling constant volumes of ethanal using calibrated flasks (3.1). To prepare the stock solution, measure 10 mL pure ethanal in a calibrated flask, transfer the pure ethanal to another 20-mL calibrated flask and make up to the mark with ultra-pure, HPLC-grade water. The solutions diluted are prepared by making up to volume with ultra-pure, HPLC-grade water in calibrated flasks of greater capacity. Calibration solutions, with concentrations of 10 mg/L, 30 mg/L, 50 mg/L, 70 mg/L and 100 mg/L, are obtained through dilution of the stock solution in 50-mL calibrated flasks. The volumes required are sampled from the stock solution using a precision micropipette (3.2), for example, and made up to volume with ultra-pure, HPLC-grade water in calibrated flasks (3.1).

Preparation of solvent A for HPLC analysis

2.14. 0.5% (v/v) formic acid solution obtained by diluting concentrated formic acid (2.4) in ultra-pure, HPLC-grade water (2.7).

3. APPARATUS

3.1. Everyday laboratory glassware, including class-A calibrated flasks of 10, 20 and 50 mL, 2-mL vials and 1-L containers for the solvents, and Pasteur pipettes
3.2. Precision micropipettes
3.3. Vortex-type stirrer
3.4. 0.45-µm membrane filters for sample preparation, certified for use in HPLC
3.5. 1-L vacuum flask (where the automatic degasser of the solvents is not provided with the HPLC apparatus)
3.6. Vacuum pump (where the automatic degasser of the solvents is not provided with the HPLC apparatus)
3.7. Analytical balance with precision of ± 0.0001 g
3.8. Natural convection oven with precision of ±1 °C at 65 °C
3.9. HPLC apparatus with UV detector, equipped with two gradient pumps and an oven for the heating of the column
3.10. C18 column (250 x 4.6 mm, particle diameter: 4 µm)

Note: Any other system may be used on the condition that the ethanal is well separated from the other derivatised carbonyl compounds. The chromatographic resolution between the ethanal peak and the greatest neighbouring peak on the chromatogram should be higher than 1.

4. SAMPLING

The wine sample should be taken and stored in a glass container sealed with a Teflon stopper in an inert atmosphere (nitrogen or argon).

5. PROCEDURE

Derivatisation

The derivatisation takes place in 2-mL glass vials sealed with Teflon stoppers, inside which the following is successively added: 100 µL wine or standard solution filtered at 0.45 µm, 20 µL freshly-prepared sulphur dioxide solution (2.2) at a concentration of 1120 mg/L SO_2, 20 µL 25% sulphuric acid (2.3) and 140 µL freshly-prepared solution of 2,4-dinitrophenylhydrazine in acetonitrile at a concentration of 2 g/L DNPH (2.12).
After these additions, the solution is immediately vortex stirred and placed in the oven at 65 °C for 15 minutes before being cooled at room temperature.

Once the reaction is completed, the solution is cooled at room temperature for 15 minutes before being injected into the HPLC apparatus. The samples should be injected in less than 10 hours from the end of the derivatisation reaction.

HPLC analysis

The specific HPLC-analysis parameters are provided below by way of example.

Normal operating conditions:

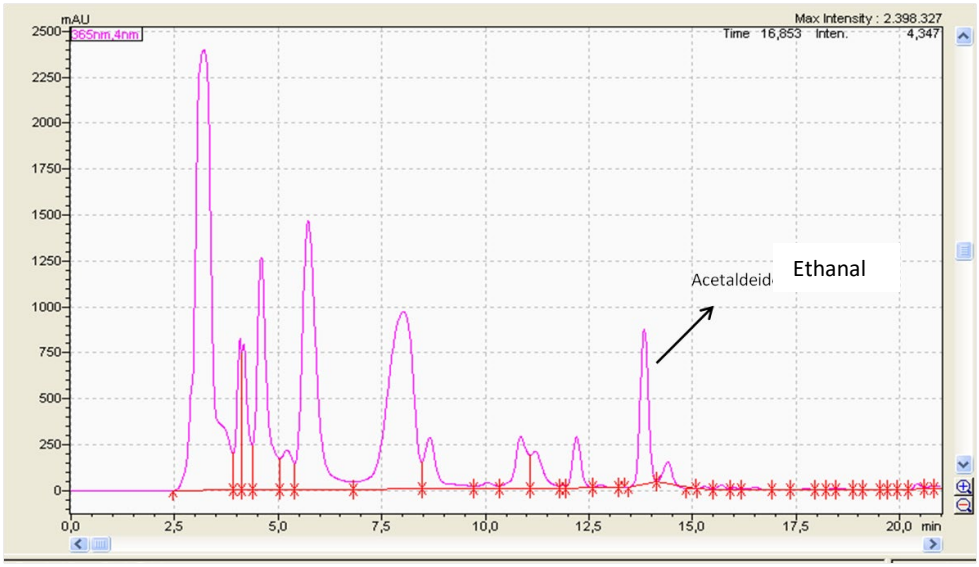
- Injection volume: 15 µL
- Flow rate: 0.75 mL/min
- C18 column (5.10)
- Solvent for cleaning the injector: acetonitrile
- Column temperature: 35 °C
- Mobile-phase-A solvent: 0.5% formic acid in ultra-pure, HPLC-grade water
- Mobile-phase-B solvent: acetonitrile
- Detection at 365 nm

Elution gradient

The elution programme provides for:

35% eluent B (0.1 s)	65% eluent A (0,1 s)
60% eluent B (8 min)	40% eluent A (8 min)
90% eluent B (13 min)	10% eluent A (13 min)
95% eluent B (15 min)	5% eluent A (15 min)
95% eluent B (17 min)	5% eluent A (17 min)
35% eluent B (21 min)	65% eluent A (21 min)
35% eluent B (25 min)	65% eluent A (25 min)

Example chromatogram



6. CALCULATION

The ethanal concentration is calculated based on the equation of the calibration curve obtained after injection of the calibration solutions (2.13).

7. PRECISION AND VALIDATION PARAMETERS

The coefficient of variation for the analyses repeated within the same laboratory should be less than 6% (for a concentration interval of between 10 mg/L and 100 mg/L). The repeatability standard deviation is 2.7% for a concentration of 14 mg/L, 2.98% for a concentration of 18 mg/L, 4.8% for a concentration of 22 mg/L and 1.3% for a concentration of 60 mg/L. The linearity range is between 0.2 and 80.0 mg/L. The limit of detection is 0.1 mg/L. The recovery rate in wine is between 92% and 102% (m/m).

8. RESULTS

The results are expressed in mg total ethanal / L to 1 decimal point.

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Elias, R. J., Laurie, V. F., Ebeler, S. E., Wong, J. W., and Waterhouse, A. L., 'Analysis of selected carbonyl oxidation products in wine by liquid chromatography with diode array detection', *Analytica chimica acta*, 626 (1), 2008, pp. 104-110.

Han, G., Wang, H., Webb, M. R., and Waterhouse, A. L., 'A rapid, one step preparation for measuring selected free plus SO 2-bound wine carbonyls by HPLC-DAD/MS', *Talanta*, 134, 2015, pp. 596-602.

Method OIV-MA-A313-25 Type III method

Determination of L-Lactic acid in wines by automated enzymatic method

(Resolution OIV-OENO 599-2018)

1. Scope of application

This method makes it possible to determine L-lactic acid in wine by specific enzyme analysis using an automatic sequential analyser.
In this document a collaborative study is reported which demonstrates application of the method for measurement of L-lactic acid in the range from 0.06 to 1.43 g/L.

Note: Where necessary each laboratory using this method may refine, and potentially widen, this range through a validation study.

2. Standard references

- OIV *Compendium of International Methods of Analysis*: Lactic acid – enzymatic method, OIV-MA-AS313-07,
- ISO 78-2: Chemistry – Layouts for standards.

3. Reaction principles

In the presence of nicotinamide adenine dinucleotide (NAD), L-lactic acid is oxidised to pyruvate in a reaction catalysed by L-lactate dehydrogenase (L-LDH). Since the equilibrium reaction is in favour of the lactate, it is necessary to remove the pyruvate formed which is converted into L-alanine in the presence of L-glutamate. This reaction is catalysed by glutamate pyruvate transaminase (GPT).

$$L-lactate \; + \; NAD^+ \overset{L-LDH}{\longleftrightarrow} pyruvate \; + \; NADH + H^+$$

$$pyruvate + L-glutamate \overset{GPT}{\longleftrightarrow} L-alanine + \propto -ketoglutarate$$

The reduced nicotinamide adenine dinucleotide (NADH) produced is measured based on its absorption at 340 nm. It is proportional to the quantity of L-lactic acid.

4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical usage (ISO 3696 standard)

4.1.2. Glycylglycine (CAS no. 556-50-3)

4.1.3. Glutamic acid (CAS no. 56-86-0)

4.1.4. NAD (nicotinamide adenine dinucleotide) (CAS no. 53-84-9)

4.1.5. L-LDH (L-lactate dehydrogenase) (CAS no. 9001-60-9)

4.1.6. GPT (glutamate pyruvate transaminase) (CAS no. 9000-86-6)

4.1.7. L-lactic acid of purity ≥ 98% (CAS no. 79-33-4)

4.1.8. Polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8)

4.1.9. Sodium hydroxide (CAS no. 1310-73-2)

Note 1: There are commercial kits for the determination of L-lactic acid. The user needs to check the composition to ensure it contains the above-indicated reagents.

Note 2: The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme's activitiy. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

4.2. Working solutions

4.2.1. A pH 10 buffer (0.60 M glycylglycine, 0.1 M L-glutamic acid).

The preparation may be as follows:

- glycylglycine (4.1.2): 4.75 g,
- glutamic acid (4.1.3): 0.88 g,
- PVP (4.1.8): 1 g,
- water for analytical usage (4.1.1): 50 mL.

The mixture is adjusted to pH 10 using a 10 M sodium hydroxide solution, then made up to 60 mL with water for analytical usage. This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.2. R1 working solution (example):

- water for analytical usage (4.1.1): 12 mL,
- NAD (4.1.4): 420 mg.

This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.3. R2 working solution (example):

- water for analytical usage (4.1.1): 1.2 mL,
- L-LDH (4.1.5): 7600 U,
- GPT (4.1.6): 2200 U.

This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.3. Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of L-lactic acid prepared by weighing and covering the measurement range.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with a UV detector. The reaction temperature should be tightly controlled (generally 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software ensuring its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature is 37 °C. Certain pieces of apparatus may use slightly different values.

5.1.5. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm.

5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

5.3. pH meter

5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

6. Sampling

6.1. Preparation of samples of musts and still wines

In most cases, wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

- Filtration or centrifugation should be used for highly turbid samples.
- Sample dilution (manual or automatic) with water for analytical usage should be used for values exceeding the measurement range.

6.2. Preparation of samples of sparkling wine containing CO_2

Sparkling wine samples containing CO_2 may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer be strictly observed. This also applies to the various enzymatic kits available on the market.

The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction takes place.

By way of example, the quantities of different elements may be as follows:

- sample: 2.5 µL,
- buffer (80%) and R1 (20%): 120 µL,
- R2: 15 µL.

The equipment takes regular measurements that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

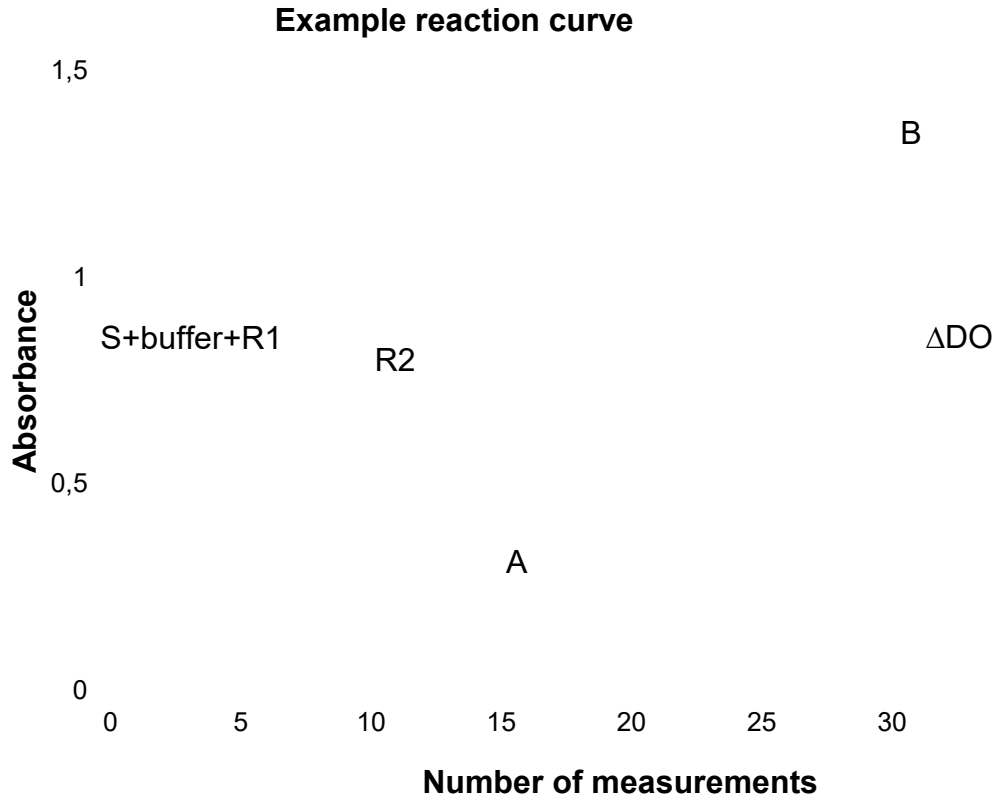


Figure 1: Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

8. Calculation of results

The measurement used for the determination of the result is as follows:

$$\Delta DO = (\text{Absorbance } B - \text{Absorbance } A)$$

In order to correlate this ΔDO value with the desired concentration of L-lactic acid, regular calibration of the apparatus is carried out using the calibration solutions at a minimum of 3 points covering the measurement range used. In the example given in Figure 2, the calibration curve is a straight line for values between 0 to 1.22 g/L L-lactic acid. In this case, for higher values, it is preferably to carry out a dilution. In

addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

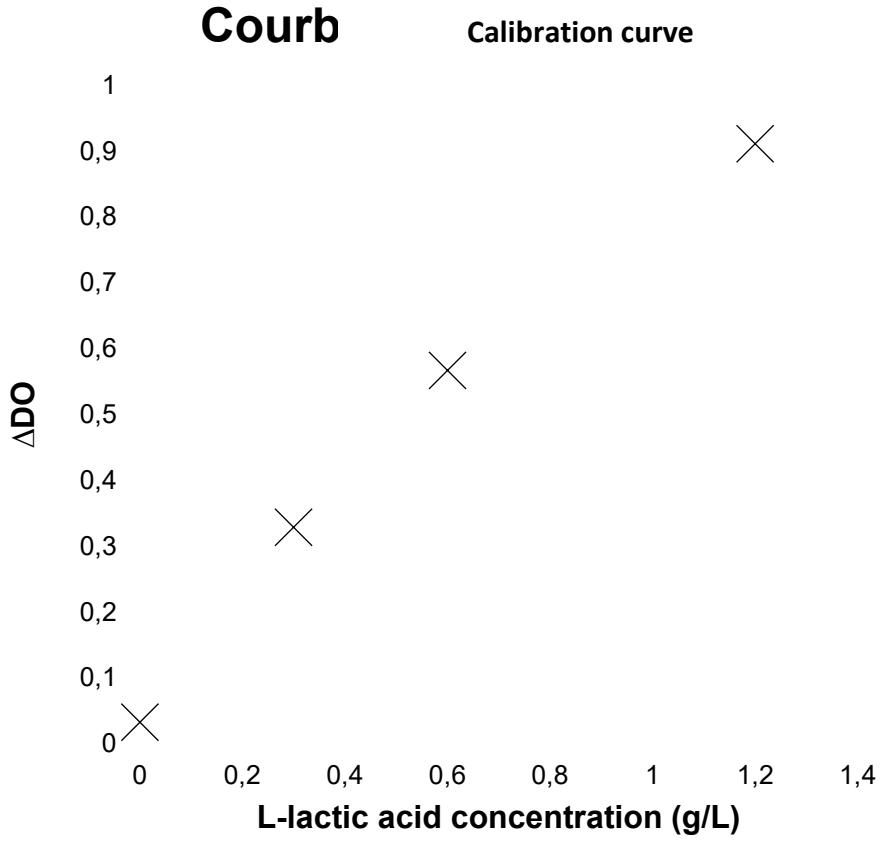


Figure 2: Calibration curve

The calibration curve can be order 1 ($Concentration = a.\Delta DO + b$), yet in this method it is more generally order 2 ($Concentration = a.\Delta DO^2 + b.\Delta DO + c$). If using a calibration curve of order 2, the laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve with high concentrations).

The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The results are expressed in g/L of L-lactic acid to 2 d.p.

10. Precision

Interlaboratory reproducibility

RSD_R = 7% (from 0.5 g/L)
CV_R% (k=2) = 2·RSD_R = 14%, (from 0.5 g/L)

Repeatability

RSD_r = 2% (from 0.5 g/L)
CV_r% (k=2) = 2·RSD_r = 4% (from 0.5 g/L)

Limit of quantification

Validated LOQ = 0.06 g/L
(Concentration where CV_R% (k=2) = 60%)

ANNEX
Results of the interlaboratory tests

Collaborative study

A total of 16 laboratories from different countries participated in the collaborative study organised in 2016.

Labo	Country
Miguel Torres S.A.- Finca Mas La Plana	SPAIN
Estación Enológica de Castilla y León	SPAIN
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	SPAIN
Estación Enológica de Haro	SPAIN
Comissão de Viticultura da Região dos Vinhos Verdes	PORTUGAL
Laboratoires Dubernet	FRANCE
Laboratoire Dicœnos Rhône	FRANCE
Laboratoire Natoli	FRANCE
SCL Montpellier	FRANCE
Agricultural institute of Slovenia	SLOVENIA
Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	GERMANY
HBLAuBA Wein - und Obstbau	AUSTRIA
Landesuntersuchungsamt Mainz	GERMANY
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	GERMANY
Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach	ITALY
Unione Italiana Vini soc. Coop.	ITALY

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur wine

Sample		A (liqueur wine)		B (dry wine)		C (dry wine)		D (dry wine)		E (dry wine)		F (dry wine)		G (dry wine)		H (sweetened wine)		I (sweetened wine)		J (sweetened wine)	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Labo3	rep#1	0.17	0.14	0.04	0.04	**0.49**	**0.49**	0.68	0.73	**1.03**	**1.06**	*1.03*	*1.09*	1.34	1.31			**0.12**	**0.12**	1.36	1.34
	rep#2	0.18	0.17	0.01	0.02	**0.43**	**0.50**	0.68	0.68	**1.01**	**1.04**	*1.05*	*1.07*	1.28	1.26			**0.12**	**0.11**	1.24	1.32
Labo6	rep#1	0.19	0.19			**0.55**	**0.55**	0.77	0.73	**1.15**	**1.11**	*1.09*	*1.09*	1.42	1.39	0.04	0.03	0.14	0.13	**1.47**	**1.43**
	rep#2	0.14	0.14			**0.52**	**0.52**	0.75	0.71	**1.16**	**1.11**	*1.09*	*1.10*	1.44	1.41	0.03	0.03	0.08	0.07	**1.50**	**1.45**
Labo7	rep#1	0.16	0.17	0.04	0.04	0.51	0.52	0.72	0.73	1.09	1.05	*1.01*	*1.07*	1.33	1.36	0.03	0.03	0.12	0.13	1.42	1.42
	rep#2	0.17	0.17	0.03	0.04	0.50	0.50	0.72	0.72	1.05	1.09	*1.04*	*1.08*	1.34	1.32	0.02	0.03	0.12	0.12	1.44	1.43
Labo9	rep#1	0.15	0.14			0.50	0.54	0.70	0.70	1.09	1.10	*1.06*	*1.06*	1.31	1.27			0.11	0.07	1.41	1.43
	rep#2	0.14	0.15			0.50	0.52	0.69	0.74	1.10	1.08	*1.01*	*1.01*	1.29	1.27		0.01	0.10	0.12	1.37	1.33
Labo12	rep#1	0.17	0.18	0.08	0.08	**0.51**	**0.51**	0.74	0.75	1.06	1.04	*1.04*	*1.02*	1.34	1.34			0.16	0.16	1.40	1.40
	rep#2	0.17	0.18	0.08	0.08	**0.51**	**0.51**	0.74	0.76	1.04	1.00	*1.03*	*1.00*	1.34	1.36			0.17	0.18	1.40	1.42
Labo13	rep#1	0.16	0.16	0.05	0.05	0.49	0.48	0.71	0.72	**1.00**	**1.08**	*1.01*	*1.01*	1.30	1.42	0.03	0.03	0.12	0.12	1.39	1.41

	rep#2	0.16	0.17	0.05	0.05	0.49	0.50	0.71	0.73	**1.03**	**1.05**	*1.01*	*1.05*	1.29	1.33	0.02	0.02	0.13	0.12	1.37	1.37
Labo14	rep#1	0.15	0.14	0.02	0.02	0.61	0.61	0.79	0.79	1.20	1.21	*1.23*	*1.24*	1.53	1.49	0.01	0.01	0.10	0.10	1.59	1.58
	rep#2	0.14	0.14	0.02	0.02	0.60	0.61	0.79	0.79	1.22	1.21	*1.24*	*1.24*	1.53	1.52	0.01	0.01	0.10	0.10	1.59	1.59
Labo15	rep#1	**0.40**	**0.43**	0.09	0.08	1.00	0.98	1.03	1.07	1.69	1.68	*1.60*	*1.60*	2.04	2.10	0.06	0.07	0.24	0.19	2.07	2.06
	rep#2	**0.43**	**0.36**	0.03	0.03	0.95	0.99	1.05	1.09	1.64	1.68	*1.62*	*1.63*	2.04	2.03	0.04	0.01	0.16	0.19	2.17	2.01
Labo16	rep#1	**0.17**	**0.17**	0.03	0.03	0.53	0.53	0.73	0.75	1.14	1.12	*1.08*	*1.09*	1.37	1.38	**0.03**	**0.02**	0.12	0.13	**1.50**	**1.44**
	rep#2	**0.17**	**0.17**	0.03	0.05	0.55	0.53	0.74	0.74	1.15	1.13	*1.06*	*1.08*	1.40	1.37	**0.04**	**0.02**	0.12	0.12	**1.46**	**1.45**
Labo17	rep#1	0.12	0.13	0.02	0.02	0.46	0.45	0.65	0.71	1.01	1.01	*1.03*	*1.07*	1.20	1.18	0.01	0.01	0.10	0.09	1.36	1.36
	rep#2	0.12	0.13	0.03	0.03	0.46	0.48	0.69	0.70	1.05	1.07	*1.04*	*1.03*	1.29	1.32	0.01	0.01	0.08	0.10	1.37	1.37
Labo18	rep#1	0.16	0.15	0.04	0.04	0.44	0.43	0.64	0.63	0.95	0.95	***0.92***	***0.92***	1.19	1.17	0.03	0.03	0.11	0.11	1.23	1.25
	rep#2	0.14	0.15	0.04	0.04	0.44	0.45	0.63	0.63	0.94	0.95	***0.92***	***0.91***	1.18	1.17	0.03	0.03	0.11	0.11	1.26	1.26
Labo19	rep#1	0.16	0.16	0.04	0.04	0.55	0.58	0.79	0.80	1.18	1.21	***1.12***	***1.14***	1.42	1.45	0.02	0.02	0.13	0.13	1.49	1.50
	rep#2	0.16	0.16	0.04	0.04	0.56	0.56	0.78	0.74	1.13	1.15	***1.13***	***1.14***	1.43	1.46	0.03	0.02	0.13	0.13	1.51	1.51
Labo20	rep#1	0.17	0.18	0.04	0.04	0.53	0.53	0.74	0.75	1.11	1.09	*1.06*	*1.07*	1.30	1.32	0.03	0.03	0.13	0.13	1.39	1.42

	rep#2	0.17	0.18	0.04	0.04	0.53	0.53	0.74	0.75	1.11	1.09	*1.06*	*1.07*	1.30	1.32	0.03	0.03	0.13	0.13	1.39	1.42
Labo21	rep#1	0.18	0.18	0.05	0.05	0.56	0.56	0.79	0.80	1.17	1.22	*1.19*	*1.20*	1.46	1.50	0.05	0.05	0.13	0.13	1.54	1.58
	rep#2	0.17	0.18	0.05	0.05	0.56	0.56	0.79	0.81	1.19	1.21	*1.20*	*1.23*	1.47	1.48	0.05	0.05	0.13	0.13	1.55	1.57
Labo22	rep#1	0.24	0.23	0.05	0.06	0.53	0.51	0.70	0.72	1.04	1.10	*1.01*	*1.00*	1.29	1.28	0.05	0.04	**0.13**	**0.16**	**1.38**	**1.40**
	rep#2	0.24	0.22	0.05	0.05	0.52	0.52	0.70	0.74	1.04	1.06	*1.06*	*1.03*	1.29	1.29	0.05	0.04	**0.15**	**0.14**	**1.43**	**1.41**

Table of the data obtained. The values in bold correspond with the values rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

Sample	A	B	C	D	E	F	G	H	I	J
No. of laboratories selected	12	12	13	14	14	14	12	12	12	12
No. of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	0.13	0.02	0.44	0.63	0.95	0.92	1.18	0.01	0.09	1.25
Max.	0.18	0.08	0.61	0.80	1.21	1.24	1.52	0.05	0.17	1.59
Overall average	0.16	0.04	0.52	0.73	1.09	1.07	1.36	0.03	0.12	1.43
Repeatability variance	0.00005	0.00003	0.00011	0.00028	0.00053	0.00035	0.00032	0.00002	0.00004	0.00028
Inter-labo stand. dev.	0.02	0.02	0.04	0.04	0.07	0.08	0.10	0.01	0.02	0.09
Reproducibility variance	0.0003	0.0003	0.0020	0.0021	0.0057	0.0067	0.0093	0.0002	0.0004	0.0083
Repeatability standard dev.	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.00	0.01	0.02

r limit	0.02	0.02	0.03	0.05	0.06	0.05	0.05	0.01	0.02	0.05
Repeatability RSD_r	4.3%	14.1%	2.0%	2.3%	2.1%	1.8%	1.3%	16.9%	5.0%	1.2%
Reproducibility stand. dev.	0.02	0.02	0.04	0.05	0.08	0.08	0.10	0.01	0.02	0.09
R limit	0.05	0.05	0.13	0.13	0.21	0.23	0.27	0.04	0.06	0.26
Reproducibility RSD_R	10.5%	40.0%	8.5%	6.4%	7.0%	7.6%	7.1%	50.8%	16.5%	6.4%
Horwitz RSD_r	4.92	6.03	4.12	3.92	3.69	3.70	3.57	6.46	5.11	3.54
$Horrat_r$	0.87	2.33	0.49	0.59	0.57	0.48	0.37	2.62	0.98	0.33
Horwitz RSD_R	7.46	9.13	6.24	5.93	5.58	5.60	5.40	9.79	7.74	5.36
$Horrat_R$	1.41	4.38	1.36	1.07	1.25	1.36	1.32	5.19	2.13	1.19

Table of the results obtained

Note: The results from samples B and H should be taken with caution due to the very low concentration levels, which are below the laboratories' limit of quantification.

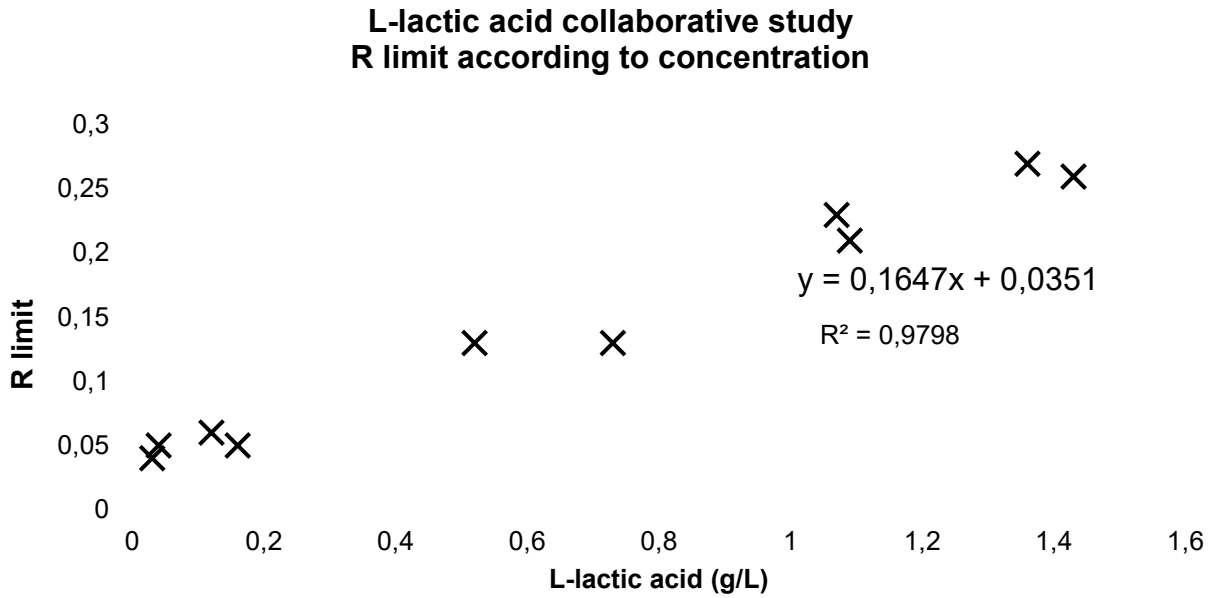


Figure 3: R limit according to concentration

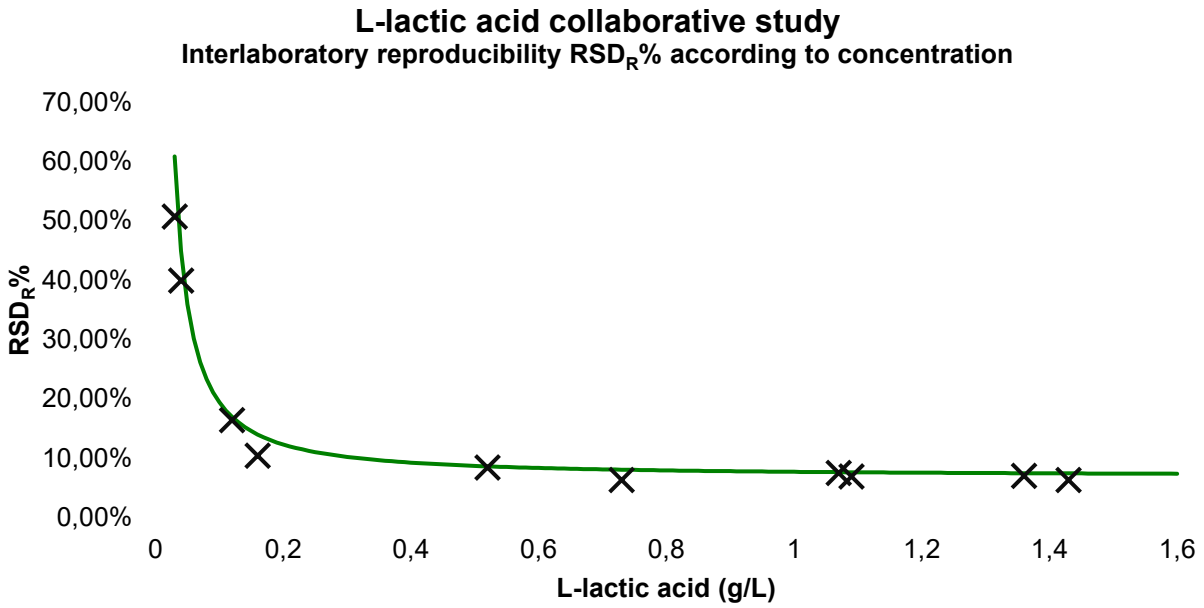


Figure 4: Interlaboratory $RSD_R\%$ according to concentration.
Modelling: $\mathbf{RSD_R\% = 0.758 \cdot C^{(-1.216)} + 7}$

Method OIV-MA-A313-26 Type III method

Determination of L-malic acid in wine by automated enzymatic method

(Resolution OIV-OENO 599-2018)

1. Scope of application

This method makes it possible to determine L-malic acid in wine by specific enzyme analysis using an automatic sequential analyser.
In this document, a collaborative study is reported which demonstrates application of the method for measurement of L-malic acid in the range from 0.12 to 2.3 g/L.

Note: Where necessary, each laboratory using this method may refine, and potentially widen, this range through a validation study.

2. Standard references

- OIV *Compendium of International Methods of Analysis*: L-malic acid – enzymatic method, OIV-MA-AS313-11,
- ISO 78-2: Chemistry – Layouts for standards.

3. Reaction principles

In the presence of nicotinamide adenine dinucleotide (NAD), L-malic acid is oxidised to oxaloacetate in a reaction catalysed by L-malate dehydrogenase (L-MDH). Since the equilibrium reaction is in favour of the malate, it is necessary to remove the oxaloacetate formed which is converted into L-aspartate in the presence of L-glutamate. This reaction is catalysed by glutamate oxaloacetate transaminase (GOT).

$$L-malate + NAD^{+} \overset{L-MDH}{\longleftrightarrow} oxaloacetate + NADH + H^{+}$$

$$oxaloacetate + L-glutamate \overset{GOT}{\longleftrightarrow} L-aspartate + \propto -ketoglutarate$$

The reduced nicotinamide adenine dinucleotide (NADH) produced is measured based on its absorption at 340 nm. It is proportional to the quantity of L-malic acid.

4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical usage (ISO 3696 standard);

4.1.2. Glycylglycine (CAS no. 556-50-3);

4.1.3. Glutamic acid (CAS no. 56-86-0);

4.1.4. NAD (nicotinamide adenine dinucleotide) (CAS no. 53-84-9);

4.1.5. L-MDH (L-malate dehydrogenase) (CAS no. 9001-64-3);

4.1.6. GOT (glutamate oxaloacetate transaminase) (CAS no. 9000-97-9);

4.1.7. L-malic acid, purity ≥ 95% (CAS no. 97-67-6);

4.1.8. Optional: polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8) or potentially PVPP (CAS no. 25249-54-1);

4.1.9. Sodium hydroxide (CAS no. 1310-73-2).

Note 1: There are commercial kits for the determination of L-malic acid. The user needs to check the composition to ensure it contains the above-indicated reagents.

Note 2: The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme protein molecules. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

4.2. Working solutions

4.2.1. A pH 10 buffer (0.60 M glycylglycine, 0.1 M L-glutamic acid).

The preparation may be as follows:

- glycylglycine (4.1.2): 4.75 g,
- glutamic acid (4.1.3): 0.88 g,
- PVP (4.1.8): 1 g,
- water for analytical usage (4.1.1): 50 mL.

The mixture is adjusted to pH 10 using a 10 M sodium hydroxide solution, then made up to 60 mL with water for analytical usage. This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.2. R1 working solution (example):

- water for analytical usage (4.1.1): 12 mL,
- NAD (4.1.4): 420 mg.

This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.3. R2 working solution (example):

- water for analytical usage (4.1.1): 1.2 mL,
- L-MDH (4.1.5): 4800 U,
- GOT (4.1.6): 320 U.

This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.3. Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of L-malic acid prepared by weighing and covering the measurement range.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with a UV detector. The reaction temperature should be tightly controlled (generally 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software ensuring its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature is 37 °C. Certain pieces of apparatus may use slightly different values.

5.1.5. Use of a reagent blank

The results are read by comparing the light intensity absorbed at the chosen wavelength between a cuvette in which the reaction is carried out and a cuvette in which the reaction does not take place (blank reagent).

5.1.6. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm.

5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

5.3. pH meter

5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

6. Sampling

6.1. Preparation of samples of musts and still wines

In most cases, wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

- filtration or centrifugation should be used for highly turbid samples,
- sample dilution (manual or automatic) with water for analytical usage should be used for values exceeding the measurement range.

6.2. Preparation of samples of sparkling wine containing CO_2

Sparkling wine samples containing CO_2 may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer be strictly observed. This also applies to the various enzymatic kits available on the market.
The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction takes place.

ADO

By way of example, the quantities of different elements may be as follows:

- sample: 2.5 µL,
- mixture of 80% buffer (3.2.1) and 20% R1 (3.2.2): 120 µL,
- R2 (3.2.3): 15 µL.

The equipment takes regular measurements that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

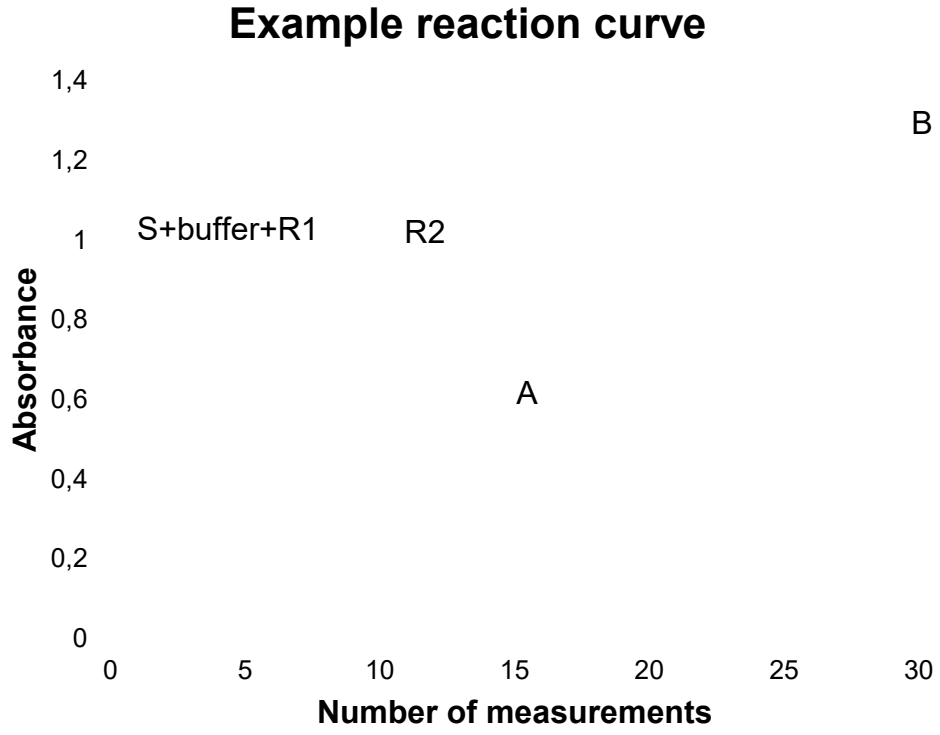


Figure 1: Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

8. Calculation of results

The measurement used for the determination of the result is as follows:

$$\Delta DO = (\text{Absorbance } B - \text{Absorbance } A)$$

In order to correlate this ΔDO value with the desired concentration of L-malic acid, regular calibration of the apparatus is carried out using the calibration solutions (§4.3) at a minimum of 3 points covering the measurement range. In the example given in Figure 2, the calibration curve obtained is a straight line for values between 0 and 2 g/L L-malic acid. In this case, for higher values, it is preferable to dilute the sample. In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

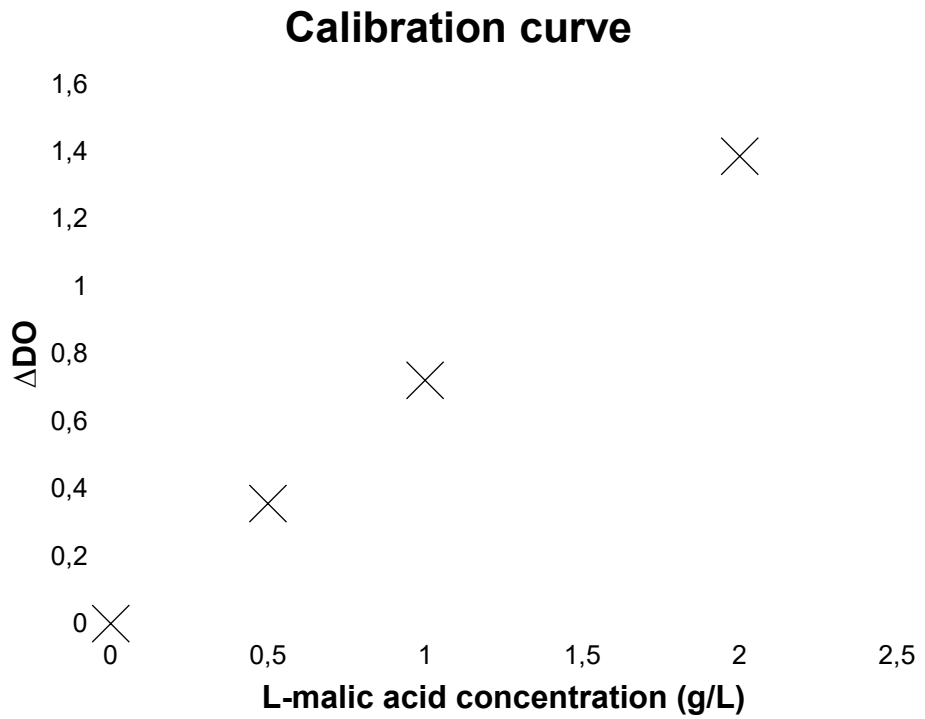


Figure 2: Calibration curve

The calibration curve can be order 1 (*Concentration* = $a.\Delta DO + b$) or even order 2 (*Concentration* = $a.\Delta DO + b.\Delta DO + c$). If using a calibration curve of order 2, the

laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve with high concentrations).

The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The results are expressed in g/L L-malic acid to 2 d.p.

10. Precision

Interlaboratory reproducibility

RSD_R = 5% (from 1 g/L)
CV_R% (k=2) = 2·RSD_R= 10%, (from 1 g/L)

Repeatability

RSD_r = 2% (from 1 g/L)
CV_r% (k=2) = 2·RSD_r = 4% (from 1 g/L)

Limit of quantification

Validated LOQ = 0,12 g/L
(Concentration where CV_R% (k=2) = 60%)

ANNEX

Results of the interlaboratory tests

Collaborative study

A total of 16 Laboratories from different countries participated in the collaborative study organised in 2016.

Labo	Country
Miguel Torres S.A.- Finca Mas La Plana	SPAIN
Estación Enológica de Castilla y León	SPAIN
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	SPAIN
Estación Enológica de Haro	SPAIN
Comissão de Viticultura da Região dos Vinhos Verdes	PORTUGAL
Laboratoires Dubernet	FRANCE
Laboratoire Dicœnos Rhône	FRANCE
Laboratoire Natoli	FRANCE
SCL Montpellier	FRANCE
Agricultural institute of Slovenia	SLOVENIA
Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	GERMANY
HBLAuBA Wein - und Obstbau	AUSTRIA
Landesuntersuchungsamt Mainz	GERMANY
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	GERMANY
Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach	ITALY

Unione Italiana Vini soc. Coop.	ITALY

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur wines.

Sample		A		B		C		D		E		F		G		H		I		J	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Labo3	rep#1	1.12	1.09	2.16	2.21	**0.08**	**0.08**	1.38	1.39	**0.16**	**0.16**	*0.05*	*0.04*	0.07	0.06	1.31	1.35	**1.24**	**1.27**	0.07	0.07
	rep#2	1.15	1.11	2.18	2.11	**0.08**	**0.09**	1.35	1.41	**0.16**	**0.16**	*0.05*	*0.04*	0.06	0.06	1.32	1.30	**1.23**	**1.24**	0.07	0.07
Labo6	rep#1	0.99	0.95	2.28	2.31	**0.04**	**0.02**	1.44	1.47	**0.05**	**0.08**	*0.04*	*0.04*	0.06	0.04	1.38	1.46	1.34	1.19	**0.04**	**0.03**
	rep#2	0.93	0.93	2.15	2.16	**0.05**	**0.03**	1.23	1.37	**0.08**	**0.09**	*0.03*	*0.05*	0.03	0.04	1.28	1.30	1.20	1.28	**0.03**	**0.03**
Labo7	rep#1	1.10	1.10	2.28	2.33	0.01		1.44	1.52	0.12	0.10		*0.01*			1.38	1.41	1.33	1.30	0.01	0.02
	rep#2	1.13	1.13	2.35	2.33	0.03	0.04	1.47	1.49	0.14	0.13				0.02	1.40	1.43	1.32	1.33	0.02	0.03
Labo9	rep#1	1.14	1.10	2.27	2.21	0.06	0.10	1.43	1.48	0.19	0.19	*0.02*	*0.02*	0.05	0.06	1.35	1.38	1.27	1.28	0.05	0.08
	rep#2	1.15	1.20	2.20	2.23	0.10	0.06	1.50	1.51	0.15	0.14	*0.03*	*0.01*	0.09	0.11	1.31	1.37	1.22	1.28	0.04	0.06
Labo12	rep#1	1.12	1.12	2.34	2.32	**0.06**	**0.02**	1.47	1.54	0.14	0.14	*0.04*	*0.03*	0.03	0.03	1.37	1.37	1.29	1.31	0.06	0.04

	rep#2	1.12	1.12	2.38	2.34	**0.07**	**0.01**	1.46	1.46	0.16	0.19	*0.04*	*0.05*	0.03	0.04	1.32	1.33	1.28	1.30	0.06	0.04
Labo13	rep#1	1.11	1.09	2.12	2.26	0.04	0.02	1.44	1.45	**0.12**	**0.14**	*0.01*	*0.01*	0.03	0.03	1.45	1.33	1.29	1.29	0.02	0.02
	rep#2	1.18	1.17	2.20	2.29	0.03	0.01	1.47	1.50	**0.11**	**0.12**			0.02	0.03	1.39	1.38	1.32	1.33		0.01
Labo14	rep#1	1.32	1.30	2.66	2.68			1.69	1.67	0.08	0.08					1.65	1.60	1.51	1.52		
	rep#2	1.30	1.30	2.63	2.68			1.67	1.67	0.08	0.08					1.61	1.60	1.51	1.53		
Labo15	rep#1	**1.18**	**1.19**	2.29	2.42	0.06	0.05	1.41	1.50	0.16	0.16	*0.04*	*0.04*	0.08	0.08	1.33	1.39	1.26	1.30	0.07	0.07
	rep#2	**1.13**	**1.20**	2.21	2.42	0.05	0.05	1.49	1.52	0.16	0.16	*0.04*	*0.04*	0.08	0.08	1.38	1.39	1.27	1.28	0.07	0.07
Labo16	rep#1	**1.22**	**1.22**	2.52	2.48			1.63	1.62	0.09	0.09					**1.50**	**1.53**	1.47	1.45		
	rep#2	**1.21**	**1.20**	2.45	2.55			1.62	1.61	0.09	0.09		*0.03*			**1.52**	**1.51**	1.44	1.45		
Labo17	rep#1	1.14	1.14	2.22	2.22	0.07	0.08	1.51	1.50	0.17	0.17	*0.07*	*0.07*	0.11	0.11	1.34	1.35	1.28	1.27	0.09	0.09
	rep#2	1.13	1.15	2.17	2.20	0.08	0.08	1.46	1.47	0.18	0.17	*0.08*	*0.07*	0.12	0.12	1.31	1.34	1.28	1.29	0.09	0.09
Labo18	rep#1	1.10	1.11	2.14	2.13	0.18	0.18	1.42	1.52	0.28	0.28	***0.16***	***0.16***	0.19	0.19	1.33	1.38	1.27	1.31	0.17	0.18
	rep#2	1.10	1.13	2.19	2.16	0.18	0.18	1.44	1.51	0.27	0.27	***0.16***	***0.16***	0.19	0.19	1.33	1.38	1.29	1.33	0.17	0.18

Labo19	rep#1	1.13	1.19	2.20	2.23	0.08	0.08	1.47	1.44	0.17	0.19	***0.07***	***0.08***	0.11	0.11	1.36	1.30	1.27	1.26	0.10	0.10
	rep#2	1.15	1.22	2.21	2.23	0.08	0.09	1.48	1.45	0.18	0.19	***0.07***	***0.08***	0.12	0.11	1.33	1.30	1.28	1.24	0.09	0.10
Labo20	rep#1	1.17	1.20	2.27	2.27	0.04	0.05	1.50	1.48	0.17	0.17	*0.04*	*0.03*	0.07	0.07	1.33	1.34	1.28	1.29	0.07	0.06
	rep#2	1.17	1.20	2.27	2.27	0.04	0.05	1.50	1.48	0.17	0.17	*0.04*	*0.03*	0.07	0.07	1.34	1.34	1.28	1.29	0.07	0.06
Labo21	rep#1	1.10	1.12	2.30	2.47	0.05	0.05	1.45	1.49	0.11	0.10	*0.05*	*0.05*	0.05	0.05	1.46	1.44	1.33	1.41	0.05	0.05
	rep#2	1.09	1.16	2.28	2.50	0.05	0.05	1.49	1.51	0.09	0.11	*0.05*	*0.05*	0.05	0.05	1.43	1.46	1.34	1.37	0.05	0.05
Labo22	rep#1	1.08	1.07	2.35	2.31	0.04	0.03	1.46	1.45	0.11	0.12	*0.01*	*0.02*	0.03	0.03	1.35	1.35	**1.26**	**1.31**	**0.03**	**0.02**
	rep#2	1.06	1.08	2.30	2.31	0.04	0.04	1.51	1.46	0.12	0.12	*0.01*	*0.03*	0.04	0.03	1.35	1.34	**1.28**	**1.25**	**0.01**	**0.01**

Table of the data obtained. The values in bold correspond with the values rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

Sample	A	B	C	D	E	F	G	H	I	J

No. of laboratories selected	15	15	12	14	13	13	12	12	12	12
No of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	0.95	2.15	0.03	1.38	0.08	0.01	0.02	1.32	1.25	0.02
Max.	1.31	2.66	0.08	1.68	0.28	0.08	0.19	1.45	1.36	0.10
Overall average	1.14	2.30	0.05	1.49	0.14	0.04	0.07	1.36	1.29	0.05
Repeatability variance	0.001	0.003	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.000
Inter-laboratory stand. dev.	0.08	0.14	0.02	0.07	0.05	0.02	0.05	0.04	0.03	0.03
Reproducibility variance	0.006	0.021	0.001	0.006	0.003	0.000	0.002	0.002	0.001	0.001
Repeatability stand. dev.	0.03	0.05	0.01	0.03	0.01	0.01	0.01	0.03	0.02	0.01
r limit	0.07	0.15	0.04	0.08	0.02	0.02	0.01	0.07	0.06	0.02
Repeatability RSD_r	2.2%	2.4%	25.0%	2.0%	6.2%	15.4%	7.4%	1.9%	1.6%	14.5%
Reproducibility stand. dev.	0.08	0.14	0.02	0.08	0.05	0.02	0.05	0.04	0.04	0.03

R limit	0.22	0.41	0.07	0.21	0.16	0.06	0.14	0.12	0.10	0.08
Reproducibility RSD_R	6.9%	6.2%	46.9%	5.0%	39.1%	57.5%	71.2%	3.2%	2.8%	53.1%
Horwitz RSD_r	3.66	3.29	5.81	3.51	5.02	6.13	5.57	3.56	3.59	5.81
$Horrat_r$	0.61	0.72	4.30	0.57	1.24	2.52	1.33	0.53	0.45	2.49
Horwitz RSD_R	5.55	4.99	8.80	5.32	7.60	9.29	8.44	5.40	5.44	8.81
$Horrat_R$	1.25	1.25	5.33	0.95	5.14	6.19	8.43	0.59	0.51	6.02

Table of the results obtained

Note: The results from samples C, F, G and J should be taken with caution due to the very low concentration levels, which are below the laboratories' limit of quantification.

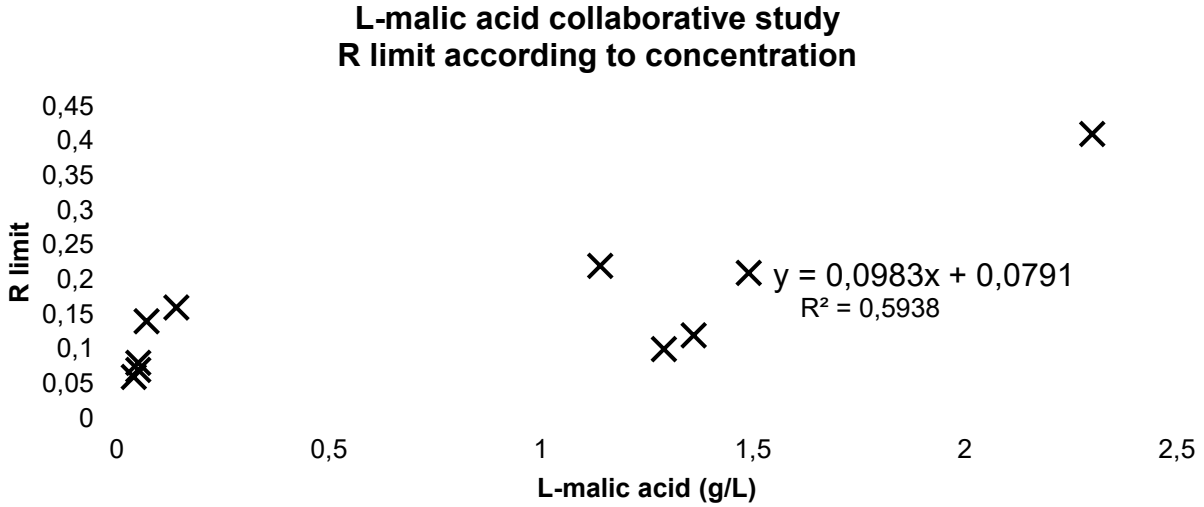


Figure 3: R limit according to concentration

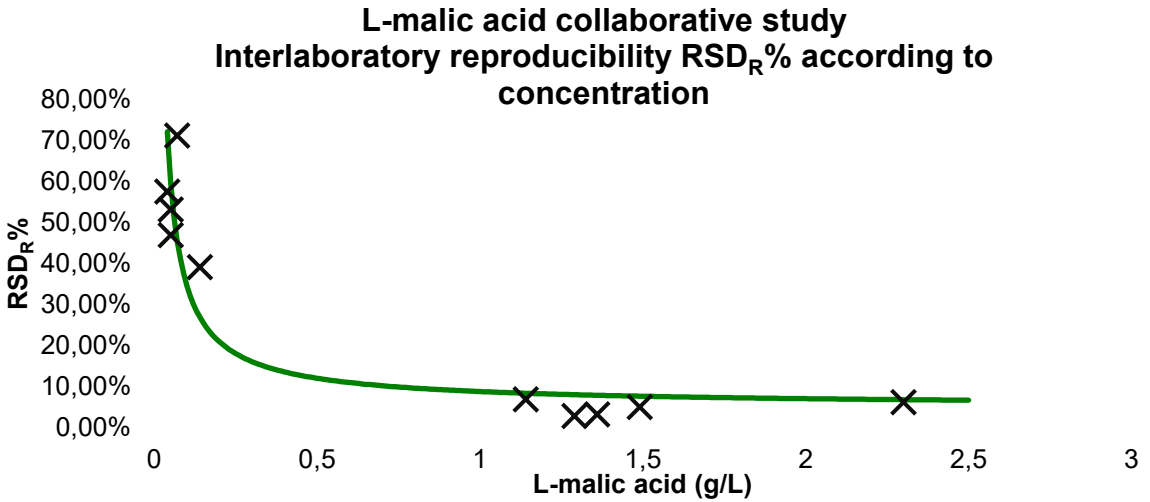


Figure 4: $RSD_R\%$ according to concentration
Modelling: $\mathbf{CV\% = 3.763 \cdot C^{(-0.895)} + 5}$

Method OIV-MA-AS313-27 Type II method

DETERMINATION OF ACETIC ACID IN WINES BY AUTOMATED ENZYMATIC METHOD

OIV-OENO 621-2019

1. Scope of application

This method makes it possible to determine acetic acid in wines using an automatic sequential analyser and specific enzyme analysis. The measurement range, which was the object of the current interlaboratory validation, is from 0.2 to 1.14 g/L acetic acid.

NOTE: A range of higher values may be analysed with the introduction of a sample dilution.

2. Standard references

- ISO 78-2: Chemistry – Layouts for standards

3. Reaction principles

In the presence of ATP, acetic acid is converted into acetyl phosphate in a reaction catalysed by acetate kinase.

(1) Acetate + ATP $\xrightarrow{\text{Acetate kinase}}$ Acetyl phosphate + ADP

The ADP formed by this reaction is reconverted into ATP in a reaction with phosphoenolpyruvate catalysed by pyruvate kinase.

(2) ADP + Phosphoenolpyruvate $\xrightarrow{\text{Pyruvate kinase}}$ Pyruvate + ATP

Pyruvate is reduced to L-lactate by reduced nicotinamide adenine dinucleotide (NADH) catalysed by lactate dehydrogenase.

(3) Pyruvate + NADH + H^+ $\xrightarrow{\text{Lactate dehydrogenase}}$ Lactate + NAD^+ + H_2O

The quantity of oxidised NADH in reaction (3) is determined by the absorbance measurement at 340 nm, and is proportional to the concentration of acetic acid in the wine.

Note 1:

When this enzymatic analysis is conducted manually, the reading is carried out once the stabilised, final plateau is reached. To achieve this, a fourth reaction makes it possible to completely push the equilibrium of reaction 1 towards the formation of acetyl phosphate through elimination of the latter.

(4) Acetyl phosphate + CoA $\xrightarrow{\text{Phosphotransacetylase}}$ **Acetyl-CoA + Inorganic phosphate**

In the case of analysis by an automated method, which has a shorter analysis time, it is not necessary to reach a stabilised, final plateau, and this reaction is pointless. The use of phosphotransacetylase is therefore not necessary, and is not described here.

Note 2:

The enzymatic reaction chain involves pyruvate. The low quantities of pyruvate (several tens of mg/L) normally present in wines do not have a significant impact on the result. In the rare cases, the presence in wine of an untypical quantity of pyruvate is likely to produce a method bias.

4. Reagents and working solutions

During analysis – unless otherwise indicated – use only quality, recognised analytical reagents and distilled or demineralised water, or water of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical use (ISO 3696 standard)

4.1.2. 3-(N-Morpholino)propanesulfonic acid (MOPS): CAS no. 1132-61-2

4.1.3. Magnesium chloride **hexahydrate**: CAS no. 7791-18-6

4.1.4. Potassium chloride: CAS no. 7447-40-7

4.1.5. ß-Nicotinamide adenine dinucleotide (NADH): CAS no. 53-84-9, purity ≥ 98%

4.1.6. Adenosine-5'-triphosphate, disodium salt (ATP): CAS no. 56-65-5

4.1.7. Potassium hydroxide: CAS no. 1310-58-3

4.1.8. Phosphoenolpyruvate tri(cyclohexylammonium) salt: CAS no. 35556-70-8 or monosodium phosphoenolpyruvate: CAS no. 138-08-9 (PEP)

4.1.9. Acetate kinase (AK): CAS no. 9027-42-3

4.1.10. Pyruvate kinase (PK): CAS no. 9001-59-6

4.1.11. Lactate dehydrogenase (LDH): CAS no. 9001-60-9

4.1.12. Polyvinylpyrrolidone (PVP): CAS no. 9003-39-8

4.1.13. Acetic acid: purity ≥ 99.5%; CAS no. **64-19-7**

4.1.14. Sodium chloride: CAS no. 7647-14-15

4.1.15. Bovine serum albumin (BSA): CAS no. 9048-46-8

Note 3: There are commercial kits for the determination of acetic acid. The user needs to check the composition to ensure it contains the above-indicated reagents. These kits are sometimes supplied with phosphotransacetylase (redundant when using an automated method).
Note 4: The use of PVP is recommended to eliminate any possible negative action of tannins in wine on the enzyme protein molecules. In the event that the use of PVP is not effective, the laboratory should ensure the absence of interference of wine tannins on the enzymes.
Note 5: BSA is an agent used for the stabilisation of enzymes in solution.

4.2. Working solutions

4.2.1. MOPS buffer

The preparation may be as follows:

- 13 g MOPS (3-(N-Morpholino)propanesulfonic acid) (4.1.2),
- 0.5 g magnesium chloride hexahydrate (4.1.3),
- 1.5 g potassium chloride (KCl) (4.1.4),
- 1.3 g PVP (4.1.12),
- 250 mL water for analytical usage (4.1.1).

Adjust the pH to 4.75 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Wait 5 minutes and readjust the pH to 7.45 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Make up to 300 mL with water for analytical use (4.1.1).

The buffer can be kept for at least 60 days at 2-8 °C (approximately).

4.2.2. Working solution 1 (R1)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),
- 300-350 mg adenosine-5'-triphosphate, disodium salt (ATP) (4.1.6),
- 50 mg phosphoenolpyruvate (PEP) tri(cyclohexylammonium) salt (4.1.8),
- 40 mg β-nicotinamide adenine dinucleotide (reduced form) (NADH) (4.1.5).

Working solution R1 can be kept for at least 30 days at 2-8 °C (approximately).

4.2.3. Working solution 2 (R2)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),

- approx. 40 units of pyruvate kinase (PK) (4.1.10),
- approx. 40 units of lactate dehydrogenase (LDH) (4.1.11),
- 50 units of acetate kinase (AK) (4.1.9),
- 300 mg BSA (4.1.15).

Working solution R2 can be kept for approximately 48 hours at 2-8 °C (approximately).

Note: When preparing these solutions, they should be mixed gently to avoid the formation of foam. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

4.3. Calibration solutions

In order to ensure the closest possible connection to the International System of Units (SI), the calibration range should be made up of pure solutions of acetic acid (4.1.13). It is recommended to prepare a stock solution (e.g. 1.5 $g.L^{-1}$ acetic acid) by weight, then the rest of the calibrations are obtained from the stock solution to cover the measurement range.
A "zero" value may be obtained using a 9‰ sodium chloride solution (4.1.14) or equivalent saline solution.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with UV detector. The reaction temperature should be stable (at around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software that handles its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU).

The absorbance values should not be saturating for the spectrometer used.

5.1.3. Precision of sampled volumes

The volumes of reagents and samples taken by the pipettes of the analyser should be of sufficient precision so as not to have a significant impact on the measurement result.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature 37 °C. Some pieces of apparatus may use slightly different values.

5.1.5. Use of a reagent blank

The results are read by comparing the light intensity absorbed at the chosen wavelength between a cuvette in which the reaction is carried out and a cuvette in which the reaction does not take place (reagent blank).

5.1.6. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm. For spectrophotometers in general use, this wavelength is to be selected.
A secondary wavelength is programmed in order to correct a potential matrix effect; this wavelength is 410 nm.
The measurement is conducted based on the DO_{340} - DO_{410} absorbance difference.

5.2. Balance

This should be calibrated to the SI and have a 1 mg resolution.

5.3. pH meter

5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

6. Sample preparation

6.1. Test samples

6.1.1. Preparation of samples of still wines

The majority of wine samples may be analysed without preparation. In some cases, a preparation may be introduced:

- Filtration should be used for highly turbid samples.
  Sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values higher than the measurement range.

6.1.2. Preparation of samples of sparkling wines

Sparkling wine samples should be subjected to a preliminary degassing by stirring under vacuum, ultrasonic treatment or any method that allows for the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly respected. The same goes for different enzymatic kits that are available on the market. The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. Homogenisation takes place. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1 to 5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction is triggered.

   By way of example, the quantities of the different elements may be as follows:
   - sample: 3 µL,
   - R1: 120 µL, at T_0 (start of sequence),
   - R2: 60 µL, at T_0 + 3 min 40 sec.

The equipment carries out regular measurements that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

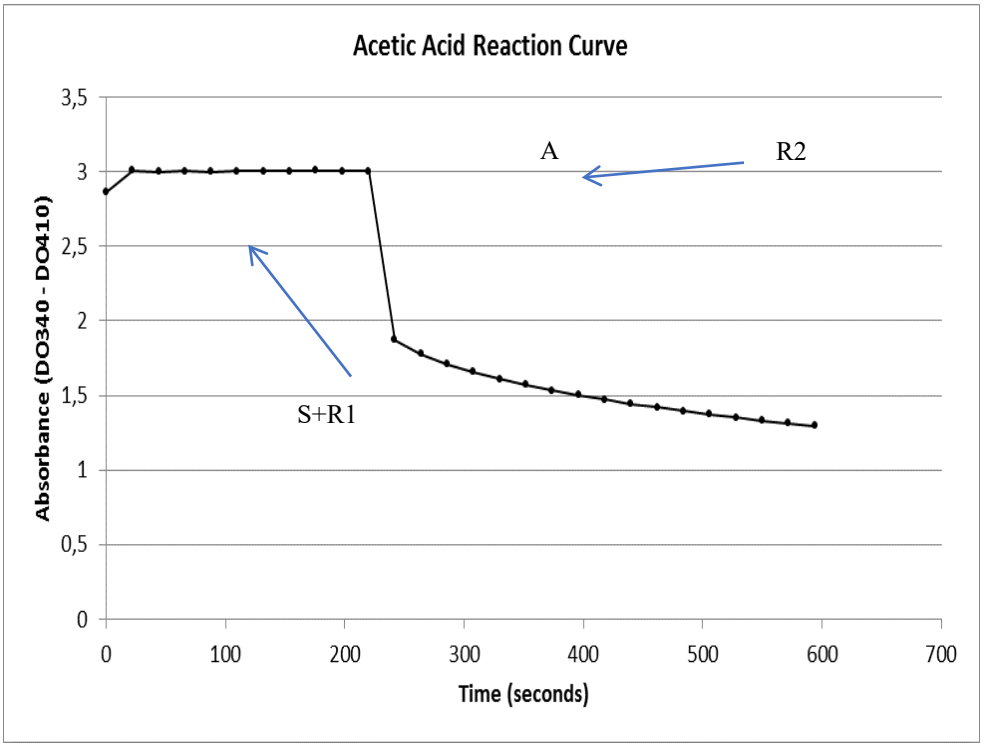


Figure 1. Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

In order to correlate this value with the concentration of acetic acid, regular calibration of the apparatus is carried out using the calibration solutions at a minimum of 3 points covering the measurement range used. The calibration curve obtained is near to a straight line. Nevertheless, a second-degree equation may be used. An example is given in Figure 2.

In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of calibration).

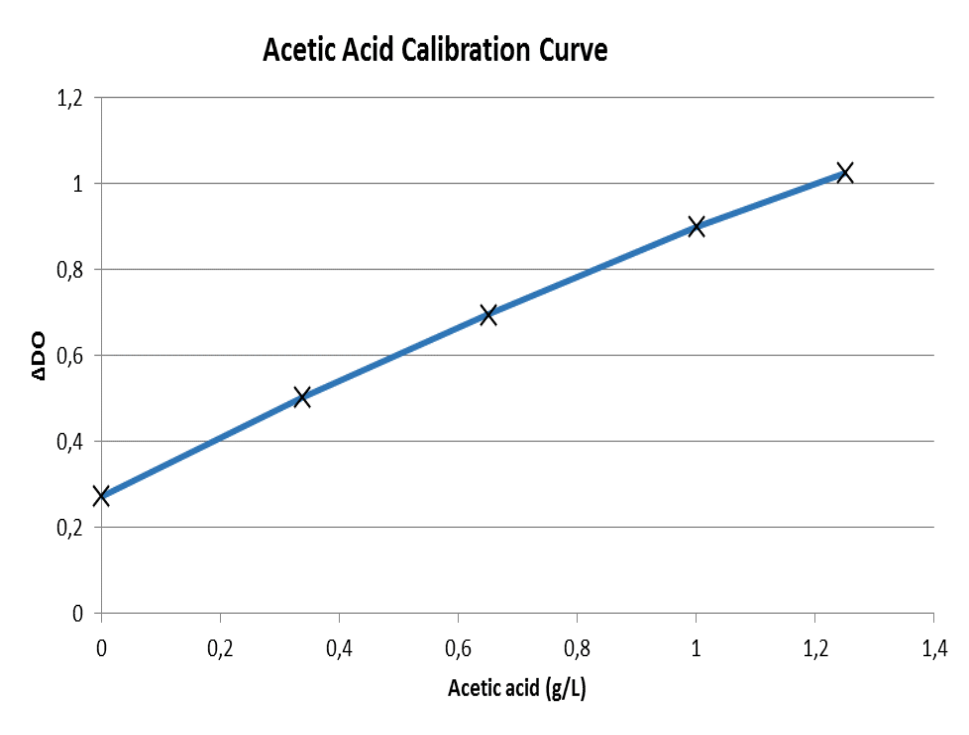


Figure 2. Calibration curve

8. *Calculations*

For each measurement, the result is given according to the following formula:

$$R = |Absorbance\ B - Absorbance\ A|$$

The values thus obtained are recorded on the calibration curve to obtain the acetic acid concentration. The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The results for acetic acid are expressed in g/L of acetic acid, calculated to two decimal places, or in another unit according to usage (meq/L). The expression of the result should be consistent with the measurement uncertainty.

10. Automated enzymatic method characteristics

Interlaboratory reproducibility

$RSD_R = 10\%$
$CV_R\%\ (k=2) = 2 \cdot RSD_R = 20\%$

Repeatability

$RSD_r = 4\%$
$CV_r\%\ (k=2) = 2 \cdot RSD_r = 8\%$

Limit of quantification

Validated LQ < 0.2 g/L
Not determined in the collaborative study

11. Bibliography

- McCLOSKEY Leo P., 'An Improved enzymatic assay for acetate in juice and wine', *Am. J. Enol. Vitic.*, Vol. 31, No. 21980.

ANNEX

Method performance studies

Collaborative study

In total, 11 laboratories from 5 different countries took part in the collaborative study.

Laboratory	Country
Miguel Torres S.A.- Finca Mas La Plana	Spain
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	Spain
Estación Enológica de Haro	Spain
Laboratoires Dubernet	France
Laboratoire Diœnos Rhône	France
Laboratoire Natoli	France
SCL Montpellier	France
Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	Germany
HBLAuBA Wein - und Obstbau	Austria
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	Germany
Unione Italiana Vini soc. Coop.	Italy

Table 1. Participating laboratories

In total, 2 x 10 samples prepared as blind duplicates were analysed, with 1 repetition. The wines analysed were dry wines, sweetened wines, and liqueur wines, wines originating from France and Portugal.

Sample		A		B		C		D		E		F		G		H		I		J	
		Port wine		Sweetened wine		Dry wine		Dry wine		Sweetened wine		Dry wine		Dry wine		Sweetened wine		Sweetened wine		Sweetened wine	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Lab3	rep#1	0.24	0.27	0.20	0.21	0.65	0.65	0.47	0.49	0.54	0.52	*1.28*	*1.30*	0.64	0.63	0.29	0.31	0.39	0.37	0.63	0.62
	rep#2	0.25	0.26	0.20	0.21	0.67	0.65	0.46	0.50	0.56	0.53	*1.29*	*1.33*	0.65	0.67	0.29	0.28	0.36	0.37	0.65	0.61
Lab7	rep#1	0.20	0.20	0.22	0.23	0.62	0.62	0.45	0.46	0.50	0.50	*1.25*	*1.30*	0.61	0.62	0.28	0.28	0.34	0.35	0.62	0.60
	rep#2	0.20	0.21	0.21	0.22	0.63	0.64	0.45	0.46	0.53	0.52	*1.20*	*1.20*	0.61	0.64	0.29	0.28	0.35	0.37	0.60	0.61
Lab9	rep#1	0.17	0.18	0.18	0.19	0.57	0.52	0.40	0.40	0.41	0.43	*1.18*	*1.18*	0.57	0.54	0.24	0.29	0.36	0.32	0.53	0.51
	rep#2	0.17	0.19	0.16	0.17	0.59	0.57	0.39	0.43	0.44	0.41	*1.16*	*1.14*	0.55	0.55	0.25	0.29	0.30	0.33	0.55	0.51
Lab12	rep#1	0.17	0.18	0.20	0.20	0.56	0.53	0.40	0.41	0.44	0.44	*1.02*	*1.01*	0.53	0.53	0.27	0.28	0.36	0.33	0.49	0.51
	rep#2	0.17	0.18	0.20	0.21	0.55	0.54	0.40	0.41	0.44	0.44	*1.02*	*1.01*	0.52	0.52	0.28	0.29	0.36	0.34	0.48	0.51
Lab13	rep#1	0.22	0.19	0.23	0.20	0.50	0.51	0.40	0.40	0.42	0.44	*0.95*	*0.97*	0.48	0.49	0.27	0.28	0.32	0.32	0.48	0.50
	rep#2	0.20	0.19	0.23	0.21	0.52	0.52	0.39	0.39	0.43	0.42	*0.97*	*0.96*	0.51	0.48	0.28	0.28	0.32	0.33	0.50	0.51
Lab14	rep#1	0.17	0.17	0.20	0.19	0.56	0.57	0.42	0.41	0.46	0.45	*1.10*	*1.14*	0.55	0.54	0.27	0.26	0.34	0.32	0.53	0.51
	rep#2	0.17	0.17	0.20	0.19	0.56	0.57	0.42	0.41	0.45	0.44	*1.12*	*1.10*	0.53	0.55	0.26	0.26	0.33	0.31	0.53	0.53
Lab15	rep#1	0.22	0.23	0.28	0.27	0.68	0.68	0.52	0.52	0.56	0.56	*1.20*	*1.23*	0.69	0.73	0.35	0.34	**0.47**	**0.42**	0.60	0.62
	rep#2	0.22	0.22	0.26	0.26	0.68	0.63	0.53	0.50	0.52	0.54	*1.18*	*1.13*	0.65	0.67	0.34	0.34	**0.42**	**0.41**	0.59	0.64
Lab17	rep#1	0.20	0.19	0.26	0.25	0.54	0.52	0.41	0.42	0.39	0.39	*1.01*	*1.00*	**0.49**	**0.45**	0.32	0.29	0.34	0.35	**0.44**	**0.43**
	rep#2	0.20	0.20	0.27	0.27	0.53	0.55	0.43	0.43	0.43	0.43	*1.03*	*1.05*	**0.49**	**0.49**	0.31	0.32	0.37	0.38	**0.44**	**0.46**
Lab18	rep#1	0.27	0.25	**0.35**	**0.33**	0.69	0.68	**0.53**	**0.56**	**0.59**	**0.59**	*1.24*	*1.21*	0.66	0.68	**0.43**	**0.41**	**0.50**	**0.51**	**0.65**	**0.63**
	rep#2	0.28	0.27	**0.36**	**0.36**	0.68	0.69	**0.55**	**0.57**	**0.60**	**0.60**	*1.26*	*1.23*	0.68	0.71	**0.44**	**0.43**	**0.50**	**0.52**	**0.63**	**0.65**
Lab20	rep#1	0.23	0.20	0.29	0.29	0.58	0.57	0.49	0.47	0.47	0.47	*1.15*	*1.13*	0.55	0.58	0.34	0.35	0.39	0.40	0.55	0.52
	rep#2	0.23	0.20	0.29	0.29	0.58	0.57	0.49	0.47	0.47	0.47	*1.15*	*1.13*	0.55	0.58	0.34	0.36	0.39	0.40	0.55	0.52
Lab22	rep#1	0.17	0.16	0.20	0.18	0.60	0.60	0.43	0.44	0.47	0.47	*1.20*	*1.20*	0.58	0.58	0.26	0.26	0.32	0.32	0.55	0.58
	rep#2	0.17	0.17	0.19	0.19	0.61	0.61	0.43	0.43	0.48	0.47	*1.21*	*1.22*	0.59	0.58	0.26	0.27	0.31	0.33	0.54	0.59

Table 2. Table of data obtained (in g·L^{-1} of acetic acid). The values in bold correspond to the values rejected by the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with a significance level of 2.5% (two-tailed test).

Sample	A	B	C	D	E	F	G	H	I	J
No. of laboratories selected	11	10	11	10	10	11	10	9	9	9
No. of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	0.17	0.18	0.51	0.40	0.41	0.96	0.49	0.26	0.32	0.50
Max.	0.27	0.29	0.69	0.52	0.55	1.30	0.69	0.35	0.39	0.63
Overall average	0.20	0.22	0.59	0.44	0.47	1.14	0.59	0.30	0.35	0.55
Repeatability variance	0.0001	0.0001	0.0002	0.0001	0.0002	0.0006	0.0003	0.0001	0.0003	0.0003
Inter-lab. stand. dev.	0.03	0.04	0.06	0.04	0.05	0.11	0.07	0.03	0.03	0.05
Reproducibility variance	0.001	0.001	0.003	0.002	0.002	0.012	0.005	0.001	0.001	0.003
Repeatability stand. dev.	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.02
r limit	0.03	0.03	0.04	0.03	0.04	0.07	0.05	0.02	0.05	0.05
Repeatability RSD_r	4.5%	4.5%	2.4%	2.7%	2.9%	2.1%	3.1%	2.8%	4.8%	3.2%
Reproducibility stand. dev.	0.03	0.04	0.06	0.04	0.05	0.11	0.07	0.03	0.03	0.05
R limit	0.10	0.11	0.17	0.12	0.14	0.31	0.19	0.09	0.08	0.15
Reproducibility RSD_R	16.8%	17.4%	9.9%	9.5%	10.6%	9.5%	11.7%	11.1%	8.5%	9.5%
Horwitz RSD	4.74	4.68	4.04	4.22	4.18	3.66	4.04	4.49	4.38	4.08
$HorRat_r$	0.96	0.97	0.60	0.63	0.70	0.58	0.77	0.62	1.10	0.79
Horwitz RSD	7.18	7.09	6.12	6.40	6.34	5.54	6.13	6.80	6.63	6.18
$HorRat_R$	2.34	2.45	1.63	1.49	1.67	1.71	1.91	1.63	1.29	1.53

Table 3. Table of results obtained

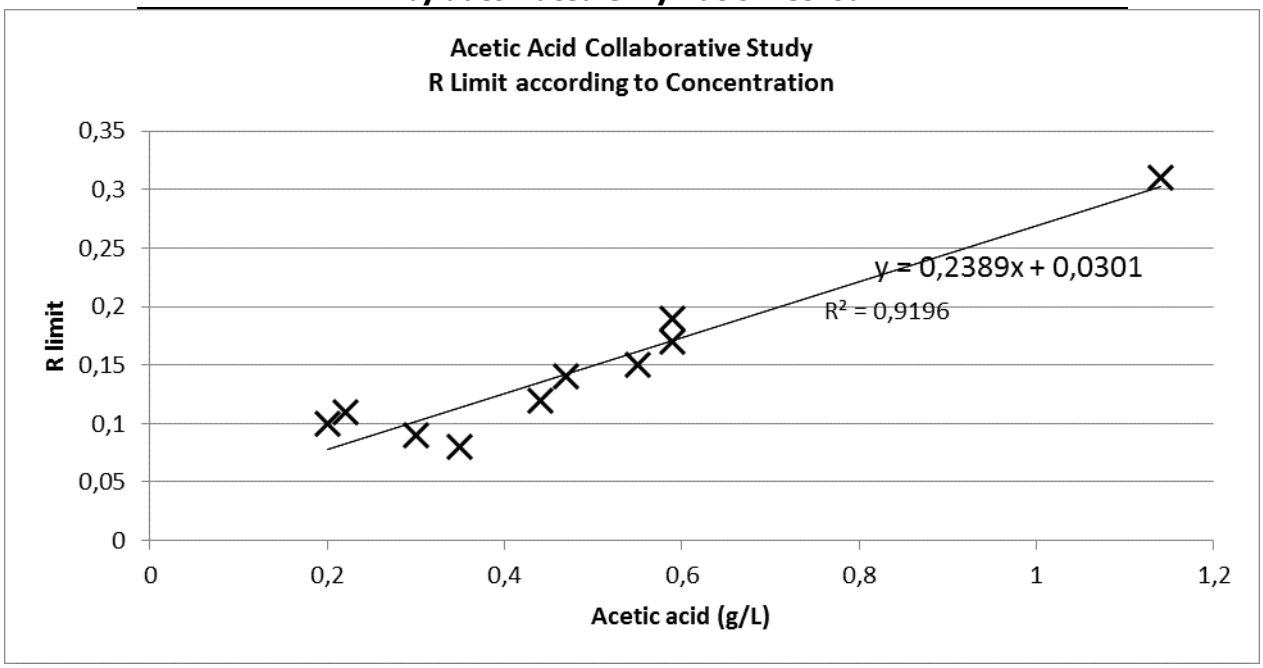


Figure 3. R limit according to concentration

Method OIV-MA-AS313-28 Type II method

Determination of D-gluconic acid in wines and musts by automated enzymatic method

OIV-OENO 622-2019

1. Scope of application

This method makes it possible to determine D-gluconic acid in wines and musts by specific enzymatic analysis using an automatic sequential analyser, with concentrations of 0.06 g/L to 5.28 g/L of analyte (taking into account that the sample may be diluted).

2. Principle

The D-gluconate present in the sample is phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by gluconate kinase (GK), to produce D-gluconate 6-phosphate and adenosine diphosphate (ADP).

$$\text{D-gluconate} + \text{ATP} \xrightarrow{\text{GK}} \text{D-Gluconate-6-P} + \text{ADP}$$

In the presence of nicotinamide adenine dinucleotide phosphate (NADP), D-gluconate 6-phosphate oxidises to form ribulose 5-phosphate through the action of enzyme 6-phosphogluconate dehydrogenase (6-PGDH). The quantity produced of reduced nicotinamide adenine dinucleotide phosphate (NADPH) corresponds to that of D-gluconate-6-phosphate and, as such, of D-gluconic acid.

$$\text{D-gluconate-6-P} + \text{NADP}^{+} \xrightarrow{\text{6-PGDH}} \text{Ribulose-5-P} + \text{NADPH} + \text{CO}_2 + \text{H}^{+}$$

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is determined by spectrophotometry at 340 nm (the wavelength of maximum

absorption of NADPH). The NADPH concentration is proportional to the concentration of D-gluconic acid.

3. Reagents and working solutions

3.1. Reagents:

3.1.1. Distilled water for laboratory use, certified to the EN ISO 3696 standard

3.1.2. PIPES (Piperazine-1,4-bis[ethanesulfonic acid]) (CAS No. 5625-37-6)

3.1.3. β-NADP-Na_2 (β-Nicotinamide adenine dinucleotide phosphate, disodium salt) (CAS No. 24292-60-2)

3.1.4. $MgCl_2 \cdot 6H_2O$ (Magnesium chloride hexahydrate) (CAS No. 7791-18-6)

3.1.5. ATP-Na_2 (Adenosine 5'-triphosphate disodium salt) (CAS No. 987-65-5)

3.1.6. Gluconate kinase (GK) (EC 2.7.1.12)

3.1.7. 6-phosphogluconate dehydrogenase (6-PGDH) (EC 1.1.1.44)

3.1.8. D-gluconic acid sodium salt (CAS No.527-07-1), minimum purity ≥ 99%

3.1.9. NaOH (Sodium hydroxide) (CAS No. 1310-73-2)

3.1.10 . PVP K-90 (Polyvinylpyrrolidone K-90) (CAS No. 9003-39-8)

3.2. Working solutions

3.2.1. Reagent 1: dissolve 30.2 g PIPES (3.1.2) (100 mmol/L), 1 g β-NADP-Na_2 (3.1.3) (1.3 mmol/L), 5.28 g NaOH (3.1.9) and 5 g PVP K-90 (3.1.10) in 1 L distilled water (3.1.1). The pH should be in the 6.3-6.4 range. This solution is stable for at least 4 weeks at 2-8 °C.

3.2.2. Reagent 2: dissolve 30.2 g PIPES (3.1.2) (100 mmol/L), 1 g $MgCl_2 \cdot 6H_2O$ (3.1.4) (1.3 mmol/L), 4.84 g ATP-Na_2 (3.1.5) and 7.6 g NaOH (3.1.9) in 1 L distilled water (3.1.1). The pH should be in the 7.0-7.2 range. Add 10 KU kinase glutonate (3.1.6) and 10 KU 6-

phosphogluconate dehydrogenase (3.1.7). This solution is stable for at least 4 weeks at 2-8 °C.

3.3. Calibration solutions
Calibration solutions are prepared from the D-gluconic acid sodium salt (3.1.8), by weighing, in concentrations that cover the linear range of the method (0.06-2 g/L).

Note 1: The formulations described above are for preparing 1 L of reagent. Other volumes may be prepared according to the needs of the laboratory.

Note 2: Commercial kits are available for the determination of D-gluconic acid. The user should check that the kit includes the reagents mentioned above.

4. Apparatus

4.1. Sequential automatic analyser with temperature control (approximately 37 °C), adjusted to measure absorbance at 340 nm. The apparatus should have software that facilitates data acquisition and carries out the necessary calculations.
4.2. Spectrophotometer or photometer to measure absorbance at 340 nm
4.3. Glass, quartz or methacrylate cuvettes
4.4. Class-A glassware for regular laboratory use (flasks, pipettes, etc.)
4.5. Micropipettes
4.6. Analytical balance with a resolution of ± 0.0001 g
4.7. pH meter

5. Sample preparation

If necessary, follow the procedure for preparation of the corresponding sample:

5.1. Filter or centrifuge the samples if they contain suspended particles.

5.2. Degas samples that contain carbon dioxide through stirring under vacuum, an ultrasonic bath or any other means that makes it possible to reach the required level of degasification.
5.3. Samples with a concentration higher than the specified limit of linearity (2 g/L) should be diluted with distilled water (3.1.1). Multiply the concentration obtained by the dilution factor.

6. Procedure

Given that different types of analysers may be used, it is recommended to strictly follow the manufacturer's instructions. This is also applicable to commercial enzymatic kits.

The procedures are those detailed below (volumes are given by way of example).

6.1. Manual procedure

6.1.1. Preheat the reagents and photometer to 37 °C.

6.1.2. Add the following to a cuvette using a pipette:

	Reagent blank (RB)	Standard / Sample
Standard / Sample	-	33 µL
Distilled water	33 µL	-
Reagent 1	800 µL	800 µL

6.1.3. Mix and incubate for 1 min at 37 °C. Read the absorbance (A1) at 340 nm.

6.1.4. Add the following to the cuvette using a pipette:

Reagent 2	200 µL	200 µL

6.1.5. Mix and incubate for 10 min at 37 °C. Read the absorbance (A2) of the reagent blank, standard and sample at 340 nm.

6.2. Automated procedure

6.2.1 Introduce the following parameters into the automatic analyser (which complies with the requirements in paragraph 4.1):

Wavelength:	340 nm
Temperature:	37 °C
Analysis mode:	2 points (differential)
Sample volume:	10 □L
Volume of Reagent 1:	240 □L
Volume of Reagent 2:	60 □L

6.2.2 Programme an application in the analyser so that it performs the following sequence:

	Reagent blank (RB)	Standard / Sample
Standard / Sample	-	10 µL
Distilled water	10 µL	-
Reagent 1	240 µL	240 µL

Mix, incubate for 1-5 min and read the absorbance (A1). Then add:

Reagent 2	60 µL	60 µL

Mix, incubate for 10 min and read the absorbance (A2).

The apparatus takes regular measurements, which makes it possible to obtain reaction kinetics (Fig. 1).



Figure 1: Example of reaction kinetics

6.2.3. It is advisable to check the calibration by carrying out three controls spread out over the measurement range. Each laboratory should establish its own internal quality-control programme, as well as correction procedures in case the controls do not comply with the acceptable tolerance levels.

7. Calculations

Calculate the D-gluconic acid concentration using the following formula:

- If the calibration is carried out with one point (standard) and the blank:

$$\frac{(A2 - 0.81 \times A1)_{Sample} - (A2 - 0.81 \times A1)_{RB}}{(A2 - 0.81 \times A1)_{Standard} - (A2 - 0.81 \times A} \times F \times g/L_{Standard} = g/L_{Sample}$$

- If the calibration is with a calibration line:

$$A = (A2 - 0.81 \times A1)_{Sample} - (A2 - 0.81 \times A1)_{RB}$$

The absorbance calculated (A) is interpolated on the calibration line (Fig. 2) to obtain the D-gluconic acid concentration. Multiply the concentration obtained by the dilution factor (F).

A1: absorbance of the Blank/Standard/Sample + Reagent 1
A2: absorbance of the Blank/Standard/Sample + Reagent 1 + Reagent 2
RB: reagent blank
0.81: factor of correction of the dilution of Reagent 1 (this may vary depending on the volumes used according to the formula [Sample vol. + Reagent 1] / [Sample vol. + Reagent 1 + Reagent 2]).
F: factor of dilution of the sample (to be applied if necessary)

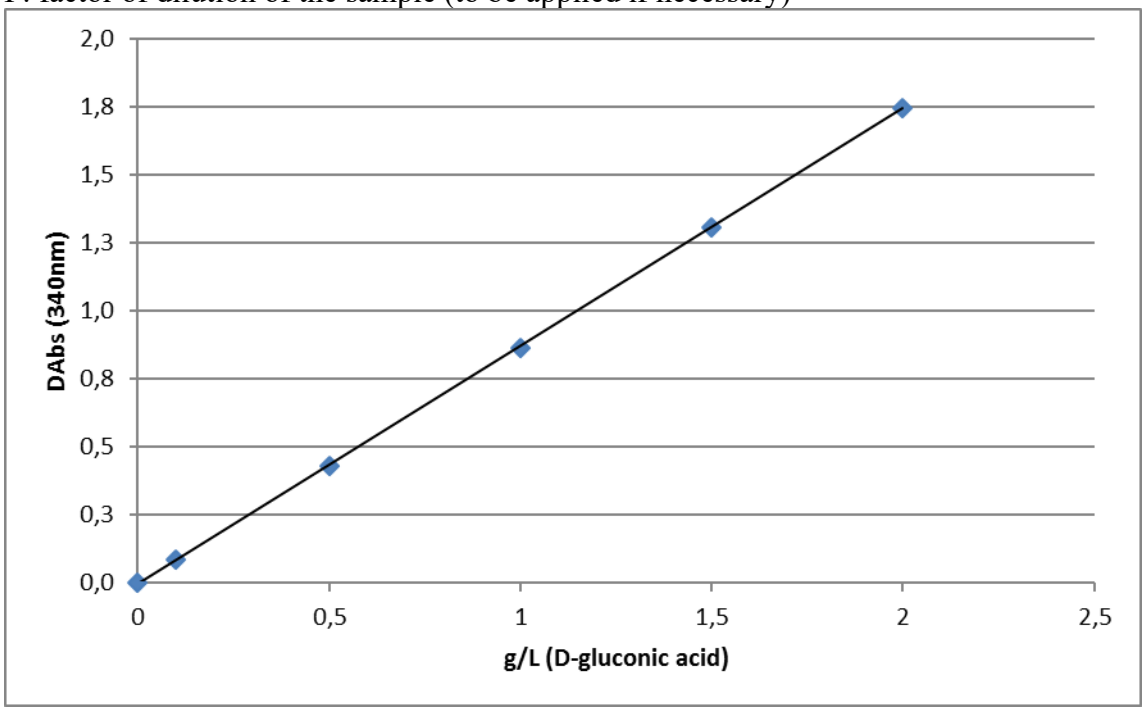


Figure 2: Example of a calibration line

8. Expression of results

The results are expressed in g/L to 2 decimal points, or in accordance with the uncertainty.

9. Automated enzymatic method characteristics

9.1. Repeatability

$r = 0.0396x + 0.0098$

With *x* representing the concentration of gluconic acid in g/L.

9.2 Reproducibility
$R = 0.1226x + 0.0237$
With *x* representing the concentration of gluconic acid in g/L.

9.3 Limit of quantification
Validated LoQ = 0.06 g/L

ANNEX

Results of the inter-laboratory study

1. Collaborative study

1.1.Participating laboratories: 19 laboratories participated from 6 different countries.

Laboratory	Country
Agroscope	Switzerland
Biosystems S.A	Spain
Bundesamt für Weinbau	Austria
Bundesinstitut für Risikobewertung (BfR)	Germany
Centrolab 2006, S.L	Spain
Comité Champagne Comité Interprofessionnel du vin de Champagne (CIVC)	France
Estación de Viticultura y Enología de Navarra (EVENA)	Spain
Estación de Viticultura y Enología Alcázar de San Juan	Spain
Estación Enológica de Castilla y León (ITACyL)	Spain
Estación Enológica de Haro	Spain
Federal College and Research Institute for Viticulture and Pomology (HBLA)	Austria
Freixenet S.A	Spain
Institut Català de la Vinya i el Vi (INCAVI)	Spain
Instituto dos Vinhos do Douro e do Porto (IVDP)	Portugal
Laboratoires Dicœnos Rhône	France
Laboratoires Dubernet	France
Laboratorio Arbitral Agroalimentario	Spain
Landesuntersuchungsamt, Institut für Lebensmittelchemie und Arzneimittelprüfung	Germany
Miguel Torres, SA	Spain

For analysis, use 2 x 10 blind duplicate samples, with 1 repetition.

1.2. Samples

Sample	Vial	Type of sample
A	1 / 12	Moscatel
B	2 / 11	Concentrated must
C	3 / 13	Sulphited must
D	4 / 15	White wine
E	5 / 14	White wine
F	6 / 16	Rosé wine
G	7 / 10	Red wine
H	8 / 19	Red wine
I	9 / 18	Red wine
J	17 / 20	Synthetic matrix

1.3. Automated method results

		A		B		C		D		E		F		G		H		I		J	
		1	12	2	11	3	13	4	15	5	14	6	16	7	10	8	19	9	18	17	20
1	Rep #1	2.10	2.00	1.00	1.00	0.23	0.23	0.29	0.28	0.10	0.11	2.72	2.72	5.20	5.25	0.15	0.15	0.49	0.48	0.06	0.06
	Rep #2	2.01	2.02	0.99	1.00	0.22	0.23	0.30	0.29	0.11	0.10	2.70	2.68	5.13	5.18	0.14	0.14	0.48	0.49	0.05	0.06
	□ (1)	**2.06**	**2.01**	**1.00**	**1.00**	**0.23**	**0.23**	**0.30**	**0.29**	**0.11**	**0.11**	**2.71**	**2.70**	**5.17**	**5.22**	**0.15**	**0.15**	**0.49**	**0.49**	**0.06**	**0.06**
	Rep #1	1.95	2.02	1.02	0.99	0.23	0.23	0.29	0.30	0.10	0.09	2.79	2.72	5.27	5.24	0.13	0.13	0.47	0.46	0.05	0.05
2	Rep #2	2.00	2.10	1.03	1.01	0.23	0.23	0.29	0.30	0.11	0.10	2.75	2.80	5.30	5.20	0.13	0.13	0.47	0.45	0.05	0.05
	□ (2)	**1.97**	**2.06**	**1.03**	**1.00**	**0.23**	**0.23**	**0.29**	**0.30**	**0.11**	**0.10**	**2.77**	**2.76**	**5.28**	**5.22**	**0.13**	**0.13**	**0.47**	**0.45**	**0.05**	**0.05**
	Rep #1	2.19	2.19	1.06	1.07	0.27	0.28	0.34	0.33	*0.13*	*0.13*	2.95	3.06	5.54	5.63	0.08	0.08	0.51	0.50	0.06	0.06
3	Rep #2	2.21	2.30	1.09	1.07	0.27	0.28	0.34	0.34	*0.13*	*0.13*	2.95	2.99	5.51	5.68	0.07	0.08	0.49	0.50	0.06	0.06
	□ (3)	**2.20**	**2.24**	**1.07**	**1.07**	**0.27**	**0.28**	**0.34**	**0.34**	***0.13***	***0.13***	**2.95**	**3.02**	**5.53**	**5.66**	**0.07**	**0.08**	**0.50**	**0.50**	**0.06**	**0.06**
	Rep #1	2.10	2.10	1.02	1.05	0.23	0.24	0.28	0.29	0.10	0.10	2.70	2.80	*3.78*	*3.94*	0.13	0.12	0.45	0.46	*0.10*	*0.10*
4	Rep #2	2.08	2.12	1.03	1.04	0.24	0.25	0.29	0.29	0.10	0.11	2.72	2.82	*3.80*	*3.98*	0.13	0.13	0.45	0.45	*0.10*	*0.10*
	□ (4)	**2.09**	**2.11**	**1.03**	**1.05**	**0.24**	**0.25**	**0.29**	**0.29**	**0.10**	**0.11**	**2.71**	**2.81**	***3.79***	***3.96***	**0.13**	**0.13**	**0.45**	**0.46**	***0.10***	***0.10***
	Rep #1	1.88	1.94	0.98	0.96	*0.22*	*0.33*	0.27	0.26	0.10	0.09	2.68	2.58	4.95	4.90	0.11	0.08	0.43	0.42	0.04	0.05
5	Rep #2	2.06	2.12	0.99	1.00	*0.24*	*0.23*	0.29	0.29	0.11	0.09	3.08	2.84	5.40	5.30	0.12	0.15	0.47	0.48	0.07	0.06
	□ (5)	**1.97**	**2.03**	**0.99**	**0.98**	***0.23***	***0.28***	**0.28**	**0.28**	**0.11**	**0.09**	**2.88**	**2.71**	**5.18**	**5.10**	**0.12**	**0.12**	**0.45**	**0.45**	**0.06**	**0.06**
	Rep #1	2.06	2.02	1.00	1.01	0.22	0.23	0.28	0.29	0.10	0.10	2.80	2.78	5.22	5.22	0.12	0.12	0.46	0.46	0.05	0.05
6	Rep #2	1.98	2.01	0.99	1.00	0.22	0.22	0.27	0.28	0.09	0.10	2.75	2.75	5.22	5.22	0.12	0.11	0.45	0.46	0.05	0.05
	□ (6)	**2.02**	**2.02**	**0.99**	**1.00**	**0.22**	**0.23**	**0.28**	**0.29**	**0.09**	**0.10**	**2.78**	**2.77**	**5.22**	**5.22**	**0.12**	**0.12**	**0.45**	**0.46**	**0.05**	**0.05**
	Rep #1	2.02	2.02	0.98	0.99	0.23	0.23	0.29	0.29	0.11	0.11	2.74	2.75	5.28	5.16	0.13	0.13	0.46	0.47	0.05	0.06
7	Rep #2	2.01	2.01	0.98	0.99	0.23	0.23	0.30	0.29	0.11	0.12	2.75	2.74	5.28	5.22	0.14	0.14	0.46	0.47	0.05	0.05
	□ (7)	**2.02**	**2.02**	**0.98**	**0.99**	**0.23**	**0.23**	**0.30**	**0.29**	**0.11**	**0.12**	**2.75**	**2.75**	**5.28**	**5.19**	**0.14**	**0.14**	**0.46**	**0.47**	**0.05**	**0.06**
	Rep #1	2.09	2.11	1.02	1.01	0.24	0.24	0.29	0.29	0.10	0.09	2.83	2.84	5.20	5.25	0.10	0.10	0.47	0.46	0.05	0.05
8	Rep #2	2.10	2.09	1.00	1.01	0.24	0.23	0.29	0.29	0.10	0.10	2.80	2.78	5.18	5.18	0.10	0.08	0.47	0.47	0.05	0.05
	□ (8)	**2.10**	**2.10**	**1.01**	**1.01**	**0.24**	**0.24**	**0.29**	**0.29**	**0.10**	**0.10**	**2.82**	**2.81**	**5.19**	**5.22**	**0.10**	**0.09**	**0.47**	**0.47**	**0.05**	**0.05**
	Rep #1	1.94	2.02	1.00	1.00	0.24	0.24	0.29	0.29	0.11	0.11	2.74	2.74	5.15	5.25	0.15	0.15	0.47	0.48	0.06	0.06
9	Rep #2	1.94	2.00	1.00	1.00	0.24	0.24	0.29	0.29	0.11	0.11	2.76	2.74	5.10	5.10	0.14	0.15	0.47	0.47	0.06	0.06
	□ (9)	**1.94**	**2.01**	**1.00**	**1.00**	**0.24**	**0.24**	**0.29**	**0.29**	**0.11**	**0.11**	**2.75**	**2.74**	**5.13**	**5.18**	**0.15**	**0.15**	**0.47**	**0.48**	**0.06**	**0.06**
	Rep #1	1.95	2.05	1.00	1.00	0.23	0.23	0.29	0.29	0.11	0.10	2.70	2.65	5.20	5.30	0.14	0.14	0.48	0.45	0.05	0.05
10	Rep #2	2.00	2.00	0.99	1.00	0.23	0.25	0.28	0.29	0.10	0.11	2.70	2.75	5.40	5.30	0.13	0.13	0.48	0.46	0.05	0.05
	□ (10)	**1.98**	**2.03**	**1.00**	**1.00**	**0.23**	**0.24**	**0.29**	**0.29**	**0.11**	**0.11**	**2.70**	**2.70**	**5.30**	**5.30**	**0.14**	**0.14**	**0.48**	**0.46**	**0.05**	**0.05**
	Rep #1	*0.10*	*<0.05*	*<0.05*	*<0.05*	0.23	0.25	0.29	0.33	0.08	0.10	*0.13*	*0.26*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	0.06	0.05
11	Rep #2	*0.10*	*<0.05*	*<0.05*	*<0.05*	0.25	0.24	0.28	0.27	0.08	0.10	*0.13*	*0.26*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	0.05	0.05
	□ (11)	***0.10***	**-**	**-**	**-**	**0.24**	**0.25**	**0.29**	**0.30**	**0.08**	**0.10**	***0.13***	***0.26***	**-**	**-**	**-**	**-**	**-**	**-**	**0.06**	**0.05**
	Rep #1	*2.49*	*2.47*	*1.17*	*1.16*	0.29	0.27	*0.38*	*0.38*	*0.14*	*0.13*	*3.51*	*3.42*	*6.37*	*6.12*	0.18	0.18	*0.61*	*0.60*	0.06	0.05
12	Rep #2	*2.56*	*2.53*	*1.16*	*1.20*	0.30	0.28	*0.38*	*0.37*	*0.14*	*0.13*	*3.43*	*3.32*	*6.28*	*6.24*	0.18	0.18	*0.61*	*0.60*	0.06	0.05
	□ (12)	***2.53***	***2.50***	***1.17***	***1.18***	**0.30**	**0.28**	***0.38***	***0.38***	***0.14***	***0.13***	***3.47***	***3.37***	***6.33***	***6.18***	**0.18**	**0.18**	***0.61***	***0.60***	**0.06**	**0.05**
	13a	2.05	2.06	0.98	0.98	0.22	0.22	0.28	0.27	0.10	0.10	2.76	2.74	5.27	5.18	0.13	0.15	0.46	0.45	0.05	0.05
13	13b	2.05	2.06	0.98	0.98	0.22	0.22	0.28	0.27	0.10	0.10	2.76	2.74	5.27	5.18	0.13	0.15	0.46	0.45	0.05	0.05
	□ (13)	**2.05**	**2.06**	**0.98**	**0.98**	**0.22**	**0.22**	**0.28**	**0.27**	**0.10**	**0.10**	**2.76**	**2.74**	**5.27**	**5.18**	**0.13**	**0.15**	**0.46**	**0.45**	**0.05**	**0.05**
	Rep #1	1.86	1.86	0.96	0.96	0.23	0.24	*0.29*	*0.32*	0.11	0.10	2.57	2.64	4.99	5.19	*0.17*	*0.11*	*0.50*	*0.39*	0.05	0.09
14	Rep #2	1.88	1.86	0.95	0.97	0.23	0.23	*0.29*	*0.32*	0.15	0.1	2.56	2.65	5.00	5.10	*0.15*	*0.11*	*0.45*	*0.34*	0.06	0.05
	□ (14)	**1.87**	**1.86**	**0.96**	**0.97**	**0.23**	**0.24**	***0.29***	***0.32***	**0.13**	**0.10**	**2.57**	**2.65**	**5.00**	**5.15**	***0.16***	***0.11***	***0.48***	***0.37***	**0.06**	**0.07**
	Rep #1	1.91	1.98	1.11	1.12	0.30	0.31	0.32	0.33	0.10	0.11	3.23	3.13	5.88	6.01	*0.13*	*0.16*	0.50	0.52	*0.02*	*0.04*
15	Rep #2	1.93	1.99	1.12	1.13	0.31	0.32	0.32	0.34	0.09	0.12	3.24	3.14	5.90	6.08	*0.12*	*0.17*	0.51	0.53	*0.02*	*0.04*
	□ (15)	**1.92**	**1.99**	**1.12**	**1.13**	**0.31**	**0.32**	**0.32**	**0.34**	**0.10**	**0.12**	**3.24**	**3.14**	**5.89**	**6.05**	***0.13***	***0.17***	**0.51**	**0.53**	***0.02***	***0.04***
	Rep #1	1.98	1.99	1.00	1.00	0.23	0.23	0.28	0.28	0.10	0.10	2.78	2.82	5.27	5.34	0.13	0.13	0.47	0.47	0.05	0.05
16	Rep #2	2.04	2.08	0.99	1.00	0.23	0.23	0.28	0.28	0.10	0.10	2.79	2.81	5.30	5.28	0.14	0.14	0.47	0.47	0.05	0.05
	□ (16)	**2.01**	**2.04**	**1.00**	**1.00**	**0.23**	**0.23**	**0.28**	**0.28**	**0.10**	**0.10**	**2.79**	**2.82**	**5.29**	**5.31**	**0.14**	**0.14**	**0.47**	**0.47**	**0.05**	**0.05**
	Rep #1	2.27	2.22	*1.17*	*1.20*	0.27	0.27	0.30	0.29	0.12	0.10	2.75	2.72	5.20	4.95	0.16	0.17	0.46	0.47	0.07	0.07
17	Rep #2	2.24	2.21	*1.19*	*1.19*	0.29	0.29	0.29	0.28	0.12	0.11	2.77	2.79	5.05	4.90	0.16	0.12	0.46	0.42	0.08	0.06
	□ (17)	**2.26**	**2.22**	***1.18***	***1.20***	**0.28**	**0.28**	**0.30**	**0.29**	**0.12**	**0.11**	**2.76**	**2.76**	**5.13**	**4.93**	**0.16**	**0.15**	**0.46**	**0.45**	**0.08**	**0.07**

19	Rep #1	2.08	2.08	1.02	1.00	0.24	0.23	0.29	0.28	0.11	0.10	2.80	2.82	5.40	5.37	0.14	0.15	0.48	0.49	0.06	0.05
	Rep #2	2.08	2.08	1.01	1.00	0.23	0.24	0.28	0.29	0.10	0.11	2.82	2.80	5.28	5.40	0.14	0.14	0.47	0.47	0.05	0.05
	□ (19)	**2.08**	**2.08**	**1.02**	**1.00**	**0.24**	**0.24**	**0.29**	**0.29**	**0.11**	**0.11**	**2.81**	**2.81**	**5.34**	**5.39**	**0.14**	**0.15**	**0.48**	**0.48**	**0.06**	**0.05**

Table of data obtained. The values in italics are the results removed due to outliers from individual values according to the simple 2-tail Grubbs test and the double Grubbs test (2-tail, P = 2.5%), and according to the Cochran test (1-tail test where P = 2.5%).

Sample	A	B	C	D	E	F	G	H	I	J
Accepted labs	16	15	17	16	16	16	15	15	15	16
Repetitions	4	4	4	4	4	4	4	4	4	4
Minimum value	1.87	0.96	0.22	0.28	0.09	2.61	5.03	0.07	0.45	0.05
Maximum value	2.24	1.12	0.31	0.34	0.12	2.81	5.97	0.18	0.52	0.07
Mean value (g/L)	**2.04**	**1.01**	**0.25**	**0.29**	**0.10**	**2.79**	**5.28**	**0.13**	**0.47**	**0.06**
S_r	0.03	0.01	0.01	0.01	0.01	0.05	0.08	0.01	0.01	0.01
r limit = 2□2* S_r	0.09	0.02	0.02	0.02	0.03	0.13	0.22	0.02	0.02	0.02
RSD_r	1.48%	0.76%	2.13%	1.93%	8.53%	1.70%	1.50%	3.99%	1.70%	9.86%
S reproducibility (S_R)	0.09	0.04	0.03	0.02	0.01	0.13	0.24	0.03	0.02	0.01
R limit = 2□2* S_R	0.28	0.11	0.07	0.05	0.06	0.38	0.67	0.07	0.05	0.02
RSD_R	4.63%	3.96%	10.57%	5.89%	8.91%	4.81%	4.50%	19.21%	4.09%	12.49%
Horwitz RSD_r (%)	3.39%	3.77%	4.66%	4.54%	5.31%	3.23%	2.94%	5.12%	4.22%	5.84%
$HorRat_r$	0.44	0.20	0.46	0.43	1.61	0.53	0.51	0.78	0.40	1.69
Horwitz RSD_R (%)	5.08%	5.65%	6.99%	6.81%	7.96%	4.85%	4.40%	7.68%	6.34%	8.75%
$HorRat_R$	0.91	0.70	1.51	0.86	1.12	0.99	1.02	2.50	0.65	1.43

S: Standard deviation / RSD: Relative standard deviation / r: Repeatability limit / R: Reproducibility limit

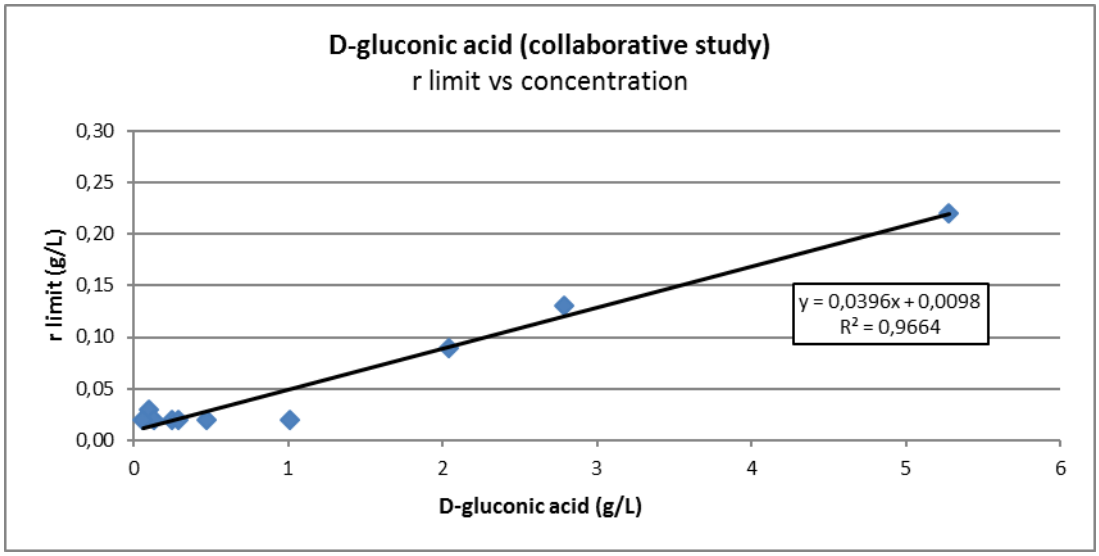


Figure 3: Repeatability limit according to concentration

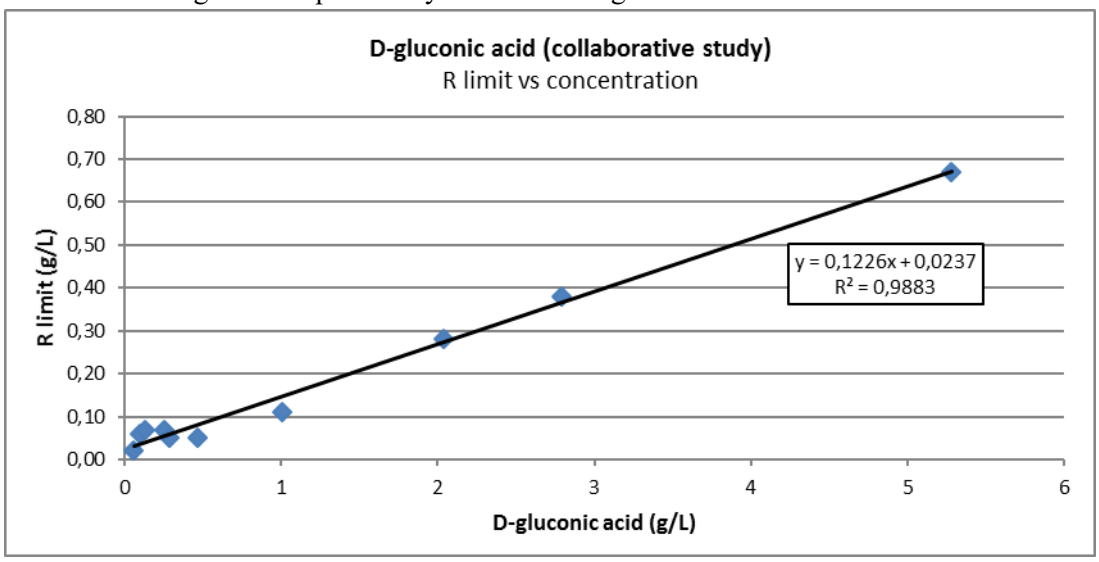


Figure 4: Reproducibility limit according to concentration

1.4. Manual method results

		A		B		C		D		E		F		G		H		I		J	
		1	**12**	**2**	**11**	**3**	**13**	**4**	**15**	**5**	**14**	**6**	**16**	**7**	**10**	**8**	**19**	**9**	**18**	**17**	**20**
	Rep #1	2.05	2.09	1.06	0.99	0.25	0.25	0.34	0.33	*0.10*	*0.12*	2.85	2.84	5.32	5.34	0.14	0.13	0.45	0.46	0.05	0.05
2	Rep #2	2.08	2.10	1.03	1.02	0.23	0.26	0.35	0.32	*0.09*	*0.10*	2.83	2.86	5.34	5.36	0.15	0.13	0.44	0.45	0.05	0.05
	□ (2)	**2.07**	**2.10**	**1.05**	**1.01**	**0.24**	**0.26**	**0.35**	**0.33**	**0.10**	**0.11**	**2.84**	**2.85**	**5.33**	**5.35**	**0.15**	**0.13**	**0.45**	**0.46**	**0.05**	**0.05**
	Rep #1	2.24	2.11	1.01	1.04	0.26	0.26	0.34	0.33	0.11	0.11	3.05	3.19	5.64	5.68	0.14	0.16	0.34	0.41	0.05	0.05
10	Rep #2	2.37	2.24	1.01	1.06	0.25	0.26	0.35	0.34	0.12	0.11	3.10	3.02	5.65	5.78	0.14	0.15	0.33	0.42	0.05	0.05
	□ (10)	**2.31**	**2.18**	**1.01**	**1.05**	**0.26**	**0.26**	**0.35**	**0.34**	**0.12**	**0.11**	**3.08**	**3.11**	**5.65**	**5.73**	**0.14**	**0.16**	**0.34**	**0.42**	**0.05**	**0.05**
	Rep #1	2.61	2.54	1.04	0.99	0.27	0.28	0.34	0.34	0.13	0.12	3.44	3.38	5.97	6.22	0.21	0.23	0.44	0.47	0.05	0.05
18	Rep #2	2.57	2.54	0.97	1.01	0.28	0.28	0.35	0.35	0.12	0.12	3.32	3.42	6.04	6.31	0.21	0.21	0.51	0.53	0.05	0.05
	□ (18)	**2.59**	**2.54**	**1.00**	**1.00**	**0.28**	**0.28**	**0.34**	**0.34**	**0.12**	**0.12**	**3.38**	**3.40**	**6.00**	**6.26**	**0.21**	**0.22**	**0.48**	**0.50**	**0.05**	**0.05**

Table of data obtained. The values in italics are the results removed due to outliers from individual values according to the simple 2-tail Grubbs test and the double Grubbs test (2-tail, P = 2.5%), and according to the Cochran test (1-tail test where P = 2.5%).

Sample	A	B	C	D	E	F	G	H	I	J
Accepted labs	3	3	3	3	3	3	3	3	3	3
Repetitions	4	4	4	4	4	4	4	4	4	4
Minimum value	2.05	0.97	0.23	0.32	0.09	2.83	5.32	0.13	0.33	0.05
Maximum value	2.61	1.06	0.28	0.35	0.13	3.44	6.31	0.23	0.53	0.05
Mean value (g/L)	**2.29**	**1.02**	**0.26**	**0.34**	**0.11**	**3.11**	**5.72**	**0.17**	**0.44**	**0.05**
S_r	0.06	0.02	0.01	0.01	0.01	0.02	0.11	0.01	0.03	-
r limit = 2□2* S_r	0.16	0.07	0.02	0.03	0.02	0.04	0.31	0.03	0.10	-
RSD_r	0.03%	0.02%	0.03%	0.03%	0.06%	0.01%	0.02%	0.06%	0.08%	-
S reproducibility (S_R)	0.25	0.02	0.02	0.01	0.01	0.27	0.41	0.04	0.06	-
R limit = 2□2* S_R	0.70	0.07	0.05	0.03	0.03	0.77	1.14	0.12	0.17	-
RSD_R	0.11%	0.02%	0.06%	0.03%	0.10%	0.09%	0.07%	0.26%	0.14%	-
Horwitz RSD_r (%)	3.33%	3.76%	4.62%	4.44%	5.24%	3.18%	2.90%	4.94%	4.27%	-
$HorRat_r$	0.77	0.60	0.55	0.61	1.09	0.16	0.67	1.21	1.82	-
Horwitz RSD_R (%)	4.99%	5.64%	6.92%	6.66%	7.86%	4.77%	4.35%	7.41%	6.41%	-
$HorRat_R$	2.18	0.42	0.93	0.47	1.30	1.85	1.63	3.46	2.22	-

S: Standard deviation / RSD: Relative standard deviation / r: Repeatability limit / R: Reproducibility limit. The statistical parameters were calculated taking into account the results of the 3 laboratories.

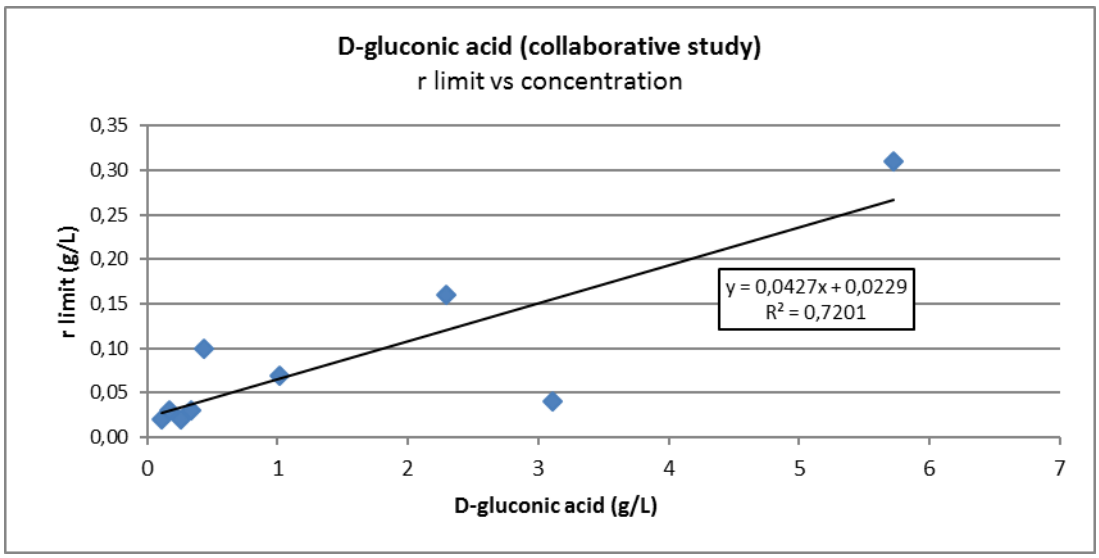


Figure 5: Repeatability limit according to concentration

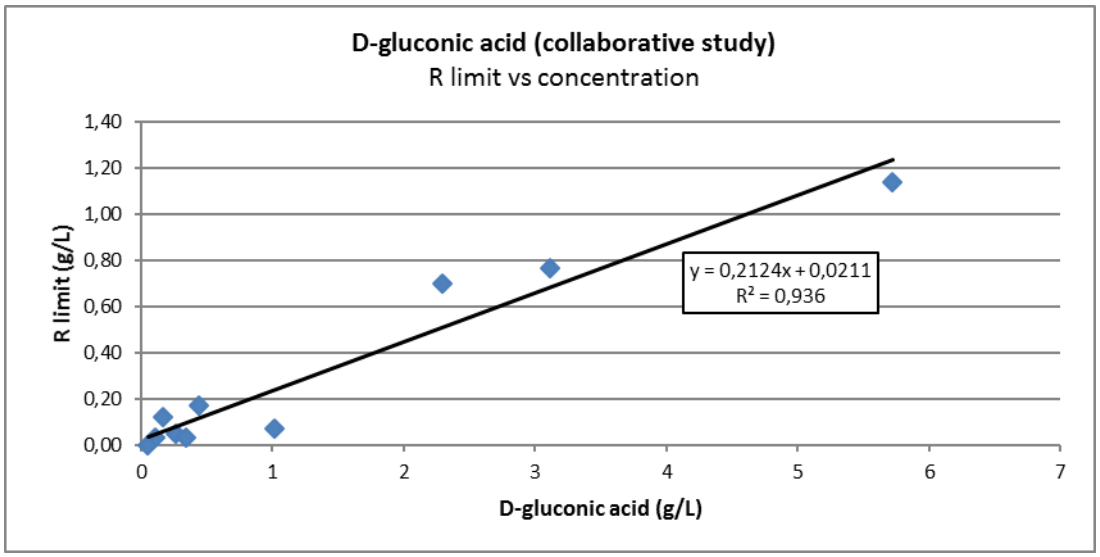


Figure 6: Reproducibility limit according to concentration

2. Bibliography

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Method OIV-MA-AS314-01 Type II method

Carbone Dioxide

With a range of concentration up to 1.5 g/L
A 39 modified by OENO 21/2003
OENO 3/2006
OIV/OENO 377/2009

1. Principle

1.1 Still wines (CO_2 over pressure ≤ 0.5 x 10^5 Pa*)

The volume of wine taken from the sample is cooled to around 0°C and mixed with a sufficient quantity of sodium hydroxide to give a pH of 10-11. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid needed to change the pH from 8.6 (bicarbonate form) to 4.0 (carbonic acid). A blank titration is carried out in the same conditions on decarbonated wine in order to take account of the volume of sodium hydroxide solution taken up by the wine acids.

1.2 Sparkling and semi-sparkling wines

The sample of wine to be analyzed is cooled near to the freezing point. After removal of a sub-sample to be used as a blank after decarbonation, the remainder of the bottle is made alkaline to fix all the carbon dioxide in the form of Na_2CO_3. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid solution needed to change the pH from 8.6 (bicarbonate form) to 4.0 (carbonic acid). A blank titration is carried out in the same conditions in decarbonated wine in order to take account of the volume of sodium hydroxide taken up by the wine acids.

2. Description of the method

2.1 *Still Wines*

(CO_2 over pressure ≤ 0.5 x 10^5 Pa)

2.1.1 Apparatus

- Magnetic stirrer
- pH meter

2.1.2 Reagents

* 1 bar = 10^5 Pascal (Pa)

- Sodium hydroxide solution, 0.1 M
- Sulfuric acid solution, 0.05 M
- Carbonic anhydrase solution, 1 g/L

2.1.3 Procedure

Cool the wine sample together with the 10 mL pipette used for sampling to approximately 0°C.

Place 25 mL sodium hydroxide solution, 0.1 M, in a 100 mL beaker; add two drops of carbonic anhydrase solution, 1 g/L. Introduce 10 mL of wine using the pipette cooled to 0°C.

Place the beaker on the magnetic stirrer, immerse the pH electrode and magnetic rod, and stir moderately.

When the liquid has reached room temperature, titrate slowly with the sulfuric acid solution, 0.05 M, until the pH reaches 8.6. Note the burette reading.

Continue titrating with the sulfuric acid until the pH reaches 4.0. Let n mL be the volume used between pH 8.6 and 4.0.

Remove CO_2 from approximately 50 mL of the wine sample by shaking under vacuum for three minutes, the flask being heated in a water bath to about 25 °C.

Carry out the above procedure on 10 mL of the decarbonated wine. Let n' mL be the volume used.

2.1.4 Expression of results

1 mL of the titrated sodium hydroxide solution, 0.05 M, corresponds to 4.4 mg of CO_2. The quantity of CO_2 in grams per liter of wine is given by:

$$0.44\ (n - n')$$

The result is quoted to two decimal places.

Note: For wines which contain little CO_2 (CO_2 < 1 g/L), the addition of carbonic anhydrase to catalyze the hydration of CO_2 is unnecessary.

2.2 *Sparkling and semi-sparkling wines*

2.2.1 Apparatus

- Magnetic stirrer
- pH meter

2.2.2 Reagents

- Sodium hydroxide, 50% (*m/m*)
- Sulfuric acid solution, 0.05 M
- Carbonic anhydrase solution, 1 g/L

2.2.3 Procedure

Mark the level of wine in the bottle and then cool until freezing begins.

Allow the bottle to warm up slightly, while shaking, until ice crystals disappear.

Remove the stopper rapidly and place 45 to 50 mL of wine in a measuring cylinder for blank titration. The exact volume removed, *v* mL, is determined by reading on the measuring cylinder after it has returned to room temperature.

Immediately after the blank sample has been removed, add 20 mL of the sodium hydroxide solution for a 750 mL bottle.

Allow the wine to reach room temperature.

Place 30 mL of boiled distilled water and two drops of the carbonic anhydrase solution into a 100 mL beaker. Add 10 mL of wine that has been made alkaline.

Place the beaker on the magnetic stirrer, set up the electrode and magnetic rod and stir moderately.

Titrate with the sulfuric acid solution, 0.05 M, slowly until the pH reaches 8.6. Note the burette reading.

Continue titrating slowly with the sulfuric acid, 0.05 M, until the pH reaches 4.0. Let *n* mL be the volume added between pH 8.6 and 4.0.

Remove CO_2 from the *v* mL of wine placed on one side for the blank titration by agitating under vacuum for three minutes, the flask being heated in a water bath at about 25 °C. Remove 10 mL of decarbonated wine and add to 30 mL of boiled distilled water, add two to three drops of sodium hydroxide solution, 50%, to bring the pH to 10 to 11. Then follow the above procedure. Let *n*' mL be the volume of sulfuric acid added, 0.05 M.

2.2.4 Expression of results

1 mL sulfuric acid, 0.05 M, corresponds to 4.4 mg of CO_2.

Empty the bottle of wine which has been made alkaline and determine to within 1 mL the initial volume of wine by making up to the mark with water, say V mL. The quantity of CO_2 in grams per liter of wine is given by the following formula:

$$0.44 \left(n - \frac{V - v + 20}{V - v}\right.$$

The result is quoted to two decimal places.

2.3 *Expression of Results*

The excess pressure at 20°C ($Paph_{20}$) expressed in Pascals is given by the formula:

$$Paph_{20} = \frac{Q}{1{,}951 \times 10^{-5}\,(0{,}86 - 0{,}01A)\,(1 - 0{,}00144\,S)} - Patm$$

where:
Q = CO_2 content in g/L of wine,
A = the alcoholic strength of wine at 20 °C,
S = the sugar content of the wine in g/L,
Patm= the atmospheric pressure, expressed in Pascals.

2.4 *Note*

The procedure described below can be used as the usual method for wines containing less than 4 g per liter of carbon dioxide.

Prepare two samples of wine for analysis.

Open one of the samples after it has been cooled to approximately 5°C and immediately add 5 mL of a sodium hydroxide solution, 50% (*m/m*), for 375 mL of sample. Stopper immediately and mix. Place 10 mL of wine so processed into a beaker containing 40 mL of water and add 3 drops of carbonic anhydrase solution, 0.1 mg/mL. Titrate with a sulfuric acid solution, 0.02275 M, until reaching a pH of 8.6, then continue titrating to a pH of 4.0. The volume used to change the pH from 8.6 to 4.0 is *n* mL.

Remove the carbon dioxide from about 25 mL of wine, taken from the second sample, by agitation under a vacuum for about 1 min. into a 500 mL flask containing 3 drops of carbonic anhydrase solution. Add 0.33 mL of sodium hydroxide, 50% (*m/m*). Apply the above titration procedure to 10 mL decarbonated wine. Let *n*' mL be the volume of H_2SO_4, 0.02275 M used. 1mL corresponds to 200 mg of carbon dioxide per liter. The amount of wine analyzed for carbon dioxide, in milligrams per liter:

$$(n - n') \times 200 \times 1.013$$

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Reference method:

CAPUTI A, UEDA M., WALTER P. & BROWN T., *Amer. J. Enol. Vitic.,* 1970, **21,** 140-144.
SUDRAUD P., *F.V., O.I.V.,* 1973, n° 350.
GORANOV N., *F.V., O.I.V.,* 1983, n° 758.
BRUN S. & TEP Y., *F.V., O.I.V.,* 1981, n° 736 & 1982, n° 736 (bis).

- Collaborative Study -

Titrimetric determination of carbon dioxide in sparkling and semi-sparkling wines
- Report on Results -

Goal of the study

The objective of the study is to determine the repeatability and reproducibility characteristics of the reference method (MA-E-AS314-01-DIOCAR) for the titrimetric CO_2 determination in sparkling and semi-sparkling wine.
O.I.V. definitions and limits for the CO_2 content are given with resolution OENO 1/2002.

Needs and purpose of the study

The reference method for the CO_2 determination includes no precision data. This collaborative trial was thus conducted.
Due to the analytical particularity, theconventional validation protocol was not able to be completely respected. Out of one bottle of sample only one independent determination could be done. Each bottle had to be considered as

individual. Therefore homogeneity testing within the pre-investigations for collaborative studies was impossible. In order to provide homogenous test material close co-operation with producers was necessary. Samples were obtained during the filling of the bottles on the filling line in a very short time space, thus that it must be assumed that the CO_2 is homogeneously distributed in all bottles.
This study was designed to be a blind duplicate test. The complete anonymity of the samples could not be guaranteed because the partners involved used different types of bottles and/or stoppers for the different samples. Therefore we had to rely on the honesty of the participating laboratories which were requested to perform the data analysis independently without any data modification.

Scope and applicability

1. The method is quantitative.
2. The method is applicable for the determination of CO_2 in sparkling and semi-sparkling wines to check that standards are respected.

Materials and matrices

The collaborative study included 6 different samples. All were sent in blind duplicate, so that in total 12 bottles were distributed to the participants.

Table 1. Samples and coding.

Sample	Bottle Code	Type
SAMPLE A	(Code 1 + 9)	sparkling wine
SAMPLE B	(Code 2 + 5)	semi-sparkling wine ("petillant")
SAMPLE C	(Code 3 + 4)	sparkling wine
SAMPLE D	(Code 6 + 10)	semi- sparkling wine ("petillant")
SAMPLE E	(Code 7 + 11)	semi- sparkling wine ("petillant")
SAMPLE F	(Code 8 + 12)	sparkling wine (red)

Control measures

The method considered is already approved in practice. Only the missing precision data had to be determined within the collaborative study. A pre-trial was not required because most of the laboratories had been already using the reference method in routine analysis.

Method to be followed and supporting documents

. Supporting documents were given to the participants (Covering letter Reference for method of analysis, Sample Receipt Form and Result Sheet).
. The determination of CO_2 content in g/l should be expressed in g/l.

Data analysis

1. Determination of outliers was assessed by Cochran, Grubbs and paired Grubbs tests.
2. Statistical analysis was performed to obtain repeatability and reproducibility data.
3. HORRAT values were calculated.

Participants

13 laboratories from several different countries participated in the collaborative study. Lab-Code numbers were given to the laboratories. The participating laboratories have proven experience in the analysis of CO_2 in sparkling wine.

Table 2. List of participants.

Landesuntersuchungsamt D-56068 Koblenz GERMANY	Institut für Lebensmittelchemie und Arzneimittelprüfung D-55129 Mainz GERMANY
Landesuntersuchungsamt D-67346 Speyer GERMANY	Institut für Lebensmittel, Arzneimittel und Tierseuchen D-10557 BERLIN GERMANY
Servicio Central de Viticultura y Enologia E-08720 Villafranca Del Pendes SPAIN	Landesuntersuchungsamt D-54295 Trier GERMANY
Landesuntersuchungsamt D-85764 Oberschleißheim GERMANY	Instituto Agrario di S. Michele I-38010 S. Michele all Adige ITALIA
Chemisches Landes- u. Staatl. Veterinäruntersuchungsamt D-48151 Münster GERMANY	Ispettorato Centrale Repressione Frodi I-31015 Conegliano (Treviso) ITALY
Bundesamt für Weinbau A-7000 Eisenstadt AUTRIA	BgVV D-14195 Berlin GERMANY
Chemisches und Veterinäruntersuchungsamt D-70736 Fellbach GERMANY	

Results

The uncertainty data are directly calculated for the CO_2 determination from the results submitted. For the assessment of the collaborative trial the Horrat-ratio is of relevance. For all samples a ratio of < 2 was obtained for r and R, convincing for a collaborative study. Table 3 shows the results of the CO_2 titration for each sample.

Table 3. Summarised results of the CO_2 determination.

CO_2	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE E	SAMPLE F
Mean [g/l]	9.401	3.344	9.328	4.382	4.645	8.642
r [g/l]	0.626	0.180	0.560	0.407	0.365	0.327
sr [g/l]	0.224	0.064	0.200	0.145	0.130	0.117
RSDr %	2.379	1.921	2.145	3.314	2.803	1.352
Hor	0.893	0.617	0.804	1.109	0.946	0.501
R [g/l]	1.323	0.588	0.768	0.888	0.999	0.718
sR [g/l]	0.473	0.210	0.274	0.317	0.357	0.256
RSDR %	5.028	6.276	2.942	7.239	7.680	2.967
HoR	1.245	1.331	0.728	1.599	1.711	0.726

Method OIV-MA-AS314-03 Type II method

Determination of the carbon isotope ratio $^{13}C/^{12}C$ of CO_2 in sparkling wines Method using isotope ratio mass spectrometry (IRMS)

(OENO 7/2005
OIV/OENO 377/2009
OIV-OENO 512-2014)

Foreword

The following standard method has been prepared with the agreement of all the laboratories participating in the OIV Collaborative study: ^{13}C-IRMS analyses of CO_2 in sparkling wine (2003-2004).

Introduction

The headspace in a bottle of sparkling wines contains a CO_2-rich gaseous phase in equilibrium with the CO_2 dissolved in the liquid phase. This gas evolves during the second fermentation, induced by the addition of sugar from grape, beet, sugar cane or maize. However, the CO_2 content of sparkling wines may also be increased artificially with industrial CO_2.

In 1997, an off-line method for the determination of the $^{13}C/^{12}C$ isotopic ratio of CO_2 from sparkling wines by isotope mass spectrometry (IRMS) was presented to the OIV. This method led on to new procedures based on automated on-line techniques, developed in some European laboratories. One of these procedures was presented to the OIV in 2001. Technical progress in the next few years may well lead to new procedures for determining reliably and rapidly the $^{13}C/^{12}C$ isotopic ratio of numerous samples of CO_2. An exhaustive description of all applicable procedures for different techniques runs the risk of the method being rapidly superseded. The following method takes this into account and describes the basic principles for the correct measurement of the carbon-13 content in CO_2 from sparkling wine and includes a brief description of the procedures used nowadays and, by way of examples, some exhaustive descriptions of procedures based on off-line and on-line techniques.

1. Scope

This method determines by isotope mass spectrometry (IRMS) the stable carbon isotope ratio ($^{13}C/^{12}C$) of CO_2 in sparkling wines. The method includes a range of procedures whose use depends on the instruments available.

2. Normative references

ISO 5725-2:1994 "Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method".

ISO 78-2:1999 "Chemistry - Layouts for standards - Part 2: Methods of chemical analysis".

3. Definitions

$^{13}C/^{12}C$: Isotope ratio of carbon 13 to carbon 12 for a considered sample;

$\delta^{13}C$: Carbon 13 (^{13}C) content expressed in parts per mill (‰);

V-PDB: Vienna-Pee-Dee Belemnite. The PDB standard is a fossil calcium carbonate from South Carolina in USA, with an isotope ratio ($^{13}C/^{12}C$ or R_{PDB}) = 0.0112372. This value is the reference point for the common international PDB scale for $\delta^{13}C$ values expressed in parts per mill (‰).

m/z: mass to charge relationship

S_r: Repeatability standard deviation. The standard deviation of test results obtained under repeatability conditions (conditions where independent test results are obtained with the same method on identical test samples in the same laboratory by the same operator using the same equipment within short intervals of time).

r: Repeatability limit. Value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95%; $r=2.8 \cdot S_r$.

S_R: Reproducibility standard deviation. The standard deviation of test results obtained under reproducibility conditions (conditions where test results are obtained with the same method on identical test samples in different laboratories with different operators using different equipment).

R: Reproducibility limit. Value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95%; $R=2.8 \cdot S_R$

4. Principle

Plants are classified as C3 and C4 depending on the route followed for sugar synthesis. The sugar from C3 plants, such as grape and beet, has lower ^{13}C content than the sugar from C4 plants like cane sugar and maize. This difference is maintained in the ^{13}C content of the fermentation products of sugars such as ethanol and CO_2. Moreover, the industrial CO_2 used in the food industry and that comes from the combustion of fossil fuels or from the thermal treatment of carbonates has ^{13}C content different from the products of C3 and C4 plants. Consequently, the $^{13}C/^{12}C$ isotope ratio of CO_2 from sparkling wine is governed by the type of sugar used in the second fermentation (C3 or C4) or by the isotopic composition of the industrial CO_2 added.

The studies performed till now on the ^{13}C content of CO_2 from sparkling wine have shown that the CO_2 obtained by fermentation of sugar from C3 plants has $\delta^{13}C$ in the range of -17‰ to -26‰, whereas CO_2 obtained by fermentation of sugar from C4 plants has $\delta^{13}C$ in the range of -7‰ to -10‰. Gasified wines have their $^{13}C/^{12}C$ isotope ratio below -29‰ or above -10‰, depending on the carbon dioxide source[1-4]. Therefore, the measurement of the stable carbon isotope ratio ($^{13}C/^{12}C$) of CO_2 from sparkling wines can be a good method for finding the origin of the gas.

^{13}C content is determined from carbon dioxide gas obtained from sparkling wine. The various possible combinations of the ^{18}O, ^{17}O, ^{16}O and ^{13}C, ^{12}C isotopes lead to mass 44 corresponding to the $^{12}C^{16}O_2$ isotopomer, mass 45 corresponding to $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ species, and mass 46 for the $^{12}C^{16}O^{18}O$ isotopomer ($^{13}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can be ignored due to their very low abundance). The corresponding ion currents are determined on the three different collectors. The ionic current m/z 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the intensity current measured for m/z 46 by including the relative abundance of ^{18}O and ^{17}O (Craig correction). Comparison with a reference calibrated against the international standard V-PDB then allows the calculation of the ^{13}C content on the $\delta^{13}C$‰ relative scale.

5. Reagents and material

The materials and consumables depend on the equipment used in the laboratory.

When the separation and purification of the CO_2 samples is performed by cryotrapping in a vacuum line the following reagents are used:

- Liquid nitrogen
- Ethanol
- Solid CO_2

In general, the following consumables are used for the analysis with any Continuous Flow system (EA-IRMS or GC-C-IRMS). Other materials of similar quality can replace any product on this list:

- Helium for analysis (CAS 07440-59-7)
- Oxygen for analysis (CAS 07782-44-7)
- Carbon dioxide for analysis used as a secondary reference gas for carbon-13 content (CAS 00124-38-9).
- Oxidising reagent for the furnace of the combustion system, such as cupper oxide for microanalysis (CAS 1317-38-0).
- Desiccant to remove water produced by combustion: for example, magnesium perchlorate for microanalyses (CAS 10034-81-4). This is not necessary when the EA-IRMS or the GC-C-IRMS systems remove water by cryotrapping.
- Capillary column and the Naphion membrane to remove water produced by combustion in GC-C-IRMS systems.

The Reference Gas used in the measurements can be a certified gas or a working reference gas calibrated compared to international references with known delta values (certified gases or reference materials). Some international reference materials that can be used for gas reference calibration and for control of the gas reference calibration are the following:

Code sample	Material	$\delta^{13}C_{PDB}$	
IMEP-8-A	CO_2	-6.40‰	from Messer Griesheim
ISO-TOP	CO_2	-25.7‰	“
BCR-656	Ethanol	-20.91‰	from IRMM
BCR-657	Glucose	-10.76‰	“
SAI-692C	CO_2	-10.96‰	from Oztech Trading Coorporation (USA)

NBS-22	Oil	-29.7‰	from IAEA
IAEA-CH-6 (ANU)	Sucrose	-10.4‰	"
NBS-18	Calcite	-5.1‰	"
NBS-19	TS-limestone	+1.95‰	"

FID-Mix	mixture of n-alkanes in isooctanol	from Varian
C14	-29.61‰	
C15	-25.51‰	
C16	-33.39‰	

6. Apparatus

The usual laboratory apparatus for carbon isotope ratio measurements and, in particular, the following:

- Isotopic ratio mass spectrometry (IRMS), with the ability to determine the ^{13}C content of CO_2 gas at natural abundance with an internal precision of 0.05 ‰ or better (expressed in relative □ value). The internal precision is defined here as the difference between two measurements of the same CO_2 sample.

  The mass spectrometer will generally be fitted with a triple collector to measure simultaneously the current intensities for m/z 44, 45 and 46. The mass spectrometer should either be fitted with a dual-inlet system, for alternating measurement of the unknown sample and a standard, or use a continuous-flow technique (CF-IRMS).

- Continuous-flow systems (CF-IRMS). Continuous-flow systems with an automated gas sampling system can be used. Several commercially available CF-IRMS techniques suitable for the scope of the present method are:

  - GC-C-IRMS (Gas chromatography – combustion- IRMS)
  - EA-IRMS (Elemental analyser equipped for liquid or solid injection)

  These systems separate and purify CO_2 and elute the resulting carbon dioxide to the ionisation chamber of the spectrometer.

- Gas Sampler-IRMS**.** A peripheral system may be used for the on-line gas preparation, isolation of CO_2 and introduction of CO_2 into the isotope ratio mass spectrometer.

- Glass or steel vacuum line, with cryogenic traps and connected to a pump able to obtain a pressure lower than 5.10^{-3} mbar.

- Gas sampling devices, commercially available (such as syringe for gas samples) or designed in-house, able to extract a CO_2 aliquot from the sparkling wine without isotopic fractionation.
- Sealed vials for gas samples, adaptable on gas autosampler to the continuous-flow systems.
- Sealed vials for sparkling wine aliquots, adaptable on vacuum line and/or on gas autosampler to the continuous-flow systems.

7. Procedure

The proposed method includes three steps: CO_2 sampling, CO_2 purification and separation, and $^{13}C/^{12}C$ ratio measurement. These steps can be totally independent (off-line system) or fully or partially connected on-line (on-line system). Any procedure that avoids isotopic fractionation of the CO_2 sample during the three steps of the method may be used. Details on particular procedures based on off-line and CF systems are given in Annexes A, B and C.

The following description refers to the procedures used for the participant laboratories in the inter-laboratory test.

7.1. CO_2 sampling procedures:

a. Sampling the CO_2 at room temperature from the headspace of the bottle by plugging a special device through the cork, or

b. Sampling the CO_2 from the headspace of the bottle after removing the cork and sealing the bottle with a gas-tight precision lock connected to a sampling device. The sparkling wine bottle should be cooled to under 0ºC before changing the locking device and then warmed to room temperature. An aliquot of gas collected in the sampling device is removed by a gas-tight syringe and injected into a sealed GC-vial, or

c. Sampling the CO_2 from an aliquot of sparkling wine. The sparkling wine bottle should be cooled to 4º-5ºC before removing the cork. The wine aliquots are placed in a special bottle adaptable to a glass vacuum line or to a gas autosampler.

d. Refrigerate the sample at 4-5 °C, before quickly transferring the liquid into a vial and sealing it with a Teflon-silicone septum cap. Then 50 □L of liquid is then transferred into a 10 mL vial and analysed. If necessary, the vial should be filled with helium in order to remove the atmospheric CO_2.

e. After refrigerating the sample, the bottle is opened at room temperature and a sample of 200 µL of liquid is taken using a pipette and placed in suitable

vials. The vials are immediately resealed then placed in an ultrasonic bath for 10 min prior to analysis.

The statistical results of the inter-laboratory test for sampling procedures 7.1.d and 7.1.e are given in ANNEX E.

7.2. CO_2 purification and separation procedures

a. Uncondensed gases and water present in the gas sample are removed in a vacuum line by use of cryogenic traps, or

b. Gas samples are purified and CO_2 separated by different on-line systems, which are connected to the IRMS by means of continuous-flow or a cryogenic trap. Some of the on-line systems that can be used are the following:

- a water cryogenic trap on-line with a continuous-flow system

- a water trap (magnesium perchlorate) followed by a gas chromatograph

- a gas chromatograph connected either directly to the IRMS or by means of a combustion interface.

7.3. $^{13}C/^{12}C$ ratio measurement:

The carbon isotope ratio of CO_2 obtained from sparkling wine is measured by using an isotopic ratio mass spectrometer.

8. Calculation

Express the $^{13}C/^{12}C$ isotope ratio of the CO_2 from sparkling wine as the deviation from a working standard ($\delta^{13}C$) previously calibrated in relation to the international standard PDB (Pee Dee Belemnite). This parameter is defined as the relative difference per thousand between the ^{13}C and ^{12}C ratios of a sample in relation to the PDB Standard. The PDB standard is a fossil calcium carbonate from South Carolina in USA, with an isotope ratio (R_{PDB}) = 0.0112372. This value is the reference point of the common international PDB scale for $\delta^{13}C$ values expressed in parts per mill (‰).

The $\delta^{13}C$ values expressed in relation to the working standard are calculated with the following equation:

$$\delta^{13}C_{sam/ref}\ (‰) = 1000 \times (R_{sam} - R_{ref}) / R_{ref}$$

where

R_{sam} is the $^{13}C/^{12}C$ isotope ratio of the test portion;

R_{ref} is the $^{13}C/^{12}C$ isotope ratio of the working standard.

The $\delta^{13}C$ values expressed in relation to the PDB standard are calculated using the following equation:

$$\delta^{13}C_{sam/V\text{-}PDB} \text{ (‰)} = \delta^{13}C_{sam/ref} + \delta^{13}C_{ref/V\text{-}PDB} + (\delta^{13}C_{sam/ref} \times \delta^{13}C_{ref/V\text{-}PDB}) / 1000$$

where

$\delta^{13}C_{ref/V\text{-}PDB}$ is the isotopic deviation of the working standard previously determined from the PDB standard expressed in parts per mill (‰).

Express the results to two decimal places.

9. Precision

Details of the inter-laboratory test on precision of the method are given in annex D and E.

9.1. Repeatability

The absolute difference between two single results found on identical test sample by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in no more than 5% of the cases.
The accepted mean values of the standard deviation of repeatability (S_r) and repeatability limit (r) are equal to:

$S_r = 0.21‰ r = 0.58‰$
Characteristics of sampling procedures 7.1.a-c

$S_r = 0.21‰ r = 0.56‰$
Characteristics of sampling procedures 7.1d and 7.1e

9.2. Reproducibility

The absolute difference between two single results found on identical test sample reported by two laboratories will exceed the reproducibility *R* in not more than 5% of the cases.
The accepted mean values of the standard deviation of reproducibility (S_R) and reproducibility limit (R) are equal to:

S_R = 0.47‰ R = 1.33‰
Characteristics of sampling procedures 7.1.a-c

S_R = 0.68‰ R = 1.91‰
Characteristics of sampling procedures 7.1d and 7.1e

10. Test report

The test report shall contain the following data:

- all the information necessary for the identification of the sample tested;
- a reference to the International Standard Method;
- the method used, including the procedure for sampling and measurement and the instrument system used;
- the results of the test and units, including the results of the individual determinations and their mean, calculated as specified in clause 8 (“Calculation”);
- any deviations from the procedure specified;
- any unusual features observed during the test;
- the date of the test;
- whether repeatability has been verified;
- a description of the procedure for the reference gas calibration used to measure the test portions.

Annexes (A,B,C,D, E)

11. Bibliography

1. *Mesure du rapport isotopique $^{13}C/^{12}C$ du gaz carbonique des vins mousseux et des vins gazéifiés. J. Merin and S. Mínguez. Office International de la Vigne et du Vin. Paris. F.V. 1039, 2426/200297 (1997).*
2. *Examination of the $^{13}C/^{12}C$ isotopes in sparkling and semi-sparkling wine with the aid of simple on-line sampling. M. Boner and H. Förstel. Office International de la Vigne et du Vin. Paris. F.V. 1152. (2001).*
3. *Use of $^{13}C/^{12}C$ ratios for studying the origin of CO_2 in sparkling wines. J.Dunbar. Fresenius Z. Anal. Chem., 311, 578-580 (1982).*
4. *Contribution to the study of the origin of CO_2 in sparkling wines by determination of the $^{13}C/^{12}C$ isotope ratio. I. González-Martin, C. González-Pérez, E. Marqués-Macías. J. Agric. Food Chem. 45, 1149-1151 (1997).*
5. *Protocol for Design, Conduct and Interpretation of Method-Performance studies. Pure Appl. Chem., 1995, 67, 331-343*.

ANNEX A

Experimental procedure based on off-line systems for sampling and measurement
("in-house" sampling device, off-line vacuum line and dual-inlet IRMS)

1. Material

- Sampling device. The device that will be used to extract gas aliquots from the bottle consists of a hollow punch (steel needle) with three lateral orifices through which the gas enters. It is connected to a valve system composed of two valves connected in sequence and has a capacity of about 1 mL. One valve is attached to the punch (Valve 1) and the other is attached to a steel tube (Valve 2), which connects the device to a vacuum line. For a glass vacuum line an adapter with a flexible steel tube will be necessary. Figure shows the device for gas collection.
- Off-line vacuum line with two cryogenic traps (P<0.05 mbar). Two types of vacuum line can be used, a glass or steel vacuum line.
- Dual-inlet - Isotope ratio mass spectrometer with the ability to determine the ^{13}C content of CO_2 gas at natural abundance with an internal precision of 0.05‰ or better (expressed in relative δ value). Internal precision is here defined as the difference between two measurements of the same CO_2 sample.

2. Procedure (see Figure)

2.1. CO_2 sampling:

1. Connect the sampling device to vacuum line and test its seal capacity.
2. Punch the sampling device with the valves closed into the bottle cork by means of a circular movement whilst maintaining the device vertical.
3. Connect the sampling device–wine bottle assembly to the vacuum line and evacuate the line and the reservoir delimited by the two valves (Valve 2 opened and Valve 1 closed).
4. Once a vacuum has been created in the reservoir, close valve 2, open valve 1 and maintain this configuration for 1 min. After the equilibration time, close valve 1. The gas retained in the reservoir is then purified.

2.2. CO_2 purification and separation:

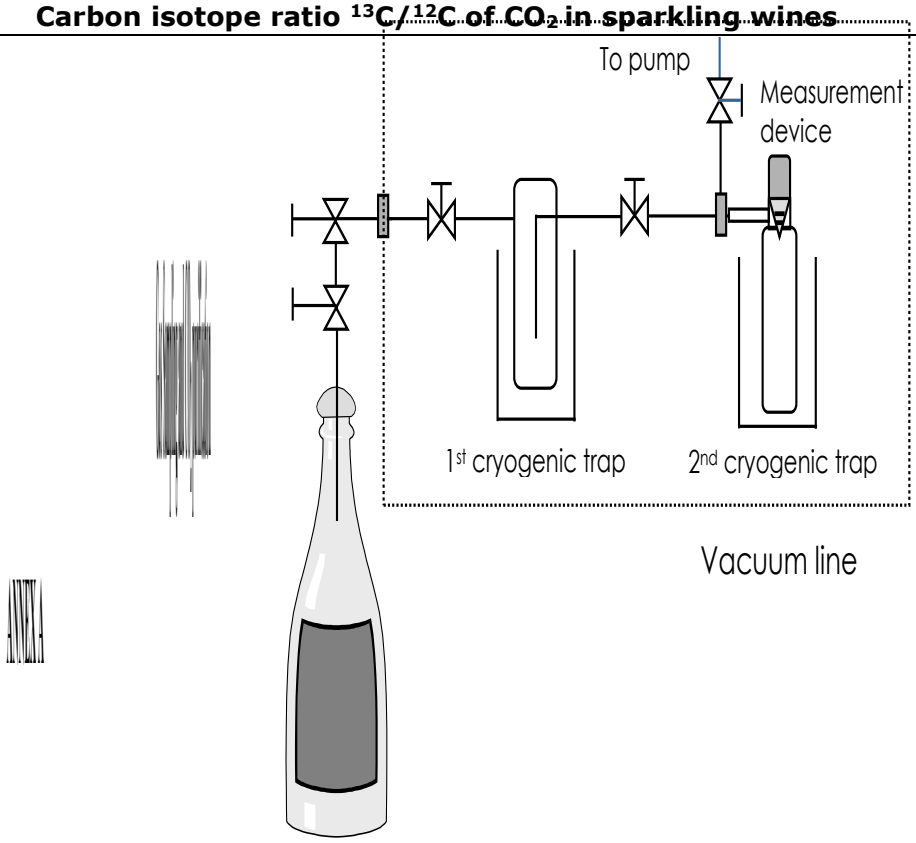
1. Transfer the CO_2 collected in the reservoir to the first cryogenic trap by liquid nitrogen for at least 1 min, then pump the uncondensed gas until a pressure of less than 0.05 mbar is reached.
2. Transfer the CO_2 sample to the measurement device by using liquid nitrogen in the second cryogenic trap and by changing the liquid nitrogen in the first cryogenic trap for a water trap at -80 ± 5 ºC. Maintain this for at least 1 min.
3. Pump the uncondensed gas (until a pressure of less than 0.05 mbar is reached) before closing the measurement device.

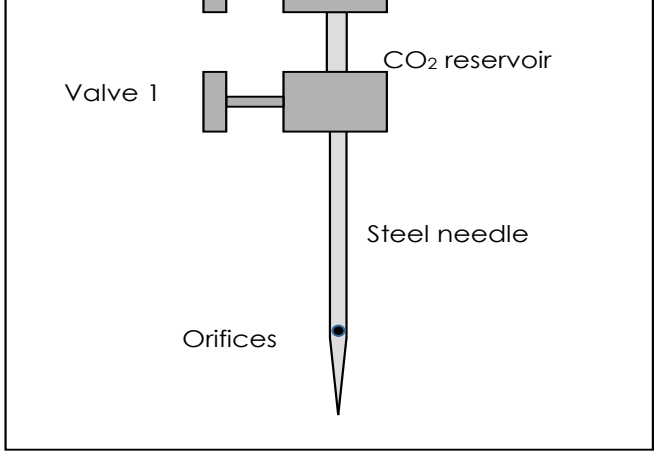
2.3. $^{13}C/^{12}C$ ratio measurement

The carbon isotope ratio of CO_2 obtained is measured by using a dual-inlet IRMS.

3. Reference

Mesure du rapport isotopique $^{13}C/^{12}C$ du gaz carbonique des vins mousseux et des vins gazeifiés. J.Merín, S.Mínguez. Office International de la Vigne et du Vin, F.V. 1039, 2426/200297.


To pump
Measurement device
1st cryogenic trap
2nd cryogenic trap
Vacuum line


Connector to vacuum line
Valve 2
CO2 reservoir
Valve 1
Steel needle
Orifices

ANNEX B

Experimental procedure based on the on-line systems for sampling and measurement (CF-IRMS)

1. Sampling technique

At first the sampling system is evacuated, the carbon dioxide is extracted from the bottle using a "sampling device", and a specific quantity is transferred to the storage vessel. After applying an overpressure, a small quantity of sample gas is introduced into the on-line helium flow with the aid of a restrictor. The sampling system is illustrated in Figure 2.

There is now a continuous carbon dioxide flow present in the helium flow (sample flow). The remaining helium flow is free from carbon dioxide and acts as the zero flow. Artificial "switching peaks" are generated by temporarily switching from the zero flow to the sample flow (switching time: 2 seconds), which are measured in the MS for their isotopic ratio.

2. Procedure (see Figure):

2.1. Evacuation of the sampling system

The entire sampling system is evacuated to a negative pressure of 1 mbar (V3 closed)

2.2. Sampling

The closure is pierced with a "sampling device" and the bottle atmosphere is transferred into the gas storage vessel (GV) with the aid of the negative pressure (pressure increase to approx. after 50 mbar). The fine adjustment valve (VF) permits a controlled and slow transfer of the gas. The gas is purified in the cryotrap during transfer.

2.3. Feeding

After sampling (V3, V2 closed, V4 open), an overpressure of 1,5 bar is built up with the aid of helium. The gas to be measured is fed to the CF-IRMS by opening V3. The measurement can be performed after a pre-run of 150 seconds. A capillary is integrated as a restrictor which only allows the feeding of a very small carrier gas quantity (10mL/min).

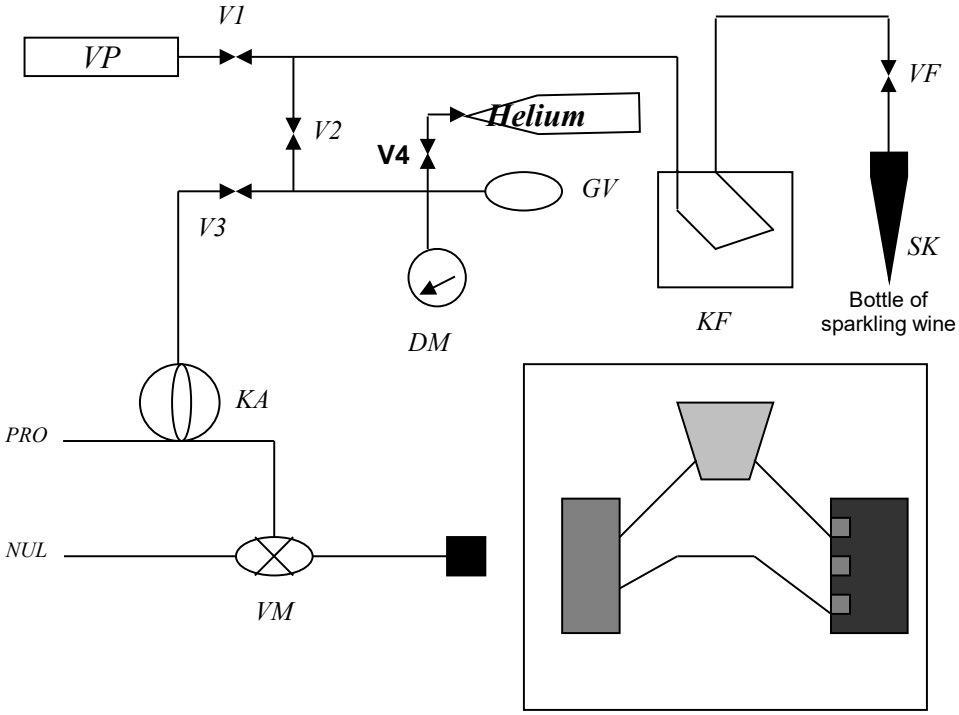
2.4. Measurement

A carbon dioxide flow is now continuously present in the helium sample flow (PRO). Switching from the sample flow (PRO) to the pure helium flow (NUL) permits the generation of artificial switching peaks.
Switching on the sample side: 2 seconds (zero side: 10-30 seconds).

3. Reference

Examination of the $^{13}C/^{12}C$ isotopes in sparkling and semi-sparkling wine with the aid of simple on-line sampling. M. Boner and H. Förstel. Office International de la Vigne et du Vin, FV 1152.

Diagram of the on-line system



V1-V4	check valve
VP	vacuum pump
VF	fine adjustment valve
SK	sampling device
PRO	helium sample flow (50 mL/min)
NUL	helium (zero) flow (60mL/min)
KF	water trap propanol at – 90ºC
GV	250 ml gas storage vessel
DM	pressure gauge
KA	restrictor capillary (10cm, 150µm)
VM	2/4-way valve

ANNEX C

Experimental procedure based on the GC-C-IRMS technique

1. Instrument characteristics

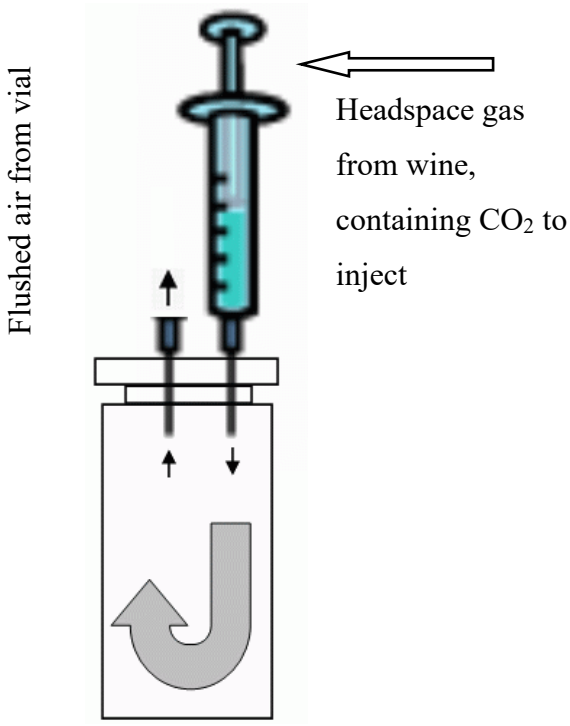
- Gas Chromatograph: GC Varian 3400
- Capillary Column: HP-INNOWax (Crosslinked Polyethylene Glycol), 30 m x 0.25 mm ID, film thickness 0.5 μm
- Combustion interface by ThermoFinnigan-MAT, with oxidation oven set at 940°C or off; reduction oven at 640°C or off
- Mass Spectrometer: DeltaPlus ThermoFinnigan-MAT.
-

2. Procedure

2.1. CO_2 sampling:

1. Aliquots of gas were collected through a 25cc syringe, by plugging a long iron needle through the cork. CO_2 pressure filled the syringe with the headspace gas spontaneously.
2. Transfer the gas in already crimped vials for subsequent analysis. The vials used to store the gas are previously crimped with Teflon-silicone septum caps. To flush out the air inside – and thus the atmospheric CO_2 – a second needle is plunged into the septum, to guarantee that headspace gas from wine pushes out the air in vial. See figure below.

NOTE: A bigger syringe is used, in line with vial volume, to make sure the vial is clean. In our case, a 25cc (or even bigger) syringe for a 2 ml vial.

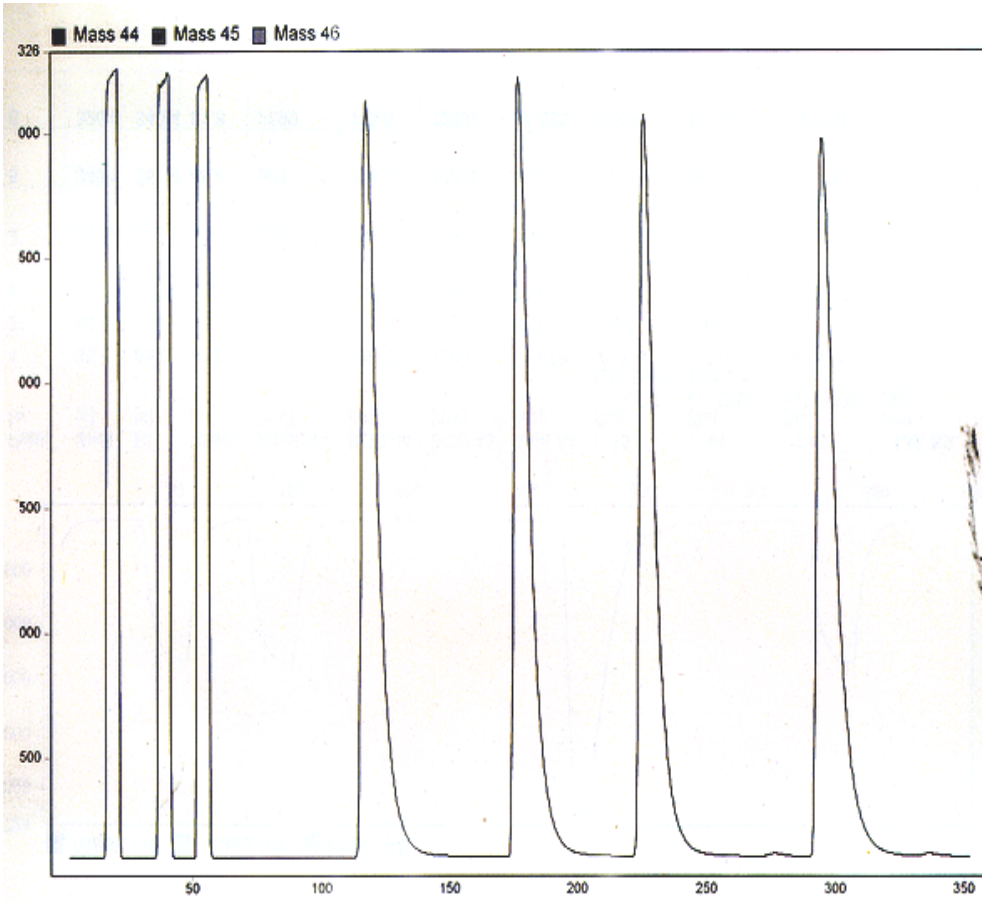


* Note that vial is not in scale with syringe.

2.2. GC-IRMS analyses: CO_2 injection and $^{13}C/^{12}C$ ratio measurement

A very few µL of gas were directly injected into the column with a 10 µL cemented-needle Hamilton syringe. Split conditions of high flow were set up. The carrier helium was at 20 PSI.

4 injections were carried out in each run for each sample. Total run time for the analysis was 6 minutes. See chromatogram below.



2.3. Processing of results

The software used to record and elaborate signals from the mass spectrometer, was version 1.50 of Isodat NT, from ThermoFinnigan-Bremen, running under MS-Windows NT OS.

For each sample, the mean $\delta^{13}C$ value is calculated as the average value of the last 3 injections. The $\delta^{13}C$ value of the first injection is systematically discarded.

ANNEX D
(informative)

Statistical results of the inter-laboratory test

In accordance with ISO 5725:1994, the following parameters were defined in an inter-laboratory test conducted by 11 European laboratories and a Mexican laboratory.

Year of the inter-laboratory test	2003-2004
Number of laboratories	12
Number of samples	5 in blind duplicates
Parameter	$\delta^{13}C$ of CO_2

Sample identification	A	B	C	D	E
Number of participating laboratories	12	12	12	12	12
Number of laboratories retained after eliminati outliers	12	11	12	12	12
Number of replicates per laboratory	2	2	2	2	2
Number of accepted test results	24	22	24	24	24
Mean ($\delta^{13}C$) ‰	-9.92	-20.84	-23.66	-34.80	-36.43
s_r^2	0.057	0.031	0.119	0.006	0.044
Repeatability standard deviation (S_r) ‰	0.24	0.18	0.35	0.08	0.21
Repeatability value, r (2.8 x S_r) ‰	0.67	0.49	0.97	0.21	0.58
S_R^2	0.284	0.301	0.256	0.140	0.172
Reproducibility standard deviation (S_R) ‰	0.53	0.55	0.51	0.37	0.41
Reproducibility value, R (2.8 x S_R) ‰	1.49	1.54	1.42	1.05	1.16

Sample types:
- A Sparkling wine - C_4 sugar
- B Sparkling wine - C_3 sugar
- C Sparkling wine - C_3 sugar
- D Gasified wine
- E Gasified wine

ANNEX E

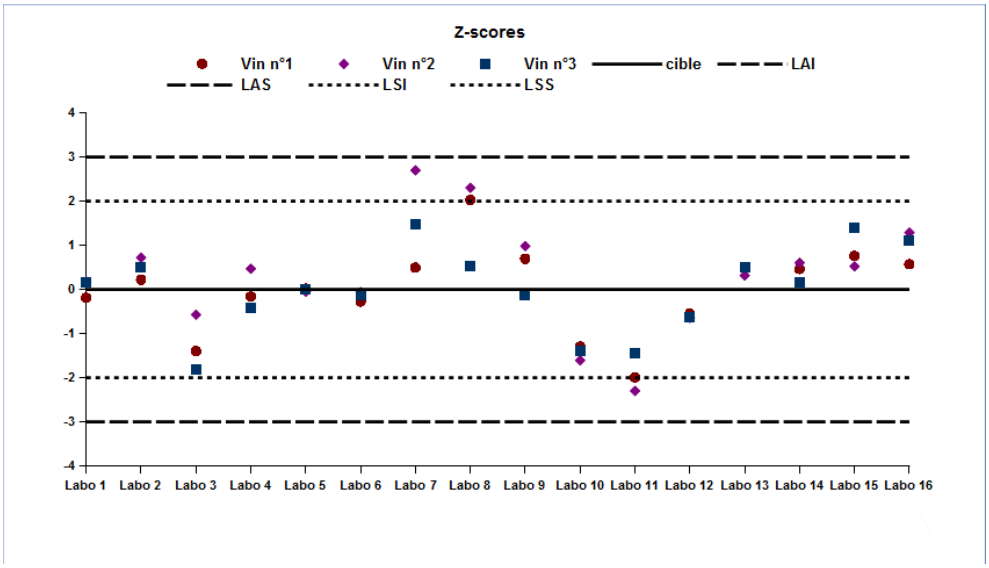
Statistical results of the inter-laboratory test on sparkling and gasified wines
Sampling procedures 7.1.d and 7.1.e

In accordance with method **OIV-MA-AS1-09: R2000**, the following parameters were defined as part of an inter-laboratory test conducted with 16 laboratories.

Year of the inter-laboratory test: 2013-2014
Number of laboratories: 16
Type of samples: Sparkling and gasified wines
Number of samples: 3, as blind duplicates
Parameter measured: $\delta^{13}C$

INDICATORS	WINE NO. 1	WINE NO. 2	WINE NO. 3
Number of laboratories	16	14	16
Number of repetitions	2	2	2
Minimum	-32.90	-33.10	-23.64
Maximum	-29.83	-30.97	-20.57
Repeatability variance s_r^2	0.0467	0.0118	0.0648
Inter-group variance s_L^2	0.43853	0,29762	0.51616
Reproducibility variance s_R^2	0.4852	0.3094	0.5810
Overall average	-31.42	-31.83	-22.15
Repeatability standard deviation	0.22	0.11	0.25
r limit	0.612	0.307	0.720
Reproducibility standard deviation	0.70	0.56	0.76
R limit	1.971	1.574	2.157

Laboratory Code	A	B	A	B	A	B	Wine No. 1	Wine No. 2	Wine No. 3
Lab 1	-31.40	-31.69	-31.56	-31.88	-21.93	-22.12	-0.18	-0.19	0.16
Lab 2	-31.23	-31.29	-31.43	-31.41	-21.46	-22.04	0.23	-0.73	0.52
Lab 3	-32.65	-32.12	-32.15	-32.13	-23.41	-23.64	-1.39	-0.56	-1.81
Lab 4	-31.55	-31.50	-31.46	-31.66	-22.40	-22.54	-0.15	0.48	-0.42
Lab 5	-31.50	-31.30	-31.80	-31.90	-22.00	-22.30	0.03	-0.04	0.00
Lab 6	-31.46	-31.75	-31.96	-31.75	-22.39	-22.10	-0.27	-0.05	-0.13
Lab 7	-31.48	-30.66	-31.29	-29.35	-21.47	-20.57	0.50	2.71	1.48
Lab 8	-29.83	-30.17	-29.73	-31.35	-21.50	-21.96	2.04	2.31	0.55
Lab 9	-30.96	-30.90	-31.34	-31.21	-22.22	-22.27	0.70	0.99	-0.13
Lab 10	-32.34	-32.29	-32.68	-32.75	-23.25	-23.14	-1.29	-1.60	-1.37
Lab 11	-32.90	-32.70	-33.10	-33.10	-23.00	-23.50	-1.98	-2.29	-1.45
Lab 12	-31.91	-31.68	-32.22	-32.14	-22.58	-22.66	-0.54	-0.63	-0.62
Lab 13	-31.03	-31.10	-31.61	-31.68	-21.78	-21.74	0.51	0.33	0.51
Lab 14	-31.25	-30.93	-31.43	-31.54	-22.01	-22.02	0.57	0.62	0.17
Lab 15	-30.89	-30.88	-31.59	-31.47	-21.08	-21.07	0.76	0.53	1.41
Lab 16	-31.05	-30.98	-31.24	-30.97	-21.090	-21.490	0.58	1.30	1.13



Biblipgraphy

1. Ana I. Cabañero, Tamar San-Hipólito and Mercedes Rupérez, GasBench/isotope ratio mass spectrometry: a carbon isotope approach to detect exogenous CO2 in sparkling drinks Rapid Commun. Mass Spectrom. 2007; 21: 3323–3328.
2. Laetitia Gaillard, Francois Guyon⁄, Marie-Hélène Salagoïty, Bernard Médina, Authenticity of carbon dioxide bubbles in French ciders through multiflow-isotope ratio mass spectrometry measurements. Food Chemistry. 2013, 141: 2103–2107

Method OIV-MA-AS314-04 Type II method

Determination of carbon dioxide in wine by manometric method

For a range of concentration from 0.5 g/L to 7 g/L

A39; OENO 21/2003;
OENO 2/2006
OIV/OENO 377/2009

1. PRINCIPLE

The carbon dioxide in the sample is bound with 10 M sodium hydroxide. An Erlenmeyer flask with a side arm is connected to a manometer and the carbon dioxide is released with sulphuric acid from the prepared sample. The resultant increase in pressure is measured. It allows quantifying carbon dioxide content.

2. REAGENTS

2.1. Freshly distilled or deionised water;
2.2. Sodium hydroxide (purity >98%);
2.3. Sulphuric acid (purity >95-97%);
2.4. Sodium carbonate anhydrous (purity >99%).

Preparation of the reagents

2.5. 10 M Sodium hydroxide: dissolve 100 g of sodium hydroxide (2.2) in 200 ml water (2.1) and make up to 250 ml in a volumetric flask.

2.6. Sulphuric acid, about 50% (v/v): cautiously add concentrated sulphuric acid (2.3) to an equal volume of water (2.1). Mix well by stirring. Cool to room temperature.

2.7. Carbon dioxide standard solution 10 g/l: dry anhydrous sodium carbonate (2.4) in an oven at 260°C-270°C over night, and cool to room temperature in a desiccator. Dissolve 6.021 g of dry sodium carbonate in water (2.1) and make up to 250 ml in a volumetric flask.
2.8. Carbon dioxide calibration solutions 0.4; 1; 2; 4 and 6 g/l: with pipettes take 2, 5, 10, 20 and 30 ml of the standard solution (2.7) in separate 50 ml volumetric flasks and make up to 50 ml with water (2.1).

3. APPARATUS

3.1. 250 ml and 50 ml volumetric flasks;
3.2.Oven;
3.3. Dessicator;
3.4. Balance with an accuracy of ± 0.1 mg;
3.5. Refrigerator or water-ethylene glycol bath, -4°C;
3.6. Electronic density meter or pycnometer and thermostatic water bath, 20°C;
3.7. Pipettes 0.5, 2, 3, 5, 10, 20 and 30 ml;
3.8. 100 ml cone-shaped vial, large ground-glass mouth;
3.9. Digital manometer (allowing measures up to 200 kPa with an accuracy of 0.1kPa);
3.10. Reaction flask: 25 ml Erlenmeyer flask with a 3 ml side arm and a three-way valve (see figure 1);
3.11. Vacuum system (i.e. water suction pump).
3.12 Separation funnel

4. PROCEDURE

4.1. Sample preparation

Prepare the sample in duplicate. Cool the sample in a refrigerator overnight or in a -4°C water-ethylene glycol bath for 40 min. Place 3 ml of 10 M sodium hydroxide solution (2.5) in a 100 ml cone-shaped vial. Weigh the flask with contents at an accuracy of 0.1 mg. Pour approximately 75 ml of the cooled sample in the cone-shaped vial containing the sodium hydroxide solution. Weigh the flask with contents at an accuracy of 0.1 mg. Mix and allow to warm up to room temperature.

4.2. Determination of carbon dioxide content

Transfer 2 ml of the prepared sample (4.1) into the reaction flask. Connect the flask to the manometer via the open three-way valve. Pipette 0.5 ml of 50% sulphuric acid (2.6) into the side arm. Secure the three-way valve and the side arm stopper with clips. Note the air pressure. Close the three-way valve. Mix the contents by tilting and shaking vigorously. Note the pressure. The prepared sample can be diluted with water
if necessary.

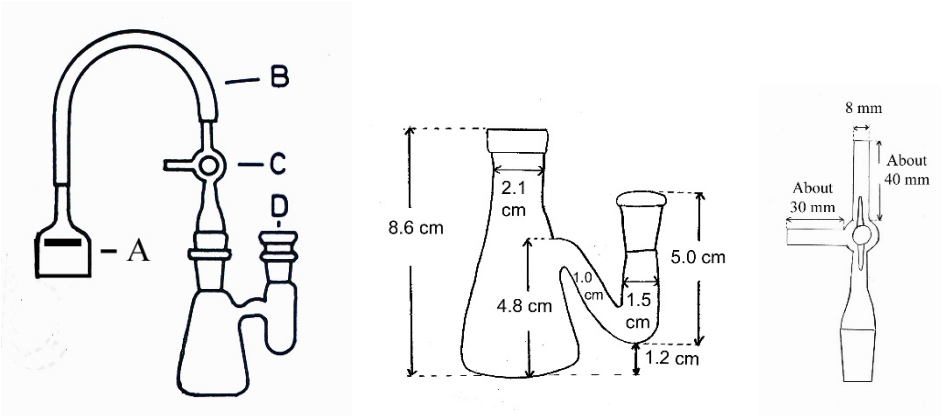


Fig.1 Apparatus. A manometer, B rubber hose, C three-way valve, D reaction flask (left) and approximate measures of the glassware (centre and right).

4.3. Calibration

Determine the carbon dioxide content of the calibration solutions as described above (4.2). Measure three calibration solutions which are within the expected concentration range of the sample. These calibration solutions are measured in duplicate.

4.4. Measurement of the density of the sample

Remove carbon dioxide from the sample by shaking the sample first in a separation funnel and then for 3 min in a vacuum generated by a water suction pump. Measure the density of the sample either with an electronic density meter or a pycnometer.

5. CALCULATION

Calculate the pressure increase caused by the carbon dioxide released from each calibration solution and construct a calibration graph.

Calculate the slope (a) and bias (b) of the calibration graph.

Volume V (ml) of the prepared sample:

$V = [(m2-m1) \times 1000]/d$ (1)
where
m1 (g)= weight of (flask + 3 ml NaOH);
m2 (g) = weight of (flask + 3 ml NaOH + sample);
d (kg/m^3) = density of sample.

Pressure increase p_i caused by the carbon dioxide released from the prepared sample:

$$pi = ps - pap \quad (2)$$

where
p_s = manometer reading after releasing the carbon dioxide from the sample
p_{ap} = manometer reading before addition of H_2SO_4 (i.e. air pressure)

Concentration of carbon dioxide, C, in the sample (g/l) is given by:

$$C = [(pi - b) / a] \times [(V + 3)/V] \times L \quad (3)$$

where
p_i = increase of pressure (equation 2)
a = slope of calibration graph
b = bias of calibration graph
V = sample volume (equation 1)
L = dilution factor in case the sample is diluted after sample preparation

Content of carbon dioxide in % by weight:

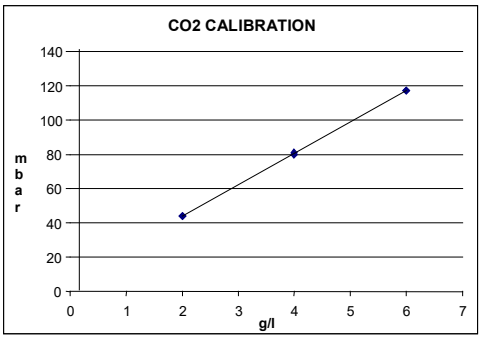
$$CO2\ \%\ (w/w) = C \times 100/d \qquad (4)$$

Example of the calculation of the content of carbon dioxide:

Calibration

Conc of STD g/l	Air pressure mbar	Pressure std mbar	Pressure increase mbar
2	1021	1065	44
2	1021	1065	44
4	1021	1101	80
4	1021	1102	81
6	1021	1138	117
6	1021	1138	117

slope	18.25000
intercept	7.50000
correlation	0.99995



Calculation of the content of CO_2

SAMPLE	Density d (kg/m^3)	Flask + NaOH m1 (g)	Flask NaOH+ sampe m2 (g)	Air pressure p_{ap} (mbar)	Sample pressure p_s (mbar)	p_s-p_{ap}	Sample V (ml)	CO2 g/l	Mean CO2 g/l
Sparkling wine 1	1027.2	84.6287	156.162	1021	1112	91	69.64	**4.77**	
Sparkling wine 1	1027.2	84.6287	156.162	1021	1113	92	69.64	**4.83**	**4.80**
Sparkling wine 2	1025.3	86.1066	153.4407	1021	1118	97	65.67	**5.13**	
Sparkling wine 2	1025.3	86.1066	153.4407	1021	1118	97	65.67	**5.13**	**5.13**

6. VALIDATION

6.1. Performance criteria

- Standard deviation estimated from duplicates, s_o = 0.07 g/l
- Relative standard deviation, RSD = 1.9%
- Repeatability, r = 5.6 %
- Expanded measurement uncertainty (k = 2), U = 3.8%
- Calibration range 0.4-6 g/L
- Determination range 0.3 -12 g/L (samples with concentration above 6 g/L should be diluted 1:2 with water to fit the calibration range)
- Detection limit 0.14 g/L
- Quantification limit 0.48 g/L

Annex A

Literature

European Brewery Convention Analytica-EBC, Fourth edition, 1987, 9.15 Carbon dioxide.

OIV, SCMA 2002, FV N° 1153, determination of carbon dioxide in alcoholic beverages by a modified EBC method

OIV, SCMA 2004, FV N° 1192, determination of carbon dioxide in alcoholic Beverages by a modified EBC method, Statistical results of the collaborative study

OIV, SCMA 2005, FV N° 1222, comparison of the titrimetric method and the modified EBC method for the determination of carbon dioxide in alcoholic beverages

Ali-Mattila, E. and Lehtonen, P., Determination of carbon dioxide in alcoholic beverages by a modified EBC method, Mitteilungen Klosterneuburg 52 (2002): 233-236

Annex B

Statistical results of the collaborative study

DETERMINATION OF CARBON DIOXIDE IN ALCOHOLIC BEVERAGES BY A MODIFIED EBC METHOD

1. Goal of the study

The objective of the study was to determine the repeatability and reproducibility of the modified EBC method for the determination of carbon dioxide in wines, sparkling wines, ciders and beers.

2. Needs and purpose of the study

Fermentation produces carbon dioxide in alcoholic beverages. In the production of sparkling wines, carbon dioxide is one of the most essential products and it can also be added to certain alcoholic beverages. Carbon dioxide modifies the taste and aroma and is a preserving agent in alcoholic beverages.

In accordance with the definitions of the International Code of Oenological practices, sparkling wine should have an excess pressure of not less than 3 bar due to carbon dioxide in solution, when kept at a temperature of 20°C in closed containers. Correspondingly semi-sparkling wine should have an excess pressure of not less than 1 bar and not more than 2,5 bar. Excess pressure of, 3 bar, 2.5 bar and 1 bar correspond at 20°C about, 5.83 g/L, 5.17 g/L and 3.08 g/L of carbon dioxide in solution, respectively.

There is currently no practical and reliable method for the determination of carbon dioxide in alcoholic beverages. The wide variation in carbon dioxide results in international proficiency tests is a clear indication of the fact that there is a need for a reliable method.

3. Scope and applicability

The proposed method is quantitative and it is applicable for the determination of carbon dioxide in alcoholic beverages. This method was validated in a collaborative study for the determination of carbon dioxide in wine, beer, cider and sparkling wine via the analyses at levels ranging approximately from 0.4 g/L to 12 g/L (Note: the actual calibration level ranges from 0,4 g/L to 6 g/L. The samples should be diluted with water to this level in case the carbon dioxide content is higher than 6 g/L).

4. Materials and matrices

The collaborative study consisted of 6 different samples. All except the beer samples were sent in blind duplicate, so that in total 12 bottles were distributed to the participants: two beers, two ciders, two red wines, two white wines, two pearl wines and two sparkling wines. Each bottle was coded individually for each participant. All samples were delivered in original bottles and the labels were removed from all samples except the sparkling wine samples. Measuring the amount of carbon dioxide in 10 bottles of the same lot number tested the homogeneity of the samples.

5. Practice samples

Four control samples were sent to participants to familiarize them with the method. . These samples included one beer, one wine, one pearl wine and one sparkling wine sample.

6. Method to be followed and supporting documents

The method and an Excel table for the calculation of results were sent to participants. Supporting documents were also given, including the covering letter, sample receipt form, and result sheets.

7. Data analysis

7.1. Determination of outliers was assessed by Cochran's test, Grubbs' test and bilateral Grubbs test.
7.2. Statistical analysis was performed to obtain repeatability and reproducibility data.

8. Participants

Nine laboratories in different countries participated in the collaborative study. Lab-codes were given to the laboratories. The participating laboratories have proven experience in the analysis of alcoholic beverages.

Alcohol Control Laboratory
Alko Inc.
P.O.Box 279
FIN-01301 Vantaa
Finland

Altia Ltd
Valta-akseli
Rajamäki
Finland

Arcus AS
Haslevangen 16
P.O.Box 6764 Rodeløkka
0503 Oslo
Norway

ARETO Ltd
Mere pst 8a
10111 Tallinn
Estonia

Bundesamt für Weinbau
Gölbeszeile 1
A-7000 Eisenstadt
Austria

Comité Interprofessionnel du Vin de Champagne
5, rue Henri MARTIN
BP 135
51204 EPERNAY CEDEX
France

High-Tec Foods Ltd
Ruomelantie 12 B
02210 Espoo
Finland

Institut für Radioagronomie
Forschungszentrum
Jülich GMBH
Postfach 1913
52425 JÜLICH
Germany

Systembolagets laboratorioum
Armaturvägen 4,
S-136 50 HANINGE
Sweden

9. Results

The homogeneity of the samples was determined by measuring the carbon dioxide content in 10 bottles of the same lot number at the Alcohol Control Laboratory (Finland). Samples with the corresponding lot numbers were sent to the participants:

CO_2 g/L	Beer 1	Beer 2	Cider	White wine	Red Wine	Pearl wine	Sparkling wine
Means	5.191	5.140	4.817	1.337	0.595	5.254	7.463
	0.020	0.027	0.025	0.036	0.038	0.022	0.046

According to the homogeneity test the CO_2 content in the two beers was the same and therefore they were considered as blind duplicates.

The individual results for all samples and laboratories of the collaborative study are given below.

Lab code	Beer 1	Beer 2	Cider 1	Cider 2	White wine 1	White wine 2	Red wine 1	Red wine 2	Pearl wine 1	Pearl wine 2	Sparkling wine 1	Sparkling wine 2
A	5,39	5,08	4,75	4,91	1,25	1,11	0,54	0,54	5,15	5,22	6,93	6,91
B	4,76	5,53	4,71	4,70	1,90[3]	1,78[3]	0,73[2]	1,19[2]	5,85[3]	5,93[3]	7,66[3]	7,72[3]
C	5,15	5,14	4,93	4,94	1,36	1,41	0,51	0,48	5,23	5,33	7,33	7,36
D	3,13[1]	3,95[1]	4,36[1]	0,38[1]	1,11[1]	1,11[1]	0,43[1]	0,38[1]	4,47[1]	4,29[1]	5,54[1]	5,52[1]
E	4,87	4,73	4,96	4,78	1,52	1,52	0,78[3]	0,80[3]	4,98	4,94	5,83	6,17
F	5.34	4.91	4.71	5.01	1.33	1.40	0.46	0.57	5.22	4.95	6.52	6.67
G	5,18	5,15	4,82	4,86	1,37	1,36	0,56	0,59	5,22	5,27	7,54	7,47
H	5,42	5,40	5,05	5,12	1,15	1,30	0,52	0,53	5,12	5,10	7,25	7,34
I	5,14	5,13	4,65	4,76	1,16	1,19	0,47	0,61	5,16	5,06	6,88	6,48

1. Removed because of large systematic error obviously due to poor calibration
2. Outlier by Cochran's test
3. Outlier by Grubbs' test

Statistical results of the collaborative test are summarised below.

	Beer	Cider	White wine	Red Wine	Pearl wine	Sparkling wine
Mean (g/L)	5.145	4.859	1.316	0.532	5.139	6.906
Mean rep. 1 (g/L)	5.156	4.833	1.306	0.510	5.154	6.897
Mean rep 2 (g/L)	5.134	4.885	1.327	0.553	5.124	6.914
s_r (g/L)	0.237	0.089	0.060	0.053	0.086	0.149
s_R (g/L)	0.237	0.139	0.135	0.059	0.124	0.538
SDR_r (%)	4.597	1.821	4.562	9.953	1.663	2.163
RSD_R (%)	4.611	2.855	10.22	11.07	2.407	7.795
r (2,8*s_r) (g/L)	0.662	0.248	0.168	0.148	0.239	0.418
R (2,8*s_R) (g/L)	0.664	0.388	0.377	0.165	0.346	1.507
HORRAT R	1.043	0.640	1.883	1.779	0.544	1.843

Conclusion
The Horrat values are < 2 indicating an acceptable method. The Horrat values are, however, a little bit high. In five of the nine participating laboratories these tests were made almost with no previous experience. Therefore the results can be considered at least as very satisfactory.

The method gives the results in g/L but the results can be converted to pressure units. [1]

1. Troost, G. and Haushofer, H., Sekt, Schaum- und Perlwein, Eugen Ulmer Gmbh & Co., 1980, Klosterneuburg am Rhein, ISBN 3-8001-5804-3, Diagram 1 on the page 13.

Annex C

Validation at low carbon dioxide levels

1. The detection and the determination limit

A sample of white wine was analysed in duplicate ten times. The statistical data was as follows:

Replicates	10
Mean CO_2 (g/L)	0.41
Standard deviation of the mean, s (g/L)	0.048
Detection limit 3 x s	0.14
Determination limit 6 x s	0.48

2. Standard addition

Standard additions in five different concentrations in duplicates were made into the same wine which was used for the determination of the detection and determination limits. The corresponding concentrations of CO_2 were also added to water. The linear regressions of these two experiments were compared.

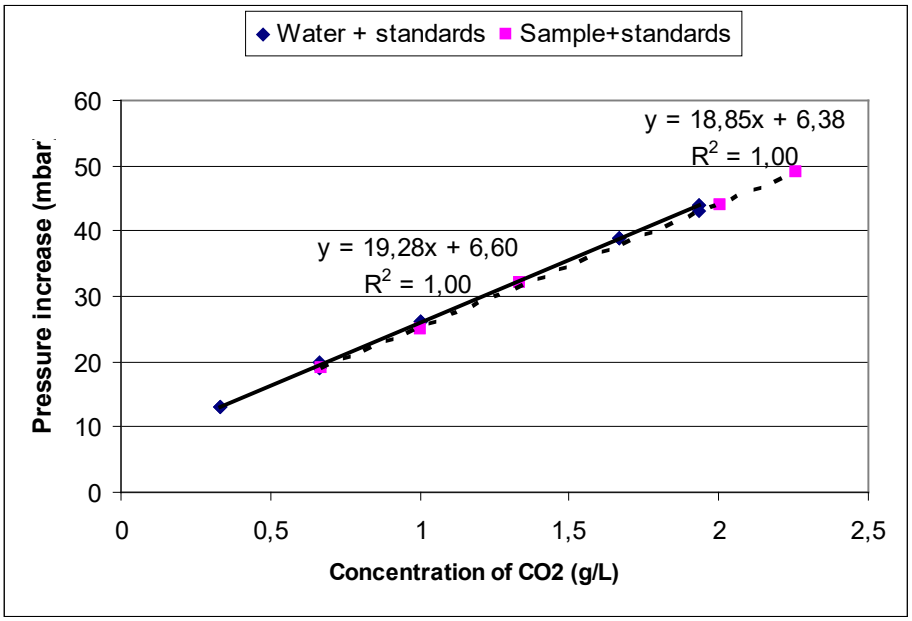


Fig. 1 Standard additions to the sample and to water.

Statistical data of the plots:

	Water+ standards	Sample+standards
Slope	19.3	18.9
Uncertainty of the slope	0.3	0.3
Intercept	6.6	6.4
Uncertainty of the intercept	0.4	0.5
Residual standard deviation	0.4	0.3
number of samples	15	10

According to statistical data the two regression lines are similar.

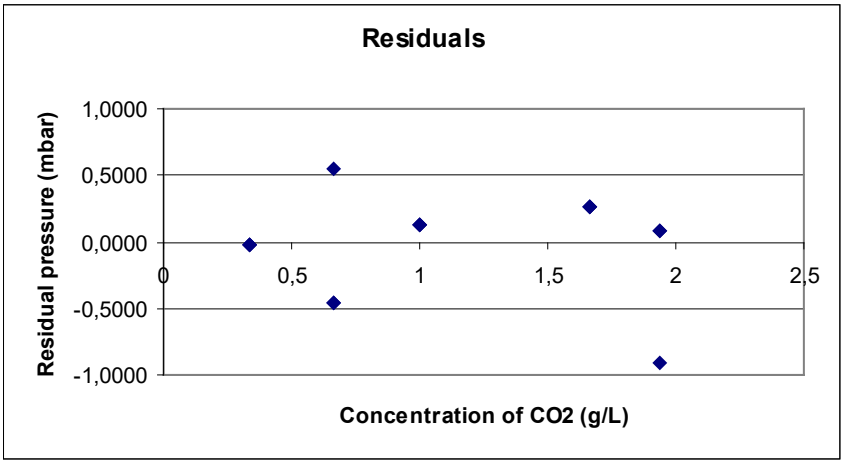


Fig. 2. The residuals of the "water+standards" equation

The residuals are dispatched on both sides of zero indicating that the regression line is linear.

Annex D

Comparison with other techniques and laboratories

1. Comparison of the modified EBC method with the commercial Anton Paar CarboQC instrument

Sample	Modified EBC method (g/L)	Anton Paar CarboQC (g/L)	Difference
Sparkling wine	9.14	9.35	-0.21
Cider	4,20	4,10	0.1
White wine	1,18	1,10	0.08
Red wine	1,08	0,83	0.25
Beer 1	5,26	5,15	0.11
Beer 2	4,89	4,82	0.07
Beer 3	4,90	4,92	-0.02
Non-alcohol Beer 1	5,41	5,33	0.08
Non-alcohol beer 2	5,39	5,36	0.03
			Mean 0.06

According to t-test there is no systematic difference in the measurements.

2. Comparison between Bfr, Germany and ACL, Finland

Bfr sent four samples to ACL, and ACL sent five samples to Bfr. These nine samples were analysed independently both by ACL using the method presented in this paper and in Germany at Bfr using the titrimetric method. Statistics of the results were as follows:

Mean of the difference	0.14 g/L
Std. of the difference	0.13 g/L
Z-score	1.04

The method presented here and the titrimetric method were also compared by Bundesamt für Weinbau in Austria using 21 samples of their own. Statistical data was as follows:

Mean of the difference	-0.01 g/L
Std. of the difference	0.26 g/L
Z-score	-0.03

Conclusion

According to this paper as well as earlier experiments this method is universal. It is suitable for the determination of the carbon dioxide content in all kinds of alcoholic beverages, e.g. beers, wines, fruit wines, ciders, pearl wines and sparkling wines with the concentration level of 0.3 g/L and higher.

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