RESOLUTION OENO 17/2001

DETERMINATION OF ISOTOPIC RATIO OF ETHANOL

THE GENERAL ASSEMBLY,

HAVING CONSIDERED Article 5 of the October 13, 1954 international convention for the unification of means of analysis and appreciation of wine,

WITH THE PROPOSAL of the Methods of Analysis and Appreciation of Wine sub-committee

DECIDES to introduce in Appendix A of the Compendium on International Methods of Wine and Musts, the following method:

DETERMINATION BY ISOTOPE RATIO MASS SPECTOMETRY 13C/12C OF WINE Ethanol OR THAT OBTAINED THROUGH THE fermentation OF MUSTS, CONCENTRATED MUSTS OR GRAPE SUGAR.

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

□^{13}C : Carbon 13 contents (^{13}C) expressed in parts per 1000 (‰)

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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

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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 ‰

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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 (□□) 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

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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 □g 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

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- Bulb sealed in glass filled with copper oxide (□□) 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

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- 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 □ 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 (□ ^{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 □ ^{13}C are expressed compared to reference work:

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- $\delta^{13}C_{ech/ref}‰ = 1000 \times (R_{ech}-R_{ref})/R_{ref}$

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

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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 □^{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:	□ ^{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%

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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 (□ ^{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

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Number of laboratories:	14 for distillation of wine and 7 for also measuring □ ^{13}C of ethanol in wine 8 for measuring □ ^{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	□ ^{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 (□ ^{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 □ ^{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

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parametres and the reproductability limit. The results obtained for the wine and distillate ethanol □ ^{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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