Method OIV-MA-BS-22 : R2009

Type II method

Determination by isotope ratio mass spectrometry of the $^{13}C/^{12}C$ ratio of wine ethanol of spirit drinks of viti-vinicultural origin

(OIV/OENO 381/2009)

1. Field of application

The method enables the measuring of the $^{13}C/^{12}C$ isotope ratio of the ethanol of spirit drinks of vitivinicultural origin.

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 (SNIF-NMR)»

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 (‰)

SNIF-NMR: Site-specific natural isotope fractionation 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 remained the primary reference to express 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 carbon dioxide 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 beet belong to the C_3 group. Sugar cane and corn belong to the C_4 group. 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 SNIF-NMR enable the quantification of mixed sugars added or alcohol of plant origin C_3 and C_4.

The carbon 13 content is determined on carbon dioxide resulting from the complete combustion of the sample. The abundance of the 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 ion currents measured by three different collectors of isotopic mass spectrometers. The contributions of isotopomers $^{13}C^{17}O^{16}O$ et $^{12}C^{17}O_2$ can be neglected because of their low abundance. The ion 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 and ^{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	□^{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 in Geel (B) (Institut des Matériaux et Mesures de Référence) :

Name	Material	□^{13}C versus V-PDB (9)
- CRM 656	Wine alcohol	-26,93 ‰
- CRM 657	glucose	-10,75 ‰
- CRM 660	Aqueous alcoholic solution (ABV 12%)	-26,72 ‰

Standard working standard with a known $^{13}C/^{12}C$ ratio calibrated with international reference materials.

A standard list of consumables established for continuous flow systems follows below:

- 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 carbon13 (CAS 00124-38-9)
- Oxidation reagent for the oven and the combustion system as follows: copper oxide (II) for elementary analysis (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 apparatus equipped with a water elimination system by cryo-trapping or through selective permeable capillaries.

6. Apparatus and materials

6.1. Isotope ratio mass spectrometry (IRMS)

Isotope ratio mass spectrometry (IRMS) enables the determination of the relative contents of ^{13}C of naturally occurring CO_2 gas 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 quantitatively convert ethanol into carbon dioxide and capable of eliminating all other combustion products including water, without any isotopic fractionation. The apparatus can be either an integrated continuous flow system integrated with mass spectrometry (6.2.1), of an autonomous combustion system (6.2.2). The apparatus must be as precise as indicated in (11).

6.2.1. Continuous flow systems

These consist of either an elemental analyser, or a gas chromatograph 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
- balance with 1 µg accuracy or better
- tool for capsule sealing
- tin capsules for liquid samples
- tin capsules for solid samples

The following laboratory material is needed when using an elemental 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 materials 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 spectrometry used by the laboratory.

6.2.2. Separate preparation systems

In this case the samples of carbon dioxide resulting from the combustion of samples to be analyzed and the reference sample are collected in ampoules which are then put in a double entry spectrometer system to carry out isotopic analyses. Several types of combustion apparatus described in the literature can be used:

- Closed combustion system filled with circulating oxygen gas
- Elemental analyser with helium and oxygen flows

- Sealed glass ampoule filled with copper oxide (II) used as an oxidation agent

7. Preparation of samples for tests

Ethanol must be extracted from the spirit drink before isotopic testing. This is carried out by distilling the beverage as described in §3.1 of the SNIF-NMR method for the determination by NMR of the deuterium distribution in the ethanol of spirit drinks of vitivinicultural origin.

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 quantitatively into carbon dioxide without the evaporation of ethanol, can use the preparation of carbon dioxide for isotopic analyses. An experimental procedure based on the use of an elemental analyser:

a. Placing the samples in capsules

- use capsules, tweezers and a clean preparation tray
- take an appropriate sized capsule using 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 spectrometer’s sensitivity.

- close the capsule with the sealing tool.
- each capsule must be completely sealed. If not, it must be discarded and a new capsule must be prepared.

- two capsules must be prepared for every sample
- place the capsules in an appropriate place on the elemental analyser sample tray. Every capsule must be carefully identified in order by number.
- systematically place capsules containing working 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 elemental analysis and mass spectometry instrumentation

- adjust the temperature of the elemental analyzer ovens and the helium and oxygen gas flow for an optimal combustion of the sample;
- check the elemental analysis system and the mass spectometry system for leaks (for example by checking the ion current where m/z = 28 corresponds to N_2);
- adjust the mass spectrometer to measure the ion current intensities for 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 on the autosampler of the elemental analyser or chromatographare introduced successively. The carbon dioxide for each sample combustion is eluted into the mass spectrometer which measures the ion current. The data system records the ion current intensities and calculates the ▯ values for each sample (9).

9. Calculation

The objective of the method is to measure the $^{13}C/^{12}C$ isotopic ratio of the ethanol extracted from spirit drinks. The $^{13}C/^{12}C$ isotopic ratio can be expressed by its deviation compared to the reference work. The carbon 13 (▯ ^{13}C) isotopic ratio is calculated on a delta scale per thousand by comparing the results obtained for the sample to be measured to the working reference calibrated previously based on the primary international reference (V-PDB). The δ^{13}C values are expressed compared to the working reference:

$$\delta^{13}\mathrm{C}_{\mathrm{ech/ref}}\% = 1000 \times (\mathrm{R}_{\mathrm{ech}} - \mathrm{R}_{\mathrm{ref}})/\mathrm{R}_{\mathrm{ref}}$$

where $\mathrm{R}_{\mathrm{ech}}$ and $\mathrm{R}_{\mathrm{ref}}$ are respectively the isotopic ratio$^{13}\mathrm{C}/^{12}\mathrm{C}$ of the sample and the working reference.

The δ^{13}C values are thus expressed using V-PDB:

$$\delta^{13}\mathrm{C}_{\mathrm{ech}/_{\mathrm{V-PDB}}}\% = \delta^{13}\mathrm{C}_{\mathrm{ech/ref}} + \delta^{13}\mathrm{C}_{\mathrm{ref}/_{\mathrm{V-PDB}}} + \left(\delta^{13}\mathrm{C}_{\frac{\mathrm{ech}}{\mathrm{ref}}} \times \delta^{13}\mathrm{C}_{\mathrm{ref}/_{\mathrm{V-PDB}}}\right)/1000$$

where $\delta^{13}\mathrm{C}_{\mathrm{ref/V\text{-}PDB}}$ is the isotopic deviation determined beforehand for the working reference to V-PDB.

Small variations may occur while measuring on line due to changes in the instrumental conditions. In this case the δ^{13}C samples must be corrected according to the difference in the δ^{13}C value from the working 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 working reference, the variation and therefore the correction applied to the sample results may be assumed to be linear. The working reference 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 between the two values (the difference between the assigned value of the working reference and the measurement values obtained).

10. Quality assurance and control

Check that the ^{13}C value for the working reference does not differ by more than 0.5‰ of the accepted value. If not, the spectrometer must be checked and possibly readjusted.

For each sample, verify that the difference in the results between the 2 capsules measured successively is under 0.3 ‰. The final result for a given sample is the average value of the 2 capsules. If the deviation is higher than 0.3 ‰ the measurement should be repeated.

Measurement condition monitoring can be based on the ion current intensity for m/z = 44 and is proportional to the quantity of carbon injected in the elemental analyzer. Under standard conditions, the ion 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 elemental analyser, or the mass spectrometer.

11. Method performance characteristics (precision)

One collaborative 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, and further information from other joint laboratory studies on wine (11.2) and in particular proficiency testing (11.3) for isotopic measurements have also been considered. The results show that different distillation systems under satisfactory conditions, and in particular those used for SNIF-NMR measurements, do not have significant variability for determining the □^{13}C of ethanol in wine. It is reasonable to suppose that this would likewise be true for the ethanol of spirit drinks. The precision parameters observed for wine are almost identical to those obtained in the joint study on distillates (11.1).

11.1. Joint study on distillates

Year of inter laboratory study	1996
Number of laboratories	20
Number of samples	6 samples in double-blind comparison
Analysis	ethanol □ ^{13}C

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 anomalous 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

Reproducibility standard deviation (S_R) ‰	0.20	0.18	0.20	0.21	0.18	0.24
Reproducibility limit R (2,8× S_R) ‰	0.55	0.9	0.55	0.60	0.50	0.68

11.2. Inter laboratory study on two wines and one alcohol

Year of inter laboratory trial:	1996
Number of laboratories	14 for distillation of wine and 7 for also measuring the δ^{13}C of ethanol in wine 8 for measuring the δ^{13}C in the alcohol sample
Number of samples	3 (White wine ABV 9.3 % vol., white wine ABV 9.6 % and alcohol of strength 93% m/m)
Analysis	ethanol δ^{13}C

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
Reproducibility variance S_R^2	0.0525	0.0740	0.0962
Reproducibility standard deviation (S_R) ‰	0.23	0.27	0.31
Reproducibility limit R (2.8× S_R) ‰	0.64	0.76	0.87

Different distillation systems were used by the participating laboratories. The ▯ ^{13}C isotopic determinations carried out in one laboratory on all of the distillates returned by the participants, did not reveal any anomalous values or significantly distinct average values. The variation in results (S^2 = 0.0059) is comparable to repeatability variances Sr^2 from the collaborative study on distillates (11.1).

11.3. Results from proficiency-testing studies

Since December 1994 international proficiency testing to determine the isotopic measurements for wine and alcohol (ABV of distillates 96 % vol.) have been organized regularly. The results enable participating laboratories to check the quality of their analyses. Statistical results enable an appreciation of the variability of derterminations under reproducibility conditions. This enables an estimation of the variance parameters and the reproducibility limit. The results obtained for the wine and ethanol distillate ▯ ^{13}C determinations are summarized in the table below:

Wines					Distillates			
Date	N	SR	S2R	R	N	SR	S2R	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 laboratorie

12. Bibliography

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