Method OIV-MA-VI-22 : R2013
Type II method

Method for $^{13}C/^{12}C$ isotope ratio determination of acetic acid in wine vinegars by isotopic mass spectrometry

(OIV-OENO 510-2013)

1. Object/Scope of application

The method enables the determination of the ^{13}C/^{12}C isotope ratio of acetic acid extracted from wine vinegar by isotope ratio mass spectrometry (IRMS).

2. Definitions

^{13}C/^{12}C: Isotope ratio of carbon 13 to carbon 12 in a given sample

□^{13}C: Isotope ratio of carbon 13 (^{13}C) to carbon 12 (^{12}C) expressed in parts per 1000 (‰)

VPDB: Vienna-Pee-Dee Belemnite, or PDB, is the main reference for measuring natural variations in the carbon isotope ratio. Calcium carbonate comes from a cretaceous belemnite from the Pee Dee formation in South Carolina (USA). Its ^{13}C/^{12}C isotope ratio or R_{PDB} is R_{PDB}= 0.0112372. PDB reserves have been exhausted for a long time, but it remains the main reference for expressing natural variations in the carbon isotope ratio. Reference materials are calibrated based on PDB and are available at the International Agency of Atomic Energy (IAEA) in Vienna (Austria). The isotopic determinations of naturally occurring carbon are expressed in terms of VPDB, as is standard practice.

m/z: Mass/charge ratio

3. Principle

The ^{13}C/^{12}C isotope ratio is determined by the CO_2 resulting from the total combustion of acetic acid extracted from the vinegar sample. An elemental analyser is generally used for the combustion, coupled with a spectrometer to determine the isotope ratios.

4. Reagents and products

The materials and the consumables depend on the apparatus (6) used by the laboratory. The systems used for the combustion of the sample are generally based on elemental analysers. These systems can be equipped to introduce 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 following reference materials, reagents and consumables can be used:

4.1. Reference materials available from the IAEA:

Name	Material	$\delta^{13}C$ according to VPDB
IAEA-CH-6	saccharose	-10.4 ‰
IAEA-CH-7	polyethylene	-32.2 ‰
NBS22	oil	-30.0 ‰
USGS24	graphite	-16.1 ‰

4.2. Working solution

4.2.1. Carbon dioxide (or carbonic anhydride) as a secondary reference gas for the measurement (CAS N° 00124-38-9).

4.2.2. Working and control solution with δ^{13}C values calibrated according to international reference materials.

4.3. Consumables

A standard list of consumables used in continuous flow systems is as follows:

- helium for analysis (CAS N° 07440-59-7),
- oxygen for analysis (CAS N° 07782-44-7),
- carbon dioxide for analysis, used as a secondary reference gas for the carbon 13 content (CAS N° 00124-38-9).
- oxidation reagent for the oven and the combustion system, such as copper oxide (II) for elemental analysis (CAS N° 1317-38-0),
- drying agent to eliminate water produced by combustion, such as Anhydrone for elemental analysis (magnesium perchlorate) (CAS N° 10034-81-8).

This is not necessary for apparatus equipped with a water-elimination system by cryo-trapping or through selective permeable capillaries.

5. Apparatus

5.1. Isotope ratio mass spectrometry

Isotope ratio mass spectrometry enables the determination of the relative content of 13C with respect to that of 12C in CO2 gas with an internal precision of 0.05‰. Internal precision is defined here as the difference between two measurements of the same sample of CO2.

The mass spectrometer used to determine 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 $^{13}C^{17}O^{16}O$)

When measuring the corresponding intensity, the $^{13}C/^{12}C$ isotope ratio is determined by the m/z=45 and m/z=44 intensity ratios after conducting a series of corrections for the $^{12}C^{17}O^{16}O$ isobaric series, whose contribution may be calculated from the intensity of the current measured by m/z=46 and from the relative abundance of ^{18}O and ^{17}O (Craig correction).

The isotope ratio mass spectrometer must either be equipped with:

- a double entry system (double input system) to alternately measure the sample and a reference standard,
- or a continuous flow system that quantitatively transfers CO_2 resulting from the combustion of the samples and of the working solution into the mass spectrometer.

5.2. Combustion apparatus

Combustion apparatus capable of quantitatively converting acetic acid into carbon dioxide and of eliminating all other combustion products, including water, without any isotopic fractionation. The apparatus can either be an integrated continuous flow system coupled to the mass spectrometer (6.2.1) or an autonomous combustion system (6.2.2). The apparatus must be as precise as indicated in (11).

5.2.1. Continuous flow system

These consist of an elemental analyser, or of a gas chromatograph equipped with an online combustion system.

The following laboratory materials are used for systems equipped for the introduction of samples contained in metallic capsules:

- volumetric micropipette with appropriate cones,

- analytical balance with 1 µg precision or better,
- pliers for encapsulation,
- tin capsules for liquid samples,
- tin capsules for solid samples.

The following laboratory materials are needed when using an elemental analyser equipped with a liquid injector or when using a gas chromatograph:

- syringe for liquids,
- flasks equipped with a seal-closure system and inert septa.

The laboratory materials indicated are listed as examples and may be replaced with other materials of equivalent performance, depending on the type of combustion and spectrometry apparatus used by the laboratory.

5.2.2. Manual preparation systems

The carbon dioxide samples to be analysed, resulting from the combustion of samples, and the reference samples are collected in bulbs which are then put in a double entry spectrometry system to carry out isotopic analysis. Several combustion apparatus, which are described in various written works, can be used:

- combustion system with circulating oxygen gas,
- elemental analyser with helium and oxygen flow,
- sealed glass bulb filled with copper oxide (II) used as an oxidation agent.

6. Preparation of samples

Acetic acid must be extracted from vinegar and purified in order to be analysed by IRMS. At least 6 mL of pure acetic acid must be recovered at the end of the extraction.

To extract and purify the acetic acid, any method that does not involve isotopic fractionation may be used. The following method (including the reagents used) and Cadiot column in Figure 1 are given as an example.

6.1. Principle

The acetic acid from vinegar is first extracted with diethyl ether. It is then purified by distillation.

6.2. Reagents

- Diethyl ether (purity ≥99.8)

6.3. Laboratory apparatus

- Liquid–liquid extractor,
- Cadiot-type extraction column,
- round-bottom flask,
- condenser,
- heating mantle.

6.4. Experimental determinations

6.4.1. Liquid–liquid extraction

Pour around 125 mL of diethyl ether into a 250 mL round-bottom flask. Use a 400 mL to 800 mL liquid–liquid extractor; depending on the acetic acid content of the vinegar (at least 6 mL of pure acetic acid must have been recovered at the end of the extraction).

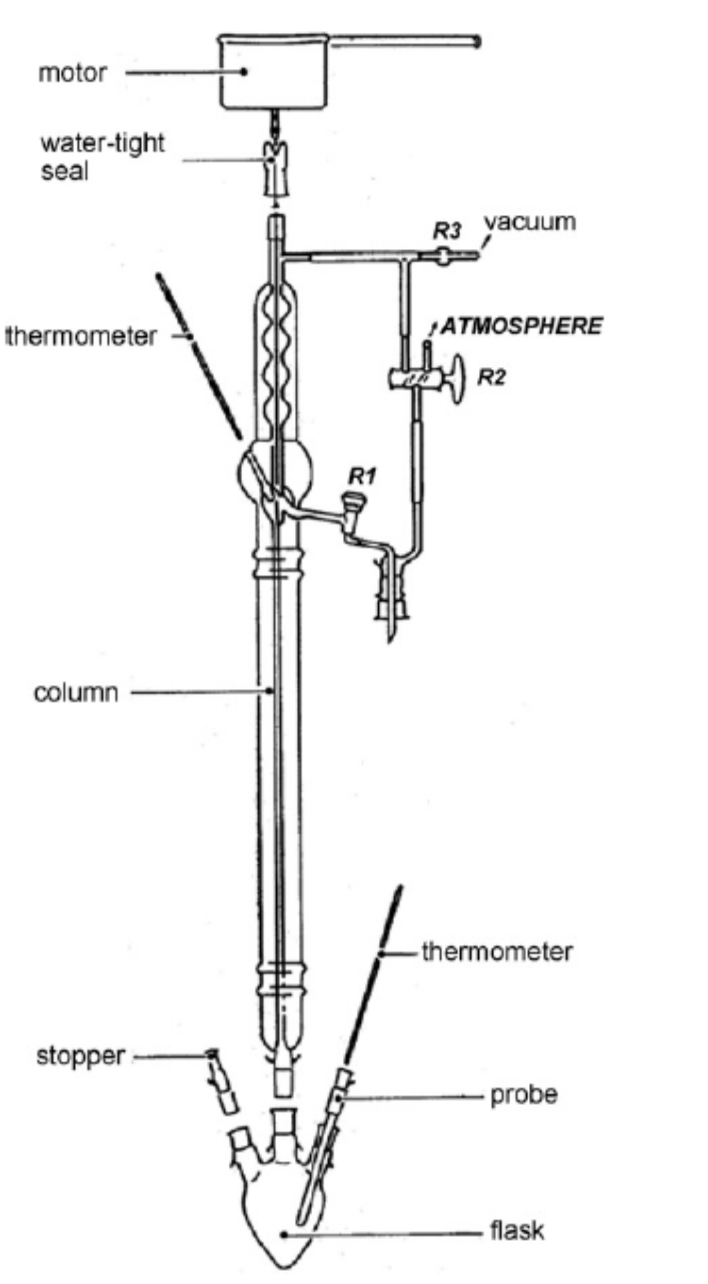
Pour the vinegar into the extractor and top up with diethyl ether. Place the round-bottom flask in the heating mantle, connect the extractor and open the water for the condenser, which is located in the upper part of the extractor. The extraction must last at least 5 hours.

After this time period, separate the aqueous and organic solutions. Add the organic solution to the extract in the round bottom flask.

6.4.2. Purification of the extract

The acetic acid contained in the round bottom flask in diethyl ether solution is distilled on a column which avoids isotopic fractionation of acetic acid, as shown in Fig. 1.

Fig. 1: Diagram of the distillation device (from Thomas and Jamin, 2009)



An appropriate 250 mL flask is used to collect the distillate.

Open the water for the condenser and switch the heating mantle on. Take care to ensure that the temperature is moderate during the distillation of the diethyl ether (which has a boiling point of 34 °C).

When most of the diethyl ether has been distilled (there is no more vapour at the top of the column), increase the temperature.

The distillation is complete when the internal temperature at the top of the column is stable at about 90°C (pure acetic acid distils at 116-117°C).

The remaining traces of diethyl ether in the acetic acid must be removed by blowing N_2 for 10 minutes on the residue at room temperature.

7. Procedure

All preparation steps must be carried out without any significant acetic acid loss through evaporation, which would change the isotopic composition of the sample.

The following description makes reference to the procedures generally used for acetic acid sample combustion using commercial automatic combustion systems. Any other method may be used, provided that it ensures that samples are converted quantitatively into carbon dioxide without losses by evaporation

7.1. Placing the samples in capsules

- Use capsules, tweezers and a clean preparation tray,
- take an appropriate-size capsule using tweezers,
- introduce the necessary amount of liquid into the capsule using a micropipette.

Note: The appropriate quantity of sample must be calculated according to the quantity of carbon necessary, depending on the mass spectrometry instrument sensitivity.

- Close the capsule using pliers,
- 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 samples drum of the elemental analyser. Every capsule must be clearly numbered in order,
- systematically place capsules containing the working solution at the beginning and at the end of the sample series,
- regularly insert a control sample in between the sample series.

7.2. Checking and adjusting the elemental analysis and mass spectrometry instruments

- Adjust the temperature of the elemental analyser ovens and the helium and oxygen gas flows for an optimal combustion of the sample,
- check the elemental analysis system and the mass spectrometry system for leaks (for example, by checking the ion current for m/z = 28 corresponding to N_2.).
- adjust the mass spectrometer to measure the intensities of ion currents for m/z = 44, 45 and 46,
- check the system using reference standards before starting to measure the samples.

7.3. Carrying out a series of measurements

The samples are successively introduced into the elemental analyser. The carbon dioxide resulting from the combustion of each sample is transferred to the mass spectrometer, which measures the ion current. The computer, which is connected to the apparatus, records the ion-current intensities and calculates the □ values for each sample (9).

8. Calculations

The objective of the method is to measure the $^{13}C/^{12}C$ isotope ratio of acetic acid extracted from vinegar. The $^{13}C/^{12}C$ isotope ratio is expressed as the deviation in relation to a working solution. The carbon (□^{13}C) isotope ratio is calculated on a delta scale per thousand by comparing the results obtained for the sample to those obtained for the working solution calibrated beforehand, based on the primary international reference (VPDB). The □^{13}C values are expressed compared to the working solution:

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

where R_{sam} and R_{ref} are, respectively, the $^{13}C/^{12}C$ isotope ratio of the sample and of the internal standard.

The values □^{13}C are thus expressed according to VPDB:

$$\delta^{13}C_{sam/VPDB}\% = \delta^{13}C_{sam/ref} + \delta^{13}C_{ref/VPDB} + (\delta^{13}C_{sam/ref} \times \delta^{13}C_{ref/VPDB})/1000$$

where □$^{13}C_{ref/VPDB}$ is the isotope value determined beforehand for the working solution to VPDB.

Small variations may occur while measuring online due to changes in the instrumental conditions. In this case, the □^{13}C of the samples must be corrected according to the difference between the □^{13}C value from the working solution and its real value, which must be calibrated beforehand against VPDB by comparison with one of the international reference materials. The correction that must be applied to the samples varies in a linear fashion according to the value of the two working solutions which precede and follow the samples. The working reference 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 value assigned to the working solution and the obtained measurements).

9. Quality control of the analyses

Check that the ^{13}C value of the control standard fits the acceptancy criteria defined by the control card of the document OIV-MA-AS1-11. If not, the spectrometer instrument adjustments must be checked and possibly readjusted.

For each sample, verify that the difference in the result between the two capsules measured successively is under 0.3‰. The final result for a given sample is the average value for the two 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 analyser. Under standard conditions, the ion-current intensity should be almost constant for the samples analysed. A significant deviation could be indicative of acetic acid evaporation (an imperfect seal on a capsule), or of instability of the elemental analyser or the mass spectrometer.

10. Precision

An international collaborative study was carried out (Thomas and Jamin, 2009) for validation of the method.

The repeatability limit (r) is, on average, 0.51‰.

The reproducibility limit (R) is, on average, 0.93‰.

The data and results of the international collaborative study are presented in Annex A.

11. Bibliography

1. OIV-MA-AS1-08: Reliability of analytical results (Resolution 5/99)
2. OIV-MA-AS1-09: Protocol for the design, conducts and interpretation of collaborative studies (Resolution 6/2000)
3. OIV-MA-AS1-11: Harmonised guidelines for internal quality control in analytical chemistry laboratories
4. OIV-MA-AS312-06: R2009 Determination by isotope ratio mass spectrometry $^{13}C/^{12}C$ of wine ethanol or that obtained through the fermentation of musts, concentrated musts or grape sugar.
5. IUPAC Protocol in W. Horwitz, *Pure Appl. Chem*. 67 (2) (1995) 331
6. Thomas, F., Jamin, E. (2009). (2)H NMR and (13)C-IRMS analyses of acetic acid from vinegar, (18)O-IRMS analysis of water in vinegar: International collaborative study report. *Analytica Chimica Acta*, 649, 98-105.

Annex A: Results of the international collaborative study

This document sets out the results of the study for the validation of the method of analysis of the $^{13}C/^{12}C$ ratio in acetic acid extracted from wine vinegar (Thomas and Jamin 2009).

The study was carried out in accordance with documents OIV-MA-AS1-08 and OIV-

MA-AS1-09.

1. Participating laboratories

14 laboratories participated in the study:

Bundesinstitut für Risikobewertung, Berlin	Germany
Central Science Laboratory, York	United Kingdom
Custom Technical Laboratory, Prague	Czech Republic
Chemical Institute of the Hungarian Customs and Finance Guard, Budapest	Hungary
Eurofins, Nantes	France
FEM-IASMA, San Michele all'Adige, Trento	Italy
Joint Research Center, Ispra	Italy
Arbitral Agroalimentario Del MAPA, Madrid	Spain
Landesuntersuchungsamt, Speyer	Germany
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Wurzburg	Germany
Service Commun des Laboratoires, Bordeaux	France
Service Commun des Laboratoires, Montpellier	France
Service Commun des Laboratoires, Paris	France
Unione Italiana Vini, Verona	Italy

2. Samples

The study was carried out on 5 double blind samples: three wine-vinegar samples (B, C, D), an alcohol-vinegar sample (A) and a wine-vinegar sample with 20% of A (Thomas and Jamin, 2009).

For each sample the acetic acid was extracted and the □^{13}C was analysed. The results are indicated in the following table.

3. Statistical evaluation

Statistical calculations were performed according to the OIV-MA-AS1-09 protocol, which is based on Horwitz' IUPAC protocol. Outliers were removed in the following way: Cochran tests for removal of the laboratories with the highest variance; single and pair value Grubbs tests for individual or paired individual outliers; then more Cochran tests, etc., keeping a proportion of outliers <2/9. The standard deviations of repeatability (sr) and of reproducibility (sR) were then calculated for each material from valid results pairs from the blind duplicates.

For more information, refer to the publication by Thomas and Jamin, 2009.

Description of the sample	N° of valid results	N° of replicates	δ^{13}C average (‰)	s_r (‰)	Repeatability limit r (2.8×S_r) ‰	s_R (‰)	Reproducibility limit R (2,8× SR) ‰
A	10	2	-29.52	-0.14	0.39	0.34	0.95
B	11	2	-26.66	0.11	0.31	0.25	0.70
C	10	2	-27.51	0.20	0.56	0.33	0.92
B+20%A	11	2	-27.15	0.14	0.39	0.29	0.81
D	7	2	-27.19	0.33	0.92	0.46	1.29

The average repeatability limit (*r*) was 0.51‰, and the average reproducibility limit (*R*) was 0.93‰, comparable to the values observed for wine alcohol (OIV-MA-AS312-06).