

## RESOLUTION OIV/OENO 343/2010

### **METHOD FOR THE DETERMINATION OF THE Method for the determination of the $^{13}\text{C}/^{12}\text{C}$ 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) ISOTOPE RATIO OF GLYCEROL IN WINES BY GAS CHROMATOGRAPHY COMBUSTION OR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO AND ISOTOPE RATIO MASS SPECTROMETRY (GC C IRMS OR HPLC-IRMS)**

THE GENERAL ASSEMBLY,

CONSIDERING Article 2 paragraph 2 iv of the agreement dated April 3, 2001 establishing the International Organization of Vine and Wine,

UPON THE PROPOSAL of the Sub-commission “Methods of Analysis”,

DECIDES to complete Annex A of the Compendium of International Methods of Analysis of Wine and Must with the following type IV method:

Title	Type of the method
Method for the determination of the $^{13}\text{C}/^{12}\text{C}$ 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)	IV

## 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  $^{13}\text{C}/^{12}\text{C}$  ratio of glycerol. If its quantification is required simultaneously with the  $^{13}\text{C}/^{12}\text{C}$  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  $^{13}\text{C}/^{12}\text{C}$  ratio.

## 2. DEFINITIONS

- $^{13}\text{C}/^{12}\text{C}$  : ratio of carbon-13 ( $^{13}\text{C}$ ) to carbon-12 ( $^{12}\text{C}$ ) isotopes for a given sample.
- $\delta^{13}\text{C}$  : carbon-13 content ( $^{13}\text{C}$ ) 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  $^{13}\text{C}/^{12}\text{C}$  isotope ratio or RPDB 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  $\text{C}_3$  (Calvin cycle) and  $\text{C}_4$  (Hatch-Slack) cycles. Most plants, such as the vine and beet, belong to the  $\text{C}_3$  group, whilst maize and cane belong to the  $\text{C}_4$  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 ( $\text{C}_4$  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  $^{13}C^{16}O$  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  $\geq 99\%$  (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<sup>-1</sup> 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  $^{13}\text{C}/^{12}\text{C}$  ratio calibrated against international reference materials.
- 4.11. Working standard samples of 1,5-pentanediol with a known  $^{13}\text{C}/^{12}\text{C}$  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  $^{13}\text{C}$  content of naturally-occurring  $\text{CO}_2$  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  $\text{CO}_2$ . 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  $\mu\text{m}$  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. 13C/12C 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  $T \leq 4^\circ\text{C}$  until analysis.

### 6.2. 13C/12C 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  $T \leq 4^\circ\text{C}$  until analysis.

### 6.3. 13C/12C 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  $T \leq 4^\circ\text{C}$  until analysis.

## 7. PROCEDURE

### 7.1. GC-C-IRMS

The following description refers to the procedures generally used for glycerol  $^{13}\text{C}/^{12}\text{C}$  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<sup>-1</sup>, 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<sup>-1</sup>).

At regular intervals (e.g. once a week) re-oxidation of the oxidation reactor with O<sub>2</sub> is required (the intervals depend on the total amount of substances that has passed through the reactor).

#### 7.1.2. $^{13}\text{C}/^{12}\text{C}$ ratio of glycerol

During each  $^{13}\text{C}/^{12}\text{C}$  analysis, at least two pulses of reference CO<sub>2</sub> gas (4.9) from the cylinder are introduced. This CO<sub>2</sub> is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference CO<sub>2</sub> 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 sampl
- Control sample

The control sample is an ethanol solution of glycerol with a known accurately-measured  $\delta^{13}\text{C}$  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. $^{13}\text{C}/^{12}\text{C}$ ratio of glycerol and its quantification

If quantification of glycerol is required at the same time as  $^{13}\text{C}/^{12}\text{C}$  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,  $\delta^{13}\text{C}$  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<sup>-1</sup>. 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 <sup>13</sup>C/<sup>12</sup>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  $\mu\text{L min}^{-1}$
- B. Flow rate of the acid and oxidant reagents in the LC interface is set at 40 and 30  $\mu\text{L min}^{-1}$ , 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  $\mu\text{L min}^{-1}$  The reagent bottles are degassed with helium during the complete chromatographic run.

### 7.2.2. <sup>13</sup>C/<sup>12</sup>C ratio of glycerol

During each <sup>13</sup>C/<sup>12</sup>C analysis, at least two pulses of reference CO<sub>2</sub> gas (4.9) from the cylinder are introduced (see example of chromatogram in 11.2). This CO<sub>2</sub> is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference CO<sub>2</sub> 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  $\delta^{13}\text{C}$  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. $^{13}\text{C}/^{12}\text{C}$ ratio

The  $^{13}\text{C}/^{12}\text{C}$  isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of carbon-13 ( $\delta^{13}\text{C}$ ) is then calculated on a delta scale per thousand ( $\text{‰}$ /1000 or  $\text{‰}$  ‰) 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  $^{13}\text{C}/^{12}\text{C}$  analyses, a reference  $\text{CO}_2$  gas is introduced, which is calibrated against other PDB-calibrated international standards.

The  $\delta^{13}\text{C}$  values are expressed in relation to the working reference as follows:

$$\delta^{13}C_{sample/ref\%} = (R_{sample}/R_{ref} - 1) \times 1000$$

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:

$$\delta^{13}C_{sample/V-PDB\%} = \delta^{13}C_{sample/ref} + \delta^{13}C_{ref/V-PDB} + (\delta^{13}C_{sample/ref} \times \delta^{13}C_{ref/V-PDB})/1000$$

where  $\delta^{13}C_{ref/V-PDB}$  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  $V \cdot s$ ) 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

$$C_{glyc_{sample}} = K.C_{1,5PD_{sample}} \cdot \frac{S_{glyc_{sample}}}{S_{1,5PD_{sample}}} \times dilution\ factor$$

Where:

$C_{x_{sample}}$  is the concentration in g L<sup>-1</sup> of the species in the sample;

$S_{x_{sample}}$  is the area of the peaks produced;

K ( the response factor) is calculated as follows:

Equation 3 (see 8.2)

$$K = \frac{C_{glyc_{st}}}{C_{1,5PD_{st}}} \cdot \frac{S_{1,5PD_{st}}}{S_{Glyc_{st}}}$$

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<sup>-1</sup> 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  $^{13}\text{C}$  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  $\delta^{13}\text{C}$  value already determined by EA-IRMS. For the 3 repetitions,  $n=3$ , using the GC-C-IRMS technique a standard deviation  $\text{SD} \leq 0.6$  ‰ 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  $\text{g L}^{-1}$  in dry wine, the 2 solutions represent this range. The first solution was 4.0  $\text{g L}^{-1}$  and gave an experimental concentration of 3.6  $\text{g L}^{-1}$  ( $\text{SD}=0.2$ ,  $n=8$ ). The second solution, 8.0  $\text{g L}^{-1}$ , gave a value of 7.9  $\text{g L}^{-1}$  ( $\text{SD}=0.3$ ,  $n=8$ ).

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 HPOLC and/or enzymatic analysis.  
Concentrations are given in  $g.L^{-1}$   $n>3$  and  $SD<0.6$

The concentrations of glycerol found by GC-C-IRMS are consistent with the values 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  $\delta^{13}C$  values of glycerol obtained by HPLC-IRMS<sup>[1]</sup>

		HPLC-IRMS							
		Day 1		Day 2		Day 3		Day 4	
Sample	Repetitions per sample	Mean $\delta^{13}C$ (‰)	SD (‰)	Mean $\delta^{13}C$ (‰)	SD (‰)	Mean $\delta^{13}C$ (‰)	SD (‰)	r (‰)	R (‰)

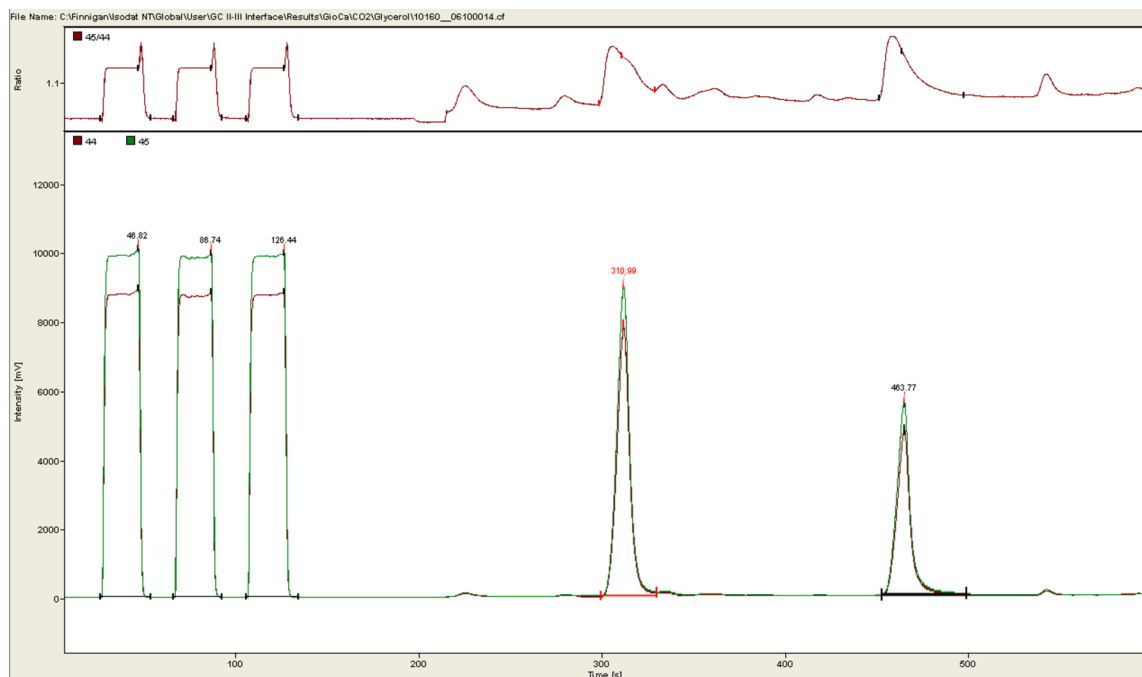
Glycerol (standard) <sup>[2]</sup>	10	-27.99	0.05	-27.94	0.04	-27.95	0.08	0.17	0.18
Synthetic wine (6 g/l)	10	-28.06	0.13	-28.14	0.12	-28.14	0.11	0.34	0.35
Synthetic wine (8 g/l)	10	-28.11	0.12	-28.18	0.07	-28.21	0.07	0.25	0.28
Synthetic wine (10 g/l)	10	-28.06	0.06	-28.06	0.09	-28.05	0.09	0.23	0.24
Wine	10	-28.88	0.10	-28.85	0.27	-28.72	0.23	0.60	0.62

The following performance parameters for determining the  $\delta^{13}\text{C}$  of glycerol were obtained from a wine sample:

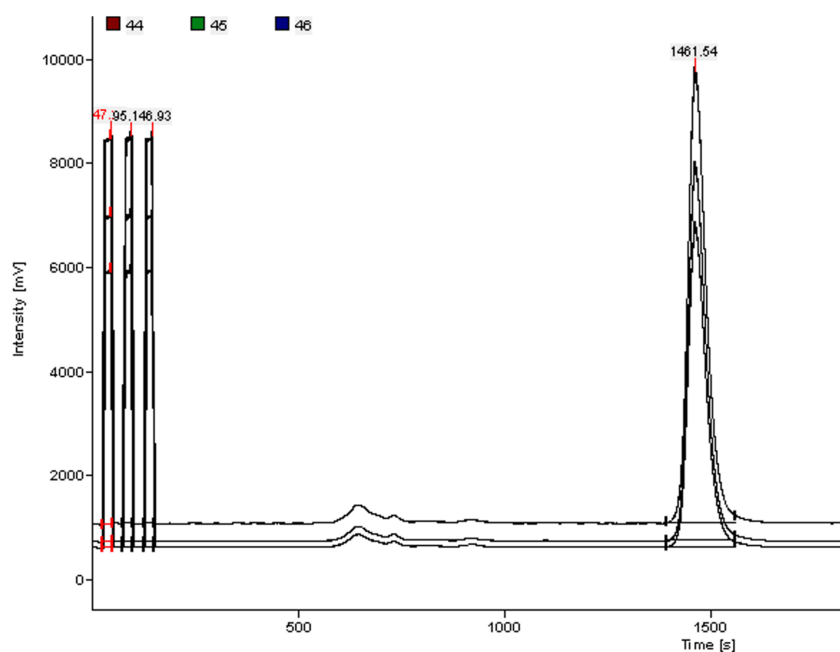
- Repeatability r: 0,60 ‰
- Reproducibility R: 0,62

## 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



## 12. BIBLIOGRAPHY

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<sup>[1]</sup> Values of  $\delta^{13}C$  are expressed in ‰ vs V-PDB

<sup>[2]</sup> EA-IRMS glycerol (standard) result:  $-28.02 \pm 0.09$  ‰