RESOLUTION OENO 7/2005

DETERMINATION OF THE CARBON ISOTOPE RATIO $^{13}C/^{12}C$ OF CO_2 IN SPARKLING WINES

METHOD USING ISOTOPE RATIO MASS SPECTROMETRY (IRMS)

THE GENERAL ASSEMBLY,

CONSIDERING Article 2 paragraph 2 iv of the agreement establishing the International Organisation of Vine and Wine,

UPON THE PROPOSAL of the Sub-commission of Methods of Analysis and Appraisal of Wine,

DECIDES to complete the Annex C of the Compendium of International Methods of Analysis of Musts and Wines by the following 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)

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

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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exhaustive description of all applicable procedures for different techniques runs the risk of the method being rapidly superseded.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.

Sparkling wines - Determination of the carbon isotope ratio $^{13}C/^{12}C$ of CO_2 in sparkling wines - Method using isotope ratio mass spectrometry (IRMS)

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”.

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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

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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

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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-675	Glucose	-10.76‰ “

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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

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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.

- 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

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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.

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

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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 x (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:

- $\delta^{13}C_{sam/V\text{-}PDB}$ (‰) = $\delta^{13}C_{sam/ref}$ + $\delta^{13}C_{ref/V\text{-}PDB}$ + ($\delta^{13}C_{sam/ref}$ x $\delta^{13}C_{ref/V\text{-}PDB}$) / 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.

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

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repeatability limit (r) are equal to:

S_r = 0.21‰ r = 0.58‰

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‰

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)

11. Bibliography

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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.

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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.

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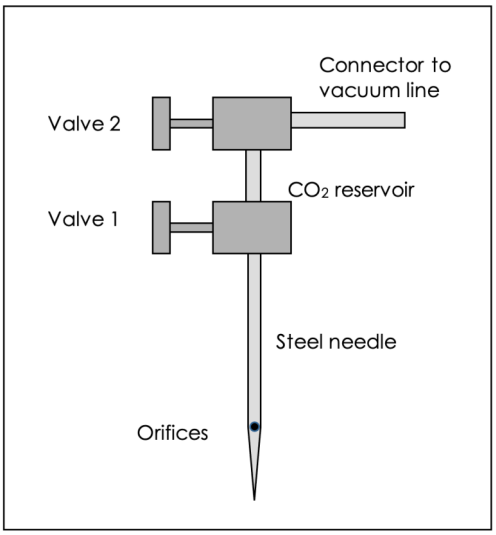
3. Reference

1. 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.

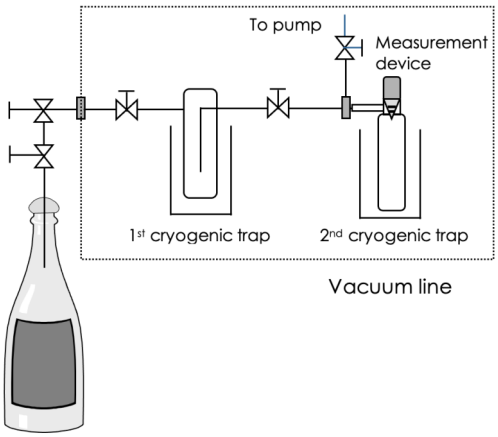
Annex A

Diagrams of the off-line system

Gas sampling device



Gas Sampling and CO_2 purification



ANNEX B

Experimental procedure based on the on-line systems for sampling and measurement

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(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).

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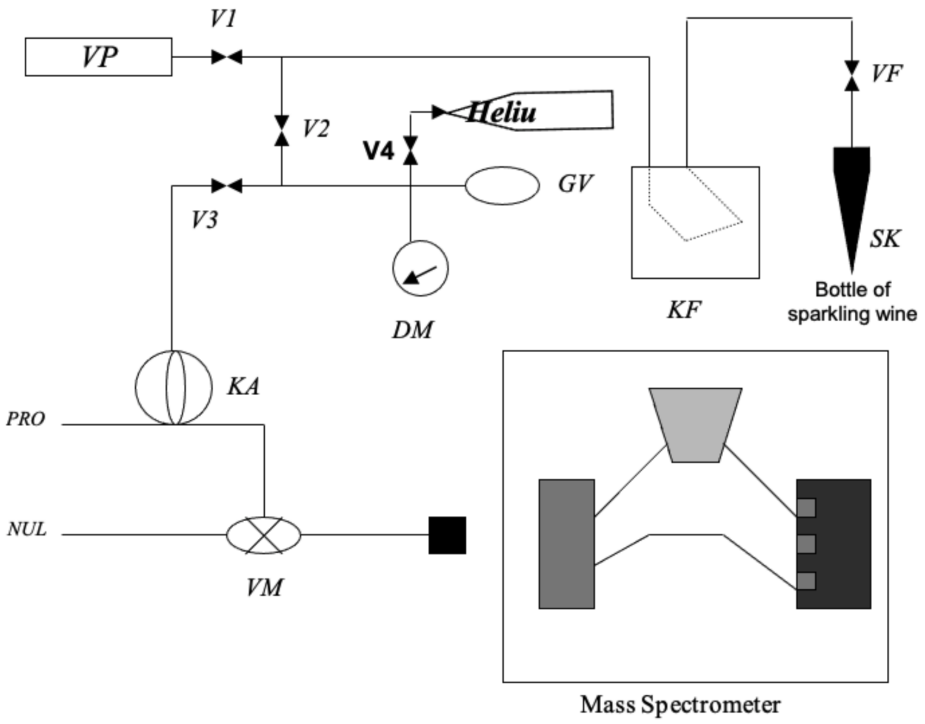
Switching from the sample flow (PRO) to the pure helium flow (NUL) permits the generation of artificial switching peaks.
Switching on the sample side: 2 seconds (zero side: 10-30 seconds).

3. Reference

1. 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.

ANNEX B

Diagram of the on-line system



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- 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

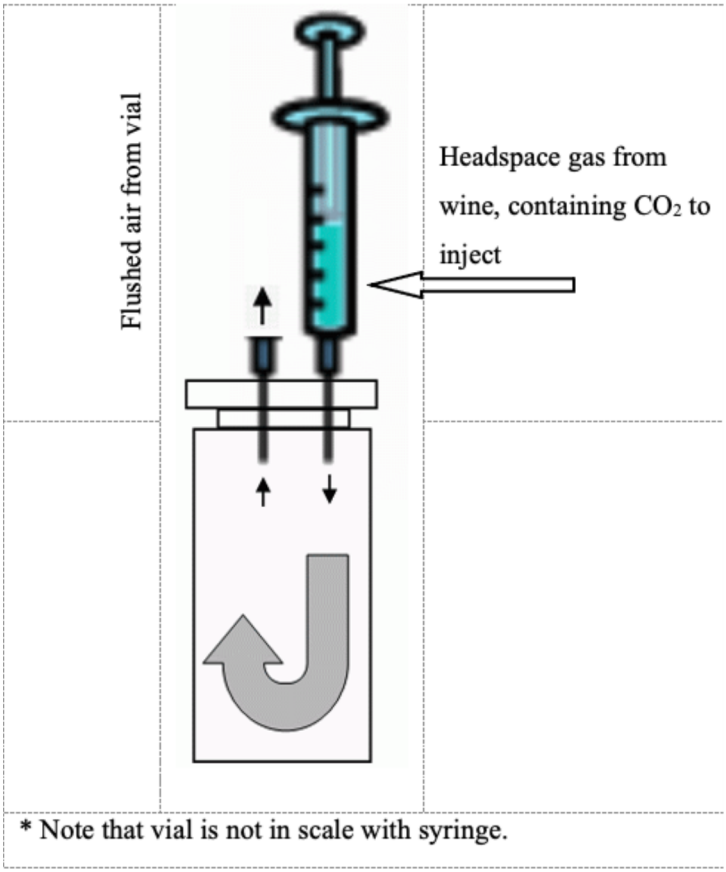
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needle through the cork. CO_2 pressure filled the syringe with the headspace gas spontaneously.

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.



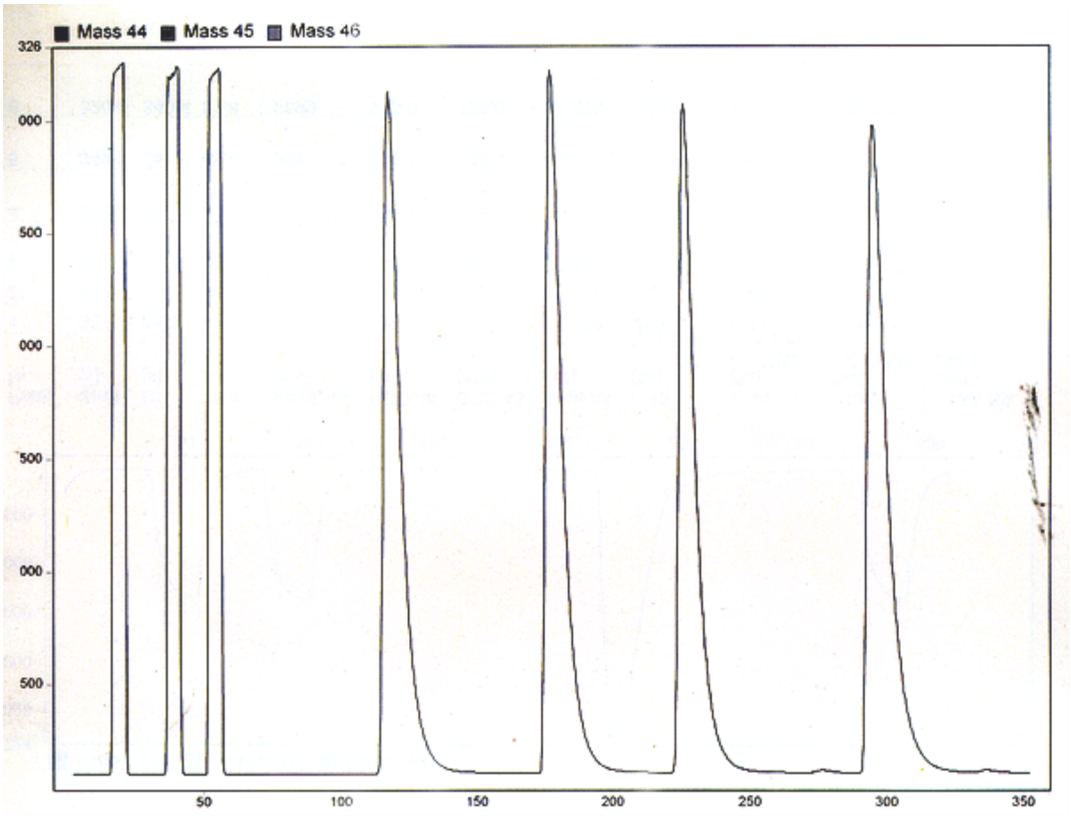
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2.2. GC-IRMS analyses: CO_2 injection and $^{13}C/^{12}C$ ratio measurement

A very few μL of gas were directly injected into the column with a 10 □L 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 □^{13}C value of the first injection is systematically discarded.

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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 eliminating 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:

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- A Sparkling wine - C_4 sugar
- B Sparkling wine - C_3 sugar
- C Sparkling wine - C_3 sugar
- D Gasified wine
- E Gasified wine

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