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ARTICLE

Simultaneous Stable Carbon Isotopic Analysis of Wine Glycerol and Ethanol by Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry

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A novel procedure was established for the simultaneous characterization of wine glycerol and ethanol $^{13}C/^{12}C$ isotope ratio, using liquid chromatography/isotope ratio mass spectrometry (LC-IRMS). Several parameters influencing separation of glycerol and ethanol from wine matrix were optimized. Results obtained for 35 Spanish samples exposed no significant differences and very strong correlations (r = 0.99) between the glycerol $^{13}C/^{12}C$ ratios obtained by an alternative method (gas chromatography/isotope ratio mass spectrometry) and the proposed new methodology, and between the ethanol $^{13}C/^{12}C$ ratios obtained by the official method (elemental analyzer/isotope ratio mass spectrometry) and the proposed new methodology. The accuracy of the proposed method varied from 0.01 to 0.19‰, and the analytical precision was better than 0.25‰. The new developed LC-IRMS method it is the first isotopic method that allows $^{13}C/^{12}C$ determination of both analytes in the same run directly from a liquid sample with no previous glycerol or ethanol isolation, overcoming technical difficulties associated with complex sample treatment and improving in terms of simplicity and speed.

KEYWORDS: Carbon isotope; glycerol; ethanol; LC-IRMS; adulteration detection

INTRODUCTION

Nowadays the wine market is an important and expanding sector of the food industry. In addition, wine is a high-volume product, governed by market rules of supply and demand. During wine elaboration (fermentation), about 92% of the sugar molecules undergo alcoholic fermentation to produce ethanol; the remaining 8% undergoes glycero-pyruvic fermentation to yield glycerol (*1*). Thus, the main components of wine are water, ethanol, and glycerol.

Adulteration of wine can happen in many ways, for example, addition of nongrape ethanol, addition of nongrape sugar (chaptalization), water, or other unauthorized substances (e.g., glycerol) (*2*). Chaptalization is used to increase the natural amount of alcohol (natural sugar) up to an amount of total alcohol that is necessary for a stable and tasty wine and to meet the legal requirements (*3*). Because such a practice is only allowed by European legislation in some zones (*4*), the addition of sugar to wine is a fraudulent practice. Glycerol could contribute to the mouth feel properties and smoothness of wine (*5*,*6*) that are often indicative of high quality wines (*1*). For this reason, glycerol production is one of the desirable features during grape must fermentation. It is also an important contributor to the sugar-free extract of wines, an index on which is based a quality scaling of wines in some European countries (*7*). Therefore, for such reasons, glycerol is sometimes fraudulently added to wine to disguise poor quality (*8*). As this practice is not permitted by European Commission (EU) regulations (*9*), it is the goal to study possible methods to detect addition of synthetic or natural but exogenous glycerol.

The combination of traditional and specialized analytical methods is the most promising way to improve food authenticity and to detect adulterations (*3*). During the past decade, analytical methods have been improved in this field. Methods of stable isotope ratio analysis (SIRA) are based on the measurement of the stable isotope contents of the biologically important elements (H, C, and O) of a product or of a specific component (*10*) and are based on the fact that the same molecule exhibits varying isotope contents depending on its origin, synthetic or from a plant which has metabolized it (*11*, *12*) (C3 or C4 photosynthetic pathway). Therefore, these methods play a key role in detecting adulterations such as addition of water and inadmissible sweetening or chaptalization with beet or cane sugar (*3*). Because of this, some of these methods have been adopted as official methods by the European Union (EU).

The potential of stable isotope techniques to detect economic adulteration is considerably improved by analyzing target components of a product. This is achieved by coupling a separation technique to isotope ratio mass spectrometry (IRMS) to enable precise compound-specific isotope analysis (CSIA) at natural isotopic abundance level.

The isotope ratio mass spectrometer was not successfully adapted as a detector for gas chromatography (GC) until the late 1970s (*13*, *14*) and commercialized in the late 1980s.

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Continuous flow gas chromatography/combustion coupled to IRMS (GC/C/IRMS) allows online measurements of the carbon-13 content of each isolated molecule (oxidized to CO_2) of many matrices in different fields (*15*). Obtaining a reliable and reproducible conversion of organic molecules into CO_2 is relatively straightforward when GC is coupled to IRMS, but coupling liquid chromatography (LC) to IRMS is much more complicated because the CO_2 has to be generated in, and extracted from, the liquid phase (*16*). The development of liquid chromatography coupled to stable carbon isotope ratio mass spectrometry has opened new perspectives for the $^{13}C/^{12}C$ isotope ratio analysis of food samples. LC-IRMS allows a one-step separation of the individual components of a sample and the online determination of their $\delta^{13}C$ values, avoiding both the disadvantages of off-line methods and the disadvantages of GC-based methods, that require derivatization steps, causing the addition of extra carbons.

Although organic carbon forms, such as volatile fatty acids (*17*), sugars (*18*, *19*), amino acids, (*17*, *20*−*22*) and ethanol (*2*) can be separated and their $\delta^{13}C$ value measured by this system without isotopic fractionation, applications for one of our target chemicals (glycerol) are not known. Previously described analytical methods to combat wine adulteration have been based on the extraction of ethanol from wine before isotopic testing. These methods required several steps for extracting ethanol from wine matrix (distillation (*23*, *24*), equilibration (*25*)) avoiding isotopic fractionation. Some of the technical difficulties were overcome by using special devices such as Cadiot columns that allow collection of ethanol free of isotopic effects (*23*). This methodology was able to measure $\delta^{13}C$ values of the wine ethanol, but it still required previous ethanol isolation from the sample. Two new procedures (LC-IRMS and GC-IRMS) were recently developed for wine ethanol $^{13}C/^{12}C$ isotope ratio determination directly from a liquid sample with no previous ethanol isolation (*2*).

On the other hand, different IRMS methods and results of glycerol have been published since 1997 (*26*−*30*) with the aim of eventually using this molecule as another independent probe in food origin determination. However, these methods were limited to measurements of highly pure glycerol, using off-line IRMS methods or the determination of stable isotope ratios via GC-IRMS after derivatization. A direct $\delta^{13}C$ GC-IRMS measurement concerning glycerol in wine has been published (*7*). However, to our knowledge, no simultaneous glycerol and ethanol $^{13}C/^{12}C$ determination on real wine samples without sample treatment has been performed directly.

To improve the currently available methodologies for wine adulteration detection and, thus, prevent wine fraud, a new method (with no sample treatment needed) based on the separation of glycerol and ethanol of the wine matrix and later isotopic characterization by LC-IRMS has been developed. This new procedure, its validation, the results obtained on commercial wines, and finally its practical application to the authenticity control of wine are described in this paper.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade and were used without further purification. For LC-IRMS studies, 0.5 M orthophosphoric acid (Fluka, Buchs, Switzerland), 0.5 M sodium peroxodisulfate (Fluka), and 5×10^{-3} M sulfuric acid (Panreac, Barcelona, Spain) were prepared in Milli-Q water (Millipore, Bedford, MA). Carbon dioxide (quality N-48), helium, and oxygen from Air Liquid (Madrid, Spain) were used as working reference gas, inert carrier gas, and regeneration gas for the combustion reactor, respectively.

Instrumentation and $^{13}C/^{12}C$ Measurement Conditions. *a. EA-IRMS.* A Carlo Erba (Milan, Italy) NC 2500 elemental analyzer (EA) was coupled to a Delta Plus isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany), via a ConFlow interface, and served for total $\delta^{13}C$ of ethanol in wine, and glycerol and ethanol in standards. The EA was operated using a flow of helium of 100 mL min^{-1}, at a temperature of 1020 °C in the oxidation tube, 650 °C in the reduction tube and 40 °C in the gas chromatographic column. The instrument was equipped with an autosampler; the cycle time for one complete determination was 400 s. Suitable control references were included in each batch.

b. LC-IRMS. A LC Isolink interface (Thermo Electron, Bremen, Germany) was coupled to a liquid chromatographic system and to a Delta-Plus Advantage isotope ratio mass spectrometer (Thermo Electron) without any modifications to evaluate simultaneously the $\delta^{13}C$ of glycerol and ethanol in wine. The eluent was delivered with a Surveyor LC pump (Thermo Electron). The LC pump and the chromatographic column were connected to a Rheodyne 7125 injection valve equipped with a 25 μL loop. For glycerol and ethanol separation from the wine matrix, a ligand-exchange column (HyperREZ Carbohydrate H^+, 30 cm, 8 mm) (Thermo, Chesire, U.K.) was used.

To connect the LC column to the Surveyor pump and the manufactured interface, PEEK tubing and nuts were used with 0.005 mm i.d. The tubes connecting the mobile phase bottles to the pump itself were manufactured in "No-Ox" material (1/8″ × 1.5″, Socochim, Lausanne, Switzerland) in order to avoid "regassing" of the eluent. Two in-line filters (0.25 μm) (Vici, Schmidlin Labor, Switzerland) were placed between the Isolink interface and column as well as between the t-piece and oxidation reactor (inside the interface) in order to prevent plugging of the oxidation reactor capillary or damage of the CO_2 membrane separation unit of the LC Isolink interface. The LC flow rate of the eluent (Milli-Q water) was 400 μL min^{-1}, and the flow rate of the acid and oxidant reagents in the LC interface were 30 and 20 μL min^{-1}, respectively. The temperatures of the interface reactor and the column were set at 99.9 and 65 °C, respectively. The helium flow rate of the separation unit was set at 1 mL min^{-1}. Eluent and reagent were thoroughly degassed in an ultrasonic bath using a water vacuum and purged with a constant flow of He to prevent CO_2 contamination from ambient air during operation. The pump heads of the oxidant and acid pumps were rinsed with water at least twice a day to avoid crystallization. The instrument was equipped with an autosampler; the cycle time for one complete determination was 3000 s. Suitable control references were included in each batch.

c. GC-IRMS. A Delta Plus XP mass spectrometer (Thermo Finnigan) was coupled in line with a Trace gas chromatograph through a Thermo Finnigan GC combustion III interface to separate the glycerol from the wine matrix and evaluate its $\delta^{13}C$ value, according to a previously developed method (*7*). The gas chromatograph was equipped with a WCOT fused silica capillary column filled with bonded polyethylene glycol (CP-WAX57CB, 25 m length, 0.25 mm i.d., 0.20 mm film thickness) and connected to a GCPal autosampler (CTC Analytics AG, Zwingen, Germany). The combustion furnace was an oxidation reactor (ceramic tube Al_2O_3 packed with Cu, Ni, and Pt wires, 320 mm × 0.5 mm i.d.). The reduction furnace was a ceramic tube packed only with Cu. The temperatures of the combustion and reduction reactor of the GC combustion III interface were 960 and 640 °C, respectively. At regular intervals, reoxidation of the oxidation reactor with O_2 is required (the intervals depend on the total amounts of substances that passed through the reactor). Sample solutions (0.3 μL) were injected (10 μL Hamilton syringe) in split mode (1:100). Helium was used as carrier gas, and the flow rate was set to 2 mL min^{-1}. The injector temperature was set to 250 °C. The GC oven was initially held at 120 °C for 2 min and then ramped at 10 °C min^{-1} to 220 °C, where it was held for 2 min. Each run took 14 min, not considering the necessary time of cooling. Water produced during the combustion was eliminated by a water-removing trap, consisting of a Nafion membrane. Suitable control references were included in each batch.

Samples. This study was conducted with 35 samples of Spanish wine, of different varieties and types.

Sample Preparation. *a. For $^{13}C/^{12}C$ Determination of Wine Ethanol by EA.* The ethanol must be extracted from the wine by distilling before isotopic testing by EA, according to EU regulations for wine analyses (Regulation (EEC) No. 2676/90 (*24*) and International Organization of Vine and Wine (OIV) - Resolution OENO 17/2001 (*23*)). Wine samples were distilled using Cadiot columns with rotating bands, that

allow to collect ethanol with a final alcoholic strength of >92% w/w. The adopted conditions of this step do not allow any isotopic fractionation. Then 1 μL of distillate, approximately, was placed into tin capsules for liquid samples with a microliter syringe. Each capsule had to be completely sealed with tweezers to avoid evaporation and subsequently a change in the isotopic composition of the sample. If not, it was discarded and a new capsule prepared. The capsules were placed in the appropriate place on the tray of the automatic sampler and analyzed by EA.

b. For $^{13}C/^{12}C$ Determination of Wine Glycerol and Ethanol by LC. The wine samples were diluted with Milli-Q water (1:750), filtered through 0.45 μm nylon filters, placed into glass vials, and analyzed by LC coupled to IRMS.

c. For $^{13}C/^{12}C$ Determination of Wine Glycerol by GC. The wine samples were filtered through 0.45 μm nylon filters, placed into glass vials, and analyzed by GC coupled to IRMS.

Calibration and Isotopic Calculation. At the beginning of each run, three pulses of CO_2 reference gas were admitted into the inlet system for about 20 s. The constant flow rate during this period gives these peaks a flat-top appearance. A level of CO_2 corresponding to 2−6 V (depending on the instrument) at m/z 44 was used to calibrate the system.

The $^{13}C/^{12}C$ abundance ratio was expressed as $\delta^{13}C$ values calibrated against the international standard Vienna Pee Dee Belemnite (VPDB). The delta notation is defined as

$$\delta^{13}C_{sample}(‰) = [(R_s/R_{st}) - 1] \times 1000$$

where R_s is the ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the ratio of the international standard used. The result of this calculation is a relative δ (‰) calibrated against the international standard.

The certified ethanol BCR-656, with $\delta^{13}C_{V\text{-}PDB} = -26.91 \pm 0.07$‰, available from the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium (http://www.irmm.jrc.be), and the certified oil NBS 22 with $\delta^{13}C_{V\text{-}PDB} = -29.7 \pm 0.2$‰ available from the International Atomic Energy Agency (IAEA) have been used to define the following secondary working standards: sucrose (analytical uncertainty of measurement of -10.2 ± 0.1‰ for carbon), a commercial ethanol sample (Panreac, Spain) (analytical uncertainty of -25.88 ± 0.10‰ for carbon), and glycerol (analytical uncertainty of -28.02 ± 0.09‰ for carbon), to correct eventual drift.

The ^{13}C value for the working reference was checked not to differ by more than 0.5‰ from the admissible value. If not, the spectrometry apparatus settings should be checked and, if necessary, adjusted. Samples were analyzed in triplicate, and the values averaged. If the difference between the standard deviation (SD) in three vials measured successively was $\geq |0.30|$, the measurement was repeated.

RESULTS AND DISCUSSION

Simultaneous Isotopic Characterization of Wine Glycerol and Ethanol by LC-IRMS. In LC, as in GC, the measurement of the ^{13}C isotope ratio must be performed ensuring that none of the steps in the analysis alter the isotopic composition of the molecule of interest. The key factor in achieving reliable isotopic measurements is to obtain baseline separation of the molecules of interest from the other molecules in the mixture (*31*).

Preliminary experiments were carried out to investigate and optimize $^{13}C/^{12}C$ simultaneous determination on glycerol and ethanol from wine samples. Several variables, such as column, temperature, mobile phase, and flow rates (mobile phase, oxidant, and acid) were evaluated. For the evaluation of the effects of one parameter on the $\delta^{13}C$ measurement, all other parameters were kept constant. The best results were found working under the conditions described in the Materials and Methods. Because all carbon-bearing compounds are oxidized in the Isolink interface and, thus, detected by the spectrometer, organic solvents, organic buffers, or other carbon-bearing compounds (including inorganic carbon) cannot be used in the separation. However, inorganic buffers such as phosphates, salt, and pH gradients can be used.

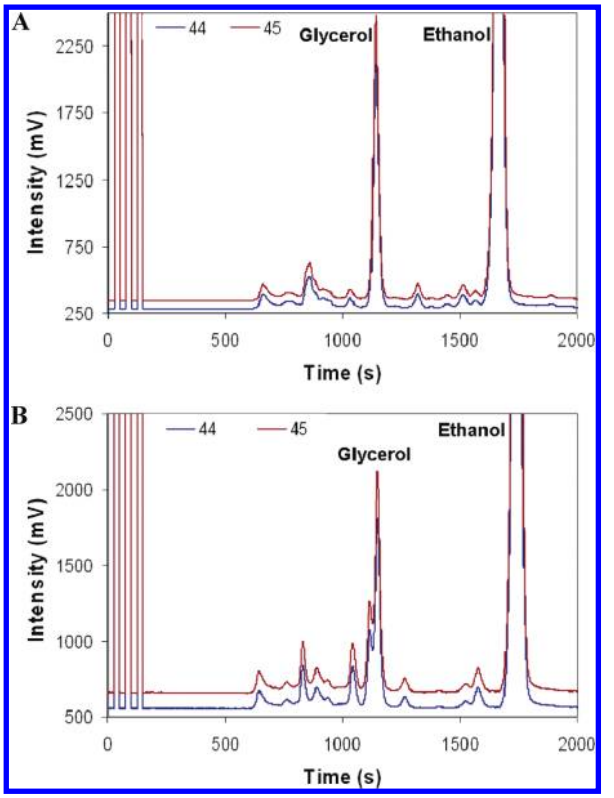

Figure 1. Chromatograms of a wine sample (close-up view of glycerol peak). Column: HyperREZ Carbohydrate H^+, (300 mm × 8 mm). Conditions: LC flow 500 μL min^{-1}, 5 × 10^{-3} M sulfuric acid mobile phase, temperature (**A**) 25 °C and (**B**) 65 °C.

Krummen et al. reported that water-compatible reversed-phased, normal-phase, ion exchange, and size-exclusion columns can be used in this system. Polymeric styrene-divinylbenzene columns loaded with cations (Ca^{2+}, Pb^{2+}, Ag^+, H^+) and operated with ultrapure water as eluent turned out to be a good alternative. Because of this, the use of a sulfonated monodisperse resin-based column in hydrogen form, especially useful for profiling oligosaccharides (*19*), organic acids, and alcohols (*2*), based on ligand-exchange and size exclusion mechanism was proposed.

In order to overcome the difficulty of developing a LC-IRMS method without organic buffer and to increase the LC efficiency, high temperature liquid chromatography (HTLC), using temperatures between 40 and 200 °C, might be a promising technique. By increasing the water temperature, the dielectric constant which is also a measure of the water polarity decreases. Then at elevated temperature, water is similar, in terms of eluotropic strength, to a mixture of organic solvent and water (*31*). The column temperature (25−65 °C) was investigated under the following conditions: mobile phase, 5 × 10^{-3} M H_2SO_4; mobile phase flow, 500 μL min^{-1}.

Results of application of different temperatures are shown in **Figure 1**. As the carbon in all eluting compounds is oxidized to CO_2, good chromatographic resolution is essential so that only the glycerol and ethanol are oxidized when the isotope amount ratios are being measured. When working at 25 °C, a baseline separation of glycerol and ethanol was achieved (**Figure 1A**). Two main peaks can be distinguished in each sample. The first peak was identified as glycerol, and the second one as ethanol. However, in some samples, the $\delta^{13}C_{glycerol}$ value obtained at 25 °C (LC-IRMS) was not consistent with the value obtained by an alternative method (GC-IRMS), showing a large shift in the glycerol isotopic ratio (from 1‰ to 2‰). These differences

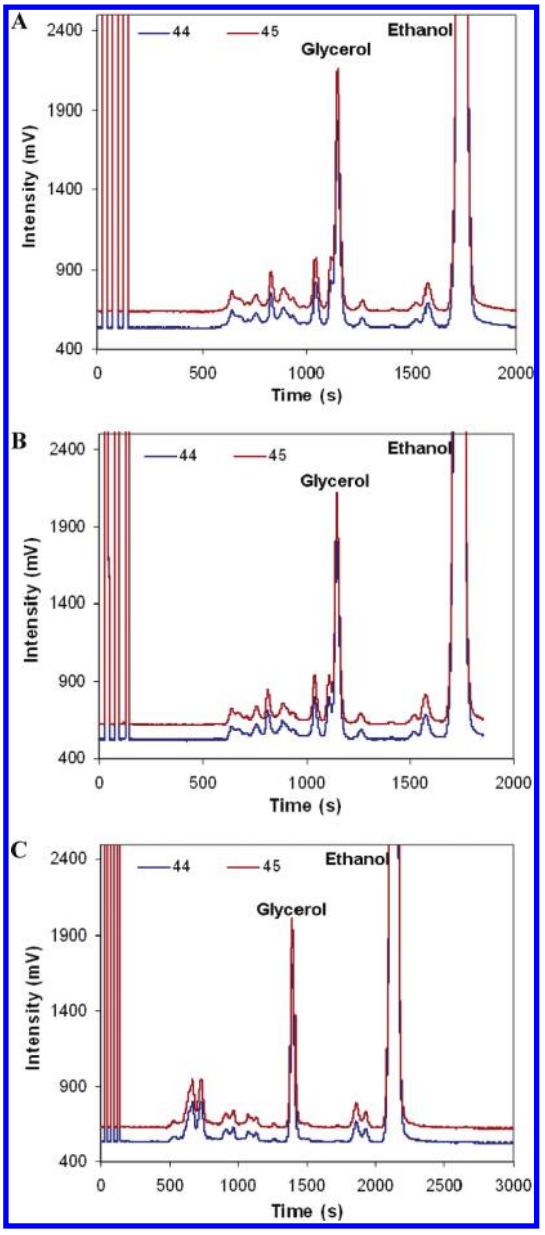

Figure 2. Chromatograms of a wine sample (close-up view of glycerol peak). Column: HyperREZ Carbohydrate H^+, (300 mm × 8 mm). Conditions: temperature 65 °C; (**A**) mobile phase, 5×10^{-3} M sulfuric acid; flow rate, 500 μL min^{-1}; (**B**) mobile phase, 2.5×10^{-3} M sulfuric acid M; flow rate, 500 μL min^{-1}; (**C**) mobile phase, Milli-Q water; flow rate, 400 μL min^{-1}.

observed at 25 °C were apparently due to a full coelution of glycerol with minor components. In fact, when working at higher temperature (65 °C) (**Figure 1B**), a compound elutes with a residence time similar to that of glycerol (partial overlap), affecting thus the δ^{13}C value and therefore the glycerol isotopic accuracy. In the case of ethanol, its characterization did not improve when the temperature was increased.

The effect of the pH of the mobile phase on wine glycerol and ethanol $^{13}C/^{12}C$ determination was also evaluated, using the following conditions: column temperature, 65 °C; mobile phase flow, 500 μL min^{-1}. As recommended by the manufacturer, 5×10^{-3} M sulfuric acid solution was used as eluent, but as stated previously a baseline separation of glycerol was not achieved (**Figure 2A**). When the pH was increased (2.5×10^{-3} M sulfuric acid), a better resolution but not baseline separation for glycerol

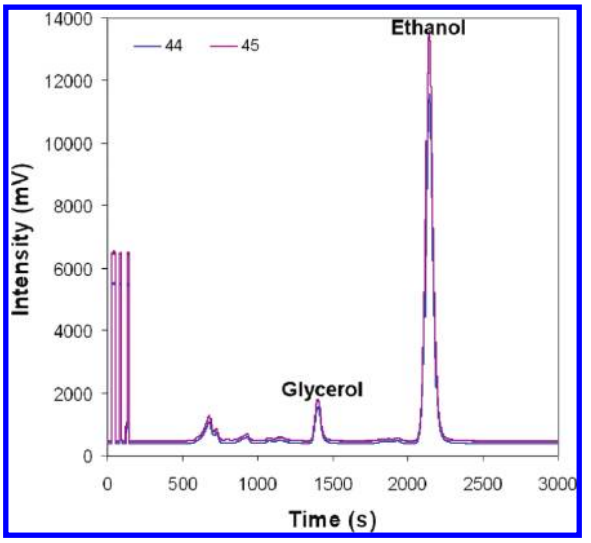

Figure 3. Chromatogram of a wine sample. Column: HyperREZ Carbohydrate H^+, (300 mm × 8 mm). Conditions: 65 °C; LC flow, 400 μL min^{-1}; Milli-Q water; oxidation and acid reagent flows, 30 and 20 μL min^{-1}, respectively.

from other minor components was achieved (**Figure 2B**). When Milli-Q water was used as eluent, a single peak for glycerol and another for ethanol were obtained (**Figure 2C**).

The optimal flow of the mobile phase (300−500 μL min^{-1}) was evaluated working under the following conditions: mobile phase, Milli-Q water; column temperature, 65 °C. The tested flow rates had no significant influence on ethanol characterization. However, the use of 400 μL min^{-1} resulted in better glycerol characterization (repeatability and reproducibility). Thus, a flow of 400 μL min^{-1} was proposed (**Figure 2C**). Consequently, the Milli-Q water mobile phase, 65 °C and 400 μL min^{-1}, provided satisfactory glycerol and ethanol determination in wine samples.

The separation of ethanol from the wine matrix was performed within 50 min, and the LC resolution achieved meets the resolution expected for this type of column and application. **Figure 3** shows the chromatographic profile of a wine sample.

In IRMS, quantitative conversion of organic components is mandatory in order to avoid fractionation. Complete conversion is achieved by adding two reagents (acid and oxidant) to the mobile phase after chromatographic separation (chemical oxidation). Because of this oxidation, after passing the hot reaction zone, surplus oxygen (O_2) remains dissolved in the liquid phase, and it is then transferred to the mass spectrometer. Since the presence of O_2 in the ion source may have detrimental effects on measurement accuracy and precision as well as on filament lifetime (*32*), an optimization of acid and oxidant flows is required to obtain the perfect conditions for glycerol and ethanol quantitative conversion into CO_2 without isotope fractionation, and to avoid a superplus of O_2 getting into the source. Particular attention was paid to m/z 32 (O_2) and m/z 44 and 45 (CO_2) ions. Different flows (10−60 μL min^{-1}) were tested, and 30 μL min^{-1} (acid) and 20 μL min^{-1} (oxidant) were chosen as optimal for a 10 V signal of oxygen in the source.

In order to study if isotopic fractionation is taking place within the stationary phase, the following steps are recommended. First, to measure a standard (as similar as possible to the analyte of interest) and a sample of known isotopic composition for each organic structure to be studied. Then, to mix the standard with the sample (with known δ^{13}C values) prior to the injection and to compare the theoretical and experimental values of the glycerol and ethanol in the mixture. A commercial glycerol

Table 1. Accuracy and Precision of $\delta^{13}C$ Values of Glycerol Obtained by LC-IRMS[a]

	glycerol						
		LC-IRMS			GC-IRMS		
	repetitions per sample	mean $\delta^{13}C$ (‰)	SD (‰)	$\lvert\Delta\delta$ (EA-LC)$\rvert$ (‰)	mean $\delta^{13}C$ (‰)	SD (‰)	$\lvert\Delta\delta$ (GC-LC)$\rvert$ (‰)
glycerol measurement (standard)[b]	10	−27.99	0.05	0.03	−27.98	0.13	0.01
wine measurement (sample)	10	−28.88	0.10		−28.79	0.16	0.09
glycerol measurement (three different days)	10	−27.96	0.06	0.06	−28.01	0.17	0.05
wine measurement (three different days)	10	−28.82	0.22		−28.63	0.19	0.19

[a] Values of $\delta^{13}C$ are expressed in ‰ vs V-PDB. [b] EA-IRMS glycerol (standard) result: -28.02 ± 0.09.

Table 2. Accuracy and Precision of $\delta^{13}C$ Values of Ethanol Obtained by LC-IRMS[a]

		ethanol		
		LC-IRMS		
	repetitions per sample	mean $\delta^{13}C$ (‰)	SD (‰)	$\lvert\Delta\delta$ (EA-LC)$\rvert$ (‰)
ethanol measurement (standard)[b]	10	−25.84	0.10	0.04
wine measurement (sample)[c]	10	−24.43	0.08	0.17
ethanol measurement (three different days)	10	−25.89	0.10	0.01
wine measurement (three different days)	10	−24.54	0.10	0.06

[a] Values of $\delta^{13}C$ are expressed in ‰ vs V-PDB. [b] EA-IRMS ethanol (standard) result: -25.88 ± 0.10. [c] EA-IRMS ethanol (wine) result: -24.60 ± 0.07.

Table 3. Stable Carbon Isotopic Characterization of Glycerol and Ethanol of Wine Samples by LC-IRMS, EA-IRMS, and GC-IRMS[a]

		LC-IRMS				EA-IRMS		GC-IRMS	
sample no.	variety	$\delta^{13}C$ glycerol (‰ vs V-PDB)	SD	$\delta^{13}C$ ethanol (‰ vs V-PDB)	SD	$\delta^{13}C$ ethanol (‰ vs V-PDB)	SD	$\delta^{13}C$ glycerol (‰ vs V-PDB)	SD
1	Monastrell	−27.81	0.18	−24.50	0.04	−24.55	0.01	−27.83	0.16
2	Monastrell	−29.54	0.21	−25.76	0.01	−25.59	0.03	−29.60	0.08
3	Tempranillo	−29.23	0.09	−25.99	0.04	−25.96	0.02	−29.53	0.13
4	Monastrell	−29.41	0.22	−26.16	0.07	−26.13	0.01	−29.32	0.05
5	Monastrell	−32.07	0.12	−27.89	0.05	−27.84	0.05	−31.90	0.24
6	Cencibel	−28.71	0.06	−25.22	0.07	−25.26	0.09	−28.69	0.11
7	Merlot	−29.71	0.25	−26.73	0.03	−26.50	0.06	−29.57	0.19
8	Cabernet Sauvignon	−27.34	0.06	−23.91	0.09	−23.96	0.10	−27.00	0.09
9	Airen	−29.04	0.10	−25.16	0.07	−25.02	0.01	−28.78	0.03
10	Airen	−27.87	0.12	−24.10	0.05	−24.15	0.06	−27.62	0.06
11	Syrah	−27.13	0.20	−23.87	0.13	−24.05	0.04	−26.73	0.06
12	Airen	−30.73	0.10	−26.92	0.02	−26.85	0.01	−30.39	0.22
13	Airen	−27.30	0.10	−24.20	0.08	−24.18	0.01	−26.98	0.07
14	Cencibel	−28.49	0.15	−25.14	0.04	−24.88	0.01	−28.20	0.15
15	Tempranillo	−29.30	0.12	−26.17	0.02	−26.11	0.01	−29.51	0.11
16	Garnacha	−27.98	0.16	−24.19	0.02	−24.16	0.04	−27.94	0.18
17	Rufete	−29.85	0.23	−26.36	0.03	−26.23	0.01	−29.87	0.20
18	Tempranillo	−29.72	0.16	−26.62	0.02	−26.42	0.04	−29.53	0.02
19	Garnacha	−28.09	0.12	−24.29	0.09	−24.44	0.01	−27.79	0.04
20	Tempranillo	−28.52	0.20	−24.48	0.06	−24.83	0.11	−28.36	0.05
21	Viura	−30.63	0.18	−26.92	0.06	−26.81	0.01	−30.70	0.03
22	Tempranillo	−30.47	0.16	−26.41	0.04	−26.63	0.11	−30.32	0.09
23	Monastrell	−32.68	0.19	−28.46	0.06	−28.32	0.05	−32.34	0.08
24	Moscatel	−31.22	0.20	−26.75	0.05	−26.69	0.04	−31.14	0.08
25	Bobal	−29.20	0.10	−25.24	0.06	−25.40	0.08	−28.90	0.05
26	Garnacha	−28.31	0.12	−24.67	0.06	−24.72	0.03	−28.07	0.29
27	Picapoll	−26.92	0.03	−24.00	0.05	−23.88	0.02	−26.70	0.10
28	Cabernet Sauvignon	−27.62	0.15	−23.70	0.08	−23.62	0.10	−27.40	0.10
29	Trepat	−29.68	0.21	−26.70	0.10	−26.71	0.02	−29.57	0.08
30	Garnacha	−29.83	0.24	−25.70	0.06	−25.67	0.08	−29.62	0.12
31	Mar Majuelo	−29.02	0.15	−25.93	0.05	−25.87	0.04	−29.11	0.04
32	Listán Blanco	−30.60	0.14	−27.12	0.07	−27.28	0.08	−30.46	0.20
33	Negramoll	−29.02	0.12	−25.10	0.11	−25.33	0.02	−29.02	0.13
34	Malvasía	−28.21	0.10	−25.30	0.13	−25.31	0.04	−28.42	0.08
35	Listán Negro	−31.63	0.05	−27.62	0.06	−27.23	0.01	−31.77	0.09

[a] Values of $\delta^{13}C$ are expressed in ‰ vs V-PDB, $n = 3$.

and ethanol sample, a wine sample and a mixture of them were prepared and injected into the LC column, under identical conditions. The theoretical glycerol and ethanol values (−27.91‰ and −25.85‰, respectively) and the experimental ones

(−27.70 ± 0.01‰ and 25.89 ± 0.01‰, n = 2) in the mixture showed that the analytes (glycerol and ethanol) in a wine matrix did not suffer from isotopic fractionation during the entire procedure.

It is also important to remark that, when applying the optimal conditions shown previously, no problems on analyte peak resolution or fractionation effects have been observed. No extra care, apart from flushing the column with storage solvent, has to be taken to keep the optimum separation properties of the LC column.

The precision of the measurement for glycerol and ethanol was determined by repeating the analysis 10 times on the standards and 10 times on a wine sample, under repeatability conditions, and by performing 10 independent analyses on the same glycerol, ethanol, and wine sample on different days, under reproducibility conditions (**Tables 1** and **2**). The reproducibility in both cases (glycerol and ethanol) was very good, with a standard deviation (SD) ≤ 0.22‰, indicating the robustness of the method, which is required for routine analysis. The accuracy of this method was expressed as the difference of the value obtained by EA-IRMS or GC-IRMS and LC-IRMS (**Tables 1** and **2**). Analysis of the glycerol on the same samples via EA-IRMS or GC-IRMS shows very close agreement, with $\Delta\delta$ (GC-LC) < 0.2%, serving to validate the methodology for glycerol determination. Analysis of the ethanol on the same samples via EA shows very close agreement, with $\Delta\delta$ (EA-LC) < 0.2%, serving to validate the methodology for ethanol determination.

Application to Samples. In the case of ethanol, the δ^{13}C values of the wine samples (n = 35) were determined by the official method (EA-IRMS) in order to evaluate and validate, subsequently, the chromatographic developed method in this paper for isotopic characterization of wine ethanol. The results found for the samples are shown in **Table 3**. The carbon isotope ratios of samples ranged from −23.62 to −28.32‰, with a mean of −25.62 ± 1.21‰.

In the case of glycerol, and due to the lack of an official method, the results obtained must be compared with the results from an alternative GC-IRMS procedure in order to evaluate and validate, subsequently, the chromatographic developed method in this paper for isotopic characterization of wine glycerol. The results found for the samples are shown in **Table 3**. The carbon isotope ratios of samples ranged from −26.70 to −32.34‰, with a mean of −29.11 ± 1.46‰.

Next, the developed method (LC-IRMS) was applied to all wine samples. To demonstrate the capability of the proposed LC procedure to efficiently perform separation and detection of glycerol and ethanol of real samples, without previous sample treatment (e.g., distillation), 35 samples were analyzed. The real samples were diluted, filtered, and processed through the liquid chromatographic column and later measured by IRMS to study the isotopic values of two main components, glycerol and ethanol. All the samples showed an appropriate chromatographic profile (baseline separation of analyte peaks) and appropriate isotopic values (according to the photosynthetic pathway used by grapes, a C3 plant). As can be seen in **Figure 4**, the δ^{13}C values of glycerol obtained by GC-IRMS and LC-IRMS as well as the δ^{13}C values of ethanol obtained by EA-IRMS and LC-IRMS are strongly correlated (r = 0.99). This confirms that the developed method is free of any isotopic fractionation, and thus, it is suitable for wine glycerol and ethanol $^{13}C/^{12}C$ isotope ratio determination.

The ethanol carbon isotope ratios of samples (**Table 3**) ranged from −23.70 to −28.46‰, with a mean of −25.64 ± 1.27‰, and the glycerol carbon isotope ratios of samples ranged from −26.92 to −32.68‰, with a mean of −29.23 ± 1.42‰. As expected, glycerol is more depleted in δ^{13}C than ethanol, because of a major isotope fractionation on position 1 during the biosynthesis (*26*).

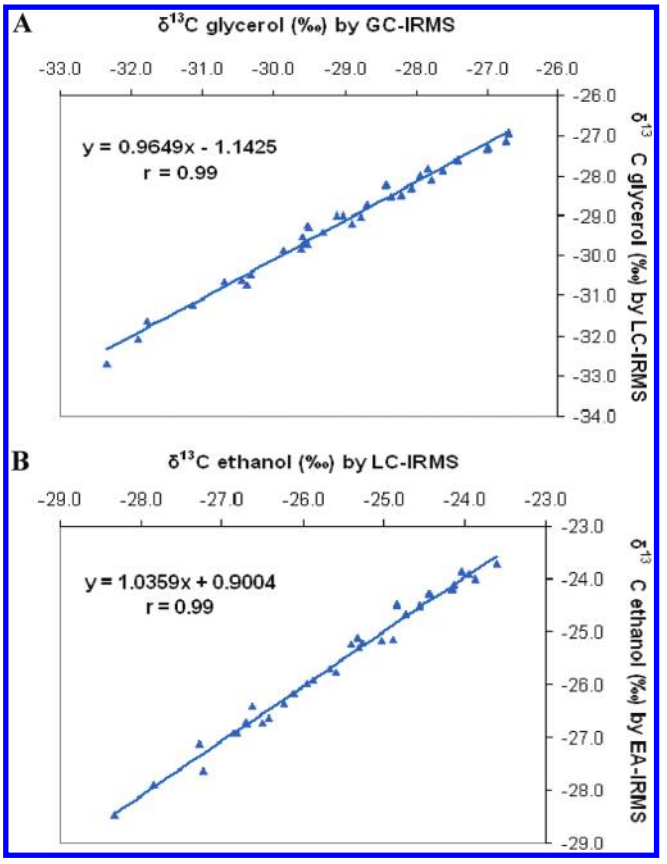

Figure 4. Correlation of (**A**) δ^{13}C glycerol (‰) of wine samples by LC-IRMS versus δ^{13}C glycerol (‰) by GC-IRMS and (**B**) δ^{13}C ethanol (‰) of wine samples by EA-IRMS versus δ^{13}C ethanol (‰) by LC-IRMS.

The results of the present study could represent a new approach for determining the adulteration of wine with glycerol or ethanol coming from synthesized products or plants (C4). In fact, the values obtained for wine samples (glycerol and ethanol) differ from those previously described in the literature of glycerol, from oils (maize oil, fish oil) (*7*, *28*) or industrial synthesis (petrochemicals) (*28*), and non-grape-ethanol (derived from C4 plants) (*23*). In addition, the average difference $\delta^{13}C_{glycerol} - \delta^{13}C_{ethanol}$ obtained (−3.59‰) is in the range observed previously by Calderone et al. (*7*). This correlation cannot exist between the same compounds from a foreign source. Therefore, further investigations on the use of $\Delta\delta^{13}$C systematic differences could provide useful information about wine authenticity criteria.

Therefore, in this paper, we demonstrate the applicability of LC-IRMS for carbon stable isotope analysis on target molecules under study (glycerol and ethanol), not on a bulk product, which could include impurities. The developed method allowed us to improve the currently available methodologies for wine adulteration detection. The major advantage is that isotopic determination of glycerol and ethanol can be performed simultaneously in the same run. In addition, from the results of this study, it can be concluded that the new developed method (LC-IRMS) provides several additional benefits over previous isotopic off-line methods: straightforward preparation (dilution, filtration, and injection), avoiding the complex sample preparation procedures needed for EA-IRMS analysis (e.g., distillation or extraction); simplicity of the operative procedure (avoiding determination of water content by the Karl Fischer method and calculation of alcoholic strength); good repeatability (SD ≤ 0.10); and no isotopic fractionation. Injections are fully automated, so that the analysis is feasible on a routine scale. The described method

can be used for simultaneous and highly precise measurements of the carbon isotopic composition of glycerol and ethanol in wine, and thus, it could be considered as a potential routine method for control laboratories equipped with suitable IRMS instruments.

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