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Characterization of European Wine Glycerol: Stable Carbon Isotope Approach

GIOVANNI CALDERONE,*,†,§ NORBERT NAULET,§ CLAUDE GUILLOU,† AND FABIANO RENIERO†

Commission of the European Communities, Joint Research Centre, Institute for Health and Consumer Protection, Physical and Chemical Exposure Unit, Ispra, Italy, and Laboratoire d'Analyse Isotopique et Electrochimique de Métabolismes (LAIEM), CNRS, Nantes, France

Glycerol of about 170 European wines was analyzed using gas chromatography−combustion−isotope ratio mass spectrometry technique. $^{13}C/^{12}C$ isotopic ratio measurements were performed to characterize glycerol's $\delta^{13}C$ values of genuine wine samples from European Union wine-producing countries. Glycerol was also successfully dosed using an internal reference, 1,5-pentanediol.

KEYWORDS: Authenticity proof; stable isotope; carbon-13; glycerol; wine; GC-C-IRMS; *Botrytis cinerea*

INTRODUCTION

Glycerol, the IUPAC name of which is 1,2,3-propanetriol, commercially known as glycerin, is the simplest triol (see **Figure 1**) and occurs in nature both in a bound form and as a free molecule. It is present mostly in the form of a triglyceride in all natural fats and oils. As a free molecule, it is also produced during alcoholic fermentation: the degradation of sugars by yeast yields ethanol, carbon dioxide, and glycerol. In wine, it is the most abundant product after water and ethanol and is predominant (*1*) among several polyols found there, including erythritol, arabitol, mannitol, sorbitol, *meso*-inositol, and 2,3-butanediol.

Glycerol concentrations in wine typically range from 4 to 16 g/L (*2*), but higher values, up to 21 g/L (*3*), are not unusual, especially in white wines from "noble rot" infected grape musts. It is well-known that grapes infected with some fungi, *Botrytis cinerea* in particular, produce musts rich in glycerol, when normal musts contain glycerol in only trace amount.

Glycerol could contribute to the mouthfeel properties and smoothness of wine (*4*, *5*). It is also an important contributor to the sugar-free extract of wine, an index on which is based a quality scaling of wines in some European countries.

Therefore, for such reasons, glycerol is sometimes fraudulently added to wine to disguise poor quality (*6*). As this practice is not permitted by European Commission (EC) regulations (*7*), the European "Glycerol Project" (*8*) aimed to study possible methods to detect addition of synthetic or natural—but exogenous—glycerol. Besides classical chemical analysis, the project tested methodologies based on stable isotope ratio measurements.

* Address correspondence to this author at TP740 BEVABS/IHCP, JRC-Ispra, via Fermi 2, 21027 Ispra (VA), Italy (telephone +39 0332785668; e-mail giovanni.calderone@jrc.it).

† Institute for Health and Consumer Protection.

§ Laboratoire d'Analyse Isotopique et Electrochimique de Métabolismes (LAIEM).

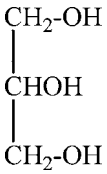

Figure 1. Glycerol structure.

Stable Isotope Ratio Methods in Food. Extensive studies on plant metabolism (*9*−*11*) and natural fractionation of stable isotopes enabled access to an extremely useful tool in the fight against fraud in the food products domain. Stable isotope ratio methods are based on the measurement of stable isotope contents (^{2}H, ^{13}C, ^{15}N, ^{18}O, ^{34}S) of a product or of a specific component such as an ingredient or a target molecule of the product. The determinations, carried out using isotope ratio mass spectrometry (IRMS) and/or nuclear magnetic resonance (NMR) techniques, can provide information on botanical and geographical origins, which are often considered to be important characteristics of many food products both by the consumer and by European regulations.

Routinely, isotopic ratio analyses to test origin of flavors and aromas, honeys (*12*, *13*), and fruit juices have been adopted in industrial production as well (*14*). The European Union (EU) has adopted analyses of $^{2}H/^{1}H$, $^{13}C/^{12}C$, and $^{18}O/^{16}O$ in its regulations (*15*, *16*) as official methods to test the authenticity of wine and alcoholic beverages.

Pioneer studies on stable isotope properties of glycerol have been performed (*17*−*20*) with the aim of eventually using this molecule as another independent probe in food origin determination. Our study is the first to perform $\delta^{13}C$ analyses on a very large variety of wine samples from all of the different EU wine-producing countries and to test the routine use of the GC-C-IRMS technique.

EU Wine Data Bank: BEVABS Role. BEVABS is the Bureau Européen des Vins, Alcools et Boissons Spiritueuses

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Table 1. EU Wine-Producing Countries and Composition of Our Samples[a]

	country of origin								
vintage	Austria	France	Germany	Greece	Italy	Luxembourg	Portugal	Spain	U.K.
1998	6/1/0	13/1/1	10/1/0	3/0/2	10/4/1	4/0/0	4/6/0	3/5/2	4/0/0
1999	7/5/0	5/3/6	9/0/1	3/3/0	11/3/2	4/0/0	7/7/0	5/5/0	4/0/0

[a]Number of genuine wines, expressed as white/red/rosé. Total: 81 samples for 1998 and 90 for 1999.

or European office for Wine, Alcohol and Spirit Drinks, which has, among others, the task to put together the data measured by each country, validate them, and build and maintain the so-called EU Wine Data Bank. This Wine Data Bank is a very rich collection (12000 samples through 1998) constituted of experimental wines, starting from vintage 1991 to now. Wines are produced by official laboratories, collecting grapes from their vine-growing areas and fermenting them in compliance with EC Regulation 2347/91. For each vintage, BEVABS performs on the 10% of reference samples measurements of D/H ratio, by ^{2}H NMR, and of ^{13}C/^{12}C ratio, by elemental analyzer IRMS (EA-IRMS), on distilled ethanol, and of ^{18}O/^{16}O ratio on the wine water, to perform the wine-DB validation.

The wine samples investigated in this work belongs to the EU Wine Data Bank.

MATERIALS AND METHODS

Samples. δ^{13}C measurements were performed on 171 wines, chosen and collected among the samples of the BEVABS Wine Data Bank. Wines have been selected following several criteria: the geographical origin inside each country; a representation, if possible, of different types—white and red varieties; and the widest possible range of δ^{13}C values measured on correspondent ethanol. Two vintages have been considered, 1998 and 1999.

The official wine-producing EU countries are nine and are listed in **Table 1**, which also shows the composition of our sampling.

Sample Preparation. The preparation of wine samples was as follows: after filtration over Waters 0.2 μm filters and a dilution (ratio 1:4) with a solution of ~0.7 g/L of 1,5-pentanediol (1,5-PD) (Lancaster) in ethanol, samples were ready for injection. The use of the diol allowed the accurate determination of the glycerol concentration.

Continuous Flow GC-C-IRMS. The separation of glycerol from the wine matrix was achieved using a Varian 3400 gas chromatograph, equipped with a Chrompack WCOT fused silica capillary column filled with bonded polyethylene glycol (CP-WAX57CB, 25 m length, 0.25 mm i.d., 0.20 μm film thickness). Isotope ratio measurements were performed, in continuous flow, using a FinniganMat Delta Plus (Bremen, Germany) mass spectrometer, coupled in line with the gas chromatograph through a FinniganMat GC combustion interface. Helium Alphagaz 2 was used as carrier gas, and the pressure at the head of the column was set to 20 psi. Particular attention was paid to using only a deactivated silica capillary, deactivated press fit connectors, and a three-way splitter ("Y" splitter, instead of a T-Valco valve) manufactured by BGB Analytik-AG, as suggested by Thermo of Bremen, to avoid effluent interaction with metals, reducing isotopic fractionation.

Sample solutions (0.3 μL) were injected (10 μL Hamilton syringe) in the column, using an A200S FinniganMat autosampler, in split mode. The injector temperature was set to 270 °C. The temperature program was set as follows: initial column temperature, 120 °C; holding time, 2 min; temperature increased at a rate of 10 °C/min, until the final value of 220 °C; final holding time, 2 min. Each run took 14 min, not considering the necessary time of cooling.

After the chromatographic separation, the effluent undergoes a combustion and a reduction step, passing through the oxidation and the reduction ovens. Components other than the glycerol, namely, the solvent, were vented with a back-flush valve during the run, to avoid oven spoiling and interferences in chromatograms.

The combustion oven consisted of a 30 cm long ceramic tube in which three thin (0.125 mm diameter) braided wires were placed: one of nickel, one of copper, and one of platinum. This tube was kept at a temperature of 960 °C, and a flow of oxygen was periodically introduced inside the oven to reoxidize the copper. The task of this oven was to combust the organic compounds coming from the gas chromatograph to CO_2, H_2O, and, if they contained nitrogen, NO_x. Eventual nitrogen oxides were reduced to N_2 in the reduction oven, at 640 °C, which consisted ofan identical ceramic tube, but filled only with copper. Water produced during the combustion was eliminated by a water-removing trap, consisting of a Naphion membrane. In our case, only glycerol was oxidized, producing CO_2 and H_2O. Carbon dioxide, carried by the helium stream, arrived at the IRMS source for ^{13}C/^{12}C analysis. Here CO_2 molecules were ionized and ions collected by three different Faraday cups depending on their m/z of 44, 45, or 46. **Figure 2** shows a schematic drawing of the GC-C-IRMS configuration we used.

Isotope ratios are usually expressed as relative deviations δ per mille (‰) with respect to a standard:

$$\delta^{13}C_{PDB} = \frac{(^{13}C/^{12}C)\text{sample} - (^{13}C/^{12}C)_{PDB}}{(^{13}C/^{12}C)_{PDB}} \times 1000 \qquad (1)$$

For ^{13}C/^{12}C the international standard is PDB (Pee Dee Belemnite, limestone), a calcium carbonate. During ^{13}C/^{12}C analyses, a reference CO_2 gas is introduced, which is calibrated against other PDB-calibrated international standards, the PDB itself being exhausted for many years. Our CO_2 was formerly calibrated against international IAEA standards for ^{13}C using our EA-IRMS system, which consists of a Carlo Erba EA (Milan, Italy) coupled to a Finnigan Mat Delta S mass spectrometer.

The gas chromatograph and the mass spectrometer system were piloted using the Thermo Isodat NT 1.5 software suite. The same software processed data acquired to calculate the δ^{13}C values against our CO_2 gas reference. CO_2 pulse peaks were introduced into the IRMS source, five at the start and one at the end of each injection of wine. The software applied by default the "CO_2 Santrock" correction to calculate the deviations.

Each wine sample was injected three times, and a BIPEA (Bureau Interprofessionnel d'Etudes Analytiques) wine was included as reference to bracket every batch of six wines, to evaluate eventual drift of values. In the case of glycerol, we accepted as repeatable a standard deviation (SD) value of <0.6‰. Thus, samples with SD ≥ 0.6 ‰ were repeated.

Another control over each single run was made using the 1,5-PD, the internal reference already used to determine the concentration of glycerol. This compound, showing a very good repeatability for ^{13}C/^{12}C measurements, hinted at the validity of the injection and combustion step. The δ^{13}C value for our 1,5-PD was −31.2‰ by GC-C-IRMS and −30.52‰ by EA-IRMS analysis. An incoherent value for the internal reference was considered to be a clue to a problem occurring in that run.

Determination of Concentration of Glycerol. We successfully solved the problem of easily dosing glycerol in a huge quantity of wine samples, using in the analytical method the "internal standard" 1,5-pentanediol. This compound is, in fact, structurally similar to glycerol, but less polar. Its retention time was 500 s, whereas that for glycerol was 680 s.

For calibration measurements, final concentrations in samples were $C_{1,5PDSt} = 0.053\%$ and $C_{glycSt} = 0.098\%$. During the routine measurements, $C_{1,5PDSample} = 0.053\%$.

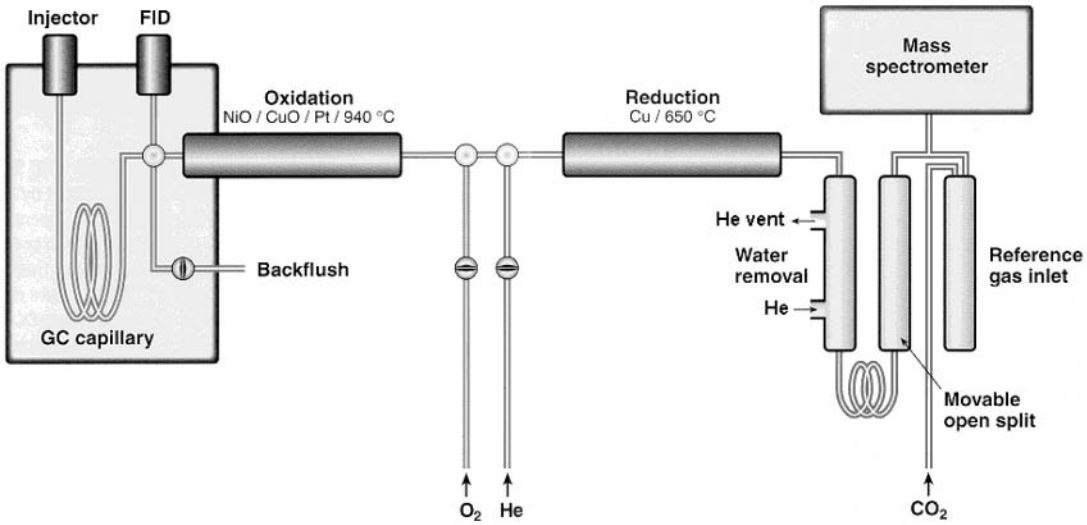


Figure 2. Scheme of GC-C-IRMS configured for $\delta^{13}C$ analyses.

Table 2. Comparison of Concentrations with Five BIPEA Wines[a]

BIPEA wine	Feb 1999	Nov 1999	Feb 2000	Sept 2001	Nov 2001	Mar 1997	May 1997
type	white	rosé	white	red	white	red	red
BIPEA range	6.2–8.4	4.8–6.6	5.7–7.7	6.3–8.5	4.6–6.2	nd	nd
BIPEA mean value	7.3	5.4	6.7	7.4	5.4	7.2[b]	6.6[b]
concn by GCC-IRMS	6.4	5.4	6.7	7.8	5.4	7.4	6.2

[a] BIPEA determinations were performed by HPLC and/or enzymatic analysis. Concentrations are given in g/L. n >3 and SD < 0.6. [b] Personal communication from BIPEA's scientific and technical staff.

Table 3. Comparison of Glycerol $\delta^{13}C$ Values by GC-C and EA-IRMS Techniques[a]

origin	commercial glycerin	vegetal glycerin	maize oil	cheese
GC-C	–27.04	–22.65	–15.39	–28.33
EA	–27.67	–22.60	–15.22	–28.13

[a] Values of $\delta^{13}C$ are expressed in ‰ vs PDB. n > 3, SD < 0.6 for GC-C-IRMS; n = 3, SD < 0.1 for EA-IRMS.

Table 4. Wines Particularly Rich in Glycerol Present an Inversion of Isotopic Fractionation

country	grape variety	glycerol	$\delta^{13}C_{glycerol}$ (n = 3)	$\delta^{13}C_{ethanol}$	$\Delta\delta$
Austria	Gewürztraminer	19.8	–28.4	–28.70	0.35
Austria	Gewürztraminer	23.3	–27.5	–28.10	0.65
France	Semillon	22.8	–24.5[b]	–25.30	0.85
Italy	Frappato	18.0	–25.65	–25.94	0.30

[a] Concentrations are given in g/L; $\delta^{13}C$ is in ‰ vs PDB. [b] SD > 0.6.

Two glycerol solutions have been used to test this method. Assuming that the typical concentration of glycerol is 4−10 g/L in dry wine, the two solutions prepared should represent this range. The first solution, of 4.0 g/L, actually poorer in glycerol than the most of the wines, gave an experimental concentration of 3.6 g/L (SD = 0.2, n = 8). The second solution (8.0 g/L), the glycerol content of which was more similar to that of real wines, gave a value of 7.9 g/L (SD = 0.3, n = 8).

Also, seven genuine, already dosed, wines (**Table 2**) were injected to test the method. These wines were chosen among our BIPEA alcoholic beverage sample data bank.

RESULTS

Preliminary study has been performed on four synthetic wine solutions (water−ethanol−glycerol), prepared using glycerol samples from different origins and with $\delta^{13}C$ values already determined by EA-IRMS. Values obtained by GC-C- and EA-IRMS are reported in **Table 3**.

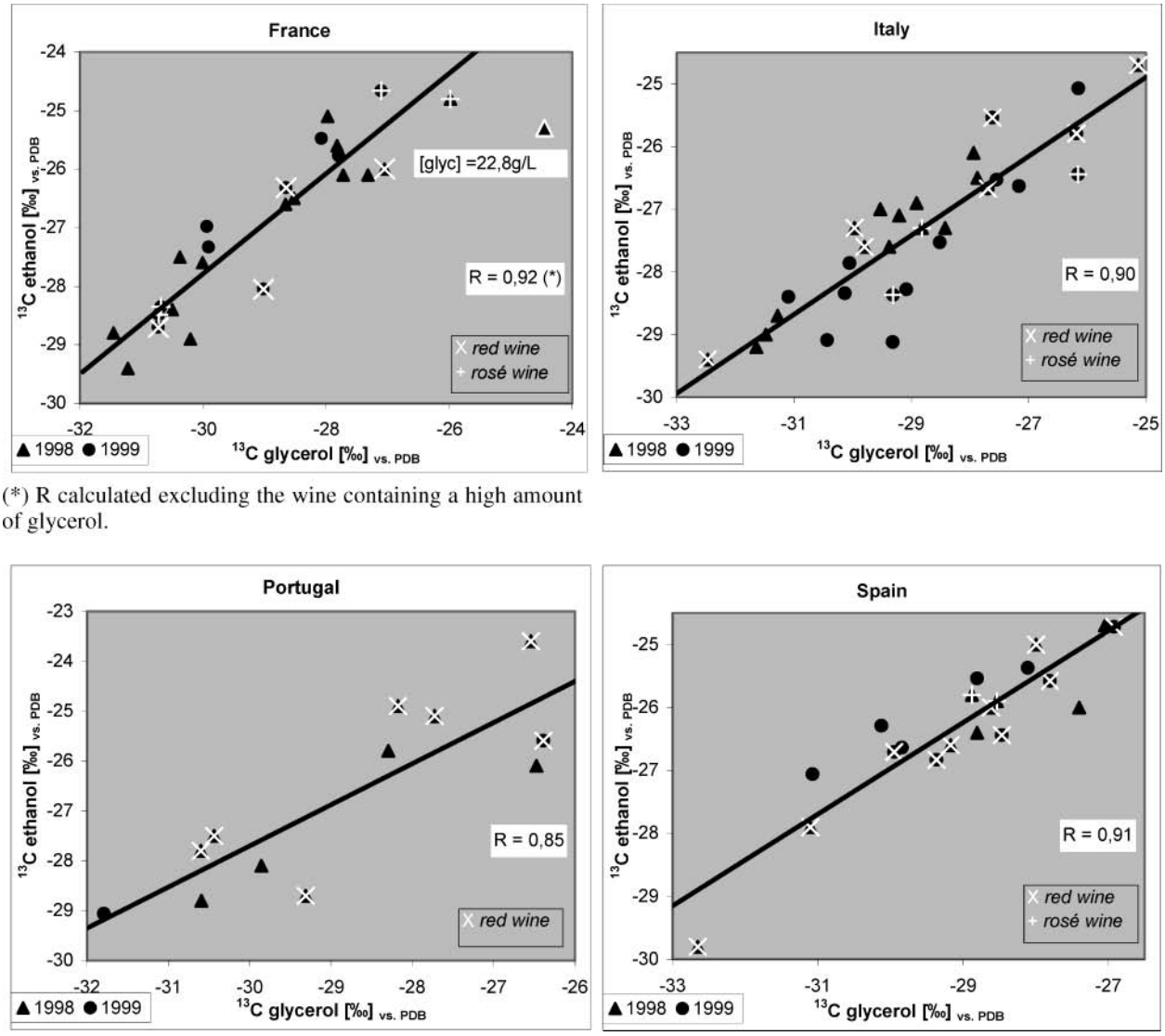
Almost the totality of wines showed no overlapping between 1,5-PD and other wine components, except sometimes in sweet wines.

The range of glycerol concentration observed in our samples was quite typical, mostly 5−15 g/L. Just few remarkable samples gave very low—from 1.5 to 5 g/L—or very high values—from 18 to 23 g/L—value (**Table 4**).

Not surprisingly, these wines were produced from Semillon and Gewürztraminer varieties that are, as is well-known, among the grapes that most benefit from the action of the *B. cinerea* fungus.

Two of the BIPEA wines were considered to be blind samples, as their concentration was not available in archives. Our results have recently been confirmed by the BIPEA's scientific and technical staff.

Figure 3 shows that for genuine wines the correlation between $\delta^{13}C$ of glycerol and $\delta^{13}C$ of ethanol from the same source is quite good (R = 0.90). As expected, glycerol is more depleted in $\delta^{13}C$ than ethanol, because of a major isotope fractionation on position 1 during the biosynthesis (*17*). The average difference $\delta^{13}C_{glycerol} - \delta^{13}C_{ethanol}$ is −2.1‰, very close to the −2.3‰ that is the value reported by Weber in a controlled and not inhibited fermentation medium, but the range we observed is very wide, from −4.1 to −0.1‰. Nevertheless, we observed an enrichment in ^{13}C in wines with a high content of glycerol. In these cases, in fact, the difference $\delta^{13}C_{glycerol} - \delta^{13}C_{ethanol}$ is not negative. For a few wines, very rich in glycerol, the differences we observed were in the range of 0.3−0.85‰, positive values. In these cases a strong contribution is supposed to be given by exogenous glycerol, a metabolite of *B. cinerea*,



(*) R calculated excluding the wine containing a high amount of glycerol.

Figure 3. Correlation of $\delta^{13}C$ of glycerol versus $\delta^{13}C$ of ethanol for 1998 and 1999 wines of four EU countries. The reported values of $\delta^{13}C$ for ethanol were those measured on distilled ethanol, using EA-IRMS.

through a different biosynthetic pathway (as explained below), affecting the global $\delta^{13}C$ value. **Figure 4** shows that glycerol concentration and ethanol content of wines are in good correlation. Logarithmic trend is slightly ($R = 0.75$) better than linear one ($R = 0.70$).

In conclusion, the advantages of the online GC-C-IRMS technique to measure $\delta^{13}C$ on glycerol, with respect to offline methods, are the following:

- GC-C-IRMS offers easy sample preparation, it is not time-consuming, it has good repeatability, and it does not show isotopic fractionation.
- Injection in column is totally machine-controlled, so that the analysis is feasible on a routine scale.
- Measurements of $^{13}C/^{12}C$ are truly performed on target molecule under study, not on a bulk product, which could include impurities.
- Furthermore, we expect that the same sample preparation used for GC-C-IRMS could be suitable to perform online measurements of $\delta^{2}H$ and $\delta^{18}O$ on glycerol with a gas chromatography−total combustion−elemental analyzer (based on pyrolysis) system.

The use of an internal reference such as 1,5-PD offers the double benefit of allowing in the same measurement both the determination of glycerol concentration and a control over the validity of the run itself.

For the purpose of this research, we did not separate the isotopic contribution of glycerol produced as metabolite by *Botrytis* and that from normal alcoholic fermentation, by *Saccharomyces cerevisiae*. We performed measurements on the sum of endogenous and exogenous glycerol. To discern the single contribution, an indirect analysis on the basis of the amount of gluconic acid, which is a specific metabolite produced by *Botrytis*, would allow the proportions of glycerol (*21*) produced, respectively, by *Saccharomyces* and *Botrytis* to be determined.

As glycerol $\delta^{13}C$ values are more positive for Semillon and Gewürztraminer wines with respect to those of nonbotrytized wines, glycerol produced by *B. cinerea* should have a higher content in ^{13}C, with the effect of increasing the overall $\delta^{13}C$ value of glycerol in wine.

This could be explained by the fact that *B. cinerea* uses tartaric and malic acids (*22*, *23*) of the grape for its metabolism, to synthesize gluconic acid and then to excrete glycerol. In fact, tartaric and malic acids contained in must and wine have higher $\delta^{13}C$ values (*24*) than sugars metabolized by yeast to produce ethanol.

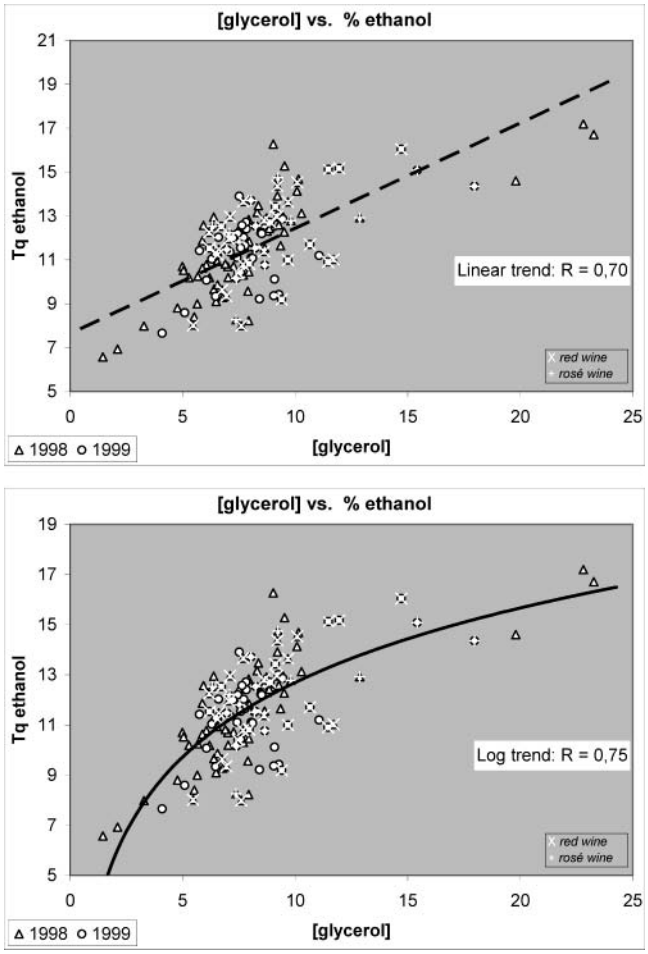


Figure 4. Correlations of glycerol concentration (g/L) versus ethanol content (% v/v) of wines.

In the case of sweet wines, gas chromatography was not sufficient to adequately separate glycerol from other components that become as concentrated as glycerol. This issue should be solved by optimizing the chromatographic conditions for the specific cases.

Even if these parameters do not seem adequate to determine the geographical origin or the adulteration of a wine, this work shows interesting correlations between glycerol and ethanol, and as already observed for the multielement stable isotope approach (*18*), it could be a relatively reliable technique to check if a wine has not been adulterated.

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