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ORIGINAL PAPER

Determination of Glycerol in Wines Using ^{31}P-NMR Spectroscopy

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Abstract ^{31}P-NMR spectroscopy was employed to detect and quantify glycerol in red wines from various regions of Greece. This novel analytical method was based on the derivatization of the hydroxyl groups of glycerol with 2-chloro-4,4,5,5-tetramethyl dioxaphospholane, and identification of the phosphitylated compound on the basis of ^{31}P chemical shifts. Quantification of glycerol in wines was accomplished by integration of appropriate signals in the ^{31}P-NMR spectrum and the use of the phosphitylated cyclohexanol as the internal standard. The method was reproducible (CV (%) = 2.35) and accurate (CV (%) = 1.34). Its applicability to glycerol quantification in wines was tested against a weighted amount of a glycerol-model compound by linear regression analysis ($R = 0.999$; intercept = 0.074 ± 0.078; slope = 0.998 ± 0.003; $p = 0.000$). Furthermore, the NMR method was compared to the AOAC official method (HPLC) using the Bland and Altman statistical analysis. The distribution of the data points in the bias plot showed that 100% of the measurements of glycerol in 16 wine samples from various regions of Greece were within the limits of agreement of the two methods.

Keywords Glycerol · Wine · ^{31}P-NMR spectroscopy

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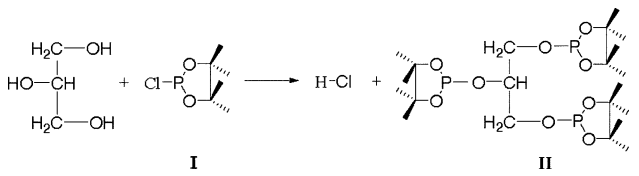
Introduction

Glycerol in wines is considered as a by-product of the alcoholic fermentation. Its presence in wines influences their sensory characteristics contributing to the richness of taste [1] and aroma perception [2]. The glycerol content in wines is used for quality scaling in several European countries. Hence, quantification of glycerol in wines represents an important step to provide quality certification. To meet this requirement, reliable analytical techniques and procedures must be used.

Several analytical approaches employing immobilized enzymes in combination with various analytical techniques have been used for the determination of glycerol in wines [3]. Infrared spectroscopy [4], HPLC [5, 6], and ^{1}H-NMR spectroscopy [7] have been employed for the determination of minor constituents in wines including glycerol. Also, glycerol in wines has been determined using the stable carbon isotope approach [8].

In this work, an alternative methodology based on ^{31}P-NMR spectroscopy is evaluated for the quantification of glycerol in wines. This method, introduced in an earlier publication [9], is based on the derivatization of the labile hydrogens of the primary and secondary hydroxyl groups of glycerol by the phosphitylating reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**I**) according to the reaction scheme shown in Fig. 1, and the use of ^{31}P chemical shifts to identify the labile centers (compound **II**). Compound **I** reacts rapidly and quantitatively under mild conditions with the hydroxyl groups. This method has been already applied to determine polyphenols [10], diacylglycerols, total free sterols, and free acidity in virgin olive oil [9, 11]. The NMR data were compared with the data obtained using the AOAC official method [5].



I II

Fig. 1 Reaction of hydroxyl groups of glycerol with 2-chloro-4,4,5,5-tetramethyl dioxaphospholane (**I**)

Materials and Methods

Samples, Reagents and Reference Compounds

Sixteen red wines from various regions of northern, middle and southern Greece (Macedonia, Thessaly, Peloponnesus, Aegean Islands and Crete) and different grape varieties (Agiorgitiko, Kotsifali, Mantilaria, Xynomavro, Liatiko, Krasato, Merlot, Syrah, Cabernet Sauvignon, and/or mixtures of two of the aforementioned varieties) were used in this study. All wine companies, from which the wines were purchased, used the standard technology of wine making. Wines were filtered using 2-μm membrane filters (Acrodisk, Pall, USA) and kept at 4 °C prior to NMR and HPLC analyses.

Glycerol model compound used in both NMR and HPLC analyses was purchased from Sigma-Aldrich (Athens, Greece). Pinacol, phosphorus trichloride, protonated solvents (analytical grade) for synthesis, pyridine solvent 99%, and deuterated chloroform were purchased from Sigma-Aldrich (Athens, Greece). The derivatizing phosphorus reagent was synthesized from pinacol and phosphorus trichloride in the presence of triethylamine adapting a method described in the literature with following modifications [12]. We replaced benzene and triethylamine used in the original method with hexane and pyridine, respectively. This modification resulted in ~45% yield of the product against ~20% yield obtained with the original method.

Sample Preparation for ^{31}P-NMR Experiments

A standard solution was prepared by dissolving 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$, (0.165 μM) and 13.5 mg cyclohexanol (13.47 mM) in 10 mL of a solvent containing pyridine and $CDCl_3$ (1.6:1.0, v/v). This solution was protected from moisture with 5-Å molecular sieves. Wine sample (0.3 g) was placed in a 5 mL round bottomed flask and connected to a vacuum line equipped with an MD 4C vacuum pump (Vacuubrand, Wertheim, Germany), and dried at 40 °C. To the dried sample, 0.4 mL of the standard solution and 100 μL of the phosphorus reagent were added. The mixture was left at room temperature for 15 min for completion of the reaction, and transferred to a 5 mm NMR tube for conducting ^{31}P-NMR experiments. It should be noted that the ^{31}P-NMR methodology is not influenced at all by the presence of water. Water was removed prior to the analysis in order to avoid wasting the phosphorus reagent which reacts with water.

^{31}P-NMR Spectra

The ^{13}P-NMR spectra were obtained on a Bruker AMX500 spectrometer operating at 202.2 MHz for the phosphorus-31 nucleus at room temperature. The spectra were recorded by employing the inverse gated decoupling technique in order to suppress NOE effects. Typical parameters for quantitative studies were: 90° pulse width 12.5 μs, sweep width 10 kHz, relaxation delay 25 s, memory size 16 K (zero-filled to 32 K). To ensure quantitative spectra, the magnitude of the relaxation delay adopted was more than five times the relaxation time (4.57 s) of the phosphitylated cyclohexanol and the phosphitylated glycerol (2.47 and 1.88 s for the primary and secondary phosphitylated hydroxyl groups, respectively). Line broadening of 1 Hz was applied and drift correction was performed prior to Fourier transform. A polynomial fourth-order baseline correction was performed before integration. For each spectrum, 32 transients were accumulated. All ^{31}P chemical shifts were relative to the product of the reaction of **1** with water (trace of water contained in all samples), which gave a sharp signal in pyridine/$CDCl_3$ at δ 132.20. It should be noted that the presence of the paramagnetic metal center of $Cr(acac)_3$ in the samples lowers the relaxation times of the phosphorus nuclei, thus shortening the duration of the measurements significantly.

HPLC System

The HPLC apparatus (Shimadzu VP, Japan) consisted of a LC-10AD VP isocratic pump (Shimadzu, Japan), an injection valve (7725, Rheodyne, USA) (injection volume 20 μL), a column furnace (CTO-10VP, USA), a refraction index detector (Shimadzu RID-10A, Japan) and a data processing system (CLASS VP, Germany).

HPLC Separation

To separate glycerol from other wine constituents, the AOAC official method [5] was followed, although a different column was used. The wine samples were injected directly through a 20-μL loop into a column EC 250/4.6 Nucleosil 100-5 C18 (Supelco, Hora, USA) controlled by thermostat at 60 °C. The flow rate was 0.4 mL/min. In addition, five standard solutions were prepared for calibration by dissolving the appropriate amounts of glycerol in distilled water.

Results and Discussion

Figure 2a shows a typical ^{31}P-NMR spectrum of a wine sample in the region where the signals of the phosphitylated hydroxyl groups of glycerol appear. The signals at δ 147.35 and δ 146.31 belong to the phosphitylated primary and secondary hydroxyl groups of glycerol, respectively (integral ratio 2:1), whereas that at δ 145.13 belongs to the phosphitylated hydroxyl group of the internal standard cyclohexanol. The concentration of glycerol in the wine samples was calculated from the following equation:

$$\text{glycerol}\,(\text{mg/mL}) = \frac{\{[(I_p/2) + I_s]/2I_{CH}\} \times A \times 10^{-3} \times M_W}{V = m/d} \quad (1)$$

where A, M_W, and I_{CH} are the μmol, molecular mass, and integral of the internal standard cyclohexanol, respectively; I_p and I_s are the integrals of the signals corresponding to the primary and secondary phosphitylated hydroxyl groups of glycerol, respectively; V, m, and d are the volume (mL), mass (mg) and density (mg/mL) of the wine samples.

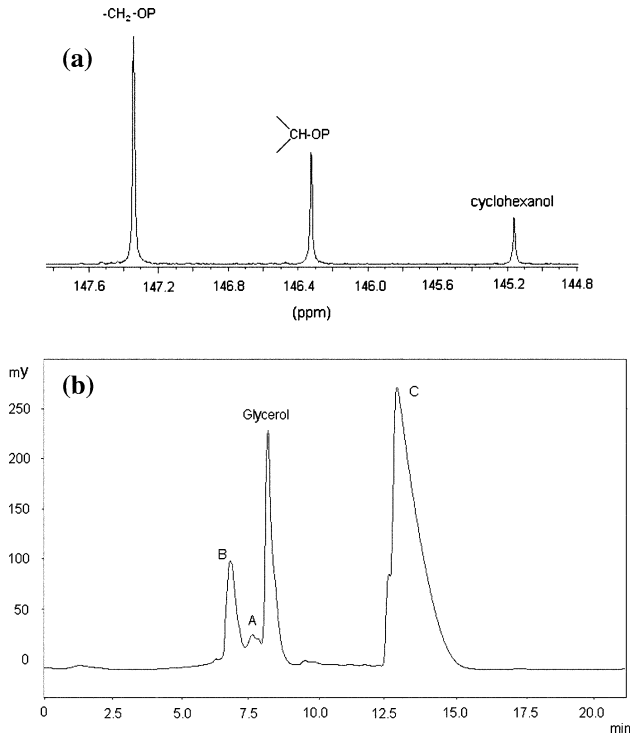


Fig. 2 **a** 202.2 MHz ^{31}P-NMR spectrum of a red wine sample in the region where the phosphitylated hydroxyl groups of glycerol appear. The peak denoted by CH at δ 145.13 belongs to the phosphitylated hydroxyl group of the internal standard cycloexanol. **b** Chromatogram of a red wine sample using an EC 250/4.6 nucleosil 100-5 C18 column at 60 °C with a mobile phase of water at a flow rate of 0.4 mL/min and using a refractive index detector. Peaks A = compounds with acid character, peaks B = glucose, fructose, peaks C = ethanol and methanol

A typical HPLC chromatogram of a wine sample is given in Fig. 2b. Glycerol eluted at 8.02 min, later than peaks A and B, and earlier than peaks C. The glycerol peak was verified by spiking a wine sample with standard glycerol. No attempt was made to verify the remaining peaks in the chromatogram, since they did not interfere in the determination of glycerol. Nevertheless, peaks A could be attributed to the sugars D-glucose or fructose; peaks B could be ascribed to compounds with acid character, whereas peaks C could result from ethanol or methanol [6, 13]. The concentration of glycerol was calculated based on the integration of the respective peak in the chromatogram in combination with the standard calibration.

Calibration with Standard Solutions

The performance of the HPLC system for glycerol quantification was examined with standard solutions having concentrations 3.00, 5.00, 7.00, 10.00, and 15.00 mg/mL. A linear five-point calibration graph was obtained with correlation coefficient 0.9995. The precision was evaluated by the standard deviation of the method S_{x0} = 0.18 mg/mL calculated as the ratio of the residual standard deviation S_y and the slope, according to ISO 8466-1 [14]. The threefold of the standard deviation (0.54 mg/mL) is considered as an estimate of the limit of detection (LOD). The corresponding absolute LOD based on the 20-μL injection volume was 10 μg/mL.

The repeatability and reproducibility between days of the HPLC method was evaluated by analysing aqueous solutions containing 7.00 mg/mL of glycerol (n = 5). The CV (%) for the repeatability was 1.45% (6.92 ± 0.10), and that of reproducibility 1.68% (6.89 ± 0.12).

Validation of the ^{31}P-NMR Method

The applicability of the present NMR method for quantitative determination of glycerol in wines was demonstrated by correlating the amount of the glycerol-model compound predicted through integration of the corresponding signals in the ^{31}P-NMR spectra to that of the weighed amount. The correlation was linear with correlation coefficient = 0.999, intercept = 0.074 ± 0.078, slope = 0.998 ± 0.003, p = 0.000 indicating that this method was reliable for quantitative analysis. The slope was very close to unity ($p < 0.05$) with insignificant dispersion; a greater dispersion is observed for the intercept. Assuming a minimum workable sample volume of 0.4 mL with a minimum signal-to-noise ratio of 3, and taking the deviation of the intercept from zero (0.074) into account, the minimum detectable amount of glycerol was on the average 0.173 μmol per 0.4 mL

or 39.8 μg/mL. The repeatability of seven consecutive spectra using the same solution of phosphitylated glycerol (10.00 mg/mL) was found to be 10.42 ± 0.14 (CV (%) = 1.34%), whereas the reproducibility was 10.23 ± 0.24 (CV (%) = 2.35%), as tested by performing same measurements on eight different glycerol solutions.

Comparison of the NMR data with those obtained using the AOAC official method for the glycerol determination in wines consisted of a more rigorous validation test. Table 1 summarizes the concentration of glycerol in 16 red wine samples from various regions of Greece, different grape varieties, and vintage as determined by ^{31}P-NMR and HPLC methods. Method comparison is usually tested by regression analysis and correlation coefficients. Nevertheless, this approach was found to be inappropriate for several reasons [15, 16]. One problem is that the degree of correlation depends on the data range of measurements. A wider range will give a better correlation, but not necessarily better agreement. Indeed, linear regression of the data in Table 1, covering a narrow range, gave a correlation coefficient 0.786, whereas the correlation coefficient increased to 0.966, when zero concentrations were included in the regression analysis.

In this study, we adopted an alternative statistical approach using difference or bias plots to compare the NMR (field method) with the HPLC (reference method), as suggested by Bland and Altman [15]. The mean value of the field and reference methods was plotted on the abscissa, and the calculated difference between measurements was plotted on the ordinate. In addition, the bias plot displayed horizontal lines for the mean difference and for the mean ±2 × standard deviations of the difference. The differences of the NMR and HPLC data for glycerol shown in Table 1 have a mean value of –0.310 mg/mL with a standard deviation of ±0.722 mg/mL. The limits of agreement, which represent the 95% confidence interval for the mean difference, were calculated to be mean ±2 × standard deviation = –0.309 ± 2 × 0.722 = +1.135 and –1.753 mg/mL. This confidence interval includes zero, so there is no evidence for systematic bias. The plot in Fig. 3 shows that all differences are distributed between the limits of agreement, and there is no any obvious relationship between the difference and the average. The 95% confidence intervals calculated from the following equations [15]:

$$95\%\ \text{CI for mean bias} = \overline{d} \pm t \times \sqrt{\mathrm{SD}^2/n} \quad (2)$$

$$95\%\ \text{CI for upper limit} = (\overline{d} + 2SD) \pm t \times \sqrt{3\mathrm{SD}^2/n} \quad (3)$$

$$95\%\ \text{CI for lower limit} = (\overline{d} - 2SD) \pm t \times \sqrt{3\mathrm{SD}^2/n} \quad (4)$$

were: –0.694 to +0.076 for the mean difference, +0.469 to +1.801 for the upper limit of agreement and –2.419 to –1.087 for the lower limit of agreement. In Eqs. 1–3, $\overline{d}$ is the mean value, SD^2 is the variance of the difference, and t is the critical value for the 5% two-sided test drawn from tables of t distribution with $n - 1$ degrees of freedom (df), where n is the sample size.

In conclusion, the data presented here showed that the field method (^{31}P NMR) was comparable with the reference method (HPLC) within the acceptable precision. The NMR method shows two major advantages when compared with HPLC. First, a calibration with standards is not

Table 1 List of wine samples and glycerol content (in mg/mL) determined by ^{31}P-NMR spectroscopy and high-performance liquid chromatography

DO	Area	Grape variety	Vintage	^{31}P NMR	HPLC
Michalakis	Heraklion	Syrah-Mandelari	2003	8.07	7.54
Sitia	Sitia	Liatiko	2003	10.71	10.23
Karabitakis	Chania	Kotsifali-Mandelari	2003	11.07	11.81
Douloufakis	Daphnes	Liatiko	2003	10.02	9.62
Miliarakis	Peza	Kotsifali-Mandelari	2003	7.98	8.57
Papaioannou	Nemea	Agiorgitiko	2000	9.70	10.38
Dereskou	Trifilia	Cabernet	2002	11.25	12.14
Merkouri	Ilia	Refosco-Mavrodaphne	2003	10.59	10.36
Xatzimichalis	Atalanti	Cabernet	2002	9.77	10.43
Paros	Paros	Mandelari-Monenvasia	2004	8.99	9.75
Giannakoxori	Imathia	Xynomavro-Merlot	2002	8.39	10.01
Gerovasiliou	Epanomi	Syrah-Merlot	2002	9.19	10.48
Pyrgos Ioulia	Drama	Merlot	2003	9.65	10.20
Archodiko	Rhodes	Mandelari-Grenache	2002	8.71	8.69
Tsadalis	Chalkidiki	Krasato-Xynomavro	2000	10.81	10.52
Biblia Xora	Paggaio	Merlot-Cabernet	2002	10.49	9.60

DO designation of origin

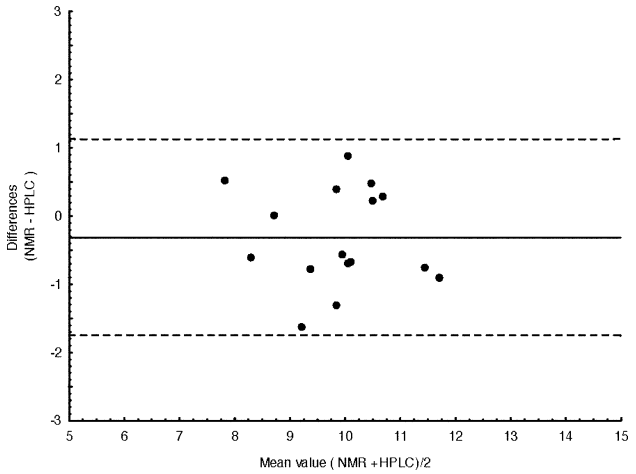


Fig. 3 Difference plot for the field method ^{31}P NMR with the reference method HPLC according to Bland and Altman. The *solid line* indicates the mean of the difference values in the ordinate. The *dotted lines* represent the limits of agreement

needed prior to the analysis which renders the NMR method somewhat faster than HPLC. The estimated duration of the analysis (removal of water, phosphitylation reaction, and recording the ^{31}P-NMR spectrum) was ~30 min; second, it gives well resolved signals in the ^{31}P-NMR spectra facilitating integration of the glycerol signals and thereby furnishing results with higher precision and accuracy. Disadvantages of the NMR methodology relative to HPLC may be its lower sensitivity and the higher cost of the analysis. The later is compensated by the fact that the ^{31}P-NMR method can be used for a simultaneous determination (a study is in progress in our laboratory) of glycerol, methanol, ethanol, organic acids and sugars (glucose, fructose), which bear readily exchangeable carboxyl and hydroxyl protons by the phosphorus reagent.

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