J Am Oil Chem Soc (2010) 87:29–34
DOI 10.1007/s11746-009-1466-4

ORIGINAL PAPER

Detection and Quantification of Free Glycerol in Virgin Olive Oil by ^{31}P-NMR Spectroscopy

Emmanuel Hatzakis · Alexia Agiomyrgianaki · Photis Dais

Received: 19 June 2009 / Revised: 13 August 2009 / Accepted: 20 August 2009 / Published online: 11 September 2009

Abstract ^{31}P-Nuclear magnetic resonance (NMR) spectroscopy was employed to detect and quantify free glycerol in virgin olive oils originating from various regions of Greece. This analytical method was based on the derivatization of the hydroxyl groups of glycerol with the tagging reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane, and identification of the phosphitylated compound on the basis of ^{31}P chemical shifts. Quantification of glycerol in olive oils was accomplished by integration of the appropriate signals in the ^{31}P NMR spectrum and the use of the phosphitylated cyclohexanol as internal standard. A linear correlation was observed between the glycerol content and 1,3-diacylglycerols and free acidity indicating that glycerol is the final product of the partial hydrolysis of triacylglycerols.

Keywords Glycerol · Olive Oil · ^{31}P-NMR Spectroscopy

Introduction

Glycerol is a chemical constituent of many food products and plays an important role in their quality. Free glycerol or in the form of triacylglycerols and phospholipids can be found in several food products, e.g., wine and other alcoholic beverages, fruit juices, honey, fats and lipids, and several other foodstuffs. Extra virgin olive oil contains glycerol mainly in the form of triacylglycerol (97–99% wt/wt) and to lesser extent in the form of diacylglycerols (up to 3% wt/wt) and monoacylglycerol (<0.3% wt/wt). Olive oil extracted from Greek olive tree varieties contains notable amounts of free glycerol, the origin of which might be attributed to enzymatic hydrolysis of its mono-, di-, and tri-esters with fatty acids.

Several methods have been developed to measure free glycerol in foodstuffs including lipids [1–10]. There are two main directions followed by these methodologies. The first involves the so-called chemical determination of glycerol, which is based on periodate oxidation of glycerol, leading to the formation of formaldehyde, which can be measured by various detection systems based on specific reactions with various reducing agents [5, 7]. An alternative procedure used for the indirect determination of glycerol, performs back-titration of periodate excess with an appropriate reducing agent [4, 8, 9]. The end point of the reaction was determined again spectrophotometrically, but at different wavelengths depending on the nature of the reaction product. The second analytical trend involves enzymatic analysis combining the selectivity of enzymes used with different detection systems [1, 10]. It should be noted that a few methods of determining glycerol [3, 6], including the official method adopted by AOAC [2], use gas and/or liquid chromatographic analyses after extraction of glycerol from the food product with an hydroalcoholic solvent.

The aim of the present study is to provide an alternative methodology based on ^{31}P-NMR spectroscopy for the detection and quantification free glycerol in virgin olive oil. This method, reported in an earlier publication [11], is based on the derivatization of the labile hydrogens of the primary and secondary hydroxyl groups of glycerol by the phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (I) according to the reaction scheme shown in Fig. 1, and the use of the ^{31}P chemical shifts to identify the labile centers (compound II). Compound I reacts rapidly

E. Hatzakis · A. Agiomyrgianaki · P. Dais (✉)
NMR Laboratory, Department of Chemistry, University of Crete, Voutes Campus, P.O. Box 2208, 71003 Heraklion, Crete, Greece
e-mail: dais@chemistry.uoc.gr

and quantitatively under mild conditions with the hydroxyl groups. This method has been applied previously to determine polyphenols [12], diacylglycerols, free sterols, and free acidity in virgin olive oil [11, 13], as well as glycerol in wine [14]. Furthermore, the origin of free glycerol in olive oil will be examined in this study by searching for possible relationships of its content with other compounds produced during the hydrolysis of triacylglycerols.

Materials and Methods

Olive Oil Samples

A total of 56 olive oil samples (harvest period 2005–2006) were obtained from two regions of Crete (Heraklion, Chania) and Peloponnesus (Messinia, Lakonia), one region of Macedonia (Chalkidiki) in northern Greece, and from the islands of Zakynthos and Lesvos. Fifty samples were extra virgin olive oils with free acidity $\leq 0.8\%$ in oleic acid, whereas the remaining six samples were lampante olive oils characterized by much higher free acidity (>0.8% in oleic acid) and/or total diacylglycerols (>3%). The choice of lampante olive oils was dictated by the need to widen the range of the glycerol content in order to perform linear regression analysis, as it will be shown later. The olive oils samples were provided by the MINERVA olive oil company (Athens, Greece). All olive oils were produced by the same method of extraction (centrifugation). Samples from Crete, Peloponnesus and Zakynthos were extracted from the olive varieties koroneiki and tsounati; samples from Lesvos were obtained from the cultivar adramitini, whereas those of northern Greece originated from different olive varieties (pasinolia, mayrolia, and a local variety). The olive oil samples were kept at −20 °C prior to analysis. All samples were analyzed twice on different days.

Chemicals

Pinacol, triethylamine, phosphorus trichloride, protonated solvents (reagent or analytical grade), and deuterated solvents used in the present study were purchased from Sigma–Aldrich (Athens, Greece). The derivatizing phosphorous reagent I was synthesized from pinacol and phosphorus trichloride following the method described in the literature [15]. However, to increase the yield of the reaction, we utilized hexane solvent instead of benzene and pyridine instead of triethylamine suggested in the original method [16]. This modification resulted in ~45% yield of the product against 19% obtained with the original method.

Extraction of Glycerol and Monoacylglycerol from Olive Oil

Glycerol and monoacylglycerols were extracted following the method developed by Montendoro et al. [17] well suited for the extraction of polar compounds in olive oil [18]. Briefly, 35 g of oil was dissolved in hexane (1:1 w/v), and extracted using 3×30 mL of methanol/water (80:20 v/v). The mixture was stirred for 2 min in a vortex apparatus and centrifuged at 3,000 rpm for 15 min. The extracts were combined and washed with 3×30 mL of *n*-hexane. The *n*-hexane was discarded, and the methanolic solution was evaporated under vacuum giving a residue (35–40 mg). The polar extracts were used immediately for sample preparation and ^{31}P-NMR measurements.

Sample Preparation for Spectral Analysis

A stock 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 mixture of pyridine and $CDCl_3$ solvents (1.6:1.0 volume ratio) and protected from moisture with Type 5A molecular sieves. 35–40 mg of the polar extract obtained from 35 g of olive oil were dissolved in 0.4 ml stock solution, in which 50 μl of the reagent I was added. The mixture was added directly into the 5-mm NMR tube and was left to react for about 15 min at room temperature). Upon completion of the reaction, the solution was used to obtain the ^{31}P-NMR spectra.

^{31}P-NMR Spectra

The ^{31}P-NMR spectra were obtained on a Bruker AMX500 spectrometer operating at 202.2 MHz for the phosphorus-

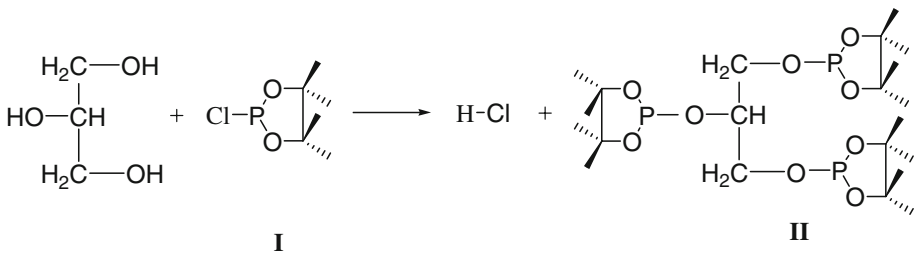

Fig. 1 Reaction of hydroxyl groups of glycerol with 2-chloro-4,4,5,5- tetramethyl dioxaphospholane (I)

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 32 K (zero-filled to 32 K). To ensure quantitative spectra, the magnitude of the relaxation delay adopted was more than five time 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, 16 transients were accumulated. All ^{31}P chemical shifts were relative to the product of the reaction of one with water (trace of water contained in all samples), which gave a sharp signal in pyridine/$CDCl_3$ at δ 132.20.

Results and Discussion

Figure 2 shows a typical ^{31}P-NMR spectrum of the polar part of olive oil in the region where the signals of the phosphitylated primary and secondary hydroxyl groups of glycerol resonate at δ 147.35 and δ 146.31, respectively [12]. Also, this spectrum shows a number of additional signals identified previously [12]. These signals belong to phenolic compounds free hydroxytyrosol, free tyrosol, and homovanillyl alcohol, the two isomers of monoacylglycerols, the triterpenic acid, maslinic acid and the two anomers of D-glucose (not assigned), all compounds co-extracted with glycerol [12]; The signal at δ 145.13 belongs to the phosphitylated hydroxyl group of the internal standard cyclohexanol which was used to quantify free glycerol according to the following equation

$$\text{Glycerol (mg/kg)} = \frac{\frac{[(I_p/2)+I_s]}{2I_{\text{cyclohexanol}}} \times A \times M_{\text{glycerol}}}{m} \quad (1)$$

where A is the weight of the internal standard (cyclohexanol) in μmol; M_{glycerol} is the molecular mass (92) of glycerol; m is the weight (35 g) of the olive oil; $I_{\text{cyclohexanol}}$, is the integral of the internal standard of cyclohexanol, I_p and I_s are the integrals of the primary and secondary phosphitylated hydroxyl groups of glycerol, respectively. Validation of the present ^{31}P-NMR methodology for the determination of glycerol in wine and several minor compounds in the polar fraction of olive oil has been performed rigorously in previous studies [11, 12, 14, 16, 19]. The amounts of various model compounds obtained from the ^{31}P-NMR spectra (in combination with the internal standard) were linearly correlated with known weighed quantities [11, 12, 14, 16] and also agreed very well with those obtained by conventional analytical methods [19], indicating that the aforementioned phosphitylation reaction is quantitative.

Table 1 summarizes the concentration of glycerol of the 56 samples of olive oil. Also, Table 1 contains the concentrations of 1,2-diacylglycerols (1,2-DGs), 1,3-diacylglycerols (1,3-DGs), total diacylglycerols (TDGs = 1,2-DGs + 1,3-DGs), the ratio D (=1,2-DGs/TDGs), 1- monoacylglycerols (1-MGs), 2-monoacylglycerols (2-MGs), total monoacylglycerols (TMGs = 1-MGs + 2-MDGs), and free acidity of the olive oil samples as determined by ^{31}P-NMR spectroscopy [11, 13]. Inspection of the data in Table 1 reveals that the glycerol concentration ranges between 0.03 and 7.46 mg/100 g oil, and it is independent of the geographical and varietal origin

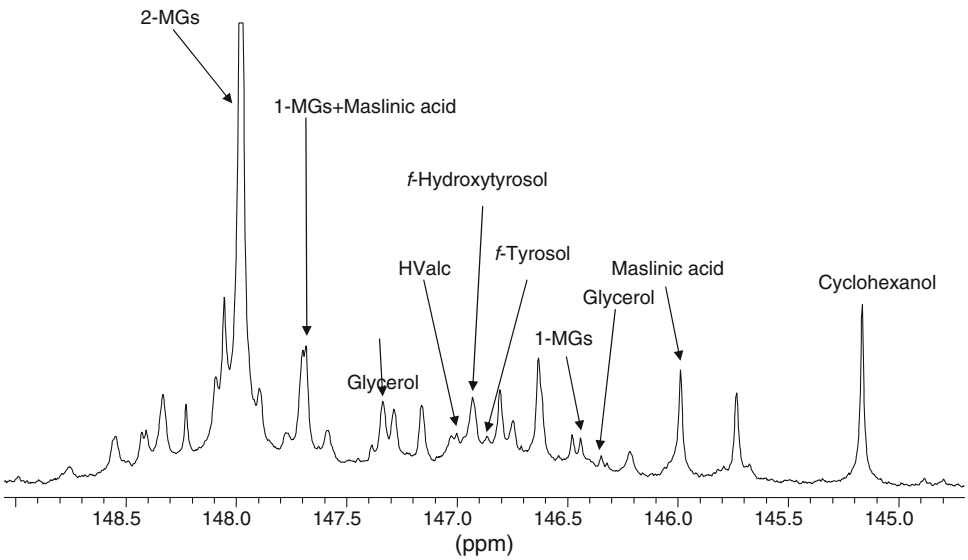

Fig. 2 202.2 MHz ^{31}P-NMR spectrum of the phosphitylated polar fraction of a virgin olive oil sample from Heraklion in chloroform/pyridine solution. The aliphatic region of the spectrum is shown. *1-MGs* 1-monoacylglycerols, *2-MGs* 2-monoacylglycerols, *f-hydroxytyrosol* free hydroxytyrosol, *f-tyrosol* free tyrosol, *HValc* homovanillic alcohol. *Cyclohexanol* is the internal standard

Table 1 Location and cultivar of olive oils

Sample	Location	Cultivar	Glycerol	1,2-DGs	1,3-DGs	TDGs	*D*	1-MGs	2-MGs	TMGs	Acidity
1	Messinia	Koroneiki	0.12	1.79	0.41	2.19	0.81	9.42	5.74	15.17	0.44
2	Messinia	MAYROLIA	0.07	2.22	0.17	2.39	0.93	4.72	3.05	7.76	0.07
3	Messinia	Mayrolia	0.09	2.04	0.17	2.21	0.92	2.91	7.31	10.23	0.04
4	Messinai	Mayrolia	0.90	1.31	0.31	1.62	0.81	4.91	10.54	15.45	0.47
5	Lesvos	Adramitini	0.28	1.18	0.14	1.32	0.89	3.32	44.16	47.48	0.13
6	Chalkidiki	Chalkidiki	0.62	1.40	0.06	1.45	0.96	7.56	65.20	72.76	0.24
7	Chalkidiki	Prasinolia	0.03	1.08	0.10	1.18	0.91	2.31	3.16	5.47	0.10
8	Chalkidiki	Prasinolia	0.03	0.96	0.11	1.07	0.90	1.86	2.65	4.51	0.09
9	Chalkidiki	Prasinolia	1.52	1.51	0.38	1.89	0.80	14.63	21.11	35.74	2.19
10	Chalkidiki	Chalkidiki	2.01	1.75	0.51	2.26	0.77	16.79	51.64	68.43	2.12
11	Chalkidiki	Chalkidiki	0.09	1.07	0.02	1.09	0.98	4.64	2.96	7.61	0.12
12	Lakonia	Koroneiki	0.19	1.70	0.11	1.81	0.94	3.12	10.21	13.32	0.24
13	Lakonia	Tsounati	0.65	1.36	0.03	1.39	0.98	3.33	52.54	55.87	0.10
14	Lakonia	Tsounati	0.22	1.40	0.02	1.42	0.98	3.85	13.37	17.22	0.12
15	Lakonia	Tsounati	0.04	1.53	0.07	1.59	0.96	2.64	17.66	20.30	0.06
16	Lakonia	Tsounati	0.47	1.27	0.05	1.31	0.97	2.82	67.41	70.23	0.07
17	Lakonia	Tsounati	0.36	1.33	0.01	1.34	0.99	2.56	36.68	39.24	0.07
18	Lakonia	Tsounati	0.72	1.43	0.11	1.55	0.93	1.57	40.47	42.04	0.08
19	Lakonia	Tsounati	0.26	1.48	0.09	1.57	0.94	1.21	92.67	93.88	0.12
20	Zakynthos	Koroneiki	0.19	1.75	0.24	1.99	0.88	8.04	9.41	17.45	0.25
21	Zakynthos	Koroneiki	0.39	1.67	0.09	1.77	0.95	4.09	82.83	86.92	0.15
22	Zakynthos	Koroneiki	0.14	1.15	0.05	1.19	0.96	5.64	4.52	10.17	0.12
23	Zakynthos	Koroneiki	0.08	1.66	0.11	1.77	0.94	1.24	14.02	15.26	0.16
24	Zakynthos	Koroneiki	0.18	1.77	0.14	1.90	0.93	5.32	11.03	16.35	0.21
25	Zakynthos	Koroneiki	0.05	1.30	0.05	1.35	0.96	7.62	8.51	16.12	0.06
26	Zakynthos	Koroneiki	0.12	1.54	0.15	1.69	0.91	6.14	80.57	86.71	0.22
27	Zakynthos	Koroneiki	0.49	1.83	0.30	2.13	0.86	4.22	36.65	40.86	0.18
28	Zakynthos	Koroneiki	0.35	1.61	0.25	1.86	0.86	3.93	17.75	21.69	0.15
29	Zakynthos	Koroneiki	0.17	1.85	0.39	2.24	0.83	4.33	8.89	13.22	0.17
30	Zakynthos	Koroneiki	0.04	1.25	0.03	1.28	0.98	3.71	9.19	12.90	0.06
31	Messinia	Koroneiki	0.13	1.68	0.17	1.85	0.91	3.21	3.21	6.42	0.04
32	Messinia	Koroneiki	0.37	2.31	0.24	2.55	0.91	3.25	18.65	21.90	0.05
33	Messinia	Koroneiki	0.05	1.70	0.07	1.77	0.96	2.53	3.76	6.29	0.07
34	Messinia	Koroneiki	0.32	1.33	0.05	1.38	0.96	3.50	2.10	5.60	0.04
35	Messinia	Koroneiki	0.05	1.22	0.04	1.26	0.97	3.53	6.01	9.54	0.03
36	Messinia	Koroneiki	0.37	1.64	0.14	1.78	0.92	4.90	8.33	13.23	0.09
37	Messinia	Koroneiki	0.38	1.52	0.11	1.62	0.93	1.32	133.86	135.19	0.06
38	Messinia	Koroneiki	0.64	1.57	0.05	1.62	0.97	3.24	14.43	17.67	0.07
39	Messinia	Koroneiki	0.33	1.68	0.17	1.84	0.91	2.82	17.88	20.70	0.06
40	Messinia	Koroneiki	0.25	1.81	0.31	2.12	0.85	3.83	3.99	7.82	0.12
41	Messinia	Koroneiki	0.15	2.26	0.25	2.51	0.90	2.87	12.62	15.49	0.20
42	Messinia	Koroneiki	0.12	1.64	0.19	1.83	0.90	3.38	12.01	15.39	0.05
43	Messinia	Koroneiki	0.18	1.93	0.30	2.22	0.87	3.11	37.85	40.96	0.13
44	Heraklion	Koroneiki	0.43	1.57	0.25	1.82	0.86	4.63	13.33	17.97	0.11
45	Heraklion	Koroneiki	0.45	1.68	0.28	1.96	0.86	6.17	14.27	20.44	0.13
46	Heraklion	Koroneiki	0.27	1.48	0.32	1.80	0.82	4.45	24.80	29.25	0.09
47	Lesvos	Adramitini	5.03	2.43	1.08	3.50	0.69	13.34	87.69	101.03	3.19
48	Lesvos	Adramitini	0.03	1.79	0.30	2.09	0.86	4.09	442.77	446.87	0.50

Table 1 continued

Sample	Location	Cultivar	Glycerol	1,2-DGs	1,3-DGs	TDGs	*D*	1-MGs	2-MGs	TMGs	Acidity
49	Lesvos	Adramitini	0.63	1.94	0.40	2.34	0.83	14.40	110.96	125.37	1.51
50	Lesvos	Adramitini	7.46	3.41	1.41	4.82	0.71	19.17	350.00	369.17	2.81
51	Lesvos	Adramitini	3.22	2.50	0.70	3.20	0.78	11.98	28.88	40.86	1.83
52	Chania	Koroneiki	0.25	1.67	0.18	1.85	0.90	2.43	20.66	23.09	0.33
53	Chania	Koroneiki	0.22	1.26	0.13	1.39	0.90	1.98	38.93	40.91	0.15
54	Chania	Koroneiki	0.16	1.27	0.09	1.36	0.94	3.11	4.24	7.34	0.08
55	Chania	Koroneiki	0.35	1.21	0.11	1.32	0.91	2.63	16.06	18.69	0.19
56	Chania	Koroneiki	0.40	1.44	0.12	1.56	0.93	3.86	74.40	78.26	0.12

Glycerol, 1-monoacylglycerols (1-MGs), 2-monoacylglycerols (2-MGs), Total monoacylglycerols (TMGs): mg/100 g oil. 1, 2-diacylglycerols (1,2-DGs), 1,3-diacylglycerols (1,3-DGs), Ratio D, Total diacylglycerols (TDGs): g/100 g oil, Free acidity: g/100 g oleic acid

of the olive oils. This observation leads to the conclusion that the content free glycerol in olive oil does not depend on environmental conditions or phylogenetic factors. The amounts of 1,2-DGs, 1,3-DGs, and TDGs are within the range expected for fresh extra virgin olive oils, except for three samples of lampante oils; TDGs in lampante olive oils exceeded the characteristic limit of 3% for extra virgin olive oils [13]. Integration of the signals of the secondary phosphitylated hydroxyl group of 1-MGs at δ 146.42 (the primary hydroxyl groups is overlapped by one of the phosphitylated hydroxyl group of maslinic acid) and 2-MGs at δ 148.06 in the ^{31}P-NMR spectrum (Fig. 2) in combination with the internal standard allowed the determination of these minor constituents of olive oils. In general, the concentration of 2-MGs is higher than that of 1-MGs, whereas the level of TMGs does not exceed the 0.3% usually measured in olive oil.

Regarding the origin of free glycerol, a logical hypothesis is to consider this compound as a product of the hydrolysis of triacylglycerols (TGs). In order to test this hypothesis, we plotted the glycerol content against the concentration of the minor compounds produced by the triacylglycerols hydrolysis, namely monoacylglycerols, and diacylglycerols, and free acidity. Plots of the glycerol concentration in 56 olive oils in Table 1 showed poor correlations with 1-MGs ($R = 0.738$), 2-MGs ($R = 0.469$) isomers, and 1,2-DGs ($R = 0.680$). Poor correlation was also observed for TDGs and TMGs against the glycerol concentration. In contrast a good linear relationship was obtained between glycerol and free acidity (free fatty acids) ($R = 0.867$), as well as between glycerol and the 1,3-DGs isomer ($R = 0.901$) demonstrating that free glycerol is the result of the partial hydrolysis of triacylglycerols. The lack of any correlation between glycerol and 1,2-DGs may be attributed to the fact that the hydrolysis of 1,2-DGs gives 2-MGs, which appear to be quite stable, not degrading to glycerol [20]. On the other hand, hydrolysis of 1,3-DGs provides directly glycerol and free fatty acids. 1,3-DGs and 1-MGs were considered as the secondary products of the isomerization of 1,2-DGs and 2-MGs by acyl migration from the *sn*-2-position to *rac*-1-position of the glycerol molecule [20, 21]. The isomerization reactions may represent the rate-determining steps of the hydrolysis of mono- and di-acylglycerols [20, 22], thus explaining the fact that the concentrations of 1,3-DGs and 1-MGs are less than those of 1,2-DGs and 2-MGs for freshly extracted olive oil (see Table 1).

Amongst the various methods for determining glycerol in food products mentioned in the introductory part only the periodate method has been applied to olive oil [4, 5]. Although analysis based on periodate oxidation of glycerol provides good linearity and low detection limits, it is not accurate especially when back-titration is used due to the complexity of the reactions. Moreover, the presence of foreign ions in foods may interfere seriously with the determination of periodate ions leading to erroneous band intensities [10]. Another problem frequently met with in the chemical analysis is the difficulty of marking the end point of titration with colored samples.

Regarding the other methods, which have not tested for the glycerol determination in fats and oils, the enzymatic methods imply the use of more than one complex enzymatic pathway and require a lengthy pretreatment, optimization of several parameters, and the preparation of many reagents. In addition, interferences resulting from reducing substances other than glycerol in most types of foods (e.g. ethanol, glucose) require further elaboration with hypochlorite solution in the extraction step [7]. Nevertheless, the use of immobilized enzymes on various supports (biosensors) appears to be very promising [16]. Chromatographic methods appear to be useful having exceptional sensitivity, but with unsatisfactory repeatability and reproducibility, since they require elaborate derivatization reactions and/or clean-up of the sample. In addition, the methods depend on the matrix in which glycerol is present and its relative concentration, thus suffering from interference from other food ingredients when applied to some kind of foods.

On the other hand, the ^{31}P-NMR methodology for the determination of glycerol in olive oil presented in this study is highly accurate and reproducible with good linearity over the concentration range of glycerol in olive oil, but higher detection limits [14] than those obtained by the periodate method [4]. In contrast to the other methods, no calibration with standards or the construction of calibration curves is required with the NMR method. Apart from glycerol, the ^{31}P-NMR analytical technique is capable of determining several minor compounds in a single experiment as shown in the present and previous studies [17, 18]. Disadvantages of ^{31}P-NMR spectroscopy may be considered the derivatization procedure, which lengthens somewhat the duration of the analysis relative to ^{1}H NMR, and the high cost of the analysis, since NMR spectrometers are quite expensive.

In summary, the main objective of the present study was the development of an easy method of determining free glycerol in virgin olive oil. This methodology can be extended to include other vegetable oils and lipid mixtures, such as biodiesel. In addition, this study suggests that glycerol may be considered as an additional indicator of hydrolysis of triacylglycerols, although further investigation is needed to unravel the hidden characteristics of hydrolysis of TGs in olive oil.

References

1. Takashi H, Mitsuhashi Y, Aoki N, Yamamoto S, Tsuji S, Ito Y, Oji Y (1991) Determination of glycerol in foods by high-performance liquid chromatography with fluorescence detection. J Chromatogr 541:265–272
2. AOAC Official Methods of Analysis 968.09, 972.10, 17th ed
3. Bonn G (1985) High-performance liquid chromatography of carbohydrates, alcohols and diethylene glycol on ion-exchange resins. J Chromatogr 350:381–387
4. Jie N, Yang D, Zhang Q, Yang J, Song Z (1998) Fluorometric determination of periodate with thiamine and its application to the determination of ethylene glycol and glycerol. Anal Chim Acta 359:87–92
5. Naviglio D, Romano R, Pizzolongo F, Santini A, De Vito A, Schiavo L, Nota G, Spagna Musso S (2007) Rapid determination of esterified glycerol and glycerides in triglyceride fat and oils by means of periodate method after transesterification. Food Chem 102:399–405
6. Bondioli P, Mariani C, Lanzani A, Fedeli E, Veronese S (1992) Vegetable oil derivatives as diesel fuel substitutes. Analytical aspects. Note 2: determination of free glycerol. Riv Ital Sostanze Grasse 69:7–9
7. Bondioli P, Bella LDella (2005) An alternative spectrophotometric method for the determination of free glycerol in biodiesel. Eur J Lipid Sci Technol 107:153–157
8. Verma K, Gupta D, Sangli S, Jain A (1987) Spectrophotometric determination of periodate with amodiaquine dihydrochloride and its application to the indirect determination of some organic compounds via the Malaprade reaction. Analyst 112:1519–1522
9. Gaikwad A, Silva M, Perez-Bendito D (1994) Sensitive determination of periodate and tartaric acid by stopped-flow chemiluminescence spectrometry. Analyst 119:1819
10. Lapenaite I, Ramanaviciene A, Ramanavicius A (2006) Current trends in enzymatic determination of glycerol. Crit Rev Anal Chem 36:13–25
11. Spyros A, Dais P (2000) Application of ^{31}P NMR spectroscopy in food analysis. Quantitative determination of the mono- and diglyceride composition of olive oils. J Agric Food Chem 48:802–805
12. Christophoridou S, Dais P (2006) A novel approach for detection and quantification of phenolic compounds in olive oil based on ^{31}P NMR spectroscopy. J Agric Food Chem 54:656–664
13. Fronimaki P, Spyros A, Christophoridou S, Dais P (2002) Determination of the diglyceride content in Greek virgin olive oils and some commercial olive oils by employing ^{31}P NMR spectroscopy. J Agric Food Chem 50:2207–2213
14. Hatzakis E, Archavlis E, Dais P (2007) Determination of glycerol in wines using ^{31}P-NMR spectroscopy. J Am Oil Chem Soc 84:615–619
15. Zwierzak A (1967) Cyclic organophosphorus compounds: synthesis and infrared spectral studies of cyclic hydrogen phosphites and thiophosphites. Can J Chem. 45:2501–2512
16. Hatzakis E, Dagounakis G, Agiomyrgianaki A, Dais P (2009) A facile NMR method for the quantification of total free and esterified sterols in virgin olive oil, *Food Chem* (submitted)
17. Montedoro G, Servili M, Baldioli M, Selvaggini R, Miniati E (1992) Simple and hydrolysable compounds in virgin olive oil: their extraction, separation and semiquantitative evaluation by HPLC. J Agric Food Chem 40:1571–1576
18. Morales MT, Tsimidou M (1999) The role of volatile compounds and polyphenols in olive oil sensory quality. In: Aparicio R, Harwood J (eds) Handbook of olive oil: analysis and properties, Chap. 12. Kluwer Academic/Plenum Publishers, Dordrecht, New York, pp 393–455
19. Dais P, Spyros A, Christophoridou S, Hatzakis E, Fragaki G, Agiomyrgianaki A, Salivaras E, Siragakis G, Daskalaki D, Tasioula-Margari M, Brenes M (2007) Comparison of analytical methodologies based on ^{1}H and ^{31}P NMR spectroscopy with conventional methods of analysis for the determination of some olive oil constituents. J Agric Food Chem 55:577–584
20. Lykidis A, Mougios V, Arzoglou P (1995) Kinetics of the two-step hydrolysis of triacylglycerol by pancreatic lipases. Eur J Biochem 230:892–898
21. Spyros A, Philippidis A, Dais P (2004) Kinetics of diglyceride formation and isomerization in virgin olive oils by employing ^{31}P NMR spectroscopy: formulation of a quantitative measure to assess olive oil storage history. J Agric Food Chem 52:157–164
22. Wang C-S, Hartsuck JA, Downs D (1988) Kinetics of acylglycerol sequential hydrolysis by human milk bile salt activated lipase and effect of taurocholate as fatty acid acceptor. Biochemistry 27:4834–4840