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^{13}C NMR Analysis of the Triacylglycerol Composition of Greek Virgin Olive Oils

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High-resolution ^{13}C NMR spectroscopy was used to study the potential of the method for the quantitative analysis of the most abundant fatty acids in the triacylglycerols of virgin olive oil samples harvested at different geographic areas of Greece. The analysis of the results was compared with data obtained with gas chromatography. Both methods showed results that differed only by 1–2 mol% of the fatty acids. NMR data provided the α/β ratio of oleic and linoleic acids. The results showed a preference for the β-position in these unsaturated acids. © 1997 John Wiley & Sons, Ltd.

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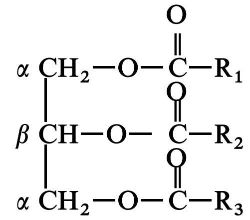
INTRODUCTION

The production of olive oil in the European Union is carried out mainly in Italy, Spain, Greece, Portugal and the southern regions of France. An added commercial incentive has been given to olive oil producers in the past few years with the considerable rise in consumer interest in olive oil as a 'health' product, in particular due to its high monounsaturated content.

Pure virgin olive oil is defined as an oil obtained only by mechanical means and this denomination is further subdivided according to free fatty acid content, expressed as oleic acid (EC Directive 136/66). Other categories for refined or solvent-treated oils are also defined. The premium attached to the higher quality categories obviously increases the risk of adulteration with cheaper products, which apart from disruption of the market by preventing fair competition, can also have severe health implications. Variations in the quality of pure virgin olive oil may also be linked to its geographical origin.

A method capable of checking whether an olive oil sample fulfils legal requirements and labelling legislation is therefore of great interest to national and EC Food Control Authorities, to the consumer and to the producer himself.

The composition of olive oil, and indeed of all vegetable oils, is generally defined in terms of the nature and distribution of the fatty acids present in the triacylglycerols and also the positions at which these fatty acids are attached to the glycerol backbone:



The acyl groups can be attached to the α (1,3-acyl) or β (2-acyl) group. In terms of triacylglycerols composition, olive oil contains mainly oleyl and linoleyl unsaturated groups in addition to palmitic and stearic saturated groups.

Both the acyl distribution and acyl positional distribution in the triacylglycerol mixtures can vary greatly between different oil varieties and, in the particular case of olive oil, may also depend on geographical and meteorological factors associated with the production area and the technique of production itself. A method which can provide both acyl distribution and acyl positional distribution would therefore be a powerful tool for olive oil characterization, either for the detection of adulteration with cheaper oils or for the authentication of geographical origin.

Gas chromatography (GC) is the standard method used so far for the quantitative determination of the triacylglycerol composition of olive oil. This is, however, a destructive method because it involves hydrolysis of triacylglycerols and methylation of the free fatty acids prior to their chromatographic analysis. Therefore, the information on the positional distribution of the individual fatty acid moieties on the glycerol backbone is

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lost. As an alternative to GC, ^{13}C NMR spectroscopy has been introduced as a technique which allows the determination of the more abundant fatty acid composition and positional distribution without any sample work-up.[1–18]

EXPERIMENTAL

Sample collection

Samples were obtained by trained samplers at the oil mills directly from the centrifugal separator during the production. This procedure eliminates any possible admixture with oils of different qualities in the storage tanks. At the same time, a questionnaire was completed containing information on the cultivar, climatic conditions, altitude of the olive oil grove, agronomic conditions, harvesting methods, type and age of the 'extraction' installation and processing conditions.

Sample sets

Virgin olive oils were obtained from different geographic areas of Greece (Peloponessos, Aegean Islands, Evia Island, Central Greece, Ionian Islands, North Greece) to cover all the important olive oil-producing areas of the country. Variations in the samples exist not only in their geographic area but also in their process and storage conditions. Samples were received in 1 l metallic cans, homogenized by shaking, poured into polypropylene flasks and stored in a dark locker at room temperature.

Fatty acid composition by GC

The fatty acid composition of the acylglycerols as their methyl esters was determined by GC. Methyl esters were prepared by *trans*-esterification of the acylglycerols with methanol using sodium methylate as catalyst and boiling under reflux for 10 min. Methyl esters were extracted from the mixture with *n*-heptane. An aliquot of 10 μl was injected in the chromatograph. The chromatographic analysis was carried out on an HP 5890 Series II Plus gas chromatograph (Hewlett-Packard) using a very polar capillary column of 50 m × 0.25 mm i.d. × 0.20 mm CP Sil 88 (Chrompack) with helium as the carrier gas. The oven temperature was isothermal at 170 °C and the injector (split) and flame ionization detector (FID) temperatures were 220 and 250 °C, respectively. Data acquisition and calculation of the results were performed with HP 3365 ChemStation software, which also controlled the instrument.

Preparation of NMR samples

A 40% (w/w) concentration of oil in $CDCl_3$ (*ca.* 1 ml) and a known amount (*ca.* 15 mg) of 1,4-diazine (pyrazine), as an internal reference, were added to a 5 mm NMR tube and mixed thoroughly by shaking several times. The complete dissolution of pyrazine is critical for NMR experiments because otherwise the peaks of the spectrum appear with wider linewidths that are difficult to simulate.

NMR spectroscopy

All spectra were recorded on a Bruker AC-300 spectrometer operating at 75.46 MHz. The temperature of 313 K was optimum since we obtained the best resolution in the spectra without significant evaporation of the deuterated chloroform solvent. Before Fourier transformation of FID, Gaussian multiplication (20% Gaussian enhancement and constructor parameter $LB = -0.092$) was applied. The tip angle used was 90°, the pulse width 6.2 μs and the acquisition time 5.44 s. The experiment was performed with an inverse gated pulse sequence in order to obtain fully decoupled spectra with no NOE. The repetition time during the experiment was 45 s (>5 times the longest T_1) and the number of scans was 300. In order to obtain a high digital resolution (0.092 Hz per point) which increases the precision of the peak simulation during the analysis of the spectra, the 32K time domain size of the FID was zero-filled to 128K.

RESULTS AND DISCUSSION

Triacylglycerol composition of the virgin olive oil

Our ^{13}C NMR study focused on the carbonyl and ethylenic carbon atom resonances of the triacylglycerols. The fatty acid carbonyl carbon atoms appear in the spectrum around 173 ppm and the ethylenic carbon atoms between 128 and 130 ppm. The spectra were recorded only in the region 105–195 ppm in order to obtain the highest resolution without excessively long acquisition. A margin of 20 ppm was left at each side of the spectrum in order for the peaks of interest not to fall at the edges of the window where they may receive less of the exciting power due to the filter applied by the spectrometer before detection.

The T_1 values for the oleoyl peaks of the virgin olive oils were measured in order to optimize the relaxation delay used in the inverse gated experiment. The values obtained (in seconds) for the different carbons are given below. The figures in parentheses correspond to those obtained by Sillerud *et al.*[19] for fat adipocytes using deuterated chloroform. They are in good agreement with those observed in our laboratory: α-C1 5.8 (5.9), β-C1 5.5 (5.95), α-C2 0.6 (0.76), β-C2 0.6 (0.72), α-C3 0.68, β-C3 0.7 (0.91), α-C9 1.5, β-C9 1.6 (1.8), α-C10 1.6, β-C10 1.6 (1.85), C16 3.2 (3.49), C17 4.0 (4.65) and C18 4.5 (5.22).

To quantify the NMR results obtained, the area of a particular peak is normalized (relative to the internal reference pyrazine) over the sum of all *n* components in the mixture according to the following general equations

$$S_i(\text{mol.\%}) = \frac{S_i}{\sum_{i=1}^{n} S_i} \times 100$$

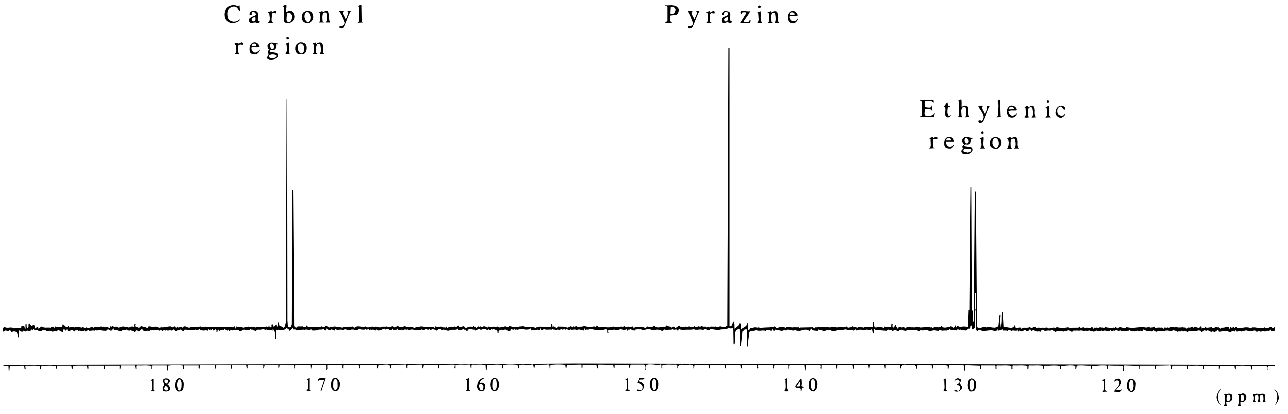

Figure 1. High-resolution ^{13}C NMR spectrum of a virgin olive oil from Chania district recorded on a Bruker AC-300 spectrometer.

Pyrazine was chosen as an internal reference, which allows the mass of the individual fatty acids to be calculated, for the following reasons: (a) it gives a readily soluble and non-volatile mixture; (b) it is a solid or non-volatile liquid; (c) it has a spin–lattice relaxation time, T_1, comparable to that of the triacylglycerol carbon atoms; (d) it has four chemically equivalent carbon atoms, which avoids the need for a large amount of substance; (e) it gives only one peak so that it can be used in isotopic NMR measurements; and (f) its chemical shift (144.8 ppm) is within the spectral window of interest and it does not coincide with the olive oil peaks. A whole ^{13}C NMR spectrum of a virgin olive oil in which the signal of the reference is prominent is shown in Fig. 1.

The spectra labelled A in Figs 2 and 3 are expansions of the ethylenic and carbonylic regions, respectively, of the ^{13}C spectrum obtained for a virgin olive oil sample from the district of Chania during the harvest period of 1996. Spectra from different other geographic regions in Greece are labelled B, C and D. The assignment of the peaks reported on the top spectrum was based on literature data.[6,7,9] Several samples from the most important olive oil-producing of various areas of Greece were analysed in order to check the reproducibility of the results. In Table 1 are given the origin, variety and physical properties of the 22 virgin olive oil samples analysed.

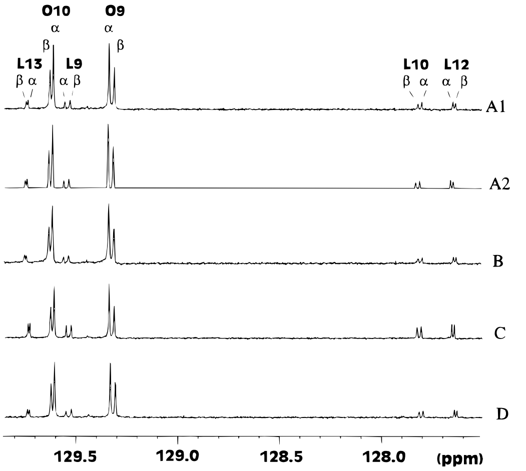

Figure 2. High-resolution ^{13}C NMR spectra of the olefinic region of virgin olive from (A) Chania district [(A1) experimental and (A2) simulated], (B) Lakonia district, (C) Evia Island and (D) Samos Island. The spectra were measured at 313 K on a Bruker AC-300 spectrometer.

Samples from the same area gave repeatably the same results within a 3 year period. Both NMR spectroscopic and GC results from sample oils collected from different geographic parts of Greece differed only by 1–2 mol% in the mean values for the major fatty acid composition. This error is reasonable if one takes into accounts that, (i) GC is a destructive method and is based on the transesterification of the fatty acids whereas NMR examines the fatty acids of the glycerol backbone intact; (ii) NMR encounters difficulties in the shimming and simulation process; (iii) difficulty in NMR analysis is also encountered with the peak which appears between the saturated fatty acids and O1α. This small peak is missing in several samples. There is some confusion as to the assignment of this small peak situated between the peak of the saturated acids and the α-oleyl

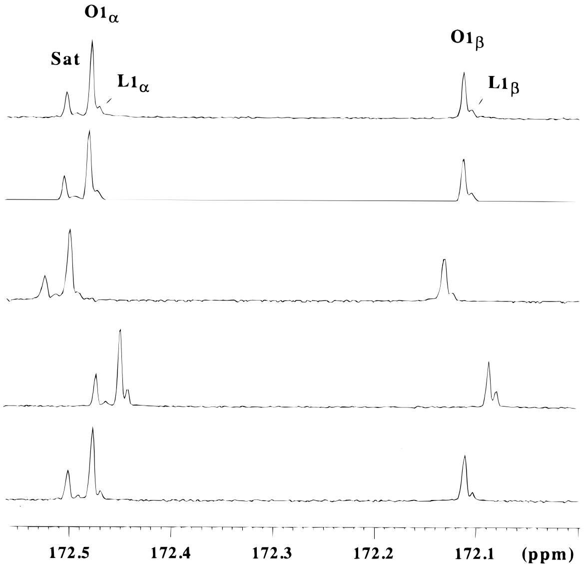

Figure 3. High-resolution ^{13}C NMR spectra of the carbonylic region of virgin olive oils from (A) Chania district [(A1) experimental and (A2) simulated], (B) Lakonia district, (C) Evia Island and (D) Samos Island. The spectra were measured at 313 K on a Bruker AC-300 spectrometer. The large expansion of the carbonylic region makes visible a one digital point shift.

Table 1. Content (mol.%) of the most abundant fatty acids of virgin olive oils harvested from different geographic areas of Greece

		Saturated		Oleic		Linoleic	
No.	Origin[a]	NMR	GC	NMR	GC	NMR	GC
1	Chania (C) (plain)	16.5	14.36	76.4	76.46	5.3	5.44
2	Chania (C) (mountain)	16.3	14.47	77.45	75.80	5.3	8.22
3	Heraklion (C) (south central, plain)	16.8	15.11	74.6	73.42	7.8	8.18
4	Heraklion (C) (south central)	18.1	16.11	70.1	70.20	9.9	10.05
5	Lassithi (C)	18.7	15.93	72.9	71.06	8.4	9.11
6	Lakonia (P) (plain)	17.6	15.08	74.8	73.96	6.2	6.74
7	Lakonia (P) (mountain)	17.3	15.26	71.2	70.04	9.6	10.27
8	Ilia (P)	13.2	14.72	76.4	74.59	6.6	6.73
9	Messinia (P) (northern)	16.8	15.54	74.2	72.85	7.5	7.85
10	Messinia (P) (southern)	17.7	15.78	72.2	71.82	7.5	7.85
11	Fthiotida (CG)	14.3	13.52	79.3	75.40	6.0	9.17
12	Lesvos (II)	16.3	14.45	75.8	73.00	6.9	10.65
13	Zakynthos (AI)	17.0	15.10	76.1	74.92	5.8	5.94
Mean		16.7	15.03	74.7	73.35	7.1	8.17
Standard deviation		1.5	0.73	2.6	2.10	1.5	1.65

[a] C, Crete; P, Peloponessos; E, Evia Island; CG, Central Greece; II, Ionian Islands (Eptanissa); AI, Aegean Islands.

peak of the carbonyl region. It was attributed to eicosenoic acid (C20:1), but quantitation of this peak in olive oil leads to a fivefold overestimation compared with the GC result. Since other monounsaturated fatty acids are present in olive oil, such as C18:1 $\omega - 7$, this peak may in fact represent several fatty acids that cannot be resolved at 75.46 MHz. This small peak makes the simulation a difficult task. Also, the analysis of the carbonyl region is difficult owing to overlap of the peaks. This difficulty could be overcome by using higher field instruments. Another issue that must be discussed in the analysis of olive oils is its time optimization in the magnetic field. If the sample remains overnight in order to increase the signal-to-noise ratio, then the drift of the magnetic field adversely affects the resolution of the carbonyl region. Thus, the increase in the linewidth of the peaks results in the disappearance of the peak between the saturated fatty acids and O1α. This shift of the resonance frequency with time is less obvious in the olefinic region.

Peak simulation

As mentioned above, the ^{13}C NMR spectrum of the carbonyl region suffers from the overlap of the peaks and does not allow their direct integration. To obtain their areas we used the 1D WIN NMR software provided by Bruker, which allows the use of different types of filter function. In our experiments we used the Lorentz Gaussian enhanced filter. Thus, the FID is multiplied by the time function $LG(t)$ which is defined as $LG(t) = EM(t)\exp[\pi LB(t_m{}^2 + t^2)/2t_m]$. $EM(t)$ stands for exponential multiplication and is given by $EM(t) = \exp(-\pi LBt)$. $t_m = GBt_{aq}$ and GB is set to 20% of the recording acquisition time (t_{aq}). The parameter LB must be entered with a negative value (the LB used was -0.092). The deconvolution of the peaks could be performed using the automatic or interactive options. In the automatic deconvolution the operation allows fitting of the spectrum with Gaussian or Lorentzian lineshapes. The region of the spectrum for the fit is selected. The individual fitted lines and the difference between the real and calculated spectra can be displayed. An option of reporting the number of peaks under fitting, their chemical shifts, their intensities and areas is available. The interactive deconvolution involves the set of initial values for the parameters controlling the iterative fitting process (position, height, width and half-height).

This analysis gave the most consistent results with GC and also the least errors in the integration of the peaks. We also applied simulation of the better resolved olefinic region (see spectra A2 in Figs 2 and 3). The mole percentage of the most abundant fatty acids derived from the peak simulation using NMR spectroscopy and GC data for the 13 samples investigated are shown in Table 1, which also contains their mean values and standard deviations from the mean values.

Calculation of the α/β ratios

The data contain information on the α/β ratio of oleic and linoleic acids of the two most abundant unsaturated acids of virgin oil. The mean values of the α/β ratio of L10α/L10β (or L12α/L12β) was calculated to be 1.1. The mean values of O9α/O9β (or O1a/O1b) were found to be 1.5. This indicates that unsaturated acids prefer the β-position. It appears that the increase in unsaturation in fatty acids parallels the preference for the β-position.

CONCLUSION

We have obtained quantitative results for the major fatty acids of virgin olive oils from different geographic areas of Greece using a combination of GC and ^{13}C NMR spectroscopy. Both methods gave similar results. GC can give more information on the fatty acid content of olive oil, whereas NMR gives additional information on the distribution of the major fatty acids present in the glycerol backbone. These α/β ratios may prove to be diagnostic parameters for the geographic origin and adulteration of olive oil with other oils. Preliminary results on these two aspects are promising and detailed results will be given in a subsequent publication. In addition, when NMR is compared with GC it has the following advantages: (a) it is not destructive, (b) it is easily quantified and (c) it is more versatile. The only disadvantage of the method is that the instrument is expensive. This disadvantage is, however, partially compensated by the fact that it does not involve any expense in the work-up of the sample. These advantages make NMR spectroscopy a promising method to study the geographic origin and adulteration of virgin olive oil.

Acknowledgement

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REFERENCES

1. D. J. Ashworth, D. O. Adams, B. Y. Giang, M. T.-H. Cheng and R. Y. Lee, *Anal. Chem.* **57**, 710 (1985).
2. F. D. Gunstone, *Chem. Phys. Lipids* **58**, 159 (1991).
3. F. D. Gunstone, *Chem. Phys. Lipids* **56**, 227 (1990).
4. F. D. Gunstone, *Chem. Phys. Lipids* **59**, 83 (1991).
5. G. Knothe, S. F. Marcel, K. L. Jie, C. C. Lam and M. O. Bagby, *Chem. Phys. Lipids* **77**, 187 (1995).
6. S. Ng and W. L. Ng, *J. Am. Oil Chem. Soc.* **60**, 7 (1983).
7. F. D. Gunstone, *Chem. Phys. Lipids* **56**, 201 (1990).
8. F. D. Gunstone, *J. Am. Oil Chem. Soc.* **70**, 361 (1993).
9. F. D. Gunstone, *Chem. Phys. Lipids* **56**, 195 (1990).
10. S. Ng, *Lipids* **20**, 778 (1985).
11. S. Ng, *J. Chem. Soc., Chem. Commun.* **00**, 179 (1983).
12. J. F. Mallet, E. M. Gaydou and A. Archavlis, *J. Am. Oil Chem. Soc.* **67**, 607 (1990).
13. F. D. Gunstone, *Chem. Phys. Lipids* **58**, 219 (1991).
14. K. F. Wollenberg, *J. Am. Oil Chem. Soc.* **67**, 487 (1990).
15. R. Sacchi, I. Medina, L. Paolillo and F. Addeo, *Chem. Phys. Lipids* **69**, 65 (1994).
16. R. Sacchi, I. Medina, S. P. Aubourg, F. Addeo and L. Paolillo, *J. Am. Oil Chem. Soc.* **70**, 3 (1993).
17. M. Yoshida, H. Kano, N. Ishida and T. Yoshida, *Agric. Biol. Chem.* **53**, 1395 (1989).
18. M. Bonnet and J. P. Renou, *Analusis* **18**, 122 (1990).
19. L. O. Sillerud, H. H. Chung, M. W. Bitensky and A. A. Francendese, *J. Biol. Chem.* **10**, 4380 (1986).