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Quantitative ^{13}C NMR Method Using the DEPT Pulse Sequence for the Detection of Olive Oil Adulteration with Soybean Oil

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The polarization transfer method DEPT (distortionless enhancement by polarization transfer) was employed to set up a quantitative method to detect olive oil adulteration by seed oils, in particular soybean oil. The DEPT pulse sequence promotes the transfer of polarization from 'concentrate spins = ^{1}H' to 'dilute spins = ^{13}C' via scalar coupling interaction. This improves the signal-to-noise ratios in the ^{13}C NMR spectra which would have to be acquired for quantitative purposes, under conditions of NOE suppression that reduce dramatically the carbon-13 resonance intensities. The resonance intensities of unsaturated carbons, C-9 for oleyl and C-10 for linoleyl and linolenyl chains, were used to detect olive oil adulteration by addition of soya oil at different levels. Calibration graphs based on a linear relationship between resonance intensities and soybean oil concentration were obtained. The limits of detection of the method were determined for each acyl chain. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Olive oil is obtained from the fruit of the evergreen tree *Olea europaea* essentially by crushing and pressing the olive paste. Unlike other vegetable oils, high-grade olive oils are obtained without further processing treatments. This peculiar property, along with nutritional benefits, makes high-grade olive oil a 'unique' commodity which, olive fruits being a very expensive fruit for growers and processors, also represents a 'high-price food.' This is the driving force behind the efforts by researchers to detect adulteration of olive oil by mixing with low-grade olive oils or with oils of different botanical origin, i.e. seed oils.

A reliable method for the detection of olive oil adulteration by seed oils is based on the prediction of the theoretical content of triglycerides of class ECN 42, where ECN is the equivalent carbon number defined as the carbon number minus two times the number of double bonds, by a computer method based on the 1,3-random-2-random distribution of fatty acids in olive oil triglycerides. The triglyceride class ECN 42, includes as the main triglyceride molecules trilinolein (LLL), 1-palmitoyl-2-linoleoyl-3-linolenoyl (PLLn), 1-linoleoyl-2-oleoyl-3-linolenoyl (LOLn) and positional isomers. The calculated value is compared with that obtained by high-resolution gas chromatography (HRGC) and reversed-phase high-performance liquid chromatography (RP-HPLC).[1] By referring to predetermined difference ranges between calculated and experimentally obtained HPLC values, the method appears to overcome the intrinsic difficulty represented by the very high variability of olive oil fatty acid compositions.

Nevertheless, in previous mono- and intervarietal stereospecific analysis studies of olive oil triglycerides, ^{13}C NMR data relative to carbonyl carbon resonances indicated that the unsaturated chains, linoleyl more than oleyl, are present in 2-positions at a higher level than predicted by 1,3-random-2-random theory.[2,3] Moreover, the long-accepted general assumption of a semi-random distribution of fatty acids in natural triacylglycerols is now being abandoned, in addition to the notion that each position of triacylglycerol is esterified independently of the other two from separate pools of fatty acids.[4]

The positional distribution of acyl chains in different vegetable oils has been defined by means of carbonyl[5,6] and olefinic carbon regions.[7] High-resolution ^{13}C NMR spectra of the olefinic and carbonyl carbons of triglycerides of different vegetable oils were run under rigorously quantitative conditions.[8] The inverse-gated proton decoupled experiment with NOE signal enhancement suppression was adopted; relaxation delays, long enough to avoid signal saturation, were set. As a consequence, acquisition times to achieve signal-to-noise ratios providing accurate integrals were extremely long.

The difficulties that have been faced in conventional quantitative ^{13}C NMR led the author to focus on polarization transfer experiments to detect protonated

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carbons at higher sensitivity resulting from spin population transfer from the abundant spin ^1H to the dilute spin ^{13}C. In particular, the ^{13}C NMR DEPT methodology was applied to measure quantitative spectra to detect olive oil adulteration with oils containing acyl chains of higher degree of unsaturation, in particular soybean oil. In addition to detecting the adulterant, this method also determines the extent of adulteration.

EXPERIMENTAL

^{13}C NMR spectra

Extra virgin olive oil samples, extracted in the Institute oil-mill, and a soybean oil obtained commercially, were mixed at different concentrations up to a final weight of 200 mg and diluted with 0.5 ml of $CDCl_3$ for NMR spectroscopy.

The distortionless enhancement by polarization transfer (DEPT) experiment was carried out to increase sensitivity of carbon-13 nuclei and the pulse sequence for *J*-coupled ^1H and ^{13}C nuclei is shown below:

^1H:D1–90–D2–180–D2–P0–D2–decoupling

^{13}C: 90 180 FID

The spectra were registered at 25 °C using a Bruker AC 300 spectrometer and run over a spectral width of 9800 Hz with 64K acquisition points zero-filled to 128K points. The proton pulse P0 was set at 45° to have positive carbon-13 CH, CH_2 and CH_3 multiplicities. The delay D2 for optimum polarization transfer was calculated on the basis of the $0.5/J_{C,H}$ relationship by using 144 Hz as the C–H coupling constant, i.e. the average of the one-bond C–H coupling values which had been determined experimentally.

To prevent signal saturation, the D1 delay was five times higher than the longest T_1 longitudinal relaxation time of proton nuclei. The inversion–recovery method was adopted to measure the proton T_1. Methyl protons were shown to have the longest T_1 (1.5 s), as a result of the increasing flexibility of the molecule moving down the chain from the glycerol backbone to the methyl end.

The DEPT spectra were measured under proton decoupling; 672 scans were registered during the 2 h experiment. Resolution enhancement was achieved by using a Gaussian filter of 0.12 Hz Lorentzian narrowing and 0.08 Gaussian broadening.

Statistical analysis

Lines of regressions were calculated with Microsoft Excel version 5.

RESULTS AND DISCUSSION

DEPT pulse sequence

The primary condition which must be fulfilled to perform quantitative carbon magnetic resonance is to accumulate noise-free spectra. Furthermore, all the resonances must have the same intrinsic intensity. In proton decoupled spectra, nuclear Overhauser enhancement (NOE) can affect the proton-substituted carbon intensities to different extents and, on the other hand, saturation decreases the intensity of carbons having long relaxation times. The distortions of carbon-13 nuclei intensities can be corrected by switching the decoupler on only during the acquisition time, i.e. by using the inverse-gated experiment to suppress NOE, and by using relaxation delays five times higher than the longest T_1 to recover all the resonances between subsequent transmitter pulses. The NOE quenching further reduced the intrinsic low sensitivity of carbon-13 resulting from a low natural abundance (1.1%) and a gyromagnetic ratio four times lower than the proton ratio. These considerations, coupled with the long carbon-13 longitudinal relaxation times which determine the repetition rate, made acquisition times which are needed to register quantitative spectra, extremely long.

The methods of carbon-13 signal enhancement known as polarization or population transfer, i.e. INEPT and DEPT methods, overcome the sensitivity limitations of conventional ^{13}C NMR spectroscopy by transferring populations from proton 'concentrate spin' to carbon-13 'dilute spin,' provided that the nuclei are *J*-coupled. Moreover, the pulse repetition rate is now determined by the T_1 relaxation times of ^1H nuclei, which are considerably shorter. Both of these facts provide improved sensitivity enhancement of the carbon-13 nucleus, in shorter experimental times, by a factor proportional to γ_H/γ_C, where γ_H and γ_C are the gyromagnetic ratios of proton and carbon-13 nuclei, respectively.

The DEPT pulse sequence was adopted to run ^{13}C quantitative and fully enhanced spectra. The DEPT experiment guarantees INEPT enhancements but, as it contains fewer pulses, the error sources are reduced, thus promising more reliable quantitative results.[9]

The drawback of the DEPT pulse sequence is that the resonances of unprotonated carbons, i.e. carbonyl carbons, of triglyceride acyl chains ranging between 172 and 174 ppm cannot be detected. The carbonyl region plays a key role in determining the triglyceride acyl chain composition except for linoleyl and linolenyl chains, which overlap, because it is the sole spectral range which permits saturated chain detection.[8]

Nevertheless, the DEPT methodology can be used in detecting olive oil adulteration with soybean oil, which is characterized by a higher content of linoleyl and linolenyl chains. DEPT took full advantage of the fact that the resonances of olefinic carbons of oleyl, linoleyl and linolenyl chains were baseline resolved (Fig. 1) thus giving the possibility of obtaining an almost complete profile of the unsaturation pattern of the oil being analysed.

Analysis of ^{13}C NMR DEPT spectra: chemical shift assignments

The resonances of unsaturated carbons in the 132–126.9 ppm range were assigned on the basis of chemical shifts of standard compounds (Table 1). The chemical shift

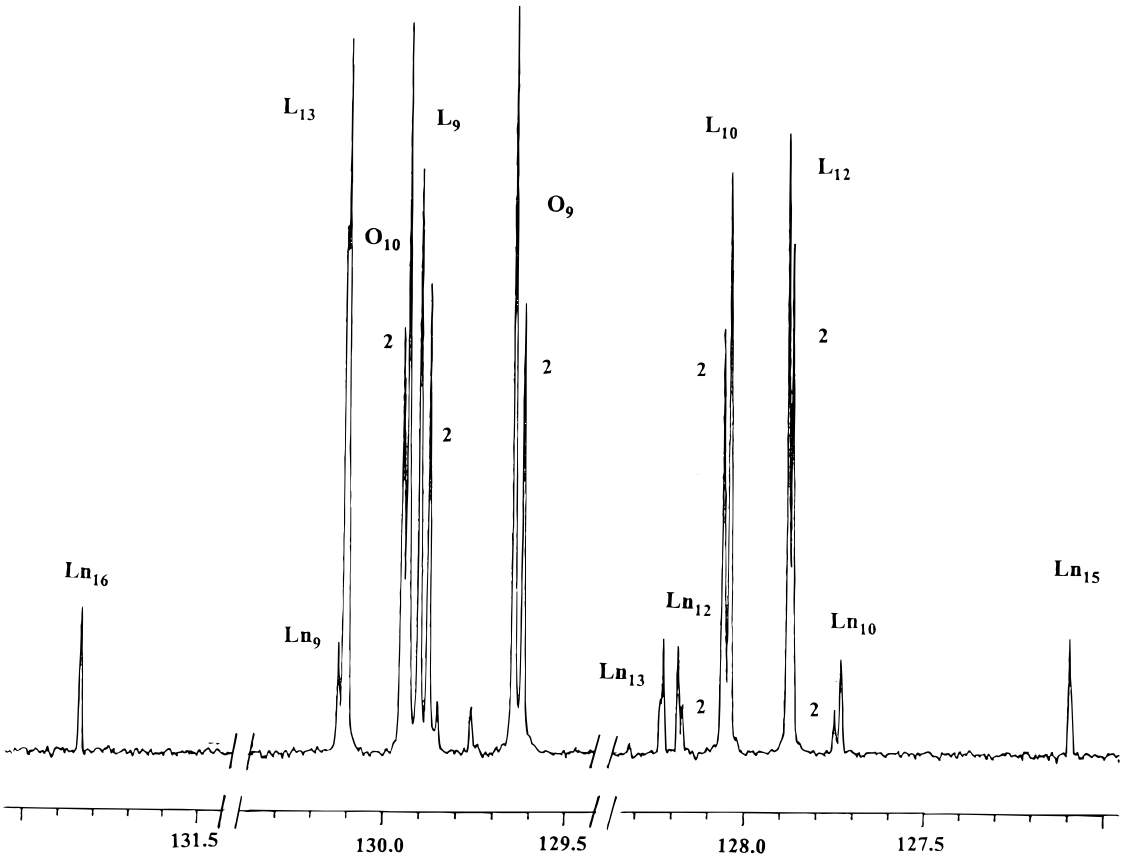

Figure 1. ^{13}C NMR DEPT spectrum of olive oil adulterated by mixing with soybean oil: olefinic spectral region. The 2-position is indicated for oleyl (O), linoleyl (L) and linolenyl (Ln) chains.

differences between each pair of double bond carbons fitted in with the values predicted by the σ-inductive through-bond mechanism of polarization transmission in non-conjugated polyenoic acids.[10]

The unsaturated carbons of oleyl, linoleyl and linolenyl chains were baseline resolved with the only exception of C-9 of the linolenyl chain, which overlaps with the linoleyl C-13.

Each unsaturated carbon signal was split depending on the chain position on the glycerol. In particular, the carbon of double-bond pairs which is closer to the carbonyl chain end, showed a positive chemical shift difference between the 1,3- and 2-positions, and this value was found to become smaller on moving down the chain towards the methyl end. The shift difference observed for the C=C carbon further from the carbonyl chain end followed the same trend but with negative values. These shift differences, registered for 1, 3- and 2-chains, seemed to be independent of acyl chains.

No splittings related to the different positions of the linolenyl chain on the glycerol molecule were detected for C-15 and C-16.

Quantitative analysis of ^{13}C NMR DEPT spectra

^{13}C NMR DEPT spectra were measured for 13 standard samples prepared by mixing high-quality olive oil with soybean oil in the 0–100% full range, keeping the final sample weight at 200 mg.

C-9 of the oleyl chain and C-10 of the linoleyl and linolenyl chains were the only resonances that showed fully resolved 1,3- and 2-splittings and they were chosen

Table 1. ^{13}C NMR chemical shifts (ppm) of double bond carbons of triglyceride acyl chains in vegetable oils

Carbon	C18:1 9c[a]			C18:2 9,12c[a]			C18:3 9,12,15c[a]		
No.	1,3-Position	2-Position	$\Delta\delta$[b]	1,3-Position	2-Position	$\Delta\delta$[b]	1,3-Position	2-Position	$\Delta\delta$[b]
9	129.641	129.616	+0.025	129.900	129.874	+0.026	130.120[c]	—	—
10	129.931	129.946	−0.015	128.037	128.056	−0.019	127.729	127.747	−0.018
12				127.876	127.864	+0.012	128.180	128.167	+0.013
13				130.097	130.105	−0.008	128.220	128.231	−0.011
15								127.093	—
16								131.836	—

[a] C18:1 9c, oleic acid; C18:2 9, 12c, linoleic acid; C18:3 9,12,15c, linolenic acid.
[b] Chemical shift differences between double bond carbon resonances of acyl chains at 1, 3- and 2-position, of glycerol.
[c] Uncertain assignment.

to assess the suitability of the DEPT pulse sequence as a quantitative methodology.

The resonances were integrated by the software provided with the spectrometer. The areas of oleyl C-9, of linoleyl, linolenyl C-10, and soybean oil percentages in olive oil were used to construct calibration graphs. The negative slope of the oleyl chain C-9 curve was explained by the different acyl chain patterns of soybean and olive oils, the former being characterized by 'high linoleic acid' and the latter by 'high oleic acid.'

The correlation coefficients r, which were found to be very close to $+1$, indicated that peak areas and soybean oil concentrations were linearly correlated.

The best straight lines were calculated using the calibration graph points by means of the least-squares method and Table 2 summarizes all the calculations. The F values were constantly higher than critical F values for the $P = 0.05$ significance level, thus guaranteeing that linear correlations were not random. The slopes of the calculated regression lines for oleyl and linoleyl chains in the -2.0×10^{-2} to $+3.0 \times 10^{-2}$ range were higher than the linolenyl chain slopes which for both 1, 3- and 2-resonances showed values below 5.0×10^{-3}. These results indicated that ^{13}C NMR detection was more sensitive for oleyl and linoleyl chains.

The limit of detection of an analyte is the minimum concentration that leads to an instrumental signal significantly different from the background signal.[11] The limits of detection for each chain were calculated. The oleyl and linoleyl chain limits were found to be half those for the linolenyl chain. These results confirmed that the sensitivity and limit of detection of unsaturated carbon resonances of the linolenyl chain made this chain less suitable than oleyl and linoleyl chains for the detection of soybean oil adulteration of olive oil.

The precision of the ^{13}C NMR DEPT method was also checked using an olive oil sample adulterated by mixing it with 17% soybean oil. The error for soybean oil concentration, calculated by using the regression lines for oleyl and linoleyl chains, was determined.

The soybean oil percentage y_0 was derived by integrating the C-9 and C-10 resonances relating to oleyl and linoleyl chains, respectively, both esterified at the 1,3-positions. The 95% confidence limits were calculated as $x_0 \pm ts_{x0}$, where t for $n-2$ degrees of freedom $= 2.201$ and the number of calibration points $n = 13$. The standard deviation of x_0, s_{x_0}, was calculated using the following equation:[11]

$$s_{x_0} = s_{y/x}/b[1/m + 1/n + (y_0 - \bar{y})^2/b^2 \times \Sigma(x_i - \bar{x})^2]^{1/2}$$

where $s_{y/x}$ = standard deviation, b = slope of the regression line, m = number of measurements required to obtain y_0, $\bar{y}$ = mean y value and $\bar{x}$ = mean x value. The confidence limits were calculated and are reported in Table 3. The confidence limits decreased by 10.8% down to 4.7% when the number of y_0 measurements m increased from 4 to 10. The confidence limit improvement resulting from more than 10 repeated measurements would not be so convenient to justify highly time-consuming NMR work. The oleyl and linoleyl chain confidence limits were almost identical.

Table 2. Parameters of regression lines calculated for oleic, linoleic and linolenic acids for an olive oil adulterated with increasing soybean oil levels

Fatty acids	Slope	Intercept	N^a	r^b	$s_{y/x}{}^c$	F^d	LOD (%)[e]
C18:1 9c:							
C-9 1,3-	$-0.030 \pm 6.4 \times 10^{-4}$	$4.41 \pm 3.6 \times 10^{-2}$	13	0.998	0.071	2211	6.9
C-9 2-	$-0.023 \pm 6.0 \times 10^{-4}$	$2.98 \pm 3.3 \times 10^{-2}$	13	0.996	0.066	1493	8.3
C18:2 9,12c:							
C-10 1,3-	$0.025 \pm 4.6 \times 10^{-4}$	$0.33 \pm 2.5 \times 10^{-2}$	13	0.998	0.050	3095	5.9
C-10 2-	$0.020 \pm 4.2 \times 10^{-4}$	$0.37 \pm 2.3 \times 10^{-2}$	13	0.998	0.046	2325	6.8
C18:3 9,12,15c							
C-10 1,3-	$0.005 \pm 1.4 \times 10^{-4}$	$0.026 \pm 8.0 \times 10^{-3}$	13	0.995	0.016	1046	10.2
C-10 2-	$0.002 \pm 1.4 \times 10^{-4}$	$0.033 \pm 7.9 \times 10^{-3}$	13	0.980	0.016	266	20.2

[a] Number of calibration points.
[b] Correlation coefficient.
[c] Standard deviation.
[d] Observed value for F statistic.
[e] Limit of detection of soybean oil in adulterated olive oil.

Table 3. Influence of measurement number on confidence limits

Repetition number	Confidence limits for oleyl C-9 1,3-position	Decrease (%)	Confidence limits for linoleyl C-10 1,3-position	Decrease (%)
4	±3.30	—	±2.81	—
6	±2.94	10.9	±2.51	10.7
8	±2.74	6.8	±2.34	6.8
10	±2.61	4.7	±2.23	4.7

In conclusion, the general principle whereby no analytical results can be considered to have a quantitative value unless the errors are estimated, was applied throughout this research. The spectroscopic data, which were obtained by applying the ^{13}C NMR DEPT method to detect the adulteration level of olive oil samples mixed with soybean oil, were used to calculate the calibration curve. The errors involved in calculating the best straight lines were estimated together with the errors we made using the curve to determine the soybean oil concentration in an adulterated olive oil. The limits of detection of the method were also estimated for the unsaturated carbons of the oleyl, linoleyl and linolenyl chains.

The results of this procedure showed the reliability of the ^{13}C NMR DEPT pulse sequence as a quantitative method to detect olive oil adulteration with soybean oil. In a more general context, this work confirmed that ^{13}C NMR spectroscopy can be regarded as a new approach to food analysis offering the advantages of no sample preparation or pretreatment and the acquisition of exhaustive structural information.

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REFERENCES

1. N. Cortesi, P. Rovellini and E. Fedeli, *Riv. Ital. Sostanze Grasse* **47**, 69 (1990).
2. G. Vlahov, *Fett*/Lipid **98**, 203 (1996).
3. G. Vlahov, A. Di Camillo and C. Masci, *J. Magn. Reson. Anal.* **2**, 253 (1996).
4. A. Kuksis, *Advances in Lipid Methodology—Three*. Oily Press Dundee (1996).
5. S. Ng, *J. Chem. Soc., Chem. Commun.*, 179 (1983).
6. S. Ng, *Lipids* **20**, 778 (1985).
7. S. Ng, *Lipids* **19**, 56 (1984).
8. K. F. Wollemberg, *J. Am. Oil. Chem. Soc.* **67**, 487 (1990).
9. M. R. Bendal, D. M. Doddrell, D. T. Pegg and W. E. Hull, *Bruker Rep*. (1983).
10. O. W. Howarth, C. J. Samuel and G. Vlahov, *J. Chem. Soc., Perkin Trans 2*, 2307 (1995).
11. J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester (1993).

