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# $^{13}$C nuclear magnetic resonance spectroscopy to check 1,3-random, 2-random pattern of fatty acid distribution in olive oil triacylglycerols

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**Abstract.** The fatty acid distribution between the 1,3- and 2-positions of triglycerides was determined in the olive oil set sampled in the Marche region, by using the $^{13}$C NMR method which applies the DEPT pulse sequence. The results confirmed that the oleic and linoleic acids were not randomly distributed at the 2-position of triacylglycerols but were lower for oleic acid and higher for linoleic acid compared to the theoretical values expected for a random distribution. Moreover, the unsaturated acids deviated from the 2-random distribution at different extents according to the acid concentration of triglyceride.

The $^{13}$C NMR results, they were obtained for three olive oil samples characterized by high, medium and low oleic acid at 2-position ($O_{found}$) corresponding to a low, medium, high deviation rate from the 2-random pattern, were compared to those calculated by the computer program based on the 1,3-random–2-random distribution of fatty acids in the triglycerides.

The molar concentrations of the triglyceride species LLL and OLL were calculated from the molar concentrations of oleic and linoleic acids at the 2- and 1,3-positions obtained by using $^{13}$C NMR and the computer method. The LLL and OLL triglycerides determined by the computer method were found to be markedly higher in the "high $O_{found}$" oil as compared to NMR. No differences were detected between the two methods for both triglyceride species in the "low $O_{found}$" oil.

## 1. Introduction

The general theories of semi-random fatty acid distribution in natural triacylglycerols appeared to be inadequate to predict their structure in vegetable oils [1].

$^{13}$C NMR spectroscopy proved to be a powerful tool for determining the triacylglycerol structures. The calculations of the composition and distribution of fatty acids between 1,3- and 2-positions of triacylglycerols can be easily performed by using the quantitative high resolution $^{13}$C NMR methodology based on carbonyl carbons of triacylglycerol acyl chains. The carbonyl carbons resonate according to both chain unsaturation degree and glycerol positions, in the frequency range from 172.5 to 173.5 ppm [2–5].

The carbonyl carbon resonances of saturated, oleate and linoleate chains attached at 1,3-glycerol positions, and those of oleate and linoleate at 2-position are almost baseline resolved with the exceptions of linoleate and linolenate carbons which overlap.

However, the quantitative NMR method based on carbonyl carbon resonances, for acquisition of proton-decoupled spectra with full NOE enhancement, does not overcome the long experiment times due to long spin lattice relaxation times $T_1$ of carbonyl carbons [6].

Nevertheless, the large $^{13}C$ NMR data set for olive oil characterization (continuously updated) was available. The quantitative $^{13}C$ NMR spectra, which are acquired by applying the DEPT (distortionless enhancement by polarization transfer) pulse sequence, do not detect carbonyl carbon resonances but in spite of this, the resolution of different chains according to their positions on glycerol can be achieved [7].

Considering that a high number of olive oil data, accounting for maximum variability of acyl chain composition of olive oil triglycerides, could safely support any evidence of new distribution patterns, the 1,3-random–2-random pattern of fatty acid distribution among the triacylglycerol positions, was checked by using $^{13}C$ NMR DEPT data.

## 2. Materials and methods

### *2.1. Materials*

Olive oils were sampled during the oil campaigns from 2000 to 2002 in four production zones of the Marche Region corresponding to the areas of the Ancona (A), Ascoli Piceno (B), Pesaro (C) and Macerata (D) provinces. The sampling rate during the three campaigns was of 54, 94, 78, and 83 olive oil samples for the areas of Ancona, Ascoli Piceno, Pesaro and Macerata, respectively, for a total of 309 oil samples.

### *2.2. $^{13}C$ NMR spectroscopy*

Two hundred milligrams of oil sample (200 mg) were dissolved in 0.5 ml of deuterated chloroform ($CDCl_3$) Sigma Aldrich, Milano, Italia. The spectra were run at 25°C on a Unity Inova Narrow Bore 500 MHz spectrometer equipped with a UNIX-based Sun Microsystems workstation (Varian NMR Instruments, Palo Alto, California).

The $^{13}C$ spectra were measured by using the quantitative $^{13}C$ NMR DEPT methodology [7]. The polarization transfer from $^{1}H$ to $^{13}C$ J-coupled nuclei applied by the DEPT pulse sequence, was optimized by setting the C–H coupling constant for the calculation of polarization transfer delay (it is equal to $1/2$ $J_{C-H}$) at 144 Hz. It was obtained by averaging the C–H couplings of acyl chain carbons (they ranged from 124 to 152 Hz for C-18 and C-9 carbons, respectively).

The spectra were acquired under proton decoupling (Waltz-16 broadband decoupling), with 128 K data points, an acquisition time of 2.9 s, and a delay between pulses of 10 s. Signal averaging was carried out for 128 transients.

The zero-filling at a power of $2^2$ and a resolution enhancement function, were applied to the free induction decays (FIDs) before Fourier transformation to improve resolution and sensitivity of $^{13}C$ resonances.

The resonance intesities of $^{13}C$ nuclei of triglyceride acyl chains were integrated by the software provided with the spectrometer.

### *2.3. Determination of the fatty acid composition by gas chromatography (HRGC)*

The methyl esters of fatty acids (they were prepared from olive oil samples by ambient temperature transmethylation with sodium methoxide [8]) were analyzed by a HRGC Mega 2 gas chromatograph

(Carlo Erba Instruments, Milano, Italy) equipped with an on-column mode injection system, flame ionization detector, and a SP-2380 fused silica capillary column ($60 \times 0.32$ mm i.d., 0.15 $\mu$m film thickness) (Supelco, Sigma Aldrich S.r.l., Milano, Italy). The oven temperature was increased at 30°C/min from 120 to 165°C, and at 5°C/min to 200°C, with detector port temperature set at 260°C and hydrogen carrier gas at flow rate of 1 ml/min.

### *2.4. Data processing*

Minitab Statistical package (Minitab, release 13.20) was used for mathematical processing and statistical analysis of the $^{13}$C NMR data set.

## 3. Results and discussion

### *3.1. $^{13}$C NMR study of olive oils sampled in the Marche region*

According to the 1,3-random–2-random pattern, two different pools of fatty acids are randomly and separately distributed between the 1,3- and 2-positions of triacylglycerols of vegetable oils, the saturated acids, i.e. palmitic and stearic acids, being esterified by almost 100% at 1,3-positions [9,10].

Considering the availability of the large set of quantitative $^{13}$C NMR data measured on the olive oils produced in the Marche region, the presumed random distribution of oleate and linoleate chains at the 2-position of olive oil triacylglycerols was checked.

The percentages of oleic ($O_{theory}$) and linoleic ($L_{theory}$) acids in the total of oleic (Otg) and linoleic (Ltg) acids in the triglyceride were compared to the percentages of oleic ($O_{found}$) and linoleic ($L_{found}$) acids in the total of these acids (O2, L2) at the 2-position [9].

The resonances of carbons C-16 and C=C carbons, were used for determining the compositional ($O,L_{theory}$) and 2-positional ($O,L_{found}$) profiles of oleic and linoleic acids of olive oil triacylglycerols, respectively. The two carbon sets were selected on the basis of the same $J_{C–H}$ coupling within each set, to overcome the resonance intensity distortions which were likely to be detected throughout the $^{13}$C spectrum. They were a consequence of the compromise which used the averaged C–H coupling constant for optimizing the DEPT pulse sequence [11].

The resonances of C-16 carbons of oleate (31.92 ppm) and linoleate (31.53 ppm) chains (which were detected as baseline resolved resonances along with saturated (31.94 ppm) and vaccenate (31.80 ppm) chains) were used to determine the percentages of each of these acids in the triglyceride ($O,L_{theory}$). The compositions (mol%) of saturated, oleic, vaccenic, and linoleic acids of whole triglyceride were also calculated on the basis of the C-16 set of resonances (Table 1).

The $O(L)_{found}$ data were calculated from the resonance intensities of C-9 of oleate (129.63 ppm) and C-10 of linoleate (128.07 ppm) chains attached at 2-position.

Since both unsaturated carbons (C-11 and C-12) of vaccenate chains at 2-positions were detected with low signal-to-noise ratios, vaccenate chain was ruled out from the calculations of unsaturated acids both in the 2-position and in whole triglyceride, to maintain a data homogeneity.

The oleate ($O_{theory}$) and linoleate ($L_{theory}$) compositions in the total oleic and linoleic acids in the triglyceride ranged from 81.2 to 94.2% and from 5.8 to 18.8%, respectively, whereas the oleate ($O_{found}$) and linoleate ($L_{found}$) compositions at the 2-position ranged from 77.4 to 93.2% and from 6.8 to 22.6%, respectively (Table 1).

Table 1
Structure of triacylglycerols of olive oils sampled in the Marche region: fatty acid composition of total triglyceride and of triglyceride 2-position

| Fatty acid[a] composition (mol%) | Mean[b] | Minimum | Maximum | StDev[c] |
|---|---|---|---|---|
| Whole triglyceride | | | | |
| S | 17.7 | 13.5 | 20.4 | 1.1 |
| O | 71.4 | 61.0 | 78.9 | 2.2 |
| L | 7.5 | 4.5 | 14.1 | 1.4 |
| Vc | 3.3 | 1.8 | 6.3 | 1.0 |
| $O,L_{theory}$ | | | | |
| O | 90.5 | 81.2 | 94.2 | 1.8 |
| L | 9.5 | 5.8 | 18.8 | 1.8 |
| $O,L_{found}$ | | | | |
| O | 88.3 | 77.4 | 93.2 | 2.4 |
| L | 11.7 | 6.8 | 22.6 | 2.4 |

[a] Acyl chains: S, saturated (n:0); O, oleate (18:1; 9*c*); L, linoleate (18:2; 9*c*12*c*); Vc, vaccenate (18:1; 11*c*).
[b] Data set was made up of 309 olive oil samples.
[c] StDev = standard deviation.

Figures 1 and 2 show the score plots of $O_{theory}$ compared to $O_{found}$ and of $L_{theory}$ compared to $L_{found}$, respectively, for all 309 olive oil samples.

A linear model was selected by regression analysis to describe the relationships between the "theoretical" and "found" percentages of oleate and linoleate chains. The equations of the fitted lines of oleic and linoleic acids were reported in Figs 1 and 2, respectively. Since the compositions of oleic and linoleic acids at 2-positions depend on each other because they were measured in percentages, the gradient for both lines was defined by the same slope value (1.27). The analysis of variance (ANOVA) evidenced that there was statistically significant relationship between $O(L)_{found}$ and $O(L)_{theory}$ at the 99% confidence level ($P$-value $< 0.01$). The coefficient of determination $R^2$ explained 95.1% of the relationship between $O(L)_{theory}$ and $O(L)_{found}$, whereas the correlation coefficient was equal to 0.975 thus indicating a strong relationship between $O(L)_{theory}$ and $O(L)_{found}$.

The oleic and linoleic acids would be randomly distributed at the 2-positions if their percentages (mol%) in the total oleic and linoleic acids in the triglyceride $O(L)_{theory}$ were equal to their percentages (mol%) in the total oleic and linoleic acids at the 2-position $O(L)_{found}$. The random distribution patterns of oleic and linoleic acids were represented by a dotted line in Figs 1 and 2. It was evident that the fitted lines deviated from "random distribution" lines at an increasing rate for decreasing values of $O_{theory}$ and for increasing values of $L_{theory}$. These findings confirmed that oleic acid and linoleic acid were not randomly distributed at the 2-position, but lower oleic acid and higher linoleic acid were detected compared to the theoretical values expected for a random distribution [9].

Nevertheless, the crucial point was that the deviation rate of the unsaturated acids from the 2-random distribution pattern varied according to the acid concentration in the triglyceride [12,13].

### *3.2. $^{13}C$ NMR to check 1,3-random–2-random distribution*

Based on these results, it appeared worthwhile to check the fatty acid distribution pattern emerging from the $^{13}C$ NMR data in comparison to that obtained by the computer method based on the

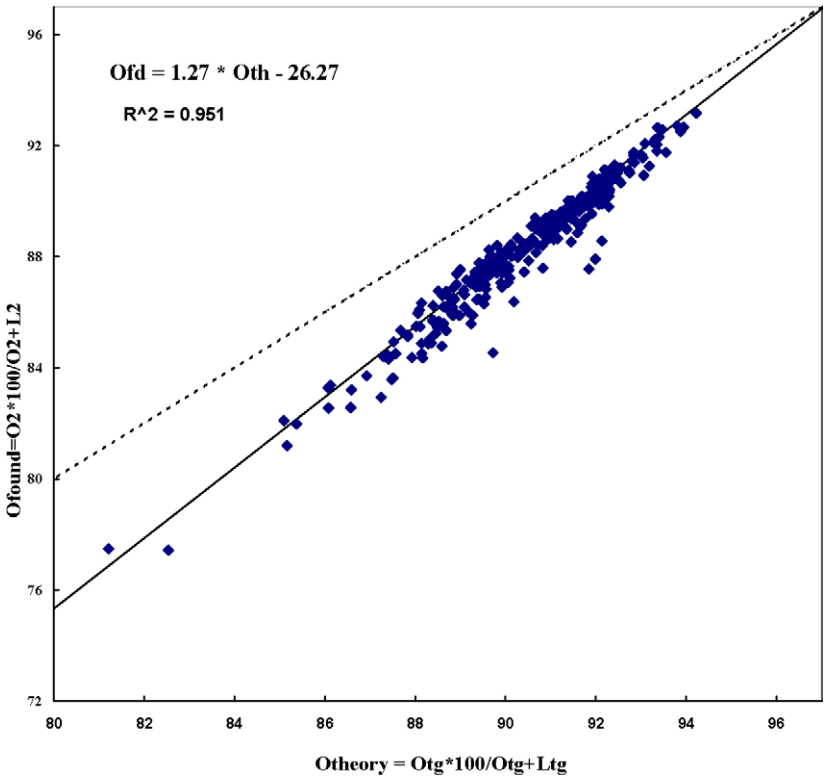


Fig. 1. The percentages of oleic acid in the total of oleic and linoleic acids in the triglyceride ($O_{theory}$) compared to the percentages of oleic acid in the total of oleic and linoleic acids at the 2-position ($O_{found}$).

1,3-random–2-random distribution. The method (HRGC/1,3-r-2-r) calculates the theoretical contents of triacylglycerols of ECN 42 and ECN 44 classes, from the fatty acid composition obtained by gas-chromatography. The method was applied to detect olive oil adulteration with seed oil by using the difference between the calculated values and those determined by liquid chromatography [14].

Three olive oil samples were selected according to low (81.2%), medium (84.5%) and high (93.2%) percentage of oleic acid at 2-position in the total of oleic and linoleic acids at 2-position ($O_{found}$) corresponding to a high, medium, low deviation rate from the 2-random distribution pattern, respectively.

The measurements of fatty acid compositions (mol%) of the whole triglyceride in the three olive oil samples performed by $^{13}C$ NMR were similar to those obtained by HRGC (Table 2). Vaccenic acid, which was determined by HRGC as a whole with oleic acid, was measured with poor accuracy by $^{13}C$ NMR (coefficient of variation of 18.0%) as expected for a resonance with low signal-to-noise ratio.

The unsaturated fatty acids at 2-position (mol%) were determined by $^{13}C$ NMR on the basis of the intensities of the C-9 and C-10 resonances of oleate and linoleate chains at 2-position, respectively.

The HRGC/1,3-r-2-r method calculated the molar percentages of the unsaturated fatty acids at 2-position by subtracting the saturated acids at 2-position (normalized values) from 100. Considering that the saturated and unsaturated acids in the 1,3-random–2-random pattern, are distributed with different coefficients between the 1,3- and 2-positions [14], the coefficient of 0.06 was used to predict the molar percentage of saturated acids at 2-position (the maximum acceptable level of saturated fatty acids in the 2-position of glycerol for virgin olive oil is $\leqslant$1.5% [15]).

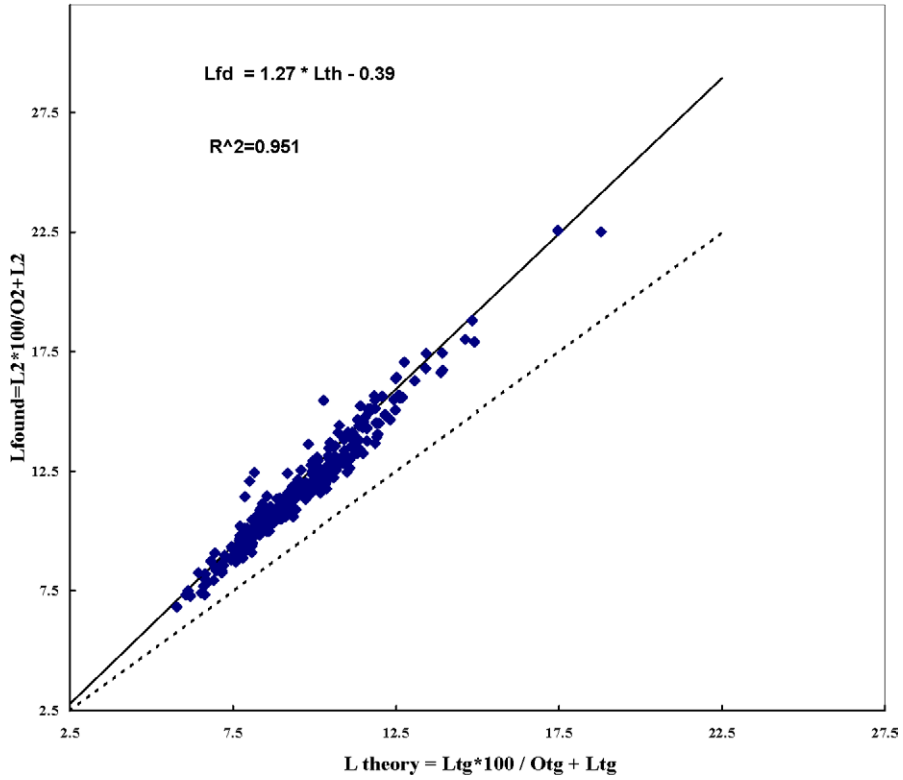


Fig. 2. The percentages of linoleic acid in the total of oleic and linoleic acids in the triglyceride ($L_{theory}$) compared to the percentages of linoleic acid in the total of oleic and linoleic acids at the 2-position ($L_{found}$).

The results obtained for the fatty acid composition at 2-position for the "high $O_{found}$" oil (Table 2) evidenced that the agreement between the 2-position data from NMR (93.2%) and HRGC/1,3-r-2-r method (91.3) was quite good as expected from an oil sample with the lowest deviation from the 2-random pattern (Fig. 1). However, the differences between NMR and HRGC/1,3-r-2-r method data increased in the "medium $O_{found}$" and "low $O_{found}$" oils where the values for oleic and linoleic acids at 2-position calculated by the computer program based on the 1,3-random–2-random distribution, were constantly higher and lower, respectively, as compared to the NMR data.

The fatty acid composition of 1,3-positions (mol%) were calculated on the basis of both NMR and HRGC/1,3-r-2-r data, as difference between the chain compositions of whole triglyceride (mol%) and of 2-position (mol%), according to the formula:

$$\text{1,3-positions} = \text{1,2,3-positions} \times 3 - \frac{\text{2-position}}{2}$$

$^{13}$C NMR enables the direct determination of the 1,3-chain composition by using the resonances of the unsaturated carbons of 1,3-positions chains, i.e. C-9 of oleate (129.65 ppm) and C-10 of linoleate (128.05 ppm) chains, the C-16 resonance of saturated chains could be used to complete the picture of 1,3-chains. Nevertheless, for the data homogeneity, the calculations according to the formula stated above were used both for NMR and computer method data.

Table 2

Determination of fatty acid distributions between the 1,3- and 2-positions of triacylglycerols of olive oils by using $^{13}C$ NMR and HRGC/1,3-random–2-random methods: calculation of triglyceride species in three olive oil samples with high, medium and low percentages of oleic acid at 2-position ($O_{found}$)

| Triacylglycerol structure | Olive oil samples | | | | | |
|---|---|---|---|---|---|---|
| | $O_{found}$ ⟨high⟩ | | $O_{found}$ ⟨medium⟩ | | $O_{found}$ ⟨low⟩ | |
| | NMR | HRGC/ 1,3-r-2-r | NMR | HRGC/ 1,3-r-2-r | NMR | HRGC/ 1,3-r-2-r |
| Fatty acid[a] composition (mol%) | | | | | | |
| Whole triglyceride | | | | | | |
| S | 18.1(1.9)[b] | 17.3(0.5)[b] | 18.5 | 16.6 | 19.3 | 17.9 |
| O | 74.0(1.7) | – | 71.0 | – | 65.9 | – |
| Vc | 3.4(18.0) | – | 2.4 | – | 3.3 | – |
| O + Vc + Po | 77.4 | 76.4(0.3) | 73.4 | 75.2 | 69.1 | 70.6 |
| L | 4.5(1.7) | 5.7(0.3) | 8.1 | 7.6 | 11.5 | 11.0 |
| Ln[c] | – | 0.6(0.3) | – | 0.6 | – | 0.5 |
| 2-position | | | | | | |
| S | – | 1.0 | – | 1.0 | – | 1.0 |
| O + Vc + Po[d] | 93.2[e] | 91.3 | 84.5 | 89.1 | 81.2 | 85.1 |
| L | 6.8 | 6.9 | 15.5 | 9.1 | 18.8 | 13.2 |
| Ln | – | 0.8 | – | 0.8 | – | 0.7 |
| 1,3-positions | | | | | | |
| S | 28.0 | 25.5 | 28.4 | 24.4 | 30.1 | 26.3 |
| O + Vc + Po[d] | 68.4[e] | 68.8 | 66.8 | 68.1 | 61.5 | 63.4 |
| L | 3.6 | 5.1 | 4.8 | 6.9 | 8.4 | 9.8 |
| Ln | – | 0.6 | – | 0.6 | – | 0.5 |
| Triacylglycerol species (mol%) | | | | | | |
| LLL | 0.0088 | 0.018 | 0.036 | 0.043 | 0.13 | 0.13 |
| OLL (all isomers) | 0.45 | 0.72 | 1.18 | 1.28 | 2.51 | 2.46 |

[a]Acyl chains: S, saturated (n:0); O, oleate (18:1; 9*c*); Vc, vaccenate (18:1; 11*c*); Po, palmitoleate (16:1; 9*c*); L, linoleate (18:2; 9*c*12*c*); Ln (18:3; 9*c*12*c*15*c*). S indicates saturated chains as a whole in NMR and the sum of palmitic (16:0) and stearic (18:0) acids in HRGC.
[b]Values are means of three replicate determinations. Coefficients of variation are given in parentheses.
[c]Linolenic acid resonances were not detected by NMR under the signal-to-noise ratio adopted.
[d]Resonances of palmitoleic and oleic acids overlapped in NMR.
[e]Calculations from NMR resonances unlike HRGC data, did not include vaccenic acid.

The molar concentrations of oleic and linoleic acids in the 1,3-positions along with that in the 2-position, were used to determine the mol% of the triglyceride species LLL (class ECN 42) and OLL (class ECN 44, all isomers, i.e. positional and optical isomers) by using the formulas [16]:

$$\mathrm{LLL} = \frac{(\mathrm{L}_1) \times (\mathrm{L}_2) \times (\mathrm{L}_3)}{10{,}000}$$

$$\mathrm{OLL\ (all\ isomers)} = \frac{(\mathrm{L}_1) \times (\mathrm{O}_2) \times (\mathrm{L}_3)}{10{,}000} + \frac{2 \times (\mathrm{O}_1) \times (\mathrm{L}_2) \times (\mathrm{L}_3)}{10{,}000}$$

The results evidenced that the differences between the mol% of LLL and OLL calculated from NMR and HRGC/1,3-r-2-r data, decreased from the maximum value in correspondence of the "high $O_{found}$"

oil sample (low deviation from random pattern) up to no differences were detected in the "low $O_{found}$" oil (high deviation from random pattern). The composition pattern of linoleate chain could account for the same values for LLL and OLL triglycerides obtained by the NMR and HRGC/1,3-r-2-r methods, for an oil sample with maximum deviation from random pattern. The linoelate percentages, they increased from "high $O_{found}$" to "low $O_{found}$" olive oil, measured by NMR were higher at 2- and lower at 1,3-positions, respectively, as compared to the percentages obtained by the HRGC/1,3-r-2-r method. They were the higher differences between the linoleate chain percentages at 2-position detected by the two methods, that made NMR and HRGC/1,3-r-2-r predict the same LLL and OLL percentages in the "low $O_{found}$" oil.

## 4. Conclusion

The determination of fatty acid composition of 2-triacylglycerol position by applying $^{13}C$ NMR methodology on a very large set of olive oil samples, confirmed that the fatty acid distribution at 2-position of triacylglycerols deviated from the 1,3-random–2-random pattern, and evidenced that the deviation extent varied according to the fatty acid concentration in the total triglyceride. As a result, the actual difficulty of the computer method based on the 1,3-random–2-random pattern in predicting the theoretical content of triacylglycerol species with ECN 42 and ECN 44, was evidenced for the oils with high–medium $O_{found}$ values.

Based on these findings, it appeared reasonable extending the research to an even larger data set of olive oils to confirm the fatty acid distribution at the 2-glycerol positions which emerged from the $^{13}C$ NMR data of the olive oils sampled in the Marche region.

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