^{13}C Nuclear Magnetic Resonance Spectroscopy to Determine Fatty Acid Distribution in Triacylglycerols of Vegetable Oils with "High - Low Oleic Acid" and "High Linolenic Acid"

Giovanna Vlahov*

CRA – OLI Centro per l'Olivicoltura e l'Industria Olearia, Sede Scientifica Città S. Angelo, Viale Petruzzi n. 75, 65013 Città S. Angelo (PE), Italy

Abstract: Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy was applied to determine the fatty acid positional composition of triacylglycerols in vegetable oils with oleic acid percentages "higher and lower than 50%" and with linolenic acid percentages higher than 5%.

In particular, conventional ^{13}C NMR spectroscopy which applies the basic one-pulse sequence for signal averaging, was used to determine on the basis of carboxy and unsaturated carbon resonances, the compositions of two different pools of fatty acids at the 1,3- and 2-positions of triacylglycerols.

The results confirmed that the chains at 1,3- and 2- triacylglycerol positions deviated from the 1,3-random-2-random distribution pattern at variable extents depending on the chain concentration in the total triglyceride of oil samples. However, two factors were likely to regulate the chain distribution between 1,3- and 2-glycerol positions, namely the concentration of fatty acids in the total triglyceride and their positional specificity.

Keywords: ^{13}C NMR, fatty acids, positional distribution, triacylglycerols, vegetable oils.

INTRODUCTION

^{13}C nuclear magnetic resonance spectroscopy was applied by Wollemberg [1] as a pioneer technique to carry out the positional analysis of fatty acids in triacylglycerols of vegetable oils. The Author proved that ^{13}C NMR detects the unsaturated fatty acids according to their unsaturation degree, namely oleic, linoleic and linonenic acids, and their 1,3- and 2- positions on glycerol backbone. Based on these results, the random distribution theories of fatty acids distribution in triacylglycerols have been verified.

The theory of random distribution, it stated that fatty acids were esterified randomly over-all hydroxyl groups, proved incorrect when analyses with lipase showed that fatty acid compositions in positions 1 plus 3 and position 2 were always different. This theory was modified by Kartha into restricted random distribution based on the observation that fully saturated triglycerides (SSS) are present to a lower extent than expected, where the saturated chains are distributed at random to form additional S_2U and SU_2 triglycerides (S= saturated, U= unsaturated chains) [2].

Among the random distribution theories, the 1,3-random-2-random theory is now widely accepted. The theory states that the glycerol is fully esterified at 2-position with a mixture of fatty acids, and at 1- and 3- positions, assumed to be identical, with another mixture of fatty acids [3]. However, the theory can only predict the fatty acid distribution between the 1,3- and 2-glycerol positions without any analytical validation.

High resolution ^{13}C NMR spectroscopy based on carboxy carbon resonances of triglyceride acyl chains was applied to determine the positional distribution of fatty acids in triacylglycerols of olive oil.

The results confirmed that two different mixtures of fatty acids esterify the 1, 3- and 2-glycerol positions where 2-position specificity values evidenced that oleate chain moved away from a random distribution pattern less than linoleate chain and that the 2-position specificity of both chains appeared to be a characteristic of the chain [4].

^{13}C NMR resonances of carboxy carbons of triglyceride acyl chains were also used to carry out the regiospecific analysis of vegetable oils of different botanical origin. The study confirmed that linoleate chain deviated from a random pattern of distribution more than oleate chain [5].

The fatty acid distribution data in olive oil triacylglycerols obtained by ^{13}C NMR spectroscopy, further confirmed that oleic and linoleic acids were not randomly distributed at the 2-position of triacylglycerols where oleic and linoleic acid contents were lower and higher, respectively, than the values predicted by the 1, 3-random-2-random theory. But the crucial point was that the unsaturated acids deviated from the 2-random distribution pattern at different extents according to the acid concentration of triglyceride [6].

These results suggested to extend the ^{13}C NMR study of positional distribution of fatty acids to triacylglycerols of vegetable oils where, unlike olive oil, linoleic acid was the major acid and linolenic acid was also present. The aim was

*Address correspondence to this author at the CRA – OLI Centro per l'Olivicoltura e l'Industria Olearia, Sede Scientifica Città S. Angelo, Viale Petruzzi n. 75, 65013 Città S. Angelo (PE), Italy; Tel: +39 (0) 85 95212; Fax: +39 (0) 85 959518; E-mail: g.vlahov@tiscali.it

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to find the distribution patterns of these acids in relation to the 1,3-random-2-random theory and to determine the extent of fatty acid deviation from the 1,3-random-2-random pattern.

MATERIALS AND METHODOLOGY

Pulp oils were extracted by cold-pressure from avocado and olive fruits, and seed oils from seeds of peanut, hazelnut (one sample from United States cultivars, three samples from Italy and in particular from Sicilia, Lazio and Piemonte regions), balanites, corn, grape, rice, sunflower (two samples), croton, and groundnut. All these seed oils along with colza and soybean oil samples, were products available on the market.

^{13}C spectra were run on a Unity Inova Narrow Bore 500 MHz spectrometer equipped with a UNIX-based Sun Microsystems workstation (Varian NMR Instruments, Palo Alto, California), and a 5-mm probe operating at 25°C.

The spin-lattice relaxation times T_1 and nuclear Overhauser enhancements (η) NOE were measured (three replicate measurements were carried out) using two hundred milligrams of soybean oil sample (200 mg) which were dissolved in 0.55 ml of deuterated chloroform ($CDCl_3$) Sigma Aldrich, Milano, Italia.

The spin-lattice relaxation times of carbon-13 nuclei of triacylglycerols were determined by means of the 180° - τ - 90° inversion-recovery pulse sequence.

The proton-decoupled spectra with full NOE were measured using a spectral width of 25,000 Hz, 256 K data points and a 80° pulse length with a 5.1 s acquisition time. The decoupler was turned on before and during acquisition. The relaxation delay was set at 50 s. The spectra were zerofilled to 512 K and a resolution enhancement function which uses the Lorentzian-to-Gaussian conversion was applied.

The spectra with suppressed NOE were measured by using the inverse-gated proton-decoupled sequence, which gated the decoupler on during the sole acquisition time. The relaxation delay was set at 50 s that is ≥ 5 times the longest T_1 (the longest T_1 close to 7 s was measured in correspondence of C-16 of linolenic acid).

The ^{13}C $\{^1H\}$ NOE enhancement η values, they are defined as the fractional change in the intensity of I (^{13}C) on saturating ^1H where I_0 is the intensity of I spin at Boltzmann equilibrium, were calculated according to the relationship:

$$\eta\ ^{13}C\ \{^1H\} = (I - I_0) / I_0$$

by using the integrated intensities of carbon resonances measured with full NOE (I) and with suppressed NOE (I_0).

The quantitative spectra of oil samples were measured using two hundred milligrams of oil sample (200 mg) which were dissolved in 0.55 ml of deuterated chloroform ($CDCl_3$) Sigma Aldrich, Milano, Italia.

The high resolution ^{13}C spectra for determining the fatty acid positional composition of triacylglycerols of vegetable oils were measured by using conventional ^{13}C NMR spectroscopy which applies the basic one-pulse sequence to carry out signal averaging.

The high resolution quantitative ^{13}C spectra for determining the fatty acid positional composition of triacylglycerols of vegetable oils were acquired under proton decoupling (Waltz-16 broadband decoupling) with full NOE, a relaxation delay of 20 s to avoid signal saturation, 256 K data points, a 80° pulse length and a 5.1 s acquisition time (AT). The accumulated FIDs were zerofilled to 512 K, multiplied by a resolution enhancement function which uses the Lorentzian-to-Gaussian conversion to improve resolution and achieve a reasonable signal sensitivity, and Fourier transformed.

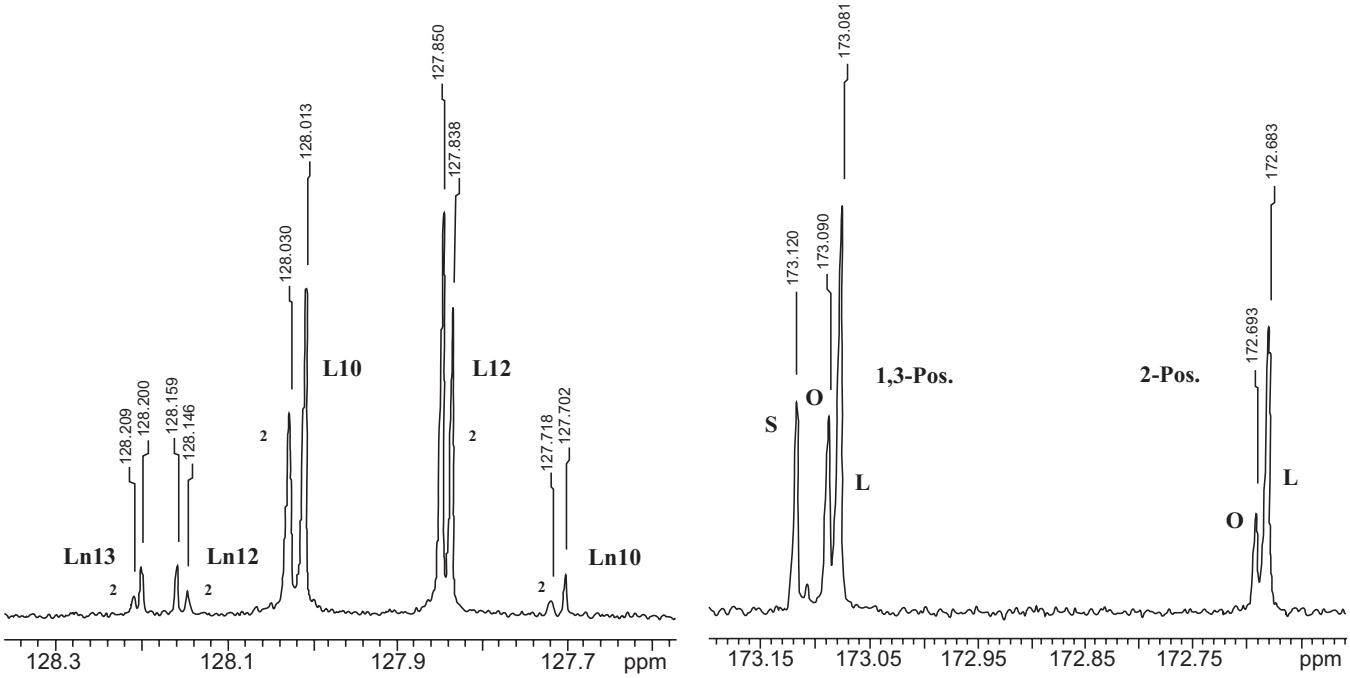


Fig. (1). ^{13}C NMR spectrum of carboxy (right trace) and unsaturated carbons (left trace) of acyl chains of soybean oil triglycerides: S, saturated chain; O, oleate chain; L, linoleate chain; Ln, linolenate chain.

Chemical shifts were given in parts per million (ppm) and calculated relative to tetramethyl silane by using the residual ^{13}C peaks of the solvent as internal standards.

RESULTS AND DISCUSSION

The determination of positional compositions of fatty acids in triacylglycerols of vegetable oils was carried out by using the frequency ranges 172.5 - 173.1 and 132.0 - 126.0 ppm where carboxy and unsaturated carbons resonate, respectively.

Only carboxy carbon frequencies allow the determination of saturated (C n:0), oleate (C 18:1 Δ9*cis*), and linoleate (C 18:2 Δ9,12*cis*) chain composition at 1,3- and 2-positions of glycerol backbone (Fig. **1**) where the 1.3-position chains were high frequency shifted by 0.40 ppm from the 2-position chains [7]. The sn-1- and sn-3- positions of triacylglycerols, which are stereospecifically numbered according to the L-form of Fisher projection of sn-glycerol [8], cannot be differentiated by NMR.

However, the overlapping of linoleate and linolenate (C 18:3 Δ9,12,15*cis*) chains in the carboxy carbon region, sug-

Table 1. Chemical Shifts, Longitudinal Relaxation Times (T_1), Nuclear Overhauser Enhancement Factors (η) of Carboxy and Unsaturated Carbons of Triglycerides Acyl Chains. Precision of ^{13}C NMR Method (CV %)

Carbon Resonance	Chemical Shift (ppm)	Longitudinal Relaxation Time T_1 (s)	Nuclear Overhauser Enhancement $\eta = I/I_0 - I$	Coefficient of Variation (CV %)
Carboxy				
S1 1,3	173.12	3.1 ± 0.1[a]	0.4 ± 0.06 [a] 5.	2[b]
O1 1,3	173.09	3.4 ± 0.05	0.5 ± 0.1	5.3
L1 1,3	173.08	3.2 ± 0.1	0.4 ± 0.07	2.6
O1 2	172.69	2.7 ± 0.2	0.5 ± 0.1	5.8
L1 2	172.68	2.5 ± 0.1	0.4 ± 0.03	2.2
Olefinic				
Ln 16	131.83	6.4 ± 0.2	1.5 ± 0.1	3.2
L13 2	130.11	2.8 ± 0.05	1.8 ± 0.1	2.2
L13 1,3	130.10	2.8 ± 0.02	1.8 ± 0.1	2.2
O10 2	129.93	1.4 ± 0.03	1.8 ± 0.2	5.6
O10 1,3	129.92	1.4 ± 0.04	1.7 ± 0.1	2.9
L9 1,3	129.89	1.9 ± 0.03	1.7 ± 0.1	2.8
L9 2	129.87	1.8 ± 0.04	1.8 ± 0.1	2.2
O9 1,3	129.63	1.4 ± 0.02	1.8 ± 0.2	2.2
O9 2	129.60	1.4 ± 0.03	1.9 ± 0.1	3.2
Ln13 2	128.21	3.1 ± 0.03	1.8 ± 0.3	12.8
Ln13 1,3	128.20	3.4 ± 0.04	1.6 ± 0.2	7.9
Ln12 1,3	128.16	3.5 ± 0.05	1.7 ± 0.2	3.6
Ln12 2	128.15	3.8 ± 0.03	1.7 ± 0.3	5.9
L10 2	128.03	1.7 ± 0.08	1.8 ± 0.2	3.8
L10 1,3	128.01	1.7 ± 0.06	1.9 ± 0.2	1.7
L12 1,3	127.85	2.2 ± 0.1	1.8 ± 0.2	2.5
L12 2	127.84	2.8 ± 0.03	1.9 ± 0.1	2.4
Ln10 2	127.72	2.1 ± 0.04	1.9 ± 0.2	5.8
Ln10 1,3	127.70	1.8 ± 0.05	1.9± 0.2	5.3
Ln15 127.	07	6.1 ± 0.1	1.9 ± 0.1	3.1

[a]Accuracies of T_1 and NOE factors are quoted as standard deviation of the mean of three replicate measurements.
[b]Precision of ^{13}C NMR method was verified by six replicate measurements of ^{13}C spectra of a soybean oil sample, resonances were integrated and the areas were checked for Coefficient of Variation .

gested the use of unsaturated carbons which, unlike carboxy carbons spreading over 300 Hz, resonated in a wider frequency range of 3000 Hz.

The unsaturated carbons of oleate, linoleate and linolenate chain at 1,3- and 2- positions of triglycerides were detected (Fig. **1**). Their chemical shifts (Table **1**) were in agreement with the σ- inductive theory of transmission of dipolar effects of C=O and C=C bonds upon other C=C bonds in polymethylene acyl chains [9, 10].

Considering that carboxy and unsaturated carbons enable the detection of all normal fatty acids, namely C n:0 (detected in carboxy carbon region), C18:1, C18:2, C18:3 (detected only in unsaturated carbon region) which are present in all living tissues [11], the C=O and C=C carbon resonances were used to determine the positional compositions of vegetable oil triacylglycerols.

The primary condition for an accurate measurement of triglyceride positional compositions requires that no intensity distortions affect the spectrum. Intensity distortions are due to signals saturation which occurs when repetition rates lower than 5 times the longest spin-lattice relaxation time (T_1) in the spectrum, are applied. A soybean oil sample was used to measure T_1s of carboxy and unsaturated carbons of triglyceride acyl chains and the results were reported in Table **1**.

Spin-lattice relaxation times of carboxy carbons of triglyceride acyl chains were in agreement with the results previously reported [12]. In particular, carboxy carbons of oleate (2.7 s) and linoleate (2.5 s) chains at glycerol 2-position relax faster than the corresponding 1,3-chains (3.4 and 3.2 s, respectively) because of their slower motion (it is more restricted in the 2-position). This results in a more efficient relaxation and shorter T_1s.

As far as T_1s of unsaturated carbons are concerned, T_1 values increased regularly from 1.9 - 1.8 s (L-9) to 2.8 s (L-13) in the linoleate chain, and from 1.8 - 2.1 s (C-10) to 6.4 s (C-16) in the linolenate chain. This pattern can be explained in terms of a less efficient relaxation due to the chain mobility which increases from the glycerol backbone to the methyl chain end [13].

C-9 of oleate, C-10 of linoleate and linolenate chains which showed the lowest T_1 values ranging from 1.4 to 2.1 s, were selected to determine the positional compositions of unsaturated chains in triglycerides.

Considering that the longest T_1 was measured for carboxy carbon of oleate chain at 1,3-glycerol position (3.4 s), a delay of 20 s (which was $> 5 \times 3.4$) was applied to avoid intensity distortions due to signal saturation.

Moreover, almost equal NOE factors (Table **1**) were measured in correspondence of C-9 of oleate and C-10 of linoleate and linolenate chains (η values ranging from 1.8 to 1.9) This result prevented intensity distortions caused by differential nuclear Overhauser enhancements and allowed the acquisition of ^{13}C spectra under full NOE thus increasing the sensitivity of carbon-13 nuclei by a factor of 3 [14].

Table 2. ^{13}C NMR Spectroscopy of Vegetable Oils with Oleate % Higher than 50% for Determining Triacylglycerol Positional Composition

Data	Avocado	Peanut	Olive Oil	Hazelnut	Hazelnut	Hazelnut	Hazelnut
Composition % of Fatty Acids from C=C Corrected for Saturated Chains by Using Carboxy Carbons							
S 18.	2	18.6	15.9	10.3	9.6	9.7	8.5
O 71.	7	64.1	76.2	81.9	81.4	83.1	85.1
L 10.	1	17.3	7.9	7.8	9.0	7.2	6.4
2-Position Specificity % of Fatty Acids							
O 37.	6	36.2	40.4	36.4	35.6	35.4	37.0
L 64.	4	57.1	50.4	49.1	54.4	49.3	56.4
Composition % of Fatty Acid Pool at 1,3-glycerol Positions							
S 27.	4	27.7	24.4	15.6	14.5	14.5	13.1
O 67.	2	61.2	69.6	78.5	79.3	80.1	82.6
L 5.	4	11.1	6.0	5.9	6.2	5.4	4.3
Composition % of Fatty Acid Pool at 2-glycerol Positions (X_{found})							
O 80.	5	70.1	88.6	88.7	85.5	89.3	89.8
L 19.	5	29.9	11.4	11.3	14.5	10.7	10.2
Composition % of Fatty Acids at 2- glycerol Positions in the Total of these Chains in Triacylglycerols (X_{theory})							
O 87.	6	78.7	90.6	91.4	90.0	92.1	93.0
L 12.	4	21.3	9.4	8.6	10.0	7.9	7.0

NOE values at carboxy carbons, confirmed to be equal for different chains and much lower (η=0.4) than those determined for the protonated unsaturated carbons. Unlike carboxy carbons for which the chemical shift anisotropy is the predominant mechanism of relaxation [12], a dipole-dipole mechanism of relaxation operates for carbons directly bonded to hydrogen atoms.

The precision of the ^{13}C NMR method was verified by six replicate measurements of proton-decoupled spectra with full NOE using a soybean oil sample. The resonances of carboxy and unsaturated carbons were integrated and the areas were checked for coefficient of variation. The results reported in Table **1**, evidenced that all the resonances showed coefficients of variation lower than 6% with the sole exception of C-13 of linolenate chain at 2-position.

The unsaturated chain percentages in the whole triglyceride and their percentages at 1, 3- and 2-positions (which measure the chain specificity for the glycerol positions) were calculated by using the selected unsaturated carbon resonances. The compositions of the two different pools of unsaturated chains at 1, 3- and 2-positions were also determined. The percentages based on unsaturated carbon resonances were corrected for saturated chain percentages which were determined by using the carboxy carbon resonances.

The assumption was also made that saturated chains esterify only the 1,3- positions of triacylglycerols [15].

The compositional data evidenced that the oil samples can be grouped according to oleate percentages in the whole triglyceride higher than 50 % and lower than 50%, and to linolenate percentages higher than 5%.

The results obtained for the oils with oleate percentages higher than 50%, they comprised avocado, peanut, olive and hazelnut (four samples with variable oleate contents were considered) oils, were reported in Table **2**.

Moreover, the triglyceride data of balanites, corn, grape, rice, sunflower, croton and groundnut oil samples with oleate percentages lower than 50% were reported in Table **3**.

Triglycerides of the oil samples are made up of saturated (n:0 where n= 16, 18, ^{13}C NMR can not differentiate saturated chains by carbon number), oleate and linoleate chains. In particular, in the oil group with oleate higher than 50%, saturated chains ranged from 8.5 to 18.6 %, oleate from 64.1 to 85.1 %, and linoleate from 6.4 to 17.3 %, whereas in the oil with oleate lower / equal than 50% saturated, oleate and linoleate chain percentages were comprised in the ranges 11.0 - 33.2%, 7.3 - 46.3 %, 33.2 - 80.6 %, respectively.

The compositional data of the oils containing percentages of linolenate chain higher than 5% were reported in Table **4**. Oleate and linoleate chains were the major chains in both oils where oleate predominated in the colza oil sample with 59.4% and linoleate in the soybean oil sample with 54.1 %. However, the linolenate chain content was higher in the colza oil with 9.0 % as compared to the 5.6 % in soybean oil.

Considering the different oil groups (comprising the oils with oleic acid higher and lower than 50% and the oils with

Table 3. ^{13}C NMR Spectroscopy of Vegetable Oils with Oleate Chain % Lower than 50% for Determining Triacylglycerol Positional Composition

Data	Balanites	Corn	Grape	Rice	Sunflower	Sunflower	Croton	Groundnut
Composition % of Fatty Acids from C=C Corrected for Saturated Chains by Using Carboxy Carbons								
S 33.	2	14.1	12.1	25.2	11.0	16.5	12.1	19.7
O 26.	8	30.4	21.7	41.6	30.5	19.6	7.3	46.3
L 40.	0	55.5	66.2	33.2	58.5	63.9	80.6	34.0
2-Position Specificity % of Fatty Acids								
O 41.	9	34.4	40.2	36.8	33.7	33.5	39.7	33.2
L 55.	7	42.2	37.9	50.2	41.2	42.1	40.6	56.1
Composition % of Fatty Acid Pool at 1,3-glycerol Positions								
S 49.	9	21.4	18.3	37.0	16.7	24.8	18.7	30.0
O 23.	4	30.1	19.6	38.7	30.8	19.5	6.8	47.2
L 26.	7	48.5	62.1	24.3	52.5	55.7	74.4	22.8
Composition % of Fatty Acid Pool at 2-glycerol Positions (X_{found})								
O 33.	4	30.8	25.8	48.0	29.9	19.6	8.1	44.6
L 66.	6	69.2	74.2	52.0	70.1	80.4	91.9	55.4
Composition % of Fatty Acids at 2- glycerol Positions, in the Total of these Chains in Triacylglycerols (X_{theory})								
O 40.	0	35.4	24.7	55.7	34.2	23.4	8.3	57.7
L 60.	0	64.6	75.3	44.3	65.8	76.6	91.7	42.3

Table 4. [13]C NMR Spectroscopy of Vegetable Oils with Linolenate Chain % Higher than 5% for Determining Triacylglycerol Positional Composition

Data Co	lza	Soybean
Composition % [a]		
S 9.	9	16.3
O 5	9.4	24.1
L 2	1.7	54.1
Ln 9.	0	5.6
2-Position Specificity %		
O 2	8.9	35.6
L 5	5.4	44.0
Ln 5	6.8	36.6
Composition % Fatty Acid Pool at 1,3-Positions		
S 1	5.1	24.8
O 6	4.3	23.6
L 1	4.8	46.2
Ln 5.	8	5.4
Composition % Fatty Acid Pool at 2-Position		
O 5	0.0	24.9
L 3	5.1	69.2
Ln 14.	9	5.9

[a]The composition % data of fatty acids were calculated from unsaturated carbon resonances and were corrected for saturated chains by using carboxy carbons.

high linolenic acid) as a whole, the compositional values evidenced that the mole percentages of saturated acids were poorly correlated with the mole percentages of oleic (coefficient of correlation r = 0.34) and linoleic (coefficient of correlation r = 0.12) acids. However, a high negative correlation (correlation coefficient r = – 0.97 where the statistic R^2 showed that the linear model explained 94.15% of the variability in the linoleic acid mole % values) was found between the mole percentages of oleic and linoleic acids according to the linear relationship here below reported:

$$\text{Linoleic acid mole \%} = (\text{Oleic acid mole \%}) \times (-0.91) + 79.45 \quad (1)$$

$$r = -0.97 \quad R^2 = 94.15$$

The negative correlation was tentatively explained by admitting that linoleic acid is generated from oleic acid by an oxygen-dependent desaturation process through the action of enzymes capable of introducing double bonds between an exhisting double bond and the methyl group [2, 16].

The specificities of oleate, linoleate and linolenate chains for triacylglycerol 2-positions were calculated by normalizing the resonance intensity value of each chain at 2-position to the 1,3- and 2-position values. The results obtained for all the oil samples were reported in Fig. (**2**). The 2-specificity values of the oleate chain (in the range from 28.9 to 41.9%) were considerably lower than those measured for the linoleate chain (in the range between 37.9 and 64.4). In particular, apart from the colza oil sample (28.9%), the 2-specificity of oleate chain confirmed to be very close (36.3% averaged value) to 33.3% which is the value expected for a random distribution, whereas the linoleate chain showed higher values (49.8% averaged value). The low variability measured in

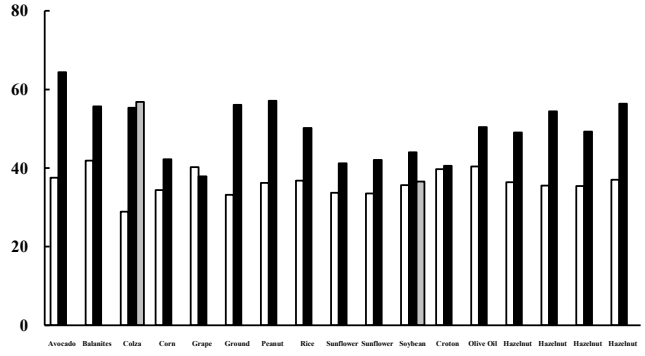


Fig. (2). The specificities of oleic (□), linoleic (■) and linolenic (▨) acids for 2-positions of triacylglycerols of the oil set comprising oil samples with oleic acid higher and lower than 50% and oil samples with linolenic acid higher than 5% were compared.

terms of relative standard deviations of 9% and 15% for 2-specificity values of oleate and linoleate chains, respectively, confirmed that the 2-specificity of a chain was likely to be the chain characteristic [4].

The 2-specificity of linolenate chain in soybean oil was very close (36.6%) to the value expected for a random distribution pattern (33.3%) whereas in colza oil it was higher (56.8%) and almost equal to the specificity of linoleate chain (55.4%).

A further inspection into the 2-specificity data of oleate and linoleate chains of the whole oil set evidenced a negative correlation (coefficient of correlation r = –0.77 where the statistic R^2 = 60.00 indicated that the linear model explained 60 % of the variability in the linoleate 2-position specificity values) between the 2-position specificity and the mole percentages of linoleate chain according to the linear relationship:

Linoleic acid 2-pos Specificity = (Linoleic acid mole %) × (-0.23) + 57.55 (2)

$r = -0.77$ $R^2 = 60.00$

This result was in agreement with the feature that whenever a chain exhibited a strong specificity, a non significant concentration effect was measured [17].

Moreover, a positive linear correlation (regression line graph was reported in Fig. (**3**)) was found between mole percentages of oleic acid and 2-position specificity of linoleic acid, the equation is here below reported:

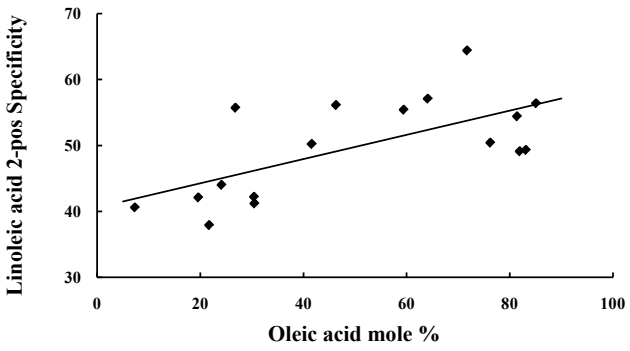


Fig. (3). Correlation of mole percentages of oleic acid and 2-position specificities of linoleic acid in the oil set comprising oil samples with oleic acid higher and lower than 50% and oil samples with linoleic acid higher than 5%.

Linoleic acid 2-pos Specificity = (Oleic acid mole %) × 0.18 + 40.59 (3)

$r = 0.66$ $R^2 = 43.26$

Even if the Linoleic acid 2-pos Specificity and the Oleic acid mole percentages were poorly correlated (correlation coefficient r = 0.66), this relationship can be tentatively explained by remembering that an increase of oleate mole percentages determines a decrease in linoleate mole percentages resulting in higher values for 2-specificity of this chain (1).

The compositions of the two different pools of fatty acids entering the 1,3- and 2- positions of triacylglycerols were calculated in the two oil sets with oleic acid higher than 50% in one set and lower than 50% in the other.

It is worth highlighting that ^{13}C NMR enables the determination of positional compositions of triacylglycerols, unlike chromatographic techniques which don't distinguish the fatty acid positions [18], and the Computer method (it is used to detect the olive oil adulteration with seed oils) which predicts the fatty acid compositions at 1,3- and 2- positions on the basis of the 1,3-random 2-random theory of fatty acid distribution in triglycerides [19].

The ^{13}C NMR data assumed also analytical relevance considering that the fatty acids at 1,3- and 2- positions were determined directly on an oil sample which was simply dissolved in a deuterated solvent without any further chemical treatment.

The distribution patterns of oleic and linoleic acids at 2-position of triacylglycerols in the oil groups with oleic acid higher and lower than 50%, were measured.

In particular, the percentages of oleic (O_{found}) and linoleic (L_{found}) acids at 2-glycerol position were compared to the percentages of oleic (O_{theory}) and linoleic (L_{theory}) acids in their total percentages in triacylglycerols, respectively. Linear relationships were calculated by regression analysis between the $O(L)_{found}$ and $O(L)_{theory}$ and the equations were here below reported:

Oils with Oleic acid %> 50%

$O\,found_{2pos} = O\,theory \times 1.45 - 44.78$ $r = 0.99$ $R^2 = 97.28$ (4)

$L\,found_{2pos} = L\,theory \times 1.45 - 0.52$ $r = 0.99$ $R^2 = 97.28$ (5)

Oils with Oleic acid %≤ 50%

$O\,found_{2pos} = O\,theory \times 0.76 + 3.37$ $r = 0.99$ $R^2 = 97.30$ (6)

$L\,found_{2pos} = L\,theory \times 0.76 + 20.28$ $r = 0.99$ $R^2 = 97.30$ (7)

The correlation coefficients r = 0.99 indicated a strong relationship between O,L_{found} and O,L_{theory} in correspondence of oleate and linoleate chains at 2-position, where the coefficient of determination R^2 showed that linear models explained ≥ 97 % of the variability in the O,L_{found} values. The observed values for *F* statistic higher than critical *F* for P=0.05 significance level, confirmed that linear correlations were not random. The slope values for oleate and linoleate chain lines were equal because the two chain compositions at 2-position were calculated in percentages thus depending on each other.

The regression lines of oleate and linoleate chains in vegetable oils with oleate percentages higher than 50% (Fig. **4**), deviated from random distribution patterns (dotted lines), where a random distribution assumed that the percentages of oleate and linoleate chains in the total of these chains in triglycerides were equal to their percentages in the total of these chains at 2-position. The deviation of oleate chain (O_{theory} ranged from 80 to 95%) from the random distribution pattern increased as the oleate percentage in the triglyceride decreased. Linoleate chain which is the minor chain in this

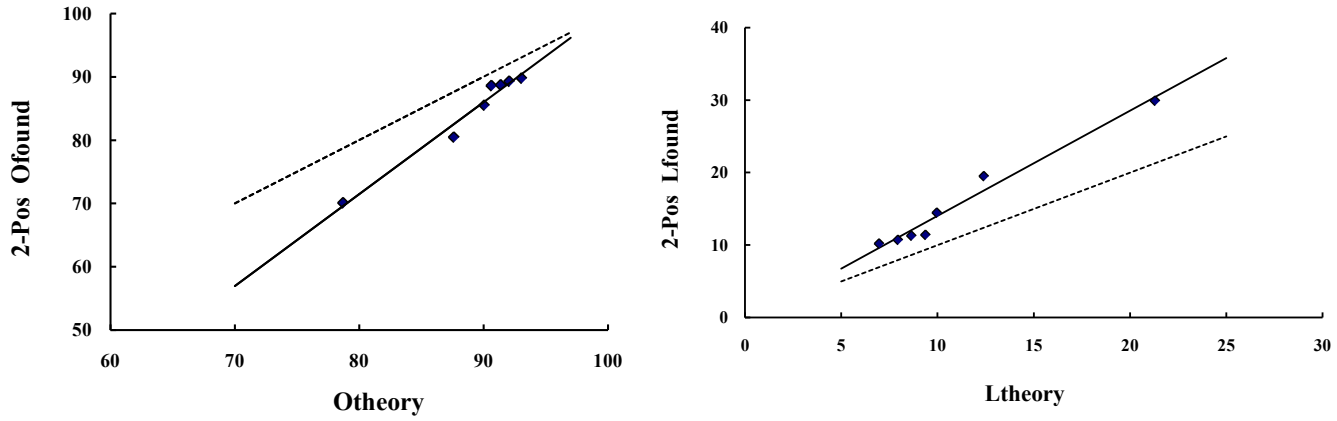


Fig. (4). Correlations of the compositions of oleic (Ofound) and linoleic (Lfound) acids at 2-glycerol position and the compositions of oleic (Otheory) and linoleic (Ltheory) acids, repectively, in the total of oleic and linoleic acids in triacylglycerols of the oil set with oleic acid higher than 50%.

oil set (L_{theory} ranged from 5 to 22%), exhibited an opposite trend.

These results agreed with the patterns already evidenced for a large set of olive oil samples where the oleate chain percentages were always higher than 50% [20].

However, in the oils with oleate chain as a minor component (L_{theory} was comprised in the range from 40 to 95%), linoleate chain deviated from the random distribution pattern at an increasing rate upon decreasing linoleate percentage in the triglyceride. Oleate chain showed an opposite trend (Fig. **5**).

It appeared evident in both oil sets that the compositions of the major chains at 2-position, i.e. oleate and linoleate in the oils with oleate higher and lower than 50%, respectively, were closer to a random distribution pattern in correspondence of the higher values of O_{theory} and L_{theory}, respectively. That is, when chain concentrations are higher, "the concentration factor" predominates over "the specificity factor" almost zeroing it in agreement with the results obtained on maize triglycerides [17].

As an example, in the croton oil (Table **3**) with a high linoleate percentage (80.6%), the linoleate chain 2-specificity lowered to 40.6. This value is close to 33.3% detected for a random distribution, and lower than the 2-specificity of the linoleate chain determined in the oils with a lower chain concentration (on average, 2-specificity of linoleate chain = 50%).

The percentages of saturated (1,3-Pos S_{found}), oleate (1,3-Pos O_{found}) and linoleate (1,3-Pos L_{found}) chains at 1,3- positions as compared to the percentages of saturated (S_{theory}), oleate (O_{theory}) and linoleate (L_{theory}) chains in their total percentages in triacylglycerols, were also checked for the two oil groups. The equations of regression lines are reported below:

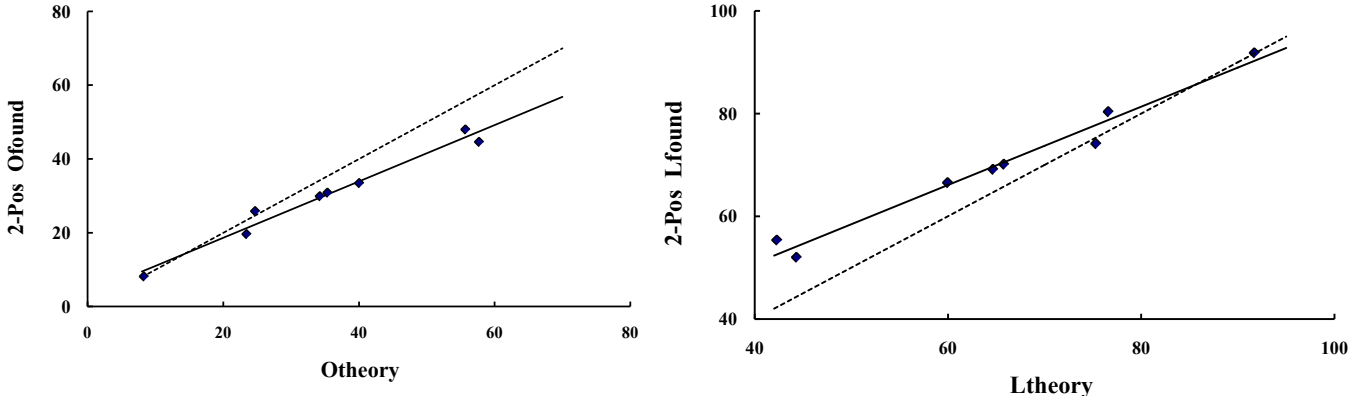


Fig. (5). Correlations of the compositions of oleic (Ofound) and linoleic (Lfound) acids at 2-glycerol position and the compositions of oleic (Otheory) and linoleic (Ltheory) acids, respectively, in the total of loeic and linoleic acids in triacylglycerols of the oil set with oleic acid lower than 50%.

Oils with Oleic acid %> 50%

$S\,found_{1,3\text{-}pos} = S\,theory \times 1.49 + 0.20$ r=0.99 R^2=99.88 (8)

$Ofound_{1,3\text{-}pos} = O\,theory \times 1.05 - 7.72$ r=0.98 R^2=96.72 (9)

$L\,found_{1,3\text{-}pos} = L\,theory \times 0.56 + 1.06$ r=0.95 R^2=90.88 (10)

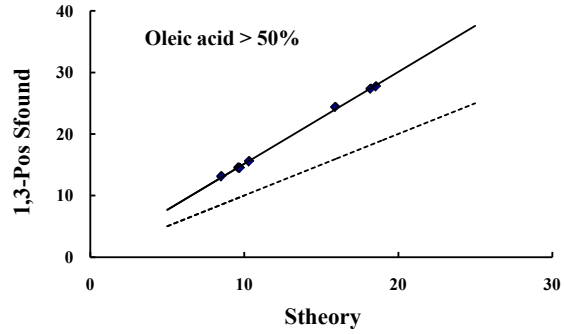
Oils with Oleic acid % ≤ 50%

$S\,found_{1,3\text{-}pos} = S\,theory \times 1.48 + 0.56$ r=0.99 R^2=99.89 (11)

$Ofound_{1,3\text{-}pos} = O\,theory \times 0.98 + 1.51$ r=0.99 R^2=98.38 (12)

$L\,found_{1,3\text{-}pos} = L\,theory \times 1.13 - 15.15$ r=0.99 R^2=98.87 (13)

The correlation coefficients r ≥0.95 confirmed that S,O,L_{found} were highly correlated to S,O,L_{theory} where the coefficient of determination R^2 showed that the linear models explained ≥ 96% of the variability in the S,O_{found} values (only 91 % in correspondence of L_{found} values).



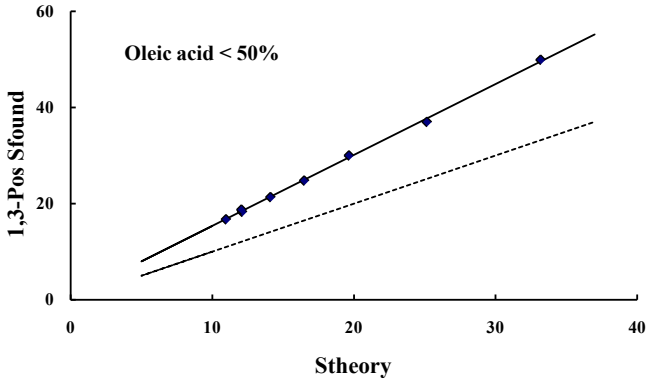
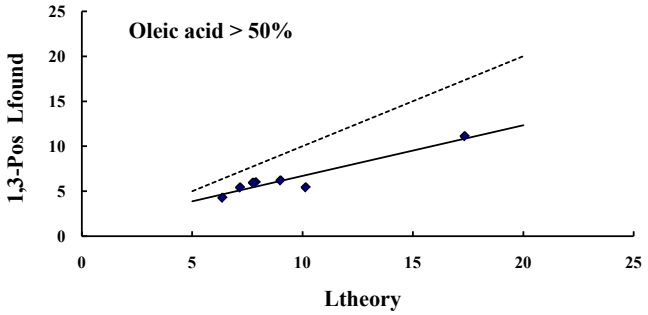


Fig. (6). Correlations of the compositions of saturated acid at 1,3-glycerol positions (Sfound) and the compositions of saturated acid in the total of saturated, oleic and linoleic acids in triacylglycerols (Stheory) of the oil sets with oleic acid higher and lower than 50%.

In both oil sets, saturated (Fig. **6**) and linoleate (Fig. **7**) chain percentages at 1,3- positions (S,L_{found}) were higher and lower, respectively, than those expected for a random distribution in agreement with the results obtained for a large olive oil set [20]. The deviations from the random patterns of the saturated chain in both oil groups, and of linoleate chain in the oil set with oleate higher than 50%, increased in correspondence of an increase of the S,L chain concentration in the triacylglycerol (S,L_{theory}).



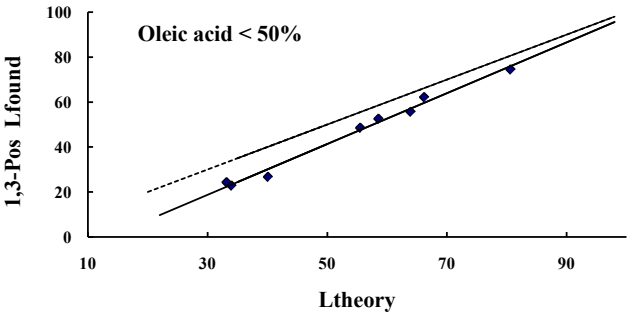


Fig. (7). Correlations of the compositions of linoleic acid at 1,3-glycerol positions (Lfound) and the compositions of linoleic acid in the total of saturated, oleic and linoleic acids in triacylglycerols (Ltheory) of the oil sets with oleic acid higher and lower than 50%.

However, the percentages of oleate chains at 1, 3-positions in the oil set with oleate higher than 50% (oleate was the major chain in these oils) were lower than those predicted by the random distribution pattern (Fig. **8**) in agreement with the results obtained for a large olive oil set where oleate was the major chain [20].

A deeper insight into the chain distribution patterns, evidenced that linear models for the chain distribution at 1,3-positions in the oil sets with oleate >50% and ≤ 50%, exhibited almost the same slope values for saturated (1.49 and 1.48, respectively), and oleate (1.05 and 0.98, respectively) chains. The higher slope values determined in correspondence of saturated chains as compared to oleate chain, suggested that the saturated chain composition at 1,3-positions was the most influenced by the chain concentration in the total triacylglycerol. The oleate chain with slope values very close to 1 (1.05 and 0.98), was almost randomly distributed like the linoleate chain (slope = 1.13) in the oils with oleic acid ≤ 50%. However, the low slope value (0.56) in correspondence of linoleate chain at 1,3-positions in the oils with oleate >50%, indicated that linoleate chain compositions at 1,3-positions were less influenced by the chain concentrations in the total triacylglycerol [20].

The compositions based on ^{13}C NMR data, of the two fatty acid pools at 1,3- and 2-positions of triacylglycerols in the oils with oleic acid higher and lower than 50%, con-

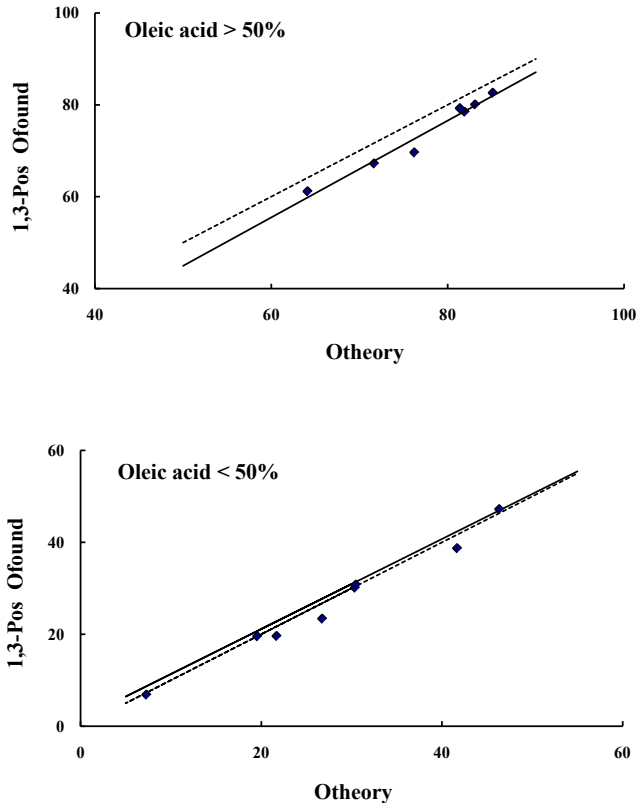


Fig. (8). Cor relations of the compositions o f ol eic acid at 1, 3-glycerol pos itions (Ofound) and the compositions o f ol eic ac id in the to tal o f s aturated, o leic and lin oleic acids in triacylglycerols (Otheory) of the oil sets with oleic acid higher and lower than 50%.

firmed t he c hain de viations from t he 1, 3-random-2-random distribution p attern. C onsidering t hat the Computer m ethod is ba sed on the 1, 3-random-2-random d istribution pa ttern, the c hain pos itional c ompositions we re m easured by us ing both, ^{13}CNMR and Computer m ethods, in order to evaluate the method differences.

The mole pe rcentages of s aturated, ole ate a nd linoleate chains in the total triglyceride determined on the basis of ^{13}C NMR d ata, were used to calculate the chain positional compositions by the Computer method.

The Computer method calculated the mole % of saturated chains a t 2-pos ition by us ing t he c oefficient 0. 06 ba sed o n the ratio between the peak threshold of saturated chains at 2-position (1. 3%) a nd t he m aximum c ontent of s aturated chains in o live oil (23.1%). The mole % of o leate and linoleate chains a t 2 pos itions a nd at 1, 3-positions were calculated by successive s ubtractions starting from the d ata o f saturated chains at 2-positions.

The percentages of s aturated, oleate and linoleate chains at 1, 3-positions a nd of ole ate and li noleate chains a t 2 -positions obt ained by us ing t he ^{13}C NMR m ethod (NMR) were re gressed ve rsus t he pe rcentages c alculated by us ing the Computer method (CM).

They were found linearly correlated according to the following equations:

$S_{1,3\text{-pos}}$ NMR = S $_{1,3\text{-pos}}$ CM × 1.01 + 0.43 r=0.99 R^2=99.90 (14)

$O_{1,3\text{-pos}}$ NMR = O $_{1,3\text{-pos}}$ CM × 1.00 + 2.02 r=0.99 R ^{2}=99.56 (15)

$L_{1,3\text{-pos}}$ NMR = L $_{1,3\text{-pos}}$ CM × 1.01 – 3.03 r=0.99 R^2=99.49 (16)

$O_{2\text{-pos}}$ NMR = O $_{2\text{-pos}}$ CM × 0.98 – 3.21 r=0.99 R^2=98.75 (17)

$L_{2\text{-pos}}$ NMR = L $_{2\text{-pos}}$ CM × 0.99 + 5.62 r=0.99 R^2=98.56 (18)

The s lope va lues w ere almost equal to 1 (t hey r anged from 0.98 to 1.01).

However, the intercept values indicated that the percentages of s aturated a nd o leate chains at 1, 3-positions and o f linoleate chain at 2-position determined by ^{13}C NMR (NMR) were h igher than those c alculated by the Computer m ethod (CM). Considering t hat t he Computer m ethod i s ba sed o n 1,3-random-2-random di stribution pa ttern, t hese re sults agreed w ith th e v alues m easured f or S_{found} at 1 ,3-positions (Fig. **6**), a nd L_{found} a t 2-pos ition (F igs. **4**, **5**) whi ch we re

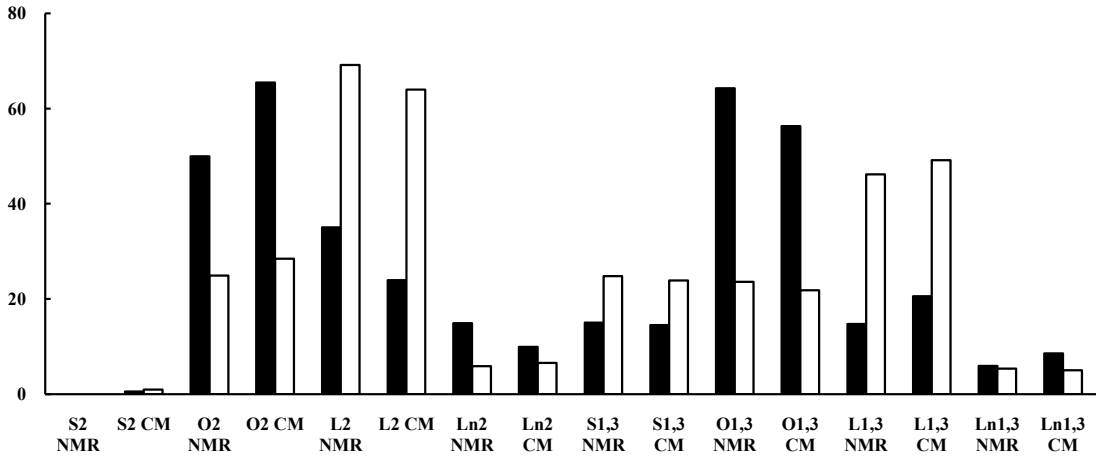


Fig. (9). Compositions of saturated (S), oleic (O), linoleic (L) and linolenic (Ln) acids at 1,3-positions and at 2-position of triacylglycerols of the colza (■) and soybean (□) oils calculated by using Carbon – 13 NMR spectroscopy (NMR) and Computer Method (CM).

higher than the values expected for a random distribution pattern. However, O_{found} values at 1,3-positions were slightly higher than the random values only in the oils with oleate lower than 50% (Fig. **8**).

On the other hand, the intercept values proved that the percentages of linoleate chain at 1,3-positions and of oleate chain at 2-position determined by the NMR method were lower than those obtained by the CM method in agreement with the values of L_{found} at 1,3- positions (Fig. **7**) and O_{found} at 2-position (Figs. **4**, **5**) which were lower than the random values in both oil sets.

The paired t-Test confirmed that the positional data obtained by NMR and CM methods were significantly different because the calculated values of $|t|$ were higher than the critical value 2.14 at P=0.05 and the null hypothesis at the 95.0% confidence level can be rejected [21].

By analogy, the acyl chain compositions of 1, 3- and 2-positions of triacylglycerols of the colza and soybean oils obtained by using the ^{13}C NMR data, were compared to those predicted by the Computer method (Fig. **9**).

In both colza and soybean oils, NMR percentages of saturated (15.1) and oleate (64.3) chains at 1, 3-positions (Table **4**) were higher than the corresponding CM percentages (14.5 and 56.3 for saturated and oleate chains, respectively). However, NMR percentages of the linoleate chain at 1,3-positions (14.8 and 46.2 for colza and soybean oils, respectively) were lower than CM percentages (20.6 and 49.2 for colza and soybean oils, respectively) in agreement with the results obtained for the oil sets with high - low oleic acid.

NMR percentages of the oleate chain at 2-position (50.0 and 24.9 for colza and soybean oils, respectively) were lower than the corresponding CM percentages (65.5 and 28.5), whereas NMR percentages of linoleate chains at 2-position (35.1 and 69.2 for colza and soybean oils, respectively) were higher than the corresponding CM values (24.0 and 64.0) which were in agreement with the results obtained for the oil sets with high - low oleic acid.

The NMR percentage of the linolenate chain at 2-position in the colza oil (14.9) was higher than the CM value (10.0) whereas in the soybean oil the NMR (5.9) and CM (6.6) data were not so different. These results considering that CM data were calculated on the basis of the 1, 3-random-2-random distribution pattern, were in agreement with a low 2-position specificity value of the linolenate chain, which in the soybean oil (36.6%) (Table **4**) was very close to the percentage of 33.3% expected for a random distribution. 2-Position specificity of the linolenate chain in colza oil was considerably higher (56.8). NMR (5.4) and CM (5.1) values of the linolenate chain at 1, 3-positions were almost equal in the soybean oil, the NMR value (5.9) in the colza oil being lower than CM value (8.6).

These results indicated that NMR and CM data were similar in the chains which exhibited a low 2-position specificity and consequently, appeared to be randomly distributed among the 1,3- and 2-glycerol positions (e.g. the linolenate chain in soybean oil).

The molar percentages of saturated, oleate and linolenate chains at 1,3- and 2-positions of triacylglycerols calculated by the NMR and CM methods, were used to calculate the compositionally different triglycerides. In particular, peanut and croton oil samples with oleate percentages higher and lower than 50%, respectively, were selected and the calculations of the mole percentages of the triglyceride species were carried out by the substitution of the appropriate values in the following equantions where a, b, and c were the mole percentages of the A,B and C, fatty acids [22]:

$$\% AAA = (\%a_1)\,(\%a_2)\,(\%a_3) / 10{,}000$$

$$\% ABA = (\%a_1)\,(\%b_2)\,(\%a_3) / 10{,}000$$

$$\% AAB = (\%a_1)\,(\%a_2)\,(\%b_3)\,(2) / 10{,}000$$

$$\% ABC = (\%a_1)\,(\%b_2)\,(\%c_3)\,(2) / 10{,}000$$

$$\% ACB = (\%a_1)\,(\%c_2)\,(\%b_3)\,(2) / 10{,}000$$

$$\% BAC = (\%b_1)\,(\%a_2)\,(\%c_3)\,(2) / 10{,}000$$

Considering that peanut and croton oils contained three fatty acids, saturated (S, given as total saturated chains because ^{13}C NMR does not detect the chains by carbon number), oleic (O) and linoleic (L) acids, the number of constitutionally different triglycerides from n = 3 fatty acids is given by $(n^3 + 3n^2 + 2n) / 6 = 10$.

The results were here below reported (CM values were in round brackets):

Pe	anut oil	Croton oil
SSS	0 (0.08)	0 (0.02)
OOS	23.8 (24.7)	0.2 (0.2)
OOL	20.7 (20.6)	1.3 (1.3)
OSS	5.4 (6.2)	0.3 (0.3)
LSS	2.3 (1.7)	3.2 (3.1)
LLO	4.9 (5.6)	13.8 (14.1)
LLS	1.8 (1.8)	25.6 (24.8)
LLL	0.4 (0.5)	50.9 (51.7)
OOO	26.2 (25.5)	0.04 (0.04)
LOS	14.5 (13.4)	4.6 (4.5)

The amounts of constitutionally different triglycerides calculated by using NMR and CM methods were similar except for SSS triglyceride which was zero because saturated acids were not detected at 2- position by NMR method.

Moreover, the amount also of positional isomers were calculated in correspondence of OOL and LLO triglyceride species.

	Peanut oil	Croton oil
OOL	9.5 (13.8)	0.8 (0.8)
OLO	11.2 (6.9)	0.4 (0.4)
Total isomers	20.7 (20.7)	1.2 (1.2)
LLO	4.1 (3.7)	9.3 (9.4)
LOL	0.9 (1.9)	4.5 (4.7)
Total isomers	5.0 (5.6)	13.8 (14.1)

As expected the compositions calculated by NMR and CM methods differed in the amounts of positional isomers.

This pattern was confirmed for triglycerides of the oils (colza and soybean) with linolenic acid higher than 5% where the number of constitutionally different triglycerides from four fatty acids, i.e. saturated (S), oleic (O), linoleic (L)

and linolenic (Ln) acids, was 20 species. The compositions of some positional isomers based on unsaturated chains, calculated by NMR and CM methods were reported:

OOL	9.5 (15.2)	5.4 (6.2)
OLO	14.5 (7.6)	3.9 (3.1)
Total isomers	24.0 (22.8)	9.3 (9.3)
OOLn	3.8 (6.3)	0.6 (0.6)
OLnO	6.2 (3.2)	0.3 (0.3)
Total isomers	10.0 (9.5)	0.9 (0.9)
LLO	6.7 (6.5)	1.5 (1.4)
LOL	1.1 (2.1)	5.3 (6.9)
Total isomers	7.8 (8.6)	6.8 (8.3)

CONCLUSION

^{13}C NMR spectroscopy can detect directly on an unreacted oil sample, the fatty acids of triglycerides according to their different unsaturation degree and their position on glycerol backbone. In particular, the chains at 1,3- and 2- glycerol positions are detected where the sn-1- and sn-3- positions can not be differentiated.

Considering these major results, conventional ^{13}C NMR spectroscopy was applied to determine the structures of triglycerides of vegetable oils with high and low oleic acid, and high linolenic acid. Acyl chain percentages in the whole triglyceride, unsaturated chain percentages at 1, 3- and 2-positions which measure the chain specificity for glycerol position, and compositions of the two different pools of fatty acids entering 1,3- and 2- positions, were determined.

The specificity values of oleate and linoleate chains for triacylglycerol 2-position, confirmed the results obtained for olive oil triglycerides. They showed that the oleate chain 2-specificity (on average 36.3%) in high and low oleic acid oils, was very close to the value expected for a random distribution pattern (33.3%), whereas the value of the linoleate chain (49.8%) was considerably higher. The low variability of the 2-positions specificities of oleate and linoleate chains over a wide range of chain compositions, made 2-position specificity be considered the chain characteristic.

Saturated, oleate and linoleate chains were not randomly distributed between 1,3- and 2-positions of triacylglycerols. They deviated from random patterns at variable extents according to the chain concentration in the total triglyceride and confirmed that both, chain concentration and specificity seemed to regulate the fatty acid distribution in triacylglycerols.

The conclusion can be drawn that the 1, 3-random 2-random distribution pattern can not adequately explain the chain distribution in triacylglycerols.

The differences between the fatty acid compositions at 1,3- and 2-positions of triacylglycerols calculated by the ^{13}C NMR and the Computer methods (the latter is based on the 1,3-random-2-random distribution pattern) reinforced this conclusion.

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