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Determination of triacylglycerols and fatty acid composition of *Chilopsis linearis* seed oil by reverse-phase high-performance liquid chromatography

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Abstract: In the present work, the compositions of the 27 triacylglycerols (TAGs) as well as fatty acid composition of *Chilopsis linearis* seed oil were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) with diode array and mass spectrometric detections. It was shown that the main conjugated acid in the seed oil is a catalpic acid, (9E,11E,13Z)-octadeca-9,11,13-trienoic acid, while two unusual fatty acids have the parameters satisfying the literature data for (10E,12E)-octadeca-10,12-dienoic and (9E,12E)-octadeca-9,12-dienoic acids. For the two conjugated acid substituents the isosbestic point (at 234 nm) was found and was used to calculate the ratios of TAGs with different chromophores in the oil, taking into account the peak areas on the oil chromatogram.

Key words: Seed oil, *Chilopsis linearis*, triacylglycerols, conjugated fatty acid

1. Introduction

Vegetable seeds are important sources of edible, industrial, and pharmaceutical oils [1]. The main components of most vegetable oils are triacylglycerols (TAGs), which consist of three fatty acid substituents attached to the glycerol backbone. Some fatty acids, known as essential, are necessary for health care since they cannot be synthesized in the body and thus must come from foods [2]. Conjugated fatty acids have unique biological activities [3,4], including anticarcinogenic and antiatherogenic effects [5], immune system strengthening [6], antidiabetic effects [7], and the ability to significantly reduce human obesity in metabolic syndrome [8]. Analysis of the composition of TAGs is of great importance, since it not only determines the fatty acid composition of the oil but also provides information on the distribution of these substituents in triacylglycerols. Nonaqueous reverse-phase high-performance liquid chromatography (RP-HPLC) is suitable for the analysis of the TAG composition of natural fats and oils without any chemical modification, which is especially important in the case of chemically highly labile conjugated unsaturated acid substituents [9]. However, the quantitative and qualitative analysis of the TAG species composition of oils is quite a difficult task, since it is necessary to achieve the separation of a large number of TAG types composed of all acid moieties of the sample under investigation. Another common problem relates to quantification because of the lack of TAG standards and the dependence of both spectrophotometric and refractometric detector responses on TAG composition [10].

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The aim of the present paper is the development of an RP-HPLC method with spectrophotometric and mass-spectrometric (MS) detections for qualitative and quantitative analysis of *Chilopsis linearis* seed oil TAG species as well as the oil fatty acid composition.

2. Results and discussion

2.1. Determination of the TAG type

Chilopsis linearis seed oil is interesting because of the known literature data [11] about simultaneous biosynthesis of one isomer of octadecatrienoic (catalpic) acid and two isomers of uncommon octadecadienoic acids, (10E,12E)-octadeca-10,12-dienoic and (9E,12E)-octadeca-9,12-dienoic, while there is no information on the TAG species composition of the oil. The literature data about the presence of the two different conjugated fatty acid substituents are readily confirmed since the n-hexane oil extract has electron-vibrational absorption spectra characteristic for conjugated trienoic (λ_{max} = 271 nm) as well as for conjugated dienoic (λ_{max} = 233 nm) compounds (Figure 1).

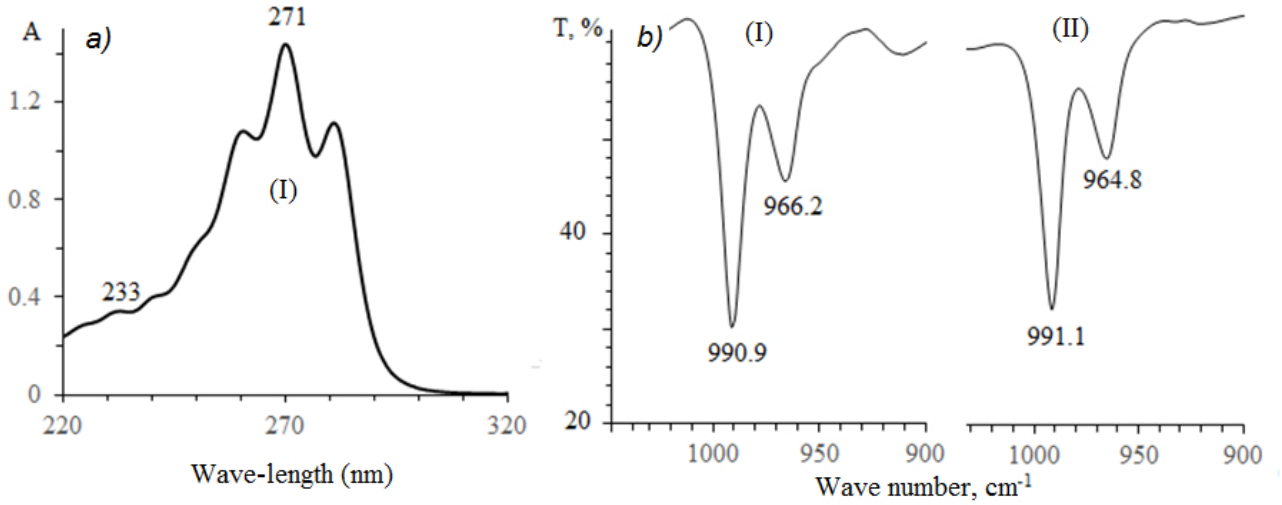

Figure 1. Electronic absorption spectrum (I) and IR spectra of fresh seed oils (I - seed oil *Chilopsis linearis*, II - seed oil *Catalpa ovata*).

Figure 1a shows the electronic absorption spectrum characteristic for *Chilopsis linearis* seed oil with three distinctly apparent absorption maxima in the range from 255 to 285 nm for conjugated triene and for conjugated diene compounds with absorption maxima around 233 nm [12]. The IR spectrum of *Chilopsis linearis* seed oil (as shown in Figure 1b) has two absorption bands, both characteristic of conjugated unsaturation [13], in the 900–1000 cm^{-1} region. The first of these bands appears at 990.9 cm^{-1} and the second at 966.2 cm^{-1}, the data being close to that for the IR spectrum of *Catalpa ovata* seed oil. These results confirm the existence of conjugated diene and triene moieties in the oil.

Due to the presence of conjugated acid substituents in the oil, we have developed an RP-HPLC method for the separation of TAGs with spectrophotometric detection. To determine the specific TAGs of *Chilopsis linearis* seed oil, chromatograms were registered at two wavelengths with subsequent comparison with the chromatogram of the catalpa seed oil. The composition of the latter oil had been determined by us earlier [14]. Additionally, the chromatograms of *Chilopsis linearis* seed oil were processed with MagicPlot Student 2.7.1 to detect individual TAGs. The chromatograms are shown in Figure 2.

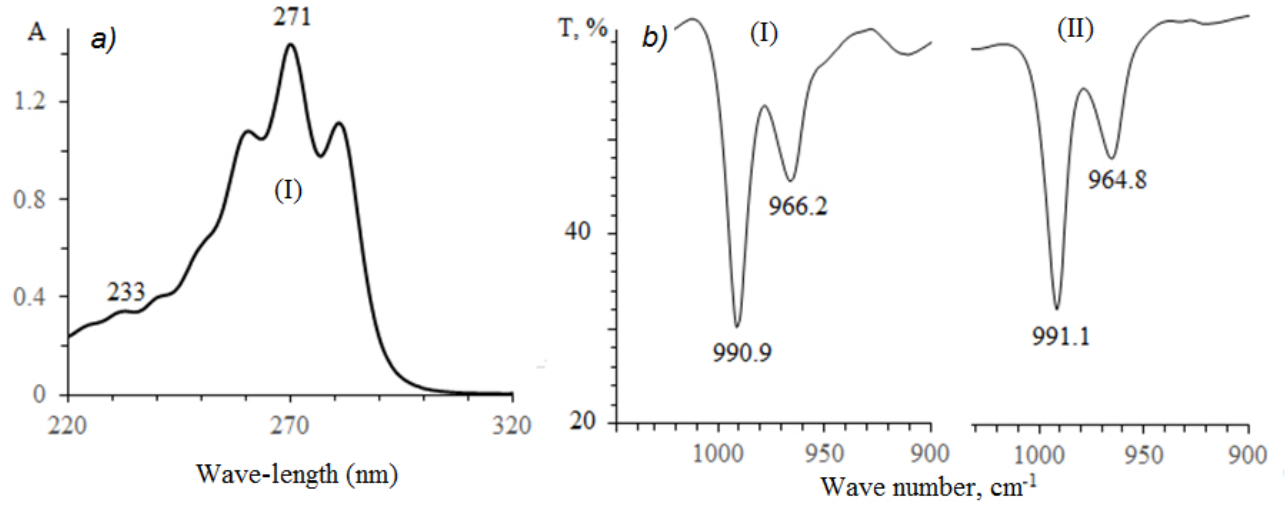

Figure 2. Separation of TAGs of *Chilopsis linearis* seed oil. Wavelengths of *Chilopsis linearis* TAGs detection: (I) - 271 nm and 233 nm, (II) - reference chromatogram of *Catalpa ovata* seed oil (271 nm). Column: 250 × 4.6 mm, Kromasil 100-5 18, mobile phase composition: 40 vol. % of 2-propanol and 60 vol. % of acetonitrile, 0.8 mL/min; temperature of column thermostat 30 °C.

There are five peak groups on the *Chilopsis linearis* seed oil chromatogram. In the case of detection at 271 nm (Figure 2, (I), 271 nm) only the peaks of TAGs that include the conjugated octadecatrienoic acid substituent are visible, while detection at 233 nm allows visibility of those with conjugated octadecadienoic acid substituents, while the sensitivity towards trienoic compounds decreases substantially. The TAGs with nonconjugated dienoic acid substituents cannot be detected directly. Using the incremental approach [15], we performed the calculation of the species composition of the studied oil (as shown in Table 1). The approach is based on the difference of logarithms of TAG retention factors (increments) for an exchange of one acid substituent by another one, while the increments do not depend on the structure of the two remaining identical substituents in the corresponding pairs of TAGs. The calculated TAG composition was confirmed by the parameters of both MS and electronic absorption spectra.

According to the presented data, the increments for TAG structure alteration in corresponding pairs are identical: for replacement of octadecatrienoic (Ca) acid substituent with the linoleic (L) one, 0.098 logarithmic units; for linoleic with oleic (O), 0.138; for oleic with palmitic (P), 0.035; and for palmitic with stearic (S), 0.128. In addition, the two increments $\Delta(L \rightarrow X)$ and $\Delta(X \rightarrow Y)$ resulted in consecutive substitution of the linoleic acid substituent by that of octadienoic (according to the MS data) acid substituents X and Y that do not correspond to any increment found for ordinary TAGs. The problem of recognition of acids X and Y is rather complicated and cannot be solved by only the chromatographic approach. Nevertheless, the composition of TAGs of *Chilopsis linearis* seed oil was solved by comparison of the increments for the oil with that for *Catalpa ovata* seed oil. Thus, first of all, octadecatrienoic acid was proved to be a catalpic one. Then the increment for the replacement of linoleic with (9-*trans*,12-*trans*)-octadeca-9,12-dienoic acid substituents, found for catalpa seed oil TAGs [14], coincides with the increment $\Delta(L \rightarrow Y)$ being the sum of two consecutive increments $\Delta(L \rightarrow X)$ and $\Delta(X \rightarrow Y)$ for *Chilopsis linearis* oil TAG. Thus, acid Y is the (9-*trans*,12-*trans*)-octadeca-9,12-dienoic acid substituent. In the case of the acid X substituents, UV-spectral properties of the corresponding TAG prove X to be a conjugated dienoic acid, namely (10E,12E)-octadeca-10,12-dienoic one, according to the literature data [11].

Table 1. The application of an increment approach to *Chilopsis linearis* seed oil TAG.

No.[a]	TAG	t_R (min)	lgk	Increment Δ(j→i) (± 0.002)						M/z [+ $^+$]
				Ca→L	L→X	X→Y	L→	→P	P→S	
1	Ca_3	17.25	0.655							873.7
2	Ca_2L	20.83	0.753	0.098						875.8
3	Ca_2X	21.57	0.771		0.018					
4	Ca_2Y	21.96	0.780			0.009				
5	CaL_2	25.33	0.852	0.098						877.8
6	CaLX	26.26	0.869		0.018					
7	CaLY	26.72	0.878			0.009				
8	Ca_2	27.46	0.891				0.138			877.8
9	CaXY	27.72	0.896		0.018					877.8
10	CaY_2	28.24	0.905			0.009				
11	Ca_2P	29.36	0.924					0.033		851.6
12	L_2X	32.14	0.968	0.098						879.8
13	CaL	33.57	0.989				0.137			
14	LXY	33.93	0.994	0.098						
15	CaX	34.92	1.007		0.019					879.7
16	CaY	35.50	1.015			0.008				
17	CaLP	36.03	1.022					0.034		853.6
18	CaXP	37.51	1.041		0.019			0.034		
19	CaYP + Ca_2S	38.29	1.051			0.010			0.127	853.7+ 881.7
20	LX	42.95	1.105	0.098			0.138			881.7
21	Ca $_2$	44.91	1.126				0.137			
22	XY	45.53	1.133				0.139			
23	LXP	46.32	1.141					0.035		855.7
24	CaLS	47.55	1.153						0.130	881.7
25	Ca P	48.48	1.162					0.036		-
26	XYP	49.13	1.168					0.035		-
27	CaYS	50.45	1.180		(L→Y) 0.027				0.129	-
Mean value				0.098	0.019	0.008	0.138	0.035	0.128	-
Catalpa seed oil				Ca→L	L→Y		L→	→P	P→S	-
Mean value				0.098	0.027		0.139	0.034	0.129	

[a]The number of peaks is shown in Figure 2.

2.2. Determination of the quantitative composition of the TAGs and fatty acid

Common simple peak area normalization procedures may be utilized for different TAG quantifications only in the case of known peak responses to the type of detection when all the peaks are visible on the same chromatogram. In the case of substituents with two different chromophores, the chromatogram must be registered at isosbestic wavelength. In the case of *Chilopsis linearis* seed oil, the wavelength of the isosbestic point may be easily calculated according to the procedure proposed in a previous paper [10]. For the calculation, three TAGs,

namely CaL_2 (peak 5, Table 1), CaXL (peak 6), and X_2L (peak 12), were taken. Normalization of the peak 5 spectrum gives a function $F_{Ca}(\lambda)$ for pure catalpic acid derivatives. A similar procedure for the peak 12 spectrum gives the second function for conjugated dienoic acid derivatives, $F_X(\lambda)$, while for peak 6 an experimental function with exact mole ratio 1:1 of the two different conjugated substituents in one TAG is obtained, $F_{CaX\,\mathrm{exp.}}(\lambda)$. On the other hand, function $F_{CaX}(\lambda)$ may be obtained by normalization of the sum of the two functions, taken with the sensitivity coefficient, g, because of the known experimental λ_{max} value:

$$F_{CaX}(\lambda) \equiv \frac{F_{Ca}(\lambda) + g \cdot F_X(\lambda)}{F_{Ca}(\lambda_{\max}) + g \cdot F_X(\lambda_{\max})} = F_{CaX(\mathrm{exp.})}(\lambda).$$

The coefficient g is determined by minimization of $\Sigma(F_{CaX}(\lambda) - F_{CaX(\mathrm{exp.})}(\lambda))^2$ calculated for each wavelength from 220 to 300 nm by 0.5 nm steps. Calculations for several different sets of spectra (for different compositions of mobile phases) allow us to obtain the coefficient g value, 0.406 ± 0.005. Building normalized spectrum $F_{Ca}(\lambda)$ and the spectrum $F_X(\lambda)$ multiplied by g on the same plot reveals the isosbestic point in the place of intersection of the two spectra, which was found to be at 243 nm.

Thus, a chromatogram should be registered at this wavelength (Figure 3) and the peak areas do not depend on the type of conjugated fatty acid substituents, still depending on the quantity of the substituents. These peak areas may be used to calculate mole fractions of TAGs of *Chilopsis linearis* seed oil (Table 2).

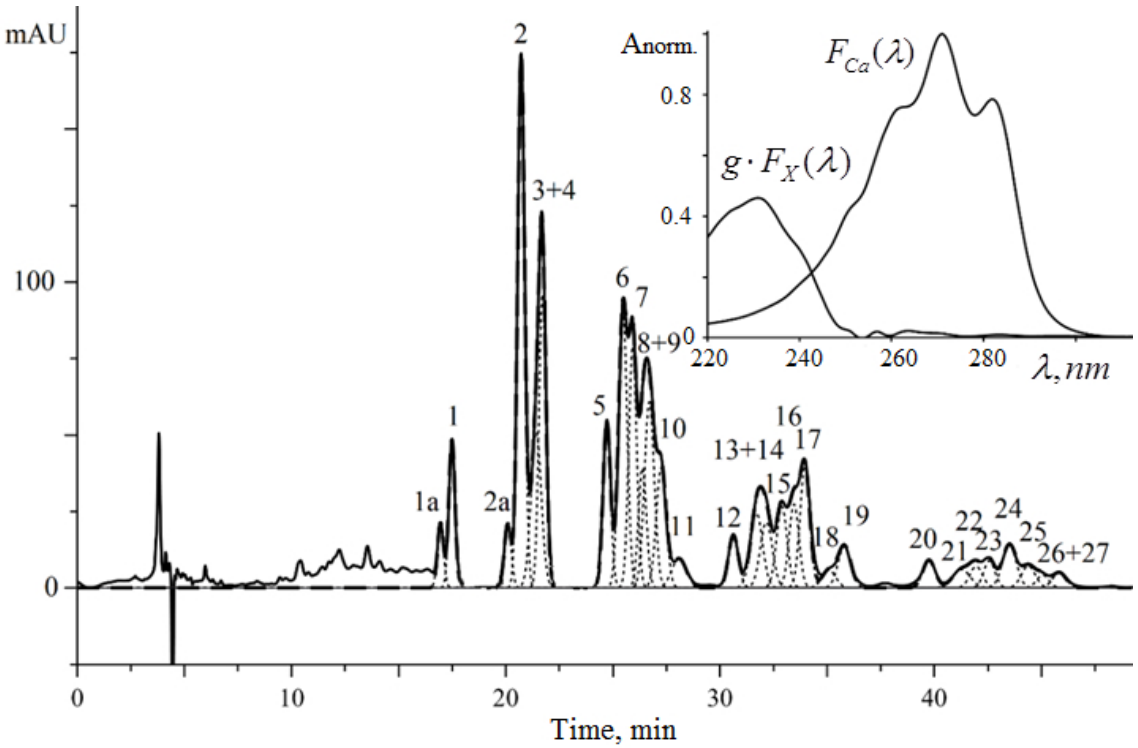

Figure 3. Chromatogram of *Chilopsis linearis* seed oil registered at an isosbestic point (243 nm). The chromatogram was processed by MagicPlot Student. For peak numbers, see Table 2.

Based on the results obtained, the fatty acid composition of this oil was calculated (Table 3). The results obtained are consistent with the results given in the literature on the direct determination of fatty acid

Table 2. The composition of the main TAGs of *Chilopsis linearis* seed oils.

No.	TAG	Mole fraction of TAG, % (± 0.3%)	No.	TAG	Mole fraction of TAG, % (± 0.3%)
1	Ca_3	1.6	*14*	LX	3.1
2	Ca_2L	11.9	*15*	CaX	3.3
3	Ca_2X	2.2	*16*	Ca	2.3
4	Ca_2	6.7	*17*	CaLP	6.2
5	CaL_2	6.7	*18*	CaXP	1.0
6	CaLX	6.4	*19*	Ca P+Ca_2S	2.2
7	CaL	11.3	*20*	LX	1.3
8	Ca_2	4.4	*21*	Ca $_2$	0.6
9	CaX	2.2	*22*	XY	1.8
10	Ca $_2$	6.2	*23*	LXP	1.3
11	Ca_2P	0.7	*24*	CaLS	3.2
12	L_2X	2.4	*25*	Ca P	1.4
13	CaL	2.8	*26*	XYP	2.8
-	-	-	*27*	Ca S	1.2

composition after oil transmethylation. Thus, *Chilopsis linearis* seed oil is a valuable oil for obtaining highly biologically active substances.

Table 3. Composition of the main acid substituents in TAGs of *Chilopsis linearis* seed oils.

Acid type	Mole fraction of acids, %	
	Present work	Literature [11]
Catalpic acid (Cal)	39.09 ± 0.45	22
Linoleic (L)	22.16 ± 0.32	28
(10E,12E)-octadeca-10,12-dienoic (D)	9.61 ± 0.78	10
(9E,12E)-Octadeca-9,12- dienoic ( )	15.57 ± 0.80	16
Oleic ( )	7.44 ± 0.22	17
Palmitic (P)	4.73 ± 0.45	7
Stearic (S)	1.42 ± 0.13	

2.3. Conclusions

In the present study, using RP-HPLC with propan-2-ol and acetonitrile as mobile phase components, combined with an incremental approach and analysis of electronic absorption spectra as well as MS, made it possible to determine the TAG and fatty acid compositions of *Chilopsis linearis* seed oil. The special procedure was applied to find the isosbestic point for oil composed of two conjugated fatty acid substituents. For the problem of peak resolution the MagicPlot Student program was utilized. The fatty acid composition was calculated on the basis of found species composition of the oil: catalpic 39.09 mole %, linoleic 22.16 mole %, (10E,12E)-octadecadienoic 9.61 mole %, (9E,12E)-octadecadienoic 15.57 mole %, oleic 7.44 mole %, palmitic 4.73 mole %, and stearic 1.42 mole %.

3. Experimental

3.1. Seed samples

Seeds of *Chilopsis linearis* Champ. ex Benth were collected in Vietnam in 2017 (Lam Dong city). *Catalpa ovata* seeds were taken from fruits grown in Belgorod, Russia, in 2017.

3.2. Sample preparation

3.2.1. Oil extraction

A portion (1 g) of plant seeds was ground in a porcelain mortar under a layer of n-hexane added by portions of 5–10 mL, the successive portions being combined. The solvent from the extract was withdrawn on a vacuum rotary evaporator.

3.2.2. Oil purification

The oil for HPLC investigation and storage was purified by solid-phase extraction. The oil solution (25 mL) in n-hexane (20 mg/mL) was passed through a syringe cartridge filled with silica. It is important to check the catalytic ability for oil destruction by HPLC of the silica before sorption for purification. This is necessary because some commercial silica trademarks were found to be not inert towards the oil even at room temperature. The oil from the cartridge was desorbed by 4 mL of acetone. After the withdrawal of acetone on a vacuum rotary evaporator, the transparent oil without heterogeneous inclusions was prepared to be ready for analysis and storage.

3.3. Chromatographic equipment

Seed oil TAG was separated by RP-HPLC on an Agilent 1200 Infinity chromatograph with diode-array and MS detectors. Chromatograms were recorded in the mobile phase of 40 vol. % propanol-2 in acetonitrile, 1 mL/min; chromatographic column: 250 × 4.6 mm Kromasil 100-5C18 (for HPLC with spectrophotometric detection) and 150 × 2.1 mm Kromasil 100-5C18 (for MS detection). MS detection was carried out in a mixed mode with chemical ionization at atmospheric pressure and ionization by electrospray under standard conditions at a fragmentor voltage of 150 V; signals were recorded for positively charged ions. $HCOONH_4$ was added to the mobile phase for MS detection with a concentration of 10 mM to enhance the formation of protonated particles. All the experiments were performed in isocratic mode. Chromatograms were recorded, stored, and processed using the specialized software product Agilent ChemStation as well as MagicPlot Student software for the resolution of "problem" (with a low value of R_S) TAG.

The retention factors were calculated using the "dead" time (t_M), which was found by a method similar to the homologous series method [16], assuming equal retention increase of capacity logarithms of factors (k) in the series Ca_3 – Ca_2L – CaL_2:

$$\frac{t_R(Ca_3) - t_M}{t_R(Ca_2L) - t_M} = \frac{t_R(Ca_2L) - t_M}{t_R(CaL_2) - t_M}.$$

3.4. Spectrophotometric equipment

The electronic absorption spectra were recorded in quartz cells (1 cm) on a Shimadzu UV 1550 spectrophotometer.

FTIR spectra were recorded on a Shimadzu IR Prestige spectrometer in thin-film KBr. The scans were achieved at a resolution of 4.0 cm^{-1} (from 450 to 4000 cm^{-1}).

3.5. Fatty acid and TAG designation

TAGs were designated by the conventional mode, with the letters representing acid substituent without specification of their position in the molecule. Letter designations of acid radicals were as follows: Ca represents the substituent of C18:$3^{9E11E13Z}$ ((9E,11E,13Z)-octadec-9,11,13-trienoic), X – (10E,12E)-octadec-10,12-dienoic (C18:2^{10E12E}), Y – (9E,12E)-octadec-9,12-dienoic (C18:2^{9E12E}), L – linoleic (C18:2^{9Z12Z}), O – oleic (C18:2^{9Z}), P – palmitic (C16:0) and stearic (C18:0) acids. For example, the formula, L_2O denotes a TAG with two substituents of linoleic acid and one of oleic acids.

3.6. Statistical analysis

In this work, to control the reproducibility of the results of parallel recordings of the chromatogram of the same oil solution, five consecutive chromatographies were performed without long pauses in order to release the substances from gallery pores [17]. As a result, it was determined that the maximum confidence interval for the mole fraction of TAG species did not exceed 0.20% for the average of five parallel results. Quantitative results of fatty acids were expressed as means ± SD of three experiments.

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