Comparison of Separation of Seed Oil Triglycerides Containing Isomeric Conjugated Octadecatrienoic Acid Moieties by Reversed-Phase HPLC

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Abstract: Relative retention analysis and increment approach were applied for the comparison of triglycerides (TGs) retention of a broad set of plant seed oils with isomeric conjugated octadecatrienoic acids (CLnA) by reversed-phase HPLC for "propanol-2-acetonitrile" mobile phases and Kromasil 100-5C18 stationary phase with diode array detection (DAD) and mass spectrometric (MS) detection. The subjects of investigation were TGs of seed oils: *Calendula officinalis*, *Catalpa ovata*, *Jacaranda mimosifolia*, *Centranthus ruber*, *Momordica charantia*, *Trichosanthes anguina*, *Punica granatum*, *Thladiantha dubia*, *Valeriana officinalis*, and *Vernicia montana*. It was found that a sequence of elution of TGs of the same types is the same without any inversions for full range of mobile phase compositions: punicic (C18:3^{9Z11E13Z}) < jacaric (C18:3^{8Z10E12Z}) < catalpic (C18:3^{9E11E13Z}) < α-eleostearic (C18:3^{9Z11E13E}) < calendic (C18:3^{8E10E12Z}) < β-eleostearic (C18:3^{9E11E13E}) < all-E calendic (C18:3^{8E10E12E}) acids. TGs and fatty acid compositions were calculated for all oil samples. Regularities of solute retentions as a function of isomeric conjugated octadecatrienoic acid moiety structure are discussed. Thus, it was proven that it is possible to differentiate TGs of complex composition with moieties of all natural CLnA by retention control accomplished by electronic spectra comparison, even though there are only three types of electronic-vibration spectra for seven isomeric CLnA.

Keywords: reversed-phase HPLC; seed oil; conjugated octadecatrienoic acids; increment approach; relative retention analysis

1. Introduction

Among a great variety of plant seed oils, oils with conjugated fatty acid moieties are of special interest because of their high biological activity [1–7]. One type of these acids includes isomeric octadecadienoic acids found mostly in the meat and dairy products derived from ruminants (conjugated linoleic acids, CLA) [8]. Mainly natural octadecatrienoic acids (conjugated linolenic acids, CLnA) [2] are synthesized in some plant seeds, being the substances under investigation in current paper. Other types of acid with more complex structure also known, for example, parinaric (octadecatetraenoic acid), some hydroxylated acids (α-kamlolenic acid) and acids with acetylenic bonds in conjugation [9].

CLnA in native seed oils are mainly in the form of triacylglycerols (TGs). Currently, there exist two ways for oil analysis. The first way implies transmethylation procedure of initial oils to obtain methyl esters of the fatty acids suitable for subsequent analysis by gas chromatography [10]. This method permits determination the oil main fatty acids as well as minor components. However, it should be

taken into account that during transesterification some labile acids may undergo transformation or be lost entirely. Moreover, the procedure leads to loss of information about fatty acids distribution among TGs species as a hardly falsifiable parameter. According to an alternative method, non-modified TGs are analyzed by HPLC separations.

The most commonly used method explores reversed-phase HPLC for TGs separation [11]. In this case, some problems arise concerning solutes resolution especially in the case of some TGs pairs [12]. Indeed, if only four types of fatty acids compose the oil, the number of resulting possible different types of TGs is 20 (without specifying the position of the acid moieties in TG). Some of these solutes are easily separated but, for another pair, retentions are close and base-line separation is almost unachievable. Thus, results of calculation of fatty acid composition of the oil by the second method may have some errors.

Another common problem is connected with solutes detection, but TGs with conjugated acid substituents are easily detectable using conventional spectrophotometric detection, while diode-array detectors are favorable due to a possibility of electronic spectra registration being the orthogonal (to retention times) properties for solute identification. Mass-spectrometric detection is highly desirable orthogonal method of solute properties control and for seed oils method APCI mass-spectrometry (atmospheric pressure chemical ionization) was developed [13].

It is commonly known that solute retention coincidence is not a proof of solute identity. However, there exist strict regularities in TGs retention. Equivalent carbon numbers (ECN) connect solute retentions with that of synthetic TGs composed by saturated fatty acids substituents [14,15]. ECN depend upon structure of all moieties, are additive and so their retention is predetermined. Thus, ECN is really an orthogonal property of the solute and so it may be used for tentative solute identification. Meanwhile ECN depends upon mobile phase composition for a given chromatographic system complicating the method utilization. Development of relative retention analysis [16] was directed to escape the dependence upon mobile phase composition. In this approach, retention of solute has two parametric dependence:

$$\log k(i) = a_0 + a_1 \log k(A) \tag{1}$$

where $k(i)$ is capacity factors of solute, while $k(A)$ is that of reference solute A.

Increment approach implies to use group contribution factor to calculate the solute retention alteration for the exchange of two fatty acids in pair of TGs is the other two moieties remain unchanged:

$$\Delta(i{\rightarrow}j) = \log k(j) - \log k(i) \tag{2}$$

The increment $\Delta(i{\rightarrow}j)$ does not depend upon nature of the remaining two unchanged substituents, but it depends upon mobile phase composition. Equation (1) describes the retention of solutes i in a broad composition of given mobile phase system and given stationary phase. For non-polar solutes, the equation may be transferred upon solute separation for other stationary reversed phases trademarks.

The aim of the present investigation was to compare retentions of TGs of oils with isomeric conjugated octadecatrienoic acids in reversed-phase HPLC to elucidate the possibilities of solute identification.

2. Materials and Methods

2.1. Plant Seed Material

Oils were extracted for plant seeds: *Calendula officinalis*, *Catalpa ovata*, *Momordica charantia* and *Punica granatum* grown in Belgorod; *Thladiantha dubia* and *Valeriana officinalis*, bought in Belgorod market for gardeners; and *Trichosanthes anguina*, *Jacaranda mimosifolia* and *Vernicia montana*, grown in Vietnam in 2016.

2.2. Oil Extraction and Purification

Oils were extracted from 2 g of seeds in porcelain mortar by 5 portions (for exhausting extraction) each of 20 mL of hexane at room temperature. The portions were combined and the solvent was withdrawn on the rotary evaporator at 30 °C.

The liquid residue was dissolved in hexane to prepare solution with oil concentration of 10 mg/mL.

The oil was refined by solid phase extraction on silica (in syringe cartridges), the silica being checked for the absence of catalytic activity. The oil was washed from sorbent with dichloromethane, solvent was evaporated and the oil was stored in a refrigerator at 4 °C.

2.3. Chromatography

For reversed-phase high-performance liquid chromatograpy (RP HPLC) we used an Agilent 1260 Infinity chromatography system with diode array and mass spectrometric detectors (Agilent Technologies, 76337 Waidbronn, Germany). The following chromatographic columns were applied: Kromasil 100-5C18 4.6 × 250 mm, and Kromasil 110-3.5C18 2.1 × 150 mm (for mass spectrometric detection).

Mass spectrometric detection was carried out in a mixed mode: atmospheric pressure chemical ionization and electrospray ionization under conventional conditions at a fragmentor voltage of 50 V; signals of positively charged ions were recorded.

2.4. Calculations and Designations

All experiments were carried out in an isocratic mode; chromatograms were recorded, stored, and processed using a specialized ChemStation software products. Non-resolved peaks were handled by Magicplot Student 2.7.2 software.

TGs were denoted in a conventional way, indicating radicals of acids with letters without specifying of their position in the molecule. The letter designations of the acid substituents: L, linoleic acid; O, oleic acid; P, palmitic acid; and S, stearic acids. For conjugated octadecatrienoic acid designations, see text. The formula L_2O means TG with two substituents of linoleic acid and one substituent of oleic acid.

The column void time (t_M) was calculated by the retention times of a series of TGs, assuming that the retention factors (k) in the series of $X_3 \rightarrow X_2L \rightarrow XL_2$ increase by the same value of logarithmic units of retention or:

$$\frac{t_R(X_3) - t_M}{t_R(X_2L) - t_M} = \frac{t_R(X_2L) - t_M}{t_R(XL_2) - t_M} \tag{3}$$

Mole fraction of TGs, α(TGs), was calculated taking into account peak area (S_i) on the chromatogram and number (n_i) of conjugated octadecatrienoic acid substituents in it:

$$\propto (TG_i) = \frac{\frac{S_i}{n_i}}{\sum_i \frac{S_i}{n_i}} \cdot 100\% \tag{4}$$

For acid j mole fraction in the oil calculation, mole fractions of all TGs were used taking into account the numbers of the acid substituents (n_{ij}), in each TGs:

$$\propto (Acid_j) = \frac{\sum_i \propto (TG_i) \cdot n_{ij}}{\sum_j \sum_i \propto (TG_i) \cdot n_{ij}} \cdot 100\% \tag{5}$$

3. Results

3.1. UV Spectroscopic Properties of Conjugated Octadecatrienoic Acids

Theoretically, there exist many isomers for octadecatrienoic acids with different position of double bonds in a carbon atom chain as well as with different *cis-trans* configuration of these bonds.

Only restricted number of the natural isomers was found in plant sources. First, in the isomers, the middle C=C-bond has only *trans*-configuration [9]. Moreover, only two positional isomers are synthesized in plant seeds: octadeca-9,11,13-trienoic and octadeca-8,10,12-trienoic acids [17]. Thus, only three types of chromophores may be responsible for the appearance of UV-spectra due to conjugation of the three C=C-bonds (Figure 1).
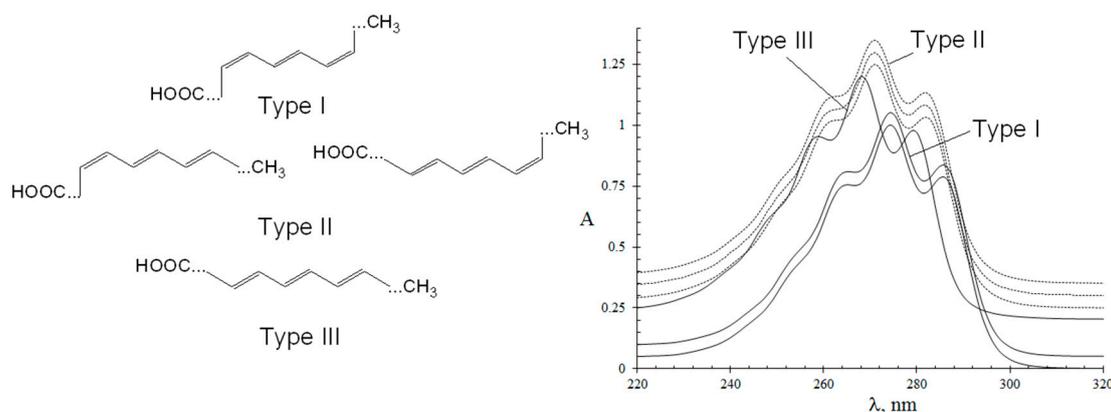
Figure 1. Three types of cis/trans configurations in natural conjugated octadecatrienoic fatty acids and their electronic spectra: Type I, punicic and jacaric acids; Type II, catalpic, α-eleostearic and calendic acids; and Type III, β-eleostearic and all-*trans*-calendic acids.

All oils with conjugated octadecatrienoic acids have electronic-vibration spectra with three apparently visible overlapping bands. The band's maxima wavelength depends upon type of *cis-trans* configuration [18]: exchange of *trans*- by *cis*-configuration causes bathochromic shift. In addition, since chromophore group is far away from carboxylic group in natural octadecatrienoic acid isomers only three different spectra may be registered (Figure 1). Thus, comparison of electronic spectra is a valuable instrument in solute structure differentiation at least into three types. It was proven in present investigation that the differentiation is favored by the absence of solvatochromic effects for the spectra for full range of mobile phase compositions suitable for TGs separation, at least for 34–60% propanol-2 in acetonitrile.

3.2. Retention Analysis of TGs of Plant Sources Seeds with Isomeric Octadecatrienoic Acids

3.2.1. Triglycerides of Oil with (9Z,11E,13E)-octadeca-9,11,13-trienoic Acid Moieties

From the series of plant sources with seed oils enriched with (9Z,11E,13E)-octadeca-9,11,13-trienoic or α-eleostearic acid [17], *Momordica charantia* was chosen as a source with high content of the acid simultaneously with other ordinary acids being suitable for increments calculation. The oil is known [19] to be built with linoleic, oleic, palmitic, stearic and α-eleostearic (α-El) acid.

Mainly 10 types of TGs with different retentions (Figure 2) compose the oil. A search of equal increments of TGs retention alteration together with mass-spectra consideration permits solute composition elucidation, as proposed in Table 1. The results are in a good agreement with published data [20,21].

The main TGs component of the oil is composed of two substituents of α-eleostearic and the third one, stearic acids (α-El$_2$S). The mole fraction of this component (38.2 mol. %) was calculated taking into account peak areas in Figure 2 and the number of α-eleostearic acid moieties in the TGs (Table 1). Calculated by the same way, fatty acid composition shows the predominant acid to be α-eleostearic (as a sum of all octadecatrienoic acids) (57.7 mol. %), followed by stearic (16.3%), linoleic (14.2%), oleic (7.6%) and palmitic (4.1%) acids.
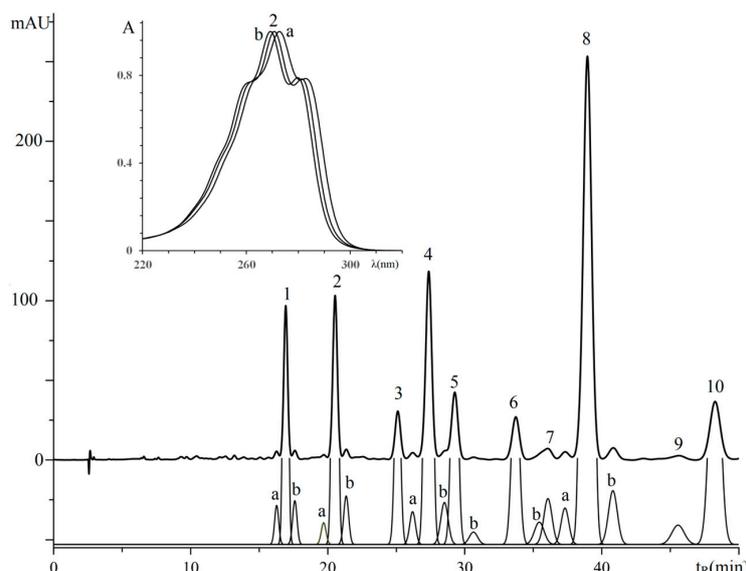
Figure 2. Separation of TGs of *Momordica charantia* seed oil. Column 4.6 mm × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 1.

Table 1. Retention parameters, mass-spectral signals and content of TGs of *Momordica charantia* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values				Mole Fraction of TG, %	*M/z* [M + H$^+$]
				X→L	L→O	O→P	P→S		
1	X[b]$_3$	16.94	0.761					4.8	873.8
2	X$_2$L	20.54	0.858	0.097				9.1	875.8
3	XL$_2$	25.11	0.956	0.098				5.6	877.7
4	X$_2$O	27.37	0.998		0.140			13.3	877.7
5	X$_2$P	29.27	1.030			0.032		4.6	851.8
6	XLO	33.72	1.097	0.099	0.140			6.9	879.8
7	XLP	36.05	1.128	0.098		0.031		2.8	n.d. [c]
8	X$_2$S	38.95	1.164				0.134	38.2	879.8
9	XO$_2$	45.65	1.237		0.141			1.5	n.d.
10	XLS(+XOP)	48.27	1.263	0.099			0.135	13.2	881.7 + 855.7
	Middle value			0.098	0.140	0.031	0.134		

[a] numbers of TG are the numbers of peaks in Figure 2; [b] X, moiety of conjugated octadecatrienoic acid (α-eleostearic); [c] n.d., not determined.

The increments calculated in the case of this seed oil were used for the calculation of the compositions of TGs of the other oils discussed below.

3.2.2. Triglycerides of Oil with (9Z,11E,13Z)-octadeca-9,11,13-trienoic Acid Moieties

Punica granatum is a prominent plant source of the oil of punicic acid type, but it has been intensively investigated in a series of papers [19]. Thus, we used a different natural source of the oil enriched with punicic (9Z,11E,13Z)-octadeca-9,11,13-trienoic acid, Pu (*Trichosanthus anguina*) seed oil. According to literature data [22], the seed oil of *Trichosanthus kirilowii* is composed of moieties of linolenic (38.2%), punicic, (38.0%), and oleic (11.8%) acids. In the case of *Trichosanthus anguina* seed oil, we may see five groups of peaks on the chromatogram (Figure 3). The composition of ten main peaks with identical electronic spectra refer to Type I (Figure 1), and hence composed with participation of punicic acid as the only conjugated one, and is calculated by the increment approach (Table 2). The increments for common acids substitutions linoleic by oleic, Δ(L→O), oleic by palmitic, Δ(O→P), and palmitic by stearic, Δ(P→S), are the same as for *Momordica* seed oil, despite different TGs compositions (another isomer of octadecatrienoic acid moieties).
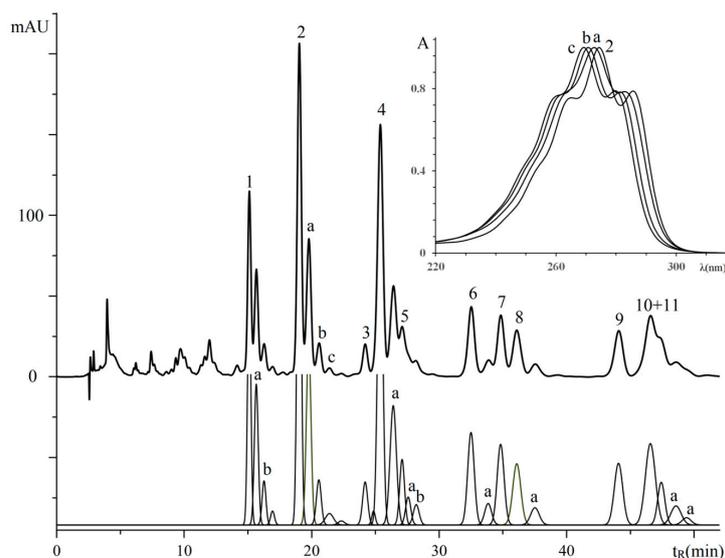
Figure 3. Separation of TGs of *Trichosanthus anguina* seed oil. Column 4.6 mm × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 2.

Table 2. Retention parameters, mass spectral signals and content of TGs of *Trichosanthes anguina* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values				Mole Fraction of TG, %	M/z [M + H$^+$]
				X→L	L→O	O→P	P→S		
1	X [b] $_3$	15.11	0.703					6.18	873.8
2	X$_2$L	19.04	0.820	0.118				18.43	875.8
3	XL$_2$	24.20	0.939	0.118				2.96	877.7
4	X$_2$O	25.39	0.962		0.141			17.18	877.7
5	X$_2$P	27.10	0.993			0.031		4.33	851.8
6	XLO	32.52	1.080	0.118				10.25	879.8
7	XLP	34.84	1.112			0.032		8.14	853.7
8	X$_2$S	36.09	1.128				0.135	4.26	879.8
9	XO$_2$	44.09	1.221		0.142			7.39	881.8
10	XLS(+XOP)	46.59	1.246	0.118			0.135	20.24	881.7 + 855.7
	Mean value:			0.118	0.141	0.032	0.135		

[a] Numbers of TGs are the numbers of peaks on the Figure 3; [b] X, moiety of conjugated octadecatrienoic acid (punicic).

In calculated fatty acid composition, the predominant acid was punicic (as a sum of all octadecatrienoic acids) (51.7 mol. %), followed by oleic (15.1%), linoleic (11.9%), palmitic (9.5%) and stearic (5.8%) acids.

3.2.3. Triglycerides of Oil with (8E,10E,12Z)-octadeca-8,10,12-trienoic Acid Moieties

Calendula officinalis seed represents a unique source of one of the octadecatrienoic isomers: calendic, (8E,10E,12Z)-octadeca-8,10,12-trienoic acid, Cal. According to literature data [23], this acid is predominant in different varieties of the plant (fatty acid composition (40–57%), while the content of other acids are: linoleic (30–37%), oleic (3.6–5.8%), palmitic (3.4–5.4%) and stearic (1.7–4.5%). Chromatogram of our sample of *Calendula officinalis* seed oil indicates the only predominant peak corresponds to Cal$_2$L composition of TGs (Figure 4). The composition of all TGs types with electronic spectra of the Type II (Figure 1) is presented in Table 3. The increments for common acids substitutions Δ(L→O), Δ(O→P) and Δ(P→S) remain unchanged (see Tables 1 and 2). The proposed types of TGs were supported by TGs' mass spectra (Table 3).
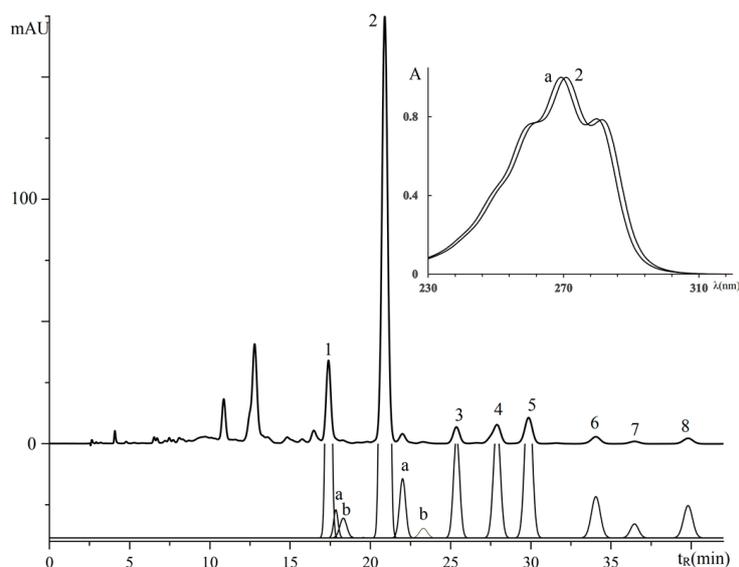
Figure 4. Separation of TGs of *Calendula officinalis* seed oil. Column 4.6 mm× 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 3.

Table 3. Retention parameters, mass spectral signals and content of TGs of *Calendula officinalis* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values				Mole Fraction of TG, %	M/z [M + H$^+$]
				X→L	L→O	O→P	P→S		
1	X[b]$_3$	17.39	0.775					8.25	873.7
2	X$_2$L	20.90	0.867	0.092				68.91	875.8
3	XL$_2$	25.36	0.961	0.094				6.06	877.7
4	X$_2$O	27.89	1.007		0.140			4.66	877.7
5	X$_2$P	29.86	1.039			0.032		5.42	851.8
6	XLO	34.05	1.101	0.094	0.140			3.75	879.8
7	XLP	36.46	1.133			0.032		1.37	853.8
8	X$_2$S	39.80	1.174				0.135	1.58	879.8
				0.093	0.140	0.032	0.135		

[a] Numbers of TGs are the numbers of peaks on the Figure 4; [b] X, moiety of conjugated octadecatrienoic acid (calendic).

In calculated by TG area fatty acid composition, the highest content is calendic (as a sum of all octadecatrienoic acids) (65.69 mol. %), followed by linoleic (28.72%), oleic 2.80%) palmitic (2.26%), and stearic (0.53) acids.

3.2.4. Triglycerides of Oil with (9E,11E,13Z)-octadeca-9,11,13-trienoic Acid Moieties

Catalpa species seed are interesting for the present investigation due to the biosynthesis of one octadecatrienoic acid isomer: catalpic, (9E,11E,13Z)-octadeca-9,11,13-trienoic acid, Cat [24]. According to literature data [24], this acid fraction in fatty acids composition is 42.25%, while the contents of other acids are as follows: linoleic (39.95%), oleic (7.71%), palmitic (2.77%), and stearic (2.65%). In addition, it is important to mention that α-linolenic acid was also found (0.56%), along with some other acid. Chromatogram of our sample of *Catalpa ovata* seed oil is somewhat more complicated compared to previously discussed chromatograms (Figure 5). All 15 numbered peaks have the same electronic spectra of Type II (Figure 1).
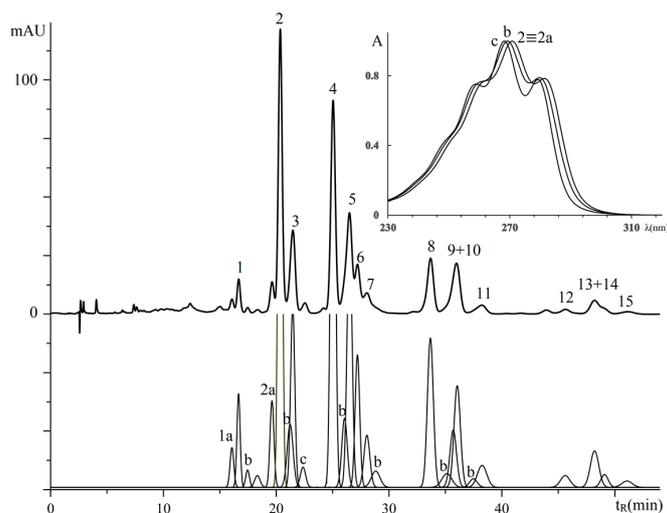
Figure 5. Separation of TGs of *Catalpa ovata* seed oil. Column 4.6 mm × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 4.

Table 4 contains some pairs of TGs known from the previous tables, but, to fit the increment approach as well as mass-spectrometric data, we must include not only one new conjugated octadecatrienoic acid (catalpic) but also another one—an uncommon octadecadienoic acid of unknown structure, non-distorting the electronic spectra of Type II.

Table 4. Retention parameters, mass spectral signals and content of TGs of *Catalpa ovata* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values					Mole Fraction of TG, %	M/z [M + H+]
				X→L	L→Y	L→O	O→P	P→S		
1a	LnX [b]$_2$	16.08	0.735						0.56	873.7
1	X$_3$	16.67	0.753						1.08	
2a	LnXL	19.63	0.836						2.56	
2	X$_2$L	20.36	0.854	0.100					12.02	875.8
3	X$_2$Y [c]	21.50	0.881		0.027				2.87	
4	XL$_2$	25.04	0.955	0.101					42.24	
5	XYL	26.50	0.982		0.027				9.74	877.8
6	X$_2$O	27.19	0.995			0.141			2.28	877.8
7	XY$_2$	28.05	1.009		0.027				4.62	877.8
8	XLO	33.67	1.096	0.101					7.69	
9	XYO	35.69	1.123		0.027				2.84	879.7
10	XLP	36.03	1.127				0.032		5.40	
11	XYP	38.20	1.155		0.027				1.56	853.7
12	XO$_2$	45.63	1.237			0.141			0.84	881.7
13	XLS	48.11	1.261					0.134	2.31	881.7
14	XOP	48.91	1.269				0.032		0.87	855.8
15	XYS	51.10	1.289		0.028				0.5	881.7
				0.101	0.027	0.141	0.032	0.134		

[a] Numbers of TGs are the numbers of peaks on the Figure 5; [b] X, moiety of conjugated octadecatrienoic acid (catalpic); [c] Y, moiety of uncommon octadecadienoic acid.

3.2.5. Triglycerides of Oil with (8Z,10E,12Z)-octadeca-8,10,12-trienoic Acid Moieties

Fatty acid composition of *Jacaranda* seed oil, determined in Reference [25], includes linoleic (41.4%), jacaric (as (8Z,10E,12Z)-octadeca-8,10,12-trienoic (Jac)) (30.9%), with two isomers ((8Z,10E,12E)-octadeca-8,10,12-trienoic (1.1%) and (8E,10E,12Z)-octadeca-8,10,12-trienoic (1.9%)), oleic (11.1%), stearic (4.3%) and palmitic (3.5%) acids. On the chromatogram of our sample of *Jacaranda mimosifolia* seed oil (Figure 6), there ten main TGs are found (Table 5), with electronic spectra corresponding to Type I (Figure 1). The value of increment Δ(Jac→L) is 0.009 points smaller

than that of increment $\Delta(\mathrm{Pu}{\rightarrow}\mathrm{L})$. Thus, the difference of retention (as logarithms of capacity factors) of corresponding TGs with punicic and jacaric acids is similar to the TGs with another pair of conjugated octadecatrienoic acids with the same structure alteration, $\Delta(\mathrm{Cat}{\rightarrow}\mathrm{Cal}) = 0.008$. In this case, this increment may be common for all pairs of TGs for which CH_2-group is shifting form inner to outer part of the acid substituent. It may be proposed that, for substitution of all-*trans* octadeca-9,11,13-trienoic acid moiety by all-trans octadeca-8,10,12-trienoic, one increment must be around 0.009.
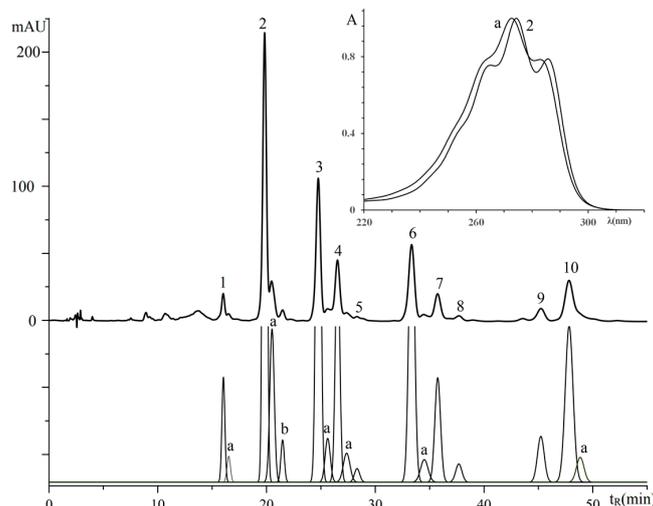
Figure 6. Separation of TGs of *Jacaranda mimosifolia* seed oil. Column 4.6 mm × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 5.

Table 5. Retention parameters, mass spectral signals and content of TGs of *Jacaranda mimosifolia* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values				Mole Fraction of TG, %	M/z [M + H$^+$]
				X→L	L→O	O→P	P→S		
1	X [b]$_3$	16.02	0.733					1.36	873.8
2	X$_2$L	19.83	0.841	0.108				22.50	875.8
3	XL$_2$	24.76	0.950	0.109				24.78	877.7
4	X$_2$O	26.52	0.983		0.142			6.25	877.7
5	X$_2$P	28.31	1.014			0.031		0.42	851.8
6	XLO	33.34	1.091	0.108	0.142			18.01	879.8
7	XLP	35.73	1.124	0.110		0.032		6.42	853.7
8	X$_2$S	37.69	1.148				0.135	0.75	n.d. [c]
9	XO$_2$	45.33	1.234		0.143			3.83	879.7
10	XLS(+XOP)	47.82	1.258	0.110			0.135	15.67	881.7 + 855.7
	Mean value:			0.109	0.142	0.032	0.135		

[a] Numbers of TGs are the numbers of peaks on the Figure 6; [b] X, moiety of conjugated octadecatrienoic acid (jacaric);
[c] n.d., not determined.

Calculated fatty acid composition of the oil is as follows: jacaric (as a sum of all octadecatrienoic acids) (44.30%), followed by stearic (5.69%), linoleic (37.17%), oleic (10.58%) and palmitic (2.27%) acids.

3.2.6. Triglycerides of Oil with (9E,11E,13E)-octadeca-9,11,13-trienoic Acid Moieties

In the first paper with results of investigation of *Centranthus ruber* seed oil, only α-eleostesric acid was found [26,27]. Meanwhile, detection of the mixture of this acid with its all-*E* isomer was reported later [28]. In our case, the seeds were found to be a wonderful source of oil composed by two isomeric octadecatrienoic acid isomers: α-eleostearic and β-elleostearic (9E,11E,13E)-octadeca-9,11,13-trienoic acid (β-El).

The oil is an interesting example with comparable amounts of isomeric octadecatrienoic acids resulting in appearance of thee peaks in region of elution X2L TG species (Figure 7). Opposite to the former cases, peaks have different electronic spectra: Type II, Type III and mixed type. Thus, Table 6 contains more TGs species than the other tables (except of *Catalpa* seed oil). This is just an example to calculate increment for exchange of the two isomeric acids:

$$\Delta(\alpha El \rightarrow \beta\text{-}El) = 0.021 \tag{6}$$
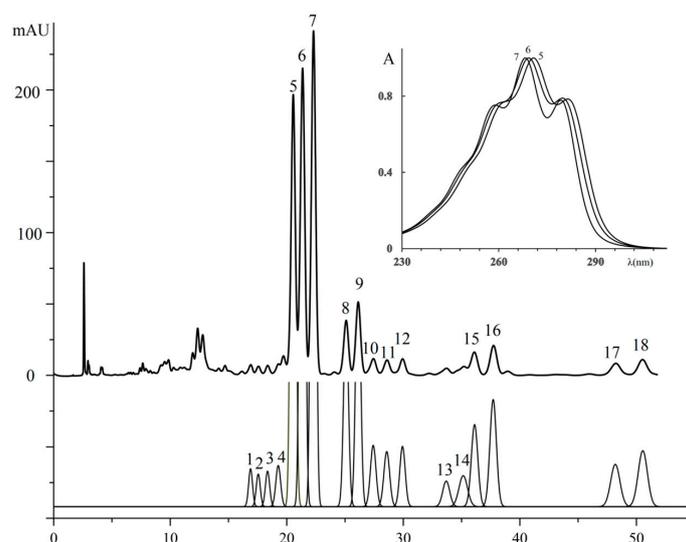
Figure 7. Separation of TGs of *Centranthus ruber* seed oil. Column 4.6 mm × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 6.

Table 6. Retention parameters, mass spectral signals and content of TGs of *Centranthus ruber* seed oil for the mobile phase composition 35% propanol-2 and 65% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$	Increment Values					Mole Fraction of TG, %	*M/z* [M + H$^+$]
				$\alpha El \rightarrow \beta El$	$\alpha El \rightarrow L$	$L \rightarrow O$	$O \rightarrow P$	$P \rightarrow S$		
1	αEl [b] $_3$	16.94	0.761						0.28	
2	$(\alpha El)_2 \beta El$	17.62	0.782	0.020						873.8
3	$\alpha El(\beta El)_2$	18.36	0.802	0.021						
4	βEl [b] $_3$	19.28	0.827	0.022					0.41	
5	$\alpha El_2 L$	20.56	0.859		0.098				15.35	
6	$\alpha El \beta ElL$	21.39	0.878	0.020					16.66	875.7
7	$\beta El_2 L$	22.30	0.899	0.020					21.40	
8	αElL_2	25.10	0.956		0.097				6.88	
9	βElL_2	26.14	0.976	0.020					10.19	877.7
10	$\alpha El_2 O$	27.44	0.999			0.140			2.34	
11	$\alpha El \beta ElO$	28.60	1.019	0.020		0.140			2.28	877.7
12	$\beta El_2 O$	29.94	1.040	0.022					3.17	
13	$\alpha ElLO$	33.71	1.096		0.097				1.41	
14	$\beta ElLO$	35.21	1.117	0.020					2.07	879.8
15	$\alpha ElLP$	36.10	1.128				0.032		4.09	
16	$\beta ElLP$	37.74	1.149	0.021			0.032		5.52	853.7
17	$\alpha ElLS$	48.24	1.262					0.134	3.01	
18	$\beta ElLS$	50.53	1.284	0.021				0.134	4.35	881.8
	Mean value:			0.021	0.097	0.140	0.032	0.134		

[a] Numbers of TGs are the numbers of peaks on the Figure 7; [b] αEl and βEl, α-eleostearic and β-eleostearic acids, respectively.

Calculated by the same way, fatty acid composition is as follows: α-eleostearic (23.8%), β-eleostearic (30.8%), linoleic (36%), oleic (3.8%), palmitic (3.2%) and stearic (2.5%) acids. This is close to data published in [28]: α-eleostearic (28%), β-eleostearic (17%), linoleic (36%), oleic (4%), and saturated (18%).

3.2.7. Triglycerides of Three Plant's Seed Oil

For determination of relative retentions parameters of TGs of all basic seed oils, the retention of Pu_3 of *Punica granatum* seed oil [29–31] was chosen as a reference. The reasons for the choice are: (a) the fruit is available all year round in the fruit market and thus the oil may be easily prepared; and (b) there is only one main peak within the seed oil chromatogram (peak of Pu_3), while the others have much smaller peak areas, thus not disturbing the retention of reference solute determination.

The relative retention was determined for three of four different mobile phase compositions for eluent system "propanol-2-acetonitrile". The retentions were registered only for chromatographic isocratic runs performed at chromatographic system equilibrium. The latter was controlled by complete coincidence of two consecutive chromatograms of the sample.

The equations obtained were used to calculate the TGs composition of the three seed oils (*Valeriana officinalis*, *Vernicia montana* and *Thladiantha dubia*) by minimization of differences of experimental retention parameters and calculated values, while considering the type of electronic spectra (Figures 8–10 and Tables 7–9).
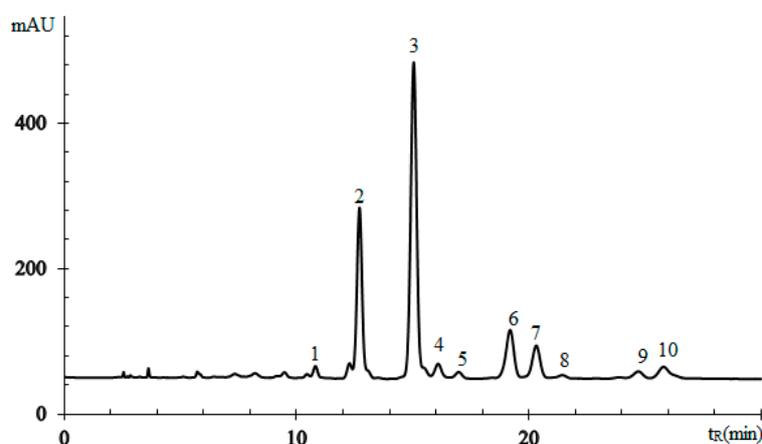
Figure 8. Separation of TGs with α-eleostearic acid moieties of *Valeriana officinalis* seed oil. Column 4.6 mm $\times$ 250 mm Kromasil 100-5C18; mobile phase, 42% propanol-2 and 58% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 7.

Table 7. Experimental retention parameters for TGs of *Valeriana officinalis* seed oil versus of relative retention for the mobile phase composition 42% propanol-2 and 58% acetonitrile calculated by equations.

No. [a]	TG Composition	t_R (min)	Log$k$(i) Experimental	Log$k$(i) Calculated	Content, Mol. %
1	X^{b}_3	10.81	0.522	0.521	0.6
2	X_2L	12.71	0.611	0.609	13.12
3	XL_2	15.04	0.700	0.699	56.12
4	X_2O	16.09	0.735	0.736	1.66
5	X_2P	17.08	0.766	0.767	0.79
6	XLO	19.19	0.825	0.826	12.15
7	XLP	20.42	0.855	0.857	8.19
8	X_2S	21.44	0.879	0.887	0.6
9	XO_2	24.82	0.951	0.953	2.24
10	XLS	26.09	0.975	0.976	4.52

[a] Numbers of TGs are the numbers of peaks on the Figure 8; [b] X, moiety of α-eleostearic acid.
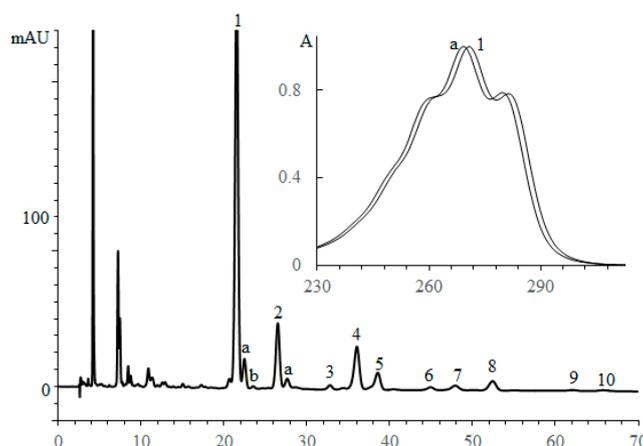
Figure 9. Separation of TGs of *Vernicia montana* seed oil. Column 4.6 × 250 mm Kromasil 100-5C18; mobile phase, 33% propanol-2 and 67% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 8.

Table 8. Experimental retention parameters for TGs of *Vernicia montana* seed oil vs. calculated by equations of relative retention for the mobile phase composition 33% propanol-2 and 67% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$(i) Experimental	Log$k$(i) Calculated	Content, Mol. %
1	X^b_3	21.55	0.882	0.880	48.44
2	X_2L	26.51	0.982	0.982	16.89
3	XL_2	32.81	1.084	1.085	3.63
4	X_2O	36.05	1.128	1.129	12.17
5	X_2P	38.56	1.159	1.161	5.28
6	XLO	45.09	1.231	1.233	2.56
7	XLP	48.51	1.265	1.265	4.52
8	X_2S	52.62	1.302	1.303	4.09
9	XO_2	62.29	1.379	1.380	1.17
10	XLS	66.13	1.406	1.408	1.23

[a] Numbers of TGs are the numbers of peaks on the Figure 9; [b] X, moiety of α-eleostearic acid.
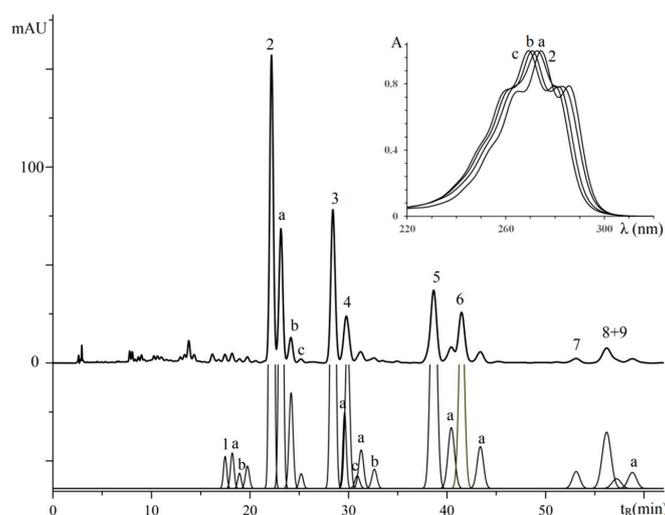
Figure 10. Separation of TGs of *Thladiantha dubia* seed oil. Column 4.6 × 250 mm Kromasil 100-5C18; mobile phase, 34% propanol-2 and 66% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm. For peak number composition, see Table 9.

Table 9. Experimental retention parameters for TGs of *Thladiantha dubia* seed oil vs. calculated by equations of relative retention for the mobile phase composition 34% propanol-2 and 66% acetonitrile.

No. [a]	TG Composition	t_R (min)	Log$k$(i)		Content, Mol. %
			Experimental	Calculated	
1	$X^{\,b}_{\,3}$	17.46	0.777	0.777	1.14
2	X_2L	22.18	0.896	0.898	29.14
3	XL_2	28.71	1.021	1.021	27.82
4	X_2O	30.08	1.043	1.045	4.56
5	XLO	38.99	1.164	1.166	17.18
6	XLP	41.88	1.197	1.199	12.34
7	XO_2	53.79	1.312	1.313	1.18
8 + 9	XLS + XOP	56.82	1.337	1.339	6.65

[a] Numbers of TGs are the numbers of peaks on the Figure 10; [b] X, moiety of punicic acid.

α-Eleostearic acid moieties were the main conjugated acid moieties involved in TG structures in the two oils. However, for *Valeriana* seed oil, we did not calculate fatty acid composition because of existence of valuable amounts of TGs without conjugated acid moieties, which was revealed by refractive index detection not included into results of the current paper. *Vernicia montana* seed oil composition: 78.4% of α-eleostearic, 10.8% of linoleic, 5.7% of oleic, 3.3% of palmitic and 1.8% of stearic acid. *Thladiantha* seed oil contains simultaneously α-eleostearic (9.5%), punicic (35.5%), β-eleostearic (0.3%), linoleic (40.1%), oleic (8.3%), palmitic (4.4%) and stearic (2.0%) acids.

4. Discussion

4.1. Some Remarks About Increment Approach

For a given chromatographic system, according to the principle of additivity, the retention (relative to trioleate retention, α, or selectivity) of TG composed by acids A, B and C may be described by Equation [15]:

$$\log\alpha(ABC) = \log\alpha(A) + \log\alpha(B) + \log\alpha(C) \tag{7}$$

For acid C moiety replacement by acid D moiety, we may write Equation (8):

$$\log\alpha(ABD) = \log\alpha(A) + \log\alpha(B) + \log\alpha(D) \tag{8}$$

Subtracting the Equation (7) from Equation (8) we get:

$$\log\alpha(ABD) - \log\alpha(ABC) = \log\alpha(D) - \log\alpha(C) \tag{9}$$

Since, for the given chromatographic system in the state of equilibrium, the retention of trioleate is constant, Equation (9) may be exchanged by Equation (10), where increment is calculated as difference between capacity factors of two TGs:

$$\log k(ABD) - \log k(ABC) = \Delta(C{\rightarrow}D) \tag{10}$$

Thus, for calculation of retention of all 20 TGs composed by acids A, B, C and D, it is necessary and sufficient to point out retention of a reference solute (e.g., A_3) and values of three increments: $\Delta(A{\rightarrow}B)$, $\Delta(B{\rightarrow}C)$ and $\Delta(C{\rightarrow}D)$.

Utilization of difference values $\Delta(A{\rightarrow}B)$ instead of partial values of Equation (7) is favorable because they are directly calculated for a set of logarithms of experimental capacity factors responsible for substituents exchange, revealing a dependence between solute structure and retention.

4.2. Comparison of Retention of TGs with Isomeric Conjugated Octadecatrienoic Acid Moieties

Summarizing the results obtained for oil separation in mobile phase containing 35 % propanol-2 and 65% acetonitrile we may built a final table of increments (Table 10).

Table 10. Increments ($\pm$0.001) of TGs retention for conjugated octadecatrienoic acid moieties Δ(top$\rightarrow$bottom) exchange for mobile phase composition 35% propanol-2 and 65% acetonitrile.

		Pu	Jac	Cat	αEl	Cal	βEl	βCal
Pu	9Z11E13Z							
Jac	8Z10E12Z	0.009						
Cat	9E11E13Z	0.017	0.008					
αEl	9Z11E13E	0.020	0.011	0.003				
Cal	8E10E12Z	0.025	0.016	0.008	0.005			
βEl	9E11E13E	0.041	0.032	0.024	0.021	0.016		
βCal	8E10E12E	0.049	0.040	0.032	0.029	0.024	0.010	

For the same conjugated double bonds position (9,11,13) along the carbon chain, the sequence of elution times is:

$$\text{Pu} < \text{Cat} < \alpha\text{El} < \beta\text{El}, \tag{11}$$

that is, retention becomes greater when *cis*-double bond is substituted by *trans*-configuration, the increase being somewhat greater for the substitution of the outer double bond. The difference of TGs retention with catalpic and α-eleostearic acid is not great but enough to differentiate TGs with two or three of these acid moieties. There are some problems for differentiation of TGs with one moiety of the acids (Figure 11).
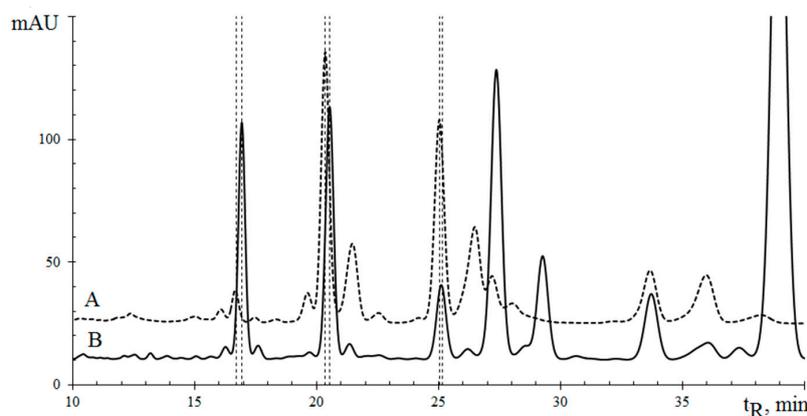
Figure 11. Overlapping of chromatograms of *Catalpa ovata* (A) and *Momordica charantia* (B) seed oil. Column 4.6 × 250 mm Kromasil 100-5C18; mobile phase, 35% propanol-2 and 65% acetonitrile, 1 mL/min; column thermostat temperature, 30 °C; detector, 270 nm.

Exchange of double bonds position from 9,11,13 to 8,10,12 is equal to movement of one CH_2-group from inner part of molecule to the outer one. For mobile phase 35% propanol-2 and 65% acetonitrile, increments for exchange punicic by jacaric acids as well as that for pairs β-eleostearic—β-calendic and catalpic—β-calendic acids are similar:

$$\Delta(\text{Pu}\rightarrow\text{Jac}) = 0.009 \tag{12}$$

$$\Delta(\beta\text{El}\rightarrow\beta\text{Cal}) = 0.010 \tag{13}$$

$$\Delta(\text{Cat}\rightarrow\text{Cal}) = 0.008 \tag{14}$$

Finally, the complete series of acid moieties for the increase of TGs retention time is as follows:

$$\text{Pu} < \text{Jac} < \text{Cat} < \alpha\text{El} < \text{Cal} < \beta\text{El} < \beta\text{Cal.} \tag{15}$$

4.3. Minor Seed Oil Components Identification

4.3.1. *Momordica charantia* Seed Oil

Pairs of tiny peaks accomplish the main peaks in Figure 2 with electronic spectra differing from it. The positions of all (including non-separated) peaks in Figure 1 was revealed by utilization of MagicPlot Student 2.7.2 program for chromatogram handling. The peaks that are eluted before the main one (marked with *a*) have bathochromic shifts of electronic spectra maxima, while peaks eluted after the main one (marked with *b*) have hypochromic shifts. By taking into account the previously found increments, it is possible to conclude that the oil contains not only α-eleostearic acid moieties but also earlier eluted TGs with punicic (a) acid moiety:

$$\Delta(\alpha\text{El} \rightarrow \text{Pu}) = \log k(\text{a}) - \log k(2) = -0.020 \tag{16}$$

TGs with β-eleostearic (b) acid moiety have greater retention:

$$\Delta(\alpha\text{El} \rightarrow \beta\text{E}) = \log k(\text{b}) - \log k(2) = 0.021 \tag{17}$$

These results are in a good agreement with the conclusions in Reference [21], although the authors were somewhat uncertain about presence of β-eleostearic acids moieties in the oil.

4.3.2. *Trichosanthus anguina* Seed Oil

The oil contains not only punic acid moieties but also later eluted TGs with one punicic acid moiety substitution with α-eleostearic (a):

$$\Delta(\alpha\text{E} \rightarrow \text{Pu}) = \log k(2) - \log k(\text{a}) = -0.020 \tag{18}$$

and TGs with two consecutive substitutions:

$$2\Delta(\alpha\text{E} \rightarrow \text{Pu}) = \log k(2) - \log k(\text{b}) = -0.039 \tag{19}$$

The validity of increment approach is supported with electronic spectra alteration (Figure 3). Meanwhile, the appearance of tiny peak *c* indicates existence of some other minor acid component, presumably β-eleostearic, but in small quantities.

4.3.3. Calendula Officinalis Seed Oil

The data in Table 3 indicated that calendic acid is distributed between TGs types apparently non-statistically because, for example, the ratio between peak areas (S) of the three TGs composed by moieties of calendic (Cal) and linoleic (L) acids:

$$S(\text{Cal}_3){:}S(\text{Cal}_2\text{L}){:}S(\text{CalL}_2) \tag{20}$$

is 0.50:1.00:0.08 for experimental data instead of calculated for statistical distribution as 0.97:1.00:0.25.

Meanwhile, the oil also contains isomeric to (α-)calendic acid moiety, leading to later elution of TGs with evident hypochromic shift of spectral maxima with a high value of increment per one new acid permitted to refer the acid as to β-calendic (8E,10E,12E)-octadeca-8,10,12-trienoic (1.56%).

$$\Delta(\alpha\text{Cal} \rightarrow \beta\text{Cal}) = \log k(\text{a}) - \log k(2) = 0.024 \tag{21}$$

$$2\Delta(\alpha Cal \rightarrow \beta Cal) = \log k(b) - \log k(2) = 0.049 \tag{22}$$

4.3.4. *Catalpa ovata* Seed Oil

In Figure 5, for the two main TGs types (No. 1 and No. 2), earlier eluting components are rather noticeable. The increment for exchange of catalpic acid by corresponding acid for this TGs does not fit any increment found for CLnA:

$$\Delta(X \rightarrow Cat) = \log k(1) - \log k(1a) = \log k(2) - \log k(2a) = 0.018 \tag{23}$$

Electronic spectra of all mentioned peaks are the same, as are their mass-spectra. Thus, the acid X may be non-conjugated α-linolenic, (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (Ln). The proposition was confirmed by analysis of fatty acids obtained by alkaline hydrolysis of this oil and flaxseed oil. Moreover, peaks No. 3 and No. 5 have the same electronic and mass spectra as peaks No. 2 and No. 4, respectively. This demands proposing the existence of some uncommon octadecadienoic acid, Y, in the oil. The corresponding exchange is perfectly described by increment model,

$$\Delta(L \rightarrow Y) = \log k(3) - \log k(2) = \log k(5) - \log k(4) = \ldots = 0.027 \tag{24}$$

The most probable solution that satisfies the parameters for unknown acid structure may be linoleic acid isomer with double bonds system shifted towards carboxylic group by 3 CH_2-group because for one CH_2-group shift an increment was found above to be around 0.009 (Equations (11)–(13)):

However, the real structure of the acid will be established in upcoming research.

4.3.5. *Jacaranda mimosifolia* Seed Oil

Considerations of electronic spectra and retentions of tiny peaks on the chromatogram of the oil leads to the conclusion that these peaks may appear due to synthesis of calendic and/or isomeric to the calendic—not found in other natural sources (8Z,10E,12E)-octadeca-8,10,12-trienoic acid—as was supposed in Reference [25]. Indeed, the spectrum of peak 2a is non-distinguishable from spectrum of peak 2a of trichosanthus seed oil chromatogram.

4.4. Relative Retention Analysis of TGs with Conjugated Seed Oils

Coefficients of relative retention (Equation (1)) of all TGs of all seed oils under investigation in current paper are listed in Table 11. The data may be explored for seed oil TGs determination by choice of the equations of relative retention fitting experimental data within discrepancy of no more than 0.002 of logarithmic units. The approach was successfully applied to three plant types of seed oils (Tables 8–10).

Table 11. Coefficients of relative retentions of TGs vs. retention of Pu_3 (Equation (1)) for chromatographic system "pronanol-2-acetonitrile" and stationary phase Kromasil 100-5C18.

TG Structure	Coefficients of Equation (1)	X, Conjugated Octadecatrienoic Acid						
		Pu	Jac	Cat	αEl	Cal	βEl	βCal
X_3	a_1	1.000	1.011	1.014	1.010	1.021	1.035	1.054
	a_0	0.000	0.022	0.041	0.052	0.058	0.100	0.113
X_2L	a_1	1.042	1.048	1.052	1.047	1.054	1.062	n.d.
	a_0	0.089	0.104	0.116	0.123	0.127	0.154	n.d.
XL_2	a_1	1.091	1.086	1.090	1.085	1.092	1.087	n.d.
	a_0	0.174	0.187	0.190	0.195	0.195	0.212	n.d.
X_2O	a_1	1.101	1.106	1.112	1.104	1.115	1.120	n.d.
	a_0	0.189	0.206	0.215	0.224	0.225	0.255	n.d.
X_2P	a_1	1.105	1.103	n.d.	1.107	1.118	n.d.	n.d.
	a_0	0.218	0.239	n.d.	0.253	0.256	n.d.	n.d.
XLO	a_1	1.147	1.144	1.151	1.144	1.152	1.135	n.d.
	a_0	0.276	0.281	0.289	0.295	0.294	0.317	n.d.

Table 11. *Cont.*

TG Structure	Coefficients of Equation (1)	X, Conjugated Octadecatrienoic Acid						
		Pu	Jac	Cat	αEl	Cal	βEl	βCal
XLP	a_1	1.148	1.146	1.155	1.146	1.156	1.146	n.d.
	a_0	0.307	0.319	0.318	0.325	0.323	0.346	n.d.
X$_2$S	a_1	n.d.	1.170	n.d.	1.171	1.183	n.d.	n.d.
	a_0	n.d.	0.327	n.d.	0.343	0.345	n.d.	n.d.
XO$_2$	a_1	1.209	1.206	1.213	1.200	n.d.	n.d.	n.d.
	a_0	0.374	0.387	0.387	0.396	n.d.	n.d.	n.d.
XLS	a_1	1.216	1.210	1.218	1.214	n.d.	1.216	n.d.
	a_0	0.394	0.408	0.408	0.412	n.d.	0.434	n.d.

The relative retention plot (separation map) for jacaranda seed oil TGs (vs. Jac$_3$) is presented in Figure 12.
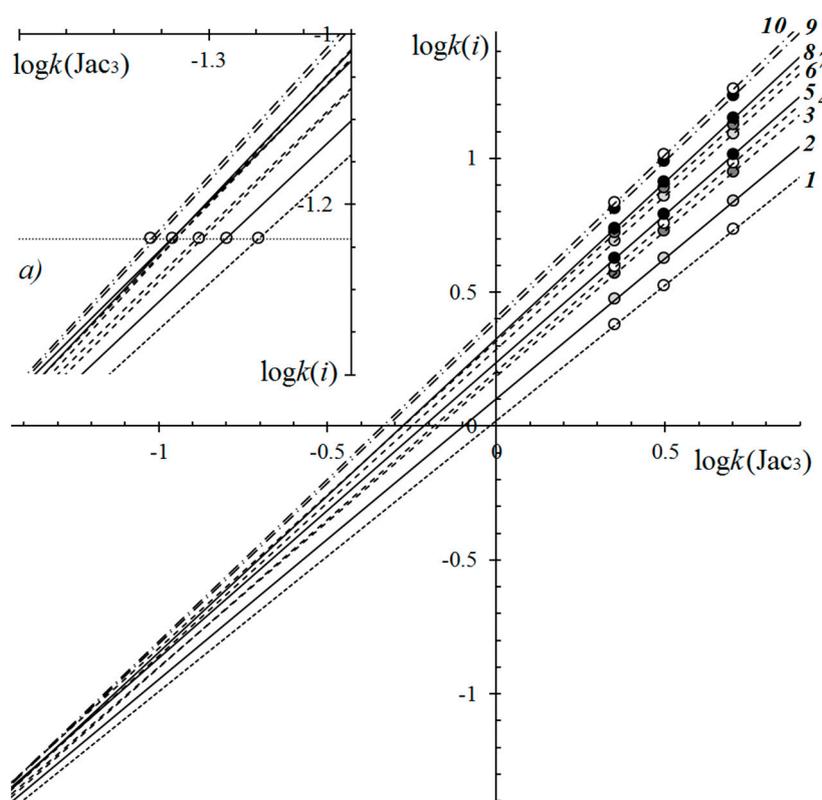
Figure 12. Separation map for *Jacaranda mimosifolia* seed oil. Insert is the left lower part of the map with "zero" points. Column 4.6 × 250 mm Kromasil 100-5C18; mobile phase system "propanol-2-acetonitrile", column thermostat temperature, 30 °C; detector, 270 nm (numbers of lines are numbers of substances in Table 5).

The straight lines of the relationships have a trend to intersect in lower left side corner of the plot (for imaginable mobile phase composition with high elution strength). The first consequence of the plot type is the dependence of increments upon eluent composition: increments decrease with elution strength increase. The second consequence is that selectivity parameter of the pair of solutes separation in the general case is also not a constant for wide range of mobile phase compositions. The valuable property of two-parameter relative retention equations is a non-sensitivity to mobile phase composition.

Another advantage of the relative retention plot in the present case is the possibility of differentiation of TGs by the number of double bonds in a molecule. Indeed, if the mobile phase

elution strength is rising, for some mobile phase composition hydrophobic (Van der Waals interactions), solvation properties may become equal for any compound of homologues series [32]. The retention in this case points out "zero" point on the plot. However, because of the existence of another type of interaction, this point moves to the right or left; for example, the more double bonds exist in a molecule, the more to the right the point must be shifted.

For the Jacaranda seed oil, the position of the "zero" point may be determined by intersection of lines for relative retention of the pair of homologous TGs, Jac_2P and Jac_2S (Figure 12). On the plot lines of relative retention of all ten TGs intersect, the horizontal line (for "zero" points positions on the plot) is almost proportional to the number of double bonds in a molecule, despite possible errors at approximation far beyond the compositions of mobile phase used for formation of the plot.

5. Conclusions

The sequence of retention times of the same types of TGs is constant for reasonable compositions of propanol-2-acetonitrile mobile phases and Kromasil 100-5C18 stationary phases: punicic ($C18:3^{9Z11E13Z}$) < jacaric ($C18:3^{8Z10E12Z}$) < catalpic ($C18:3^{9E11E13Z}$) < α-eleostearic ($C18:3^{9Z11E13E}$) < calendic ($C18:3^{8E10E12Z}$) < β-eleostearic ($C18:3^{9E11E13E}$) < all-E calendic ($C18:3^{8E10E12E}$) acids. Meanwhile, the retention alteration in series catalpic→α-eleostearic→calendic moieties for TGs of similar structures are quite small but sufficient for solute differentiation when TGs contain at least two moieties of the acids. The migration of CH_2-group from the inner part of the fatty acid moiety to the outer one leads to rise of solute retention by similar values of logarithms of retention capacity (log$k$) for any starting *cis-trans* stereoisomers. Exchange of stereo configurations in direction *cis→trans* also results in increase of retention being slightly different for inner and outer *cis*-double bond. Thus, it was proven that it is possible to differentiate TGs of complex composition with moieties of all natural CLnA by retention control accomplished by electronic spectra comparison, even though there are only three types of electronic-vibration spectra for seven isomeric CLnA. Equations of TGs relative retention were found to be useful for preliminary TGs identification.

References

1. De Carvalho, E.B.T.; de Melo, I.L.P.; Mancini-Filho, J. Chemical and physiological aspects of isomers of conjugated fatty acids. *Ciênc. Tecnol. Aliment. Campinas* **2010**, *30*, 295–307. [CrossRef]
2. Yuan, G.-F.; Chena, X.-E.; Li, D. Conjugated linolenic acids and their bioactivities: A Review. *Food Funct.* **2014**, *5*, 1360–1368. [CrossRef] [PubMed]
3. Tanaka, T.; Hosokawa, M.; Yasui, Y.; Ishigamori, R.; Miyashita, K. Cancer Chemopreventive Ability of Conjugated Linolenic Acids. *Int. J. Mol. Sci.* **2011**, *12*, 7495–7509. [CrossRef] [PubMed]
4. Tsuzuki, T.; Tokuyama, Y.; Igarashi, M.; Miyazawa, T. Tumor growth suppression by α-eleostearic acid, a linolenic acid isomer with a conjugated triene system, via lipid peroxidation. *Carcinogenesis* **2004**, *25*, 1417–1425. [CrossRef] [PubMed]
5. Aruna, P.; Venkataramanamma, D.; Singh, A.K.; Singh, R.P. Health Benefits of Punicic Acid: A Review. *Compr. Rev. Food Sci. Food Saf.* **2016**, *15*, 16–27. [CrossRef]
6. Suzuki, R.; Yasui, Y.; Kohno, H.; Miyamoto, S.; Hosokawa, M.; Miyashita, K.; Tanaka, T. Catalpa seed oil rich in 9t,11t,13c-conjugated linolenic acid suppresses the development of colonic aberrant crypt foci induced by azoxymethane in rats. *Oncol. Rep.* **2006**, *16*, 989–996. [CrossRef] [PubMed]
7. Shinohara, N.; Ito, J.; Tsuduki, T.; Honma, T.; Kijima, R.; Sugawara, S.; Arai, T.; Yamasaki, M.; Ikezaki, A.; Yokoyama, M.; et al. Jacaric acid, a linolenic acid isomer with a conjugated triene system, reduce stearoyl-CoA desaturase expression in liver of mice. *J. Oleo Sci.* **2012**, *61*, 433–441. [CrossRef] [PubMed]

8.　Benjamin, S.; Spener, F. Conjugated linoleic acids as functional food: An insight into their health benefits. *Nutr. Metabol.* **2009**, *6*, 1–13. [CrossRef] [PubMed]

9.　Hopkins, C.Y.; Chisholm, M.J. A Survey of the Conjugated Fatty Acids of Seed Oils. *J. Am. Oil Chem. Soc.* **1968**, *45*, 176–182. [CrossRef] [PubMed]

10.　Ichihara, K.; Fukubayashi, Y. Preparation of fatty acid methyl esters for gas-liquid chromatography. *J. Lipid Res.* **2010**, *51*, 635–640. [CrossRef] [PubMed]

11.　Frede, E. Improved HPLC of Triglycerides by Special Tempering Procedures. *Chromatographia* **1988**, *21*, 29–36. [CrossRef]

12.　Barrón, L.J.R.; Santa-Maria, G. Non-Aqueous Reverse-Phase HPLC Analysis of Triglycerides. *Chromatographia* **1987**, *23*, 209–214. [CrossRef]

13.　Li, L.; Han, J.; Wang, Z.; Liu, J.; Wei, J.; Xiong, S.; Zhao, Z. Mass Spectrometry Methodology in Lipid Analysis. *Int. J. Mol. Sci.* **2014**, *15*, 10492–10507. [CrossRef] [PubMed]

14.　Podlaha, O.; Toregerd, B. A System for Identification of Triglycerides in Reversed Phase HPLC Chromatograms Based on Equivalent Carbon Numbers. *J. Sep. Sci.* **1982**, *5*, 553–558. [CrossRef]

15.　Sempoore, G.; Bezard, J. Qualitative and quantitative analysis of peanut oil triacylglycerols by reversed-phase liquid chromatography. *J. Chromatogr.* **1986**, *366*, 261–282. [CrossRef]

16.　Deineka, V.I. Experimental substantiation of a method of relative analysis of retention in HPLC. *Russ. J. Phys. Chem.* **2006**, *80*, 425–428. [CrossRef]

17.　Hennessy, A.A.; Ross, P.R.; Fitzgerald, G.F.; Stanton, C. Sources and Bioactive Properties of Conjugated Dietary Fatty Acids. *Lipids* **2016**, *51*, 377–397. [CrossRef] [PubMed]

18.　Hopkins, C.Y.; Chisholm, M.J.; Orgodnik, J.A. Identity and Configuration of Conjugated Fatty Acids in Certain Seed Oils. *Lipids* **1969**, *4*, 89–92. [CrossRef]

19.　Chisholm, M.J.; Hopkin, C.Y. Fatty Acid Composition of Some *Cucurbitaceae* Seed Oils. *Can. J. Chem.* **1964**, *42*, 560–564. [CrossRef]

20.　Chang, M.-K.; Conkerton, E.J.; Chapital, D.C.; Wan, P.J.; Vadhwa, O.P.; Spiers, J.M. Chinese Melon (*Momordica charantia* L.) Seed: Composition and Potential Use. *J. Am. Oil Chem. Soc.* **1996**, *73*, 263–265. [CrossRef]

21.　Joh, Y.-G.; Kim, S.-J. Analysis of Molecular Species of Triacylglycerols from Vegetable Oils Containing Fatty Acids with Non-Methylene-Interrupted Double Bonds, by HPLC in the Silver-ion Mode. *J. Jpn. Oil Chem. Soc.* **1998**, *47*, 927–936. [CrossRef]

22.　Joh, Y.-G.; Kim, S.-L.; Christie, W.W. The Structure of the Triacylglycerols, Containing Punicic Acid, in the Seed Oil of *Trichosanthes kirilowii*. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1037–1042. [CrossRef]

23.　Król, B.; Paszko, T.; Król, A. Conjugated linolenic acid content in seeds of some pot marigold (*Calendula officinalis*) cultivars grown in Poland. *Farmacia* **2016**, *64*, 881–886.

24.　Takagi, T.; Itabashi, Y. Occurrence of mixtures of geometrical isomers of conjugated octadecatrienoic acids in some seed oils: Analysis by open-tubular gas liquid chromatography and high performance liquid chromatography. *Lipids* **1981**, *16*, 546–551. [CrossRef]

25.　Yamasaki, M.; Motonaga, C.; Yokoyama, M.; Ikezaki, A.; Kakihara, T.; Hayasegawa, R.; Yamasaki, K.; Sakono, M.; Sakakibara, Y.; Suiko, M.; et al. Induction of apoptotic call death in HL-60 cells by Jacaranda seed oil derived fatty acids. *J. Oleo Sci.* **2013**, *62*, 925–932. [CrossRef] [PubMed]

26.　Hopkins, C.Y.; Chisholm, M.J. Identification of conjugated triene fatty acids in certain seed oils. *Can. J. Chem.* **1962**, *40*, 2078–2082. [CrossRef]

27.　Radunz, A.; Heb, P.; Schmid, G.H. Analysis of the Seed Lipids of *Aleurites montana*. *Z. Naturforsch.* **1998**, *53c*, 305–310.

28.　Tulloch, A.P. ^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis of Seed Oils Containing Conjugated Unsaturated Acids. *Lipids* **1982**, *17*, 544–550. [CrossRef]

29.　Turtygin, A.V.; Deineka, V.I.; Deineka, L.A. Determination of the triglyceride composition of pomegranate seed oil by reversed-phase HPLC and spectrophotometry. *J. Anal. Chem.* **2013**, *68*, 558–563. [CrossRef]

30.　Boroushaki, M.T.; Mollazadeh, H.; Afshari, A.R. Pomegranat seed oil: A comprehensive review on its therapeutic effects. *Int. J. Pharm. Sci. Res.* **2016**, *7*, 430–442.

31. Topkafa, M.; Kara, H.; Sherazi, S.T.H. Evaluation of the Triglyceride Composition of Pomegranate Seed Oil by RP-HPLC Followed by GC-MS. *J. Am. Oil Chem. Soc.* **2015**, *92*, 791–800. [CrossRef]

32. Deineka, V.I. Relative retention analysis in HPLC: The correlation between incremental relationships. *Russ. J. Phys. Chem.* **2006**, *80*, 605–608. [CrossRef]