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Simple complementary liquid chromatography and mass spectrometry approaches for the characterization of triacylglycerols in *Pinus koraiensis* seed oil

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ABSTRACT

A new simple strategy to identify triacylglycerols (TAGs) in oils and fats was performed using on line coupling of non aqueous reversed phase chromatography-electrospray ionization–mass spectrometry (NARP-LC-ESI–MS^2) with silver nitrate ($AgNO_3$) as post-column additive, and chromatographic data (partition number information and both the graphs of $\log k$ vs. number of double bond (DBN) and carbon number (CN)). NARP liquid chromatography permitted to separate TAGs composed of $\Delta 5$ and $\Delta 9$ but not from $\Delta 11$ double bond location on alkyl chain of fatty acid residues. Silver cationization improved the sensitivity by a factor one hundred. MS^2 information gave unambiguously the nature of three fatty acid residues bonded to glyceryl backbone of TAGs while $\log k$ against DBN and CN curves discriminated between the same molecular mass TAG isomers (whose constitutive fatty acid residues are double bond position and configuration isomers). Combination of structural information given by MS with chromatographic retention laws led to the development of a general methodology for determination of the structure of TAGs in lipids. This methodology was applied to *Pinus koraiensis* seed oil for which some uncommon TAGs are present. It permitted the identification of 58 TAGs in this oil. The experimental proof of 29 uncommon TAGs as component of this oil is demonstrated. Among them 26 were minor constituents.

1. Introduction

Natural oils and fats are complex mixtures consisting primarily of triacylglycerols (TAGs) with the number of TAGs being 50 or more. A triacylglycerol is formed almost invariably by long chain fatty acids linked to a glycerol molecule and is defined by the following attributes:

1. Total carbon number (CN), equal to the sum of the carbon atoms contained in the three alkyl chains.
2. Fatty acid (FA) alkyl chain length (odd and even numbered with two to almost one hundred carbon atoms).
3. FA position on the glycerol backbone (*sn-1/3*, *sn-2*).
4. The total number of double bonds (DBN).
5. Double bond position and configuration in each fatty acid.
6. In addition there can be a host of further structural features including branch points, 3, 5, 6 or 7 membered-rings, acetylenic and allenic bonds, oxygenated functions and many more [1].

As it is well known, the common fatty acids of animals and plants consist of even linear chains of 16–22 carbons atoms with zero to six double bonds of the cis (Z) configuration. Polyunsaturated fatty acids have methylene interrupted double bond system in general. More rarely these double bonds are conjugated [1].

The separation of TAGs from vegetable oil is quite a challenging task because of the presence of the numerous TAGs species with similar physicochemical properties. Retention of TAG is a sum of opposite effects determined by the two elements of the structure: the carbon skeleton and the unsaturation and/or the presence of polar functional groups.

Various analytical methods are therefore being employed to overcome the challenge.

High Temperature Capillary Gas Chromatography (HT-CGC) is one such analytical method being used. Optimizing TAG separation by CGC is well documented in the literature [2,3].

A second one consists by using high performance liquid chromatography (HPLC) which may be considered as the method of choice for the usual separation of TAGs. There are two main HPLC methods for the separation of TAG species: non aqueous reversed-phase (NARP-LC) and silver-ion (Ag-LC) liquid chromatography. In NARP-LC, the elution order is based on increasing partition numbers PN = CN – 2DBN (partition number = carbon number – twice

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the double bond number) [4–6]. In fact because of the higher resolving power of RPLC, it was found later that the coefficient is close but not equal 2 for all acid residues depending on their number and position of double bonds and their chain length. Precisely, it is not constant but decreases with the increasing number of double bonds, and also when CN decreases [7]. Consequently with the efficient stationary phases available nowadays, separation of great number of TAGs with same PN is easily obtained. For example there is easy separation of groups such as OOO, OOP, OPP and PPP (with same PN). Also separation has been achieved for pairs such as LLL–OLLn (with same PN, CN and DBN) [8,9]. Finally the more difficult closest structural pairs such as LnLnγLn–LnLnLn (with same PN, CN and DBN but differently located along the same hydrocarbon chain: Δ6 or Δ9) has been resolved after chromatographic optimization [10], leading to the identification of TAGs of both borage and black currant stone oils. The separation of double bond configuration TAGs isomers was also reported using these optimized conditions: all TAGs of *cherry nut* oil possessing FA residue of α-eleostearic acid (a conjugated Δ9 FA residues for which two double bonds are in trans (E) configuration) could be perfectly separated from the non-conjugated ones possessing FA residues with same PN, CN and DBN (Ln or γLn) but all in cis configuration at their location (Δ6 and Δ9) on the alkyl chain (which could be found in both borage or black currant stone and linseed or rose hips oils) [8].

Ag-LC is also commonly used in TAG separations. In this case, the elution order is related to an increasing total number of double bonds and the separation of unsaturated TAG of different geometrical configuration is achieved. There is no selectivity for TAGs differing only in the length of alkyl chains (with same DBN but different CN). The separation mechanism is based on specific silver ion/double bond interactions and chromatographic retention increases with the number of double bonds [4,5,11]. Practically, Ag-LC can be performed in two different experimental conditions. In the first case, silver ions are bound unto the stationary phase by using cation exchange resins as packing. In the second case, silver ions are loaded unto a silica gel. Even though there is a marked difference between the two methods, both Ag-LC systems separate TAGs in order of unsaturation: i.e. SSS, SMS, SMM + SSD, etc. (where S = saturated FA, M = monounsaturated FA and D = di-unsaturated FA, etc.). Silica stationary phase systems coated with $AgNO_3$ (Ag-LC) offer also a separation of regio-isomeric TAGs (*sn-1/3*, *sn-2*), for instance, the separation of SMS and SSM [12]. In conclusion Ag-LC allows the separation of TAGs with the same PN but different DBN. It does not allow the separation of certain class of TAG which could be easily separated using reversed phase. Therefore NARP-LC usually provides a better resolution of complex TAG mixtures such as plant oils [13], Ag-LC is of special value when, used to complement NARP.

Lastly, a third method of choice for separating TAGs is reversed phase SuperCritical Fluid Chromatography (RP-SFC) and reversed phase-SubCritical Fluid Chromatography (RP-SbFC). By combination of temperature and pressure effects selectivities were modified, permitting best separation of some TAGs pairs which were difficult to separate in NARP-LC [14–16]. However, there was a loss of separation for other pairs of TAGs. From this point of view NARP-LC and RP-SFC or RP-SbFC are complementary techniques for analysing TAGs.

Although the use of monodimensional chromatographic techniques can often provide useful information on TAG profiles in lipidic matrixes, a fully comprehensive analytical view of these samples may be attained by combining two independent separation steps with different selectivities. Examples are Ag-LC/NARP-LC [17], NARP-LC/Ag-LC [18]. Several multi-modal chromatographic tandems for the analysis of TAGs have been reported [19,20]. Sandra et al [20] developed an automated on line comprehensive RP-SFC × Ag-SFC system using octadecyl bonded silica and silver loaded stationary phase in the first and second dimensions respectively. Lastly comprehensive NP-SFC × RP-LC which is a valuable alternative for TAGs separations by the implementation of normal phase SFC and RP-LC in a comprehensive configuration has been developed [21]. The most common use of multidimensional chromatography for TAGs is for the pre-treatment of a complex matrix in an off-line mode.

Whatever the chromatographic techniques used, the methodology for identification of TAG follows in general the three steps:

1. The determination of fatty acid (FA) composition [22,23]. The determination of double bond locations on FA residue by CGC–MS is achieved by using specific derivatives for mass spectrometry of fatty acids [1,24]. With the inclusion of optical isomers, there are at least theoretically 3*n* different TAGs species (where n is the number of fatty acids more generally obtained from fatty acid methyl esters (FAME) CGC analysis), and this aids evaluation of TAG structure. But this is very often insufficient because all the theoretically 3*n* TAGs are not biosynthesized, the amount of each TAG is not totally correlated to the amount of fatty acids, and finally the insufficient resolution power of used chromatographic techniques.
2. The identification by comparison with standard analysed in the same chromatographic conditions. Unfortunately, compared to all possible TAGs there are far fewer number of available pure TAGs to be used for the comparison. To overcome these limitations, supplementation of unknown fats by another oil of well known composition as standard mixture could be used [10]. Unfortunately, even using these two methods leave a lot of TAGs unidentified.
3. Use of predicting diagrams $\log k$ vs. CN, $\log k$ vs. PN and $\log k$ vs. DBN from a general scheme first published by Goiffon et al. [25,26] for identification of TAGs composed of saturated fatty acids residues (with $10 < CN < 18$) which was extended later by Stolyhwo et al. [27] for identification of TAGs composed of higher fatty acid residues (with $10 < CN < 24$).

However, none of the three methods cited when used alone, permits unambiguous identification of all TAGs. This is because TAGs are in great number in oils and fats and not always well separated, especially those with similar structures (same CN, same PN, same DBN) because their retention factor (or gradient retention times) could be close together. These methods must be used to complement each other in order to ensure complete and unambiguous characterization of TAGs. As the CGC of FA derivatives helps to identify FAs, the position of double bonds on the carbon chain and configurations, comparison with standard TAGs or oils of well known composition analysed in the same chromatographic conditions helps to identify some TAGs in the oil which are present in the standards or the oil of well known TAGs composition. A combination of the information obtained from the two methods together with the predicting diagrams help to identify the remaining TAGs in the oil.

To overcome the inconvenience of unavailability of a large number of TAG standards, the major technologies used for analysis of non-volatile lipids are liquid chromatography coupled with mass spectrometry (LC/MS), because it provides both structural information and usually also a very high sensitivity [28].

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two popular ionization techniques used in LC/MS for qualitative and quantitative analysis of these lipids [29–32]. Based on their ionization mechanisms, ESI is more suitable for ionization of polar and ionic compounds and is capable of ionizing both small and large biomolecules. APCI can ionize less polar and neutral compounds more efficiently than ESI. Consequently

TAGs are very often analysed by RPLC-APCI-MS [29,31,33] and less commonly by ESI-MS [34–36].

Using only mass spectrometry, it is not possible to identify isomers of TAGs composed of fatty acids residues with the same CN, the same DBN but differing by the position or the configuration of double bond along the chain. Thus identification of all TAGs necessitates the combination of all the methods described above together with the information given by on line mass spectrometry.

In this article a simple strategy to characterize the TAGs has been developed. *Pinus koraiensis* seed oil was chosen as a sample for the studies because the plant belongs to the large taxonomical group of coniferophytes (and gymnosperm) seed oils which is known for the presence of a mixture of usual and unusual FAs [37,38]: the Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5 UPIFA) with the first site of unsaturation at the fifth carbon atom and *cis* (Z) configuration, such as *Z*5, Z9-octadecadienoic (taxoleic), *Z*5, Z9, Z12-octadecatrienoic (pinolenic), *Z*5, Z11-eicosadienoic (keteleeronic), *Z*5, Z11, Z14-eicosatrienoic (sciadonic) which belong to the ω6 and ω9 series [39,40]. Generally there is only one Δ5 fatty acid linked on TAG which is mainly bonded on sn-1/sn-3 position on TAG skeleton [41]. This oil exhibits peculiar biochemical properties such as hypocholesterolemic and hypo-triglyceridemic at least in the rat. These effects have been attributed to the presence of Δ5 UPIFA in these oils [42].

The strategy developed here utilises a NARP-ESI-MS^2 method as well as chromatographic retention laws. The MS^2 helped to unambiguously identify the three fatty acid residue of each TAG at each retention factor. Knowing the FAs nature determined by CGC, chromatographic laws helped to precisely distinguish between TAG isomers of the same molecular mass whose position of double bond along the alkyl chain of fatty acid residue is not the same. The overall proposed methodology can be used to find the TAGs composition of any oil and fat. The method has been applied to *P. koraiensis* seed oil.

2. Experimental

2.1. Materials

Acetonitrile and acetone were HPLC grade from Carlo Erba (Rodano, Italy). The *P. koraiensis* seed oil was kindly supplied by late R. Wolff (Laboratoire de Lipochimie Alimentaire ISTAB, Talence, France). The $AgNO_3$ was of analytical grade and was obtained from Sigma–Aldrich (Logistik GmbH, Germany). The TAG standards 1,3-dilinoleoyl-2-oleyl-glycerol (LOL), 1,2-dioleyl-3-linoleoyl-glycerol (OOL), 1,2,3-trilinoleoyl-glycerol (LLL), 1-palmitoyl-2-oleyl-3-linoleoyl-glycerol (POL), 1,2-dilinoleoyl-3-palmitoloyl-glycerol (LLP), 1,2-dioleyl-3-palmitoyl-glycerol (OOP) and 1,2,3-trioleyl-glycerol (OOO) were obtained from Larodan Fine Chemicals AB (Malmö, Sweden). Soya bean oil was obtained from Guerbet laboratory (Aulnay sous bois, France) and calophyllum oil was obtained from Institut de Recherche Pierre Fabre (Vigoulet-Auzil, France).

2.2. Abbreviations

The TAGs are defined by means of three letters, each corresponding to the trivial name of a fatty acid residue linked to the glycerol backbone. In this article no distinction were made between sn-1/sn-3 and sn-2 configuration. The position of the FAs in a TAG has been ordered by the following classical rules: FAs of smaller CN come first, and if the FAs are of the same CN the one with smaller DBN comes first (e.g. we have PSO no matter the position of the three fatty acid residues on glycerol). The abbreviations used in this paper for FAs are shown in Table 1.

2.3. Sample preparation

The *P. koraiensis* seed oil extraction was done as previously reported [43].

The *P. koraiensis* seed oil was dissolved in acetonitrile/acetone (50/50, v/v) to prepare an initial solution with a concentration of 10.6 mg/ml.

Initial concentrations of 5 mg/ml were prepared for the soya and calophyllum oils.

Fatty acids methyl esters composition of this sample of *P. koraiensis* seed oil was obtained using a previously reported standard CGC procedure [40,44]. It is given in Table 1. In another study, the same authors reported that 0.34% (w/w) of total FA acids of *P. koraiensis* seed oil were unidentified [45].

2.4. Preparation of TAG standards

1.5 mg/ml solution of each standard TAG was prepared using acetonitrile/acetone (50/50, v/v). 20 μl of each prepared solution was then taken and mixed together to form a mixture of standard TAGs with each TAG having a concentration of 0.214 mg/ml.

2.5. LC–MS instrument

HPLC system: A quaternary HPLC system consisting of Spectra System P 1000 XR pump (Thermo Scientific, Boston, MA, USA) was used for pumping the acetonitrile/acetone mobile phase whereas Hewlett Packard 1050 series (Agilent Technology, Santa Clara, CA, USA) was utilised to pump the $AgNO_3$ on line after the column via a T connector (Interchim, Montlucon, France). A 250 × 2.0 mm × 5 μm Kromasil C18 (Eka Nobel, Bohus, Sweden) column was used throughout the experiment. Mobile phase used in isocratic mode were acetonitrile: acetone (47:53) with a flow rate of 0.25 ml/min. $AgNO_3$ of concentration 100 μM was introduced into the LC effluent after the column at a flow rate of 0.05 ml/min to increase the sensitivity [46]. Injections of 5 μl of analytical samples were done by Thermo Separation Products AS 100 XR autosampler. The silver containing solutions were freshly prepared and protected from light during the experiments.

The dead time t_0 of the column was determined by injecting pure acetonitrile.

MS system: The column was connected to a Thermo-Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher, Boston, USA) equipped with an ESI ion source. The entire volume of the column effluent was directed to the mass spectrometer. Data acquisition and processing were performed using Xcalibur data system (Woburn, MA, USA). The source voltage was 4.5 kV and the capillary temperature was 250 °C. The capillary voltage was 25 V and the lens offset 20 V. Nitrogen was used both as sheath gas and as an auxiliary gas at a flow rate of 75 and 50 (arbitrary units), respectively. The ESI was in positive mode and mass range was set at 50–2000 amu. For MS^2 analysis, collision energy used was 35% and the isolation width was 3.

To perform MS^2 analysis, the chromatogram was explored to identify all the *m*/*z* of the TAGs silver adducts, which were then mass selected in different scan events and fragmented. To complete all the analyses three or four injections had to be done.

The mixture of TAG standards made up of OOO, OOL, OLL, LLL, POO, POL, PPP and PLL was injected and run using the LC–MS conditions described above (see Table 4). Soya bean oil and calophyllum oil were also injected in the same conditions to identify other classical TAGs which are present in them (LLnLn, LLLn, LLL, OLnLn, OLLn, OOLn, OLL, OOL, OOO, SLL, SOL, SOO, SSO, PLnLn, PLLn, PLL, POLn, POL, PPL, PSL, PPO, POO, PSO, SSL) [6,8]. MS/MS analysis was later performed.

Table 1
The trivial names of identified fatty acids found in *Pinus koraiensis* seed oil, their abbreviations, carbon number (CN), number of double bonds (DBN) molecular masses (M) and their % composition [42,45].

Trivial name	Abbreviation	CN:DBN	*M* (g/mol)	Composition (mole %)
Palmitic acid	P	16:0	255.2	4.2
Palmitoleic acid	Po	16.1 (Z9)	253.2	Traces
14-Methyl-hexadecanoic acid (anteisomargaric acid)	aM	17:0 anteiso	269.2	Traces
Margaroleic acid	Mo	17:1 (Z9)	267.2	Traces
Stearic acid	S	18:0	283.3	1.8
Oleic acid	O	18:1 (Z9)	281.2	24
Asclepic acid (cis-vaccenic acid)	As	18:1 (Z11)	281.2	1
Linoleic acid	L	18:2 (Z9, Z12)	279.2	48.4
Taxoleic acid	T	18:2 (Z5, 9)	279.2	1.8
Linolenic acid	Ln	18:3 (Z9, Z12, Z15)	277.2	0.2
Pinolenic acid	Pi	18:3 (Z5, Z9, Z12)	277.2	14.9
Arachidic acid	A	20:0	311.2	Traces
Gondoic acid	Go	20:1 (Z11)	309.3	1
Eicosa dienoic acid	20:2	20:2 (Z11, 14)	307.3	0.5
Keteleeronic acid	Ke	20:2 (Z5, 11)	307.3	Traces
Sciadopinolenic acid	Sc	20:3 (Z5, 11, 14)	305.2	0.9
Bis HomoPinoleic acid	Hp	20:3 (Z7, 11, 14)	305.2	Traces
Behenic acid	B	22:0	339.2	Traces

Full scan analysis was done on *P. koraiensis* seed oil after which MS/MS analysis was performed on the produced masses corresponding to the TAG and silver adducts.

2.6. Methodology

The quantities of fatty acids determined by CGC, enabled the prediction of TAGs that are in high or low amounts in the oil [47]. From the quantitative FA composition of the analysed *P. koraiensis* seed oil (Table 1) all the possible structures of TAGs as well as their statistical amounts were determined using the method of calculation based on randomly distributed FA residues on glyceryl backbone [47]. These results (not reported here) permitted the grouping together of all TAGs possessing the same molecular mass. Considering their theoretically calculated amount helped to provide a theoretical picture of the most represented TAGs. But this is very insufficient for identification, because all these theoretically calculated TAGs are not biosynthesized and the amount of each TAG is not totally correlated to the amount of fatty acids.

Another problem occurs if analyst wants to correlate this theoretical percentage of TAGs with observed percentage area in NARP chromatography. In quantitative analysis the response coefficient of all TAGs present in an oil cannot be determined due to the lack of standard. Consequently, it is not possible to determine the percentage TAGs from the area percentage. Moreover these response coefficients are not identical for each TAG. Thus, it is impossible to use the theoretical percentage of TAGs for precise assignments of structures of a TAG using the report of the normalized integration report of an analysis.

However two principal conclusions could be deduced from such calculations:

- the more abundant detected TAGs in NARP-ESI-MS^2 must possess in their structure, at least, one of the most abundant FA residue obtained in CGC but should not possess in their structure a fatty acid residue with low abundance (less than 5%).
- a detected less abundant TAG in NARP-ESI-MS^2 could correspond to any theoretical structure independent of the more or less abundant nature of their constituting FA residue.

In conclusion, comparing the calculated theoretical percentage of TAGs to the chromatographic area percentage could only help in the attribution of a structure when it concerns TAGs with high area percentage but not identified with a standard. For such chromatographic peak (high area percentage), corresponding TAGs could be either a TAG composed of (or having) one of the most abundant FA residue (obtained in CGC) or a mixture of the previous one and coeluted TAGs composed of (or having) less abundant FA residues. This underlines the need for NARP-ESI-MS^2 to ensure precise identification of TAGs in fats and oils.

3. Results and discussion

3.1. Silver cationization and identification of TAGs in ESI mass spectra

Silver cationization offered the advantage of improving the sensitivity by a factor 100 for TAGs [46]. For each chromatographic peak, individual TAGs were identified on the basis of their positive ion ESI mass spectra using $[M+Ag]^+$ and $[M+Ag+AgNO_3]^+$ adducts for their molecular weight determination. Silver has two isotopes of similar abundance, i.e. ^{107}Ag (52%) and ^{109}Ag (48%). Consequently, the $[M+Ag]^+$ forms a doublet of ratio of abundance 1:1 (Fig. 1). The Ag adduct $[M+Ag+AgNO_3]^+$ forms a triplet of ratio of abundance 1:2:1 (Fig. 1).

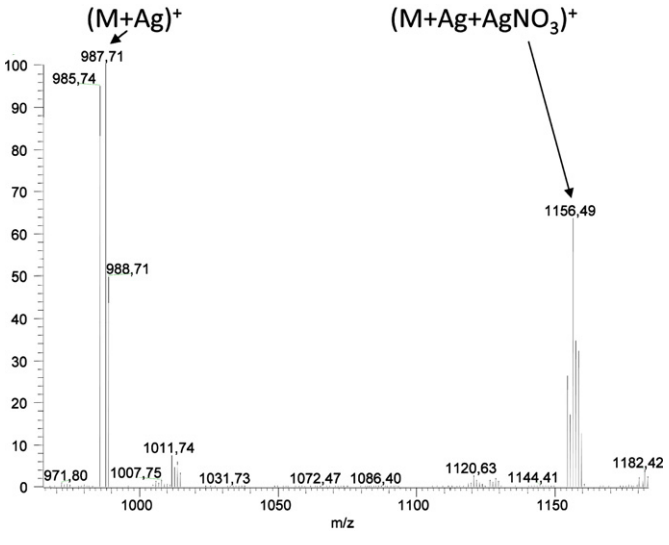


Fig. 1. The $(M+Ag)^+$ doublet and $(M+Ag+AgNO_3)^+$ triplets ions of LLL.

Table 2
The nature and constituting fatty acid residues of TAGs, their partition number (PN), the molecular masses of their adducts $(TAG+Ag)^+$ and diacylglycerols $(DAG-H+Ag)^+$, and the retention factor (k) at which they occur.

Ret. factor, k	Mass of TAG adduct $(TAG+^{107}Ag)^+$	PN	$(DAG-H+^{107}Ag)^+$ fragment masses produced in MS^2			Nature of TAG	Mass and structure of fatty acid residues broken off		
			1	2	3		M_{FA} (FA_1)	M_{FA} (FA_2)	M_{FA} (FA_3)
10.6	981.7	38	701.5	703.5		XXY	279.2 (18:2)	277.2 (18:3)	
11.4	981.7	38	701.5	703.5		XXY	279.2 (18:2)	277.2 (18:3)	
13.6	983.7	40	703.5	705.5		XXY	279.2 (18:2)	277.2 (18:3)	
14.0	957.7	40	679.5	701.5		XXY	277.2 (18:3)	255.2 (16:0)	
14.7	1009.7	40	703.5	729.5	731.5	XYZ	305.2 (20:3)	279.2 (18:2)	277.2 (18:3)
14.8	983.7	40	703.5	705.5		XXY	279.2 (18:2)	277.2 (18:3)	
16.8	957.7	40	679.5	701.5		XXY	277.2 (18:3)	255.2 (16:0)	
16.8	985.7	42	705.5			XXX	279.2 (18:2)	279.2 (18:2)	
18.3	1011.7	42	705.5	731.5		XXY	305.2 (20:3)	279.2 (18:2)	
18.5	985.7	42	705.5			XXX	279.2 (18:2)		
19.0	985.7	42	703.5	705.5	707.5	XYZ	281.2 (18:1)	279.2 (18:2)	277.2 (18:3)
20.8	959.7	42	681.5	679.5	703.5	XYZ	277.2 (18:3)	279.2 (18:2)	255.2 (16:0)
20.8	999.7	43	703.5	719.5	721.5	XYZ	295.2 (19:1)	279.2 (18:2)	277.2 (18:3)
22.7	999.7	43	705.5	719.5		XXY	293.2 (19:2)	279.2 (18:2)	
22.7	973.7	43	693.5	695.5	703.5	XYZ	279.2 (18:2)	277.2 (18:3)	269.2 (17:0)
22.7	1013.7	44	705.5	733.5		XXY	307.2 (20:2)	279.2 (18:2)	
23.5	987.7	44	705.5	707.5		XXY	281.2 (18:1)	279.2 (18:2)	
25.2	1013.7	44	707.5	731.5	733.5	XYZ	305.2 (20:3)	281.2 (18:1)	279.2 (18:2)
26.0	961.7	44	681.5	705.5		XXY	279.2 (18:2)	255.2 (16:0)	
26.0	987.7	44	705.5	707.5		XXY	281.2 (18:1)	279.2 (18:2)	
26.0	1013.7	44	703.5	733.5	735.5	XYZ	309.2 (20:1)	279.2 (18:2)	277.2 (18:3)
27.0	987.7	44	705.5	709.5		XXY	281.2 (18:1)	277.2 (18:3)	
28.8	935.7	44	657.5	679.5		XXY	277.2 (18:3)	255.2 (16:0)	
28.8	975.7	45	695.5	705.5		XXY	279.2 (18:2)	269.2 (17:0)	
29.1	961.7	44	679.5	683.5	705.5	XYZ	281.2 (18:1)	277.2 (18:3)	255.2 (16:0)
29.1	987.7	44	703.5	705.5	709.5	XYZ	283.2 (18:0)	281.2 (18:1)	277.2 (18:3)
32.0	975.7	45	693.5	697.5	705.5	XYZ	281.2 (18:1)	277.2 (18:3)	269.2 (17:0)
32.6	935.7	44	657.5	679.5		XXY	277.2 (18:3)	255.2 (16:0)	
32.6	1015.7	46	705.5	735.5		XXY	309.2 (20:1)	279.2 (18:2)	
33.3	989.7	46	707.5	709.5		XXY	281.2 (18:1)	279.2 (18:2)	
36.0	963.7	46	681.5	683.5	707.5	XYZ	281.2 (18:1)	279.2 (18:2)	255.2 (16:0)
36.0	989.7	46	705.5	709.5		XXY	283.2 (18:0)	279.2 (18:2)	
36.0	1015.7	46	709.5	733.5		XXY	305.2 (20:3)	281.2 (18:1)	
36.6	989.7	46	707.5	709.5		XXY	281.2 (18:1)	279.2 (18:2)	
37.8	1015.7	46	709.5	731.5		XXY	305.2 (20:3)	283.3 (18:0)	
40.1	977.7	47	695.5	697.5	707.5	XYZ	281.2 (18:1)	279.2 (18:2)	269.2 (17:0)
40.3	937.7	46	657.5	681.5		XXY	279.2 (18:2)	255.2 (16:0)	
41.0	963.7	46	679.5	685.5	707.5	XYZ	283.2 (18:0)	277.2 (18:3)	255.2 (16:0)
41.0	989.7	46	705.5	707.5	711.5	XYZ	283.2 (18:0)	281.2 (18:1)	277.2 (18:3)
45.5	1017.7	48	707.5	735.5	737.5	XYZ	309.2 (20:1)	281.2 (18:1)	279.2 (18:2)
47.0	991.7	48	709.5			XXX	281.2 (18:1)		
50.5	965.7	48	683.5	709.5		XXY	281.2 (18:1)	255.2 (16:0)	
50.5	991.7	48	707.5	709.5	711.5	XYZ	283.2 (18:0)	281.2 (18:1)	279.2 (18:2)
50.5	1017.7	48	705.5	737.5		XXY	311.2 (20:0)	279.2 (18:2)	
54.9	965.7	48	681.5	685.5	709.5	XYZ	283.2 (18:0)	279.2 (18:2)	255.2 (16:0)
55.9	939.7	48	657.5	683.5		XXY	281.2 (18:1)	255.2 (16:0)	
55.9	1043.7	48	703.5	763.5	765.5	XYZ	339.2 (22:0)	279.2 (18:2)	277.2 (18:3)
64.7	1019.7	50	709.5	737.5		XXY	309.2 (20:1)	281.2 (18:1)	
71.6	993.7	50	709.5	711.5		XXY	283.2 (18:0)	281.2 (18:1)	
71.6	1019.7	50	707.5	737.5	739.5	XYZ	311.2 (20:0)	281.2 (18:1)	279.2 (18:2)
71.6	1045.7	50	705.5	765.5		XXY	339.2 (22:0)	279.2 (18:2)	
78.8	967.7	50	683.5	685.5	711.5	XYZ	283.2 (18:0)	281.2 (18:1)	255.2 (16:0)
96.8	1073.7	52	705.5	793.5		XXY	367.2 (24:0)	279.2 (18:2)	
99.4	1021.7	52	709.5	739.5		XXY	311.2 (20:0)	281.2 (18:1)	
99.4	1047.7	52	707.5	765.5	767.5	XYZ	339.2 (22:0)	281.2 (18:1)	279.2 (18:2)
109.0	995.7	52	711.5	713.5		XXY	283.2 (18:0)	281.2 (18:1)	
137.2	1049.7	54	709.5	767.5		XXY	339.2 (22:0)	281.2 (18:1)	
137.2	1075.7	54	707.5	793.5	795.5	XYZ	367.2 (24:0)	281.2 (18:1)	279.2 (18:2)

3.2. Identification of the chemical composition of TAGs

The $[TAG+Ag]^+$ adducts (above named $[M+Ag]^+$) were mass selected and fragmented to obtain the masses of the fragment ions of the diacylglycerols $[DAG-H+Ag]^+$ (see Table 2: 4th, 5th and 6th columns). During this MS^2 analysis, the TAGs gave ions that helped to predict the structure of the TAGs. The difference in mass between $(TAG+Ag)^+$ and $(DAG-H+Ag)^+$ gives the mass of the FA broken off the glycerol backbone of a TAG (see Table 2: 8th, 9th and 10th columns). This permitted the determination of the structure (see Table 2: 7th column) and the composition of a TAG which could be confirmed by knowing the molecular mass of the TAG (obtained by subtracting the mass of ^{107}Ag) from the mass of $(TAG+^{107}Ag)^+$ adduct (Table 3: 4th column).

Also it was observed by MS^2 that a singlet ion corresponded to a TAG composed of three identical FAs. Such TAGs have the structure XXX. For example when $k = 16.8$, TAG of mass 985.7 yielded a single ion with mass 705.5 $((18:2/18:2\text{-}H)+Ag)^+$ corresponding to the loss of a C18:2 fatty acid. When two ions were observed in the MS^2, it corresponded to TAGs with two different FAs. It had the gen-

Table 3
The nature and chemical composition of TAGs, the molecular masses of their adducts $(TAG+Ag)^+$, their structure as determined by various methods, and the retention factor at which they occur.

k	Mass of TAG adduct $(TAG+^{107}Ag)^+$	Nature of TAG	Determination of TAG Structure by			
			MS	MS + CGC	Comparison with standard TAGs, TAGs in known oils and reference	Hypothesis
10.6	981.7	XXY	18:2/18:3/18:3		(not LLnLn)	
11.4	981.7	XXY	18:2/18:3/18:3		(not LLnLn)	
13.6	983.7	XXY	18:2/18:2/18:3		LLPi[a]	LLLn/LLPi?
14.0	957.7	XXY	16:0/18:3/18:3	P/18:3/18:3	(not PLnLn)	PPiPi/PLnPi?
14.7	1009.7	XYZ	18:2/18:3/20:3			
14.8	983.7	XXY	18:2/18:2/18:3		(not LLPi not LLnLn)	LTPi/LTLn?
16.8	957.7	XXY	16:0/18:3/18:3	P/18:3/18:3	(not PLnLn)	PPiPi/PLnPi?
16.8	985.7	XXX	18:2/18:2/18:2		LLL	
18.3	1011.7	XXY	18:2/18:2/20:3			
18.5	985.7	XXX	18:2/18:2/18:2		(not LLL)	LLT?
19.0	985.7	XYZ	18:1/18:2/18:3		OLPi[a]	
20.8	959.7	XYZ	16:0/18:2/18:3	P/18:2/18:3	PLPi[a]	
20.8	999.7	XYZ	18:2/18:3/19:1	18:2/18:3/19:1		
22.7	999.7	XXY	18:2/18:2/19:2	18:2/18:2/19:2		
22.7	973.7	XYZ	17:0/18:2/18:3	aM/18:2/18:3		
22.7	1013.7	XXY	18:2/18:2/20:2			LL20:2?
23.5	987.7	XXY	18:1/18:2/18:2		OLL	
25.2	1013.7	XYZ	18:1/18:2/20:3			
26.0	961.7	XXY	16:0/18:2/18:2	P/18:2/18:2	PLL	
26.0	987.7	XXY	18:1/18:2/18:2		(not OLL)	OLT?
26.0	1013.7	XYZ	18:2/18:3/20:1	18:2/18:3/Go		
27.0	987.7	XXY	18:1/18:1/18:3		OOPi[a]	
28.8	935.7	XXY	16:0/16:0/18:3	P/P/18:3	PPLn	PPLn
28.8	975.7	XXY	17:0/18:2/18:2	aM/18:2/18:2		
29.1	961.7	XYZ	16:0/18:1/18:3	P/18:1/18:3	POPi[a]	
29.1	987.7	XYZ	18:0/18:2/18:3	S/18:2/18:3	SLPi[a]	
32.0	975.7	XYZ	17:0/18:1/18:3	aM/18:1/18:3		
32.6	935.7	XXY	16:0/16:0/18:3	P/P/18:3	(not PPLn)PPPi[a]	
32.6	1015.7	XXY	18:2/18:2/20:1	18:2/18:2/Go		
33.3	989.7	XXY	18:1/18:1/18:2		OOL	
36.0	963.7	XYZ	16:0/18:1/18:2	P/18:1/18:2	POL	
36.0	989.7	XXY	18:0/18:2/18:2	S/18:2/18:2	SLL	
36.0	1015.7	XXY	18:1/18:1/20:3			
36.6	989.7	XXY	18:1/18:1/18:2		Not OOL	OOT?
37.8	1015.7	XXY	18:0/18:2/20:3	S/18:2/20:3		
40.1	977.7	XYZ	17:0/18:1/18:2	aM/18:1/18:2		
40.3	937.7	XXY	16:0/16:0/18:2	P/P/18:2	PPL	
41.0	963.7	XYZ	16:0/18:0/18:3	P/S/18:3	PSLn	
41.0	989.7	XYZ	18:0/18:1/18:3	S/18:1/18:3		SOLn/SOPi?
45.5	1017.7	XYZ	18:1/18:2/20:1	18:1/18:2/Go		
47.0	991.7	XXX	18:1/18:1/18:1		OOO	
50.5	965.7	XXY	16:0/18:1/18:1	P/18:1/18:1	POO	
50.5	991.7	XYZ	18:0/18:1/18:2		SOL	
50.5	1017.7	XXY	18:2/18:2/20:0	18:2/18:2/A		LLA?
54.9	965.7	XYZ	16:0/18:0/18:2	P/S/18:2	PSL	
55.9	939.7	XXY	16:0/16:0/18:1	P/P/18:1	PPO[a]	
55.9	1043.7	XYZ	18:2/18:3/22:0	18:2/18:3/B		
64.7	1019.7	XXY	18:1/18:1/20:1	18:1/18:1/Go		
71.6	993.7	XXY	18:0/18:1/18:1	S/18:1/18:1	SOO[a]	
71.6	1019.7	XYZ	18:1/18:2/20:0	18:1/18:2/A		OLA?
71.6	1045.7	XXY	18:2/18:2/22:0	18:2/18:2/B		LLB?
78.8	967.7	XYZ	16:0/18:0/18:1	P/S/18:1	PSO[a]	
96.8	1073.7	XXY	18:2/18:2/24:0	18:2/18:2/Lg		LLLg?
99.4	1021.7	XXY	18:1/18:1/20:0	18:1/18:1/A		OOA?
99.4	1047.7	XYZ	18:1/18:2/22:0	18:1/18:2/B		OLB?
109.0	995.7	XXY	18:0/18:0/18:1	S/S/18:1	SSO[a]	
137.2	1049.7	XXY	18:1/18:1/22:0	18:1/18:1/B		OOB?
137.2	1075.7	XYZ	18:1/18:2/24:0	18:1/18:2/Lg		OLLg?

[a] From [52].

eral structure XXY. For example when $k = 26$, a TAG of mass 961.7 yielded two ions of masses 681.5 and 705.5, corresponding to the loss of C18:2 and C16:0 residues. The difference in the molecular weights of the ions as well as the number of ions produced, helped to determine that the TAG is composed of two residues of C18: 2 and a single residue C16:0. As the FAME analysis of the *P. koraiensis* seed oil showed, the residue C16:0 corresponds to only palmitic acid but there are two acids of structure C18:2, i.e. linoleic or taxoleic acid (Table 1). So, we can say that the structure of the TAG is P-18:2–18:2 with C18:2 being either L or T (Table 3). When three ions were produced in the MS^2, it corresponded to TAG with three different FAs with structure XYZ. For example, at $k = 20.8$, a TAG whose mass of adduct is 959.7 yielded three fragments of masses 679.5, 681.5 and 703.5 corresponding to the loss of C18:3, C18:2, C16:0. There is

only one C16:0 (P) in the oil, hence the structure of such a TAG is P-C18:2/C18:3 (Table 3). Also at $k = 26$, a TAG whose mass of adduct is 1013.7 yielded three fragment ions of masses 703.5, 733.5, and 735.5 corresponding to the loss of C18:2, C18:3 and C20:1 residues (Table 2). From the analysis of FAMEs of *P. koraiensis* seed oil, the residue or FA C20:1 corresponds to only gondoic acid (Go), but the structures C18:2 and C18:3 each has two FAs corresponding to it (Table 1). Hence the MS^2 permitted the identification of the TAG as 18:2/18:3-Go (Table 3) without knowing which isomers of C18:3 and C18:2 are present.

For a given retention factor, MS^2 helped to distinguish between co-eluted adducts of the different molecular mass and how each TAG is made up of different fatty acids residues. For example both TAGs with $k = 22.7$ have respectively 973.7 and 1013.7 as molecular mass of adduct. MS^2 analysis clearly permitted the identification of 18:2–18:2–20:2 and 17:0–18:2–18:3 as co-eluted TAGs (Table 3). The MS^2 thus helped to unambiguously identify co-eluting TAGs. For a given molecular mass at different retention factors MS^2 helped to eliminate possibilities available for a particular molecular mass of adduct if TAG is made up of different fatty acid residues. For example both TAG with $k = 32.6$ and $k = 36$ have the same molecular mass of 1015.7 but MS^2 analysis clearly helped to distinguish between 18:2/18:2/20:1 and 18:1/18:1/20:3. The residues 20:1 and 20:3 respectively refer to Go and Sc deduced from FAME analysis. At retention factor 36 the fragment masses obtained from the mass 1015.7 were 709.5 and 733.5 corresponding to $((18:1/18:1\text{-}H)+Ag)^+$ and $((18:1\text{-}Sc\text{-}H)+Ag)^+$ (Table 2), meaning it is the 18:1/18:1-Sc that elutes at this retention time and not the 18:2/18:2-Go which elutes at $k = 32.6$ (Table 3).

However, the MS^2 could not distinguish between TAGs of the same molecular mass and same FA chain length but differing in positions of double bond or configuration. For example at $k = 33.3$ and $k = 36.6$, the molecular mass of the Ag adduct is 989.7 and the MS^2 showed that the structure is XXY and it composed of 18:1/18:2/18:2. According to the FAME qualitative composition, there are 6 possible TAGs: OLL, OLT, OTT and AsLL, AsLT, AsTT which corresponds to a 18:1/18:2/18:2 structure.

In the same manner, the structure of all the 58 TAGs as given by the MS^2 could be determined. These results are reported in Table 3 column 4.

It has been shown by our results that some TAGs have lignoceric acid C24:0 ($k = 96.8$ and 137.2) in their structure. This is in agreement with work done by Lisa and Holčapek in which they found TAGs composed of lignoceric acid (Lg, C24:0) residues in some resinous seed oils (*Picea*, *Larix*, *Abies*) using APCI-MS [48].

In the same way, two other TAGs containing rare fatty acid residues C19:1 ($k = 20.8$) and C19:2 ($k = 22.7$) were observed. Finding a C19:1 fatty acid residue is in agreement with work done by Wolff and Bayard in FAME analysis-CGC on *P. koraiensis* seed oil [45]. In addition to all the fatty acids reported in Table 1 [40,44], they reported in this other publication [45] the presence of two supplementary unknown FAMEs on CPSil 88 stationary phase, and the equivalent chain length (ECL) for one of them is compatible with a C19:1 ω8 fatty acid structure.

3.3. Identification of the structure of TAGs

As the MS^2 information alone could not help to completely give attribution to all TAGs, there was the need to explore other options to complement the MS^2 results to assure complete attribution. In this present work, in order to identify the unresolved and structurally undetermined TAGs, the chromatographic data were exploited by using comparison of retention factor of some TAGs standards, TAGs in well known oils and chromatographic rules.

Table 4
The standard TAGs, the mass of their silver adducts $(M+^{107}Ag)^+$, retention factor (k), carbon number (CN), and double bond number (DBN).

TAG standards	$(M+^{107}Ag)^+$	k	CN	DBN
LLL	985.7	16.9	54	6
OLL	987.7	23.6	54	5
PLL	961.7	26.1	52	4
OOL	989.7	33.5	54	4
POL	963.7	36.1	52	3
OOO	991.7	47.0	54	3
POO	965.7	50.6	52	2
PPP	913.7	58.7	48	0

3.4. Identification of common FAs in TAGs (composed of P, S, O, L, Ln, residues)

Initially TAG standards OOO, OOL, OLL, LLL, POO, POL, PPP and PLL were mixed and run under the NARP-LC–MS condition discussed above. Table 4 gives the standard TAGs, their retention time, retention factor, carbon number (CN) and double bond number (DBN). All these standard TAGs are present in the *P. koraiensis* seed oil (Table 3 column 6). As all of these TAGs are also present in soybean oil and calophyllum [6,8], analysing these two oils in the same chromatographic conditions and comparing the retention factors of TAGs as well as the chromatograms with that of *P. koraiensis* seed oil, allowed the identification of some TAGs corresponding to this class of TAGs (SLL, SOL, SOO, PPL, PSL, PPO, PSO, SSL, PSLn) present in the *P. koraiensis* seed oil (Table 3 column 6). This helped for example to identify which of the two C18:1/C18:2/C18:2 is eluted at $k = 23.5$, and that is OLL. It also enabled the ascribing of SLL to C18:0/C18:2/C18:2 ($k = 36$).

3.5. Identification of unclassical TAGs composed of rare fatty acid residues (composed of As, T, Pi, Sc, Hp, Go, aM, 19:1, 19:2, 20:2, Ke) and minor fatty acid residues (composed of Lg, B and A)

As well known, the triacylglycerols eluted according to the PN values [4–8]. Among the TAGs with the same PN, separation was achieved and is approximately dependent on the DBN (Table 5). This is supported by work done by Dugo et al. and other authors [7,18]. However, often there are overlaps between the last TAGs of a given PN and the first TAG of next PN [25,26]. Thus, to ensure a precise identification, there is the need to compare the plots of $\log k$ vs. CN and $\log k$ vs. DBN. These two kinds of plots give complementary information [1,2,8,25,26].

The principles of identification are based on the following two thermodynamic rules. Considering hydrophobic theory and for homologues such as CN > 6, Martin [49] made the hypothesis that the variation of change in free enthalpy of transfer of a chain compound from the mobile phase to the stationary phase at infinite dilution ($\Delta\Delta G$) is constant when the chain length is enhanced by a methylene group. Consequently, when analysing homologues, their chromatographic behaviour must be such that the plots of $\log k$ vs. CN would be linear. It was experimentally verified by a lot of authors and reported in a previous review [47]. In the case of TAGs, containing fatty acids like P, S, A, B, and Lg which are fatty acid homologues, this leads to the assurance that the $\log k$ of each constituting TAG of the following two series of TAGs: [PXX, SXX, AXX, BXX, LgXX] and [PXY, SXY, AXY, BXY, LgXY] must fall on two different straight lines independently of the nature of X and Y residues. This was experimentally verified [25,26,47,50,51]. Following the same reasoning, independently of the respective structure of the three fatty acid residues (X, Y and Z) bonded to a TAG backbone, it was proposed and afterward experimentally verified, that the $\log k$ of two other series of TAGs: [XXX, XXY, XYY, YYY] as well as [XXY, XZY, ZZY] fall on two different straight lines [25–27]. Also

Table 5
The TAGs, their carbon number (CN), partition number (PN), double bond number (DBN), their structure determined by log *k* vs. CN and log *k* vs. DBN graphs, and the retention factor at which they occur.

k	log *k*	DBN	CN	PN	Structure of TAG identified by		
					MS, CGC, standards, comparison with reference oils	(log *k* vs. CN)	(log *k* vs. DBN)
10.6	1.03	8	54	38	18:2/18:3/18:3	LLnPi and TLnLn	
11.4	1.06	8	54	38	LPiPi	TLnPi and LPiPi	LPiPi
13.6	1.13	7	54	40	LLPi	LLPi	LLPi
14.0	1.15	6	52	40	PPiLn or PPiPi	PLnPi	
14.7	1.17	8	56	40	P/18:3/20:3	LPiSc	LPiSc
14.8	1.17	7	54	40	LTPi	LTPi	LTPi
16.8	1.23	6	52	40	PPiLn or PPiPi	PPiPi	PPiPi
16.8	1.23	6	54	42	LLL	LLL	LLL
18.3	1.26	7	56	42	18:2/18:2/20:3	LLSc	LLSc
18.5	1.27	6	54	42	LLT? (not LLL)	LLT	LLT
19.0	1.28	6	54	42	OLPi	OLPi	OLPi
20.8	1.32	5	52	42	PLPi	PLPi	PLPi
20.8	1.32	6	55	43	18:2/18:3/19:1		
22.7	1.36	6	55	43	18:2/18:2/19:2		
22.7	1.36	5	53	43	aM/18:2/18:3	aMLPi	aMLPi
22.7	1.36	6	56	44	LL20:2 or LT20:2	LLC20:2	
23.5	1.37	5	54	44	OLL	OLL	OLL
25.2	1.40	6	56	44	18:1/18:2/20:3	OLSc	OLSc
26.0	1.41	4	52	44	PLL	PLL	PLL
26.0	1.41	5	54	44	OLT? (not OLL)	OLT	OLT
26.0	1.41	6	56	44	18:2/18:3/Go	LPiGo	LPiGo
27.0	1.43	5	54	44	OOPi	OOPi	OOPi
28.8	1.46	3	50	44	PP/18:3	PPLn	
28.8	1.46	4	53	45	aM/18:2/18:2	aMLL	aMLL
29.1	1.46	4	52	44	POPi?	POPi/PTL	POPi
29.1	1.46	5	54	44	SLPi	SLPi	SLPi
32.0	1.50	4	53	45	aM/18:1/18:3	aMOPi	aMOPi
32.6	1.51	3	50	44	PPPi/PPLn	PPPi	PPPi
32.6	1.51	5	56	46	18:2/18:1/Go	LLGo	LLGo
33.3	1.52	4	54	46	OOL	OOL	OOL
36.0	1.56	3	52	46	POL	POL	POL
36.0	1.56	4	54	46	SLL	SLL	SLL
36.0	1.56	5	56	46	18:1/18:1/20:3	OOSc	OOSc
36.6	1.56	4	54	46	OOT (not OOL)	OOT	OOT
37.8	1.58	5	56	46	S/18:2/20:3	SLSc	SLSc
40.1	1.60	3	53	47	aM/18:1/18:2	aMOL	aMOL
40.3	1.60	2	50	46	PPL	PPL	PPL
41.0	1.61	3	52	46	PS/18:3	PSLn	
41.0	1.61	4	54	46	S/18:1/18:3	SOPi	SOPi
45.5	1.66	4	56	48	18:1/18:2/Go	OLGo	OLGo
47.0	1.67	3	54	48	OOO	OOO	OOO
50.5	1.70	2	52	48	POO	POO	POO
50.5	1.70	3	54	48	SOL	SOL	SOL
50.5	1.70	4	56	48	18:2/18:2/A	LLA	LLA
54.9	1.74	2	52	48	PSL	PSL	PSL
55.9	1.75	1	50	48	PPO	PPO	PPO
55.9	1.75	5	58	48	18:2/18:3/B	LPiB	LPiB
64.7	1.81	3	56	50	18:1/18:1/Go	OOGo	OOGo
71.6	1.85	2	54	50	SOO	SOO	SOO
71.6	1.85	3	56	50	18:1/18:2/A	OLA	OLA
71.6	1.85	4	58	50	18:2/18:2/B	LLB	LLB
78.8	1.90	1	52	50	PS/18:1	PSO	PSO
96.8	1.99	4	60	52	18:1/18:2/Lg	LLLg	LLLg
99.4	2.00	2	56	52	18:1/18:1/A	OOA	OOA
99.4	2.00	3	58	52	18:2/18:2/B	OLB	OLB
109.0	2.04	1	54	52	SSO	SSO	SSO
137.2	2.14	2	58	54	18:1/18:1/B	OOB	OOB
137.2	2.14	3	60	54	18:1/18:2/Lg	OLLg	OLLg

when considering two different series of TAGs such as [XXX, XXY, XYY, YYY] and [XXX, XXZ, XZZ, ZZZ] the two corresponding straight lines are convergent at the same point corresponding to log *k* of the TAG: XXX [25–27]. We used these three chromatographic laws for the identification of TAGs composed of rare and minor fatty acid residues

3.5.1. Use of log *k* vs. CN plots

A graph of log *k* vs. CN was plotted for all the standard TAGs to assure that the chromatographic rules explained in Section 3.5 are valid under these chromatographic conditions (Fig. 2A). Drawing a line between PPP, PPO, POO and OOO means replacing 'P' with 'O' from one TAG to the next. Two parallel lines could be drawn for this line (i.e. PPL to OOL via POL and PLL to OLL). Also drawing a line between POO and OOL meant replacing 'P' with 'L'. Two more parallel lines could be drawn for this line, i.e. (PPO, POL to OLL) and (PPP, PPL, PLL to LLL) (Fig. 2A). These plots confirm the fact that a straight line can be drawn through the log *k* points for a series of TAGs for which one FA residue is replaced by another, for example 'P' in one TAG is replaced with 'L' in the other, and that this straight line will be parallel to all other straight lines linking other series of TAGs for which the same FA residue 'P' is replaced with 'L' on

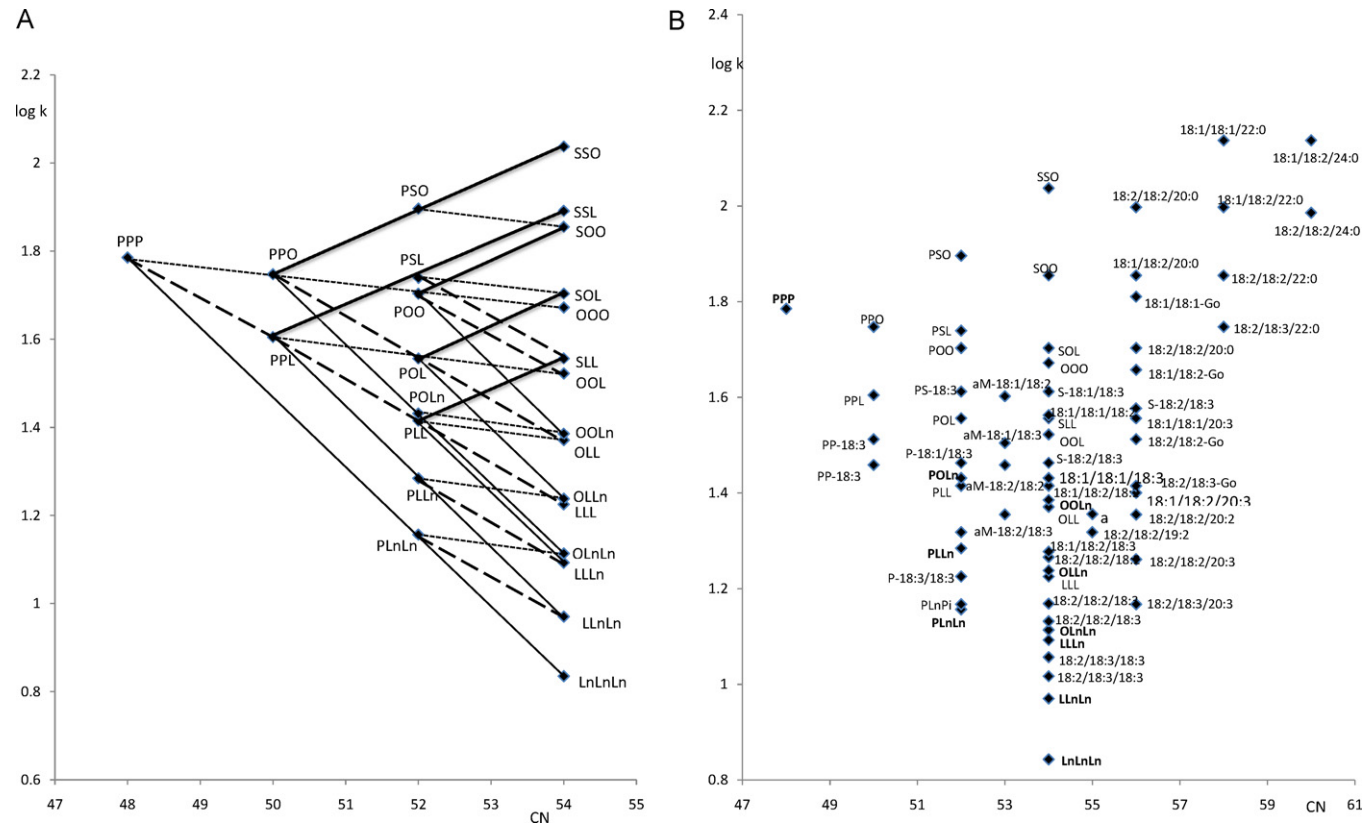


Fig. 2. (A) Plots of log *k* vs. CN for standard TAGs. (B) All identified TAGs in this study.

the log *k* vs. CN graphs (Fig. 2A). Fig. 2 gives the plot of log *k* vs. CN for all TAGs in this study (pure standard and as components of *P. koraiensis* seed oil).

3.5.1.1. Identification of TAGs with A, B, Lg residues. The plots of log *k* vs. CN gave straight parallel lines for homologous series which are constituents of seven different homologous series of TAGs including P in their structure. In this work it was possible to draw seven different parallel lines (Fig. 3A). For example PPO, PSO and SSO fall on the same line because they form a homologous series as P is replaced with S. From PPO to PSO and PSO to SSO there is a replacement of C16:0 (P) with C18:0 (S), which indicates a difference of an ethylene ($-CH_2CH_2-$) group from one TAG to the next. Likewise. PPL and PSL were on a second line parallel to the first one. POO (18:1/18:1/16:0) and SOO (18:1/18:1/18:0) fall on a third line. But on this third line there are also two other TAGs which are 18:1–18:1–20:0 and 18:1–18:1–22:0 whose structure can be respectively OOA and OOB because the only FA with structure 20:0 is 'A' and the only FA with structure 22:0 is 'B' (Table 1).

With the same reasoning, we identified OLA, OLB and OLLg from the extrapolated fourth parallel: POL, SOL, 18:1/18:2/A, 18:1/18:2/B and 18:1/18:2/Lg. The extrapolated fifth parallel PLL, SLL, 18:2/18/2/A, 18:2/18:2/B and 18:2/18:2/Lg helped to identify LLA, LLB and LLLg.

3.5.1.2. Identification of FA residue with CN = 17. Two TAGs with the saturated FA residue of 17 carbon (CN = 53) do not align with the TAGs consisting of residues corresponding to FA peer counterparts (Fig. 3A: aM/18:1/18:2 at $k = 40.1$ is below the third parallel drawn from POL to OLLg. as well as aM/18:2/18:2 at $k = 28.8$ which is below the fifth parallel drawn from PLL to LLLg). This confirms a structure of branched fatty acid residue of 17 carbon atoms confirmed by CGC–MS analysis of the corresponding FAME showing the presence of ante-iso-margaric acid i.e. 14-methyl hexadecanoic (aM) in *P. koraiensis* seed oil [43]. This may not be margaric acid which is saturated linear with 17 carbon atoms and which would align with TAG with the same structure substituted by P, S, B, A or Lg.

3.5.1.3. Identification of TAGs with Pi residues. As previously explained, plots of log *k* vs. CN give convergence points on homogeneous TAGs (XXX). For instance OOO, POO and PPO fall on a straight line on which PPP must also fall. Like LLL, PLL, PPL fall on another line, the intersection of these two last lines confirms the retention factor of PPP.

The knowledge of log *k* of PPP leads to the identification of TAGs possessing Pi or Ln fatty acid residues in their structure. PP-C18:3 ($k = 28.8$ and $k = 32.6$) can either be PPPi or PPLn since there are only two possibilities for C18:3, i.e. Pi or Ln. PPPi was identified when the PPPi, POPi, OOPi was found to be parallel to the PPO, POO and OOO line (Fig. 3B). With one P/P/18:3 ascribed to PPPi (at $k = 32.6$), the other (at $k = 28.8$) was ascribed to PPLn since there are only two possibilities for C18:3, Pi or Ln. The retention of PPLn was confirmed by similar plots done for the TAGs in soya oil which contains high amounts of Ln. Knowing the positions of PPP, PPLn, PLnLn, PLLn, PPPi, LLnLn, OOLn, OLLn and OLnLn on the graph, log *k* vs. CN could then help to find PLnPi, PLPi, LLPi, OLPi, OOPi, POPi using the beams of lines derived from PPP (Fig. 3B). This confirms the results reported in our previous work where we demonstrated that there is no TAGs corresponding to LnLnLn, LLnLn and LLLn in *P. koraiensis* seed oil [52].

Accordingly to what has been previously reported [52] and applying the chromatographic laws of retention, it was demonstrated that two pairs of TAGs could be co-eluting: LLnPi with TLnLn ($k = 11.4$) as well as LPiPi with TLnPi ($k = 10.6$). Considering the relative FAME amount of L and T as well as Ln and Pi (Table 1), it

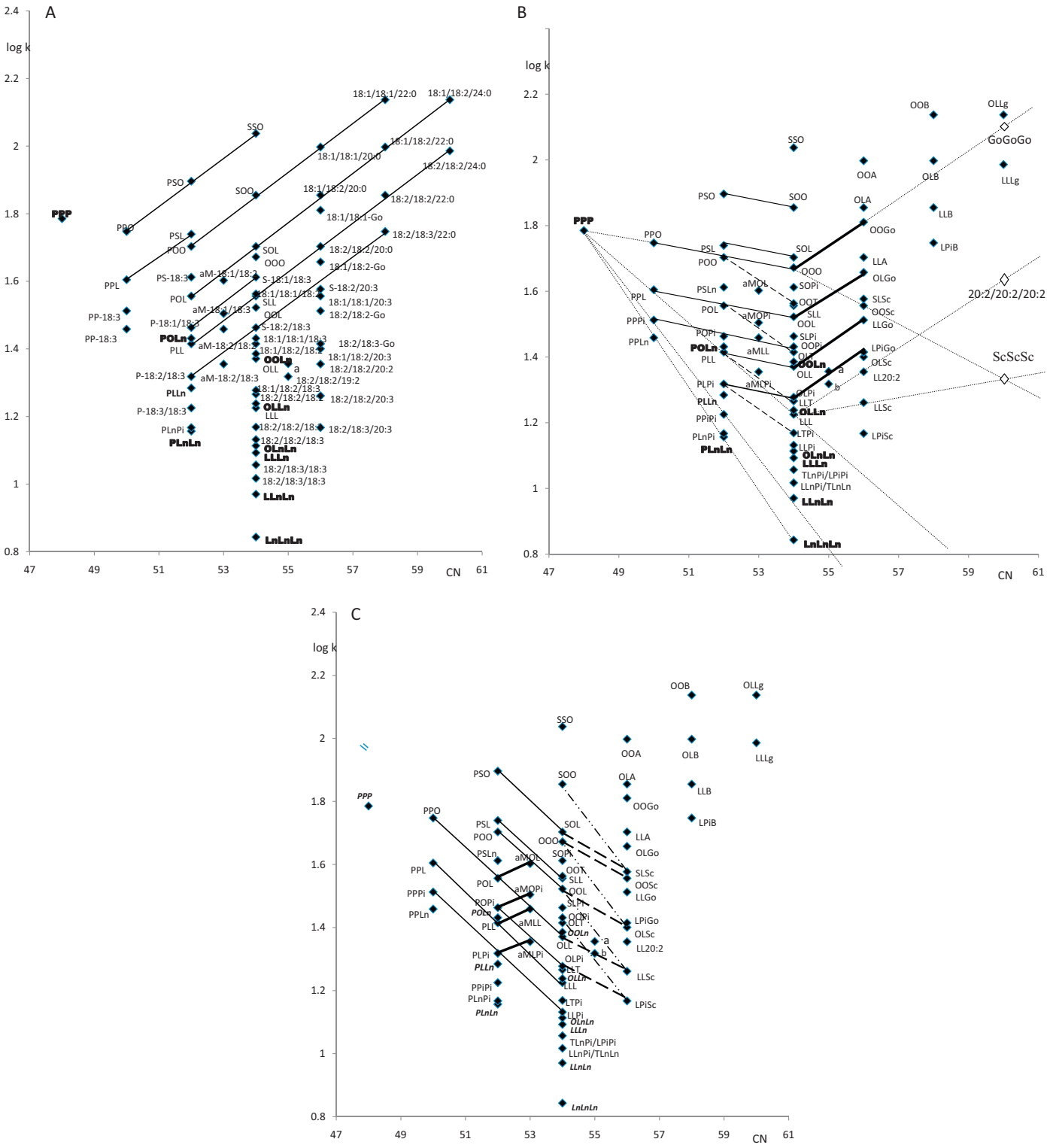


Fig. 3. (A) A plot of log *k* vs. CN showing parallel lines of TAGs that form homologous series. (B) A plot of log *k* vs. CN in which parallel lines helped to identify TAGs with 'Go', 'C20:2', 'O', and 'L' fragments. (C) Plot of log *k* vs. CN in which parallel lines helped identified TAGs with aM, Sc and Pi fragments.

could be presumed that the most probable structures of these TAGs may be respectively LPiPi and LLnPi. They are present in minute quantities.

In addition, this confirms the previously reported results [8,10,52] for which the TAG with FA residue having one double bond in Δ5 are systematically more retained than the corresponding TAG with FA having a double bond in Δ9.

3.5.1.4. Identification of TAGs with T residues. The MS^2 results showed the presence of two TAGs with same structure 18:1/18:1/18:2 with retention factors 33.3 and 36.6. Given the qualitative and quantitative composition of FAs in this oil (Table 1), it can correspond to OOL or OOT. OOL was identified at k 33.3 by comparison with standard TAGs. The other 18:1/18:1/18:2 which is more retained (k = 36.6) has been attributed to OOT. Using the same

reasoning, if we consider replacing 'P' with 'T', we can draw parallel lines (POO–OOT), (POL–OLT), (PLL–LLT) and (PLPi–LTPi). With these parallel lines we could assign the structures OOT, OLT, LLT and LTPi (Fig. 3B).

Identification of these TAGs is confirmed by the fact that the TAGs with FA residue having a double bond in Δ5 (T) are systematically more retained than TAGs with FA having double bond in Δ9 (L) as previously seen with Ln and Pi.

With similar reasoning (Fig. 3C) it was possible to identify the TAGs with residues Sc, aM, Go and C20:2.

3.5.1.5. Identification of TAGs with Sc residues. To help identify TAGs with Sc residue, we used the knowledge that OO-C20:3 is OOSc since the quantity of Sc is far higher than Hp in the *P. koraiensis* seed oil (Table 1). When a line is drawn to join OOO to OOSc, we replace O with Sc. We could then find a series of parallel lines which are parallel to the OOO to OOSc line (i.e. OOL to OLSc, OLL to LLSc, SOL to SLSc, and OLPi to LPiSc). These attributions were confirmed by a second series of parallel lines when residues OO were replaced by LSc residues (SOO to SLSc, OOO to OLSc, OOL to LLSc, and OOPi to LPiSc). This gave four series of parallel lines and four parallel beams (Fig. 3C).

3.5.1.6. Identification of TAGs with aM residues. The structures of TAGs with aM residue were confirmed when parallel lines were drawn for TAGs in which 'P' is replace by 'aM'. A series of four parallel lines could be drawn (POL–aMOL), (POPi–aMOPi), (PLL–aMLL) and (PLPi–aMLPi) (Fig. 3C).

3.5.1.7. Identification of TAGs with Go or As residues. Finally, we are sure (with previous CGC analysis) that C20:1 is Go. We confirmed the location of TAGs with Go residue by obtaining four parallel lines when we replace O with Go, i.e. (OOO–OOGo) parallel to the lines (OOL–OLGo), (OLL–LLGo) and (OLPi–LPiGo) (Fig. 3B).

Knowing the retention of TAGs with a Go residue it became possible to determine where some TAGs with an As residue fall on a chromatogram. This is due to the fact that Go (20:1) and As (18:1) are homologous Δ11 fatty acids. Thus, from the log *k* of OOGo, by drawing a parallel line to the seven straight lines of the plots of log *k* vs. CN for the homologous TAGs (Fig. 3A) the value of k for OOAs could be determined at the intercept of this parallel line with a CN value of 54. When this was done, we found that OOAs must be co-eluted with OOO. In conclusion it could be deduce that when using the present experimental conditions as well as previous ones [8], it is impossible to determine for all the TAGs possessing an O residue if they are pure TAGs or co-eluted with all similar TAGs possessing As (18:1, Z11) residues instead of O (18:1, Z9) residues.

In terms of separation power of TAGs, it became evident in our NARP conditions that it is possible to chromatographically separate TAGs with Δ5 fatty acids from classical Δ9 fatty acids. Conversely, the TAGs with Δ9 and Δ11 fatty acids were co-eluted. This original results obtained in the case of TAGs is identical to the ones previously reported for the analysis of corresponding fatty acid methyl esters in classical RPLC [1].

3.5.1.8. Identification of TAGs with 20:2 residues. It was then left with determination of the structure of TAG 18:2/18:2/20:2. The CGC analysis gave two possibilities for C18:2 as L or T and C20:2 as Ke (Z5, 11) or eicosadienoic acid (Z11, 14). The structure 18:2/18:2/20:2 can therefore be LL-20:2, LT-20:2 or TT-20:2. TAGs with L residues have been well identified. For example, we know without ambiguity the retention of LLL, OLL and OOL. If we suppose that 18:2/18:2/20:2 is LL-C20:2, extrapolation of the line joining LLL to LL-20:2 coincide with the retention of 20:2/20:2/20:2 which is consistent with the retention of GoGoGo and ScScSc. The retention of 20:2/20:2/20:2 is between that of GoGoGo and ScScSc. On the other hand, if we suppose that 18:2/18:2/20:2 is LT-20:2 or TT-20:2, the retention of 20:2/20:2/20:2 is found to be lower than that of ScScSc which is inconsistent with chromatographic laws (Fig. 3B).

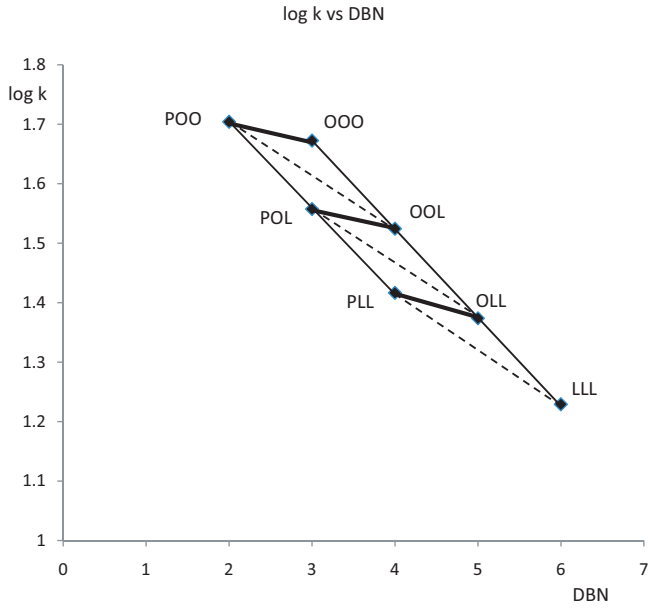


Fig. 4. A plot log *k* vs. DBN for standard TAGs.

Therefore the structure of the corresponding TAG has been attributed to LL20:2 instead of LT20:2 or TT20:2 (Fig. 3B). With Ke present only in trace amounts in this oil, the C20:2 is more likely to be eicosadienoic acid (Z11, 14). This is consistent with the work of Gresti et al. [42].

3.5.1.9. Identification of TAGs with Hp and Ke residues. For each well determined chemical composition TAG possessing one 20:3 or one 20:2 fatty acid residue in its structure (see Section 3.2) we have only detected a single TAG and not two. Contrary to Sc (20:3, Z5, Z11, Z14) and 20:2 (Z11, Z14) FAs which are respectively 0.9% and 0.5% abundant, Hp (20:3, Z7, Z11, Z14) and Ke (20:2, Z5, Z11) were present as traces in *P. koraiensis* seed oil (see Table 1).

Compared to the TAGs possessing either Sc or 20:2 residue in their structure, corresponding TAGs possessing either Hp or Ke residue must be in very low amount in the oil. In the presently used analytical conditions, according to both the detected TAGs and the FAME composition, it is impossible to determine if all TAGs with Sc residue in their structure were co-eluted with the corresponding TAG having an Hp residue or were separated but below the limits of detection of our analytical system.

Same remark is valid when considering TAGs with a 20:2 or a Ke residue.

3.5.1.10. Identification of TAGs with 19:1 and 19:2 and residues. TAGs 18:2/18:2/19:2 (k = 22.7) and 18:2/18:3/19:1 (k = 20.8) are present in minute quantity. Considering the relative FAME amount of L and T as well as Ln and Pi (Table 1), it could be presumed that the most probable structures of these TAGs may be respectively LL19:2 and LPi19:1.

3.5.2. Use of log k vs. DBN plots

A plot of the graph of log *k* vs. DBN using the standard TAGs possessing P, L and O in their structure (as an example) gave straight lines through the points corresponding to same CN but with different DBN, such as OOO, OOL, OLL and LLL, thereby establishing a complementary identification (Fig. 4). Similarly, there was a linear relation among POO, POL, and PLL (Fig. 4). The two lines were

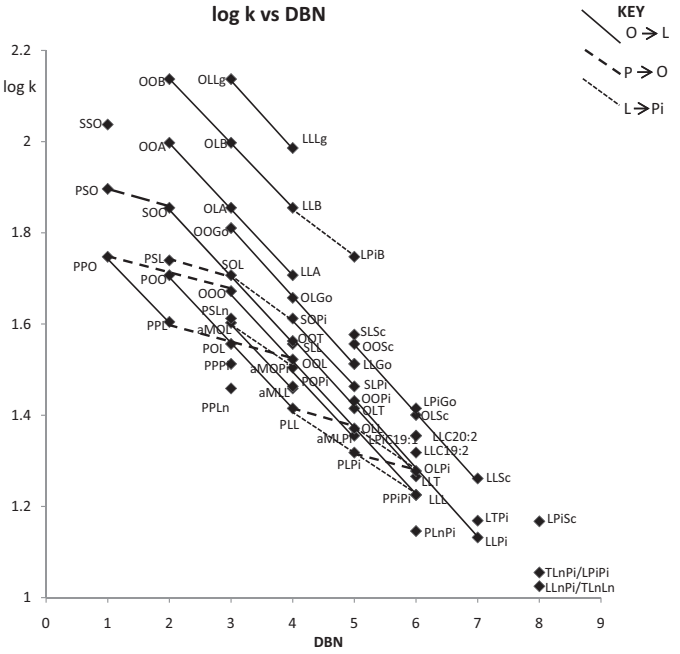
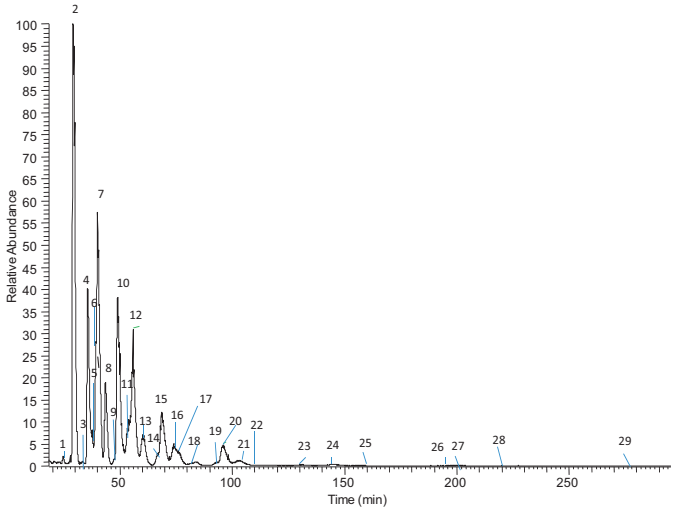


Fig. 5. A graph of log *k* vs. DBN for TAGs in *Pinus koraiensis* seed oil.



1. TLnPi/TLnLn + LPiPi/LLnPi
2. LLPi
3. PLnLn + LPiSc + LTPi
4. LLL + PiPi
5. LLSc
6. LLT
7. OLPi
8. PLPi + LLC19:2
9. aMLPi + LLC20:2 + LPiC19:1
10. OLL
11. OLSc+PLL+LPiGo+ OLT
12. OOPi
13. POPi + SLPi + aMLL + PPLn
14. LLGo + aMOPi + PPPi
15. OOL
16. SLL + POL + OOSc + OOT
17. SLSc+PPL+aMOL
18. SOPi+PSLn
19. OLGo
20. OOO
21. POO + LLA + SOL
22. PSL + PPO + LPiB
23. OOGo
24. SOO+OLA+LLB
25. PSO
26. LLLg
27. OOA + OLB
28. SSO
29. OOB+OLLg

Fig. 6. Chromatographic separation of *Pinus koraiensis* seed oil. For chromatographic conditions see Section 2.5.

found to be parallel to each other. In each case, oleic acid 'O' of the preceding TAG is replaced by linoleic acid 'L' in the next TAG (OOO–OOL–OLL–LLL). This confirmed the work done by Goiffon et al. [25]. Similar plots were made for TAGs in the *P. koraiensis* seed oil (Table 5 and Fig. 5). As expected, OOSc, OLSc and LLSc fall on the same line and their line was parallel to POO–PLL and OOO–LLL lines. All attributions/predictions made from the curves of log *k* vs. CN were confirmed by the plots of log *k* vs. DBN.

This strategy helped to identify and characterize the TAGs unambiguously. The molecular mass of $[M+Ag]^+$, $[DAG-H+Ag]^+$, the chromatographic rule, the graphs of log *k* vs. DBN and log *k* vs. CN allowed the identification of the TAGs. This strategy permitted the identification of 58 TAGs in *P. koraiensis* seed oil.

Present results could be compared to previous published ones on TAGs composition of *P. koraiensis* seed oil. Gresti et al. reported the fractionation of TAGs of *P. koraiensis* oil using semi-preparative NARP chromatography and analysed the FA composition of each fraction by CGC [42]. As expected, complete separation of the TAGs was not achieved. Theoretically, each HPLC fraction consisted up to five components which were reconstructed on the basis of FA composition of the fractions. A total of 50 TAG for 16 peaks was described. Among them 17 were higher than 1% [42]. On their part, Imbs et al. quantitatively determined 26 TAGs of *P. koraiensis* seed oil using Ag-LC and NARP-LC for the separation of TAGs. The identification of the TAGs was carried out by combining TLC, CGC, NARP-LC, and calculated equivalent carbon number of TAGs standards [53].

Lastly, using NARP-LC, and the same sample of *P. koraiensis* seed oil, Héron et al. identified 27 TAGs from supplementation method [52] and predicting diagrams (graphs), log *k* vs. DBN and log *k* vs. CN [25]. They proved that TAGs possessing Δ5 fatty acid residues were eluted after the TAGs isomers possessing classical Δ9 fatty acid residue, but no experimental verification using MS was published [52] Moreover, using the same optimized conditions and without MS confirmation of their TAG identification they also predicted and showed that TAGs possessing Δ6 fatty acid residues could be separated from TAGs possessing Δ5 fatty acid residue. Both were eluted after the TAGs isomers possessing classical Δ9 or Δ11 fatty acid residue. In the same way they also predicted that TAGs with a Z fatty acid residue instead of a E fatty acid residue in same location on the hydrocarbonaceous chain are more retained [8].

Table 6 gives a summary of the chromatographic conditions utilised by these different authors for separation of TAGs in *P. koraiensis* seed oil, their major peaks obtained as well as the number of major TAGs identified.

Fig. 6 gives the chromatographic separation achieved when the *P. koraiensis* seed oil was ran under the chromatographic conditions described earlier with post-column addition of $AgNO_3$.

Out of the 58 TAGs identified by our method, only 4 contained more than one Δ5-olefinic acid (LPiPi, LPiT, LPiSc and PPiPi). The results showed that the Δ5-olefinic acids 'Pi', 'T' and 'Sc' are preferentially associated with two linoleic acids (e.g. LLPi, LLSc, LLT), and to a lesser extent, to one molecule of linoleic acid and one molecule of oleic acid (e.g. OLSc, OLPi, etc.), or two oleic acid molecules (e.g. OOPi, OOSc, OOT). Other combinations were also found (e.g. PPPi and PPiPi) but were in very low amounts. This confirms what was reported by Gresti et al [42]. Gresti et al reported the presence of trace amounts of ante-iso heptadecanoic acid (C17:0), i.e. 14-methyl hexadecanoic acid (aM) but did not identify any TAG with aM. However, our method identified aMLPi, aMLL, aMOPi, aMOL to be present. We have the cause to believe that the mass corresponding to C17:0 is 14-methyl hexadecanoic acid also named ante-iso-margaric acid (aM) as indicated by Gresti et al and not margaric acid (Ma) since the PN of the TAGs containing this fatty acid does not always fit into the elution order of PN (Table 5). All the 19 major TAGs experimentally determined by Gresti et al have been identified by our method.

Also all the 26 TAGs identified and quantified by Imbs et al. [53] were identified and characterized by our method. Due to the use of the online coupling of NARP-LC-ESI–MS^2 and post column addition of $AgNO_3$ which greatly increased the sensitivity leading to the identification of TAGs that are in small amounts, TAGs that were

Table 6
Summary of previous work done on *Pinus koraiensis* seed oil.

Authors	Chromatographic conditions	Number of major peaks	Major TAGs identified[a]	Total TAGs identified
Gresti et al. [42]	Acetone/acetonitrile (55/45, v/v), flow rate: 1 ml/min; ODS (250 × 4.0 mm × 4 μm)	16	17	50
Héron et al [52]	Dichloromethane/acetonitrile (31/69, v/v), flow rate: 1 ml/min; Brownlee ODS (250 × 4.6 mm × 5 μm)	15	17	27
Imbs et al. [53]	Ag-TLC fractions of TAGs separated by HPLC with acetone (100%); flow rate: 4.5 ml/min; Supelco ODS (250 × 9.5 mm × 5 μm)	17	16	26

[a] Well separated or co-eluted.

labelled as unknown by Imbs et al. have been positively identified by our method. Also TAGs identified as LLEm, OOEm, SLEm, OLEm (where Em is a C20 acid) by Imbs et al., have been positively identified by our method as LLSc/LLGo/LLA (for LLEm), OOSc/OOA/OOGo (for OOEm), SLSc (SLEm), and OLSc/OLA/OLGo (for OLEm) respectively.

In a previous studies 17 major TAGs were identified among predicted 27 using theoretical calculations and predicting diagrams [52]. Interestingly, all the 27 TAGs have been identified by our method in addition to 31 other TAGs not experimentally identified previously. Our present results are thus supported by experimental proof from MS and theoretical calculations.

22 new TAGs were identified by this method to be present in *P. koraiensis* seed oil which were not identified by either Gresti et al. or Imbs et al. Most of these new TAGs found in *P. koraiensis* seed oil by this method have also been identified by Lisa et al. [33] to be present in other studied conifer seed oils by using APCI-MS.

4. Conclusion

This study shows that both chromatographic data (log *k* vs. DBN and log *k* vs. CN) and mass spectrometry information (molecular weight with good sensitivity) were necessary to fully characterize oil sample.

Liquid chromatography permitted the separation of TAGs composed of Δ5 or Δ6 from those containing Δ9, but it was not able to separate the TAGs with Δ9 from those with Δ11 double bond location on alkyl chain of fatty acid residues. Mass spectrometry permitted the detection of the TAGs through the molecular weight information which was made possible by using silver nitrate as post-column additive which improved the sensitivity by a factor of 100 for some TAGs. Therefore, it was possible to detect all TAGs even those at low level. After having the molecular weight information full scan and MS^2, it was possible to differentiate the isomers of TAGs by utilising log *k* vs. CN and log of k vs. DBN. Co-eluting TAGs could be identified without any ambiguity. This strategy is simple and easy to handle and permitted routine use as it allowed the differentiation of 58 TAGs in the *P. koraiensis* seed oil sample. Also it could be used in the absence of standard TAGs to identify those present in an oil.

The strategy also demonstrated that simple post-column additive Ag-LC–MS^n method could be sufficient to identify the TAGs present in an oil without looking for the best optimized chromatographic conditions.

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