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High-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry and gas chromatography–flame ionization detection characterization of Δ5-polyenoic fatty acids in triacylglycerols from conifer seed oils

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Abstract

Edible conifer seeds can serve as a source of triacylglycerols (TGs) with unusual Δ5 unsaturated polymethylene interrupted fatty acids (UPIFAs), such as *cis*-5,9-octadecadienoic (taxoleic), *cis*-5,9,12-octadecatrienoic (pinolenic), *cis*-5,11-eicosadienoic (keteleeronic) and *cis*-5,11,14-eicosatrienoic acids (sciadonic). Conifer seed oils from European Larch (*Larix decidua*), Norway Spruce (*Picea abies*) and European Silver Fir (*Abies alba*) have been analyzed by non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) with atmospheric pressure chemical ionisation (APCI)-MS detection. The influence of different positions of double bonds in Δ5-UPIFAs on the retention and fragmentation behavior is described and used for the successful identification of TGs in each oil. TGs containing Δ5-UPIFAs have a higher retention in comparison with common TGs found in plant oils with single methylene interrupted Δ6(9)-FAs and also significantly changed relative abundances of fragment ions in APCI mass spectra. Results obtained from HPLC/MS analyses are supported by validated GC/FID analyses of fatty acid methyl esters after the transesterification. The total content of Δ5-UPIFAs is about 32% for European Larch, 27% for Norway Spruce and 20% for European Silver Fir. In total, 20 FAs with acyl chain lengths from 16 to 24 carbon atoms and from 0 to 3 double bonds have been identified in 64 triacylglycerols from 3 conifer seed oils.
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Keywords: Conifer seed oil; Triacylglycerol; Triglyceride; Delta 5-Olefinic; Polyenoic fatty acid; HPLC/MS

1. Introduction

Triacylglycerols (TGs) are natural compounds consisting of saturated and unsaturated fatty acids (FAs) differing in their acyl chain lengths, in the number and positions of double bonds (DBs), in the *cis*/*trans* configuration of DBs, in positional isomerism and *R*/*S* optical isomerism of TGs containing three different FAs. The standard notation of TGs is based on the initials of FA trivial names (see Table 1), arranged in the order of their stereochemical positions on the glycerol skeleton. In addition to saturated and monounsaturated FAs, common plant oils contain polyunsaturated FAs most frequently with 18 carbon atoms and single methylene interrupted DBs [1–5], i.e. *cis*-9,12-octadecadienoic (linoleic acid) and *cis*-9,12,15-octadecatrienoic (linolenic acid). TGs with unusual FAs, such as those containing conjugated DBs (e.g. conjugated linolenic acids) [6] or unsaturated polymethylene interrupted fatty acids (UPIFAs) [7–14], can be found in some taxonomical groups.

Gymnosperms (*Gymnospermae*) includes trees and shrubs and they are commonly known as conifers. This large taxonomical group is known for the presence of unusual FAs with the first site of unsaturation at the fifth carbon atom (Δ5-UPIFAs) and *cis* (Z) configuration, such as *cis*-5,9-octadecadienoic (taxoleic), *cis*-5,9,12-octadecatrienoic (pinolenic),

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Table 1
Systematic and trivial names of fatty acids found in triacylglycerols of studied conifer seed oils listed with their abbreviations, carbon numbers (CN), double bond (DB) numbers and equivalent carbon numbers (ECN)

Systematic name	Trivial name	Abbreviation	CN:DB	ECN
Hexadecanoic	Palmitic	P	C16:0	16
cis-9-Hexadecenoic	Palmitoleic	Po	C16:1	14
Heptadecanoic	Margaric	Ma	C17:0	17
Octadecanoic	Stearic	S	C18:0	18
cis-9-Octadecenoic	Oleic	O	C18:1	16
cis-11-Octadecenoic	Vaccenic	Va[a]	C18:1	16
cis-9,12-Octadecadienoic	Linoleic	L	C18:2	14
cis-5,9-Octadecadienoic	Taxoleic	Ta[a]	C18:2	14
cis-9,12,15-Octadecatrienoic	Linolenic	Ln	C18:3	12
cis-5,9,12-Octadecatrienoic	Pinolenic	Pi[a]	C18:3	12
Nonadecanoic	–	–	C19:0	19
Eicosanoic	Arachidic	A	C20:0	20
cis-9-Eicosenoic	Gadoleic	G	C20:1	18
cis-11,14-Eicosadienoic	–	–	C20:2	16
cis-5,11-Eicosadienoic	Keteleeronic	Ke[a]	C20:2	16
cis-8,11,14-Eicosatrienoic	–	–	C20:3	14
cis-5,11,14-Eicosatrienoic	Sciadonic	Sc[a]	C20:3	14
Docosanoic	Behenic	B	C22:0	22
Tricosanoic	–	–	C23:0	23
Tetracosanoic	Lignoceric	Lg	C24:0	24

[a] Suggested abbreviations used in this work.

cis-5,11-octadecadienoic (ephedrenic), *cis*-5,11-eicosadienoic (keteleeronic), *cis*-5,11,14-eicosatrienoic (sciadonic), *cis*-5, 9,12,15-eicosatetraenoic (coniferonic) and *cis*-5,11,14,17-eicosatetraenoic (juniperonic) acids. Sciadonic acid has been identified in *Podocarpus nagi* seed oil as early as in 1962 [15]. The presence of the following Δ5-UPIFAs has been reported in Gymnosperm plants: keteleeronic and sciadonic acids in *Ginkgo biloba* [16,17], pinolenic acid in *Larix leptolepsis* [18] and *Pinus koraienesis* [19], taxoleic and sciadonic acids in *Taxus baccata* [20], sciadonic acid in *Toreya nucifera* [19,21], ephedrenic, keteleeronic, sciadonic and juniperonic acids in *Ephedra campylopoda* [22], ephedrenic and sciadonic acids in *Ginkgo biloba* [23], pinolenic acid in *Picea abies* [24], taxoleic, pinolenic, coniferonic, sciadonic and juniperonic acids in many species of conifers [25].

In contrast to several papers dealing with methyl esters of Δ5-UPIFAs (e.g. reviews [7,8,11]), much less attention has been paid to intact TGs that contain those Δ5-UPIFAs. ^{13}C nuclear magnetic resonance (NMR) spectroscopy of oils from three *Taxus* and one *Torreya* species has confirmed that Δ5-olefinic acids are apparently excluded from the *sn*-2 position which is characteristic for all *Gymnosperm* species analyzed so far [12]. ^{13}C NMR spectroscopy of the seed oil from two *Ephedra* species shows that Δ5-UPIFAs are excluded from the *sn*-2 position, a characteristic common to all analyzed *Conipherophytes* species (more than 30 species), with the possibility of an exclusive esterification at the *sn*-3 position [13]. The chemical degradation of conifer seed oils followed by gas chromatographic analysis of dibutyroyl derivatives of monoacylglycerols reveals that seed oils of 18 species from 5 conifer families contain Δ5-olefinic acids esterified mainly at primary positions (i.e. *sn*-1 and *sn*-3) of the glycerol backbone, whereas less than 8% of Δ5-olefinic acids are esterified in the secondary position (i.e. *sn*-2) [14].

Non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) has been widely used for the separation of complex natural lipid samples [1–5,26–41]. The retention in NARP-HPLC increases with increasing equivalent carbon number (ECN) defined as the total carbon number (CN) in all acyl chains minus two times the number of DBs, i.e. ECN = CN − 2DB. Under optimized separation conditions, the separation of most TGs within the same ECN group is also possible, for example the critical pair LLL/OLLn or the group of OOO, OOP, OPP and PPP can be well resolved [1,2,4,5,26,27]. The separation of TGs differing in the positions of DBs is also feasible [28]. On the other hand, NARP-HPLC is not suitable for the separation of three types of isomerism, i.e. regioisomers, *R*/*S* isomers and *cis*/*trans* isomers. Various mobile phase systems, mostly in gradient elution mode, are described in the literature, such as 2-propanol/acetonitrile [1–3,27,29], 2-propanol/acetonitrile/hexane [30,31], acetone/acetonitrile [26,31-34], 100% propionitrile [4], acetonitrile/chloroform [5], acetonitrile/dichloromethane [35–41], etc.

The coupling of HPLC and mass spectrometry (MS) is a powerful tool in lipid analysis, because it provides both structural information and usually also the highest sensitivity among all available chromatographic detectors [30]. Atmospheric pressure chemical ionization (APCI) is the most frequently used ionization technique for TG analysis because of easy coupling to non-aqueous mobile phase systems and high ionization efficiency for non-polar species. The presence of both protonated molecules $[M+H]^+$ and fragment ions $[M+H-R_iCOOH]^+$ is important for structure elucidation [1–5,26–42]. Electrospray ionization (ESI) mass spectra exhibit $[M+Na]^+$ and $[M+K]^+$ adduct ions instead of protonated molecules and also some fragment ions, such as $[M+Na-R_iCOOH]^+$ and $[M+Na-R_iCOONa]^+$, but with lower relative abundances

[1,42]. If ammonium ion is added to the eluent, then the formation of $[M+NH_4]^+$ adduct ions is preferred, which simplifies the subsequent fragmentation in comparison to alkali metal adducts [40,41]. Coupling with silver-ion HPLC may require the use of post-column make-up flow of polar solvent both for ESI and APCI, because typical mobile phases contain more than 98% of hexane [34] or other non-polar solvent, which is not favorable for the ionization process. APCI mass spectra provide information on the predominant FA in the *sn*-2 position [1–5,29–33]. The precise ratio of regioisomers can also be obtained by the measurement of calibration curves with both positional isomers [5,33].

The main goal of our work is the identification of TGs from three representative conifer seed oils containing Δ5-UPIFAs isolated in our laboratory—European Larch (*Larix decidua*), Norway Spruce (*Picea abies*) and European Silver Fir (*Abies alba*). First, the retention behavior of TGs with unusual Δ5-UPIFAs in NARP-HPLC and the fragmentation behavior in positive-ion APCI mass spectra are described and used for the positive identification of TGs in each oil. Then, the pine seed oils are transesterified using a standard procedure with sodium methoxide and analyzed by a validated gas chromatography—flame ionization detection (GC/FID) method for the analysis of FAMEs and concentrations of individual FAs and also average parameters are calculated and used for the characterization of individual oils.

2. Experimental

2.1. Materials

Acetonitrile, 2-propanol, hexane and standards of 1,2,3-tri-(*cis*-9,12-octadecadienoyl)glycerol (trilinolein) and 1,2,3-tri-(*cis*-9,12,15-octadecatrienoyl)glycerol (trilinolenin) were purchased from Sigma-Aldrich (St. Louis, USA). The solvents were filtered through a 0.45 μm Millipore filter and degassed by continuous stripping with helium. The standards of *cis*-5,9-octadecadienoic (taxoleic) methyl ester, *cis*-5,9,12-octadecatrienoic (pinolenic) methyl ester and FAMEs standard mixture Me100 (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0. C17:1, C18:0, C18:1, C18:1T, C18:2, C18:2T, C18:3, C18:3γ, C20:0, C20:1, C20:2, C20:3, C20:3γ, C20:4, C20:5, C21:0, C22:0, C22:1, C22:2, C22:6, C23:0, C24:0 and C24:1) were purchased from Larodan Fine Chemicals (Mälmo, Sweden).

2.2. HPLC conditions

The chromatographic apparatus consisted of a Model 616 pump with a quaternary gradient system, a Model 996 diode-array UV detector, a Model 717 + autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). The HPLC conditions: two chromatographic columns Nova-Pak C_{18} (300 mm × 3.9 mm and 150 mm × 3.9 mm, 4 μm, Waters) connected in series, a flow rate of 1 mL/min, an injection volume of 10 μL, a column temperature of 25 °C and a mobile phase gradient with a slope of 0.65%/min: 0 min—100% acetonitrile, 106 min—31% acetonitrile—69% 2-propanol, 109 min—100% acetonitrile. The injector needle was washed with the mobile phase before each injection. The column hold-up volume, t_M, was 3.20 min for the system with 300 + 150 mm Nova-Pak C_{18} columns. The UV detection at 205 nm and positive-ion APCI-MS were coupled in series. The Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) in the mass range *m/z* 50–1200 was used with the following setting of tuning parameters: the pressure of the nebulizing gas of 70 psi, the drying gas flow rate of 3 L/min, temperatures of the drying gas and APCI heater were 350 and 400, respectively. The isolation width *m/z* = 2 and the collision amplitude 0.8 V were used for HPLC/MS/MS experiments. The reconstructed ion current (RIC) chromatograms in the region *m/z* 300–1200 were used for the peak integration. Presented peak areas correspond to averaged values from three consecutive chromatographic runs. Individual reconstructed ion current chromatograms were used to support the identification and apportionment of coeluting peaks.

2.3. Sample preparation

The conifer seeds from European Larch (*Larix decidua*), Norway Spruce (*Picea abies*) and European Silver Fir (*Abies alba*) were collected in early summer 2005 in the Bohemian Forest. Ten to fifteen grams of seeds were weighed and then carefully crushed in a mortar to fine particles, which were mixed with 15 mL of hexane, and the mixture was stirred occasionally for 15 min. The solid particles were filtered out using a rough filter paper and then the extract was filtered again using a fine filter with 0.45 μm pores. From the filtered extract, hexane was evaporated at room temperature yielding a pure plant oil. The oil samples were dissolved in an acetonitrile–2-propanol–hexane mixture (2:2:1, v/v/v) to prepare 3% solution (w/v), 10 μl of this solution was injected for HPLC analysis.

2.4. Preparation of fatty acid methyl esters and their GC/FID analysis

Fatty acid methyl esters (FAMEs) were prepared from TGs in conifer seed oils using a standard procedure with sodium methoxide [43]. FAME mixtures were analyzed by GC/FID on a Varian CP 3800 with an autosampler CP-8410 and an injector CP-1177 (Varian Analytical Instruments, Walnut Creek, CA, USA) using silica capillary column BTR-Carbowax-30 W-0.5 F, 30 m length, 0.32 mm I.D., 0.5 μm film thickness (Quadrex, Woodbridge, CT, USA). GC conditions were as follows: the injection volume was 1 μL, the split ratio was 1:40, the flow rate of nitrogen as a carrier gas was 0.7 ml/min, the temperature program was as follows: the initial temperature was 160 °C, held for 6 min, then a ramp to 200 °C at 20 °C/min, held for 10 min, ramp to 240 °C at 5 °C/min, held for 28 min with the total analysis time of 54 min. Injector and detector temperatures were 250 and 270 °C, respectively.

3. Results and discussion

3.1. Nomenclature for TGs containing common FAs and unusual Δ5-UPIFAs

Table 1 summarizes all FAs identified in individual TGs in conifer seed oils together with their trivial names, abbreviations, carbon numbers (CN), double bond (DB) numbers and equivalent carbon numbers (ECN). Table 2 lists ECNs, nominal molecular weights (MWs), retention times t_R, relative retention r and retention factors k measured using the HPLC method with 45 cm total column length for 64 TGs identified in three conifer seed oils consisting of 20 FAs. The masses and structures of fragment ions have been described in our previous works [1,2,27]. Plant oils usually contain a mixture of regioisomers. Three identical acyl chains on the glycerol backbone (single-acid $R_1R_1R_1$ type) provide only a single ion $[M+H-R_1COOH]^+$, while mixed-acid $R_1R_1R_2$ type produces two different $[M+H-R_1COOH]^+$ and $[M+H-R_2COOH]^+$ ions with the statistical abundance ratio 2:1, and the $R_1R_2R_3$ type has three different $[M+H-R_1COOH]^+$, $[M+H-R_2COOH]^+$ and $[M+H-R_3COOH]^+$ ions with the statistical abundance ratio 1:1:1. The neutral losses of R_iCOOH from the primary positions *sn*-1 and *sn*-3 are preferred, compared to the cleavage from the secondary *sn*-2 position. This characteristic can be used for the determination of the predominant FA in the *sn*-2 position for plant oils containing common unsaturated FAs [1–5,33]. This approach works well for polyenoic FAs with common DB positions, but for unusual DB positions (e.g. Δ5-UPIFAs) the regioisomeric standards are needed for reliable identification of sn-2 FA or even the quantitation of the ratio of individual FAs in sn-2 position. The presence of Δ5-UPIFAs changes the observed ratio of $[M+H-R_iCOOH]^+$ ions. In this work, the determination of *sn*-2 FAs on the basis on well established rules [1,33,25,42] is done for TGs consisting of common FAs. However, the assignment of FA prevailing in individual stereochemical positions for TGs containing Δ5-UPIFAs is based only on previous literature evidence [9,10] that Δ5-UPIFAs preferentially occupy the *sn*-3 position, but the analytical data supporting this assignment are not available because of the total absence of mixed-acid TG standards with Δ5-UPIFAs.

The positions *sn*-1 and *sn*-3 are considered as equivalent, because these regioisomers cannot be distinguished by NARP-HPLC or mass spectrometry, so acids in primary *sn*-1 and *sn*-3 positions are ordered only by decreasing mass, i.e. SLO but not OLS. An exception is Δ5-UPIFAs assigned preferentially to *sn*-3 position based on earlier data [9,10,12–14]. The term “common FAs” is used throughout this work for FAs with even number of carbon atoms and sites of unsaturation at positions 9, 12 and 15 (e.g. oleic acid C18:1Δ9, linoleic acid C18:2Δ9,12 or linolenic acid C18:3Δ9,12,15) or saturated FAs.

3.2. Chromatographic behavior of TGs containing Δ5-UPIFAs

It is well known that the retention of TGs in NARP systems depends on the acyl chain lengths and the number of double

Table 2
Triacylglycerols (TG) identified in studied conifer seed oils listed with their equivalent carbon numbers (ECN), molecular weights (MW), retention times (t_R), relative retention r, retention factors (k) and differences in retention factors (Δk)

TG	ECN	MW[a]	t_R	r[b]	k[c]	Δk[d]
LnLnLn[e]	36	872	48.3	0.800	14.09	0
PiLnPi		872	51.1	0.849	14.97	0.88
PiPiPi		872	52.4	0.872	15.38	1.29
LnLLn[e]	38	874	54.0	0.901	15.88	0
LnLPi		874	55.4	0.926	16.31	0.43
PiLPi		874	56.8	0.950	16.75	0.87
LLLn	40	**876**	**59.6**	**1.000**	17.63	0
LnOLn[e]		876	60.6	1.018	17.94	0
LLPi		876	61.0	1.025	18.06	0.43
C20:3LPi		902	61.6	1.035	18.25	–
C20:3LnTa		902	62.1	1.044	18.41	–
LnLnP[e]		851	62.1	1.044	18.41	0
TaLPi		876	63.1	1.062	18.72	1.09
PiOPi		876	63.5	1.069	18.84	0.90
PPiPi		850	64.9	1.094	19.28	0.87
LLL	42	**878**	**65.3**	**1.000**	19.41	0
C20:2LLn[e]		904	65.4	1.002	19.44	0
OLLn		878	66.4	1.018	19.75	0
C20:3LL		904	66.2	1.014	19.69	–
C20:2LPi		904	66.7	1.023	19.84	0.40
LLTa		878	67.1	1.029	19.97	0.56
LOPi		878	67.6	1.037	20.13	0.38
PLLn[e]		852	67.8	1.040	20.19	0
SLnLn[e]		878	68.5	1.052	20.41	0
PLPi		852	69.0	1.019	20.56	0.37
TaOPi		852	69.5	1.068	20.72	0.97
PTaPi		852	70.9	1.090	21.16	0.97
SPiPi		878	71.2	1.095	21.25	0.84
LnLMa	43	866	70.7	1.087	21.09	0
C20:2LL	44	906	70.8	0.985	21.13	0
OLL		**880**	**71.8**	**1.000**	21.44	0
GLLn[e]		906	72.1	1.004	21.53	0
C20:3LO		906	72.5	1.010	21.66	–
OOLn[e]		880	72.6	1.012	21.69	0
LLP		854	73.1	1.019	21.84	0
GLPi		906	73.3	1.022	21.91	0.38
OLTa		880	73.5	1.025	21.97	0.53
SLLn[e]		880	73.8	1.029	22.06	0
OOPi		880	73.9	1.031	22.09	0.40
LnOP[e]		854	74.0	1.032	22.13	0
PLTa		854	74.9	1.045	22.41	0.57
SLPi		880	75.1	1.048	22.47	0.41
POPi		854	75.4	1.052	22.56	0.43
C19:0LPi	45	894	76.7	1.071	22.97	–
C20:2LO	46	908	77.0	0.988	23.06	–
GLL		908	77.2	0.991	23.13	0
OLO		**882**	**77.9**	**1.000**	23.34	0
C20:3OO		908	78.6	1.009	23.56	–
SLL		882	79.0	1.015	23.69	0
OLP		856	79.3	1.019	23.78	0
OOTa		882	79.7	1.024	23.91	0.57
SOLn[e]		882	80.0	1.028	24.00	0
BLnLn[e]		934	80.1	1.029	24.03	0
SOPi		882	81.3	1.046	24.41	0.41
BPiPi		934	82.7	1.064	24.84	0.81
C19:0OPi	47	896	82.7	1.064	24.84	–
GLO	48	910	83.1	0.989	24.97	0
OOO		**884**	**84.0**	**1.000**	25.25	0
ALL		910	84.8	1.010	25.50	0
SLO		884	85.1	1.014	25.59	0
BLLn[e]		936	85.1	1.014	25.59	0

Table 2 (*Continued*)

TG	ECN	MW[a]	t_R	r[b]	k[c]	Δk[d]
OOP		858	85.4	1.017	25.69	0
BLPi		936	86.3	1.028	25.97	0.38
AOPi		910	86.8	1.035	26.13	–
POP		832	87.0	1.037	26.19	0
PPP		806	88.7	1.058	26.72	0
C23:0LLn[e]	49	950	87.8	1.047	26.44	0
C23:0LPi		950	88.9	1.061	26.78	0.34
GOO	50	912	89.0	0.979	26.81	0
BLL		938	90.0	0.991	27.13	0
LgLLn[e]		964	90.2	0.993	27.19	0
ALO		912	90.4	0.995	27.25	0
SOO		**886**	**90.8**	**1.000**	27.38	0
LgLPi		964	91.5	1.008	27.59	0.40
SOP		860	92.3	1.017	27.84	0
LgLL	52	966	94.9	0.988	28.66	0
BLO		940	95.5	0.995	28.84	0
AOO		**914**	**96.0**	**1.000**	29.00	0
LgLO	54	968	100.5	1.048	30.41	0
BOO		942	101.0	1.054	30.56	0

[a] For simplicity, nominal masses are listed in this table and throughout the text.

[b] Relative retention $r = (t_R - t_M)/(t_S - t_M)$, where t_M is 3.20 min and t_S are retention times of standards for particular ECN groups (printed in bold), i.e. LLLn for ECN = 41 and lower, LLL for ECN = 42 and 43, OLL for ECN = 44 and 45, OLO for ECN = 46 and 47, OOO for ECN = 48 and 49, SOO for ECN = 50 and 51, AOO for 52 and higher.

[c] Retention factor $k = (t_R - t_M)/t_M$, where t_M is 3.20 min.

[d] Differences between retention factors (Δk) for TGs containing FAs with common DB positions (e.g. Δ-9,12,15) and unusual DB position in Δ5. Value "0" means that this TG has only FAs with common DB positions.

[e] TGs from different plant oils [2,44] not identified in conifer seed oils, but listed here because of the calculation of Δk.

bonds according to the equation ECN = CN − 2DB, but this equation does not reflect the fact that optimized separation systems also enable quite good resolution within particular ECN groups. The retention order of TGs consisting of common FAs within one ECN group is also known [1,2,27,37], in contrast to unusual TGs containing Δ5-UPIFAs measured in this work. Fig. 1 illustrates the chromatograms of analyzed conifer seed oils with APCI-MS detection. The average differences in retention factors Δk for the presence of Δ5-UPIFAs are the following: $\Delta k = 0.40$ for one, $\Delta k = 0.86$ for two and $\Delta k = 1.29$ for three pinolenic acids and $\Delta k = 0.56$ for one taxoleic acid (all data are shown in Table 2). For the presence of both pinolenic and taxoleic acids in one TG species, the observed shift $\Delta k = 0.97$ for TaOPi and PTaPi fits well with the sum of contributions for Pi and Ta, $\Delta k = 0.40 + 0.56 = 0.96$. Retention differences are used for the identification of these TGs together with the determination of MWs based on $[M+H]^+$ ions and the characteristic ratios of diagnostic ions, as discussed later in more detail. It is likely that relative retention is similar in other NARP chromatographic systems and hence it may be used for the identification of Δ5-UPIFAs by analogy. To support the hypothesis that the composition of mobile phases in different NARP systems using C18 column packing has a negligible effect on the retention order, we have compared the retention order of TGs in several tens of publications published by different research groups (all references in this and our previous publications) and we have not found even a single case where the retention order is changed regardless of the NARP solvent system. Of course, the resolution and the number of coelutions depend strongly on the separation system, but the order of TG peaks is never changed. This may be explained by the fact that TG molecules do not contain any polar or ionizable functional groups and therefore changed chromatographic conditions have comparable effects on all TGs, because the only functionalities are triester groups and double bonds for unsaturated acyl chains. This information is very useful for transferring relative retention characteristics among different NARP systems applicable for the identification of unknown TGs. It has initiated our effort to collect relative retention data for an extensive range of TGs consisting of common, unusual and also very rare FAs [44].

Table 3 shows average relative peak areas from three consecutive runs for 64 identified TGs in pine seed oils. It should be emphasized that these values are calculated without the use of response factors (unlike our previous work [2]), because the standards of Δ5-UPIFAs are not available, therefore these values are only semi-quantitative. Unfortunately, the extrapolation of response factors for TGs with common positions of DBs (e.g. LLL and LnLnLn) is impossible due to the unknown response differences between common FAs and Δ5-UPIFAs. Peak areas are integrated in the reconstructed ion current chromatograms in the range 300–1200 m/z, but for strongly overlapping peaks (e.g. PLTa, SLPi and POPi in Fig. 1A), the RIC chromatograms of individual $[M+H]^+$, $[M+H-R_iCOOH]^+$ and $[R_iCO]^+$ ions are used to distinguish contributions of particular TGs to such unresolved chromatographic peaks. Peak areas calculated this way are reproducible among different runs and the same way of data processing enables the direct comparison of results with our other works [2,44].

GC/FID data (Table 4) show the presence of other Δ5-UPIFAs (keteleeronic and sciadonic), but these acids are not identified in any TG, because the trace relative concentrations of these acids are distributed among different TGs and therefore the HPLC/MS technique cannot detect such trace FAs in TG combinations. Moreover, potential coelutions with dominant TGs may complicate their identification. The comparison of our data with previous work [8] shows a very good correlation.

3.3. APCI mass spectra of TGs containing Δ5-UPIFAs

The APCI ionization and fragmentation behavior of TGs with common positions of DBs is well known from numerous publications (e.g. [1,2] and citations therein). Briefly, the protonated molecule $[M+H]^+$ is the base peak for TGs with many DBs, while the fragment ion $[M+H-R_iCOOH]^+$ is the base peak for more saturated TGs. Relative abundances of $[M+H-R_iCOOH]^+$ ions can be used for the determination of the prevailing FA in the *sn*-2 position, because the neutral loss from this position is energetically disfavored, hence the corresponding fragment ion $[M+H-R_2COOH]^+$ has a decreased relative abundance in comparison to the statistically expected value (Table 5). For TGs containing only common FAs, this rule can be roughly applied even without regioisomeric standards which are rather expensive or often unavailable. Some

Fig. 1. Chromatographic separation of conifer seed oils: (A) European Larch (*Larix decidua*), (B) Norway Spruce (*Picea abies*), (C) European Silver Fir (*Abies alba*). Experimental conditions: 30 + 15 cm Nova-Pak C_{18} columns connected in series, APCI-MS detection, flow rate 1 mL/min, column temperature 25 °C, injection volume 10 μl, the mobile phase gradient 0 min—100% acetonitrile, 106 min—31% acetonitrile—69% 2-propanol, 109 min—100% acetonitrile. Numbers correspond to ECNs.

differences may be found for different instruments [2,5,33,42] and different ionization techniques [2]. Unfortunately, this rule can fail for TGs containing unusual TGs, for example Δ5-UPIFAs. Figs. 2 and 3 highlight the differences between standards of common TGs (LLL in Figs. 2A and 3A, LnLnLn in Figs. 2B and 3B) and their isomers with the shifted position of DB from Δ6 to Δ5 identified in European Larch (LLTa in Figs. 2C and 3C, PiPiPi in Figs. 2D and 3D) both in

Table 3
Average relative peak areas from three consecutive runs in percent for 64 triacylglycerols (TG) identified in studied conifer seed oils using HPLC/APCI-MS

TG	European Larch (*Larix deciduas*)	Norway Spruce (*Picea abies*)	European Silver Fir (*Abies alba*)
PiLnPi	0.2	–	–
PiPiPi	0.5	–	–
LnLPi	1.3	–	–
PiLPi	6.6	4.2	0.7
LLLn	0.6	–	1.2
LLPi	22.8	29.5	19.3
C20:3LPi	0.8	–	0.7
C20:3LnTa	1.3	–	–
TaLPi	1.6	0.6	0.6
PiOPi	3.4	0.9	0.3
PPiPi	0.4	–	–
LLL	5.1	7.3	0.9
OLLn	1.0	–	4.2
+C20:3LL[a]		2.5	
C20:2LPi	0.7	1.4	0.4
LLTa	2.8	4.1	6.6
LOPi	16.9	16.6	14.0
PLPi	4.3	4.0	2.5
TaOPi	1.3	0.7	0.5
PTaPi	0.7	0.8	–
+LnLMa	0.7		
+C20:2LL[a]			
SPiPi	0.3	–	–
OLL	4.7	5.9	11.0
C20:3LO	0.6	0.8	2.8
LLP	0.6	1.7	1.5
GLPi	1.1	1.6	1.6
OLTa	1.4	1.3	3.3
OOPi	7.4	3.9	6.5
PLTa	0.6	0.4	0.5
SLPi	2.0	3.0	1.7
POPi	1.6	1.2	1.0
C19:0LPi	0.1	–	0.1
C20:2LO	0.1	<0.1	0.3
GLL	0.5	0.4	0.4
OLO	1.9	1.8	4.9
C20:3OO	0.2	0.1	0.7
SLL	0.4	0.8	1.0
OLP	0.7	0.9	1.0
OOTa	0.9	0.6	2.5
SOPi	0.9	0.8	0.6
BPiPi	0.1	–	–
+C19:0OPi[a]		–	–
GLO	0.1	0.1	0.3
OOO	0.6	0.5	2.4
ALL	0.1	0.2	0.3
SLO	0.2	0.3	0.5
OOP	0.2	0.2	0.8
BLPi	0.1	0.2	0.3
AOPi	0.2	0.2	0.4
+POP[a]			
PPP	–	–	<0.1
C23:0LPi	<0.1	<0.1	–
+GOO[a]		0.1	
BLL	<0.1	0.1	0.1
ALO	<0.1	0.1	0.2
SOO	0.1	0.1	0.3
LgLPi	<0.1	<0.1	–
SOP	<0.1	<0.1	<0.1
LgLL	<0.1	<0.1	<0.1
BLO	<0.1	<0.1	0.1
AOO	<0.1	<0.1	0.1
LgLO	<0.1	<0.1	<0.1
BOO	<0.1	<0.1	<0.1

Value "<0.1" means that the peak area is lower than 0.1%, but TG is still positively identified.
[a] Sum of coeluting peaks.

first-order (Fig. 2) and tandem (Fig. 3) mass spectra. The first-order mass spectra of unusual Δ5-UPIFAs show a significantly changed ratio of $[M+H]^+/[M+H-R_iCOOH]^+$ ions. This ratio is changed from 100/42% for LLL (Fig. 2A) to 74/100% for LLTa (Fig. 2C), and from 100/25% for LnLnLn (Fig. 2B) to 87/100% for PiPiPi (Fig. 2D). TGs containing unusual FAs also provide increased relative abundances of fragment ions $[B]^+$, $[B-H_2O]^+$, $[C]^+$ and $[C-H_2O]^+$, other examples are listed in Table 5. The differences observed in tandem mass spectra of $[M+H]^+$ ions are even more pronounced (Fig. 3), because the base peaks for common TGs (Figs. 3A and B) are $[B-H_2O]^+$ ions, but $[B]^+$ ions are base peaks for TGs containing Δ5-UPIFAs (Fig. 3C and D). The same difference of relative abundances is observed for $[C]^+/[C-H_2O]^+$ pair of fragment ions. Summarizing the information from the retention times, MW determination and fragmentation behavior in first-order and tandem mass spectra, Δ5 positional isomers can be unambiguously identified in unknown samples of natural TGs mixtures.

Fig. 4 depicts APCI mass spectra of $R_1R_2R_3$ type (LOPi) and $R_1R_1R_2$ type (LLPi) TGs containing one Δ5-UPIFA from the analysis of European Larch. In case of TGs containing only common FAs, the statistical ratio 1:1:1 for $R_1R_2R_3$ type and 2:1 for $R_1R_1R_2$ type would be shifted towards lower relative abundance of $[R_1R_3]^+$ ion, because the neutral loss from *sn*-2 position is energetically disfavored and therefore the fragment ion corresponding to the neutral loss from sn-2 position has a lower relative abundance than statistically expected. Thanks to the literature data [8,9] showing that Δ5-UPIFAs occupy almost exclusively *sn*-3 position, one may expect either OLPi or LOPi species. The relative ratio of $[LPi]^+/[OPi]^+/[OL]^+ = 9/11/100\%$ (Fig. 4A) indicates that both species are present at comparable relative abundances with small preference for LOPi. The precise ratio of LOPi/OLPi may be obtained only if both stereochemical standard are available. If the mixed spectrum of LOPi/OLPi is compared with the TG analogue with common DB positions (i.e. LOLn/OLLn) measured on the same instrument under identical conditions (Table 5), then observed relative abundances for $[OL]^+/[OLn]^+/[LLn]^+$ are in the ranges 60–100/84–100/43–100% in contrast to values for LOPi/OLPi mixtures—$[OL]^+/[OPi]^+/[LPi]^+ = 100/14–24/13–17\%$. The relative abundance of the fragment ion corresponding to the neutral loss of Δ5-UPIFA is significantly higher (approximately four times, see data in Table 5) compared to TGs containing only common FAs. Similar behavior is observed for all TGs containing pinolenic or taxoleic acid (Table 5).

3.4. Comparison of HPLC/APCI-MS and GC/FID results of conifer seed oils

To verify the validity of HPLC/MS results on TGs, conifer seed oils have been transesterified with sodium methoxide to obtain methyl esters of individual FAs, which are subsequently analyzed by a validated GC/FID method. Standards of the whole range of FAMEs are used to obtain response factors according to the previously described method [45]. Table 4 shows quantitative results on FAs relative contents determined in this work

Table 4
Relative concentrations of individual FAMEs in weight percent calculated from GC/FID according to Ref. [45]

Fatty acid	European Larch (*Larix deciduas*)		Norway Spruce (*Picea abies*)		European Silver Fir (*Abies alba*)
	Our results	Wolff et al. [8]	Our results	Wolff et al. [8]	
16:0	2.28	2.80	2.73	2.78	2.69
Δ9-16:1	0.06	0.43	0.10	0.20	<0.06
17:0	<0.06	–	<0.06	–	<0.06
18:0	1.26	1.46	1.76	1.49	1.71
Δ9-18:1	17.59	18.76	13.68	13.41	23.68
Δ11-18:1	0.81	0.97	1.08	1.55	0.13
Δ9,12-18:2	43.30	43.10	46.61	49.89	42.23
Δ5,9-18:2	2.73	2.20	3.42	3.25	5.09
Δ9,12,15-18:3	0.52	0.56	0.22	0.21	0.83
Δ5,9,12-18:3	28.45	27.39	22.81	24.67	12.61
19:0	0.08	–	<0.06	–	0.11
20:0	0.18	0.23	0.30	0.24	0.50
Δ9-20:1	0.39	0.40	0.37	0.31	0.50
Δ11,14-20:2	0.36	0.35	0.61	0.58	0.32
Δ5,1 1-20:2	0.16	0.14	0.08	0.05	0.36
Δ8,11,14-20:3	0.28	–	0.15	–	0.16
Δ5,11,14-20:3	0.58	0.51	0.82	0.94	1.91
22:0	0.07	0.10	0.12	0.19	0.71
23:0	<0.06	–	<0.06	–	0.06
24:0	<0.06	–	<0.06	–	<0.06
Others	0.83	0.60	5.03	0.24	6.40
Σ of Δ5-UPIFAs	31.92	30.24	27.13	28.91	19.97

Value "<0.06" means that this TG is positively identified in this sample, but the peak area is lower than 0.06%.

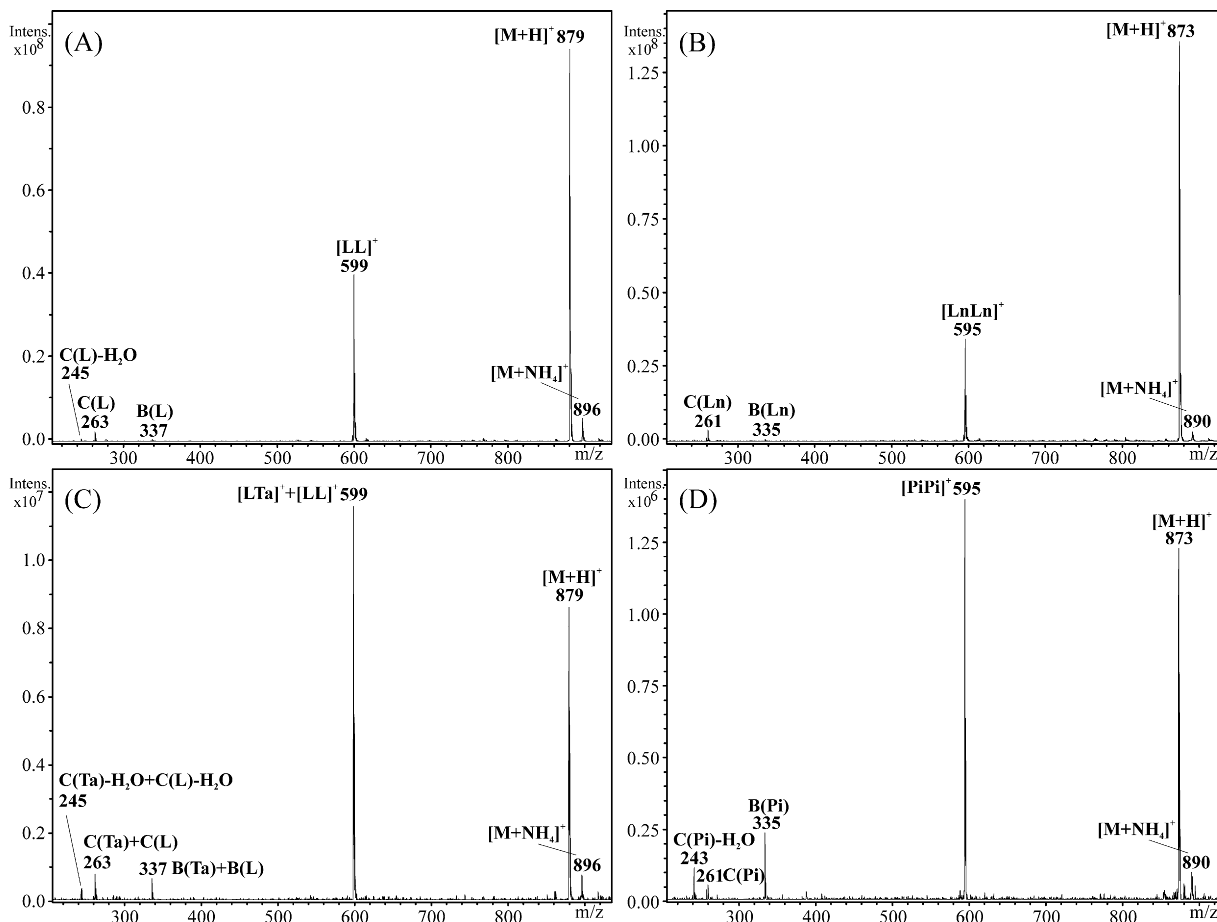

Fig. 2. Comparison of APCI first-order mass spectra of TG isomers with different double bond positions: (A) LLL standard, (B) LnLnLn standard, (C) LLTa from European Larch, and (D) PiPiPi from European Larch. Notation of ions is explained in the text and ref. [1].

Table 5
Comparison of relative ratios of fragment ions A ($[M + H - R_iCOOH]^+$) and $[M + H]^+$ ions for TGs containing only common polyunsaturated FAs (linolenic acid C18:3Δ-9,12,15 and linoleic acid C18:2Δ-9,12) vs. TGs containing unusual Δ5-UPI FAs (pinolenic acid C18:3Δ-5,9,12 and taxoleic acid C18:2Δ-5,9)

TGs	Relative ratios of fragment ions A (%)						Relative ratios of $[M+H]^+$ and most abundant fragment ion A (%)	
$R_1R_1R_2$[a]	R_1R_1	50	R_1R_2	100			$[M+H]^+$	Ion A
OOP[b]	OO	68	OP	100			9	100
OPO[b]	OO	21	OP	100			9	100
OPP[b]	PP	80	OP	100			6	100
POP[b]	PP	24	OP	100			2	100
LnLLn[c]	LnLn	42–50	LLn	100			100	13–32
PiLPi[d]	PiPi	9–13	LPi	100			90–93	100
LLLn[c]	LL	57–74	LLn	100			100	14–37
LLPi[d]	LL	100	LPi	26–33			100	68–83
OLnLn[c]	LnLn	40–56	OLn	100			100	17–47
PiOPi[d]	PiPi	9–11	OPi	100			63–83	100
OOLn[c]	OO	40–71	OLn	100			100	39–64
OOPi[d]	OO	100	OPi	21–32			36–45	100
OLO[c]	OO	47–75	OL	100			33–45	100
OOTa[d]	OO	100	OTa	35–40			10–12	100
$R_1R_2R_3$[a]	R_1R_2	100	R_1R_3	100	R_2R_3	100	$[M+H]^+$	Ion A
OLLn[c]	OL	60–100	OLn	84–100	LLn	43–100	100	12–30
OLPi[d]	OL	100	OPi	14–24	LPi	13–17	74–86	100
LnLP[c]	LP	71–95	LnP	52–76	LLn	100	100	14–30
PLPi[d]	LP	100	PiP	11–14	LPi	21–24	82–84	100
OLnP[c]	OP	62–100	LnP	46–72	OLn	95–100	100	28–69
POPi[d]	OP	100	PiP	8–11	OPi	22–23	20–29	100
SOLn[c]	SO	95–100	SLn	62–93	OLn	78–100	100	33–61
SOPi[d]	SO	100	SPi	14–27	OPi	14–19	19–23	100

[a] Theoretical (i.e. statistical) ratio of fragment ions.
[b] Regioisomeric TG standards.
[c] TGs containing only common polyunsaturated FAs (linolenic acid C18:3Δ-9,12,15 and linoleic acid C18:2Δ-9,12), data obtained from measurements of other 13 plant oils [2,44].
[d] TGs containing unusual Δ5-UPIFAs (pinolenic acid C18:3Δ-5,9,12 and taxoleic acid C18:2Δ-5,9).

for European Larch, Norway Spruce and European Silver Fir. The comparison with earlier published data [8] confirms a good mutual agreement. The results of GC/FID are very important for the identification of TGs with different position of DBs, because the relative concentrations of pinolenic and taxoleic acids can be correlated with their occurrence in TGs. Such TGs have identical MWs and observed ions as for analogues with common FAs (i.e. L and Ln), but the retention times both for GC and HPLC are shifted and the ratio of characteristic ions in APCI mass spectra is changed.

Our last work on the quantitation of TGs by HPLC/MS and transesterified FAMEs by GC/FID has shown [2] that both methods provide comparable results in the calculation of average parameters. In this work, only GC/FID average parameters (Table 6) are calculated because of the absence of Δ5 TGs standards, so the response factors can not be determined for TGs containing one, two or three Δ5-UPIFAs.

For the identification of methyl esters of Δ5-UPIFAs, the standards are available only for pinolenic and taxoleic acids, but keteleeronic and sciadonic acids are identified on the basis of

Table 6
Average carbon numbers (aCN), average equivalent carbon numbers (aECN) and average double bond (aDB) numbers, and the number of identified TGs in three conifer seed oils calculated from GC/FID analyses

Conifer seed oil source	Latin name	No. of TGs	Average parameter		
			aCN	aECN	aDB
European Larch	*Larix decidua*	63	17.85	13.82	2.01
Norway Spruce	*Picea abies*	51	17.09	13.32	1.89
European Silver Fir	*Abies alba*	53	16.90	13.56	1.67

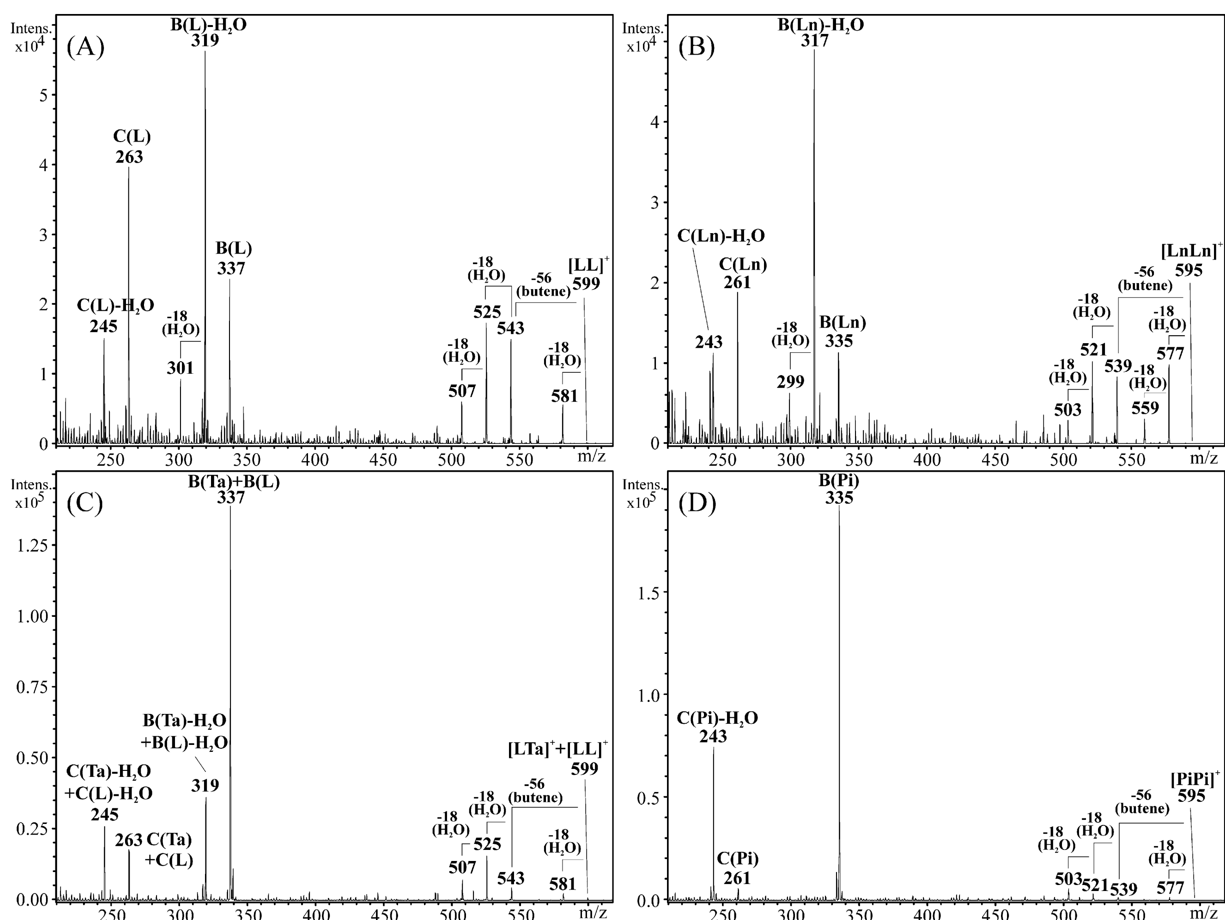

Fig. 3. Comparison of APCI tandem mass spectra of TG isomers with different double bond positions: (A) MS/MS of ion $[LL]^+$ at *m/z* 599 for LLL standard, (B) MS/MS of ion $[LnLn]^+$ at *m/z* 595 for LnLnLn standard, (C) MS/MS of ions $[LTa]^+$ and $[LL]^+$ at *m/z* 599 for LLTa from European Larch, and (D) MS/MS of ion $[PiPi]^+$ at *m/z* 595 for PiPiPi from European Larch. Notation of ions is explained in the text and ref. [1].

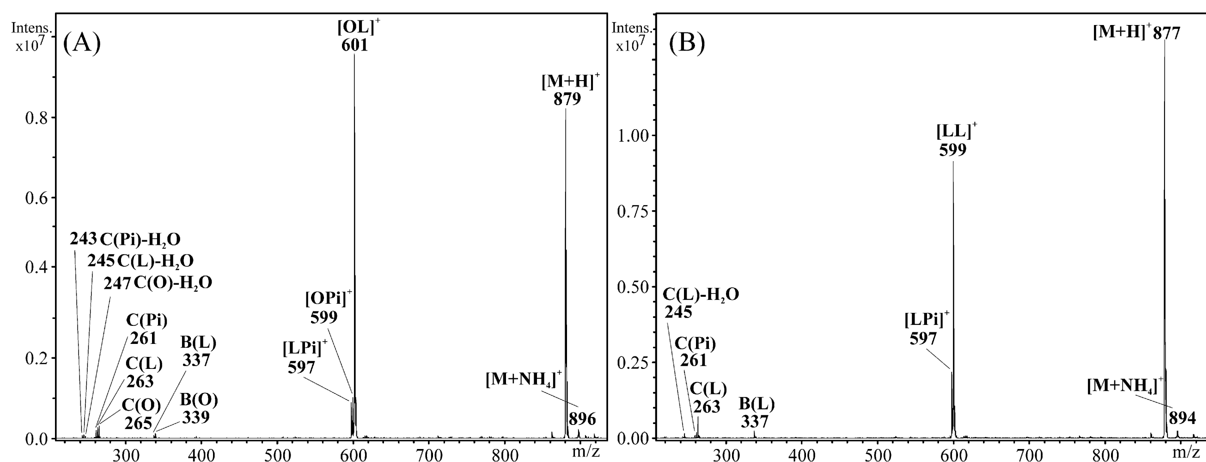

Fig. 4. Comparison of APCI first-order mass spectra of: (A) a mixture of LOPi/OLPi from European Larch, (B) LLPi from European Larch. Notation of ions is explained in the text and ref. [1].

characteristic shifts in retention times and determined MWs in accordance with previous literature data [11–14]. The relative concentrations of Δ5-olefinic FAs in pine seed oils are rather high (32% for European Larch, 27% for Norway Spruce and 20% for European Silver Fir). The dominant Δ5-UPIFA is pinolenic acid which represents approximately 90% of total Δ5 content in studied conifer seed oils.

4. Conclusions

Our HPLC/APCI-MS method has been successfully applied for the identification of 64 TGs consisting of 20 FAs in three conifer seed oils (European Larch, Norway Spruce and European Silver Fir) from the Gymnosperms taxonomical group known for a high content of D-5 olefinic acids (i.e. taxoleic, pinolenic,

keteleeronic and sciadonic). These unusual Δ5-UPIFAs are found in 32 identified TGs. A validated GC/FID method of transesterified oils has been used to confirm HPLC/MS results on TGs and for the calculation of average parameters useful for the characterization of a given plant oil. This is the first time when the study of intact TGs containing Δ5-UPIFAs in conifer seed oils is reported together with their retention characteristics in addition to previously known FA concentrations measured after the transesterification. APCI mass spectra of TG containing Δ5-UPIFAs provide useful information for distinguishing them from common polyenoic FAs in addition to the shifts in retention times. Differences in relative retention, characteristic features in first-order and tandem mass spectra and molecular weight determination can be applied for the positive identification of unknown TGs containing Δ5-UPIFAs in complex natural samples.

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