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Strategy for stereospecific characterization of natural triacylglycerols using multidimensional chromatography and mass spectrometry

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ABSTRACT

Stereoisomeric determination of individual triacylglycerols (TAGs) in natural oils and fats is a challenge due to similar physicochemical properties of TAGs with different fatty acid combinations. In this study, we present a strategy to resolve the enantiomeric composition of nutritionally important TAGs in sea buckthorn (*Hippophaë rhamnoides*) as an example food matrix. The targeted strategy combines 1) fatty acid profiling with GC, 2) separation of TAGs with RP-HPLC, 3) stereospecific separation with chiral-phase HPLC and 4) structural characterization with MS. Three major asymmetric diacid- and triacid-TAG species were analyzed in sea buckthorn pulp oil. Off-line coupling of RP-HPLC and chiral-phase HPLC allowed separation of several TAG regioisomers and enantiomers, which could not be resolved using one-dimensional techniques. Enantiomeric ratios were determined and specific structural analysis of separated TAGs was performed using direct inlet ammonia negative ion chemical ionization method.

Of the TAG 16:0/16:1/16:1 palmitic acid (C16:0) was located predominantly in a primary position and the enantiomeric ratio of TAG *sn*-16:1-16:1-16:0 to *sn*-16:0-16:1-16:1 was 70.5/29.5. Among the TAGs 16:0/16:0/18:2 and 16:0/16:0/16:1, only ca 5% had C16:0 in the *sn*-2 position, thus, ca 95% were symmetric *sn*-16:0-18:2-16:0 and *sn*-16:0-16:1-16:0. The enantiomeric ratio of triacid-TAGs containing C16:0 and two unsaturated fatty acids (palmitoleic C16:1, oleic C18:1 or linoleic acids C18:2) could not be resolved due to lack of commercial enantiopure reference compounds. However, it became clear that the targeted strategy presented offer unique and convenient method to study the enantiomeric structure of individual TAGs.

1. Introduction

Scientists over decades have investigated research strategies on the fatty acids in the three *sn*-positions of triacylglycerols (TAGs) in natural fats and oils [1]. These methods of analysis are of importance, as both the fatty acids and their specific distribution in TAGs influence the nutritional value as well as the biochemical and technological properties of fats and oils [2–7]. Unique, species-specific structures are formed in TAG biosynthesis, in which three acyltransferases specifically esterify each of the hydroxyl positions of the glycerol backbone resulting in non-random fatty acid combinations [8].

Studies introducing methods for stereospecific analysis of TAGs rely largely on enzymatic [9] and chemical [10–12] hydrolysis followed by different chromatographic and chiral separations of mono- or diacyl-*sn*-glycerols [13–15]. As a result, these methodologies unveil the total fatty acid composition in each stereospecific positions, *sn*-1, *sn*-2 and *sn*-3 [1], and the composition of individual TAGs is neglected. If the TAG species are pre-fractioned before stereospecific analysis, the method will also give information about stereochemistry of the selected TAG species [16,17]. However, the amount of sample required is quite high due to several analytical pretreatment steps followed by gas chromatographic analysis, and the acyl migration after enzymatic hydrolysis poses a serious risk for compromised results [13,18]. Yet, analysis of the enantiomeric proportions of the individual TAGs is still an extremely challenging task due to the enormous number of different TAG species with similar physicochemical properties (including fairly similar or identical chromatographic and spectroscopic behavior), characteristic in natural fats and oils.

The direct separation of enantiomers using chiral stationary phases has been primarily applied to separate asymmetric TAGs

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in previous studies [19]. It is commonly known that enantiomeric separation of the two isomers is generated when there is a three-point interaction between the compounds and chiral stationary phase [20]. However, the chiral interactions and the retention mechanisms are not fully understood. Furthermore, the chiral behavior of TAGs and their elution order are still to be investigated. Gotoh and co-authors screened nine chiral stationary phases for the enantiomeric separation of *rac*-16:0-16:0-18:1 [21]. Only CHIRALCEL OD-RH silica column coated with cellulose tris-(3,5-dimethylphenylcarbamate) resulted in enantiomeric separation when methanol was used as the mobile phase. Baseline separation was not achieved without sample recycling. The same chiral selector has been applied to study different TAG structures by other researchers [19,22–27] using methanol or hexane-isopropanol as eluents. However, a limited number of studies have been carried out by using natural TAGs as sample material [23,24,26,27].

Recently, researchers have shown increasing interest in simultaneous separation of TAGs enantiomers and regioisomers [24,28,29]. Due to the co-elution, several columns in series or sophisticated stationary phases have been used. Nevertheless, it is impossible to determine the enantiomeric ratio of all enantiomers even when extracted ion chromatograms of each mass-to-charge ratio are used [28]. Despite progress in stereospecific analysis of TAGs, no universal column-solvent combination for complete chromatographic separation of TAGs in mixtures has been introduced. Thus, to study the enantiomeric ratio of selected TAGs, fractions as pure as possible are required. Multidimensional techniques are gaining importance also in chiral separations [30]. RP-HPLC as the first dimension and silver ion chromatography as the second dimension has enabled excellent resolution of the TAG regioisomers [31]. Before chiral-phase HPLC, an achiral RP-HPLC separation is commonly used, and also sample recycling HPLC has been applied to enhance the separation efficiency [23,25,26]. For complete separation, on-line two dimensional HPLC, i.e. RP-HPLC or silver ion HPLC together with chiral-phase HPLC is suggested [32] but no practical application of such approach has been published so far.

Recently, we demonstrated a direct chiral-phase recycling HPLC method that enabled the enantiomeric separation of 17 intact racemic TAGs with fatty acids of C12–C22 and 0–6 double bonds without any derivatization steps [22,25]. The purpose of the present study was to prove that the chiral-phase recycling HPLC method together with RP-HPLC and MS detection can be used as a targeted strategy to resolve the stereospecific composition of asymmetric TAGs extracted from natural samples. Pulp oil of sea buckthorn (*Hippophaë rhamnoides* L.) was selected as an example of oil of plant origin. All parts of the sea buckthorn plant are rich in bioactive fractions, of which the seed and pulp oils are among the most valuable products [33,34]. The pulp oil contains more saturated fatty acids than the seed oil, but has still rather high concentration of palmitoleic acid (C16:1) [35]. Evidently, pulp oil contains asymmetric TAGs with saturated fatty acids, mostly palmitic acid (C16:0), and the unsaturated C16:1. C16:1, not common in plant oils, is considered to be a bioactive molecule with potential significance for dietary management of obesity, fatty liver, insulin resistance, and diabetes [36]. Our special aim was to determinate the enantiomeric ratio of *sn*-16:0-16:1-16:1 to *sn*-16:1-16:1-16:0 and *sn*-16:0-16:0-16:1 to *sn*-16:1-16:0-16:0 using a multidimensional chromatographic approach with MS. These methodologies are crucial in order to unearth the molecular level information of TAG structures supporting the research in chemistry and biochemistry of lipids. In addition, they are also important to facilitate the research in human nutrition and metabolism as well as the development of tailored food products and nutraceuticals.

2. Experimental methods

2.1. Sample materials

Three Russian cultivars ('Prozcharachnaya', 'Botanicheskaya' and 'Trofimovskaya') of sea buckthorn (*Hippophaë rhamnoides* L. ssp. *mongolica*) grown in Finland (60° 23' N 22° 09' E, altitude 2 m, Satava, Turku) were selected as samples. The berries were picked optimally ripe, frozen and stored at –20°C immediately after harvesting until analysis. Two fatty acid methyl ester mixtures, GLC reference standard 68D (Nu-Chek-Prep, Elysian, MN) and Supelco 37 Component Fatty Acid Methyl Ester Mix (Sigma-Aldrich, St. Louis, MO) were used as external standards in the fatty acid analyses. The TAG reference compounds containing TAGs with ECN (equivalent carbon numbers = total number of acyl carbons – 2 × number of double bonds [37]) 40–48 are listed in the supplementary material (SM) **Table S1**. A/B/C denotes a TAG containing three different fatty acids in unknown stereoisomeric (*sn*) positions. *Sn*-A-B-C indicates a TAG in which the stereospecific positions *sn*-1, *sn*-2 and *sn*-3 of fatty acids are defined to be A, B, and C, respectively. *Rac*-A-B-C indicates a racemic mixture of enantiomers *sn*-A-B-C + *sn*-C-B-A in equimolar ratios. A-B-C, again, denotes a stereoisomeric pair of TAGs in which the fatty acids A and C in the outer positions can occupy randomly the *sn*-1 and *sn*-3 positions, not necessarily in equimolar ratios as in *rac*-A-B-C. All solvents were either pro analysis, HPLC grade or MS grade and used without further purification.

2.2. Extraction of pulp oil and isolation of triacylglycerols

Whole seeds of sea buckthorn berries were separated from pulp (fruit flesh and skin) with tweezers after breaking the berries gently under liquid nitrogen. Extraction of oil from lyophilized berry pulp was performed according to the Folch procedure [38] with modifications described in our previous study [39]. TAGs were solid-phase extracted from the total lipids using Sep-Pak Vac 6 cc silica (500 mg) cartridges (Waters, Dublin, Ireland) [39].

2.3. Fatty acid analysis

The TAG fractions of the pulp oil samples were transesterified into fatty acid methyl esters by a sodium methoxide method [40] and the fatty acid methyl esters were analyzed using a gas chromatograph coupled with a flame ionization detector. Instrumentation and chromatographic settings were as previously described [39] with slightly different column oven programming; hold 130°C for 1 min, increase to 170°C at 4.5°C/min, with no hold, then increase to 220°C at 10°C/min, then hold for 3.5 min, increase to 230°C at 10°C/min, then hold for 11 min, and finally increase to 240°C at 60°C/min, and hold for 3 min. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of reference mixtures mentioned in subsection "Sample materials" and the quantitative correction factors were calculated. All chromatographic data was analyzed with LabSolutions v. 5.93 (Shimadzu, Kyoto, Japan). Quantification was carried out by using the correction factors by calculating the area percentages (%) of each fatty acid methyl ester.

2.4. Multidimensional liquid chromatography

To increase the number of the resolved TAG species, a combination of the two different separation modes and recycling HPLC were applied. An achiral-chiral two-dimensional HPLC in off-line

mode utilizing one achiral C_{18} column and two polysaccharide-based chiral stationary phases were used to obtain as pure TAG fractions as possible.

In the first dimension, TAGs were separated by an achiral Ascentis C_{18} column (250 × 4.6 mm, 5 µm, Supelco, Bellefonte, PA) using Shimadzu Prominence preparative HPLC-UV instrumentation consisting of a SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column oven, and a DGU-20A5 degasser (Kyoto, Japan). Injection volume was 20 µL, and TAGs were separated at 25°C using a flow rate 1 mL/min. In a linear solvent gradient the amount of acetone in acetonitrile was increased from 40 to 90% in 50 min. TAGs were recorded at 205 nm with SPD-20A UV-detector, and the fractions were collected automatically using a fraction collector FRC-10A. In order to have sufficient amount of material, the fractionation was repeated up to five times and the corresponding fractions were pooled, evaporated to dryness and re-dissolved in an appropriate volume of hexane.

Three main fractions (**Fig. 2**) collected were selected according to mass spectral characterization for analysis on the second dimension, which was chiral-phase recycling HPLC. The HPLC instrumentation used for chiral separation was the same as that used in the RP-mode, and the instrument parameters were the same as previously published [25]. Briefly, separations were carried out with two CHIRALCEL OD-RH [cellulose tris (3,5-dimethylphenylcarbamate), 150 × 4.6 mm, 5 μm, Chiral Technologies Europe, Illkirch, France] columns. The CS3080 Sample Peak Recycler™ (Chiralizer Services, Newtown, PA) including a controlling device and a high-pressure 10-port valve was connected to the system to improve the enantiomeric separation by rerunning the partially separated compounds after the first column *via* the UV detector to the second column. After recycling system, another absorbance detector (Waters 486 Tunable Absorbance Detector, Millipore Corp., Milford, MA) was connected in line to ensure that only the peaks of interest were collected (SM **Figure S1**). Due to the unforeseeable chromatographic resolution of the fractions analyzed, a manual switching between the columns was used, and fractionation result was simulated on chromatogram afterwards. Once a desired resolution was attained and the later peak in the peak pair was passed through the UV detector, the position of valve was not changed allowing the separated peaks to elute through the later detector after which they were collected. Before fractionation of the actual samples, performance of the fraction collecting procedure was ensured with reference compounds, and the chromatogram is presented in SM **Figure S2**.

2.5. Mass spectrometric analyses

Before the stereospecific analysis, structural features of TAGs extracted from the sea buckthorn pulp oil, were preliminary characterized to select asymmetric TAGs. An optimized UHPLC-MS method with atmospheric pressure chemical ionization (UHPLC-APCI-MS) [41] was applied to analyze the ECNs and the regioisomeric composition of TAGs. The instrumentation consisted of a Waters Acquity UHPLC coupled to a Waters Quattro Premier triple quadrupole MS equipped with an APCI source (Waters Corp., Milford, MA). RP-UHPLC separation was carried out using BEH C_{18} column (100 × 2.1 mm, 1.7 µm, Waters Corp., Milford, MA). The flow rate was 0.4 mL/min and in a binary solvent gradient the amount of acetone in acetonitrile was increased from 0 to 70% in 22 min. For the MS detection, the effluent was entirely directed to the APCI ion source. The MS working conditions were as follows: corona current 15 µA, cone voltage 30 V, extractor 5, the cone and desolvation gas flows were 47 and 250 L/h, respectively, and the probe and source temperatures were 450°C and 120°C, respectively. Full scan mass spectra (*m/z* 400–1000) were collected in positive ion mode and the scan time was set at 400 ms. This method produces diagnostically useful molecular and fragment ions, $[M–RCO_2]^+$, for structural characterization. For each chromatographic peak, TAGs were identified using reference compounds (SM **Table S1**), $[M+H]^+$ and $[M–RCO_2]^+$ ions, and the area of the eleven most abundant TAG species in the chromatogram were calculated as a percentage of the total area of all TAG peaks.

TAG molecular weight distribution of sea buckthorn pulp oil was determined with the direct inlet ammonia negative ion chemical ionization MS (NICI-MS). The NICI-MS scan data was also used to create a product ion scan method for each selected pseudomolecular $[M–H]^-$ ion. A Thermo Scientific TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was used as described previously [42]. Briefly, 1 µL of the TAG fraction obtained from the solid phase extraction was pipetted onto the rhenium wire tip of the direct exposure probe. The probe tip containing the sample was placed directly inside the ion source of the mass spectrometer via the vacuum interlock. MS scans between *m/z* 400–1000 were acquired in quadruplicate. The relative molar proportions of different molecular weight species were calculated using abundances of the $[M–H]^-$ ions. The amount of naturally occurring ^{13}C was taken into account when the proportions of TAGs were calculated.

The TAG molecular weight distribution of sea buckthorn pulp oil determined with direct inlet ammonia NICI-MS was compared to the random distribution of fatty acids in TAGs. The random distribution of fatty acids in the glycerol molecule was mathematically modelled using a RANDTAGS calculation program [43]. In practice, the amount of the most prevalent fatty acids presented in **Fig. 1A** was entered into RANDTAGS (v 1.0) program, which computed abundance TAGs based on random combination and distribution of fatty acids.

The positional distribution of fatty acids in ten sea buckthorn pulp oil TAG sub-fractions obtained after separation with achiral-chiral two-dimensional HPLC were analyzed with the ammonia NICI-MS/MS as described previously [42]. Fragmentation of $[M–H]^-$ ions was performed using collision-induced dissociation with argon gas, which favors dissociation of fatty acids from *sn*-1/3 positions. Product ions were scanned between *m/z* 100–650 to determine the primary (*sn*-1/3) and secondary (*sn*-2) positions of fatty acids. Calculations of the TAG regioisomer abundances were based on the relative proportions of $[M–H–FA–100]^-$ and $[RCO_2]^-$ ions, and the results were calculated using MSPECTRA 1.4 software [44,45].

3. Results and discussion

3.1. Fatty acid composition

Resolution and identification of the nine most abundant fatty acid methyl esters derived of the sea buckthorn pulp oil TAGs are presented in SM **Figure S3**. Because the fatty acid compositions of the three different cultivars were relatively similar (SM **Figure S4A**), the fatty acid compositions were averaged for subsequent interpretations (**Fig. 1A**). The fatty acid profile of sea buckthorn pulp oil of *H. rhamnoides* ssp. *mongolica* was similar to that determined by Yang & Kallio, 2006 [46]. The most abundant fatty acid was C16:1, which comprised over 40% of the total fatty acid pool, followed by C16:0 (32%). Only fatty acids present at > 0.2% (area percentage) of total fatty acids are reported, including an unidentified fatty acid (on average, 1.5%) with average retention time of 15.8 min (SM **Figure S3**). The nine fatty acids result in a total number of 729 ($x=y^3$) theoretically possible TAGs including all regio- and enantiomers [19], which demonstrates the complexity of TAGs.

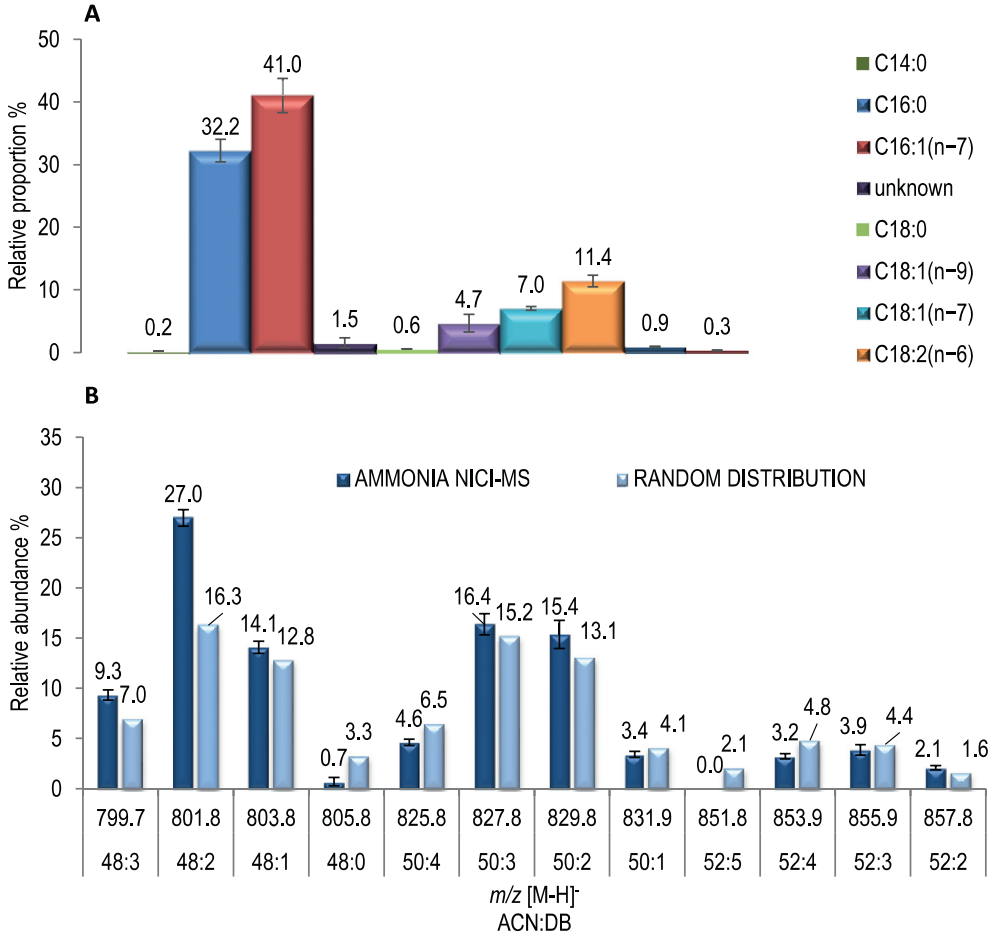

Fig. 1. Characterization of triacylglycerol composition of sea buckthorn pulp oil. (A) Average fatty acid composition of sea buckthorn pulp oil of the three cultivars of H. rhamnoides ssp. mongolica. (B) Molecular weight distribution of sea buckthorn pulp oil from the direct inlet ammonia NICI-MS results and calculated as random distribution of fatty acids (RANDTAGS).

3.2. Triacylglycerol composition of sea buckthorn pulp oil

The separation of TAGs of sea buckthorn pulp oil into eleven ECN groups in the first dimension is illustrated in **Fig. 2**. It shows a representative total ion chromatogram obtained with the UHPLC-APCI-MS method and proportions of the eleven most abundant molecular weight (ECN) species (area-%). ECN 44 and ECN 46 were the most abundant ECN species composing 72.2% of all TAGs. Identifications are listed in **Table 1.** The TAG profiles of the three different cultivars were relatively similar (SM **Figure S4B**).

The chromatographic profile and the mass spectral data revealed the existence of overlapping TAGs (**Table 1**). The UHPLC-APCI-MS method resulted in relatively fast but tentative identification of each TAG species of the sea buckthorn pulp oil. Analysis of the positive ion APCI mass spectra confirmed the TAGs with C16:1. All mass spectral data was in agreement with the results of the gas chromatographic analysis of fatty acid methyl esters. From the four most abundant TAG species, fraction 2 contained only all unsaturated TAGs (16:1-16:1-16:1, 16:1-18:2-16:1 and 18:2-18:2-16:1), fraction 5 contained mainly 16:1-16:1-16:0 and 16:0/16:1/18:2, fraction 8 composed mostly of 16:0/16:1/18:1 and 16:0/18:1/18:2, and the main TAG species in fraction 9 were 16:0/16:0/16:1 and 16:0/16:0/18:2 (**Table 1**). Less abundant TAG species or fractions which comprised of only unsaturated fatty acids (like fraction 2) were not further analyzed. Thus, based on mass spectral data, TAG fractions which contained asymmetric TAGs with saturated and unsaturated fatty acids i.e. fractions 5, 8 and 9 indicated by red lines in **Fig. 2** were subjected to chiral separation.

Ammonia NICI-MS data was compared with random distribution of fatty acids (see SM **Table S2**) according to ECN values in **Fig. 1B**. The main difference of molecular weight distribution in sea buckthorn pulp oil analyzed with direct inlet ammonia NICI-MS, compared to random distribution of fatty acids, was seen in the amount of ACN:DB (number of acyl carbons:number of double bonds) 48:2 (ECN 44). The major ACN species of TAGs were 48, 50 and 52. Similar pattern of molecular weight distribution was presented by Yang & Kallio, 2006 [46]. The major ACN:DB species of TAGs of sea buckthorn pulp oil were 48:2 (ECN 44), 50:3 (ECN 44), 50:2 (ECN 46) and 48:1 (ECN 46), respectively, which constitute 72.9% of all TAG species detected. The same major ACN:DB species resulted from the RANDTAGS calculation constituted 57.4% of the total TAGs. The total number of possible TAGs calculated by RANDTAGS [43] was 84 (SM **Table S2**) with the most abundant TAGs including 16:0/16:1/16:1, 16:0/16:0/16:1, 18:1/16:0/16:1 and 18:2/16:0/16:1 (47.3%).

3.3. Chiral resolution and identification of targeted triacylglycerols

Fractions 5, 8 and 9 were further separated by using chiral columns and the sample recycling system. Progress of enantiomeric separation is illustrated in **Fig. 3**. The chiral-phase recycling HPLC method enabled successfully the further separation of

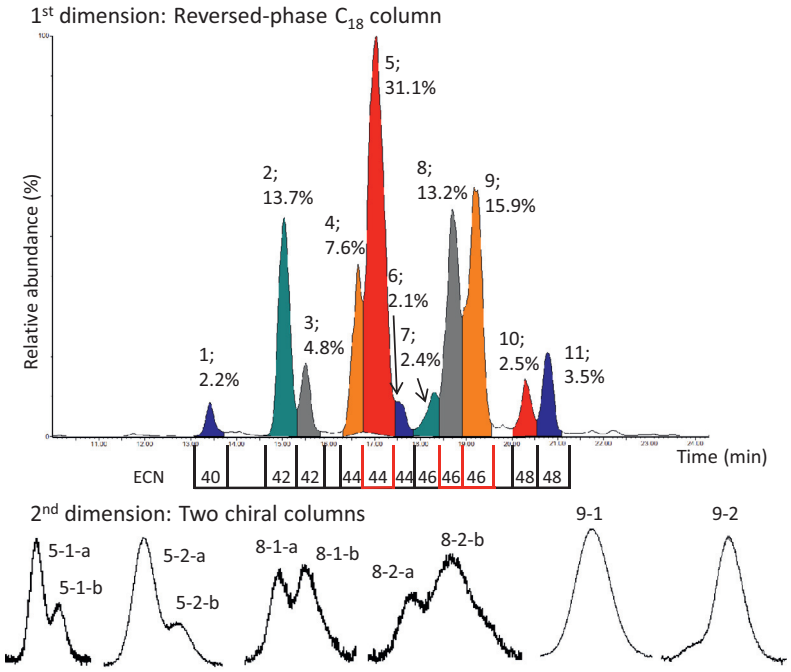

Fig. 2. 1st dimension: Separation of TAGs using reversed-phase column (UHPLC-APCI-MS total ion chromatogram). The fractions indicated by red lines were collected on the first dimension and re-injected to the enantioselective column on the second dimension. 2nd dimension: Stereospecific separation of fractions using chiral columns with recycling HPLC. Sub-fractions are marked with corresponding numbers and letters.

all TAGs examined. Depending on the number of peaks in each fraction and their separation efficiency, they were either separated all in one run or in the separate runs. From the three TAG fractions collected in the first dimension, altogether 10 new sub-fractions were obtained after enantiomeric separations in the second dimension (**Fig. 2**).

From fraction 5 altogether four sub-fractions were obtained and collected in two separate enantioselective runs. With fraction 8 the separation was faster and all four new fractions were collected in one run (**Fig. 3**). After seven column passes there were two clear peaks separated from fraction 9 and the sub-fraction 9-1 was collected. The sample recycling was continued but after 14 column passes there was only small shoulder detected and also the sub-fraction 9-2 was collected. Molecular weight distribution of TAGs in all the collected fractions were confirmed with ammonia NICI-MS, and the regiospecific composition of TAGs of all the sub-fractions were determined with ammonia NICI-MS/MS (**Table 2**, SM **Table S3**).

Some of the fragments listed in SM **Table S3** were not abundant (seen) in all parallel spectra due to the compromised sample amount, which prevented the calculations with MSPECTRA for sub-fraction 5-2-b. Both sub-fraction 5-1-a and 5-1-b composed mostly of 16:1-16:1-16:0 indicating that they contain asymmetric TAGs. According to our previous study, *sn*-16:1-16:1-16:0 elutes before *sn*-16:0-16:1-16:1 [25] and the chiral chromatographic elution behavior and separation profile of the sub-fractions 5-1-a and 5-1-b was similar with the reference compound *rac*-16:1-16:1.16:0 [25]. Thus, the sub-fraction 5-1-a contained mainly *sn*-16:1-16:1-16:0 and 5-1-b *sn*-16:0-16:1-16:1 with enantiomeric ratio 70.5/29.5.

The main $[M–H]^-$ ion in sub-fractions 8-1-a and 8-1-b was 829.8 indicating TAGs with 50 acyl carbons and 2 double bonds. Again, in sub-fractions 8-2-a and 8-2-b the main $[M–H]^-$ ion was 855.8, which indicates TAGs with 52 acyl carbons and 3 double bonds. Also all these fractions contained triacid-TAG species, in which unsaturated fatty acids (C16:1 or C18:1 or C18:2 or C18:1, respectively) were in the *sn*-2 position. As previously known, in plant oils mainly unsaturated fatty acids are esterified in the position *sn*-2, whereas in animal fats *sn*-2 position contains mainly saturated fatty acids [47]. There is only a limited amount of information available related to the elution order and chiral chromatographic behavior of triacid-TAGs [28]. In theory, they contain six isomers, which include three regioisomers, each consisting of a pair of enantiomers, thus complicating the enantioseparation. Due to the lack of knowledge related to enantioseparation of triacid-TAGs and the incomplete separation of the sub-fractions between 8-1-a and 8-1-b as well as 8-2-a and 8-2-b, no stereospecific identification can be made on the TAG molecular species in these fractions based on current chiral chromatographic results.

Separation of fraction 9 was effective. Already after seven column passes there was two peaks separated. Their MS/MS analysis was also quite straightforward probably due to the symmetric TAGs, which contained only two fatty acids, unsaturated fatty acids (C16:1 or C18:2) predominantly in the *sn*-2 position and saturated C16:0 in the positions *sn*-1 and *sn*-3. Only ca 5% had C16:0 in the *sn*-2 position. The sub-fraction 9-1 contained mainly 16:0-18:2-16:0 and the sub-fraction 9-2 contained 16:0-16:1-16:0.

It is apparent according to **Table 2** that sea buckthorn pulp oil contains many asymmetric TAGs with three different fatty acids. Chiral chromatographic studies with structured ABC-type TAG reference compounds are needed to obtain more information about elution order and chromatographic behavior of TAGs with three different fatty acids. Another improvement to study enantiomeric ratio of individual TAGs would be the multidimensional chromatography in online mode. However, there may be restrictions for mobile phase to be used in such analysis, because many commonly used LC eluents are not applicable with chiral columns. In future research, concentration of the fraction has to be higher in order to obtain sufficient amount of samples for further analytical steps. Also separation of symmetric TAG from asymmetric TAGs prior to chiral analysis would simplify the enantiomeric resolution.

Table 1
Ions observed in the UHPLC-APCI-MS spectra and their possible identifications. Numbers of fractions further analyzed are bolded.

#	t_R (min)	$[M+H]^+$ m/z			Fragments $[M-RCO2]^+$ and possible DAGs *m/z*						Possible TAGs
1	13.42	825.7	799.6		545.8 [14:0-18:3]	547.7 [16:1-16:1]; [14:0-18:2]	571.8 [16:1-18:3]	597.7 [18:2-18:3]			14:0-18:3-18:2; 16:1-16:1-18:3
2	15.04	801.7	827.7	853.7	547.8 [16:1-16:1]	573.8 [16:1-18:2]	599.7 [18:2-18:2]				16:1-16:1-16:1; 16:1-18:2-16:1; 18:2-18:2-16:1
3	15.50	827.7	801.7	853.7	547.7 [16:1-16:1]; [14:0-18:2]	545.7 [14:0-18:3]	549.7 [16:0-16:1]	573.7; [16:0-18:3]; [16:1-18:2] 575.7 [16:0-18:2]; [16:1-18:1]	571.8; 521.7	[16:1-18:3]; [14:0-16:1]	16:0/16:1/18:3; 14:0/16:1/18:2; 16:0/18:2/18:3; 16:1/18:1/18:3
4	16.65	855.7	881.7	829.7	575.8 [16:0-18:2]; [16:1-18:1]	547.8 [16:1-16:1]; [14:0-18:2]	601.8 [18:1-18:2]; [18:0-18:3]	573.8 [16:0-18:3]; [16:1-18:2]	575.7; 599.7	[16:1-18:1]; [18:2-18:2]	16:1/18:1/18:2; 18:1/18:2/18:2; 16:1/16:1/18:1
5	17.03	**803.7**	829.7		549.8 [16:0-16:1]	547.8 [16:1-16:1]	573.8 [16:1-18:2]	575.8 [16:0-18:2]			**16:1-16:1-16:0; 16:0/16:1/18:2**
6	17.46	829.7			547.8 [16:1-16:1]; [14:0-18:2]	549.7 [16:0-16:1]	551.7 [16:0-16:0]	573.8 [16:0-18:3]; [16:1-18:2]	521.7; 575.7	[14:0-16:1]; [16:0-18:2]; [16:1-18:1]	16:0/16:1/18:2; 16:1/16:1/18:1; 16:0/16:0/18:3
7	18.30	883.7	857.7		601.8 [18:1-18:2]	603.8 [18:1-18:1]	575.8 [16:0-18:2]; [16:1-18:1]	549.7 [16:0-16:1]			18:1/18:1/18:2; 18:1/18:1/16:1
8	18.71	**831.7**	857.7		577.8 [16:0-18:1]; [16:1-18:0]	575.8 [16:0-18:2]; [16:1-18:1]	549.8 [16:0-16:1]	601.8 [18:1-18:2]			**16:0-16:1/18:1; 16:0/18:1/18:2**
9	19.20	**805.7**	831.7		549.8 [16:0-16:1]	551.7 [16:0-16:0]	575.8 [16:0-18:2]; [16:1-18:1]				**16:0/16:0/16:1; 16:0/16:0/18:2**
10	20.30	859.7			577.8 [16:0-18:1]	603.8 [18:1-18:1]					16:0-18:1-18:1
11	20.79	833.7			577.8 [16:0-18:1]	551.8 [16:0-16:0]	549.8 [16:0-16:1]				16:0/16:0/18:1

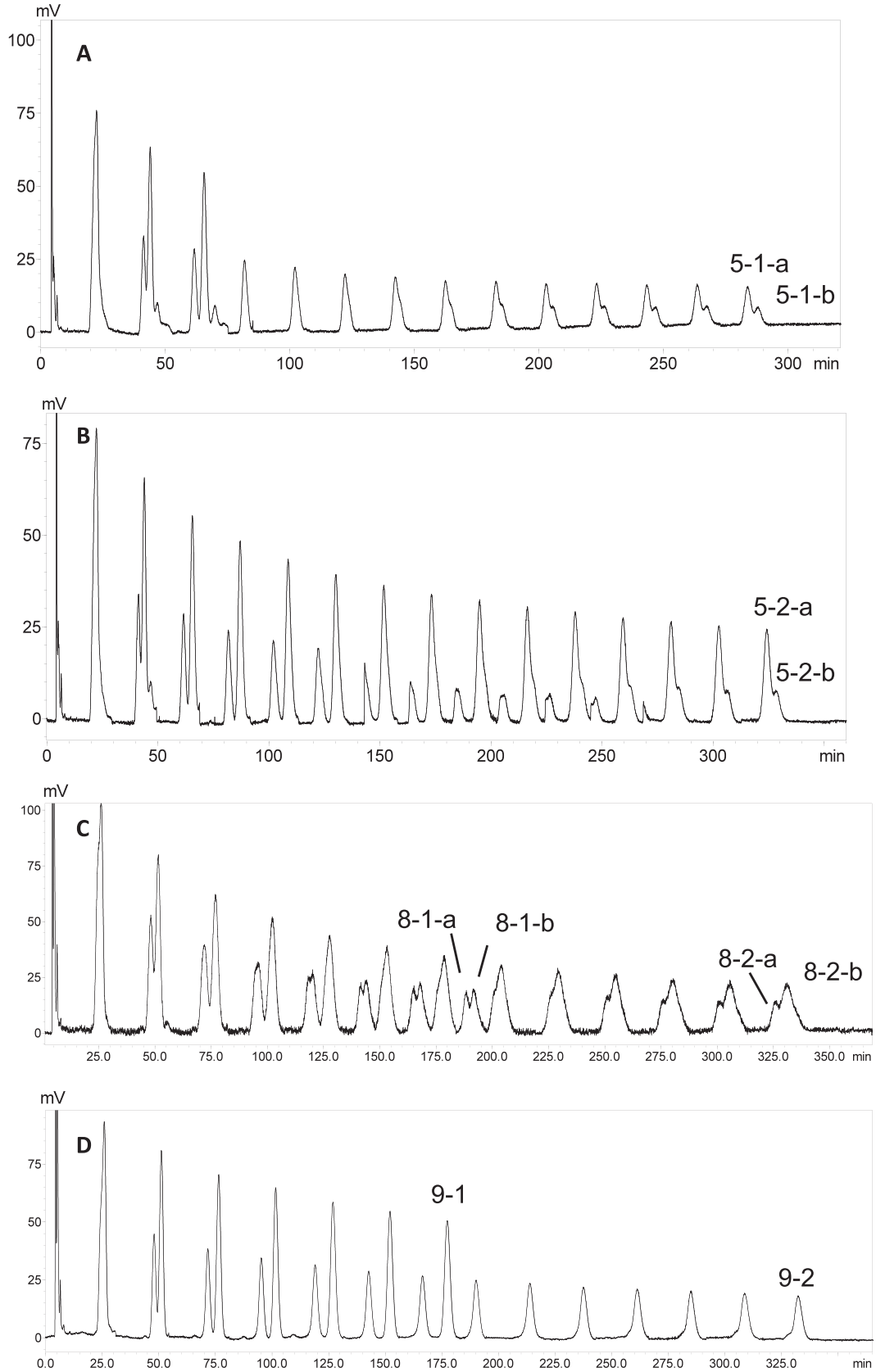

Fig. 3. HPLC-UV chromatograms of chirally separated fractions 5, 8 and 9. (A) Fraction 5 separated and collected fractions 5-1-a, 5-1-b. (B) Fraction 5 separated and collected fractions 5-2-a and 5-2-b. (C) Fraction 8 separated and collected fractions 8-1-a, 8-1-b, 8-2-a and 8-2-b. (D) Fraction 9 separated and collected fractions 9-1 and 9-2.

Table 2
Collected fractions, chiral chromatographic and NICI-MS results, and proposed enantiomers with relative abundances calculated using MSPECTRA software.

Fract.	t_R of Collected Peak (min)	No of Column Passes	Enantiomeric Ratio (Area-%)	$[M–H]^-$ACN:DB	$[M–H]^-$ACN:DB	Proposed Enantiomers	Relative Abundance ± Std
5				801.8 48:2	827.8 50:3		
5-1-a	283.7	14	70.5	801.8 48:2		*sn*-16:1-16:1-16:0	97.0 ± 6.0 (n=4)
						16:1-16:0-16:1	3.0 ± 6.0
5-1-b	288.1	14	29.5	801.8 48:2		*sn*-16:0-16:1-16:1	96.5 ± 6.0 (n=3)
						16:1-16:0-16:1	3.5 ± 6.0
5-2-a	324.1	15	76.0	827.8 50:3		16:1-16:0-18:2	2.2 ± 4.3 (n=4)
						16:0-16:1-18:2	30.1 ± 8.9
5-2-b	328.3	15	24.0	827.8 50:3		16:1-18:2-16:0	67.7 ± 6.9
							traces of the same enantiomers
8				829.8 50:2	855.8 52:3		
8-1-a	188.5	8	43.1	829.8 50:2		16:1-16:0-18:1	0 (n=4)
						16:0-16:1-18:1	27.1 ± 1.3
						16:1-18:1-16:0	72.9 ± 1.3
8-1-b	191.7	8	56.9	829.8 50:2		16:1-16:1-18:0	0.8 ± 0.9 (n=4)
						16:1-18:0-16:1	0.05 ± 0.09
						16:1-16:0-18:1	0.001 ± 0.001
						16:0-16:1-18:1	52.3 ± 8.9
						16:1-18:1-16:0	46.9 ± 18.6
8-2-a	326.5	13	25.0	855.8 52:3		16:0-18:2-18:1	33.8 ± 15.2 (n=3)
						18:2-16:0-18:1	0
						16:0-18:1-18:2	66.2 ± 15.2
8-2-b	330.9	13	75.0	855.8 52:3		16:1-18:2-18:0	0
						18:2-16:1-18:0	0.6 ± 1.2 (n=4)
						16:1-18:0-18:2	0.3 ± 0.6
						16:1-18:1-18:1	1.4 ± 2.8
						18:1-16:1-18:1	1.2 ± 2.4
						16:0-18:2-18:1	51.9 ± 32.8
						18:2-16:0-18:1	11.8 ± 16.6
						16:0-18:1-18:2	32.8 ± 21.9
9				803.8 48:1	829.8 50:2		
9-1	177.4	7	65.7	829.8 50:2		16:0-16:0-18:2	6.7 ± 5.6
						16:0-18:2-16:0	93.3 ± 5.6
9-2	332.3	14	34.3	803.8 48:1		16:1-16:0-16:0	4.2 ± 2.4
						16:0-16:1-16:0	95.8 ± 2.4

4. Conclusions

A targeted strategy using mass spectral characterization and achiral-chiral off-line two-dimensional HPLC for analysis of stereospecific structures of individual triacylglycerols in nutritionally important natural oils was demonstrated. The methodology was applied to study enantiomeric composition of targeted TAGs extracted from sea buckthorn pulp oil. The first dimension (RP-HPLC) separated the TAGs into eleven fractions based on ECN values. UHPLC-APCI-MS was applied to determine the molecular weight distribution, relative abundance of each of these fractions and preliminary regiospecific composition. The three TAG fractions, representing 60% of the total TAGs, were further separated into ten subfractions on the second dimension (chiral-phase recycling HPLC), although baseline separation was not achieved between all the sub-fractions. Analysis of these sub-fractions with direct inlet NICI-MS/MS enabled determination of regioisomeric and enantiomeric composition of TAGs. The enantiomeric ratio of TAG *sn*-16:1-16:1-16:0 to TAG *sn*-16:0-16:1-16:1 was 70.5/29.5. Another prevalent palmitoleic acid-containing TAG species 16:0/16:0/16:1, contained mainly (96%) symmetric TAG 16:0-16:1-16:0. The results are consistent with results of earlier study by Yang and Kallio, which suggested that palmitic acid mainly locate in the positions *sn*-1 or *sn*-3 in sea buckthorn plant oil [46].

This analytical strategy leads to revelation of stereospecific structural information of natural TAGs – impossible to obtain by other methods available so far. The methodology can still be further developed by improving the chromatographic separation of regioisomers of TAGs before stereospecific separation. Consequently, the use of presented methodology to study enantiomeric composition of individual TAGs can remarkably contribute to the field of analytical chemistry of lipids and lipidomics as well as improve the knowledge of the detailed molecular structure of nutritionally important TAGs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Marika Kalpio: Formal analysis, Investigation, Methodology, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Kaisa M. Linderborg:** Supervision, Resources, Writing - review & editing, Funding acquisition. **Mikael Fabritius:** Formal analysis, Methodology, Writing - review & editing. **Heikki Kallio:** Conceptualization, Supervision, Writing - review & editing. **Baoru Yang:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

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Supplementary materials

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