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Analysis of triacylglycerol regioisomers in plant oils using direct inlet negative ion chemical ionization tandem mass spectrometry

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ABSTRACT

Triacylglycerols (TGs) are the primary lipids of plant oils and the positional distribution of fatty acids (FAs) is essential to physicochemical, functional, and nutritional qualities of oils. Most studies have reported TG species in plant oils. In some studies, FA combinations in each TG species have been reported still neglecting the regioisomer composition of TGs. In this study, a fast direct inlet negative ion chemical ionization tandem mass spectrometric (NICI-MS/MS) method and optimization algorithm were applied to study the regioisomerism of TGs in 18 different plant oils. According to FA composition results, oleic, FA 18:1(9); linoleic, FA 18:2(9,12); palmitic, FA 16:0 and stearic acid, FA 18:0 were the most abundant FAs, composing mainly TG species having acyl carbon numbers 50, 52 and 54 and 1–4 double bonds. Based on 35 detected TG species, oils were classified into five groups using clustering analysis. Each group had a different dominant TG species of which the most abundant were triunsaturated ones. In regioisomeric pairs or triplets, FA 16:0, FA 16:1(9), FA 18:0, and FA 18:2 (9,12) were more commonly in the *sn*-1/3 position, while FA 18:1 slightly preferred *sn*-2. The most abundant TG regioisomers were: TG 16:0_18:1(*sn*-2)_18:1 (52:2, mainly 18:1 in *sn*-2) especially in avocado, macadamia nut, olive, and palm oils; TG 18:2_18:2(*sn*-2)_18:1 and TG 18:2_18:1(*sn*-2)_18:2 (TG 54:5, mainly 18:2 in *sn*-2) in corn, pumpkin seed, sesame, and sunflower oils. The use of high-throughput NICI-MS/MS method to study regioisomers in commercial plant oils contributes to further studies on profiling lipid structure and developing products with specific TG compositions to meet dietary needs. The regiospecific information of TGs in edible oils is crucial for understanding their health benefits and functional properties, which are in turn needed in selecting oils for various applications.

1. Introduction

Plant oils are important raw materials for food products and play a significant role in human nutrition. The global consumption of plant oils increased by 30 % in the past decade (Statista, 2024). Triacylglycerols (TGs) are the most abundant components, accounting for over 95 % of plant oils (Wei et al., 2013). While different fatty acid (FA) combinations create various TG species and molecular species, positional distribution of different FAs in the glycerol backbone results in regioisomers within each molecular species consisting of two or three different FAs (Amaral et al., 2006). TG regio- and stereoisomer profiles influence the physicochemical properties such as crystallization and the melting point of plant oils and the sensory properties of food containing these oils (Calvo, Juárez, & Fontecha, 2021; Lee, Nagai, Gotoh, Fukusaki, & Bamba, 2014; Mizobe et al., 2013; Xu Ling et al., 2018). For example, transesterification changes the palm oil from a liquid form to a solid paste at room temperature (Linderborg & Kallio, 2005). Positional distribution of FAs in TGs may affect the digestion, absorption, and metabolism of fats and oils (Han & Ye, 2021). In *vivo*, FAs are only absorbed as free FAs or as esterified FAs in *sn*-2 (stereospecific numbering) position of TGs after digestive lipolysis (Michalski et al., 2013). Generally, terminal FAs (*sn*-1 and *sn*-3 positions) are more prone to be hydrolyzed by lipases compared with internal FAs (the *sn*-2 position) resulting in the remaining glycerol backbone being absorbed by small intestine cells (Michalski et al., 2013; Řezanka, Pádrová, & Sigler, 2017). Hence, *sn*-2 FAs play an important role in dietary TGs. As a typical example, the

Abbreviations: ACN, acyl carbon number; DB, double bond; CID, collision-induced dissociation; FA, fatty acid; FAME, fatty caid methyl ester; FID, flame ionization detector; MS/MS, tandem mass spectrometry; NICI, negative ion chemical ionization; SFA, saturated fatty acid; SFC, supercritical fluid chromatography; *sn*, stereospecific numbering; TG, triacylglycerol; UFA, unsaturated fatty acid; UHPLC, ultra-high-performance liquid chromatography.

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functional difference has been demonstrated between the human milk TG regioisomeric pairs 18:1_16:0(*sn*-2)_18:1 (FA 18:1, oleic or vaccenic acid; FA 16:0, palmitic acid) and 18:1_18:1(*sn*-2)_16:0. TG isomer 18:1_16:0(*sn*-2)_18:1 contributes to remitting constipation, optimizing calcium and fat absorption and enhancing bone development (Ghide & Yan, 2021; Mu & Høy, 2004; Mu & Porsgaard. 2005). Therefore, in-depth molecular-level knowledge of the isomeric structures of TGs in plant oils is essential for understanding the structure–function relationships and for precision product design to achieve specific composition and targeted functions.

In nature, FAs are non-randomly esterified to positions *sn*-1, *sn*-2, and *sn*-3 during biosynthesis of TGs in plants (Calvo et al., 2021; Lee et al., 2014). Generally, saturated FAs (SFAs) are preferentially esterified to the *sn*-1/3 positions, whereas unsaturated FAs (UFAs) prefer the *sn*-2 position (Amaral et al., 2006). However, the regioisomer composition of TGs in plant oils is species-specific, and there is limited knowledge on the TG regioisomer profiles of plant oils. While most studies have reported TG species (ACN:DB) in plant oils (Komaram et al., 2021; Lesellier et al., 2021; Salerno et al., 2023; Wang et al., 2023; Wei et al., 2019; Zhang et al., 2018; Zhang et al., 2022), some have reported the TG molecular species (FA combination in each TG species) of certain plant oils without resolving the regioisomeric composition (Sabzi et al., 2023; Wei et al., 2023; J. Zhang et al., 2023). A few studies focused on the regioisomer ratio of specific molecular species in plant oils such as TG 16:0_18:1_18:1, TG 16:0_16:0_18:1, TG 18:2_18:1_18:1 (FA 18:2, linoleic acid), TG 18:2_18:2_18:1, and TG 16:0_18:1_18:2 (Gros et al., 2023; Leskinen, Suomela, & Kallio, 2010; Tarvainen, Kallio, & Yang, 2019;Lísa et al., 2009b; Masuda et al., 2021; Zhang et al., 2021; Zhang et al., 2024). However, these studies investigating TG regioisomers in natural fats and oils have been quite limited in scope, not providing comprehensive regioisomer profiles of the samples.

Due to the complexity of TG profiles in natural oils and fats, qualitative and quantitative analysis of TG regioisomers presents a great challenge to the analytical methods. TG regioisomer analysis can be achieved by different analytical approaches, which have been reviewed recently (Fabritius & Yang, 2023). Briefly, ultra-high-performance liquid chromatography (UHPLC) has been used as a standalone separation method or coupled with tandem mass spectrometry (MS/MS). Additionally, shotgun (direct infusion/inlet) MS/MS analysis without chromatographic separation has been successfully utilized for TG regioisomer analysis. Depending on the overall objective of the analysis, each of the methodologies has its advantages and disadvantages. LC methods for separating TG regioisomers, especially for comprehensive profiling, typically need a long separation which is impractical for high-throughput analyses (Lísa et al., 2009b). Additionally, certain TG regioisomers such as those containing only SFAs are difficult to resolve with current chromatographic methods (Fabritius & Yang, 2023). In addition to LC methods, supercritical fluid chromatography (SFC) has been utilized to separate TGs before MS detection. Masuda et al. (2021) used SFC-MS/MS with a chiral phase column to separate and identify certain stereo- and regioisomers of TGs in olive and palm oils. While ion mobility spectrometry shows potential for separating specific TG regioisomers, it has not yet been demonstrated to be practical for comprehensive profiling of TG regioisomers in complex natural samples, as separation efficiency is highly influenced by the FA characteristics of the TGs (Bowman et al., 2017). However, this might change in the future with more advanced techniques such as the cyclic ion mobility (de Bruin et al., 2025). The MS/MS methodologies for analyzing TG regioisomers are based on the characteristic fragment ions or relative abundances of structurally informative fragments in the MS^n spectra. Among the different fragmentation techniques used in MS/MS methods for TG regioisomer analysis, collision-induced dissociation (CID) is the most commonly used, where diacylglycerol fragments of different abundance result from dissociation of *sn*-1/3 and *sn*-2 FAs (Fabritius & Yang, 2023). There are also some emerging techniques such as electron activated dissociation (EAD) (Zhang et al., 2024), and ozone-induced dissociation (OzID) (Marshall et al., 2016), yielding *sn*-specific fragments and providing possible alternatives for CID. However, practicality of these alternative fragmentation methods in resolving complex regioisomeric profiles of natural fats and oils remains to be demonstrated. Therefore, despite the effort and progress in developing analytical methods, the TG regioisomer profiles of commercially important plant oils and other fats and oils remain largely unknown.

Direct inlet ammonia negative ion chemical ionization MS/MS (NICI-MS/MS) has been developed and applied by our group over the past three decades (Kallio & Currie, 1993; Kallio et al., 2001; Leskinen et al., 2007). In this method, TGs in the sample are directly introduced into the ion source of a mass spectrometer without chromatographic separation, forming deprotonated $[M-H]^-$ ions which are then fragmented with CID into structurally informative $[M-H-RCOOH-100]^-$ ions. A calculation software (MSPECTRA) was created for automatic calculation of the TG regioisomer composition utilizing the relative abundances of the fragment ions (Kallio & Currie, 1993). The method was subsequently used to analyze for example TG regioisomers in vegetable oils and lard, but due to the low sensitivity of the instrument, only regioisomer information of several major TG molecular species such as TG 18:2_18:2_18:1, TG 18:2_18:1_18:1, TG 16:0_18:1_18:1 and TG 16:0_16:0_18:1 were quantified (Kallio & Currie, 1993; Kallio et al., 2001; Leskinen et al., 2007). Recently, our group has updated and validated the NICI-MS/MS method to operate on the more advanced instrument platform with higher sensitivity by using a total of 49 commercially available TGs , and upgraded the calculation software to MSPECTRA 1.4, achieving improved throughput of the MS/MS analysis and data processing (Fabritius et al., 2020). The optimization algorithm enables more accurate calculations of TG regioisomers in natural fats and oils, which may contain numerous overlapping isobaric TGs and their fragments. A similar optimization algorithm has also been used in some of our recent studies with a different UHPLC-ESI-MS/MS analysis platform (Sazzad et al., 2022, 2024).

In the current study, we applied the NICI-MS/MS method and the MSPECTRA 1.4 to quantify the regioisomeric compositions of TGs in 18 different plant oils. The aim was to create comprehensive knowledge on the TG regioisomer profiles of commercially important plant oils. The results of TG regioisomers obtained in this study provide an important reference for basic research in the biochemistry of lipids as well as guidance for designing food and nutraceuticals with targeted composition, quality and functionality.

2. Materials and methods

2.1. Nomenclature

The annotations are based on the LIPID MAPS guideline (Liebisch et al., 2020). Briefly, TG species refers to TGs containing specific number of ACNs and DBs without information on the FA composition (e.g. TG 50:1). Molecular species refers to TGs with specific FA composition without information on their positional distribution (e.g. TG 16:0_16:0_18:1). Regioisomers refer to TGs with defined FA in *sn*-2 position and the remaining two FAs in *sn*-1/3 positions (e.g. regioisomers TG 16:0_16:0(*sn*-2)_18:1 and TG 16:0_18:1(*sn*-2)_16:0). Enantiomeric TGs with all three FA positions defined such as TG 16:0/16:0/18:1 and TG 18:1/16:0/16:0 cannot be distinguished with the current method. Trivial names, species level and DB-position level shorthand notations of FAs are shown in Supplementary Table 1 (Table S1).

2.2. Materials and reagents

In this study, eighteen different plant oils were analyzed. Almond (*Prunus amygdalus*, expeller-pressed, refined, Finland), rice bran (*Oryza sativa*, expeller-pressed, cleaned, Finland), and safflower (*Carthamus tinctorius*, expeller-pressed, cleaned, Finland) oils were purchased from Limepop Naturals (Helsinki, Finland). Argan (*Argania spinose*, virgin

cold-pressed, Morocco), avocado (*Persea americana*, extra virgin, New Zealand), corn (*Zea mays*, refined, Turkey), hempseed (*Cannabis sativa*, virgin cold-pressed, Finland), linseed (*Linum usitatissimum*, cold-pressed, Finland), macadamia nut (*Macadamia integrifolia*, cold-pressed, New Zealand), oat (*Avena sativa*, unknown, Finland), olive (*Olea europaea*, virgin cold-pressed, Spain), peanut (*Arachis hypogaea*, refined, Germany), pumpkin seed (*Cucurbita pepo*, not cold-pressed, Austria), rapeseed (*Brassica napus*, refined, Finland), sesame (*Sesamum indicum*, cold-pressed, filtered), sunflower (*Helianthus annuus*, refined, Denmark), and walnut (*Juglans regia*, unknown, Netherlands) oils were purchased from the local markets (Turku, Finland); palm oil (*Arecaceae*, unknown, Finland) was provided by Anton's Best (Helsinki, Finland). Oils covered three fruit pulp oils (avocado, olive, and palm), five nut oils (almond, argan, macadamia nut, peanut, and walnut), and ten seed oils (corn, hempseed, linseed, oat, pumpkin seed, rapeseed, rice bran, safflower, sesame, and sunflower).

Two commercial external FA methyl ester (FAME) mixtures were used. GLC reference standard 68D was purchased from Nu-Chek-Prep (Elysian, MN, USA) and Supelco 37 Component FAME Mix from Supelco (St. Louis, MO, USA). Internal standard triheptadecanoin (TG 17:0/17:0/17:0) was purchased from Larodan (Malmö, Sweden). Acetyl chloride ($\geq$ 99.0 %), chloroform (HPLC grade, $\geq$ 99.8 %) and methanol (LC-MS grade, $\geq$ 99.9 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether (HPLC grade, $\geq$ 99.9 %) and hexane (HPLC grade, $\geq$ 97 %) were purchased from Fisher Scientific (Loughborough, UK). Potassium chloride was purchased from VWR international (Radnor, PA, USA), and potassium carbonate was from Acros Organics (Geel, Belgium).

2.3. Isolation of triacylglycerols and preparation of fatty acid methyl esters

The TG fractions were isolated using the solid phase extraction (SPE) without preliminary lipid extraction step. A Sep-Pak Vac silica 1 cc (100 mg) SPE column (Waters, Dublin, Ireland) was conditioned with 1 mL diethyl ether. The lipid sample dissolved in 1 mL diethyl ether was applied to the SPE column. The sample vial was washed with 2 mL diethyl ether, which was transferred into the column. TG fraction was eluted with 9 mL diethyl ether. Then, the solvent was evaporated to dryness under gentle nitrogen flow at 50 °C. The TG fractions were dissolved in 1 mL hexane and stored at –80 °C prior to the MS/MS analysis. 100 μL of TG fraction (about 0.5 mg) was taken for methylation using acid-catalyzed method (Y. Zhang et al., 2023). Firstly, the exact amount of internal standard TG 17:0/17:0/17:0 (5 % weight percentage of TG) was pipetted to the TG fraction. Then, 2 mL of freshly prepared acetyl chloride:MeOH (1:10, *v/v*) was added to the mixture. Reactions were performed in sealed vials in a 50 °C oven overnight. After cooling, 2 mL of potassium chloride was carefully added, followed by 1 mL hexane. The volume of hexane depended on the amount of lipids. Samples were vortexed and centrifuged for 5 min at 1000 rcf. The top layer was recovered. After the methylation, the FAMEs formed were dissolved in 1 mL hexane and were stored at –80 °C. Vials were thoroughly mixed before FA composition analysis.

2.4. Analysis of fatty acid composition

The FA composition was determined by chromatographic analysis of the FAMEs using Shimadzu GC-2010 gas chromatograph with AOC-20i auto injector and flame ionization detector (FID) (Shimadzu Corporation, Kyoto, Japan). The column was a wall-coated open tubular column DB-23 (60 m × 0.25 mm i.d., liquid film 0.25 μm, Agilent Technologies, J.W. Scientific, Santa Clara, CA, USA). Helium was used as the carrier gas. The injection temperature was 270 °C, and the injection volume was 0.5 μL. Splitless injection mode was used, and the split opened after 1 min. The column oven temperature program was as follows: initially 130 °C for 1 min, increase to 170 °C at a rate of 6.5 °C/min, increase to 205 °C at 2.75 °C/min and hold for 18 min, increase to 230 °C at 30 °C/min and hold for 2 min. The detector temperature was 280 °C. FAME37 and 68D were used as external standards for identification, and TG 17:0/17:0/17:0 was used as an internal standard for quantification. Correction factors were calculated with external standards to correct the difference in detector response between each FA and the internal standard (Fabritius et al., 2020). FA composition was expressed as a weight percentage of all FAs.

2.5. Triacylglycerol species and regioisomer composition

The TG species composition and regioisomer analyses were carried out using the same instrument and method as in the previous study of our group with some modifications (Fabritius et al., 2020). All mass spectrometric analyses of TGs were carried out using a Thermo Scientific TSQ 8000 EVO triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a direct exposure probe.

TGs were first chemically ionized to negative molecular ions $[M-H]^-$ with ammonia gas (purity 6.0), followed by the selection of TG species (ACN:DB species) in the first quadrupole. The targeted molecular weight of TG species was applied to the method program. The second quadrupole further performed fragmentation with argon gas, yielding negative ions $[M-H-RCOOH-100]^-$ and $[RCOO]^-$ which were separated in the last quadrupole. Regioisomeric information of TGs were determined with MSPECTRA 1.4 by calculating the relative proportions of the formed fragments $[M-H-RCOOH-100]^-$ and $[RCOO]^-$. More detailed information is provided in the data treatment section.

An aliquot of 2 μL of the TG fraction (5 mg/mL in hexane) of each sample was loaded onto the rhenium wire on the top of the probe with a syringe. The MS instrument parameters were set according to previous study in our group (Fabritius et al., 2020): the ion source temperature 340 °C, ammonia flow rate 1.5 mL/min, the electron energy 70 eV, emission current 300 mA, probe heating rate was 100 mA/s and the scan time 0.1 s. MS scans were acquired within *m/z* 400–1000. The *m/z* values of $[M-H]^-$ ions were used to distinguish different TG species and calculate their relative molar percentages. As several TG species can have a difference of only one DB resulting in a mass difference of a 2 Da, some of the M+2 isotopes will overlap with certain monoisotopic TG ions in the MS scan. Therefore, the amount of naturally occurring ^{13}C was taken into account when the proportions of TG species were calculated. TG species with abundance of 0.5 mol% or higher of all identified TGs were reported in the results.

The regioisomeric compositions of TG species with relative abundances above 1 mol% of total TGs (26 TG species, covered over 96 mol% in each oil) were determined. Each product ion scan method was created using the TG species information obtained in the preliminary MS scan. CID with argon as collision gas was used to fragment the pseudomolecular $[M-H]^-$ TG ions (Fabritius et al., 2020). Collision energy was 20 eV and other parameters were same as in the MS scan. Product ions were scanned between *m/z* 100–650 to identify the primary (*sn*-1/3) and secondary (*sn*-2) positions of FAs. The relative proportions of $[M-H-RCOOH-100]^-$ and $[RCOO]^-$ ions of the fragmentation spectra were used to calculate the abundances of TG regioisomers. A maximum of six different TG species and all their regioisomers were analyzed in a single run. All analyses were performed in four repetitions, and the results are expressed as average ± standard deviation. The positional and *cis* vs. *trans* information of DBs in FAs couldn't be reached with current NICI-MS/MS method.

2.6. Data treatment

FA composition was analyzed with LabSolutions software (Shimadzu Corporation, Kyoto, Japan). MS and MS/MS raw data was initially preprocessed and exported from XCalibur software. Further, MS data was calculated with Excel (Microsoft Office 2016) to obtain the TG species distribution. MS/MS raw data was first exported and converted

to ASCII format and analyzed by MSPECTRA 1.4 software to obtain quantitative profile of TG regioisomers (Fabritius et al., 2020). The TG regioisomer calculation algorithms and MSPECTRA software were first described by Kallio and Rua (1994) and Kurvinen et al. (2001). Later, they were further updated to handle data obtained with the new generation MS instrument and to resolve the regiospecific profile of TGs with TSQ 8000 EVO spectrometer (Fabritius et al., 2020). The software has an optimization algorithm to mitigate the effects of structurally informative isobaric fragment ions that are dissociated from multiple different TG molecular species with the same precursor ion mass. It is important to consider the isobaric overlap in order to achieve accurate results, something that is often overlooked in many studies with complex natural samples. The principle for TG regioisomer calculation was described briefly in a recent review (Fabritius & Yang, 2023). MSPECTRA 1.4 first used the deprotonated ion $[M-H]^-$ to obtain TG species (ACN:DB species) information according to the *m/z* ratio, followed by judging TG molecular species and *sn*-specific information with CID generated ions $[M-H-RCOOH-100]^-$ and $[RCOO]^-$. $[RCOO]^-$ intensities were used to quantify the FA combinations of TG, identifying the TG molecular species within each TG species (ACN:DB species). Further, *sn*-specific information of TG was calculated by relative abundances of $[M-H-RCOOH-100]^-$ ions. Theoretically, the relative abundance of $[M-H-RCOOH-100]^-$ missing FA anions from the *sn*-2 position was lower than that from the *sn*-1/3 positions.

Python (version 3.8.3) with clustering analysis (method, ward; metric, Euclidean) was used to classify oils into five groups according to the TG species distribution: Group 1, almond, peanut, and rapeseed oils; Group 2, avocado, macadamia nut, olive; Group 3, argan, oat, and rice bran oils; Group 4, corn, pumpkin seed, sesame, and sunflower oils, and palm oils; Group 5, hempseed, linseed, safflower, and walnut oils. The reason to choose the abundance of TG species as grouping criteria was that it would reflect the information of FA composition and directly influence the abundance of the TG regioisomers. Data visualization was realized with Origin 2016 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism 10 (GraphPad Software, Boston, USA).

The molar percentages of FAs in *sn*-1/3 or *sn*-2 positions were extracted from regioisomer results.

3. Results and discussion

3.1. Fatty acid composition

Altogether 18 FAs including 7 SFAs and 11 UFAs with the acyl chain length from 12 to 24 carbons and 0 to 4 double bonds in *cis* configuration were detected from 18 plant oils (Table S1). FA composition was expressed as relative mass percentage (%). Four main FAs among all oils were FA 18:2(9,12) (2.9–76.1 %), FA 18:1(9) (9.3–73.0 %), FA 16:0 (4.7–34.1 %), and FA 18:0 (0.5–6.5 %).

FA 18:1(9) (38.7–73.0 %) was the most abundant in FA in most of the samples (almond, peanut, rapeseed, avocado, macadamia nut, olive, palm, argan, oat, and rice bran oils). Whereas FA 18:2(9,12) (38.7–76.1 %) was the most abundant in corn, pumpkin seed, sesame, sunflower, hempseed, safflower and walnut oils. Specifically, oat oil contained the same amount of FA 18:1(9) and FA 18:2(9,12) (38.7 %). Linseed oil was the only oil that contained a significant amount of FA 18:3(9,12,15) (54.0 %).

FA 18:2(9,12) was the second most abundant FA in almond, peanut, rapeseed, argan, and race bran oils (10.5–37.1 %) and FA 16:0 in avocado, olive, and palm oils (14.0–34.1 %). Previous studies have reported 18.2–37.6 % abundance of FA 18:2(9,12) in almond, peanut, and rapeseed oils and 8.0–43.7 % FA 16:0 in avocado, olive, and palm oils, which were also the second most abundant FAs (Orsavova et al., 2015; Vingering et al., 2010; Wei et al., 2019). Palmitoleic acid [FA 16:1(9)] was the second most abundant FAs in macadamia nut oil (15.1 %), which was reported similarly as 18.9 % in a previous study (Lísa et al., 2009a). The characteristic FA composition of macadamia nut oil, clearly different from those of avocado, olive and palm oils, was emphasized in reported literature (Gros et al., 2023). Rapeseed oil contained notable amount of α-linolenic acid [FA 18:3(9,12,15)] (7.5 %) contrary to the other six oils mentioned above, and similar results of 7.7–9.6 % were reported in previous studies (Lísa et al., 2009a; Vingering et al., 2010; Wei et al., 2019), confirming rapeseed oil as a good source of *n*-3 FAs.

Altogether FA 18:1(9) and FA 18:2(9,12) covered about 80 % of all FAs in argan, oat, rice bran, corn, pumpkin seed, sesame, and sunflower oils, which contain the two FAs at the relative proportions close to each other (32.5–56.4 % of FA 18:2(9,12) vs. 31.1–47.3 % of FA 18:1(9)). Other studies also reported nearly equal amount of FA 18:1(9) (28.0–52.5 %) and FA 18:2(9,12) (33.2–54.5 %) in these oils (Fernández-Acosta et al., 2019; Orsavova et al., 2015; Vingering et al., 2010; Wei et al., 2019). FA 16:0 was the third most abundant FA in these seven oils, comprising 6.2–16.6 % of total FAs.

In contrast to the other oil species, hempseed, linseed, and walnuts contained high amount of FA 18:2(9,12) and FA 18:3(9,12,15). Safflower oil presented FA 18:3(9,12,15) only 0.2 % of the total FAs but highest amount of FA 18:2(9.12) (76.1 %). These results were in agreement with earlier studies (Golkar et al., 2011; Maguire et al., 2004; Wei et al., 2019). FA 16:0 was the third or fourth most abundant FA in these four oils accounting for 5.0–7.1 % of the total FAs. Stearidonic acid [FA 18:4(6,9,12,15)] was unique to hempseed oil, comprising 1.5 % of the total FAs. Previously, Petrović et al. (2015) reported FA 18:4 (6,9,12,15) at levels of 0.3–1.6 % of total FAs in eleven hempseed cultivars.

Among all oils, palm oil contained the highest proportions of SFAs (39.7 %), clearly higher than the levels in other oils (below 20.0 %). In contrast, walnut, linseed, almond as well as rapeseed oils with SFAs no more than 10.0 % (10.0, 9.8, 9.2 and 7.5 %, respectively) were good resources of UFAs. In addition, safflower oil contained FA 18:2(9,12) at the highest proportion (76.1 %) among all the oils studied. Regarding the balance between *n*-3 and *n*-6 PUFAs, linseed, rapeseed, hempseed, and walnut oils presented *n*-6/*n*-3 ratio of 0.3, 2.6, 3, and 4.7 respectively. A lower *n*-6/*n*-3 ratio in the diet is associated with lower risk of inflammation and other chronic diseases (Saini & Keum, 2018). Similarly, the reported ranges of the *n*-6/*n*-3 ratio in rapeseed and hempseed oils were 2.1–2.6 (Leskinen et al., 2007; Lísa et al., 2009a; Vingering et al., 2010; Wei et al., 2019) and 0.4–2.9 (Orsavova et al., 2015), respectively. Linseed, hempseed and walnut oils could be ideal choices for increasing *n*-3 FA consumption.

3.2. Triacylglycerol species composition

Clustering analysis of 18 plant oils based on TG species composition expressed as ACN:DB is presented in Fig. 1, data and grouping are shown in Table S2 and intra-group comparison is shown in Fig. S1. A total of 35 TG species were detected with ACNs ranging from 46 to 56. The number of identified TG species in each oil varied from 11 (safflower oil) to 18 (rapeseed oil). The most abundant ACNs were 50, 52 and 54, and only ACN:DBs 52:4, 52:3, 52:2, 54:4 and 54:3 were detected in all studied oils which could be explained by the abundant FA 18:2(9,12), FA 18:1(9), FA 16:0 and FA 18:0 commonly present in plant oils.

As shown in Fig. 1, although FA composition of oils in Group 1 (almond, peanut, and rapeseed oils) showed similarity to those in Group 2 (avocado, macadamia nut, olive, and palm oils), significant differences were seen in the TG species distributions between the two groups. In Group 1, the most abundant ACN was 54 (62.7–74.8 mol%) with TG 54:3 (28.5–42.5 mol%) as the dominating TG species. A previous study (Lísa & Holčapek, 2008) reported the relative concentration of TG 54:3 in almond, peanut, and rapeseed oils 12.5 %-28.5 %. The difference could be caused by different sample regions. In Group 2, ACN 52 was the most abundant ACN species (39.2–45.9 mol%). TG species 52:2 was the most abundant in avocado, olive and palm oils (25.3–32.0 mol%), whereas TG 52:3 and TG 54:3 (18.4 and 18.7 mol%, respectively) dominated in macadamia nut oil. Similar abundance of TG 52:2 in

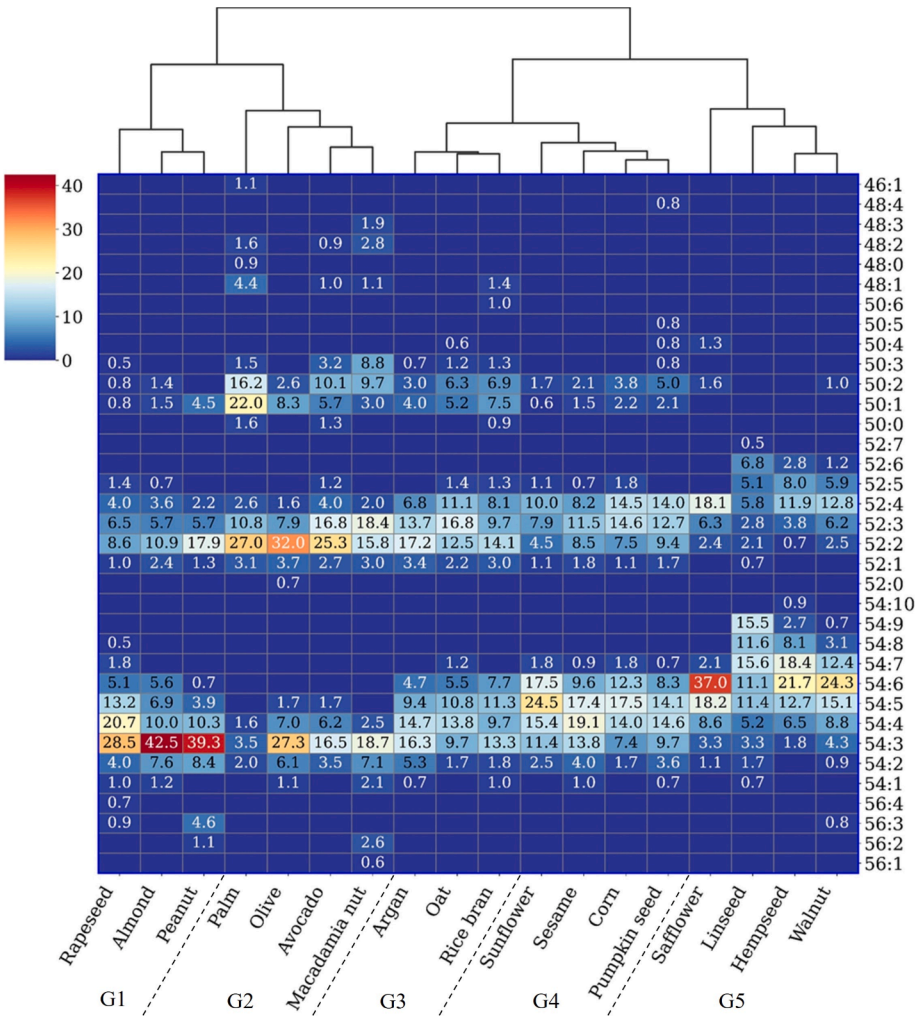

Fig. 1. Clustering analysis of 35 kinds of TG species of plant oils (mol%).

avocado, olive and palm oils (20.2–24.8 %) was reported previously (Lísa & Holčapek, 2008; Wei et al., 2019). In addition, TG 46:1 was unique for palm oil (1.1 mol%). However, TG 46:1 was not reported in a previous publication (Lísa & Holčapek, 2008; Wei et al., 2019), which might be due to the differences in samples and the detection limitation of techniques.

TGs in Group 3 (argan, oat, and rice bran oils) and Group 4 (corn, pumpkin seed, sesame, and sunflower oils) were distributed in ACN 54, 52, and 50, where ACN 54 was the dominating one (42.7–73.1 mol%). ACN 52 dominated in oat oil together with ACN 54 (44.0 mol%). Nevertheless, the most abundant TGs in either group were totally different. In Group 3, the most abundant TGs in argan and rice bran oils belonged to TG 54:3 (16.3 and 13.3 mol%) and TG 52:2 (17.2 and 14.1 mol%, respectively), and in oat oil TG 52:3 (16.8 mol%). Results in rice bran oil were reported previously (9.55 wt% for TG 54:3 and 11.69 wt% for TG 52:2) (Wei et al., 2019), and the small differences caused not only by the different units, but also by the number of detected TGs in each TG species. In Group 4, corn, pumpkin seed, sesame and sunflower oils mainly contained TG 54:5 (14.1–24.5 mol%), while TG 54:4 was most abundant in pumpkin seed oil (14.6 mol%), as well as in sesame oil (19.1 mol%). Similar results of TG 54:5 (17.2–22.1 %) in corn, sesame and sunflower oils were reported before (Lísa & Holčapek, 2008).

Oils in Group 5 (hempseed, linseed, safflower, and walnut oils) were also rich in ACN 54 (69.7–76.1 mol%), whereas ACN 50 was almost absent or much lower compared with the other four groups (0.0–2.9 vs. 2.1–41.4 mol%). Hempseed, safflower and walnut oils had TG 54:6 at considerable proportions (11.1–37.0 mol%), while linseed oil was rich in TG 54:9 and TG 54:7 (15.5 and 15.6 mol%, respectively) due to the high amount of FA 18:3(9,12,15). TG 54:6 was reported in safflower and walnut oils (43.3 % and 24.7 %, respectively) (Lísa & Holčapek, 2008). TG 54:10 was only detected in hempseed oil (0.9 mol%) due to the special FA 18:4(6,9,12,15). Contrarily, a previous study (Lísa et al., 2009a) didn't report TG 54:10 with HPLC/APCI-MS method. This may be caused by different sample sources and methods.

3.3. *Triacylglycerol regioisomer composition and positional distribution of FAs*

A total of 220 TGs regioisomers including 96 regioisomer pairs or triplets and 4 of single FAs (TG 16:1/16:1/16:1, TG 18:1/18:1/18:1, TG 18:2/18:2/18:2 and TG 18:3/18:3/18:3) were detected. Relative abundances of different regioisomers within each TG species are reported in Table S3. The same data is visualized in Fig. S2 for TG species with abundance above 5 mol% of total TGs. All the spectra including [M−H]− (TG species), [M−H−RCOOH−100] − and [RCOO] − ions in each oil (separate Excel file) was shown in Fig. S3.

To investigate the positional profiles of FAs in the TGs, the molar percentages of FAs in *sn*-1/3 or *sn*-2 positions in selected 33 TG species (>1 mol%) were extracted from the regioisomer results (Table S4), and the data visualization is shown in Fig. 2. The overall FA composition extracted from the TG results is highly consistent with the FA composition analysis performed with the GC-FID method. This shows that the

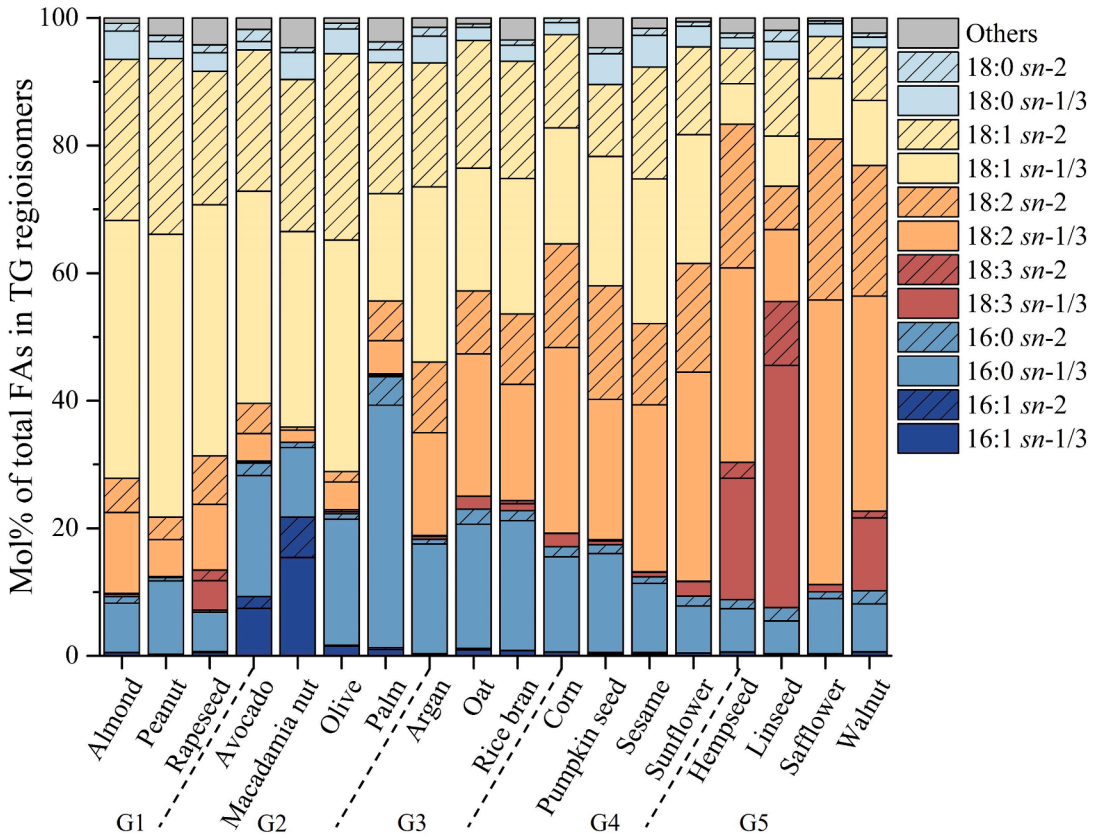

Fig. 2. Abundance of FAs in *sn*-1/3 or *sn*-2 positions (mol%). (Others included species-level FAs 12:0, 14:0, 18:4, 20:0, 20:1, 20:2, 22:0, and 22:1.).

MS/MS method can be reliably used for FA composition analysis with the disadvantage of it not providing the information on double bond locations. For example, the sum percentages of FA 18:1 in *sn*-1/3 and *sn*-2 positions in almond oil was 65.7 mol%, which was highly consistent with the sum 68.3 % of FA 18:1(9) and FA 18:1(11) in the FAs result. Some deviation may occur due to the lower abundant TGs species (<1 mol%) that were not included in TG regioisomeric analysis, different analysis techniques, and different units used for calculations (GC-FID weight%, MS mol%). FA 16:0, FA 16:1, FA 18:0, and FA 18:2 dominated in the *sn*-1/3 positions in most of the analyzed oils, with the exception that 18:0 in avocado oil preferred *sn*-2 position (Fig. 2). The location of FA 18:1 varied among samples, usually more inclined to be in *sn*-1/3 positions. In palm oil FA 18:1 was more abundant in *sn*-2 position and evenly distributed in oat and hempseed oils (Table S4). The positional distribution of FA 18:2 varied more depending on the oil.

The distribution of TGs in each oil containing SFAs and UFAs at *sn*-2 and *sn*-1/3 positions in different oils is shown in Fig. 3. TGs with three UFAs accounted for the largest proportion of TGs in most studied oils (over 40 mol%), except palm oil, where the dominating TGs were those with only one UFA (SSU/SUS). The second most abundant group was TGs containing two UFAs (SUU/USU), where SUU contributed more than USU. UFAs were more commonly distributed in *sn*-2 position when TGs contained only one UFA (SSU/SUS). The SSS type TGs only represented less than 10 mol% of total TGs. Using *sn*-specific enzymatic digestion, Wei et al. (2019) and Zhang et al. (2021) obtained the overall positional distribution of the FAs, but the enzymatic methods do not provide any information on the structures of the individual TG molecules. Our comprehensive regioisomer analysis method provides much more in-depth information on the TG composition in addition to the positional distribution of the FAs.

Relative abundances of the most abundant regioisomer pairs/triplets (at least three oils contain the pair or triplet with at least 2 mol% abundance of all TGs, covered 56.3–92.4 mol% in each oil) are shown in Fig. 4.

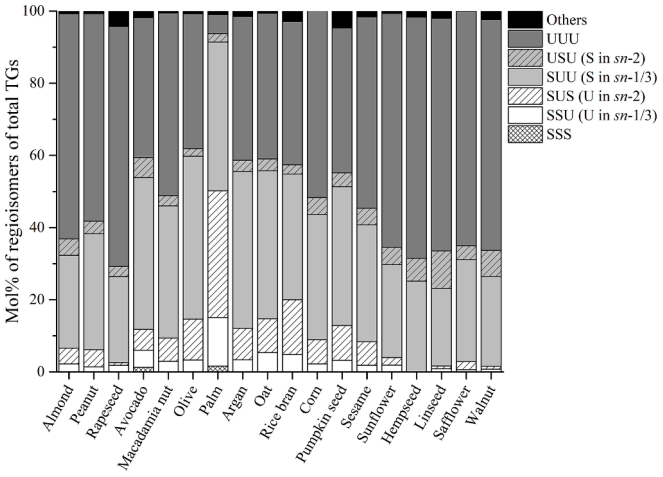

Fig. 3. Molar percentage of regioisomers of total TGs expressed as SFA and UFA (S, saturated or U, unsaturated or is not specific to a particular FA, which means that two identical letters can represent the same or different FAs. Others, the sum of TG species less than 1 mol%.).

Regioisomer information of TG in plant oils was limited. Most studies only concentrated on regiospecific information in specific TG molecular species. The comparison with seven literatures (Gros et al., 2023; Leskinen, Suomela, & Kallio, 2010; Lísa, Velínská, & Holčapek, 2009b; Masuda, Abe, & Murano, 2021; Tarvainen, Kallio, & Yang, 2019; Zhang et al., 2021; Zhang et al., 2024) were listed in Table S5, containing eight TG molecular species: TG 18:1_18:2_18:2, TG 16:0_18:2_18_3, TG 16:0_18:2_18:2, TG 16:0_18:1_18:2, TG 16:0_18:1_18:1, TG 16:0_18:1_18:0, TG 16:0_16:0_18:2, and TG 16:0_16:0_18:1. The

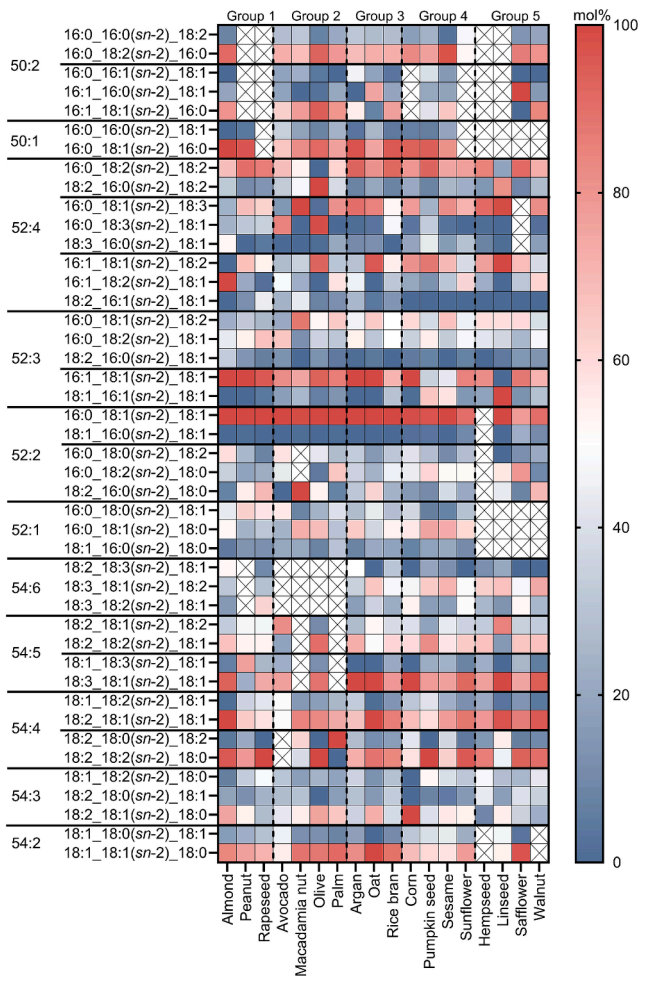

Fig. 4. Regioisomer relative abundance within the most abundant regioisomer pairs/triplets (mol%).

extracted data from literature was the TG percentage of total detected TGs with varying units among studies. The comparison was conducted by recalculating the regioisomer relative abundance in each regioisomer pair using the extracted data. Instrumental methods, as well as regional information and manufacturing methods of oils were provided.

For both Group 1 (almond, peanut, and rapeseed oils) and Group 3 (argan and rice bran oils, excluding oat oil), the most abundant TG 54:3 (according to significance analysis) was mainly comprised of TG 18:1/18:1/18:1 (89.5–97.9 mol%). The results were similar to those reported previously (77–100 %, including some studies reporting TG 18:1/18:1/18:1 as the only species in TG 54:3) (Holčapek et al., 2003; Lerma-García et al., 2011; Lísa & Holčapek, 2008; Wei et al., 2019). However, in hempseed oil in Group 5, TG 54:3 was dominated by the regioisomer triplet TG 18:2_18:1(*sn*-2)_18:0, TG 18:1_18:2(*sn*-2)_18:0, and TG 18:1_18:0(*sn*-2)_18:2 (7.6/39.9/36.3 mol%) rather than TG 18:1/18:1/18:1 (14.1 mol%). This result was consistent with the literature (Lísa et al., 2009a). In addition, TG 52:3 was the dominating species in oat oil of Group 3 with the most abundant regioisomer triplet being TG 16:0_18:1(*sn*-2)_18:2, TG 18:1_16:0(*sn*-2)_18:2, and TG 16:0_18:2(*sn*-2)_18:1 (60.9/4.1/29.0 mol%). Among all oils, FA distribution varied in this regioisomer triplet, dominated by FA 18:1 or FA 18:2 in *sn*-2 position (Fig. 4). Similar with previous literature (Gros et al., 2023; Lísa, Velínská, & Holčapek, 2009b; Zhang et al., 2024) (Table S5), TG 18:2_16:0(*sn*-2)_18:1 was the least abundant regioisomer in molecular species TG 16:0_18:1_18:2 in most of the oils. However, the relative percentages of TG 16:0_18:2(*sn*-2)_18:1 and TG16:0_18:1(*sn*-2)_18:2 varied among studies. Especially Gros et al. (2023) reported that TG 16:0_18:2(*sn*-2)_18:1 was 0.0 area% in 12 oils. This may cause by the different detection limits of techniques.

For three oils in Group 2 (avocado, olive, and palm oils), TG 16:0_18:1(*sn*-2)_18:1 and TG 18:1_16:0(*sn*-2)_18:1 was the most abundant regioisomers in the dominating TG 52:2, of which TG 16:0_18:1(*sn*-2)_18:1 accounted for 74.2–96.7 mol% within this TG species. In previous studies, 16:0_18:1(*sn*-2)_18:1 was reported as the dominating isomer in this TG ACN:DB species in olive (94.0 wt%) and in palm oils (91.3 wt%) (Masuda et al., 2021). In fact, TG 16:0_18:1(*sn*-2)_18:1 was the dominating regioisomer within the TG 16:0_18:1_18:1 molecular species in all studied oils (Fig. 4). On the other hand, TG 52:3 in macadamia nut oil in Group 2 comprised mainly of TG 16:1_18:1(*sn*-2)_18:1 and TG 18:1_16:1(*sn*-2)_18:1 (67.7/22.9 mol%). Whereas referring to the other oils among the five groups, the dominating regioisomer triplet in TG 52:3 was TG 16:0_18:1(*sn*-2)_18:2, TG 18:1_16:0(*sn*-2)_18:2, and TG 16:0_18:2(*sn*-2)_18:1 (added up to 65.9–98.1 mol%, mainly FA 18:1 or FA 18:2 in *sn*-2), except in avocado, which had TG 52:3 as the second most abundant TG species consisting mainly of regioisomer pair TG 16:1_18:1(*sn*-2)_18:1 and TG 18:1_16:1(*sn*-2)_18:1 (44.6/9.5 mol%) and triplet TG 16:0_18:1(*sn*-2)_18:2, TG 18:1_16:0(*sn*-2)_18:2, and TG 16:0_18:2(*sn*-2)_18:1 (9.7/5.8/30.3 mol%). This is likely explained by the notable amount of FA 16:1(9) in both avocado and macadamia oils (5.6 % and 15.1 %, respectively, of the total FAs in these two oils) clearly higher than the levels in other oils (less than 1 %). The results indicate again that regioisomer composition of the same TG species varies among different oils according to their different FAs compositions. Gros et al. (2023) reported only TG 16:0_18:1(*sn*-2)_18:2 in molecular TG species 16:0_18:1_18:2, which was different with the result of the current study.

For the major TG 54:5 in Group 4 (corn, pumpkin seed, sesame and sunflower oils), TG 18:1_18:2(*sn*-2)_18:2 and TG 18:2_18:1(*sn*-2)_18:2 (54.0–75.4/17.1–40.7 mol%, added up to 92.9–94.7 mol%) was the dominating regioisomer pair. However, 18:1_18:2(*sn*-2)_18:2 was not always the dominant TG in the pair among all 18 oils, such as avocado (11.6/52.0 mol%) and linseed (3.0/17.3 mol%) (Table S4). Regarding to TG percentage of total TGs, avocado and linseed 0.9 mol% 2.0 mol%. However, Lísa and Holčapek (2008) only reported molecular species TG 18:1_18:2_18:2 as 3.3 % and 2.0 % (2.3 mol% and 1.0 mol% in current study) in avocado and linseed oils, respectively. The lower abundance may have been due to the number of TG molecular species reported in each TG species, e.g. one more molecular species TG 18:3_18:2_18:0 was reported in current study. For the most abundant TG 54:4 in pumpkin seed and sesame oils, TG 18:2_18:1(*sn*-2)_18:1 and TG 18:1_18:2(*sn*-2)_18:1 (48.0/33.1 mol% in pumpkin, 64.9/19.0 mol% in sesame) were the most abundant regioisomer pairs. Similarly, regarding to TG percentage of total TGs, molecular species TG 18:1_18:2_18:1 was reported in previous study (Lísa & Holčapek, 2008) as 15.2 % (16.0 mol% in current study). In addition, FA 18:2 dominated in *sn*-2 within this pair in all 18 oils (Fig. 4).

The most abundant TG species in Group 5 (hempseed, safflower, and walnut oils, except linseed oil) was TG 54:6 with TG 18:2/18:2/18:2 as the dominating species (64.8–97.3 mol%), which is likely explained by the high amount of FA 18:2(9,12). Similarly, Zhang et al. (2023) summarized that TG 18:2/18:2/18:2 was the most abundant TG in vegetable oils with high amount of FA 18:2. However, TG 54:6 in linseed oil (11.1 mol%), as the second most abundant TG species, mainly contained regioisomers TG 18:2_18:3(*sn*-2)_18:1, TG 18:3_18:2(*sn*-2)_18:1, and TG 18:2_18:1(*sn*-2)_18:3 (11.8/7.8/40.8 mol%) and TG 18:0_18:3(*sn*-2)_18:3 and TG 18:3_18:0(*sn*-2)_18:3 (16.1/14.6 mol%). This difference is attributed to the high amount of FA 18:3 (54.0 % of total FAs) in linseed oil compared with that of FA 18:2(9,12) (54.8–76.1 % of total FAs) in the other three oils. Similar results were noticed in previous studies, where molecular species TG 18:1_18:2_18:3 and TG 18:0_18:3(*sn*-2)_18:3 dominated TG 54:5 in linseed oil (Lísa & Holčapek, 2008). These two TG species together were also reported in Wei et al.'s (2019). For the dominating TG 54:7 in linseed oil, TG 18:1_18:3(*sn*-2)_18:3 and TG 18:3_18:1(*sn*-2)_18:3 (8.9/56.8 mol%) was the most abundant

regioisomer pair. For other oils (hempseed, safflower, and walnut) in Group 5, regioisomer TG 18:3_18:2(*sn*-2)_18:2 almost completely occupied TG 54:7 (90.5–100.0 mol%). This shows that different dominating FAs could lead to distinct regioisomer compositions within the same TG species. In addition, linseed oil especially contained TG 18:3/18:3/18:3 (15.6 mol%). Similar result (14.3 % and 18.1 wt%) has been reported earlier from linseed oil (Lísa & Holčapek, 2008; Wei et al., 2019). Due to presence of FA 18:4(6,9,12,15), certain unique TG molecular species such as TG 18:4_18:3_18:2, TG 18:4_18:4_18:1, TG 18:4_18:2_18:2, TG 18:4_18:3_18:1, TG 18:4_18:3_18:0, and TG 18:4_18:2_18:1 were identified only in hempseed oil.

In UUU type TG species of all the 18 oils, FA 18:1 preferred the *sn*-2 position in most cases, which could potentially introduce biological significance. The *sn*-2 distribution feature of FA 18:1 can be noticed in TG molecular species TG 16:1_18:1_18:2 (TG 52:4), TG 16:0_18:1_18:2 (TG 52:3), TG 18:1_18:2_18:3 (TG 54:6), TG 18:1_18:1_18:2 (TG 54:4) and TG 18:0_18:1_18:2 (TG 54:3) (Fig. 4).

4. Conclusion

TG regioisomer information of plant oils is important to provide a deeper insight into their physicochemical, functional, and nutritional qualities. However, the current methodologies and data treatment result in a lack of thorough understanding of TG regioisomers in plant oils. To fill the knowledge gap, this study investigated the comprehensive regioisomer profile of TGs in 18 commercially important plant oils using direct inlet NICI-MS/MS method coupled with automated MSPECTRA software for calculations. The studied plant oils were grouped into five groups based on observed variation in TG ACN:DB species. Even though the dominant TG species varied among the groups, the regioisomeric compositions within each ACN:DB species were similar. The most abundant TG species among oils were: TG 16:0_18:1_18_1, TG 18:1_18:2_18:2, TG 18:1_18:1_18:2, TG 18:2_18:2_18:3, TG 18:1_16:1_18:1, and TG 16:0_18:1_18:2. In most TG molecular species, FA 18:1 showed a unique *sn*-2 positional preference when it was present in TG together with two other UFAs, the biological significance of which deserves further study. TG regioisomer results obtained with NICI-MS/MS method provide reliable information on FA species-level composition. This study provides a valuable reference for further studies on plant-oil-based food and nutraceuticals. Further application of the method could resolve the regioisomer profiles of a wider range of natural fats and oils, such as algae and insect oils. The method was proved to be a powerful tool for comprehensive analysis of regioisomer profiles of natural fats and oils without the need for chromatographic separation. As a major limitation, this method does not distinguish DB isomers of FAs. In addition, the method is only suitable for quantifying regioisomers of major TG species due to high deviation for low abundance TG species. These limitations further highlight the importance of alternative technologies for advanced structural analysis of TGs in natural fats and oils.

CRediT authorship contribution statement

Qizhu Zhao: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation. **Marika Kalpio:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Mikael Fabritius:** Writing – review & editing, Software, Methodology, Data curation. **Yuqing Zhang:** Writing – review & editing, Methodology. **Baoru Yang:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115710.

Data availability

Data will be made available on request.

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