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# Profiling of regioisomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry☆

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ABSTRACT

In this study, supercritical fluid chromatography (SFC) coupled with triple quadrupole mass spectrometry was applied to the profiling of several regioisomeric triacylglycerols (TAGs). SFC conditions (column, flow rate, modifier) were optimized for the effective separation of TAGs. In the column test, a triacontyl (C30) silica gel reversed-phase column was selected to separate TAG regioisomers. Multiple reaction monitoring was used to selectively quantify each TAG. Then, the method was used to perform detailed characterization of a diverse array of TAGs in palm and canola oils. Seventy TAGs (C46:0–C60:2) of these oils were successfully analyzed as a result, and twenty isomeric TAG pairs were separated well. In particular, this method provided the fast and high resolution separation of six regioisomeric TAG pairs (PPLn/PLnP, PPL/PLP, PPO/POP, SPLn/SLnP, SPO/SOP, SSO/SOS–stearic acid (S, 18:0), oleic acid (O, 18:1), linoleic acid (L, 18:2), linolenic acid (Ln, 18:3), palmitic acid (P, 16:0)) in a short time (50 min) as compared to high performance liquid chromatography. We were able to demonstrate the utility of this method for the analysis of regioisomeric TAGs in edible oils.



## 1. Introduction

In lipidomics, the profiling of triacylglycerol (TAG) can be very challenging due to the diversity and complexity of its structure [1,2]. The existence of various TAG molecular species is determined by the composition and distribution of FAs at the different stereochemical positions (*sn*-1, 2, and 3) on the glycerol backbone [3]. The distribution of FA is not particularly random; rather, it is characteristic for vegetable oils: saturated FAs occupy the *sn*-1/3 positions, and unsaturated FAs occupy the *sn*-2 position [4]. TAG is the major component of vegetable oils [4–7] and animal fats [8,9]. TAG structure determines the physical, chemical, and nutritional properties of edible oils, thus determining the quality of oils using TAG profiling is critical [10,11]. Many researchers have characterized the structure of a diverse array of TAGs in plants, animals, and fish for these reasons [12–15].

Because there are numerous isomers for TAGs, different compositions of FAs can have the same *m*/*z* ratio in mass spectrometric analysis. In particular, TAG is consisted of two acyl chain As and one acyl chain B, two regioisomers AAB and ABA, depending on which chain occupies the *sn*-2 position, exist [16,17]. Many studies have reported on the analysis of TAG regioisomers by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). Leskinen et al. quantified four regioisomeric TAG pairs based on the preferential formation of diacylglycerol (DAG) product ions by loss of *sn*-1/3 FAs from a TAG ion [18]. By way of collision-induced dissociation (CID), the loss of *sn*-1/3 FAs is more favorable than the loss of a *sn*-2 FA, which allows the composition of FAs in a TAG to be found based on the observed product ions. The intensity of the DAG ions is also used to elucidate the distribution of FAs in TAGs. In the specific case of a regioisomeric TAG pair that follows the scheme, AAB and ABA, these TAGs produce the same product ions, $[AA]^+$ and $[AB]^+$, but they differ in their intensity ratio, denoted as $[AB]^+/[AA]^+$ [19]. There was insufficient separation of the regioisomeric TAG pairs using the method by Leskinen et al.; therefore, the formation of DAG ions was not clearly dependent on the positional distribution of the FAs. Finally, it was demonstrated that the effective separation is critical to the selective quantification of TAG regioisomers.

Two HPLC techniques, reversed-phase HPLC (RP-HPLC) [18–21] and silver ion normal phase HPLC (Ag-HPLC) [22–24] have been widely applied to the separation of TAG regioisomers. In RP-HPLC, the retention of TAGs is governed by the equivalent carbon number (ECN = CN–2DB) [25]. Under the optimized RP-HPLC system, most TAGs were separated according to the ECN with the exception of regioisomers. The partial separation of regioisomers was previously achieved with very long retention times, which is not ideal for

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practical analysis [20]. In Ag-HPLC, TAGs are separated according to the number and position of DBs. In a previous study, the separation of regioisomeric TAG pairs containing up to seven DBs was performed in 90 min by Ag-HPLC/MS [17]. Furthermore, the off-line 2D coupling of RP-HPLC and Ag-HPLC provided chromatographic selectivity for various TAG regioisomers [26]. Finally, the high resolution analysis of TAG regioisomers was achieved by using HPLC. However, there still remains a need to develop a fast analytical method for TAG regioisomers, and when there are a relatively large number of samples, a high-throughput system would be required for the practical analysis.

In a previous study, the utility of supercritical fluid chromatography (SFC) was demonstrated in the analysis of diverse lipids [27–29]. Thus, SFC/MS with monolith octadecyl-silica (ODS) columns was applied to the analysis of TAGs in soybean [30]. As a result, several TAGs were effectively separated in a short analysis time (8 min) compared to conventional HPLC. Though SFC was useful for high-throughput profiling, it was not yet effective to separate and identify TAG isomers. Furthermore, the need to develop a selective and sensitive quantification method using MS/MS still remains. Therefore, the objective of this study is to develop a high-throughput and high-resolution profiling method for TAG regioisomers by SFC/MS/MS.

## 2. Materials and methods

### 2.1. Materials

Carbon dioxide (99.9% grade; Neriki Valve Co., Ltd, Amagasaki, Japan) was used as the mobile phase. HPLC-grade methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 0.1% (w/w) ammonium formate (99.99%; Sigma–Aldrich Japan K.K., Tokyo, Japan) was used as the modifier. The standard samples used in this study were as follows: 1,2-distearoyl-3-oleoyl-glycerol (SSO), 1,3-distearoyl-2-oleoyl-glycerol (SOS), 1-stearoyl-2-oleoyl-3-linoleoyl-glycerol (SOL), triolein (OOO), 1,2-dioleoyl-3-linoleoyl-glycerol (OOL), 1,2-dilinoleoyl-3-oleoyl-glycerol (LLO), 1,3-dilinoleoyl-2-oleoyl-glycerol (LOL), trilinolein (LLL), 1-stearoyl-2-oleoyl-3-palmitoyl-glycerol (SOP), 1-stearoyl-2-linoleoyl-3-palmitoyl-glycerol (SLP), 1,2-dioleoyl-3-palmitoyl-glycerol (OOP), 1-linoleoyl-2-oleoyl-3-palmitoyl-glycerol (LOP), 1,2-dilinoleoyl-3-palmitoyl-glycerol (LLP), 1,2-dipalmitoyl-3-stearoyl-glycerol (PPS), 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO), 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), and 1,2,3-triheptadecenoyl-glycerol (MoMoMo), which were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Incidentally, abbreviation of stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), margaroleic acid (17:1), and palmitic acid (16:0) are S, O, L, Ln, Mo, and P, respectively.

### 2.2. Sample preparation

Each TAG standard was prepared by dissolving it in chloroform, and these standard solutions were then stored at −30 °C. Before use, each solution was diluted to the desired concentration with chloroform. Furthermore, commercially obtained palm and canola oils were used for the analysis of biological samples. These oils were also dissolved in chloroform, and triheptadecenoin (MoMoMo, 17:1–17:1–17:1) was used as the internal standard (IS).

### 2.3. Columns

The columns used in this study were as follows: Inertsil ODS-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences Inc., Tokyo, Japan), Inertsil ODS-P column (250 × 4.6 mm ID; 5 μm, GL Sciences), Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences), Inertsil ODS-SP column (250 × 4.6 mm ID; 5 μm, GL Sciences), InertSustain C18 column (250 × 4.6 mm ID; 5 μm, GL Sciences), Sunrise C18 column (250 × 4.6 mm ID; 5 μm, ChromaNik Technologies Inc., Osaka, Japan), Sunrise C18-SAC column (250 × 4.6 mm ID; 5 μm, ChromaNik Technologies Inc.), Sunniest C18 column (250 × 4.6 mm ID; 5 μm, ChromaNik Technologies Inc.), and YMC carotenoid column (250 × 4.6 mm ID; 4 μm, YMC Co., Ltd., Kyoto, Japan). The characteristics of these columns were briefly described in Supplementary Table 1.

### 2.4. SFC/triple quadrupole (QqQ) MS conditions

SFC/QqQ MS analysis was performed using an Ultra Performance Convergence Chromatography (Waters, Milford, MA, USA) and a Xevo TQ mass spectrometer (Waters). The SFC and QqQ MS systems were controlled by MassLynx software. SFC conditions were as follows: mobile phase, supercritical carbon dioxide; modifier, methanol with 0.1% (w/w) ammonium formate; outlet pressure, 1500 psi; initial inlet pressure, 2200 psi; column temperature, 35 °C. For each run, 5 μL of the sample was injected by the full sample loop injection method. QqQ MS analysis was carried out in the positive ion mode of electrospray ionization (ESI) under the following conditions: capillary voltage, 3000 V; source temperature, 150 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h; collision gas flow rate, 12 mL/h; MS collision energy (CE), 20 eV; extractor voltage, 3 V.

## 3. Results and discussion

### 3.1. Construction of MRM transition for TAG profiling

For the detailed characterization of numerous TAGs in edible oils, a highly sensitive and highly selective detection method is required due to the complexity of the TAG structure. The large number of FA combinations on the glycerol backbone makes for a very diverse array of TAG compositions. The structures of isomeric TAGs that have the same *m/z* ratio, despite their different compositions, are especially difficult to determine using a single MS. When the regioisomeric TAG pairs are also mixed with other isomers, the MS/MS spectrum data obtained from the fragmentation is essential to analyze each TAG molecule.

For the selective detection of TAGs in this study, we applied multiple reaction monitoring (MRM), which is a non-scanning technique that can be performed on a QqQ MS. In the MRM of TAGs, two mass analyzers were used to monitor a particular product ion, DAG of a selected precursor ion, TAG. For the most effective detection, the MRM parameters, such as MRM transitions (precursor *m/z* (Q1) > product *m/z* (Q3)), cone voltage (CV), and MS/MS CE were optimized. First, the MRM transition was designed to analyze TAGs. In the positive mode of ESI, the ammoniated TAG ion ($[M+NH_4]^+$) was detected as the precursor *m/z*. In the CID-based fragmentation, TAG follows a specific pattern in its generation of product ions. With neutral losses of a FA from each TAG, the DAG ions ($[M+NH_4-RCOONH_4]^+$) were detected as the product ions of a particular *m/z*. In order to develop a TAG profiling method, 16 TAG standards (SSO, SOS, SLS, SOL, OOO, OOL, LLO, LOL, LLL, SOP, SLP, OOP, LOP, LLP, PPO, POP) were used, and MRM transitions for these TAGs were constructed (Supplementary Table 2). Based on the product ion scan, several product ions of a selected precursor ion were initially monitored (Supplementary Fig. 1). In these data, the isomeric TAG pairs, SOL/OOO and SLP/OOP, produced different detectable product ions. For example, SOL produces three DAGs, $[SL]^+$, $[LO]^+$, and $[SO]^+$, as the product ions, whereas OOO produces only $[OO]^+$ as its product ion. This makes MRM a selective detection technique because a different product ion was selected as Q3 to