Journal of Oleo Science
Copyright ©2013 by Japan Oil Chemists' Society
J. Oleo Sci. **62**, *(10) 789-794 (2013)*

JOURNAL OF OLEO SCIENCE
JOS

RAPID PAPER

Quantification of Triacylglycerol Molecular Species in Cocoa Butter Using High-Performance Liquid Chromatography Equipped with Nano Quantity Analyte Detector

Fumiaki Beppu[1], Toshiharu Nagai[2], Kazuaki Yoshinaga[2], Hoyo Mizobe[2], Koichi Kojima[2] and Naohiro Gotoh[1]*

[1] *Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan*
[2] *Tsukishima Foods Industry Co. Ltd., 3-17-9, Higashi Kasai, Edogawa-ku, Tokyo 134-8520, Japan*

Abstract: Triacylglycerol (TAG) molecular species were quantified through high-performance liquid chromatography (HPLC) equipped with a nano quantity analyte detector (NQAD). TAG standard compounds, i.e., 1,3-dipalmitoyl-2-oleoylglycerol (β-POP), 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol (β-POS), and 1,3-distearoyl-2-oleoylglycerol (β-SOS), and natural cocoa butter were used for analyses. NQAD gave the first order equation passing through the origin for all TAG standard compounds. TAG molecular species in cocoa butter were quantified using the calibration curves and the obtained values were almost the same as the reported ones of conventional cocoa butter. Furthermore, a recovery test was also carried out and the values were almost 100. Therefore, HPLC-NQAD can be successfully used for the quantification of TAG molecular species in natural fats and oils.

Key words: Calibration curve, Nano quantity analyte detector (NQAD), Quantification, Triacylglycerol molecular species

1 INTRODUCTION

The main component of lipids is triacylglycerol (TAG). TAG comprises one glycerol molecule and three fatty acid molecules[1]. As many types of fatty acids exist in nature, the combination patterns of the three fatty acids are almost infinite. The idea taking account of the combination of three fatty acids in TAG is called "TAG molecular species". Fats and oils consist of many TAG molecular species and the physical properties of each TAG molecular species are different. The physical properties of TAG molecular species significantly affect their emulsification properties during food processing[2]. Consequently, it is important to develop a method of quantifying the ratio of TAG molecular species in fats and oils for complete characterization.

To date, many methods have been developed to quantify TAG molecular species in fats and oils. A gas chromatography-flame ionization detector (GC-FID) system[3, 4] is employed in the official method for quantifying TAG molecular species in fats and oils[5]; however, this method is not suitable for the analysis of TAG molecular species with high boiling points and/or oxidatively susceptible highly unsatu-

Abbreviations: AOCS, American Oil Chemist's Society; β-POP, 1,3-dipalmitoyl-2-oleoylglycerol; β-POS, 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol; β-PPO, 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol; β-SOS, 1,3-distearoyl-2-oleoylglycerol; $C_{11}C_{11}C_{11}$, 1,2,3-triundecanoylglycerol; CAD, charged aerosol detector; ECN, equivalent carbon number; ELSD, evaporative light-scattering detector; GC-FID, gas chromatography—flame ionization detector; HPLC, high performance liquid chromatography; IS, internal standard; IUPAC, International Union of Pure and Applied Chemistry; JOCS, Japan Oil Chemists' Society; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometer; NQAD, nano quantity analyte detector; PN, partition number; POP, mixture of β-POP and β-PPO; RI, refractive index; RP-HPLC, reverse phase-high performance liquid chromatography; *s/n*, signal-noise ratio; TAG, triacylglycerol; WCPC, water-based condensation particle counter

*Correspondence to: Naohiro Gotoh, Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
E-mail: ngotoh@kaiyodai.ac.jp
Accepted July 21, 2013 (received for review June 21, 2013)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

rated fatty acids. To overcome this disadvantage, a reverse phase-high performance liquid chromatography (RP-HPLC) method was developed nearly 35 years ago for the separation of TAG molecular species. This separation method introduced the idea named "Partition Number (PN)"[6] or "Equivalent Carbon Number (ECN)"[7]. This idea has been used for the identification of fats and oils, e.g. assessment of adulteration of extra virgin olive oil[8]. The PN (or ECN) is calculated as follows: PN = CN − 2 × DB, where CN is the total number of acyl carbons and DB is the total number of double bonds in the TAG molecule. TAG molecular species elute in the order of the PN (or ECN). A silver ion column has also been used for the separation of TAG molecular species and TAG isomers based on the fact that silver ions can interact with the π electrons at double bonds in organic compounds[9−11]. The elution order of TAG molecular species depends on the number of double bonds, and the TAG molecular species with none or fewer double bonds in their structure elute earlier. Both reverse phase HPLC and silver ion column HPLC are currently very popular for the separation of TAG molecular species. However, the detectors that are used to quantify TAG molecular species have drawbacks. Many types of detectors are used with the HPLC system for the detection of TAG molecular species. The choice of detector depends on the objective of the study. Analysis of the composition of TAG molecular species requires a non-selective detector such as a refractive index (RI) detector, UV detector, evaporative light-scattering detector (ELSD), and mass spectrometer (MS). The HPLC system combined with an RI, a UV, or an MS detector is used for the analysis of TAG molecular species in natural fats and oils by American Oil Chemists' Society (AOCS) Official Methods[12, 13]. A UV detector is employed by the AOCS Official Method; however, the response of the UV detector is highly dependent on the number of double bonds[14]. For example, TAG consisting of three saturated fatty acids does not have strong UV absorption. The sensitivity of the RI detector is not sufficient for detecting minor TAG molecular species in natural fats and oils. However, RI detector is used in Japan Oil Chemists' Society (JOCS)[15], AOCS Official Methods[12, 13], and International Union of Pure and Applied Chemistry (IUPAC)[16] because of its good quantitative accuracy. Although ELSD is highly sensitive, it does not have linear calibration curves[17]. Therefore, a transport-flame ionization detector, which can theoretically quantify TAG molecular species, has been used for the detection and quantification of TAG molecular species[18, 19]. However, the use of commercial instruments leads to several problems; therefore, the transport-flame ionization detector is not widely applied. MS has also been used to analyze TAG molecular species[12, 13, 20−22]. An MS operated in selected-ion–recording or multiple-reaction-monitoring mode has a wide range of linearity that enables highly selective detection of specific TAG molecular species. However, these detection modes give different intensities for each type of TAG molecular species[22]. Although the corona charged aerosol detector (CAD) has been considered for the quantification of TAG molecular species, the obtained calibration curve is not linear[23].

Nearly 20 years ago, nano-quantity analyte detectors (NQAD), which are also known as condensation nucleation light-scattering detectors, were developed[24−26]. Similar to ELSD and CAD, this detector requires evaporation of the eluent of the column by a nebulizer. Dried analytes formed by the nebulizer are moved to a water-based condensation particle counter (WCPC) to count the number of particles. The WCPC condenses water vapor onto the particles and increases their size so that they could be detected individually using a laser-based optical sensor. This detection method has a linear response to macrolides[27], amino acids[28], aromatic compounds[29], vitamin E[29], fatty acids[29], etc. Therefore, we applied HPLC-NQAD to the quantification of TAG molecular species and confirmed the linearity of the calibration curves.

2 EXPERIMENTAL

2.1 Chemicals and materials

TAG molecular species (Fig. 1) such as 1,3-dipalmitoyl-2-oleoylglycerol (β-POP ($1)), 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol (β-POS), 1,3-distearoyl-2-oleoylglycerol (β-SOS), and 1,2,3-triundecanoylglycerol ($C_{11}C_{11}C_{11}$) and two types of cocoa butters harvested in Indonesia (Cocoa Butter Asia) and Ghana (Cocoa Butter Africa) were manufactured or obtained as purified products (Tsukishima Foods Industry Co., Ltd., Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

($1: Two types of alcohol groups, i.e., primary and secondary, are contained in the glycerol backbone of TAG. The primary and secondary alcohol groups are also discriminated by their position, i.e., α and β, respectively. There are two types of TAG molecular species that comprise two types of fatty acids ("A" and "B") with two A and one B on the glycerol backbone, i.e., TAG with A located at the β position (β-AAB) and TAG with B locating at the β position (β-ABA). The "β-" in front of three fatty acid abbreviations distinguishes the TAG positional isomers.)

2.2 Analytical conditions

An HPLC-NQAD system consisting of a pump equipped with an auto sampler (Alliance e2695, Waters Corporation, Milford, MA), NQAD (NQAD 5600, Shiseido Co., Ltd., Tokyo, Japan), column oven (MO-706, GL Sciences Inc.), and C28 column (Sunrise C28, 4.6 mm i.d. × 250 mm, 5 μm, ChromaNik Technologies Inc., Osaka, Japan), was used for quantification of TAG molecular species. A mixture of

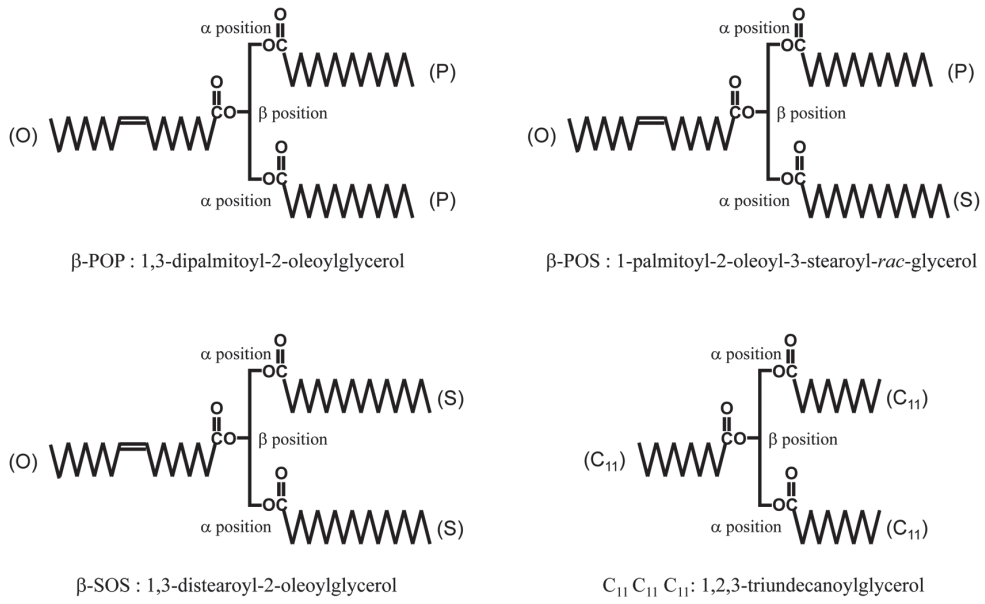

Fig. 1 Structures of TAG molecular species.

2-propanol and acetonitrile (75:25, v/v) was used as the mobile phase for separation of TAG molecular species. The flow rate, injection volume, column temperature, and autosampler temperature were 1.0 mL/min, 20 μL, 40℃, and 25℃, respectively. The nebulizer and evaporator temperatures are 30 and 90℃, respectively, under the detection conditions of the NQAD.

2.3 Preparation of calibration curves for TAG molecular species

Calibration curves for β-POP, β-POS, and β-SOS were obtained using the HPLC-NQAD chromatogram peak area. $C_{11}C_{11}C_{11}$ was used as an internal standard (IS) and the concentration ratio (sample TAG/$C_{11}C_{11}C_{11}$) and chromatogram peak area ratio (sample TAG/$C_{11}C_{11}C_{11}$) are plotted on the x- and y-axes, respectively. β-POP, β-POS, and β-SOS were mixed and dissolved in acetone at concentrations of 500, 250, 100, 50, 10, and 5 ppm. All the sample solutions were also mixed with $C_{11}C_{11}C_{11}$ at a concentration of 100 ppm. The samples were injected into the HPLC-NQAD system and the peak areas were used to prepare the calibration curves. The concentration ratio was plotted on the x-axis and the chromatogram peak area ratio was plotted on the y-axis. All the calibration curves are expressed as the first-order equation passing through the origin to determine their linearity. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated using the β-POP solution and calculated using the signal-to-noise ratio (s/n). The LOD and LOQ were 3 and 10 (s/n), respectively[30].

2.4 Quantification of TAG molecular species in cocoa butter and recovery tests

The TAG molecular species in cocoa butter were quantified using the obtained calibration curves. Correctly quantified $C_{11}C_{11}C_{11}$ was added to the two types of cocoa butter. Each cocoa butter was diluted with acetone and injected into the HPLC-NQAD system. The TAG molecular species in the cocoa butters were calculated using the calibration curves obtained previously (\$2). The accuracy of this method was evaluated using a recovery test. The correctly quantified standard compounds (i.e., β-POP, β-POS, and β-SOS) and $C_{11}C_{11}C_{11}$ were added to the two cocoa butters and diluted with acetone. The cocoa butters with and without additional standard compounds were analyzed via HPLC-NQAD, and the chromatogram peak area was used for quantification. The TAG molecular species were also quantified using the calibration curves. The amounts of TAG molecular species with and without additional standard compounds were calculated, and the calculated and actual amounts were compared. The recovery rate was obtained using the following equation:

$$\text{Recovery rate}(\%) = (\text{calculated additional standard compound amount})/(\text{actual additional standard compound amount}) \times 100(\%).$$

(\$2: The HPLC conditions employed in this study cannot separate TAG positional isomers such as β-POP and 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol (β-PPO). Consequently, the obtained HPLC chromatogram peaks could reflect a mixture of TAG positional isomers. For this reason, the TAG molecular species in cocoa butter are indicated without the "β-" symbol; therefore, POP indicates a potential mixture of β-POP and β-PPO.

3 RESULTS AND DISCUSSION

The LOD and LOQ of the NQAD were calculated to be 6 and 20 pg, respectively, using the β-POP acetone solution. Gotoh et al. reported that the LOQs of the TAG molecular species in fish oil detected by ELSD were between 476 and 936 pg, which implies that the sensitivity of the NQAD is an order higher than that of ELSD[31]. Figure 2 shows the calibration curves obtained with the IS. The slopes of the calibration curves are expressed as first-order equations passing through the origin; their R^2 values are 0.99. The slopes for β-POP, β-POS, and β-SOS are almost the same. These results are remarkable because this detector is not only highly sensitive but also gives linear calibration curves. In the food industry, the quantification of TAG molecular species in fats and oils is very important. However, HPLC-RI and RI, which are mainly used in the present official methods, are not stable and sensitive detectors. Moreover, ELSD and CAD, which are well-known sensitive detectors, are not suitable for the quantification of TAG molecular species because they do not provide linear calibration curves. These disadvantages create problems for the quantification of TAG molecular species. So far, the linearity of the calibration curves of the NQAD has been confirmed for macrolides, amino acids, aromatic compounds, vitamin E, and fatty acids. Our findings prove that the NQAD also gives linear calibration curves for TAG molecular species. Thus, the use of HPLC-NQAD would eliminate all the disadvantages of the currently used detectors.

Cocoa butter consists of three main TAG molecular species, i.e., β-POS, β-POP, and β-SOS. The structures of these TAG molecular species impart the special characteristics of chocolate melting in our mouth, namely mouth feel. Therefore, the distribution of these TAG molecular species in cocoa butter has been well examined and it is thought to be a good target oil to confirm the accuracy of the results obtained by HPLC-NQAD. The chromatogram of TAG molecular species in Cocoa Butter Asia is shown in Fig. 3. There are many reports concerning the amounts of POP, POS, and SOS in cocoa butter. For example, Pease reported that cocoa butter contains 12.0, 34.8, and 25.2% POP, POS, and SOS, respectively[32]. As quantified by HPLC-NQAD, the POP, POS, and SOS contents of Cocoa Butter Asia and Cocoa Butter Africa are almost 17, 40, and 27%, respectively (Table 1). These results are reasonable. The ratios of POP, POS, and SOS in conventional cocoa butter were reported to be 22, 46, and 32, respectively[33]. For comparison, the ratios of POP, POS, and SOS obtained in this experiment are very similar at 21, 47, and 32, respectively (Table 1). These results indicate that quantification of the TAG molecular species in natural fats and oil is possible using HPLC-NQAD. Recovery tests were performed and the results are shown in Table 2. The recovery of this method is good and all the values were approximately 100. However, some standard deviation values were not low. We examined the detection conditions of the NQAD and found that the evaporator temperature significantly affects the

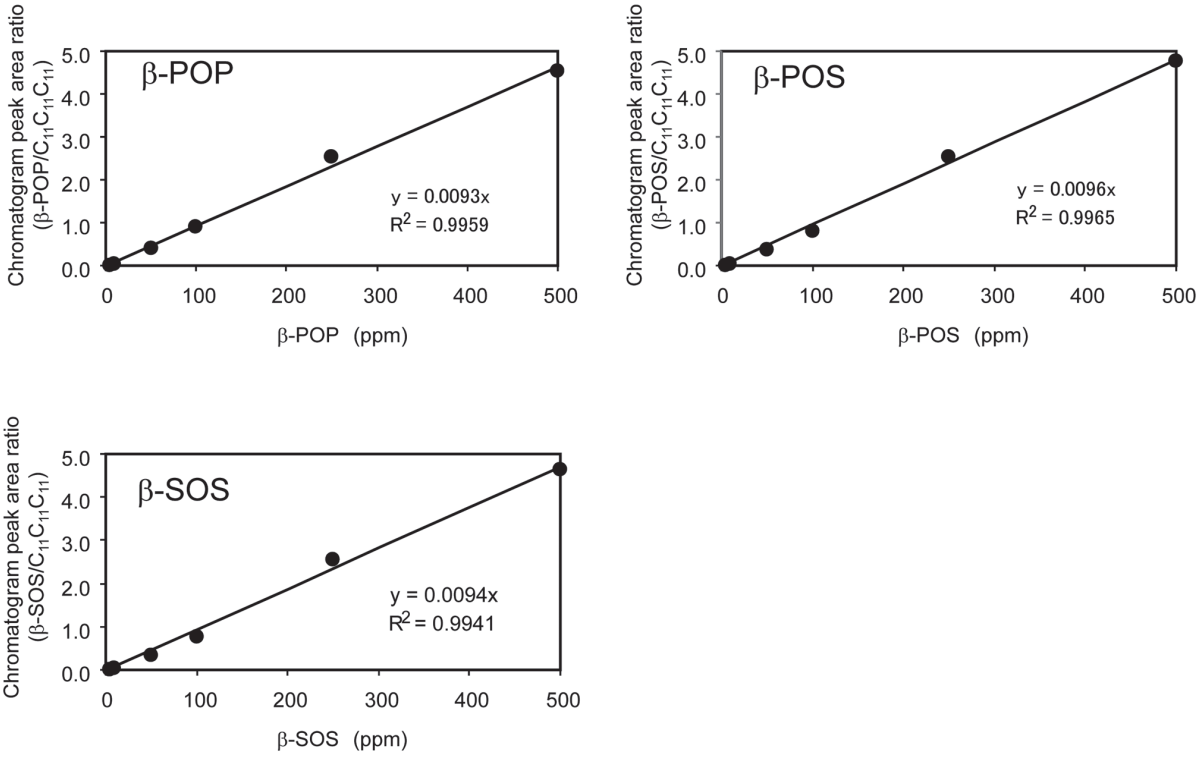

Fig. 2 Calibration curves for respective TAG molecular species.

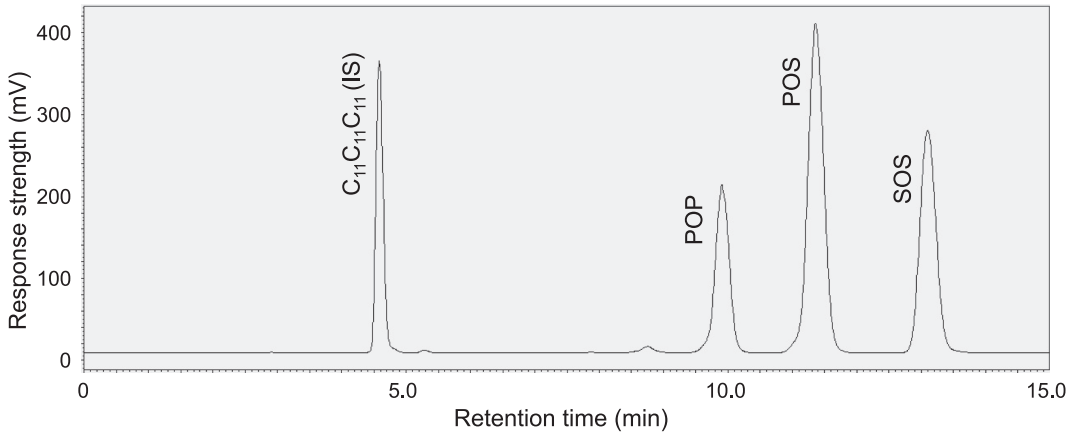

Fig. 3 Chromatogram of TAG molecular species in Cocoa Butter Asia.

Table 1 Ratio of TAG molecular species in cocoa butters and among three molecular species*.

	POP	POS	SOS	Others
Cocoa Butter Asia	17.4 ± 0.3 [21.0]*	39.4 ± 2.0 [47.5]*	26.2 ± 0.7 [31.6]*	16.9 ± 2.8
Cocoa Butter Africa	17.3 ± 0.3 [20.6]*	39.7 ± 1.1 [47.3]*	27.0 ± 0.4 [32.1]*	16.0 ± 1.6

(n = 6, mean ± SD)

Table 2 Recovery of POP, POS, and SOS in cocoa butter (%).

	POP	POS	SOS
Cocoa Butter Asia	104.6 ± 4.3	92.0 ± 13.5	112.1 ± 18.2
Cocoa Butter Africa	99.5 ± 19.6	93.6 ± 1.0	111.0 ± 8.6

(n = 3, mean ± SD)

recovery rate (data not shown). Further investigations are required to improve the accuracy of the quantification of TAG molecular species in natural fats and oils.

4 CONCLUSION

HPLC-NQAD, which is a very simple method, can be successfully applied for the quantification of TAG molecular species in natural fats and oils. This method can be used as a sensitive alternative to HPLC-RI, which is mainly employed in official methods such as JOCS official method 2.4.6.2-2013[15]. We strongly believe that the HPLC-NQAD system will be applied for the quantification of TAG molecular species.

References

1) Murray, R. K.; Granner, D. K.; Rodwell, V. W. *Harper's Illustrated Biochemistry*, 27th ed., McGraw-Hill Companies, New York. p.124 (2006).
2) Rousseau, D. Fat crystals and emulsion stability –a review. *Food. Res. Int.,* **33**, 3-14 (2000).
3) Marinetti, G. V. *Lipid Chromatographic Analysis*, 2nd ed., Marcel Dekker, New York and Basel p. 215 (1976).
4) Takagi, T.; Itabashi, Y. Gas chromatographic separation of triglycerides based on their degree of unsaturation. *Lipids,* **12**, 1062-1068 (1977).
5) Triacylglycerol composition (gas chromatography), *JOCS Official Method*, 2.4.6.1-2013.
6) Wada, S.; Koizumi, C.; Nonaka, J. Analysis of triacylglycerides of soybean oil by high-performance liquid chromatography in combination with gas liquid chromatography. *Yukagaku,* **26**, 95-99 (1977).
7) Plattner, R. D.; Spencer, G. F.; Kleiman, R. Triglycerides separation by reverse phase high performance liquid chromatography. *J. Am. Oil Chem. Soc.,* **54**, 511-515 (1977).
8) Andrikopoulos, N. K.; Giannakis, I. G.; Tzamtzis, V. Analysis of olive oil and seed oil triacylglycerides by

capillary gas liquid chromatography as a tool for the detection of the adulteration of olive oil. *J. Chromatogr. Sci.,* **39**, 137-145(2001).
9) Christie, W. W. Separation of molecular species of triacylglycerols by HPLC with a silver ion column. *J. Chromatogr.,* **454**, 273-284(1988).
10) Nikolova-Damyanova, B.; Christie, W. W.; Herslof, B. Retention properties of triacylglycerols on silver ion HPLC. *J. Chromatogr. A,* **694**, 375-380(1995).
11) Adlof, R. O. Analysis of triacylglycerol positional isomers by silver ion HPLC. *J. High Resolut. Chromatogr.,* **18**, 105-107(1995).
12) Triglycerides in vegetable oils by HPLC, *AOCS Official Method*, Ce 5b-89.
13) Individual triglycerides in oils and fats by HPLC, *AOCS Official Method*, Ce 5c-93.
14) Aoki, T.; Otake, I.; Gotoh, N.; Noguchi, N.; Wada, S. Quantification method for triglyceride molecular species in fish oil with high performance liquid chromatography-ultraviolet detector. *J. Oleo Sci.,* **53**, 285-294(2004).
15) Triacylglycerol composition(high performance liquid chromatography), *JOCS Official Method*, 2.4.6.2-2013.
16) Wolff, J. P.; Mordret, F. X.; Dieffenbacher, A. Determination of triglycerides in vegetable oils in terms of their partition numbers by high performance liquid chromatography. *Pure & Appl. Chem.,* **63**, 1173-1182 (1991).
17) Christie, W. W. Evaporative light-scattering detectors in lipid analysis. *Lipid Technol.,* **5**, 68-70(1993).
18) Pearson, C. D.; Gharfeh, S. G. Automated high-performance liquid chromatography determination of hydrocarbon types in crude oil residues using a flame ionization detector. *Anal. Chem.,* **58**, 307-311(1986).
19) Neff, W. E.; List, G. R.; Byrdwell, W. C. Analysis of triacylglycerol positional isomers in food products as brominated derivatives by high performance liquid chromatography coupled with a flame ionization detection. *J. Chromatogr. A,* **912**, 187-190(2001).
20) Byrdwell, W. C.; Emken, E. A.; Neff, W. E.; Adlof, R. O. Quantitative analysis of triglycerides using atomospheric pressure chemical ionization-mass spectrometry. *Lipids,* **31**, 919-935(1996).
21) Gotoh, N.; Matsumoto, Y.; Nagai, T.; Mizobe, H.; Yoshinaga, K.; Kojima, K.; Kuroda, I.; Kitamura, Y.; Shimizu, T.; Ishida, H.; Wada, S. Actual ratio of triacylglycerol positional isomers in milk and cheese. *J. Oleo Sci.,* **61**, 173-180(2012).
22) Holčapek, M.; Lísa, M.; Jandera, P.; Kabátová, N. Quantification of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. *J. Sep. Sci.,* **28**, 1315-1333(2005).
23) Veroort, V.; Daemen, D.; Török, G. Performance evaluation of evaporative light scattering detection and charged aerosol detection in reversed phase liquid chromatography. *J. Chromatogr. A,* **1189**, 92-100 (2008).
24) Allen, L. B.; Koropchak, J. A. Condensation nucleation light scattering: A new approach to development of high sensitivity, universal detectors for separations. *Anal. Chem.,* **65**, 841-844(1993).
25) Allen, L. B.; Koropchak, J. A.; Szostek, B. Condensation nucleation light scattering detection for conventional reversed-phase liquid chromatography. *Anal. Chem.,* **67**, 659-666(1995).
26) Koropchak, J. A.; Heenan, C. L.; Allen, L. B. Direct comparison of evaporative light scattering and condensation nucleation light scattering detection for liquid chromatography. *J. Chromatogr.,* **736**, 11-19 (1996).
27) Olsovska, J.; Kamenik, Z.; Cajthaml, T. Hyphenated ultra high-performance liquid chromatography-Nano Quantity Analyte Detector technique for determination of compounds with low UV absorption. *J. Chromatogr. A,* **1216**, 5774-5778(2009).
28) Holzgrabe, U.; Nap, C. J.; Beyer, T.; Almeling, S. Alternative to amino acid analysis for the purity control of pharmaceutical grade L-alanine. *J. Sep. Sci.,* **33**, 2402-2410(2010).
29) Hutchinson, J. P.; Li, J.; Farrell, W.; Groeber, E.; Szucs, R.; Dicinoski, G.; Haddad, P. R. Comparison of the response of four aerosol detectors used with ultra high pressure liquid chromatography. *J. Chromatogr. A,* **1218**, 1646-1655(2011).
30) Hernandez, M. J.; Garcia-Moreno, M. V.; Duran, E.; Guillen, D.; Barroso, C. G. Validation of two analytical methods for the detection of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia. *Anal. Chim. Acta,* **566**, 117-121(2006).
31) Gotoh, N.; Aoki, T.; Nakayasu, K.; Tokairin, S.; Noguchi, N.; Wada, S. Quantification method for triacylglycerol molecular species in fish oil with high performance liquid chromatography-ultraviolet detector - evaporative light scattering detector. *J. Oleo Sci.,* **55**, 457-463(2006).
32) Pease, J. J. Confectionery fats from palm oil and lauric oil. *J. Am. Oil Chem. Soc.,* **62**, 426-430(1985).
33) Sato, K.; Sasaki, M.; Ueno, S. Physical analysis of mixing phase behavior of POP/POS/SOS. *Proceedings of 101st AOCS Annual Meeting & Expo*, Phoenix, AZ (2010). http://www.aocs.org/archives/am2010/index.cfm?page=101st-AOCS-Annual-Meeting-Program-Tech-Prog&interest=Edible%20Applications%20Technology(accessed June 21, 2013).