JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development of an Offline Bidimensional High-Performance Liquid Chromatography Method for Analysis of Stereospecific Triacylglycerols in Cocoa Butter Equivalents

Sheida Kadivar,[†] Nathalie De Clercq,[†] Bangun Prajanto Nusantoro,[†] Thien Trung Le,[‡] and Koen Dewettinck*[,†]

[†]Laboratory of Food Technology and Engineering, Ghent University, Coupure links 653, B-9000 Gent, Belgium

[‡]Faculty of Food Science and Technology, Nong Lam University—Ho Chi Minh City, Thu Duc District, Ho Chi Minh City, Vietnam

ABSTRACT: Acyl migration is a serious problem in enzymatic modification of fats and oils, particularly in production of cocoa butter equivalent (CBE) through enzymatic acidolysis reaction, which leads to the formation of non-symmetrical triacylglycerols (TAGs) from symmetrical TAGs. Non-symmetrical TAGs may affect the physical properties of final products and are therefore often undesired. Consequently, an accurate method is needed to determine positional isomer TAGs during the production of CBE. A bidimentional high-performance liquid chromatography (HPLC) method with combination of non-aqueous reversed-phase HPLC and silver ion HPLC joining with an evaporative light scattering detector was successfully developed for the analysis of stereospecific TAGs. The best separation of positional isomer standards was obtained with a heptane/acetone mobile-phase gradient at 25 °C and 1 mL/min. The developed method was then used in multidimensional determination of the TAG positional isomers in fat and oil blends and successfully identified the TAGs and possible isomers in enzymatically acidolyzed CBE.

KEYWORDS: *Stereospecific triacylglycerols, non-aqueous reversed-phase liquid chromatography, silver ion liquid chromatography, cocoa butter equivalent*

INTRODUCTION

Triacylglycerols (TAGs) are the main components of fats and oils. For food products containing significant amounts of oil, the functional and nutritional properties of the product is dependent upon not only their fatty acid (FA) profiles but mainly the FA distributions on the glycerol backbone.[1] Cocoa butter (CB) for example contains more than 75% of symmetrical TAGs with oleic acid in the sn-2 position, i.e., POP, POSt, and StOSt. Cocoa butter equivalents (CBEs) are non-lauric fats with similar physicochemical characteristics as CB and are therefore compatible with CB in every amount without changing the properties of CB. The development of enzyme technology makes it possible to produce CBE from other vegetable oils. Using 1,3-specific lipase in acidolysis reactions, all of the three major TAGs of CB can be produced from vegetable oils, in which the sn-2 position of their glycerol backbone is mostly oleic acid.[2]

However, acyl migration is a major problem during the production of CBEs in acidolysis reactions, which cannot be easily avoided. Generally, acyl migration can occur inter- and intramolecularly, especially in the presence of partial acylglycerols, mainly diacylglycerols (DAG), which are the inevitable intermediates in lipase-catalyzed acidolysis.[3] During the acidolysis reaction, to produce CBE, some of the symmetrical TAGs are converted to non-symmetrical TAGs, which are characterized by a different crystallization behavior, leading to different properties of chocolate. Vereecken et al. reported that fat blends containing a higher amount of asymmetric monounsaturated TAGs (SSM) have a slower crystallization rate.[4] Therefore, a reliable method is helpful to evaluate CBE in terms of the residue of these types of TAGs, which may help to improve the production process.

The knowledge of TAG stereospecific structures is also important for studies on structured lipids, such as milk fat substitutes for infant milk formulas and specific-structured lipids. Specific-structured lipids are TAGs containing both long-chain FAs (mostly essential FAs), which are located specifically at the sn-2 position, and medium-chain FAs, which are located specifically at the sn-1,3 positions of the glycerol backbone.[5−7] In most physical and nutritional purposes, a separation between the sn-2 and sn-1/sn-3 positions is required.[8−10]

Normal-phase high-performance liquid chromatography (NP-HPLC), reversed-phase HPLC (RP-HPLC), and silver ion HPLC (Ag^+-HPLC) modes have been extensively used. The latter two are the most useful techniques for the determination of TAG composition.[11]

Non-aqueous reversed-phase (NARP)-HPLC can give information about the degree of unsaturation and carbon number (CN) values of TAGs. This method determines a separation based on partition number (PN) values: PN = CN − 2DB, where DB is the total double bond number. However, using this technique is not efficient in the separation of TAGs with the same PN, e.g., with the difference in positional isomers (PIs).[12]

Received: April 10, 2013
Revised: July 13, 2013
Accepted: July 26, 2013
Published: July 26, 2013

ACS Publications © 2013 American Chemical Society

Table 1. TAG Composition of Starting Oils and Composition of Two Blends in Terms of Starting Oils

TAG/blend[a]	P_s[b]	PMF[c]	iP_s[d]	$fhSH_o$[e]	SH_s[f]	iSH_s[g]	HOSO[h]	PBB[i]	StBB[j]
SSS	76.1	4.5	13.9	94.8	2.9	5.4	2.9	9	10
SOS, SSO	13.7	82.2	69.2	2.8	84	63.5	2.9	37	37
SLS, SSL	2.7	6.7	10.8	0.6	6.8	4.8	0.3	4	2
SOO, OSO	4.3	3.7	3.8	0.6	4.6	19.9	19.2	11	14
SOL, SLO, OSL	1.6	1	1	0.2	0.6	2.7	1.4	1	1
OOO	0.6	0.3	0.2	0.3	0.4	2.6	65.4	34	32
>3 DB	0.5	0.2	0.1	0	0.1	0.8	7.4	4	4
PBB	3.5	16.5	30	0	0	0	50		
StBB	0	0	0	5	18.5	30	46.5		

[a]S, saturate; O, oleic acid; and L, linoleic acid. [b]P_s = palm stearin. [c]PMF = palm mid fraction. [d]iP_s = interesterified palm stearin. [e]$fhSH_o$ = fully hardened shea olein. [f]SH_s = shea stearine. [g]iSH_s = interesterified shea stearin. [h]HOSO = high oleic sunflower oil. [i]PBB = palmitic-based blend. [j]StBB = stearic-based blend.

In Ag^+-HPLC, the separation is based on the weak interaction between the silver ions and the π electrons of the double or triple bonds of the carbon chain of the fatty acyl moieties, excluding the impact of the particular packing material of the column. In general, the elution order of TAG using cation-exchange Ag^+-HPLC mode (ChromoSpher Lipids columns) is dependent upon the degree of saturation as follows: SSS > SSM > SSD > MMM > SMD > MMD > SDD = SST > SMT = MDD > MMT > SDT = DDD > MDT ≥ STT > DDT > MTT > DTT > TTT (where S, saturated; M, monounsaturate; D, diunsaturate; and T, triunsaturate FA residues on the glycerol backbone).[13] It was found that the saturated−monounsaturated−saturated (SMS) TAG, with a monoenoic acid (single double bond) at sn-2 and saturated components in the primary positions, makes the complex of silver ion and π electrons less strongly than the saturated−saturated−monounsaturated (SSM) TAG. This is probably because of the steric hindrance of the unsaturated FA residue when placed in this position.[13] It is expected that Ag^+-HPLC and RP-HPLC used in a complementary way could provide almost complete separation of the different TAG species.

Regio- and stereospecific determinations are very difficult to handle, often requiring several operational steps and long sample preparations. Determination of TAG PIs was first performed by enzymatic hydrolysis procedures. Pancreatic lipase hydrolysis methods were used for regiospecific analysis of FA at position sn-2.[10,14] Indirect methods, such as enzymatic and conventional chemical applications, require manual steps.[15,16] Direct methods, such as nuclear magnetic resonance spectrometry[17] and tandem mass spectrometry (MS),[18] are quite expensive and require complex calculations.[11] The separation of TAG PI pairs has been demonstrated using a RP-HPLC system equipped with a non-end-capped octadecylsilane (ODS) column. Using RP-HPLC on an ODS column, Momchilova et al. attained complete regioselective separation of five pairs of isomeric dipalmitoyl polyalkenoyl glycerols with two to six double bonds in the unsaturated acyl residues.[19] Gotoh et al. also demonstrated that non-end-capped polymeric ODS columns can separate TAG PIs consisting of two palmitic acids and one oleic acid or one palmitic acid and two oleic acids. However, non-end-capped polymeric ODS columns for the separation of TAG PIs are only applicable when the saturated FAs contain more than 12 carbons.[20] In contrast, Ag^+-HPLC can be an alternative to traditional techniques, although this method allows for the separation of PIs only when specific experimental conditions are used.[21]

The aim of this study was to develop a HPLC−evaporative light scattering detector (ELSD) method by investigating the existing methods and optimizing them to enable a quick separation of the TAG stereo species in blends of oils and fats. In the next step, the regioisomers of TAGs were determined using NARP- and Ag^+-HPLC. The sample was first fractionated in the NARP-HPLC, and each TAG fraction was then injected to a silver ion column, to gain regioisomer separation. Finally, the method was validated by possible TAG PIs in an enzymatically produced CBE sample.

MATERIALS AND METHODS

Materials and Chemicals. HPLC-grade heptane, acetone, acetonitrile, dichloroethane, and dichloromethane used as mobile phases were obtained from Acros Organics (Geel, Belgium). TAG standards, namely, triolein (OOO), tripalmitin (PPP), 1,3-dipalmitin-2-olein (POP), and 1,2-dipalmitin-3-olein (PPO), were obtained from Nu-Chek Prep, Inc. (Elysian, MN). High oleic sunflower oil (HOSO as the OOO source), palm stearin (P_s as the PPP source), palm mid fraction (PMF as the POP source), interesterified palm stearin (iP_s as the PPO source), fully hardened shea olein ($fhSH_o$ as the StStSt source), Shea stearin (SH_s as the StOSt source), and interesterified shea stearin (iSH_s as the StStO source) were provided by Loders Croklaan N.V. (Wormeveer, The Netherlands). Fatty acid mixtures (FAMs) were provided by Oleon (Oelegem, Belgium). Immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM, immobilized on ion-exchange resin, sn-1,3 specific) was purchased from Novozymes (Bagsvaerd, Denmark).

Methods. *Preparation of TAG PIs. Fat Blends.* A palmitic-based blend (PBB) and a stearic-based blend (StBB) with varying ratios of symmetric/asymmetric TAGs were formulated. Table 1 gives the formulation of the two investigated blends along with the resulting TAG composition.

Enzymatic Acidolysis. Acidolysis reaction with lipase was carried out in a glass container in a water bath. At the start of the reaction, substrates (1 mol of HOSO + 7 mol of FAM) and 1% water were mixed using a mechanical stirrer at 300 rpm and heated to 65 °C. The reaction began when enzyme (10%) was added. The reaction was stopped after 8 h by filtering out the lipase using a filter paper. The interesterified product was distilled in a short-path distillation unit (VTA, Deggendorf, Germany) at P = 0.003 mbar and T = 200 °C. At these conditions, free FAs were totally removed.

Multidimensional Analysis. First Dimension: NARP-HPLC−ELSD. Offline fractionation of TAG species was performed with an optimized method developed by Rombaut et al.[22] Separation of TAG species was performed on a Shimadzu HPLC system (Shimadzu, Japan) with four solvent lines, a degasser, an auto sampler, and LC solution software, which was coupled to an Alltech 3300 ELSD (Grace Alltech, Lokeren, Belgium) and a fraction collector (FRC-10ADvp). N_2 was used as the nebulizing gas at a flow of 1.4 mL/min and a nebulizing temperature of 38 °C. The gain was set at 1. The column was a 150 × 3.0 mm Alltima

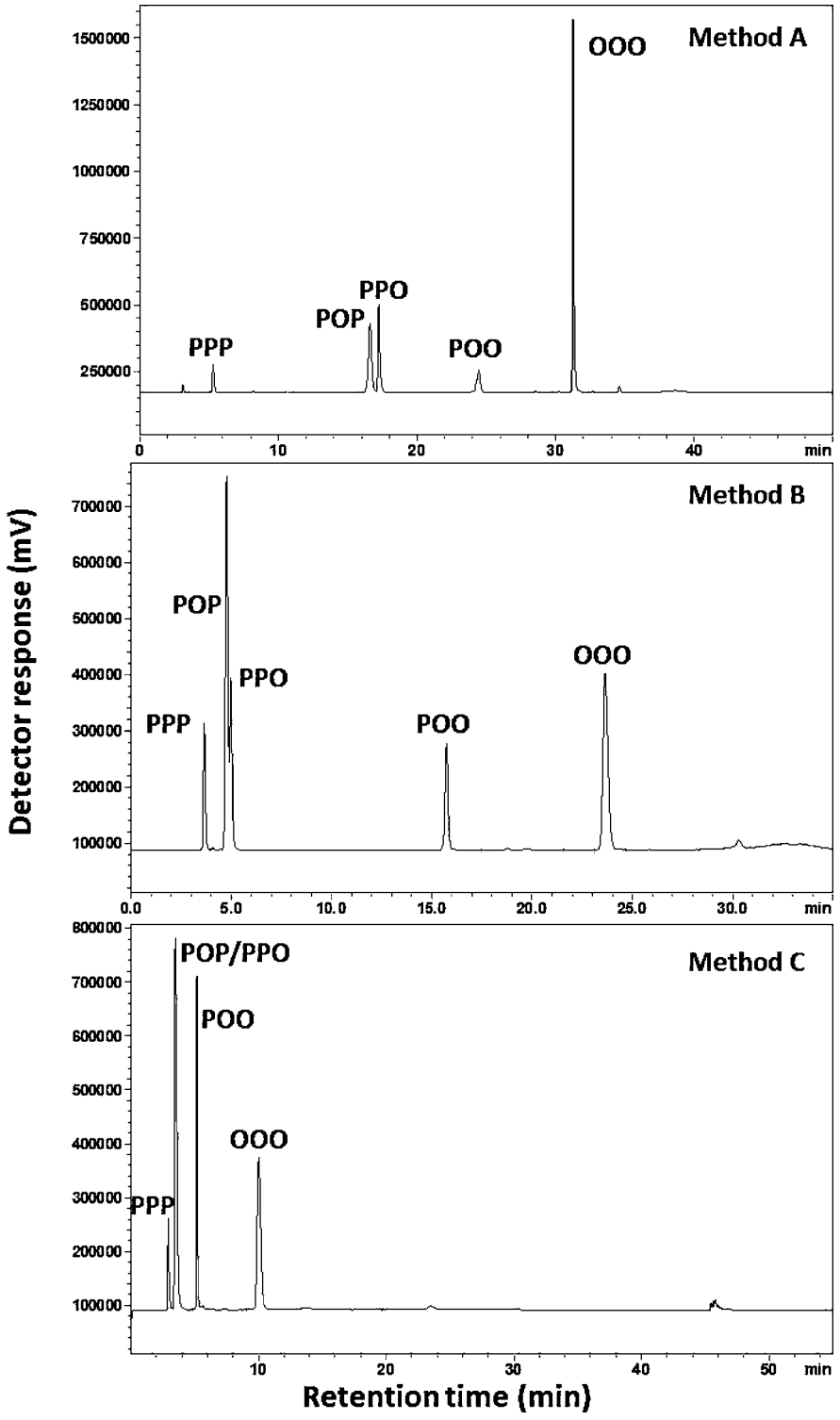

Figure 1. Influence of the mobile phase composition on the separation of stereospecific TAGs. The column temperature was set at 25 °C, and the flow of the mobile phase was maintained at 1 mL/min.

HP C18 HL with 3 μm particle diameter (Grace Alltech, Lokeren, Belgium). A precolumn (7.5 × 3.0 mm) with a silica packing was used (Grace Alltech, Columbia, MD). To obtain the appropriate quantity of each fraction, each sample was run 6 times in the injection volume of 40 μL. The solvents of all fractions were evaporated under nitrogen.

Second Dimension: Ag^+-HPLC–ELSD. Separation of stereospecific TAG species was performed on the same instrument as the previous part. The column was a 250 × 4.6 mm ChromSpher 5 Lipids (Varian, Middelburg, The Netherlands). A cation-exchange guard column (10 × 3 mm) was used to protect the column from possible contamination present in the sample and eluent (Varian, Middelburg, The Netherlands). Three different methods with different solvents were evaluated for the separation of TAG species. Varying flow rates and column temperatures were tested to obtain the optimum results. PBB was used to investigate different elution programs. The injection volume and the concentration were 25 μL and 0.5 mg/mL, respectively.

dx.doi.org/10.1021/jf401566c | J. Agric. Food Chem. 2013, 61, 7896–7903

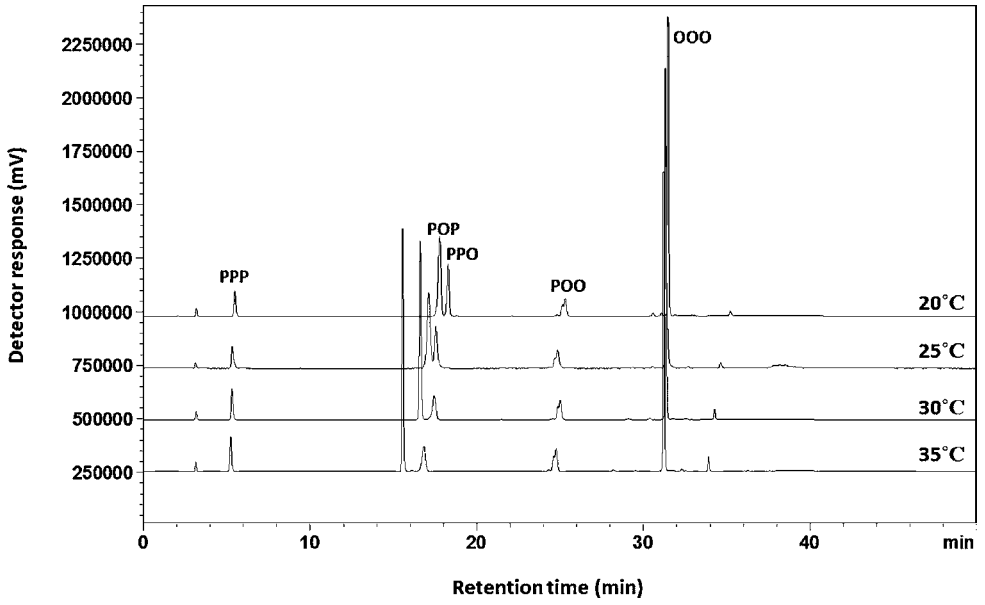

Figure 2. Influence of column temperatures on retention and separation of POP/PPO peaks using the heptane/acetone system. The flow of the mobile phase was maintained at 1 mL/min.

The optimized method was then used to separate the stereospecific TAGs. Each TAG fraction obtained from the primary column was reinjected into the secondary column for the determination of PIs. All fractions were analyzed under the same analytical conditions. With regard to the sample concentration, the fractions were properly diluted in heptane in relation to the amount of TAG contained in the relative fraction. Indeed, the sample concentration was a significant factor in Ag^+-HPLC PI separation.[21]

Method Validation. Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD and LOQ can be calculated from the regression line equation of the calibration curve of the POP and PPO standards. The formulas for calculation of LOD and LOQ are as follows:[23]

$$\text{LOD} = 3.3D/S \quad \text{and} \quad \text{LOQ} = 10D/S$$

where D is the standard error of the intercept of the regression line and S is the slope of the calibration curve.

Precision. The precision of an analysis is often expressed in terms of the relative standard deviation (% RSD) or the coefficient of variation (COV). These values are calculated from the standard deviation, S, and mean, X, of the peak area.[23]

$$\%\ \text{RSD} = \text{COV} = S/X \times 100$$

The precision of the response was tested by injecting standards of POP and PPO in the concentration of 0.1 mg/mL on the Ag^+ column for 6 repetitions.

RESULTS AND DISCUSSION

Optimizing of a HPLC Method for the Silver Ion Column. *Mobile-Phase Selection.* Three different methods were investigated for the separation of TAG species: a two-stepped linear gradient of acetone/heptane (2:98), as described by Macher and Holmqvist[24] (method A), a two-stepped linear gradient of dichloromethane/acetone (98:2), as described by Chen et al.[25] (method B), and a nonlinear gradient of acetonitrile, dichloromethane/1,2-dichloroethane (1:1, vol/vol), and acetone (0:98:2), as described by Smith et al.[6] (method C). Chromatograms with the three solvent systems are given in Figure 1. The use of a four-solvent gradient method (method C) resulted in high column back pressure and inadequate separation. As shown in Figure 1, the peaks of POP, PPO, OPO, and POO were not separated completely. The acetone/dichloromethane gradient (method B) resulted in a poor resolution of the PPO/POP pairs and PPP compared to other mobile phases. As clear from Figure 1, applying the heptane/acetone system (method A) led to a good separation of POP/PPO pairs with the acceptable retention time. Heptane/acetone (method A) was also preferred over the dichloromethane/acetone because heptane is less toxic. Consequently, the heptane/acetone gradient was selected for further analysis. However, not only is the composition of the mobile phase important in separation of PI TAGs, but also the temperature and flow rate should be accurately controlled.

Effects of Column Temperatures. The influence of column temperatures on the retention time and peak separation was investigated by analyzing the PBB at column temperatures of 20, 25, 30, and 35 °C. As shown in Figure 2, an increased column temperature corresponded with a decrease in the retention time. Consequently, the peaks of POP and PPO tended to elute faster with increasing temperatures, which resulted in a better separation of TAG. This effect can be attributed to the increased stability of the olefinic double bond complexes with Ag^+ at low temperatures.[16] It should be noted that changing the column temperature has no effect on the retention time of SSS TAGs because they have no double bonds. However, Adlof and List observed the opposite. They reported that the unsaturated fatty acid methyl ester (FAME) and TAG samples eluted more slowly at higher temperatures. They concluded that this effect in Ag^+-HPLC may be limited to hexane-based solvent systems and probably occurs with chlorinated hydrocarbon-based solvents.[26] As demonstrated in Figure 2, the column temperature of 35 °C seems to have an impact on the selectivity and sensitivity of POP/PPO. This effect is due to the fact that increasing the column temperature leads to a decrease in stability of the mobile phase/AG^+ ions complex, which, as a result, affects the column performance and the mass transfer.[27] According to Nikolova-Damyanova et al., Ag^+-HPLC is used most often at ambient temperatures.[13] Therefore, the column temperature of 25 °C was chosen

dx.doi.org/10.1021/jf401566c | J. Agric. Food Chem. 2013, 61, 7896−7903

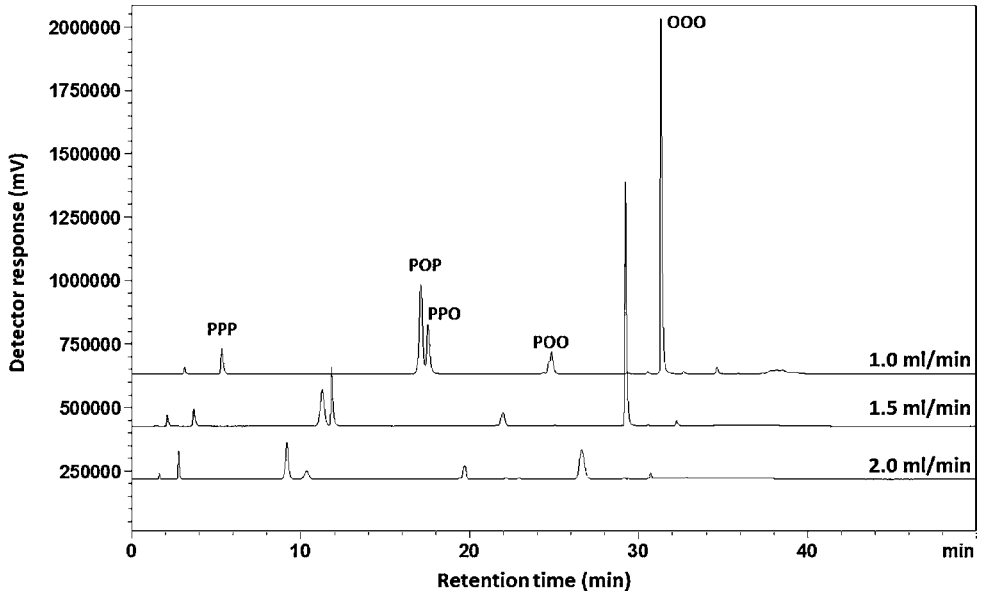

Figure 3. Influence of solvent flow rates on the retention and separation of POP/PPO peaks using the heptane/acetone system. The column temperature was maintained at 25 °C.

because efficient separation was obtained within a short retention time (38 min).

Effects of Flow Rates. The influence of flow rates on the retention time and peak separation was investigated by analyzing the blend at flow rates of 1, 1.5, and 2 mL/min. As seen from Figure 3, the retention time is highly decreased with an increase in the flow rates. However, higher flow rates resulted in a decrease in sensitivity. This phenomenon affected the separation and shape of the peaks. The increase in flow rates may result in bulk accumulation of the sample because of the evaporation in the drift tube, which could, in turn, cause a loss of sample, leading to a decrease in sensitivity.[28] Therefore, the flow rate of 1 mL/min was chosen for analysis.

Method Validation. The optimized method was validated by testing the linearity of the ELSD response, LOD, LOQ, and precision.[29]

LOD and LOQ. The LOD and LOQ were estimated for POP and PPO standards using the equations mentioned in the Methods section. The obtained LOD was 0.01 mg/mL for POP and 0.02 mg/mL for PPO standards, whereas LOQs were 0.04 and 0.06 mg/mL, respectively. The LOD value of 0.01 mg/mL means that the peak of TAG can be identified qualitatively only if the concentration of the particular TAG (POP in this case) is at least 0.01 mg/mL.

Precision. Precision is a measure of the ability of a method to generate the same result for multiple analyses of the same sample. Short-term precision is often referred to as repeatability of the method. The repeatability of the optimized method was experimentally tested by running 6 repetitions of the sample on the same day. The results showed that the % RSD on the replicated measurements for POP and PPO was 2.38 and 6.32, respectively. The lower the % RSD value means the lower variability in the data. In comparison to PPO, the POP standard had less variability in the data because of the small % RSD value.

Linearity. The ELSD typically shows a nonlinear (sigmoidal or exponential) response curve with a short linear range. This is the consequence of the fact that the analyte concentration influences the average particle size, which, in turn, affects the efficiency of the light scattering process. Because the mobile phase composition is continuously changing in gradient methods of HPLC, the response changes. As a result, the chromatogram shows different responses for the same quantities of analyte eluting through the gradient.[30] Therefore, the linearity of the ELSD should be checked. To investigate the linearity, POP and PPO standards were used. These standards were injected in different concentrations from 0.01 to 0.2 mg/mL. In Figure 4, the absolute concentration of each TAG is

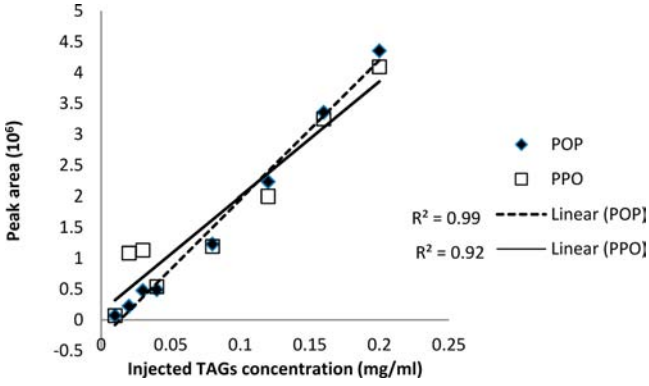

Figure 4. Peak area as a function of the injected concentration of POP and PPO. The injection volume was 25 μL. O, oleic acid; and P, palmitic acid.

plotted as a function of the peak area. From this figure, it can clearly be noticed that, in the range of 0.01−0.2 mg of TAG/mL, the ELSD response is linear and fairly uniform, irrespective of the TAG species. As such, if all TAG species would be situated in this concentration region, relative peak areas can be readily converted into relative concentrations.

Trials of Bidemensional HPLC Analysis. *TAG PIs in Fat Blends.* The palmitic- and stearic-based blends were mixed in the same ratio (w/w) to investigate the validity of the developed method. The chromatogram corresponding to this separation is presented in Figure 5. Three TAG fractions were collected through the application of a NARP- HPLC technique.

dx.doi.org/10.1021/jf401566c | J. Agric. Food Chem. 2013, 61, 7896−7903

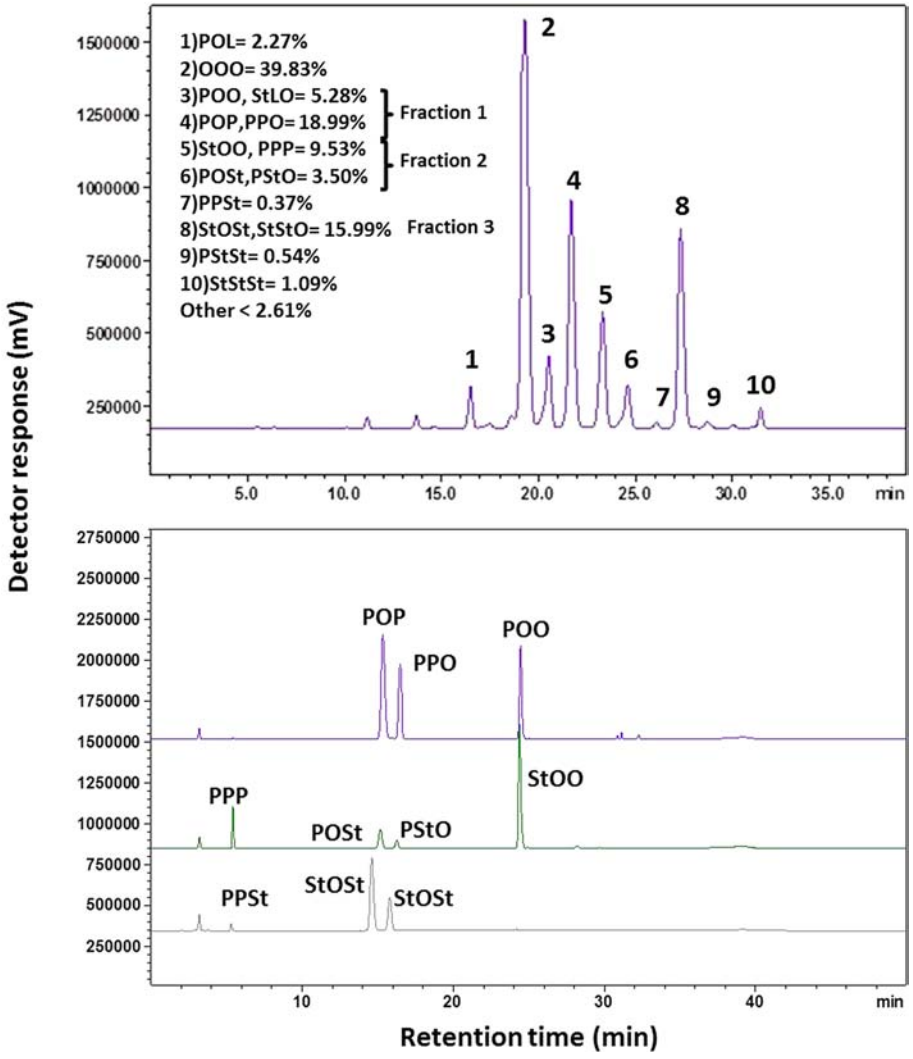

Figure 5. NARP-HPLC chromatogram of the palmitic and stearic blends (above) and Ag^+-HPLC chromatogram of TAGs from three collected fractions (below).

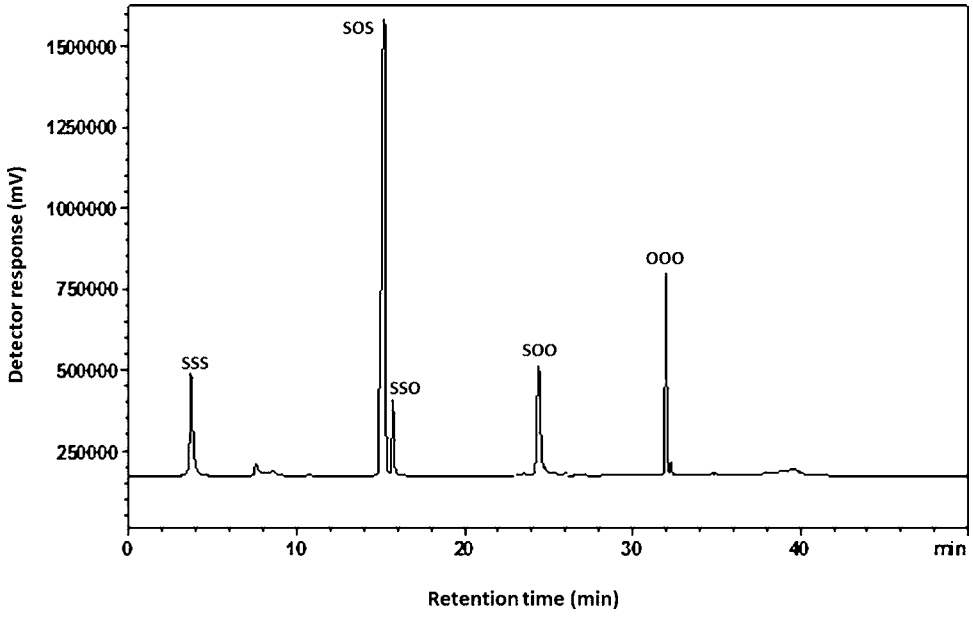

Figure 6. Ag^+-HPLC chromatogram of enzymatically produced CBE TAGs. S, saturated fatty acid; and O, oleic acid.

dx.doi.org/10.1021/jf401566c | J. Agric. Food Chem. 2013, 61, 7896−7903

The determination of the PI distribution for each TAG species would be impossible using this monodimensional approach as a result of peak coelution of TAG isomers and other TAG species. The chromatogram relative to a multidimensional NARP-Ag^+ application on TAG fractions 1,2 and 3 are shown in Figure 5. The stereospecific TAG species were separated: POP/PPO (PN = 48), StOSt/StStO (PN = 52), and POSt/StPO (PN = 50). The elution order is found to be comparable to previous studies.[1,31] Among all of the identified peaks, POP and PPO TAGs were quantified. The results for the palmitic and stearic blends showed that, at a concentration of 1 mg/mL, this sample contained 0.20 mg/mL POP and 0.15 mg/mL PPO.

TAG PIs in Enzymatically Produced CBE. As discussed earlier, it is important to have a method to investigate the existence of TAG PIs in the enzymatic acidolysis process. The enzymatically produced CBE was analyzed using the developed bidimentional HPLC method. Initially, to realize whether any acyl migration occurred during the acidolysis reaction, the sample injected to the silver ion column. As seen from Figure 6, the peak of the SSO TAG was detected. As explained in a previous part using a monodimensional method, it is not possible to identify all of the target PI TAGs (POP/PPO, POSt/PStO, and StOSt/StStO). Therefore, the CBE sample was injected to the C18 column, and three fractions were collected. In Table 2, you can find the TAG composition of the enzymatically produced CBE and three collected fractions obtaining from the C18 column. The collected fraction includes the three main TAGs of CBE (POP, POSt, and StOSt). Analyzing the collected fractions with the Ag^+ column showed 2.03, 1.12, and 0.11% for PPO, PStO, and StStO, respectively. Further purification is needed to reduce the amount of low melting TAGs (SOO and OOO) and obtain more symmetrical disaturated TAGs.

Table 2. TAG Composition of Enzymatically Produced CBE

TAG	Fraction	Relative peak area	
		C18	Ag^+
POL		0.17	
StOL		0.73	
OOO		1.89	
POO, StLO		13.68	
POP	1	13.81	11.78
PPO		-	2.03
StOO, PPP		16.82	
POSt	2	29.96	28.84
PStO		-	1.12
PPSt		3.08	
StOSt	3	16.89	16.78
StStO		-	0.11
PStSt		3.36	
StStSt		0.70	

AUTHOR INFORMATION

Corresponding Author

*Telephone: +32-0-92646165. Fax: +32-0-92646218. E-mail: koen.dewettinck@ugent.be.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Petersson, B.; Podlaha, O.; Jirskog-Hed, B. Triacylglycerol analysis of partially hydrogenated fats using high-performance liquid chromatography. *J. Chromatogr., A* **1993**, *653*, 25−35.

(2) Verstringe, S.; De Clercq, N.; Nguyen, M.; Kadivar, S.; Dewettinck, K. Enzymatic and other modification techniques to produce cocoa butter alternatives. In *Cocoa Butter and Related Compounds*; Garti, N., Widlak, N., Eds.; AOCS Press: Champaign, IL, 2012; pp 443−474.

(3) Xu, X.; Skands, A.; Høy, C.; Mu, H.; Balchen, S.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1179−1186.

(4) Vereecken, J.; Foubert, I.; Smith, K. W.; Sassano, G. J.; Dewettinck, K. Crystallization of model fat blends containing symmetric and asymmetric monounsaturated triacylglycerols. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 233−245.

(5) Xu, X. Production of specific-structured triacylglycerols by lipase-catalyzed reactions: A review. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 287−303.

(6) Smith, K.; Perkins, J.; Jeffrey, B.; Phillips, D. Separation of molecular species of *cis*- and *trans*-triacylglycerols in *trans*-hardened confectionery fats by silver-ion high-performance liquid chromatography. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1219−1222.

(7) Mukherjee, K. D.; Kiewitt, I. Structured triacylglycerols resembling human milk fat by transesterification catalyzed by papaya (*Carica papaya*) latex. *Biotechnol. Lett.* **1998**, *20*, 613−616.

(8) Senanayake, S. P. J. N.; Shahidi, F. Structured lipids via lipase-catalyzed incorporation of eicosapentaenoic acid into borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils. *J. Agric. Food Chem.* **2002**, *50*, 477−483.

(9) Kusaka, T.; Ishihara, S.; Sakaida, M.; Mifune, A.; Nakano, Y.; Tsuda, K.; Ikeda, M.; Nakano, H. Composition analysis of normal plant triacylglycerols and hydroperoxidized *rac*-1-stearoyl-2-oleoyl-3-linoleoyl-*sn*-glycerols by liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **1996**, *730*, 1−7.

(10) Neff, W. E.; El-Againy, M. Effect of linoleic acid position in triacylglycerols on their oxidative stability. *LWT—Food Sci. Technol.* **1996**, *29* (8), 772−775.

(11) Andrikopoulos, N. Chromatographic and spectroscopic methods in the analysis of triacylglycerol species and regiospecific isomers of oils and fats. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 473−505.

(12) Ruiz-Gutiérrez, V.; Barron, L. J. R. Methods for the analysis of triacylglycerols. *J. Chromatogr., B: Biomed. Sci. Appl.* **1995**, *671*, 133−168.

(13) Nikolova-Damyanova, B.; Christie, W. W.; Herslöf, B. G. Retention properties of triacylglycerols on silver ion high-performance liquid chromatography. *J. Chromatogr., A* **1995**, *694*, 375−380.

(14) Chandler, I. Determining the regioselectivity of immobilized lipases in triacylglycerol acidolysis reactions. *J. Am. Oil Chem. Soc.* **2001**, *78*, 737−742.

(15) Pchelkin, V. P.; Kuznetsova, E. I.; Tsydendambaev, V. D.; Vereshchagin, A. G. Determination of the positional-species composition of plant reserve triacylglycerols by partial chemical deacylation. *Russ. J. Plant Physiol.* **2001**, *48*, 701−707.

(16) Reske, J.; Siebrecht, J.; Hazebroek, J. Triacylglycerol composition and structure in genetically modified sunflower and soybean oils. *J. Am. Oil Chem. Soc.* **1997**, *74*, 989−998.

(17) Bergana, M.; Lee, T. Structure determination of long-chain polyunsaturated triacylgyycerols by high-resolution ^{13}C nuclear magnetic resonance. *J. Am. Oil Chem. Soc.* **1996**, *73*, 551−556.

(18) Hsu, F.-F.; Turk, J. Structural characterization of triacylglycerols as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisionally activated dissociation on a triple stage quadrupole instrument. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 587−599.

(19) Momchilova, S.; Itabashi, Y.; Nikolova-Damyanova, B.; Kuksis, A. Regioselective separation of isomeric triacylglycerols by reversed-phase high-performance liquid chromatography: Stationary phase and mobile phase effects. *J. Sep. Sci.* **2006**, *29*, 2578−2583.
(20) Gotoh, N.; Matsumoto, Y.; Nagai, T.; Mizobe, H.; Otake, I.; Ichioka, K.; Kuroda, I.; Watanabe, H.; Noguchi, N.; Wada, S. Actual ratios of triacylglycerol positional isomers consisting of saturated and highly unsaturated fatty acids in fishes and marine mammals. *Food Chem.* **2011**, *127*, 467−472.
(21) Adlof, R. O. Analysis of triacylglycerol positional isomers by silver ion high performance liquid chromatography. *J. High Resolut. Chromatogr.* **1995**, *18*, 105−107.
(22) Rombaut, R.; De Clercq, N.; Foubert, I.; Dewettinck, K. Triacylglycerol analysis of fats and oils by evaporative light scattering detection. *J. Am. Oil Chem. Soc.* **2009**, *86*, 19−25.
(23) Sadek, P. C. *The HPLC Solvent Guide*; John Wiley and Sons, Inc.: Hoboken, NJ, 2002.
(24) Macher, M.-B.; Holmqvist, A. Triacylglycerol analysis of partially hydrogenated vegetable oils by silver ion HPLC. *J. Sep. Sci.* **2001**, *24*, 179−185.
(25) Chen, M.-L.; Vali, S.; Lin, J.-Y.; Ju, Y.-H. Synthesis of the structured lipid 1,3-dioleoyl-2-palmitoylglycerol from palm oil. *J. Am. Oil Chem. Soc.* **2004**, *81*, 525−532.
(26) Adlof, R.; List, G. Analysis of triglyceride isomers by silver-ion high-performance liquid chromatography: Effect of column temperature on retention times. *J. Chromatogr., A* **2004**, *1046*, 109−113.
(27) Meyer, V. R. Theoretical principles. In *Practical High-Performance Liquid Chromatography*; John Wiley and Sons, Inc.: Hoboken, NJ, 2010; pp 17−57.
(28) Young, C. S.; Dolan, J. W. Success with evaporative light-scattering detection. *LC·GC Eur.* **2003**, *16*, 132−137.
(29) Epshtein, N. A. Validation of HPLC techniques for pharmaceutical analysis. *Pharm. Chem. J.* **2004**, *38*, 212−228.
(30) Mathews, B. T.; Higginson, P. D.; Lyons, R.; Mitchell, J. C.; Sach, N. W.; Snowden, M. J.; Taylor, M. R.; Wright, A. G. Improving quantitative measurements for the evaporative light scattering detector. *Chromatographia* **2004**, *60*, 625−633.
(31) Jakab, A.; Héberger, K.; Forgács, E. Comparative analysis of different plant oils by high-performance liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **2002**, *976*, 255−263.