Separation of Molecular Species of *cis*- and *trans*-Triacylglycerols in *trans*-Hardened Confectionery Fats by Silver-Ion High-Performance Liquid Chromatography

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Silver-phase high-performance liquid chromatography (HPLC) on silver nitrate-loaded silica achieves incomplete separation of major triacylglycerol (TAG) classes present in *trans*-hardened fats. The "ChromSpher Lipids" silver-loaded cation exchange HPLC column has been found to yield good separations of *trans*-hardened TAG, with molecular species well resolved. Separations comparable to those previously possible for nonhardened fats are now possible for *trans*-hardened fats. The separation is on the basis of number and type (i.e. *cis/trans*) of double bonds only; the position of the double bond along the acyl group appears not to influence the separation significantly. The analysis of a palm fraction, hardened to a slip melting point of 37°C and chemically randomized, is presented as an example. This technique offers a new approach to understanding and controlling the hydrogenation and processing of *trans*-hardened fats.

KEY WORDS: *Cis/trans* unsaturation, silver-ion HPLC, *trans*-hardened fats, triacylglycerols.

Triacylglycerols (TAG) that contain *trans*-fatty acyl groups are commercially important major components of many hydrogenated margarine, shortening and confectionery fats. Much of the compositional information published for such materials has been derived with nutritional aspects of their use in mind. Accordingly, many workers have published data on fatty acid composition and have sought to improve the chromatographic separation of complex mixtures that contain both geometrical and positional isomers of the same chainlength (1). For confectionery fats, interest is directed toward an understanding of melting and crystallization phenomena. To this end, structural (i.e., TAG) data are more appropriate than fatty acid data. The approach of many prior studies has been to determine TAG composition by either carbon-number gas chromatography (GC), directly by reverse-phase high-performance liquid chromatography (RP-HPLC), or by RP-HPLC in combination with GC of fatty acid methyl esters (FAME) (2). Carbon-number GC and RP-HPLC, however, offer limited discrimination between *cis* and *trans* unsaturation for molecular species of intact TAG. Argentation thin-layer chromatography (TLC) has been widely employed as part of a complex multidimensional approach to defining TAG composition (3), but argentation HPLC seems to have been little used in the characterization of intact *trans*-hardened TAG, even though by 1962 de Vries (4) had separated 1-elaido-dipalmitin and 1-oleo-dipalmitin on an 11-cm column packed with silver nitrate-impregnated silica gel G. More recently, Christie (5,6) showed that a silver ion-loaded cation exchange column (based on Nucleosil 5SA; Machery Nagel, Duran, Germany) would separate the *cis*- and *trans*-monounsaturated TAG molecular species of both sheep adipose tissue, in which the elaidyl chains are predominantly C9-unsaturated, and butterfat, in which the range of isomeric acids is greater. In *trans*-hardened fats, the range of positional fatty acid isomers is typically greater still (1,7,8). Most recently, Petersson *et al.* (9) applied a combination of RP-HPLC and RP-HPLC with silver ions in the mobile phase to the determination of TAG geometrical isomers in partially hydrogenated fats, obtaining excellent resolution overall. They reported only limited success in applying Christie's column to the direct separation of TAG in partially hydrogenated fats. Chromatograms were complex, and peaks were too broad for good identification and quantitation. Our experience has been that the silver ion-loaded ion-exchange column described by Christie (5) can indeed be a valuable tool in the analysis of partially hydrogenated fats. We now report a comparison of the separations achieved for a sample of hydrogenated palm olein (slip melting point, 37°C) on the "ChromSpher Lipids" silver-ion HPLC column and our standard silver-complexation column (10).

EXPERIMENTAL PROCEDURES

The "ChromSpher Lipids™" cation exchange column in silver-ion mode (250 mm × 4.6 mm i.d.) was obtained from Chrompack U.K. (London, England). The column was used in conjunction with a Varian 9010 solvent delivery system (Varian Ltd., Walton-on-Thames, United Kingdom) and a Varex laser light-scattering detector Mk.2 with response linearizer option (Varex Corporation, Burtonsville, MD). Detector temperature was set to maintain the exhaust at between 60 and 65°C. Nitrogen (12 psi) was used as the nebulizing gas. Our solvent system was essentially as published by Christie (5). Acetonitrile, dichloromethane/1, 2-dichloroethane (1:1 vol/vol), and acetone (HPLC grade ex; Sherman Chemicals, Sandy, United Kingdom) were used under multilinear gradient conditions at a solvent flow rate of 1 mL/min (Table 1). The solvent gradient was set up to give satisfactory separations with injection to injection times of about 1 h by using a liquid fraction of palm oil (fractionated from acetone), partially hardened to a slip melting point of 37°C and then chemically randomized. For HPLC analysis, the TAG fraction was recovered by solid-phase extraction on silica gel with dichloromethane as the eluting solvent. The dichloromethane was subsequently evaporated under nitrogen to recover the TAG, which was then redissolved at the required concentration in the appropriate injection solvent, i.e., 1,2-dichloroethane for the ChromSpher Lipids column and toluene for the silver nitrate–silica column. An example of the separation obtained on the ChromSpher Lipids column is shown in Figure 1B for comparison with that obtained from our standard silver-phase HPLC (Fig. 1A). TAG assignments in Figure 1 are not intended to imply fatty acyl positional information. Acyl group derivations used in this paper are P (for palmitic acid), St (for stearic acid), S (any saturated fatty acid), E (for elaidic acid), O (for oleic acid) and Ln (for linoleic acid). For our purposes, the gradient profile was optimized for separation of TAG

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TABLE 1

Multilinear Solvent Gradient Used with ChromSpher Lipids Column[a]

Time (min)	Acetone (%)	Acetonitrile (%)	Dichloromethane/ dichloroethane 1:1 (%)
0.0	2	0	98
5.0	3	0	97
25.0	60	0	40
32.99	80	20	0
33.0	2	0	98
55.0	2	0	98

[a]From Chrompack U.K., London, England.

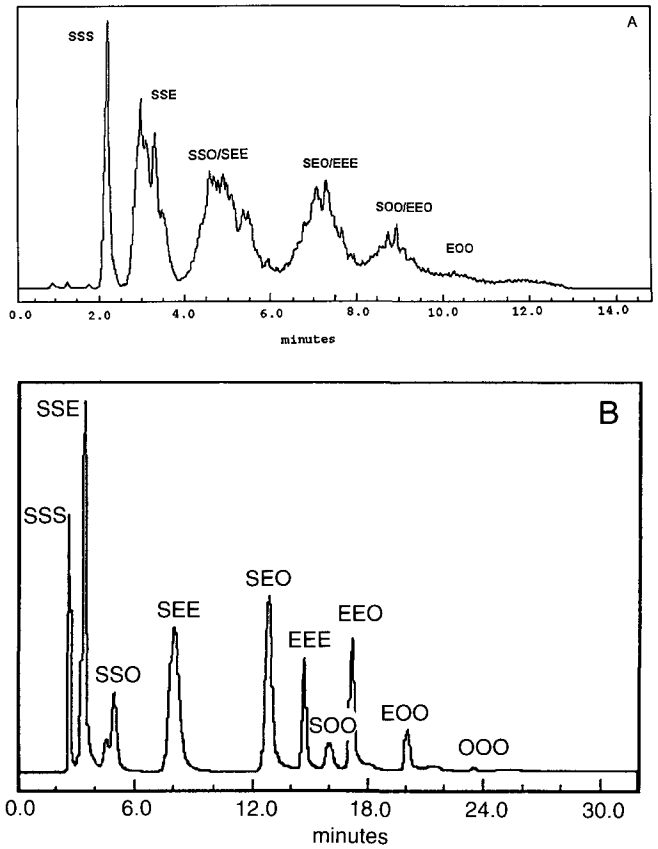

FIG. 1. Comparison of silver-complexation high-performance liquid chromatography columns: (A) silver-complexation column (silica/silver nitrate)—200 μ triacylglycerol (TAG) injected in toluene. Nonlinear gradient (1.5 mL/min) of hexane/toluene/ethyl acetate. Tracor 945 flame-ionization detector. See Experimental Procedures section for details and TAG designations. (B) Silver-ion column "ChromSpher Lipids"—200 μg TAG injected in dichloroethane. Nonlinear gradient: 1.0 mL/min of dichloromethane/dichloroethane/acetone/acetonitrile. Varex evaporative light-scattering detector (Varex Corp., Burtonsville, MD). See Experimental Procedures section for details and TAG designations. Abbreviations: O, oleic acid; E, elaidic acid; S, saturated fatty acid.

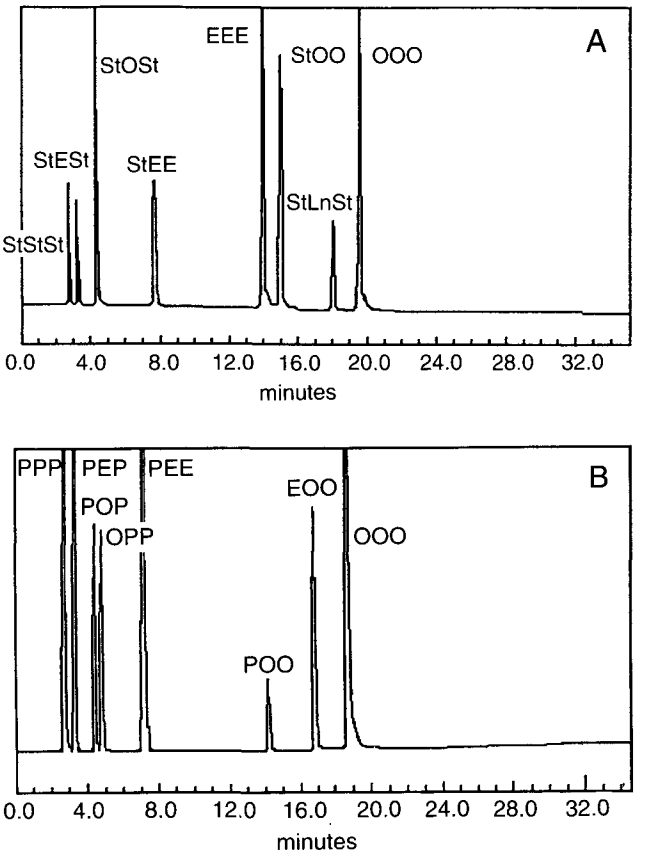

FIG. 2. Silver-ion high-performance liquid chromatography of pure TAG mixtures: 200 μg TAG injected. Flow rate, gradient and detector as for Figure 1B. See Experimental Procedures section for TAG designations. (A) Mixture of pure TAG (stearic acid-based); (B) mixture of pure TAG (palmitic acid-based). Abbreviations: St, stearic acid; Ln, linolenic acid; P, palmitic acid. See Figure 1 for other abbreviations.

with up to three double bonds. A final (equilibration) period of 20–25 min was required for the column to return to its preinjection condition. The retention of early eluted peaks was strongly affected by the length of this period of equilibration. Peak assignments were made by using pure TAG (synthesized "in-house" by Unilever Research Laboratory, Vlaardingen, The Netherlands) as primary standards (Fig. 2) and by using the interesterified hardened palm fraction, for which the TAG composition was calculated assuming a fully random fatty acid distribution. Fatty acid data (Table 2) were determined for this purpose according to the AOCS method Ce-1c-89 (11). This material was also used to determine the optimum conditions for detector operation. By simple trial and error it was quickly established that a linearizer setting of 1.3 yielded area data most closely matching the calculated composition (Table 2). Settings on either side of this value (1.2 and 1.4, respectively) gave less acceptable data. This "linearizer setting" is numerically equal to the slope of the line obtained by plotting the logarithm of the detector signal against the logarithm of component mass. Our best value of 1.3 (above) agrees well with observations made by other workers (12) who found the detector response to be proportional to solute mass raised to the power of 1.5. In use, TAG retention times on the silver-ion column decreased progressively with the number of samples processed over a period of about one month. This effect is shown in Figures 1B, 2A and 2B, where the retention of OOO decreases from 23.5

TABLE 2

Composition Calculated from FAME and Silver-Ion HPLC Analysis for *trans*-Hardened/Randomized Palm Fraction

TAG	S_3	S_2E	S_2O	SE_2	SEO	E_3	SO_2	E_2O	EO_2	O_3
Composition calculated from FAME[a] (wt %)	9.9	25.2	9.3	21.3	15.8	6.0	2.9	6.7	2.5	0.3
Composition determined by HPLC (area %)	9.1	26.3	8.1	21.5	16.7	5.6	2.0	8.4	2.2	0.2

[a]Fatty acid methyl esters (FAME) composition (wt %): 12:0, 0.4; 14:0, 1.3; 16:0, 36.7; 18:0, 5.2; 18:1*t*, 38.5; 18:1*c*, 17.6; 18:2, 0.3; 18:3, 0.0. HPLC, high-performance liquid chromatography; TAG, triacylglycerol.

to 19 min over the period of experimentation and necessitates that retention standards are chromatographed regularly throughout any work. We hypothesize that this effect is due to a slow denaturation of silver ions by materials present at low level in both samples and solvents, and which become tightly bound to the stationary phase as the column is used. Christie (5) noted no deterioration over a period of one year but did not specify the number of samples analyzed during that time. He did, however, indicate that the column could be readily regenerated by purging with methanol/acetonitrile (1:1, vol/vol) and injecting a solution that consisted of a few milligrams of silver nitrate dissolved in acetonitrile, and that no silver ions appeared to be eluted in use. The ChromSpher Lipids column could indeed be successfully regenerated to "as new" condition 2–3 times by the following procedure: The column was flushed with acetonitrile, then methanol, then water (HPLC-grade) for 20 min each at 1 mL/min flow rate. Aqueous silver nitrate solution (0.1 g in 1 mL) was then injected in 50 μL aliquots. Excess silver nitrate was eluted from the column, and was detected with sodium chloride solution. The column was flushed with water, then methanol and finally acetonitrile—as above in reverse order—followed by two blank solvent gradients (Table 1) before use. Column regeneration/conditioning can be accomplished within the working day. Silver-complexation HPLC was carried out according to our published methodology (10) with a 100 mm × 4.6 mm id column packed with "Nucleosil 100-3," three micron silica (Macherey Nagel) preloaded with 10% (w/w) AR-grade silver nitrate. The solvent system is based on toluene, hexane and ethyl acetate (HPLC-grade) mixtures, and separations are achieved by using multilinear gradients at 1.5 mL/min flow rate. Detection is established by transport FID (Tracor 945; Tremetrics Inc., Austin, TX).

RESULTS AND DISCUSSION

Silver-complexation HPLC on silver nitrate-loaded silica (10) is sensitive to fatty acyl chainlength, to number and type of double bonds and to the position of the double bond in the acyl chain. This technique produces broad, overlapping peaks when applied to *trans*-hardened fats (Fig. 1A). Some assignment is possible but only on the basis of broad classes. It has not been proven possible, for example, to separate S_2O TAG from SE_2 TAG. Tentative assignments (based upon peak collection and analysis of fatty acids) were (in elution order) S_3, S_2E, S_2O + SE_2, SEO + E_3, E_2O + SO_2. It could be argued that the resolution of TAG that contain geometrical and/or positional isomers of fatty acyls is, effectively, too good for the current purpose. During hydrogenation of liquid vegetable oils, there is not only isomerization of *cis* double bonds to the *trans* configuration, but the double bonds may also migrate to different positions along the fatty acyl chains. Thus, there are a number of possible elaidyl- and oleyl-isomers produced, each evidently differing sufficiently in bond position to be selectively complexed by the silver phase. Retention on this column is then a function both of the number and type of double bonds in the TAG, and also of the position of those double bonds in the acyl groups. Because there are a great number of possible TAG isomers, the net effect would be to produce the broad, overlapping peaks seen in Figure 1A. The ChromSpher Lipids column separates TAG on the basis of double-bond geometry (i.e., *cis* or *trans*) rather than on the basis of bond position in the acyl chain or chainlength. This leads to little or no overlap between peaks, so that S_2O is clearly resolved from SE_2 and SEO is resolved from E_3 (Fig. 1B). Figure 2 indicates that retention for palmityl- and stearyl-TAG is the same within the achievable run-to-run reproducibility. The elaidyl double bond exhibits approximately half the retention of the oleyl double bond on this column. The elution pattern observed agrees with that published by Bruhl *et al.* (13) who separated the TAG of human breast milk on a column packed with silver ion-loaded Nucleosil 10SA and a solvent system based on dichloromethane, acetone and acetonitrile. Table 2 compares calculated and found TAG compositions for the hardened/randomized palm fraction. Data agreement is acceptable, bearing in mind the well-known problem of accurately determining *cis* and *trans* fatty acid contents by gas chromatography (14) and that response of the Varex detector has not been fully calibrated in this study. The resolution achieved for *trans*-hardened TAG on the silver-ion column is not obtained on the more conventional silver-complexation column. This new approach should find application in gaining a deeper understanding and improved control of both hydrogenation and of the processing of *trans*-hardened fats.

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