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Short communication

Analysis of conjugated linoleic acid-enriched triacylglycerol mixtures by isocratic silver-ion high-performance liquid chromatography

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Abstract

Silver-ion HPLC (Ag-HPLC) was applied to the fractionation of a triacylglycerol (TAG) sample enriched (>80%) with conjugated linoleic acid (CLA). After conversion of the TAGs to fatty acid methyl esters using sodium methoxide in methanol, Ag-HPLC (dual-column; isocratic solvent system of 0.1% acetonitrile in hexane; UV detection at 233 nm) was used to determine the CLA isomer distribution (50:50 mixture of 9c,11t- and 10t,12c-18:2). Three or four Ag-HPLC columns connected in series (0.6–1.0% acetonitrile in hexane as solvent; UV detection at 206 nm) were used to analyze the sample in TAG form. Elution times for CLA-enriched TAGs averaged 30 min or less. Isocratic solvent conditions were used to eliminate the solvent equilibration times (often 30 min or more) required between sample injections when solvent programming is used. The ratio of TAGs containing three vs. only two CLA molecules was found to be approximately 3 to 1. Ag-HPLC has thus been shown to be a useful method for rapidly analyzing not only CLA isomers as esters, but also in the TAG form. Published by Elsevier Science B.V.

Keywords: Linoleic acid; Triacylglycerol; Silver

1. Introduction

While silver ion high-performance liquid chromatography (Ag-HPLC) has shown itself to be a very powerful technology for separating fatty acids [1,2] as fatty acid esters (methyl, methoxy, phenacyl, etc.) and, more recently, as the underivatized fatty acids [3], its application to the analysis of conjugated linoleic acid (CLA)-containing triacylgycerol (TAG) or phospholipid (PL) formulations has been limited primarily to prefractionation of the TAG mixture for subsequent analysis by gas chromatography (GC), Fourier transform infrared (FT-IR), mass spectrometry (MS) or nuclear magnetic resonance (NMR) [4]. Some success has been achieved utilizing Ag-HPLC for analysis of seed oils high in conjugated dienoic or trienoic fatty acids [5]. Joh and Kim [5] used a Nucleosil (TM) 5 SA column (250× 4.6 mm) saturated with silver ions and a complex solvent system of dichloromethane–dichloroethane–acetone–acetonitrile (programmed; 1 ml/min) to separate TAGs from the Chinese melon [*Momordica charantia*; 57.1 mol% conjugated triene, primarily α-eleostearic (9c,11t,13t-18:3) acid]. The authors

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also noted that conjugated trienoic fatty acids are readily oxidized/decomposed during separation on silica gel thin-layer chromatography (TLC) plates or on silver nitrate impregnated silica gel columns. Ag-HPLC has not been applied to the analysis of CLA-containing TAGs available in commercial formulations [6] or diet supplements. A better understanding of the regiodistribution of CLA isomers in TAG formulations has potential applications in the labeling of commercial CLA-containing products and, perhaps more importantly, in development of a better understanding of TAG structure as it relates to fatty acid (FA) absorption and utilization in living systems. We wished to develop a method using Ag-HPLC and an isocratic solvent system to rapidly analyze CLA-enriched TAG formulations.

2. Experimental

2.1. Materials and reagents

The CLA-enriched TAG formulation, Clarinol G-80, was obtained from Loders Croklaan, Wormerveer, The Netherlands. Hexane (Allied Fisher Scientific, Orangeburg, NY, USA) and acetonitrile (ACN; E. Merck, Darmstadt, Germany) were HPLC grade. Light petroleum and ethyl ether were obtained from Fisher Scientific, Fair Lawn, NJ, USA. All solvents were used as received.

2.2. Equipment/sample preparation

2.2.1. High-performance liquid chromatography

A Spectra-Physics P2000 solvent delivery system (Spectra-Physics Analytical, Fremont, CA, USA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20 μl injection loop, and an Isco V4 absorbance detector (Isco, Lincoln, NE, USA) at wavelengths of 206 or 234 nm were used. The ChromSpher Lipids columns (catalog No. 28313; 250×4.6 mm I.D. stainless steel; 5 μm particle size; silver ion impregnated) were purchased from Varian-Chrompack International, Middelburg, The Netherlands, and used as received. Two or more Lipids columns were connected in series to improve sample capacity and to provide the required peak-to-peak resolutions. Solvent flow-rates (1.0 to 2.0 ml/min) and compositions (0.1 to 1.0% ACN in hexane, isocratic, 23 °C) were adjusted to (a) maintain pump head pressures at ≤2500 p.s.i. and (b) maintain total sample elution times at 35 min or less (1 p.s.i.= 6894.76 Pa). Void volumes were approximately 1.0 ml per column.

2.2.2. Sample preparation

The TAG fractions isolated by Ag-HPLC were collected in scintillation vials and the solvents removed by a stream of inert gas. The TAGs were converted to fatty acid methyl esters (FAMEs) by a modification of the Glass procedure [7]. A 1.5-ml volume of benzene and 3.0 ml 0.5 *M* sodium methoxide in methanol were added to the residue and the solution was stirred (under inert gas) magnetically at 40 °C for 10 min. The sample was cooled to room temperature and 1.5 ml 1 *M* HCl solution, 3 ml saturated NaCl solution, and 4 ml hexane were added. (The solution was stirred for 15 s after each addition). The two layers were allowed to separate and the hexane layer was removed and eluted through a pre-rinsed [5 ml 10% ethyl ether (EE) in hexane] silica ''Sep-Pack'' SPE cartridge (Waters, Milford, MA, USA). The hexane addition/elution steps were repeated two more times and the cartridge was washed with a final aliquot of 12 ml 10% EE in hexane. All the eluents were combined and concentrated under a stream of inert gas. Conversion efficiency was followed by TLC [1×3 in. silica gel K6 plates (Whatman, Clifton, NJ, USA); developed in PE–EE (85:15) and visualized by iodine; 1 in.= 2.54 cm].

2.2.3. Gas chromatography

The TAG fractions were analyzed (as FAMEs) on a Varian 3400 gas chromatograph (Varian Instruments, Palo Alto, CA, USA) equipped with a 100 m×0.32 mm SP2380 (Supelco, Bellefonte, PA, USA) capillary column, flame ionization detection (FID) system and utilizing He as carrier gas. Operating conditions were: injector, 240 °C; split ratio, 100:1; oven temperature programmed from 155 °C to 220 °C at 3 °C/min with an initial hold of 15 min; detector, 280 °C.

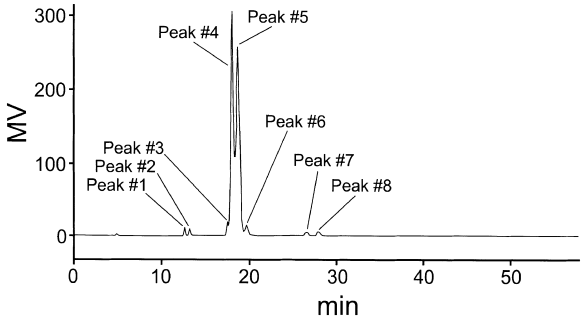

Fig. 1. Analysis of G-80 CLA isomers as FAMEs. Dual-column Ag-HPLC. Sample size: 2.5 μg. Flow-rate 2.0 ml/min, 0.1% ACN in hexane. UV detection at 233 nm. Peaks: 1=10t,12t-18:2, 2=9t,11t-18:2, 3=11c,13t-18:2, 4=10t,12c-18:2, 5=9c,11t-18:2, 6=8t,10c-18:2, 7=10c,12c-18:2, 8=9c,11c-18:2. (See footnote e of Table 1 for non-CLA fatty acids in sample).

3. Results

The elution pattern for the CLA isomers (as FAMEs) in the G-80 formulation is shown in Fig. 1 while the elution patterns for the G-80 TAGs are presented in Fig. 2. The elution patterns shown in Fig. 2 were achieved with either three (insert A) or four (Fig. 2 and insert B) Ag-HPLC columns connected in series. With three columns in series, no fractionation of the major TAG peak (peak 4) was noted. The major peak was therefore divided into three fractions (2A1, 2A2 and 2A3). The collected fractions were converted to FAMEs and analyzed by GC as noted in the Experimental section, above. Ag-HPLC solvent flow-rates and compositions were adjusted to yield total TAG elution patterns within 30–35 min and to maintain a system pressure <2500 p.s.i. Insert B of Fig. 2 illustrates the further fractionation of the major TAG peak achieved (but at the cost of increased retention times) with four columns in series and a lower percentage of ACN in the solvent. UV detection at 233 nm (specific for conjugated systems) was used to determine % CLA compositions as FAMEs; UV detection at both 206 and 233 nm could be utilized to obtain TAG elution patterns.

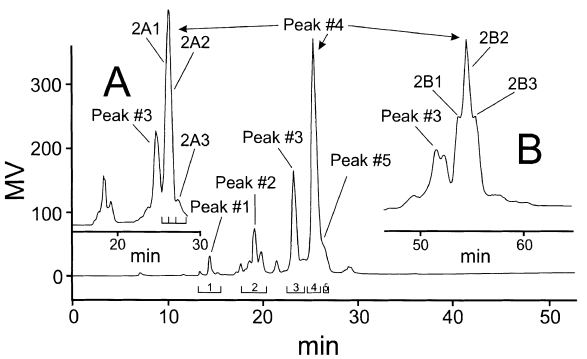

Fig. 2. Analysis of G-80 TAG formulation by four-column Ag-HPLC. Sample size: 100 μg. Flow-rate 1.5 ml/min, 1.0% ACN in hexane. UV detection at 206 nm. Peaks: 1=mono-CLA/2 misc. FAs, 2=di-CLA/mono-saturated FA, 3=di-CLA/mono 9c-18:1, 4=Tri-CLA; 5=Tri-CLA. Insert A: Three-column Ag-HPLC. Sample size: 50 μg; flow-rate 2.0 ml/min, 0.6% ACN in hexane. Fraction 2A1=54%/37%: 10t,12c-18:2/9c,11t-18:2; fraction 2A2=36%/56%: 10t,12c-18:2/9c,11t-18:2; fraction 2A3=di-9c,11t-18:2/mono-*cis*/*cis*-CLA (where *cis*/*cis* refers to 9c,11c- and 10c,12c-18:2). Insert B: Four-column Ag-HPLC. Sample size: 50 μg; flow-rate 1.5 ml/min, 0.7% ACN in hexane. Note: the two major peaks in A and B are peaks 3 and 4 of the main chromatogram and illustrate changes in separation due to changes in number of columns used or in solvent composition.

The CLA FA isomer composition for the G-80 sample and for fractions collected across the major TAG peak (peak 4, Fig. 2) isolated by Ag-HPLC of G-80 (fractions 2A1, 2A2 and 2A3) are listed in Table 1. In Table 1, part A, a G-80 sample was converted to FAMEs and the FAMEs analyzed in duplicate (runs 1 and 2) by GC and Ag-HPLC. Total % FA composition of the formulation was determined by GC and the non-CLA FAs in G-80 are listed in footnote e of Table 1. In Table 1, part B, several fractions were collected across the major TAG peak obtained from Ag-HPLC analysis of the G-80 formulation (peak 4, Fig. 2; fractions 2A1, 2A2 and 2A3) which, after conversion to FAMEs, were analyzed by GC. FA composition data was used to determine elution orders for the CLA-containing TAG isomers.

4. Discussion

Ag-HPLC has been used to analyze a variety of unsaturated lipids, but has not been applied to the analysis of CLA-enriched TAG formulations. We found Ag-HPLC and an isocratic solvent system to be a useful technique for scanning TAG isomer formulations but, as should be noted by the number of columns required, the technology may be limited

Table 1
CLA isomers present in CLA-enriched TAG formulation

	A				B		
	Gas chromatography		Ag-HPLC		Gas chromatography		
	Run 1	Run 2	Run 1	Run 2	2A1	2A2	2A3
CLA isomer[a]							
10t,12t-18:2	1.8[b]	1.8[b]	1.0	1.3	1.9[b]	0.8[b]	2.0[b]
9t,11t-18:2			1.0	1.4			
11c,13t-18:2			1.6	1.4			
10t,12c-18:2	47.3[c]	47.2[c]	45.9	45.4	53.5[c]	35.8[c]	28.2[c]
9c,11t-18:2	48.3[d]	48.2[d]	46.9	47.0	37.5[d]	55.7[d]	46.4[d]
8t,10c-18:2			1.5	1.4			
10c,12c-18:2	1.3	1.3	1.1	1.0	0.0	2.2	2.8
9c,11c-18:2	1.3	1.3	1.0	1.0	0.0	0.8	8.9
Total (normalized)[e]	100.0	99.8	100.0	99.9			
Total CLA (%)	78.2	78.0			92.9	95.3	88.3

A: Analysis of G-80 CLA isomers (as FAMEs) by GC and Ag-HPLC; duplicate runs.
B: Fatty acid composition across peak 4 from Ag-HPLC of G-80 sample (Fig. 2, insert A; fractions 2A1, 2A2, 2A3; GC analysis as FAMEs).
[a] CLA isomers listed as eluted from Ag-HPLC (as FAMEs).
[b] 9t,11t- and 10t,12t-18:2 unresolved.
[c] 10t,12c- and 11c,13t-18:2 unresolved.
[d] 9c,11t- and 8t,10c-18:2 unresolved.
[e] GC analysis; all CLA isomers. Samples also contained ca. 6.0% 16:0, 1.6% 18:0, 12.8% 9c-18:1 and 1.3% 9c,12c-18:2.

to ''simple'' (containing a limited number of TAG isomers) formulations. When compared to solvent programing, an isocratic solvent system is more rapid (does not require 20–30 min between runs for solvent equilibration), is not as dependent on instrument configuration (No. of pumps, mixing chamber configurations, etc.) and allows the use of refractive index detectors. If a simple pattern is desired, two columns in series might suffice.

Often prepared by the base-catalyzed isomerization of linoleic acid (9c,12c-18:2), the compositions of CLA formulations may vary significantly [8]. Some CLA formulations, created by the use of more extreme isomerization conditions, may contain a variety of conjugated *cis*/*trans* isomers (8t,10c-/9c,11t-/10t,12c-/11c,13t-18:2) and even significant (>10%) percentages of *trans*/*trans* or *cis*/*cis* isomers. We found CLA isomer composition of the G-80 formulation to be >91% 9c,11t- and 10t,12c-18:2 (see data in Table 1).

Analysis of the TAG formulation utilizing four Ag-HPLC columns in series resulted in a pattern of four minor and one major peak (see Fig. 2), with peak 1=mono-CLA/2 misc. FAs, peak 2=di-CLA/mono-saturated FA, peak 3=di-CLA/9c-18:1, peak 4=Tri-CLA, and peak 5=di-CLA/mono *cis*/*cis* CLA isomer. (In this instance, the term ''CLA'' is used for both the 9,11- and the 10,12-isomers). Under these conditions (2500 p.s.i. and <35 min elution time), a minimum of three columns in series were required to achieve ca. 50% separation of peaks 3 and 4. (No resolution between peaks 3 and 4 was obtained with only two Ag-HPLC columns connected in series). The last-eluting part of peak 4 was also isolated (fraction 2A3) and found to be composed predominately of TAGs with the structure di-9c,11t-18:2/mono-*cis*/*cis*-CLA (where *cis*/*cis* refers to 9c,11c- and 10c,12c-18:2).

We attempted to further fractionate the major CLA-enriched TAG peak (Fig. 2, peak 4) by using four columns in series and decreasing the percentages of ACN in the hexane. At 0.6% ACN (1.5 ml/min), the desired peak could not be eluted within 120 min. At 0.7% ACN (1.5 ml/min), peak 4 eluted at 55 min and was partially fractionated into three peaks (Fig. 2, insert B; <10% resolution; 1:2:1

ratio). Fractionation of the individual TAG isomers was improved, but at a cost of increased elution times. Yet most methods used to determine the specific FA(s) at the 1(3) or 2-position of a TAG require more time by utilizing chemical [10] or enzymatic [11] means to selectively remove FAs for further analysis by standard procedures such as GC or HPLC.

The ''partial fractionation'' of the major tri-CLA TAG peak (Fig. 2, insert B) was encouraging. Given the Ag-HPLC elution orders for CLA isomers as FAMEs (10t,12c- elutes before 9c,11t-18:2, etc.) and as TAGs (the FAs in the 1- or 3-position of the TAG exert a greater influence on TAG retention than the FAs in the 2-position), one can speculate on the composition of the 3 peaks (Fig. 2, insert B, peaks 2B1, 2B2 and 2B3) obtained by partial fractionation of peak 4. The suggested elution order would thus be 10/9/10, then 10/10/9 and 10/9/9 (unresolved), and finally 9/10/9 (where 9=9c,11t-18:2 and 10= 10t,12c-18:2). This suggested elution order is also in agreement with the fatty acid composition we found when we collected and analyzed (Table 1, part B) the first half vs. the second half of peak 4 (see insert A, Fig. 2). A higher percentage of 10t,12c-18:2 vs. 9c,11t-18:2 (54% vs. 37%) was found in the first half of the peak, while the ratio was reversed (36% vs. 56%) in the second half of the peak.

Ag-HPLC has been applied to the separation of simple mixtures of TAG isomers and to TAGs containing FAs of differing chain lengths [9], but Ag-HPLC has to date found only limited application for analysis of complex mixtures of TAGs. Ag-HPLC is often utilized as part of a ''hyphenated'' technique with reversed-phase HPLC, atmospheric pressure chemical ionization (APCI) MS, etc. [1] As demonstrated in this paper, Ag-HPLC can be used to analyze ''simple'' CLA-enriched TAG formulations. Ag-HPLC also has potential applications in the analysis of seed oils containing a limited number of FAs, and in characterization of CLA-containing TAG formulations for patent applications/diet supplements. Yet the limitations of the Ag-HPLC/isocratic solvent system for analysis of more complex TAG mixtures must also be recognized.

The potential for improved resolution of individual TAG isomers in CLA-enriched TAG formulations exists, but whether by use of multiple solvents/solvent programing, by application of a different isocratic solvent system and/or by control of column temperature remains to be determined. As we have demonstrated, Ag-HPLC may be used to rapidly screen CLA-enriched TAG formulations, but its development as a standard method for analysis of complex TAG formulations will require more research.

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