

# Analysis of triglyceride isomers by silver-ion high-performance liquid chromatography

## Effect of column temperature on retention times

R. Adlof\*, G. List

US Department of Agriculture, Food and Industrial Oil Research, National Center for Agricultural Utilization Research,  
USDA, Agricultural Research Service, Peoria, IL 61604, USA

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### Abstract

Silver ion chromatography (Ag-HPLC), utilizing columns packed with silver ions bonded to a silica or similar substrate, has proven to be a powerful technique for the analytical separation of *cis* and *trans* geometric and positional fatty acid methyl ester (FAME) and triacylglycerol (TAG) isomers. In this manuscript, we utilize an HPLC column chiller/heater to study the effects of Ag-HPLC column temperatures on elution rates of FAME and TAG isomers. Two Varian ChromSpher lipids columns connected in series and isocratic solvent systems of 1.0% or 1.5% acetonitrile (ACN) in hexane were used to analyze FAMEs (zero to six double bonds; *cis/trans* isomers), TAGs (homogeneous (triacyetyl-, tristearoyl-, trioleoyl-, trilinoeoyl- and trilinolenoyl-glycerols) and positional (1,3-distearoyl,2-monolinolenoyl- and 1,2-distearoyl, 3-monolinolenoylglycerol, etc.)) mixtures at four different temperatures (10 °C, 20 °C, 30 °C or 40 °C). Unexpectedly, the unsaturated FAME and TAG samples were found to elute more slowly at higher temperatures, a result just the opposite to the usual temperature effect (where samples elute more rapidly at higher temperatures) noted in gas and most liquid (reversed-phase or silica gel substrates) chromatography systems. This effect in Ag-HPLC may be limited to hexane-based solvent systems (it does not seem to occur with chlorinated hydrocarbon-based solvents); its magnitude is directly related to the total number of double bonds in the sample. It is also more evident with *cis* than with *trans* double bonds.

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### 1. Introduction

While temperature control and programming have become important and integral parts of gas chromatographic methodology, the methodology has found only limited application(s) in HPLC (reversed-phase [1,2], silica [3,4] and silver nitrate/silica [5]). Silver ion chromatography (Ag-HPLC), utilizing columns packed with silver ions bonded to a silica or similar packing, has proven to be a tremendously powerful technique for the analytical to semi-preparative separation/isolation of *cis* and *trans* geometric and positional fatty acid methyl ester (FAMES) and triacylglycerol

(TAG) isomers (see references [6–9] for reviews). Ag-HPLC has been applied to the separation/quantitation of *cis* and *trans* fatty acids [10,11], FAMES [12–15], positional isomers (as FAMES) from partially hydrogenated vegetable oils [15], conjugated FAMES [16], FAMES labeled with deuterium atoms on the double bond carbons [17], TAG isomers [18–20] and to the separation of FAME or TAG mixtures containing fatty acids (FAs) of widely-differing chain lengths [21].

We utilized a HPLC column chiller/heater to study the effects of column (solvent) temperature on elution rates of TAG (and later FAMES) isomers in Ag-HPLC, using a procedure similar to that employed by Smith et al. [5] who, in 1980, noted improved sample capacity and resolution of the SOS/SSO (S = stearate; O = oleate) TAG isomer pair and other TAG isomers at lower temperatures

\* Corresponding author. Tel.: +1 309 681 6278; fax: +1 309 381 6340.  
E-mail address: [adlofro@ncaur.usda.gov](mailto:adlofro@ncaur.usda.gov) (R. Adlof).

(6.5 °C versus 21.5 °C). Their system consisted of a 20 cm × 7.5 cm glass-lined steel tube slurry-packed with silver nitrate (2.5%–15%) impregnated silica, with benzene (temperature limited to benzene melting point of 5.4 °C) or toluene (less toxic than benzene, but also less efficient at separating SOS/SSO isomer pair) as solvent. We extended their concept by using commercially-available ChromSpher lipids columns and isocratic solvent systems of acetonitrile (ACN) in hexane or ACN and diethyl ether (EE) in hexane.

## 2. Materials and methods

### 2.1. Materials

Hexane (ACS grade, Allied Fisher Scientific, Orangeburg, NY, USA), diethyl ether (Fisher Scientific, Fair Lawn, NJ, USA) and acetonitrile (ACN; E. Merck, Darmstadt, Germany) were used as received. TAG isomers POP, PPO, SLnS, SSLn, LOL and LLO (where P = palmitic acid (16:0), S = stearic acid (18:0), O = oleic acid (9c-18:1), L = linoleic acid (9c,12c-18:2) and Ln = linolenic acid (9c,12c,15c-18:3)) were synthesized by the method of Kodali (see [22]) with fatty acids obtained from Nu-Chek-Prep, Elysian Fields, MN, USA, and used to prepare TAG mixtures I (POP/PPO), II (SLnS/SSLn) and III (LOL/LLO) (approximately 50/50 each isomer pair (wt.%); 1- and 3-positions of TAG assumed equal; approximately 10 mg/mL iso-octane). TAG mixture IV (approximately 20% ea. AcAcAc (where Ac = acetate), SSS, OOO, LLL and LnLnLn] and FAME mixtures I (approximately 20% ea. S, O, L, arachidonate (5c,8c,11c,14c-20:4) and docosahexaenoate (4c,7c,10c,13c,16c,19c-22:6)) and II (18:2 isomers (9t,12t- and 9c,12t-18:2)) were prepared using TAGs (excluding triacetin) and FAMES obtained from the same source. Tri-palmitin and triacetin were obtained from Sigma–Aldrich, St. Louis, MO, USA.

### 2.2. Methods

#### 2.2.1. High-performance liquid chromatography

A Spectra System (Thermo Finnigan, San Jose, CA, USA) HPLC system composed of a SCM1000 degasser, a P4000 solvent delivery system, an AS3000 autoinjector (20 µL injection loop), and a UV6000 detector were utilized with data collection via ChromQuest 3.0 software (ThermoQuest, San Jose, CA, USA). The HPLC column temperatures were controlled using a Phenomenex (Torrance, CA, USA) ThermaSphere TS-430 HPLC Column Chiller/Heater. The ChromSpher Lipids columns (Catalog no. 28313; 250 mm × 4.6 mm i.d. stainless steel; 5 µm particle size; silver ion impregnated) were purchased from Chrompack International, Middelburg, The Netherlands, and used as received. Two columns were connected in series and UV detection at 206 nm was used. The void volume of each column was approximately 2.1 mL. The isocratic solvent (1.0% or 1.5% (v/v) ACN in hexane) was prepared that morning and used during a single day for all temperature/retention studies. Solvent flow was standardized at 1.0 mL/min, 1.5 mL/min or 2.0 mL/min and a single solvent reservoir (magnetic stirring) was used to minimize possible changes in FAME or TAG retention(s) and resolution(s) due to batch-to-batch variations in mixed solvent compositions or by use of multiple pumps, reservoirs and/or different mixing chambers.

#### 2.2.2. Gas chromatography

Elution orders were confirmed by injection of known FAME or TAG standards or, in some instances, by collection of the TAG fraction(s) and, after conversion of the TAGs to FAMES [23], analysis on a Varian 3400 gas chromatograph (Varian Instruments, Palo Alto, CA, USA) equipped with a 30 m × 0.32 mm SP2380 (Supelco, Bellefonte, PA, USA) capillary column, flame ionization detection (FID) system and utilizing He as carrier gas. GC conditions were: 155 °C

Table 1  
Ag-HPLC-column temperature vs. TAG elution times

Sample	TAG column temperature (°C)	Elution times (min): TAG (# db) <sup>a</sup>				
		PPP (0)	I POP/PPO (1)	II SLnS/SSLn (3)	II SLnS/SSLn (3) <sup>b</sup>	III LOL/LLO (5)
Peak 1	10	9.9	10.0	12.2	12.4	16.8
Peak 2		10.3	12.9	13.1	16.9	
Peak 1	20	10.1	10.5	14.0	13.9	
Peak 2		10.8	14.9	14.6		
Peak 1	30	10.2	11.1	15.7	15.6	
Peak 2		11.5	16.8	16.7		
Peak 1	40	10.2	11.6	17.3	17.5	34.1
Peak 2		11.8	18.6	18.7	34.8	
ΔR (%) <sup>c</sup>		3	16	42	41	103

System: dual-column Ag-HPLC; 1.0 mL/min 1.0% ACN in hexane, detection = UV at 206 nm. Samples: TAG mixtures I, II, III and IV, 10 mg/mL, 2 µL injection.

<sup>a</sup> TAG structure(s), where (#db): number of double bonds per molecule.

<sup>b</sup> Solvent: 1.0% ACN/0.5% EE in hexane at designated temperatures/solvent flow rates.

<sup>c</sup> [(Peak 1/40 °C – peak 1/10 °C)/peak 1/10 °C] × 100%. Retention time increase of peak 1 10 °C–40 °C.

Table 2  
Ag-HPLC-column temperature vs. homogeneous TAG elution times

Run <sup>b</sup>	Column temperature (°C)	Elution times (min): TAG (# db) <sup>a</sup>			
		SSS (0)	OOO (3)	LnLnLn (9)	AcAcAc (0) <sup>c</sup>
1	10	6.7	7.5	12.6	66.5
2		6.6	7.3	11.8	
1	20	7.0	8.1	15.9	35.0
2		7.2	8.4	16.3	
1	30	7.5	9.4	21.6	26.0
2		7.5	9.4	21.6	
1	40	7.7	10.3	28.2	20.2
2		7.7	10.4	28.4	

System: dual-column Ag-HPLC; 1.0 mL/min 1.5% ACN in hexane, evaporative light scattering detection (ELSD). Samples: TAG standards I (18:0), II (18:1) and III (18:3) and IV (Ac), 10 mg/mL, 2  $\mu$ L injection.

<sup>a</sup> TAG structure(s), where (#db): number of double bonds per molecule.

<sup>b</sup> Run 1 vs. run 2 retention times result from stabilization times of 30 min and 60 min, respectively.

<sup>c</sup> 2.0 mL/min. Solvent composition/other conditions not changed.

(10 min) programmed to 200 °C at 3 °C per min (20 min hold).

Ag-HPLC analyses (see Section 2 for compositions of TAG and FAME samples):

- (a) *TAG I, II and III* (Table 1): The column temperature was set at 10 °C and, after 1 h stabilization, 2  $\mu$ L–5  $\mu$ L of TAG I was injected. After 30 min–40 min at 10 °C (to allow enough time for elution of the TAG isomer(s)), the column temperature was changed to 20 °C and again allowed to stabilize (1 h total) before re-injection of TAG I, etc. Column temperatures could be set manually or programmed; the total time required for TAG retention studies at the four temperatures was 6 h–7 h.
- (b) *TAG II: EE as co-solvent* (Table 1): To determine if the presence of EE as co-solvent had any effect on TAG retention times. Conditions were identical to those used in study (a), but a solvent system of 1.0% (v/v) ACN and 0.5% (v/v) EE in hexane was utilized.
- (c) *Homogeneous TAG mixture* (Table 2): Conditions similar to (a), but with equilibration times of 30 min, solvent composition of 1.5% ACN in hexane, and 60 min elution time for all TAG samples but AcAcAc (2.0 mL/min; 30 min equilibration; 90 min run time).

(d) *FAMEs I* (see Table 3): Conditions similar to (a).

(e) *FAMEs II* (see Table 4): Conditions similar to (a).

### 2.2.3. Reproducibility study TAG II (not shown)

The TAG standard was injected at 20 °C, then at 40 °C, and then again at 20 °C (1 h equilibration and dual injections at each temperature) to (a) confirm the observed changes in retention times were due to changes in temperature, not solvent composition, over the approximately 5 h duration of the test and (b) determine if the 1 h equilibration time at each temperature was sufficient for reproducibility.

### 2.2.4. Solvent volume/temperature study

Eluting solvent volumes (1 mL/min, 1.0% ACN in hexane) were collected (in cooled, graduated cylinders) over a timed 2 h-period at 10 °C and then at 40 °C. The collected volumes were compared to determine if solvent flow rates were influenced by solvent temperature. Total 2 h elution volumes (10 °C versus 40 °C) were identical at 119.0 mL.

### 2.2.5. Solubility of ACN in hexane study

Solvent samples (10 mL) of 1.0% (v/v) and 1.5% (v/v) ACN in hexane were placed in scintillation vials and stored

Table 3  
Ag-HPLC-column temperature vs. FAME elution times

Run	Column temperature (°C)	FAME elution times (min) <sup>a</sup>				
		18:0	9c-18:1	9c,12c-18:2	5,8,11,14–20:4	4,7,10,13,16,19–22:6
1	10	7.2	7.7	8.4	10.3	13.8
2	20	7.1	7.8	8.6	11.1	15.8
3	30	7.1	7.9	8.8	11.9	18.1
4	40	7.0	7.9	9.0	12.8	20.4
$\Delta R$ (%) <sup>b</sup>		–	3	7	24	48

System: dual-column Ag-HPLC; 1.0 mL/min 1.0% ACN in hexane, UV at 206 and evaporative light scattering detection (ELSD) detection. Sample: 18:0, 18:1, 18:2, 20:4, 22:6 FAME mix I, 5.0 mg/mL, 2  $\mu$ L injection.

<sup>a</sup> FAME retention times (min).

<sup>b</sup> [(Pk @ 40 °C – Pk @ 10 °C)/Pk @ 10 °C (in min)]  $\times$  100%. Retention time increase between 10 °C and 40 °C.

Table 4  
Ag-HPLC-column temperature vs. *cis/trans* FAME elution times

Run	Column temperature (°C)	Elution times (min) <sup>a</sup>			
		9t,12t-18:2	9t,12c-18:2	ΔT2 – T1	Resolution <sup>b</sup>
1	10	7.73	7.95	0.20	1.1
2	20	7.76	8.09	0.33	1.5
3	30	7.79	8.23	0.44	1.9
4	40	7.76	8.29	0.53	2.1

System: dual-column Ag-HPLC; 1.0 mL/min 1.0% ACN in hexane. Sample: FAME mix II, 18:2 (9t,12t- and 9c,12t-18:2), 0.25 mg/mL, 5 μL injection.

<sup>a</sup> FAME retention (min).

<sup>b</sup>  $R = (T2 - T1) / [(W2 + W1) / 2]$ , where T2, T1 = elution times (min) 9t,12c-18:2, 9t,12t-18:2, respectively at given temperature, etc. W1 is width (in min) of 9t,12t-18:2 peak at baseline, etc.

at 20 °C, 10 °C, 0 °C, and –15 °C overnight, and then examined for phase separation. Both the 1.0% and 1.5% ACN in hexane samples remained homogeneous at 20 °C and 10 °C, but phase separation was noted at 0 °C in the 1.5% ACN sample and in both samples at –15 °C.

### 3. Results and discussion

The effects of column temperatures on the elution of the SLnS/SSLn TAG isomer pair are tabularized in Table 1 (chromatograms of the SLnS/SSLn elution patterns and resolutions at 10 °C and 30 °C are illustrated in Fig. 1). The increase of column temperature from 10 °C to 40 °C unexpectedly resulted in a significant decrease in elution rate (increased retention time) for the SLnS/SSLn isomer pair of almost 50% (from 12.9 min at 10 °C to 18.6 min at 40 °C). Addition of 0.5% EE as co-solvent to the isocratic 1.0% ACN in hexane solvent system at the four temperature levels (Table 1) resulted in only a slight (<1%) increase (at 10 °C and 40 °C) or decrease (at 20 °C and 30 °C) in retention time(s). (No changes in TAG or FAME elution orders were noted during any of these studies, although normal-phase [21] contributing factors could potentially result in such changes). A study using the LOL/LLO (where L = linoleate and O = oleate) TAG isomer pair (all other conditions the same) resulted in an even more dramatic increase in elution times, from 16.8 min at 10 °C to 34.8 min at 40 °C. Stabilization times of 0.5–1.0 h were found sufficient (retention times ± 0.6%) for our studies and may perhaps be reduced even further by use of temperature-controlled solvent reservoirs,

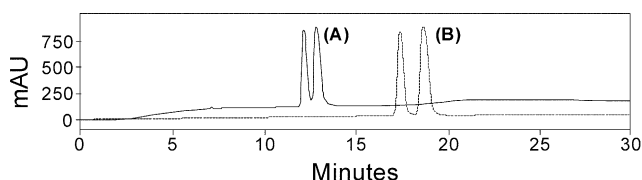


Fig. 1. Elution of SLnS/SSLn TAG standard at 10 °C (A) and 40 °C (B). Dual-column Ag-HPLC; injection = 2 μL 10 mg/mL TAG mix in isoctane; solvent: 1 mL/min of 1.0% ACN in hexane; detection: UV at 206 nm.

inlet lines and/or injectors. Neither the number of samples injected nor changes in column temperatures seemed to impact column stability/sample retention times or sample-to-sample reproducibility (see reproducibility study: TAG II in Section 2.2.3), and that solvent flow rates did not vary with temperature (see solvent volume/temperature study in Section 2.2.4).

While this interesting effect (slower sample elution at higher temperatures) has also been noted with some silica- and alumina-based HPLC systems [24], it has not been found with cyano- or diol-HPLC columns [24], and the opposite effect (more rapid sample elution at higher temperatures) has been noted with C<sub>18</sub> reversed-phase HPLC columns [25]. And, it should be emphasized again that the temperature versus TAG or FAME retention effects described in this manuscript were observed only in Ag-HPLC with hexane-based solvent systems and have not been observed with the chlorinated hydrocarbon solvent-based systems utilized by Christie [26,27].

The retention of FAME, TAG or other olefinic compounds by silver ion chromatography, considered due primarily to the interaction of the Ag<sup>+</sup> ions and the olefinic π electrons of the sample molecule(s), can be roughly correlated with the number of double bonds in the molecule. The most highly unsaturated FAMES/TAGs are usually the most strongly retained and, in our studies, exhibited the greatest temperature effect. This effect (10 °C versus 40 °C) is demonstrated in both TAG (see *R* (%), POP/PPO versus LOL/LLO (Table 1) and OOO versus LnLnLn (Table 2)) and FAME (18:1 versus 18:2 versus 20:4 versus 22:6 (Table 3)), and is more noticeable with *cis* than with *trans* double bonds (see 18:2, Table 3 and Table 4). The lack of this effect is demonstrated for saturated TAG (SSS, Table 2), while the opposite effect (a “normal phase”, more rapid sample elution at higher temperatures) is noted to a very slight degree in saturated FAME (18:0, Table 3), and significantly in the TAG AcAc (Table 2). We also noted improved resolution of the 18:2 FAME isomers (Table 4) as column temperature increased, a result similar to the improved resolution between SOS/SSO TAG isomers (silver nitrate impregnated silica gel and toluene as solvent) noted by Hammond and co-workers [5] at lower temperature(s).

In gas chromatography, changes in the distribution of the sample molecule(s) between the carrier gas and the liquid stationary phase in the GC column have been used to explain [8] in part the more rapid elution of samples with increasing column temperatures. In Ag-HPLC, solvent modifiers such as 2-propanol and acetone may reduce the interaction of the sample with silanol and other polar groups of the packing [28]. Unsaturated solvents such as benzene, toluene and ACN are assumed to complex with the silver ions, thus reducing the silver ion interaction(s) with the double bond(s)/unpaired electrons of the eluting sample [29,30]. ACN, which complexes strongly with silver ions, is often used as a phase modifier in Ag-HPLC [8]. One explanation for this temperature versus retention effect may be a temperature-induced change in the stability of the ACN/Ag<sup>+</sup> complex. The complex of ACN and Ag<sup>+</sup> ions is presumably exothermic and thereby less stable at higher temperatures, potentially allowing more interactions between Ag<sup>+</sup> ions and substrate double bond(s) and increased retention of unsaturated substrates at higher temperatures. No evidence of ACN insolubility in hexane was noted at the concentrations (1.0% and 1.5% ACN in hexane) and temperature range (10 °C to 40 °C) utilized in our studies, although phase separation was noted for 1.5% ACN at 0 °C and for both 1.0% and 1.5% ACN in hexane at –15 °C (Section 2.2.5: solubility of ACN in hexane study).

We found the isocratic ACN in hexane solvent system and the commercially available ChromSpher Lipids column(s) to be a useful combination for the separation of a variety of FAME and TAG isomers, a methodology potentially further enhanced by utilization of other hexane-based solvent systems and, unlike the silver nitrate-impregnated silica system and potential loss of silver nitrate, capable of reproducible retention times even after injection of 70+ samples over a 4-week-period. Current investigations include application of temperature programming to Ag-HPLC, from 50–60 °C to 10 °C or lower (dependent on percentage ACN in hexane), a technology similar to use of solvent programming in HPLC or temperature programming in GC for separation of complex mixtures of FAME or TAG isomers.

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