ELSEVIER

Journal of Chromatography A, 764 (1997) 337–340

JOURNAL OF CHROMATOGRAPHY A

Short communication

Normal-phase separation effects with lipids on a silver ion high-performance liquid chromatography column

Richard O. Adlof

Food Quality and Safety Research, National Center For Agricultural Utilization Research, USDA, Agricultural Research Service, 1815 N. University Street, Peoria, IL 61604, USA

Received 7 August 1996; revised 14 October 1996; accepted 17 October 1996

Abstract

The separation of saturated fatty acids (as the methyl esters and as triacylglycerols) of differing chain lengths by silver ion chromatography (ChromSpher Lipids; containing silver ions; Chrompack, Middelburg, Netherlands) was studied. The retention characteristics (isocratic solvent system of acetonitrile in hexane) of saturated fatty acid methyl esters and homogeneous, saturated triacylglycerols were found to be inversely related to the number of carbon atoms in the fatty acid(s). Thus methyl stearate eluted first and methyl acetate eluted last; for triacylglycerols, tripalmitoylglycerol eluted first while triacetin was strongly retained (11 min vs. 70 min). While mixtures of fatty acid methyl esters and of triacylglycerols could be separated, resolution was found to improve at higher concentrations of acetonitrile, a characteristic of normal-phase separations. In the absence of carbon–carbon double or triple bonds, the effects of the more polar silanol groups of the column packing tended to override the silver ion contributions (interaction of silver ions with the unpaired electrons of the carbonyl oxygens), resulting in separations similar to those observed in silica thin layer and normal-phase high-performance liquid chromatography.

Keywords: Silver ion liquid chromatography; Lipid; Fatty acid methyl ester; Triacylglycerol

1. Introduction

Silver ion high-performance liquid chromatography (Ag-HPLC) is a very versatile method for separating a wide variety of fatty acid methyl ester (FAME) and triacylglycerol (TAG) isomers [1–4]. Utilizing commercially available HPLC columns and an isocratic solvent system of acetonitrile (ACN) in hexane, we have been able to separate and quantitate both positional [5] and geometric [6] FAME isomers, to fractionate TAG [7] positional isomers and to separate monoacylglycerol (MAG) and diacylglycerol (DAG) positional isomers as the acetate derivatives [8].

The retention of MAG and DAG positional isomer pairs [as acetate(s)] by Ag-HPLC was found to be directly related to the number of acetate groups [or inversely related to the number of carbon atoms in the fatty acids (FAs)] on the TAG, with the more acetate-containing TAGs being retained longer [8]. Utilizing a dual-column Ag-HPLC system at a solvent flow of 1 ml of 1.2% ACN in hexane per minute, tripalmitoylglycerol (3×16:0) was thus found to elute first (ca. 11 min), while triacetin (3×2:0) eluted at ca. 70 min. The retention of unsaturated FAMEs and TAGs on silver ion chromatographic systems has generally been related to the stability of the complex formed between the

0021-9673/97/$17.00
PII S0021-9673(96)00886-2

silver ions and the π-electrons of the carbon–carbon double bond(s) and utilized factors related to total number, location, separation and geometry of the FA double bonds. These conditions are absent in the 16:0 series. Saturated FAs (as TAGs or FAMEs) tend to elute with or very close to the solvent front and are usually assumed to be unretained [7]. This assumption is contradicted by the large retention difference noted between 3×16:0 and 3×2:0. A study was initiated to examine this effect and its impact on the retention characteristics of unsaturated FAMEs and TAGs on silica/silver ion systems.

2. Experimental[1]

2.1. Materials and reagents

Hexane (Allied Fisher Scientific, Orangeburg, NY, USA and E. Merck, Darmstadt, Germany) were used as received. FAME standards (2:0, 4:0, 6:0, 10:0, and 16:0) and some of the TAG standards (3×6:0, 3×8:0, 3×10:0) were obtained from ChemService (West Chester, PA, USA) while the remaining TAG standards (3×2:0, 3×12:0, 3×14:0, 3×16:0 and 3×18:0) were obtained (99%+ pure) from Sigma, St. Louis, MO, USA. Specific FAME are designated 2:0 (acetate), 4:0 (butyrate), 6:0 (hexanoate), 8:0 (octanoate), 10:0 (decanoate), 12:0 (dodecanoate), 14:0 (tetradecanoate), 16:0 (hexadecanoate; palmitate) and 18:0 (octadecanoate; stearate); homogeneous TAG are denoted as 3×FAME (ie. 3×16:0). The specific FAs in the 1(3)-, 2- and 3(1)- positions of mixed TAGs are listed individually (i.e., 16:0/18:1/16:0; 18:1 defined as oleic acid). We do not differentiate between the 1- and 3- positions.

2.2. High-performance liquid chromatography

HPLC analyses were performed utilizing a Spectra-Physics P2000 solvent delivery system (Thermo Electron, Walthame, MA, USA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20 μl injection loop and an ISCO V4 Absorbance Detector (Isco, Lincoln, Nebraska, USA) set at 206 nm. Flame ionization detection (FID; Tracor Model 945; Tremetrics, Austin, TX, USA) was also used. Detector signal output was monitored by computer (Grams/386 for Chromatography, Galactic Industries, Salem, NH, USA). To improve peak resolutions and to allow the injection of larger sample sizes, two ChromSpher Lipids columns (Cat. No. 28313; 250 mm×4.6 mm I.D. stainless steel; 5 μm particle size; silver ion impregnated; Chrompack) were connected in series. Solvent flow was standardized at 1.0 ml/min.

2.3. Analyses of HPLC fractions

TAG elution patterns were determined by co-injection of purchased standards or by conversion of the isolated fractions to FAMEs [9]. FAMEs were analyzed with a Varian 3400 GC (Varian Instruments, Palo Alto, CA, USA) equipped with a 30 m×0.32 mm SP2380 (Supelco, Bellefonte, PA, USA) capillary column and FID. He was utilized as carrier gas. Methyl ester peaks were identified by comparison with standard FAME mixtures of known composition.

3. Results

The separation of a mixture composed of saturated, homogeneous TAGs is shown in Fig. 1; Insert A illustrates the optimized separation of the same TAG (without 3×2:0) mixture. The three chromatograms in Fig. 2 demonstrate the effect of increasing the percentage of ACN in the ACN–hexane solvent system on the separation of a mixture of saturated FAMEs of differing chain-lengths.

4. Discussion

The retention of unsaturated FAME and TAG isomers by HPLC, thin-layer chromatography (TLC) and on similar systems containing silver ions is usually considered to be directly related to the stability of the complex formed between the silver

[1] Names are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

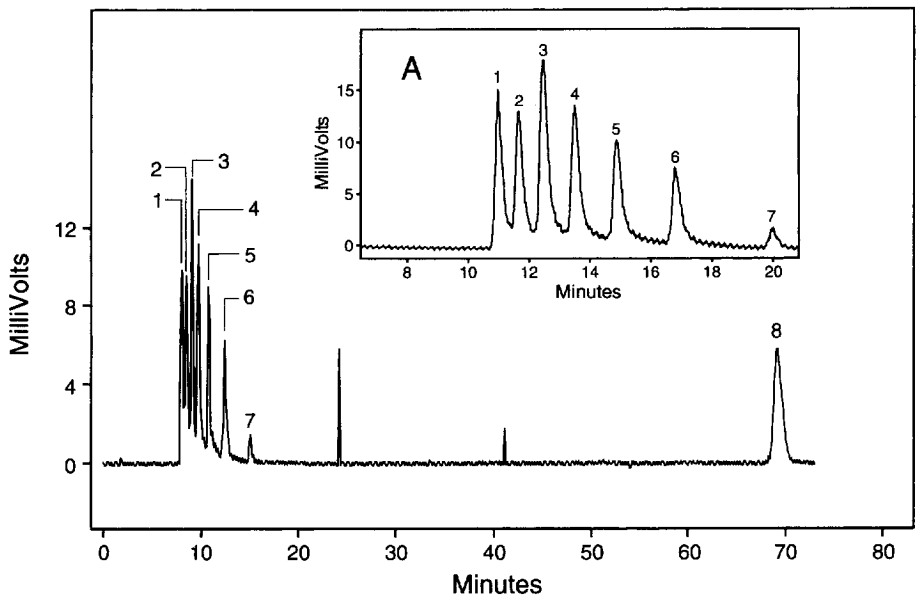

Fig. 1. Analysis of homogeneous, saturated TAG mix [3×18:0 (1), 3×16:0 (2), 3×14:0 (3), 3×12:0 (4), 3×10:0 (5), 3×8:0 (6), 3×6:0 (7)] containing 3×2:0 (8) by dual-column, Ag-HPLC. Sample size: 44 μg. Flow-rate: 1.0 ml/min 1.2% ACN in hexane. Flame ionization detection. Insert A: Optimized separation of TAG mix (no 3×2:0). Sample size: 38 μg. Flow-rate: 1.0 ml/min 0.7% ACN in hexane. Flame ionization detection.

ion and the π-electrons of the carbon–carbon double bond(s). Smaller contributions from the unpaired electrons of the carbonyl-group oxygens [2] may also influence retention. In silica-based TLC and HPLC, silanol group (normal-phase) effects [2–4,10,11] have been used to explain the elution patterns of unsaturated FAME and TAG mixtures containing

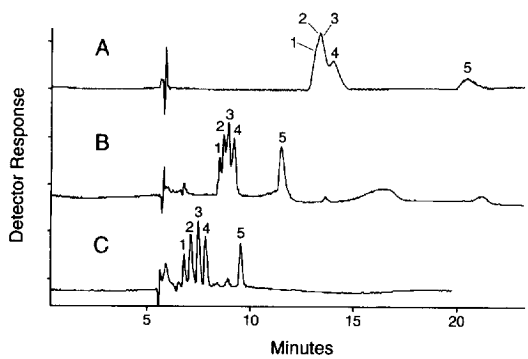

Fig. 2. Analysis of FAME mixture [16:0 (1), 10:0 (2), 6:0 (3), 4:0 (4), 2:0 (5)] by dual-column, Ag-HPLC at differing contents of ACN in hexane. Sample size: 20 μg. Flow-rate: 1.0 ml/min. Flame ionization detection. Insert A: 0.05% ACN in hexane; B: 0.2% ACN in hexane; C: 1.0% ACN in hexane.

FAs of differing chain lengths. Such effects have also been applied to the separation of unsaturated TAGs by Ag-TLC [12] and Ag-HPLC [8,12] and to TAGs separated by supercritical fluid chromatography (using a silica-based cation-exchanger in the silver ion form) [13]. The improved resolution we observed with increasing ACN content in hexane (Fig. 2) is also an effect more applicable to normal-phase systems [2,10] and, in the case of unsaturated FA and TAG, would seem to conflict with, rather than augment, the resolving power of the silver ions.

Some 30% of the original silanol groups of our Ag-HPLC column remain unreacted. In the absence of carbon–carbon double or triple bonds, substrate interaction with the more polar silanol groups of the column packing must predominate. Although silver ion effects might still be present, these effects can, in this instance, be considered minor when compared to the normal-phase contributions of the silanol groups. These contributions are most prevalent in 3×2:0, and diminish rapidly with increasing FA chain length. The TAG positional isomers 18:1/2:0/18:1 and 18:1/18:1/2:0 are readily separated by Ag-HPLC and a similar separation pattern is also observed for

16:0/2:0/16:0 and 2:0/16:0/16:0 [8]. The acetate group apparently exerts a larger influence on retention than the oleate group and may actually reduce resolution when linoleate or linolenate-containing MAGs or DAGs [converted to the acetate(s)] are analyzed by Ag-HPLC. The use of a longer-chain (5:0 or 6:0) anhydride (rather than acetic anhydride) to prepare the MAG or DAG derivatives may be necessary to optimize resolution of polyunsaturated FA-containing MAG and DAG positional isomers. Normal-phase effects must also be considered when attempting to predict the Ag-HPLC elution patterns of FAME or TAG mixtures containing FAs of widely-differing chain lengths.

It is unclear if these effects also influence the retention of saturated FAMEs and TAGs in the chlorinated hydrocarbon/ACN solvent systems utilized by Christie [3], Nikolova-Damyanova [4] and others. However, Netting and Duffield [14] demonstrated limited separation (and the same order of elution as obtained by us) of a series of straight-chain fatty acids (as pentafluorobenzyl esters) on a silica gel column using a solvent system of dichloromethane–hexane half-saturated with water (3:17).

In this research, we have expanded the applications of these versatile silver ion columns by use of their normal-phase characteristics. However, increased utilization of the dual functionality of the ChromSpher Ag-HPLC column is dependent on a more thorough understanding of the varied and complex interactions of solvent, solute, silver and silanol which characterize the concept of Ag-HPLC.

Acknowledgments

We thank T. Lamm for technical assistance.

References

[1] W.W. Christie, New Trends in Lipid and Lipoprotein Analyses, AOCS Press, Champaign, IL, USA, 1995, Ch. 6 and Ch. 7.
[2] G. Dobson, W.W. Christie and B. Nikolova-Damyanova, J. Chromatogr. B., 671 (1995) 197.
[3] W.W. Christie, High-Performance Liquid Chromatography and Lipids, Pergamon Press, Oxford, UK, 1987, p. 156.
[4] B. Nikolova-Damyanova, in W.W. Christie (Editor) Advances in Lipid Methodology - 1, Oily Press, Ayr, UK, 1992, pp. 181ff.
[5] R.O. Adlof, L.C. Copes and E.A. Emken, J. Am. Oil Chem. Soc., 72 (1995) 571.
[6] R.O. Adlof, J. Chromatogr. A, 659 (1994) 95.
[7] R.O. Adlof, J. High. Resolut. Chromatogr., 18 (1995) 105.
[8] R.O. Adlof, J. Chromatogr. A, 741 (1996) 135.
[9] R.L. Glass, J. Dairy Sci., 52 (1969) 1289.
[10] R.P.W. Scott and P. Kucera, J. Chromatogr., 112 (1975) 425.
[11] J.K.G. Kramer, R.C. Fouchard and E.R. Farnworth, Lipids, 20 (1985) 617.
[12] B. Nikolova-Damyanova, B.G. Herslof and W.W. Christie, J. Chromatogr. A, 609 (1992) 133.
[13] M. Demirbuker and L.G. Blomberg, J. Chromatogr., 550 (1991) 765.
[14] A.G. Netting and A.M. Duffield, J. Chromatogr., 257 (1983) 174.