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Short communication

Analysis of fatty acid mono- and diacylglycerol positional isomers by silver ion high-performance liquid chromatography

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Abstract

Mono- and diacylglycerol positional isomer pairs were separated (as acetates) by silver ion high-performance liquid chromatography on a commercially available column using an isocratic solvent system of 1.2% (v/v) acetonitrile in hexane and flame ionization detection. The acetate derivative(s) from the 1- and 2-monoacylglycerols and 1,2- and 1,3-diacylglycerols were prepared by acetic anhydride–pyridine. Conversion of the thermodynamically less stable 2-mono- and 1,2- diacylglycerols to triacylglycerols containing acetate(s) in the 1-, 3- and 2-positions, respectively, resulted in <3% isomerization. Conversion of the thermodynamically more stable 1-mono- and 1,3-diacylglycerol analogues yielded <1% isomers. Less than 0.5% inter-esterification by-products were noted.

The triacylglycerol, the diacylglycerol–monoacetate isomer pair, the monoacylglycerol–diacetate isomer pair and triacetin were completely separated for the 16:0 and 18:1 fatty acid series. The triacylglycerols eluted first and the triacetin eluted last. The 16:0 elution pattern is unusual, since silver ion chromatographic separations are generally ascribed to the interaction of silver ions with carbon–carbon double bond π-electrons, a condition absent in the 16:0 series.

Keywords: Monoacylglycerols; Diacylglycerols; Triacylglycerols; Lipids

1. Introduction

The synthesis of mixed fatty acid (FA) triacylglycerol (TAG) isomers of the structure ABA and AAB (where "A" and "B" are different FAs containing zero to three double bonds) for human metabolism studies required a method to determine the isomeric purity (1,2- vs. 1,3-) of the 1,2- or 1,3-diacylglycerol precursor(s). Methods for analysis [1–4] of monoacylglycerols (MAG) and diacylglycerols (DAG) without derivatization are limited, due to the ease with which MAG and DAG isomerize. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) utilizing silica gel as the sorbent can be used, but require boric acid or calcium ions to minimize isomerization [3,5,6]. Reversed-phase chromatography has been used to separate 1,3- and 1,2-DAG isomers [7], although Payne-Wahl et al. [8] obtained better DAG peak shapes and resolution on an amino-, cyano-bonded HPLC column. Silver nitrate TLC has been used to separate the acetate derivatives of MAG and DAG [9], although not as the 1,3- vs. 1,2- positional isomers. Preparation of trimethylsilyl or acetate [10] derivatives allows the MAG and DAG isomers to be analyzed by gas chromatography, but the technique requires high temperatures and on-column injection [11] for quantitation. A combination of enzymatic

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hydrolysis, methylation and GC has recently been described for determination of the positional distribution of fatty acids in TAG and DAG [12]. Underivatized acylglycerols have been analyzed by GC [13], but quantitation of the positional isomers has not been done.

Utilizing a commercially available HPLC column (ChromSpher™ Lipids) containing silver ions (Chrompack, Middelburg, Netherlands) and an isocratic solvent system of acetonitrile (ACN) in hexane, we have been able to separate and quantitate both positional [14] and geometric [15] fatty acid methyl ester (FAME) isomers and to fractionate TAG positional isomers [16], with fatty acid compositions of AAB or ABA. In this paper the concept is extended to the separation and quantitation of 1- and 2-MAG and 1,2- and 1,3-DAG positional isomers, by silver ion high performance liquid chromatography (Ag-HPLC) of their acetate derivatives.

2. Experimental

2.1. Materials and reagents

Hexane (Allied Fisher Scientific, Orangeburg, NY, USA), ACN, benzene, acetic anhydride and pyridine (all from Merck, Darmstadt, Germany) were used, as received. MAG and DAG positional isomer samples (trioleoylglycerol, 1-monooleoylglycerol, 2-monooleoylglycerol, 1,2-dioleoylglycerol, 1,3-dioleoylglycerol and the analogous palmitate (16:0) glycerols) and triacetin were obtained (99%+ pure) from Sigma (St. Louis, MO, USA). The specific FA in the 1-, 2-, 1,2- and 1,3- positions are designated by the letters P (Palmitate; hexadecanoic), O (Oleate; *cis*-9-octadecenoic), and Ac (Acetyl; ethanoic). The hydroxy function will be designated as [OH]. No differentiation is made between the 1- and 3- positions.

2.1.1. Acetate derivatives (10)

A 5–10 mg amount of acylglycerol sample was placed in a 1-dram vial and 20 drops (ca. 230 mg) of acetic anhydride and 2 drops (ca. 40 mg) of pyridine were added. The sample was capped and heated (under nitrogen atmosphere) for 1 h at 80°C, cooled to room temperature, and the acetic anhydride and pyridine were removed by a stream of nitrogen (heating to 40°C was required for 16:0). A palmitate (16:0) mixture and an oleate (18:1) mixture (both containing the TAG, the diacylglycerol–monoacetate isomer pair, the monoacylglycerol–diacetate isomer pair, and triacetin) were prepared and dissolved (ca. 10 mg of TAG mix/ml) in benzene.

2.2. High-performance liquid chromatography

A Spectra-Physics P2000 solvent delivery system (Spectra-Physics, Freemont, CA, USA) and a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20-μl injection loop were used. The Chromspher Lipids columns (Cat. No. 28313; 250×4.6 mm I.D. stainless steel; 5 μm particle size; silver ion impregnated) were purchased from Chrompack (Middelburg, Netherlands) and were used as received. To improve peak resolutions and to allow the injection of larger sample sizes, two, rather than one, Lipids columns were connected in series. Solvent flow was standardized at 1.0 ml/min; isocratic conditions (1.2% ACN in hexane; 23°C) were used to minimize variations in TAG retention(s) and resolution(s). The void volume of this pair of columns was 4.2 ml. A flame ionization detector (Model 945, Tremetrics, Austin, TX, USA) was used as the HPLC detector and an HP 3390A recording integrator (Hewlett Packard, Avondale, PA, USA) was used for quantitation. Sample mixtures of MAG and DAG in mass ratios of 2:1 to 4:1 were prepared. Pure (>99%) 1-mono-, 2-mono and 1,2-di-oleoylglycerol standards were analyzed by this procedure to determine if inter-esterification occurred or if positional isomers were generated during the acetylation step.

3. Results and discussion

Separation of the underivatized DAG isomers was possible by Ag-HPLC (dual-column, 1.0 ml/min, 1.2% ACN in hexane; data not shown), but attempts to separate monooleoylglycerol isomers (dual-column, 1.0 ml/min, 2.0% ACN in hexane) resulted in distorted and overlapping peaks. Significant isomerization (10–15%) was noted when underivatized 1- or 2-MAG samples were chromatographed, but this

phenomenon was not studied further. Possible sample losses due to silver ion–[OH] interactions were also not studied. A dual-column system was used to improve isomer separations and to allow the injection of larger sample volumes than could be applied to a single-column system.

The acetate derivatives were technically easy to prepare. The higher temperature used (vs. overnight at room temperature) did not appreciably increase isomerization, but did significantly reduce the time required to convert MAG and DAG isomers for subsequent Ag-HPLC analysis. Preparation of the acetate derivative(s) from both the 16:0 and 18:1, 1- and 2-MAG and 1,2- and 1,3-DAG resulted in <3% isomerization in the thermodynamically less stable 2-mono- and 1,2-DAG and <1% isomerization in the more stable 1- and 1,3- analogues. Inter-esterification by-products (TAG or MAG isomers created during acetylation of a DAG standard, for example) were noted at <0.5%. These results are consistent with previous observations [17].

As noted by other researchers [18], results obtained by FID can be used to quantitate MAG and DAG percentage composition data without correction factors. A prepared DAG positional isomer mixture (44.8% P[OH]P–55.2% PP[OH]; w/w) was converted to the acetate(s) and analyzed (three times) by Ag-HPLC. The DAG positional isomer composition determined by Ag-HPLC–FID (44.6% PAcP and 55.4% PPAc; S.D.±0.2) was reproducible to within 0.5% of the weighed standard. Results were within 0.5% when the analyzed standards were from just one compound class (MAG isomers or DAG isomers, for example).

The separations of 16:0 and 18:1 acylglycerol isomers (as the acetates) are illustrated in Fig. 1 and Fig. 2, respectively. Elution patterns (as acetates) were consistent, in that TAG eluted first, then the 1,3-DAG, the 1,2-DAG, the 1-MAG, the 2-MAG and then triacetin. All components, including the mono- and diacylglycerol isomer pairs, were rapidly and completely separated.

The 16:0 mixture elution pattern is unusual, since silver ion chromatographic separations are generally ascribed to the interaction of silver ions with carbon–carbon double bond π-electrons, a condition absent in the 16:0 series. Saturated FA (as TAG or FAME), which tend to elute with, or very close to, the solvent

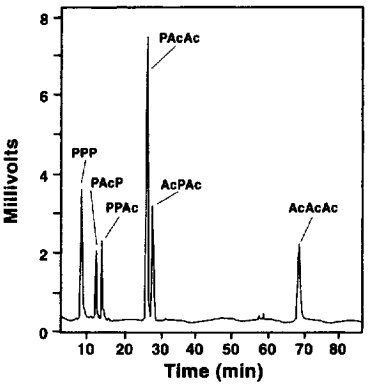

Fig. 1. Analysis of a palmitate–acetate isomer mix containing PPP, PAcP, PPAc, PAcAc, AcPAc and AcAcAc by dual column, silver ion-high performance liquid chromatography. Sample size, 20 μg. Flow-rate, 1.0 ml/min, 1.2% ACN in hexane. Flame ionization detection.

front, have been assumed to be unretained [16]. Actually, 16:0 TAG was found to be retained slightly when compared to 18:0 TAG (See Ref. [19] for similar observations). However, the relatively large retention difference between the 16:0 TAG and triacetin (8 vs. 70 min; Fig. 1) was unexpected. Complexation of carbonyl-group oxygens and silver ions has been implicated in the separation of positional isomers [19]. In TLC on silica gel G plates, TAG containing short-chain FA are retained more strongly than TAG composed of longer-chain FA [1,20]. Thus, substrate interactions with the more polar silanol groups of the Ag-HPLC column pack-

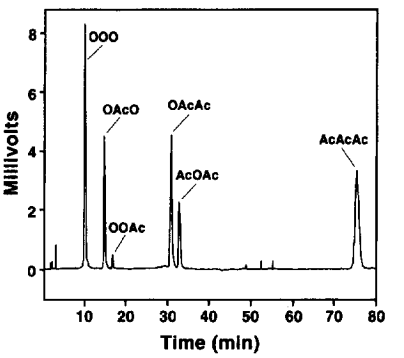

Fig. 2. Analysis of an oleate–acetate isomer mix containing OOO, OAcO, OOAc, OAcAc, AcOAc, and AcAcAc by dual column, silver ion-high performance liquid chromatography. Sample size, 17 μg. Flow-rate, 1.0 ml/min, 1.2% ACN in hexane. Flame ionization detection.

ing (some 30% of the original silanol groups of the silica remain unreacted [21]) or with the phenyl moiety of the bonded phenylsulphonic acid groups may contribute to the observed differences in retention. Steric effects have also been suggested in the separation of the 1,2- and 1,3-distearoylglycerol by reversed-phase chromatography [7]. As shown previously [16,19], the groups located on the 1(3)-position of the acetylated molecule exert a greater influence on retention than those located at the 2-position. The observed large retention difference between tripalmitin and triacetin thus seems to be due to a combination of silver ion–solvent–substrate interactions.

We have used this procedure to determine the purity of DAG synthesized in our laboratory. This procedure may also have uses in the rapid determination of the MAG and DAG configurations of formulations prepared by the confectionary, chocolate and specialty fat industries, industries in which crystallization, mouth feel and visual appeal are important factors.

Acknowledgments

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