Separation of Tripalmitin from Its Hydrolysis Products by Simple Isocratic Reversed-Phase High-Performance Liquid Chromatography

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ABSTRACT: An isocratic reversed-phase high-performance liquid-chromatographic method was developed for monitoring the lipase-catalyzed hydrolysis of solid triglycerides in supercritical carbon dioxide. The reaction products obtained consist of free fatty acids, monoglycerides, diglycerides, and unreacted triglycerides. For the method, developed with tripalmitin, a mobile phase of acetonitrile/chloroform/acetic acid (50:50:1, vol/vol/vol), a C_{18} column, and refractive index detection were used. Analysis time is 7 min. Response factors were determined for each neutral lipid class, which permitted quantitation of the hydrolysis product mixture.

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Research directed to upgrading surplus edible fats and oils by enzymatic modification in supercritical fluids required the use of a method of analysis that was capable of detecting and quantitating the hydrolysis products of tripalmitin. A quick high-performance liquid-chromatographic (HPLC) method was needed with a mobile phase that solubilized both the substrate and reaction products. Unfortunately, a search of the literature failed to uncover a simple HPLC isocratic method that was able to separate the solid neutral lipid classes, tripalmitin, dipalmitin, monopalmitin, and palmitic acid. Reversed-phase HPLC methods that employ solvent gradients with evaporative light-scattering detection (ELSD) have been described (1–4). However, these methods are more complex than isocratic methods with refractive index (RI) detection. An isocratic system with RI detection would be a better choice for this research for several reasons. Isocratic HPLC systems with RI detectors are much simpler and less expensive to operate. The isocratic system described avoids solvent gradients with their associated problems (mobile phase mixing, column reequilibration, etc.), operates at room temperature, and does not require the nebulizer gas needed with ELSD systems. It is also much easier to develop an on-line analysis method with an isocratic system and dedicate that system to a particular analysis.

Reversed phase HPLC is the most widely used HPLC method for lipid analysis (5). However, many researchers have been frustrated in attempting to use reversed-phase HPLC for separating solid mono- di-, and triglycerides as a class (6) because of the known solubility problems presented by solid glycerides. It is difficult to find a mobile phase solvent in which the solids tripalmitin, dipalmitin, monopalmitin, and palmitic acid are all soluble and also can be separated on a reversed-phase column with that mobile phase. Accordingly, the objective of this research was to develop a reversed-phase HPLC method with an isocratic mobile phase and RI detection to quantify the products from lipase hydrolysis of tripalmitin obtained under supercritical carbon dioxide conditions.

EXPERIMENTAL PROCEDURES

Materials. The lipid standards tripalmitin, dipalmitin, and monopalmitin (99%+) were obtained from Nu-Chek-Prep (Elysian, MN); palmitic acid (99%+) was purchased from Sigma (St. Louis, MO). Chloroform, HPLC-grade, was purchased from Burdick and Jackson (Muskegon, MI). Acetonitrile, HPLC-grade, was purchased from Mallinckrodt (Paris, KY). Acetic acid, reagent-grade, was purchased from J.T. Baker (Phillipsburg, NJ). The HPLC system consisted of a Beckman Model 114m HPLC pump, (Beckman Co., San Ramon, CA), a Rheodyne injector Model 7125 equipped with a 50-µL sample loop (Rheodyne, Cotati, CA), a Supelcosil LC 18 reversed-phase column (25 cm × 4.6 mm i.d.) (Supelco Co., Bellefonte, PA), a differential RI detector model R 401 (Waters Inc., Milford, MA), and an HP Model 3396 integrator (Hewlett-Packard, Avondale, PA).

Method. The individual glyceride standards (10 mg) were dissolved in 10 mL of the mobile phase ($CH_3CN/CHCl_3$/AcOH; 50:50:1, vol/vol/vol) with moderate warming to facilitate solution. The solution was then filtered through filter paper (Whatman #1; Fisher Scientific, King of Prussia, PA) to ensure that there were no undissolved solids. An aliquot (3 mL) was transferred to a tared beaker, the solvent was evaporated under nitrogen, and the sample was weighed to determine the exact concentration of sample in the mobile phase.

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Prior to analysis, the mobile phase was degassed by applying a slight vacuum for several minutes. Flow rate was usually 1 mL per min, but slower flow rates and programmed flow rates were tried. The sensitivity of the RI detector was set at 8×. The integrator was programmed to reject negative peaks, a common problem with RI detectors, and sensitivity settings were threshold = 4; peak width = 0.08; attenuation zero; and chart speed 2 cm/min.

At the end of each day, the system was flushed with methanol to remove all chlorinated solvent.

RESULTS AND DISCUSSION

Unreacted tripalmitin and its hydrolysis products from the supercritical fluid extractor were satisfactorily separated within 7 min by the HPLC method described herein (Fig. 1). The most polar lipid class, monopalmitin, eluted first, while the least polar lipid class, tripalmitin, eluted last. Peaks were identified by comparison of retention times with lipid standards. The advantage of using this mobile phase composition ($CH_3CN/CHCl_3$/AcOH; 50:50:1, vol/vol/vol) for this particular analysis is that highly crystalline solid fatty acids, such as palmitic acid, and their corresponding glycerides are soluble in this mobile phase. Tristearin is slightly less soluble (0.3 mg/mL) while tallow is soluble (1.0 mg/mL) in this mobile phase.

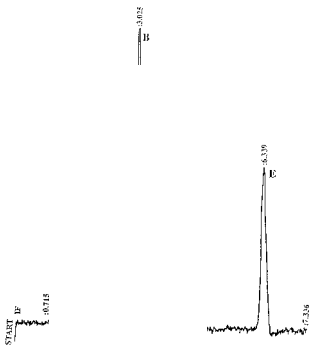

FIG. 1. HPLC analysis of partially hydrolyzed tripalmitin. Peak A = solvent; B = monopalmitin; C = palmitic acid; D = dipalmitin; E = tripalmitin. Mobile phase composition: $CH_3CN/CHCl_3$/AcOH (50:50:1, vol/vol/vol); flow rate, 1.0 mL/min; refractive index attenuation, 8×.

TABLE 1
Peak Areas, Retention Times, and Response Factors for Tripalmitin and Its Hydrolysis Products

Compound	Peak area[a]	Retention time[b]	Response factor[c]
Monopalmitin	462 ± 23	3.11 ± 0.01	0.843
Palmitic acid	270 ± 10	3.38 ± 0.01	0.493
Dipalmitin	483 ± 16	3.91 ± 0.01	0.881
Tripalmitin	548 ± 23	6.38 ± 0.02	1.000

[a]Peak areas are integrator counts per µg at refractive index detector attenuation of 8x plus standard deviation (n = 5).
[b]Retention times are in minutes and standard deviation. Flow rate of 1.0 mL/min; mobile phase composition of $CH_3CN/CHCl_3$/AcOH (50:50:1, vol/vol/vol).
[c]Relative response factors = peak area/tripalmitin peak area.

Quantitation of the lipids shown in Figure 1 required the use of response factors because the individual lipid classes did not have the same response to RI detection. Tripalmitin had the highest response, while palmitic acid had the lowest response. Table 1 lists the peak areas, retention times, and response factors for tripalmitin and its hydrolysis products. From Figure 1 it was calculated, based on three times the baseline noise level, that the minimal detectable level of tripalmitin, required for the RI detector to produce a measurable peak in the chromatogram, is 5.1 µg of tripalmitin in a 50-µL injection.

In conclusion, the reversed-phase HPLC method described herein is capable of separating and quantifying the hydrolysis products of tripalmitin. Moreover, the method is rapid, with analysis times of < 7 min, and may be adaptable to the on-line analysis of triglyceride reactions in supercritical fluids, which would be unlikely with a gradient system.

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