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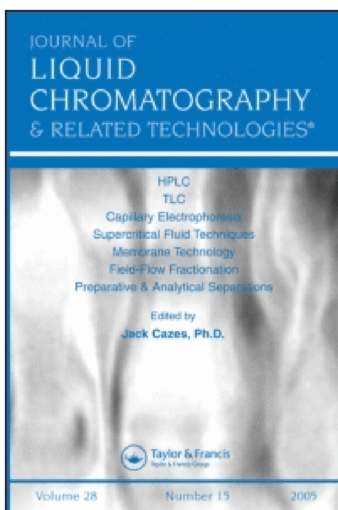

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QUANTITATION OF NEUTRAL LIPID MIXTURES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH LIGHT SCATTERING DETECTION†

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for quantifying reaction mixtures obtained from the lipase-catalyzed transesterification of fats and oils. The reaction mixtures of interest were composed of neutral lipid classes that include alkyl esters, free fatty acids, triglycerides, 1,2- and 1,3-diglycerides, and 1 (2)-monoglycerides. The method, a modification of a literature procedure, uses a binary mobile phase of hexane (A) and methyl-t-butyl ether (B) each modified with acetic acid (0.4%) and a gradient elution profile that reduced analyses' times by 25%. Lipid classes were measured by use of an evaporative light scattering detector (ELSD). Precision of injection and linearity of response of the ELSD over the range of sample amounts of interest were established for the lipid classes measured by use of standards. The method was applied to the compositional analysis of prospective biofuel alkyl esters prepared from fats, oils, and recycled greases.

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INTRODUCTION

Currently, considerable effort is being expended in studies directed to the chemistry and biocatalytic transformations of fats and oils. Among the various transformations studied, lipase catalyzed acyl exchange reactions have been investigated extensively as a way of either altering the physical properties or improving the nutritional quality of fats or oils.[1]

The enzymatic transesterification of fat or oil triglycerides (TG) in alcoholic medium produces the corresponding fatty acid alkyl esters (AE), along with free fatty acids (FFA), diglycerides (DG), monoglycerides (MG) and unreacted TG. Accordingly, a facile method of separation and quantitation for these lipid materials would be useful for the optimization of reaction conditions and or product yields.[2]

Neutral lipid classes are more amenable to separation by high performance liquid chromatography (HPLC) than gas chromatography (GC) because GC analysis requires a pre-analysis fractionation step with subsequent derivatization, whereas normal phase HPLC is able to separate all neutral lipid classes without prior derivatization.[3,4] Previous investigators have found that silica-based normal phase HPLC separation of neutral lipid classes are sometimes irreproducible due to variable amounts of water bound to the silica surface. This problem, however, has been alleviated with the advent of bonded polar phases, such as cyanopropyl.[4,5]

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Refractive Index detection is often used for isocratic HPLC quantitation of lipids. The UV detector is also widely used, but a major disadvantage of this detection method is the weak absorbance of fatty acyl groups.[6] The advent of the evaporative light scattering detector (ELSD), a mass sensitive detector, has greatly improved the usage of HPLC for lipid analysis.[7] This work describes a reproducible method for the separation of alcoholysis products of fats and oils using a cyanopropyl column along with subsequent quantitation of the separated species by ELSD after adequate optimization and calibration of the detector.

MATERIALS AND METHODS

Materials

The following lipid mixtures, purity >99%, were obtained from NuChek Prep, Inc. (Elysian, MN): i) methyl palmitate (MeP), tripalmitin (TP),

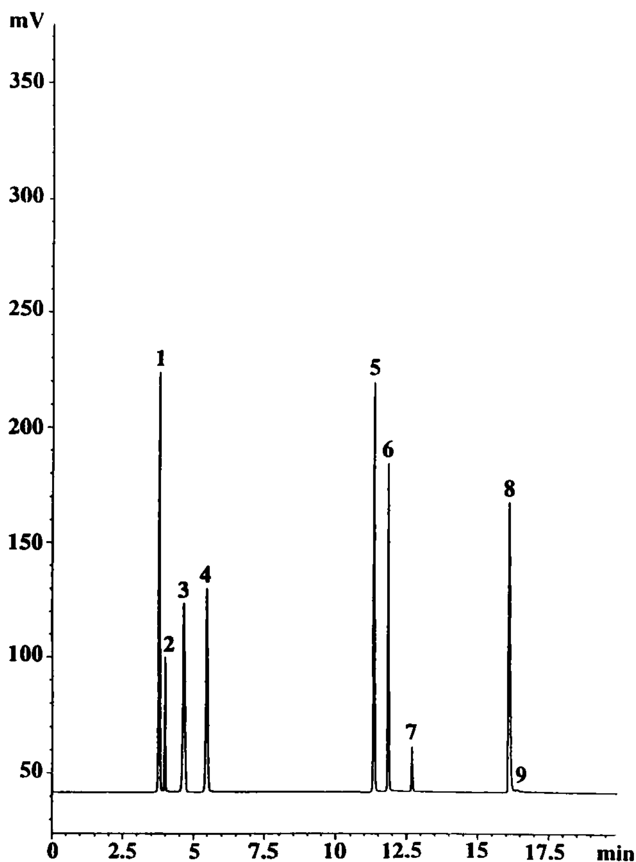

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Figure 1. HPLC of neutral lipid standards. Peak Number 1: cholesterol oleate; 2: methyl oleate; 3: oleic acid; 4: triolein; 5: cholesterol; 6: 1,3-diolein; 7: 1,2-diolein; 8: 1-monoolein; 9: 2-monoolein.

dipalmitin (DP), monopalmitin (MP); ii) methyl stearate (MeS), tristearin (TS), distearin (DS), monostearin (MS); iii) methyl oleate (MeO), triolein (TO), diolein (DO), monoolein (MO); iv) methyl linoleate (MeL), trilinolein (TL), dilinolein (DL), monolinolein (ML). Each lipid component was 25 wt% of the mixture. Stearic acid (SA), palmitic acid (PA), triolein (TO), and methyl oleate (MeO) were obtained from Sigma Chemical Co. (St. Louis, MO). Oleic acid was obtained from Applied Science (State College, PA). Linoleic acid was obtained from Nippon Oil and Fats Co. (Amagasaki, Japan). All solvents used were HPLC grade; methyl t-butyl ether (MTBE) was obtained from J.T. Baker

Table 1

HPLC Solvent Gradient Program

Time (min)	%A[a]	%B[b]
0	100	0
5	100	0
15	20	80
17	20	80
17.1	100	0
27	100	0

[a] Solvent A = hexane + 0.4% acetic acid.
[b] Solvent B = methyl t-butyl ether + 0.4% acetic acid.

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Inc. (Phillipsburg, NJ), and hexane was obtained from Burdick and Jackson (Muskegon, MI). Glacial acetic acid was analytical reagent grade obtained from Mallinkrodt (Paris, KY). Solvents used to make up HPLC gradient were degassed by helium sparge prior to chromatography.

HPLC

Separations were made on a Phenomenex (Torrance, CA) cyanopropyl (CN) column (250 x 4.6 mm i.d.) with an accompanying guard column (30x4.6 mm i.d.) of the same phase and a flow rate of 1.0 mL/min. A detailed elution scheme is given in Table 1. HPLC was conducted using a Hewlett Packard (HP) (Wilmington, DE) 1050 series liquid chromatograph with solvent cabinet, autosampler, and quaternary pump modules. A Varex (Burtonsville, MD) model IIA ELSD was used for detection. Program control, data acquisition, and analysis were carried out using H/P Chem Station software.

RESULTS AND DISCUSSION

The separation of free fatty acid (FFA) and neutral lipid standards obtained on the CN column using a mobile phase gradient of MTBE, hexane, and acetic acid is shown in Fig. 1. Separation of these compounds was initially attempted using the solvent gradient program reported by Al-Hamdy,[4] but there was inadequate resolution of peaks 1 through 4 under these conditions. We found that by decreasing the initial solvent gradient polarity profile and by

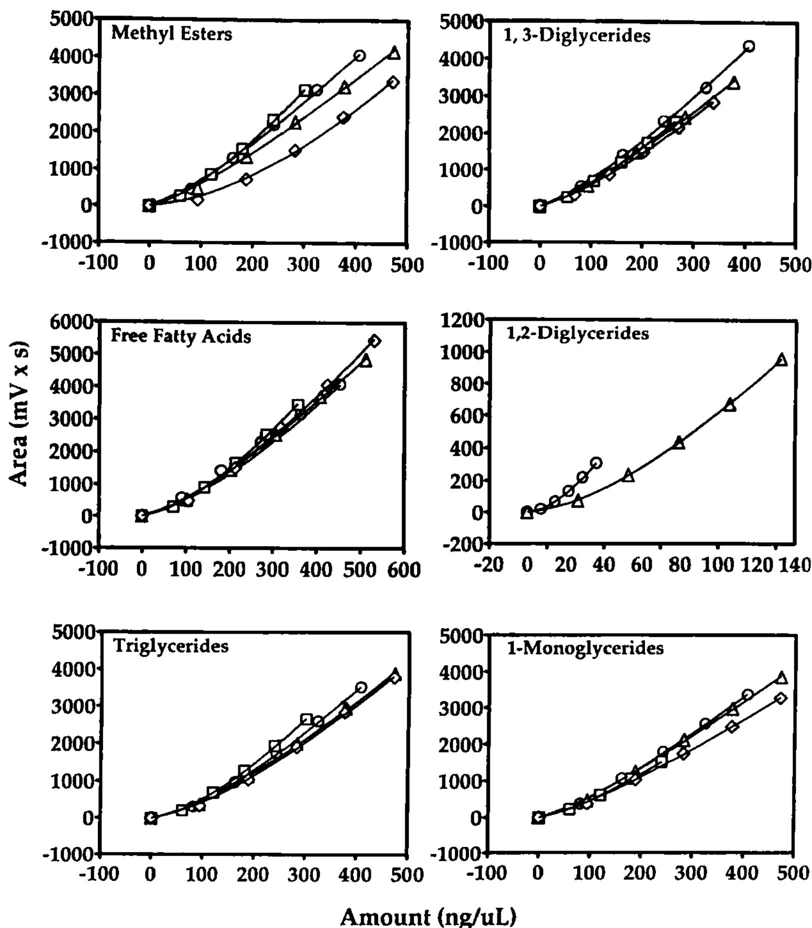

Figure 2. HPLC calibration curves of neutral lipids.

addition of acid modifier (acetic acid, 0.4%) to the mobile phase that a complete baseline separation was obtained that allowed for improved opportunities for sample analysis, automation, and quantitation (Table 1). Complete separation of all lipid classes studied was obtained within 17 minutes with an additional 10 minutes required for reequilibration of the column. Moreover, under the conditions used, there also appeared to be resolution of the 1 and 2-monoglyceride isomers.

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Table 2

HPLC Retention Times of Neutral Lipid Standards[a]

Acyl Group	Methyl Ester	Free Fatty Acid	Triglyceride	1,3 Diglyceride	1,2 Diglyceride	Monoglyceride
			Lipid Class			
C16:0	3.91;[0.12]	4.54;[0.16]	5.44;[0.34]	11.88;[0.19]	12.69;[0.11]	15.76;[0.20]
C18:0	3.87;[0.04]	4.49;[0.11]	5.32;[0.10]	11.84;[0.07]	12.67;[0.04]	16.04;[0.13]
C18:1	4.02;[0.04]	4.77;[0.58]	5.70;[0.16]	11.96;[0.07]	----	15.75;[0.14]
C18:2	4.11;[0.06]	4.87;[0.23]	6.42;[0.36]	12.28;[0.12]	----	16.25;[0.30]

[a] Values shown are average retention times (n=15) in minutes, bracketed values relative standard deviation.

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The sensitivity of an ELSD is controlled by a number of factors. Accordingly, optimization of the detector conditions was determined by repeatedly chromatographing a solution of methyl palmitate, palmitic acid, and tripalmitin at different evaporator tube temperatures (37-60°C) and nebulizer gas flows (0.8-2.35 L/min). The CN column was used with an isocratic solvent system of hexane + 0.4% acetic acid at a flow of 1.0 mL/min. From these runs the optimum detector conditions were determined to be 40°C at a nitrogen flow of 1.5 L/min.

Several papers have appeared on the principles, operation, and factors which significantly control detector response.[8,9] Previous work has shown detector response to be exponential.[10,11] In this work, linearity of detector response versus acyl moiety was determined by constructing calibration curves for all neutral lipid and FFA standards (see Figure 2). Five levels of dilution were prepared in hexane [95:5;hexane:MTBE + 0.4% acetic acid was used to facilitate dissolution of C16:0 and C18:0 series] for each set of lipid standards. Each level was chromatographed three times, sample size 20 µL per injection. The retention time variations for each lipid class studied are listed in Table 2. The data were analyzed using the HPLC data system. In all cases a power function relationship was confirmed with regression coefficients between 0.997 to 1.000.

The HPLC conditions developed for separation of lipid standards were used for the analyses of a series of transesterified triglycerides. The series of fatty acid alkyl esters was prepared by the lipase-catalyzed transesterification of rapeseed and soybean oils, tallow, and recycled greases for their prospective use

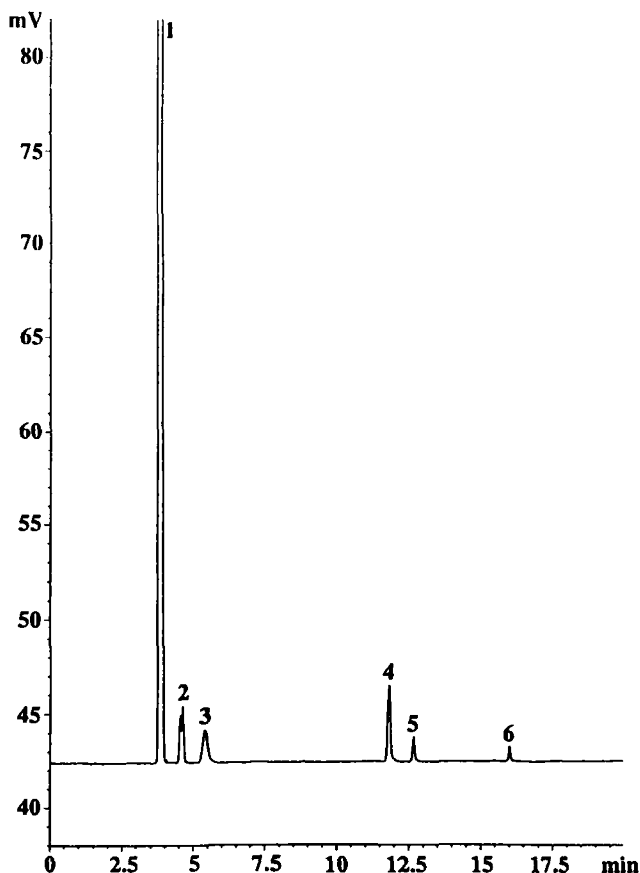

Figure 3. HPLC of tallow transesterified with isopropanol. Peak Number 1: isopropyl esters; 2: free fatty acids; 3: triglycerides; 4: 1,3-diglycerides; 5: 1,2-diglycerides; 6: 1-monoglycerides.

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as biofuel additives.[12] A typical separation obtained for an alkyl ester mixture is shown in Figure 3. Because natural oils and fats are mixed triglycerides, their alkyl ester derivatives are obtained as a mixture of fatty acid esters that vary in chain length and degree of unsaturation. This can result in the splitting of an HPLC peak for a lipid class as exemplified in the free fatty acids, peak 2, in Figure 3. The quantitative results obtained in the analyses of this ester series are listed in Table 3. The first four entries in Table 3 show that the method is suitable for detecting low levels of free fatty acids and diglycerides in intact oils

Table 3

HPLC Composition of Transesterified Fats and Oils

	Lipid Class[a]					
Lipid Sample	Alkyl Ester	Free Fatty Acid	Triglyceride	1,3 Diglyceride	1,2 Diglyceride	Monoglyceride
rapeseed oil	0.41	0.16	98.64	1.39	---	---
soybean oil	---	---	98.34	---	1.66	---
tallow	---	---	100	---	---	---
grease	---	42.24	47.6	6.99	2.52	0.65
ethyl rapeseed	97.04	2.96	---	---	---	---
ethyl soyate	95.48	3.06	---	0.89	0.34	0.22
ethyl tallowate	100	---	---	---	---	---
isopropyl tallowate	93.52	2.46	1.8	1.36	0.47	0.38
2-butyl tallowate	81.15	1.51	17.34	---	---	---
2-butyl grease	97.32	1.39	---	0.44	---	0.85
isobutyl grease	73.17	5.87	20.29	---	---	0.67

[a] Values given are expressed as wt% of lipid class in sample.

and fats, as well as the broad range of lipid classes that may be encountered in the analyses of recycled greases. The remaining entries in Table 3 give the results obtained for their ethyl, isopropyl and butyl ester derivatives. The data show that this HPLC method was effective for monitoring these lipase-catalyzed transesterifications in that it was effective in detecting minor amounts of unreacted glycerides and fatty acids in the transesterified mixtures. This is important if these esters are to meet the specifications outlined for prospective biofuels. The method enabled us to optimize the conditions for conversion of oils, fats, and greases to alkyl esters using the lipase-catalyzed procedure.

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