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Short Communication

Rapid high-performance liquid chromatographic determination of fatty acid profiles of lipids by conversion to their hydroxamic acids

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ABSTRACT

A simple and rapid procedure is described for the determination of fatty acid profiles of lipids by reversed-phase high-performance liquid chromatography with UV detection. Rapid derivatization of esterified fatty acids to their hydroxamic acids was achieved in a single step at room temperature in *ca.* 1 min by conducting the reaction in solvents of low acidity. The long-chain hydroxamic acids (C_8 to C_{24}) were separated on a commercial C_{18} column with a low Fe(III) content, which minimized peak distortion due to strong complexation. The detection limit (2 × baseline noise) was 150 pmol at 213 nm. This method was applied successfully to the analysis of various lipid samples.

INTRODUCTION

The characterization of lipid samples by their fatty acid (FA) profile has acquired considerable significance in a variety of fields. Although gas chromatography (GC) has been used predominantly for such determinations, there has been increasing interest in applying high-performance liquid chromatography (HPLC) for this purpose. In recent years, numerous HPLC methods have been reported utilizing precolumn derivatization for UV detection. A variety of UV-chromophore-containing derivatives have been prepared including phenacyl and substituted phenacyl esters [1–3], 2-nitrophenylhydrazides [4], naphthaliminoethyl [5] and *p*-nitrobenzyl esters [6]. All of the above methods involve derivatization of the *free* fatty acids, and thus require a prior saponification step when determining *esterified* fatty acids.

The ready convertibility of fatty acid esters to their hydroxamic acids (HAs), which absorb strongly in the low-wavelength UV region [7,8] (ε 1000 l mol^{-1} cm^{-1} at 213 nm, 2450 l mol^{-1} cm^{-1} at 206 nm), together with the ability to accelerate this reaction by base catalysis [9], prompted us to investigate this approach to FA profiling. Hamilton *et al.* [10] had reported a rather complex reaction scheme involving on-column conversion of fatty acids to hydroxamic acids, followed by chromatography of their ferric chelates. However, these peaks exhibited considerable tailing. The hydroxamic acids themselves were not monitored because they yielded even more distorted peak shapes, which were ascribed to iron impurities in the column packing. During this investigation, these problems were overcome, allowing the succesful chromatography of the FA hydroxamic acids.

The method described herein is rapid, uses only

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conventional chromatographic equipment, and for natural lipid samples affords results that are in good agreement with those obtained by an established GC method.

EXPERIMENTAL

Reagents and chemicals

Fatty acid methyl esters (FAMEs) and triglycerides were purchased from Sigma (St. Louis, MO, USA). The FAMEs reference mixture was obtained from Nu-Chek Prep (Elysian Park, MN, USA). Methanol and acetonitrile (HPLC grade) and 2-propanol were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Sodium methoxide (25%, w/w) and *tert.*-butyl methyl ether were purchased from Aldrich (Milwaukee, WI, USA). Hydroxylamine hydrochloride was obtained from J. T. Baker (Phillipsburg, NJ, USA). Anhydrous sodium perchlorate was purchased from G. Frederick Smith (Columbus, OH, USA). All were used without further purification.

The hydroxamation reagent, 1.5 *M* hydroxylammonium perchlorate, was prepared by adding 18.6 g of sodium perchlorate to 100 ml of *tert.*-butanol and stirring to comminute and partially dissolve the solid. Finely ground hydroxylamine hydrochloride (10.5 g) was dried for 1 h at 110°C and, after cooling, added, along with one drop of sodium methoxide solution, to the stirring sodium perchlorate suspension. After allowing the mixture to stir at least 4 h at room temperature, the suspension was centrifuged and the reagent solution decanted from the resulting sodium choride solid.

Oleo-, linoleo- and linolenohydroxamic acid were synthesized from the methyl esters following the procedure of Hung [11] with modification. All were recrystallized from hexane at −15°C.

Derivatization procedure

A sample of fat or oil (5–10 mg) was placed in a PTFE-lined screw-capped vial (45 × 10 mm) and dissolved in 1 ml of *tert.*-butyl methyl ether. A 6-μmol amount of methyl nonadecanoate was added as internal standard. A 150-μl volume of the hydroxamation reagent was pipetted in, followed by 150 μl of 25% sodium methoxide solution, and the mixture was shaken for a few seconds to ensure mixing. After 1 min, 2 ml of quench solution (5%, v/v, glacial acetic acid in methanol) was added and a 10-μl aliquot of this mixture was injected directly into the liquid chromatograph for analysis.

HPLC analysis

Analyses were carried out using a Model 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a filter-photometric detector. Separations were performed on a reversed-phase column (250 × 4.6 mm I.D.) obtained from Alltech Assoc. (Deerfield, IL, USA) packed with Nucleosil C_{18} (Macherey-Nagel, Düren, Germany), 5 μm particle size. Column temperature was maintained at 40°C. The absorbance was monitored at 213 nm, and the detector signals were fed to a Model 3392A (Hewlett-Packard) integrator. Samples were injected via a Model 7125 rotary injector valve (Rheodyne, Cotati, CA, USA) fitted with a 10-μl loop.

Analyses were performed by gradient elution, using a methanol–aqueous buffer solvent system at a flow-rate of 1.0 ml/min. The aqueous buffer was prepared as 20 m*M* sodium dihydrogenphosphate adjusted to pH 3 with concentrated phosphoric acid. A 125-μl volume of 1% (w/w) sodium nitrate per liter was added to match the absorbance of methanol at 213 nm. Solvents were degassed continuously by helium sparging.

GC analysis

Analyses were carried out using a Model 5880 gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector. FAME separations were performed on a DB-23 column (J&W Scientific, Folsom, CA, USA). Lipid samples were derivatized to methyl esters by the method of Christie [12] with modification.

RESULTS AND DISCUSSION

Reaction conditions

The conversion of esters to their hydroxamic acids is commonly conducted in hydroxylic solvents such as methanol in the presence of a strong base catalyst such as methoxide ion, and usually requires lengthly reaction times [13]. Since methanol is a relatively acidic solvent, less acidic reaction media were studied as a means of in increasing the relative base strength of methoxide and thereby accelerating

the rate of hydroxamation. The order of decreasing acidity, methanol > 2-propanol > *tert.*-butanol > *tert.*-butyl methyl ether, was accompanied by a concomitant increase in reaction rate. Hydroxamation proceeds particularly rapidly in *tert.*-butyl methyl ether, which also serves as an excellent solvent for non-polar lipids. Under the conditions recommended, the derivatization reaction is complete within 1 min, with 84% yield.

Chromatographic conditions

The elution of hydroxamic acids on commercial reversed-phase columns often gives rise to distorted peaks, which have been attributed to chelation with Fe(III) impurities in the silica support [14]. This is supported by the fact that other substances that form strong Fe(III) complexes behave similarly [15]. In our previous work [8], no appreciable peak distortion had been observed in the chromatography of hydroxamic acids on a 10-μm C_{18} reversed phase synthesized from silica that had been thoroughly acid-washed prior to bonding [16]. However, to attain the greater resolution required for complex HA mixtures, a number of commercial 5-μm reversed-phase columns were investigated. Most were found to be unsatisfactory because of peak distortion. Remedial measures for improving peak shape, such as washing the column with EDTA solution or adding it to the eluent [17], as well as by controlling eluent pH and buffer strength, failed to resolve the problem. However, a commercial reversed-phase column (Nucleosil C_{18}) based on a silica with the lowest reported metal content [18] provided satisfactory results with aqueous methanol eluents, and was utilized for the analyses reported herein. Acceptable peak shape was not obtained, however, from aqueous acetonitrile eluents.

Alternatively, a polymeric reversed-phase column (Hamilton PRP-1, 150 × 4.1 mm, 5 μm) afforded excellent peak symmetry for the individual hydroxamic acids in both methanol and acetonitrile eluents. The resolution achieved with this column, however, was marginal, particularly for complex HA mixtures. Nevertheless, if higher resolution can be achieved, polymeric reversed-phase columns offer three distinct advantages over silica-based ones for HA separations: (i) the absence of iron impurities; (ii) lack of residual hydroxyl groups; (iii) the use of acetonitrile eluents which would permit monitoring at lower wavelengths with resultant higher sensitivity (ε 5600 l mol^{-1} cm^{-1} at 195 nm).

For FA profiling, lipid samples and a standard FAME mixture were derivatized and chromatographed under identical conditions. Fig. 1 presents a chromatogram of a standard mixture of C_{10} to C_{24} FA hydroxamic acids eluted with a linear methanol–aqueous buffer gradient (70–95% methanol in 45 min). Gradients with lower initial methanol content were used for samples containing lower carbon number fatty acids.

The retention times of the HA derivatives vary directly with the number of carbon atoms and inversely with the number of double bonds in the FA moiety. In contrast to other reported FA derivatives, the HA chromophoric group adds no carbon atoms to the original FA species and therefore does not diminish the inherent reversed-phase chromatographic selectivity derived from the carbon backbone of the sample FAs. This effect is demonstrated

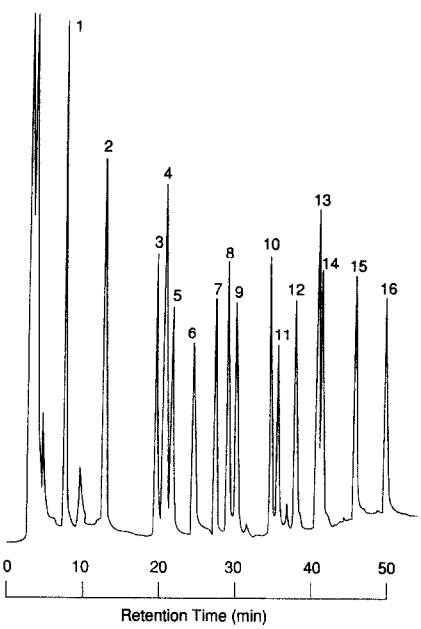

Fig. 1. Chromatogram of the hydroxamic acid derivatives of a FAME mixture separated on a reversed-phase column (Nucleosil) with a methanol–phosphate buffer gradient (70–95% methanol) and UV detection at 213 nm. Peaks: 1 = capric; 2 = lauric; 3 = myristic; 4 = linolenic; 5 = palmitoleic; 6 = linoleic; 7 = palmitic; 8 = oleic (*cis*-18:1); 9 = elaidic (*trans*-18:1); 10 = stearic; 11 = eicosenoic; 12 = nonadecanoic (internal standard); 13 = arachidic; 14 = erucic; 15 = behenic; 16 = lignoceric. Each peak corresponds to 10–30 nmol fatty acid.

TABLE I

ANALYSIS OF NU-CHEK FAME REFERENCE MIXTURE 17A

FAME	Content (%, w/w)		
	Actual	GC	HPLC
Myristic (14:0)	1.0	0.9	0.8
Palmitic (16:0)	4.0	3.7	4.2
Stearic (18:0)	3.0	2.9	2.8
Arachidic (20:0)	3.0	2.8	2.8
Behenic (22:0)	3.0	2.8	2.8
Lignoceric (24:0)	3.0	2.4	2.8
Oleic (18:1)	45.0	46.5	46.0
Linoleic (18:2)	15.0	15.0	15.2
Linolenic (18:3)	3.0	2.9	3.8
Erucic (22:1)	20.0	19.8	18.8

by the excellent resolution of the *cis–trans* isomers of $C_{18:1}$ in Fig. 1.

Quantitative analysis

Quantitative data for references and samples were calculated from individual UV response factors and/or calibration curves obtained from the derivatization of increasing amounts of a mixed FAME standard solution. Differences in individual molar absorptivities of the unsaturated species are thus easily compensated for in this simple calibration process.

The minimum detectable quantity of an individual fatty acid was determined by injecting serial dilutions of a myristic acid methyl ester derivative solution until the quantity corresponding to a peak height of 2× baseline noise was calculated (150 pmol).

In order to verify accuracy, a FAME reference mixture (Nu-Chek 17A) was analyzed by an established GC procedure [12] and this HPLC method. The results (Table I) demonstrate good agreement between the two methods.

Analysis of natural lipid samples

Lipid samples from a variety of plant and animal sources were derivatized and quantified by HPLC and GC. They included fats and oils containing a wide range of saturated and unsaturated fatty acids (C_8–C_{24}). Table II lists the FA profiles as weight percentages of four lipid samples.

In conclusion, direct derivatization of the esterified fatty acids in lipids can be used to rapidly determine FA profiles, eliminating the need for lengthly sample preparation steps. It has now been demonstrated that reversed-phase chromatography with sensitive UV detection can be successfully applied to the separation of FA hydroxamic acids.

TABLE II

DETERMINATION OF FA PROFILES OF LIPID SAMPLES BY GC AND HPLC

Fatty acid	Content (%, w/w)							
	Corn oil		Peanut oil		Coconut oil		Butter fat	
	GC	LC	GC	LC	GC	LC	GC	LC
8:0					8.0	8.4	1.4	1.0
10.0					6.0	5.9	3.0	3.5
12:0					44.9	45.2	3.5	4.3
14:0					17.9	18.4	12.6	13.7
16:0	11.1	12.4	9.8	10.6	10.0	9.4	32.6	37.0
18:0	2.0	1.7	2.6	2.2	3.3	2.5	14.0	13.3
20:0	0.5	0.8	1.3	1.5				
22:0			3.4	3.1				
24:0			1.7	1.5				
16:1							1.5	tr
18:1	25.5	27.1	48.1	48.8	7.4	7.2	30.0	25.5
18:2	60.0	56.8	31.3	30.8	2.5	2.9	2.1	0.8
18:3	0.9	1.3					1.2	0.9
20:1			1.5	1.5				

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