

Selective Derivatization and High Performance Liquid Chromatographic Analysis of Free Fatty Acids in Lipid Extracts¹

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ABSTRACT

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A procedure is reported for the determination of low levels of the free fatty acids only in cereal grain lipid extracts. High performance liquid chromatography was used for the analysis. Individual free fatty acids in the presence of the esterified form (fatty acid glycerides) were selectively derivatized under controlled conditions using *p*-bromophenacylbromide.

The free fatty acid derivatives were chromatographed on a reversed phase octylsilane bonded-phase column with water and acetonitrile/tetrahydrofuran. When the procedure was applied to lipid extracts from oat samples, free fatty acid composition as milligrams of oleic acid per milligram of lipid extract ranged from 2.89 to 4.11%.

The quantity of free acid as determined by alkaline titration has been used as an indicator of stored grain soundness (AACC 1976, Clayton and Morrison 1972, Mecham and Mossman 1974, Zeleny and Coleman 1938). Results obtained by this method are not only a function of the free fatty acid content but also all other compounds that will react with alkali (Fellers and Bean 1977). In addition, the titration end point is not distinct and is often very subjective. A number of gas chromatographic procedures have been reported (Golovna et al 1974, Phillips and Singleton 1978); however, a preliminary separation of free fatty acids from triglycerides is required.

A derivatization procedure followed by high performance liquid chromatography (HPLC) analysis was described by Tweeten and Wetzel (1979) for determination of total fatty acid composition of lipid extracts. No differentiation was made between free fatty acids and fatty acids derived from glycerides. King et al (1982) described an HPLC procedure for determining underivatized free fatty acids in natural oil. The procedure requires saponification of the lipid extract, however, and thus is not selective for initial free fatty acids in the lipid extract.

We describe here a method for determining free fatty acid in the presence of glycerides, mainly triglycerides, in lipid extracts. By carefully controlling the pH of the derivatizing reaction mixture, only free fatty acids are derivatized. Filtration of the reaction mixtures is required before HPLC analysis.

MATERIALS AND METHODS

Oat Samples

Oat samples harvested in 1979 were supplied by Quaker Oats (Cedar Rapids, IA), and obtained from different bins at their grain storage facility. Eight samples, covering a range of acid titration values, were selected for analysis by HPLC and titration procedures.

Sample Preparation for Titration

The titration procedure was an adaptation of AACC method 58-15 (1976). A 50-g sample of whole groats was ground on a Bantom micro-pulverizer (screen size: 3/64 in.). Then 40 g of the ground groats was extracted with 100 ml of toluene on a wrist-action shaker for 45 min. The amount of lipid extracted was determined by drying a 5-ml volume of the toluene extract. A 25-ml volume of the toluene extract was taken for acidity determination, and 25 ml of 2-propanol was added to it. The sample was titrated with potassium hydroxide (0.1*N*) until phenolphthalein indicator changed from colorless to pink. A sample blank of 25 ml each of toluene and 2-propanol was run for background correction. Blank

values were subtracted from sample results. Results are reported as the milliliters of 0.1*N* KOH per 10 g of lipid extract.

Sample Preparation for HPLC

The recommended procedure for preparing the lipid sample for HPLC analysis involved grinding the grain sample and immediately extracting the lipids. A quantity of grain to be ground and extracted depended on the lipid content of an individual cereal grain. Care was taken to protect the lipids from air oxidation, both before and after extraction. About 15 g of each oat sample was ground in a Wiley mill (screen size: 0.047 in.). Two 5-g samples were then prepared by drying in a moisture oven for 5 hr at 80°C under vacuum. Each sample was then extracted with 50 ml of petroleum ether (bp 30–60°C) for 4 hr on a Goldfish™ extractor (125-ml beakers). Samples were then concentrated over low heat, transferred to a 10-ml volumetric flask, and brought to volume with methylene chloride. The anticipated concentration range of total free fatty acid should be in the 1.0–5.0 µeq/ml range. This would require the lipid extract concentration to be 5–25 mg/ml, depending on the severity of seed storage conditions. One-half milliliter of sample extract was used for derivatization. It was placed in a 5-ml reaction vial with a cone-shaped bottom. Each oat sample was analyzed in duplicate.

The apparent pH of the lipid extract was adjusted to slightly basic with alcoholic potassium hydroxide (1*N*): the lipid sample changed from colorless to pink with phenolphthalein as an indicator. Excess base would cause hydrolysis of glycerides. The basic-lipid extract was brought to dryness on a rotary evaporator at an elevated temperature (40–45°C). The sample mixture in the reaction vial should change from pink to white when all solvent is evaporated from the residue. The derivatizing agent (*p*-bromophenacyl bromide, Aldrich Chemical, Milwaukee, WI 53201) with catalyst (18-Crown-6 ether, Aldrich Chemical) in acetonitrile were added to the residue in the reaction vial. The equivalence ratio of derivatizing agent to free fatty acid should be about 10:1. Details of the derivatization reaction catalyzed by crown ether had been previously reported (Durst et al 1975). The reaction mixture was heated for 15 min at 80°C, with constant stirring on a stirrer-hot plate equipped with an aluminum block drilled to accept 5-ml reaction vials (Tweeten and Wetzel 1979). After cooling, the reaction mixture was filtered (Swinney filter assembly, 0.45-µm filter) before HPLC analysis.

Derivatives of standard fatty acids (Applied Science, College Station, PA 16801) in each at a concentration of 0.5 µeq/ml were prepared using the same procedure. The calibration standard included palmitic, stearic, oleic, linoleic, linolenic, and margaric acid. The standard fatty acid derivatives were used to check calibration response factors prior to each batch of samples analyzed. Margaric acid (heptadecanoic acid) was selected as an internal standard (Phillip and Singleton 1978, Tweeten and Wetzel 1979) because it is generally not found in lipid extracts of cereal grains or oil seeds.

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Instrumentation

A model 1084B liquid chromatograph (Hewlett-Packard Company, Avondale, PA 19311) equipped with a variable wavelength UV/VIS detector, 60-position autosampler, autoinjector, and integrator was used. The analytical column was an RP-8 (10- μ m particle size, Hewlett-Packard Co.). A guard column (LiChrosorb RP-8, 10 μ m, 30 \times 4.6 mm, Brownlee Lab, Santa Clara, CA 95050) was positioned between the autoinjector and analytical column. Column temperature was 40°C. Mobile phase consisted of: A, water; and B, acetonitrile/tetrahydrofuran (20:1 by volume). Solvents were HPLC or better. Flow was 1.2 ml/min. A concave solvent gradient gave the best resolution of individual fatty acids. Solvent B started at 77%, holding for 5 min,

TABLE I
Determination of Total Free Palmitic Acid Content in Test Samples

Sample	Palmitic Acid Composition			
	Actual		Found	
	(μ eq)	(mg) ^a	(μ eq)	(mg) ^a
Palmitic acid only	3.376	0.954	3.372	0.924
Palmitic acid + oil (50 mg/ml) ^b	3.376	0.954	3.707	1.047
Palmitic acid + tristearin (0.9 mg/ml)	3.376	0.954	3.382	0.955
Palmitic acid + triolein (1.0 mg/ml)	3.376	0.954	3.351	0.947
Palmitic acid + oil (50 mg/ml) ^b	0.338	0.095	0.369	0.104
Palmitic acid + tristearin (0.9 mg/ml)	0.338	0.095	0.366	0.103
Palmitic acid + triolein (1.0 mg/ml)	0.338	0.095	0.389	0.109

^aConcentration as mg/ml.

^bCooking oil (sunflower).

TABLE II
Determination of Palmitic Acid in Presence of Tripalmitin

Trial No.	Tripalmitin ^a (TP)	Amount (μ eq/ml)		Calculated Ratio FPA/TP FPA/MA	Found Ratio ^b FPA/MA
		Palmitic Acid (FPA)	Margaric Acid (MA)		
1	2.528	0.000	1.594	0.000	0.000
2	1.264	0.126	1.594	0.099	0.079
3	0.506	0.252	1.594	0.499	0.158
4	0.253	0.503	1.594	1.988	0.327
5	0.126	1.258	1.594	9.984	0.813
6	0.063	1.887	1.594	29.952	1.220
7	0.000	2.516	1.594	∞	1.578

^aExpressed for palmitic acid in tripalmitin.

^bBased on high performance liquid chromatography analysis.

TABLE III
Free Fatty Acids in Lipid Extracts from Stored Whole Oats

Oat Sample	Fat (% db)	Total Free Fatty Acid				
		HPLC ^a (μ eq/ml)	HPLC ^a (mg/ml)	Titration Value ^b	Percent	
1	4.69	3.196	0.902	4.11	19.9	5.61
2	5.16	2.848	0.804	3.42	19.5	5.50
3	4.26	1.994	0.564	2.89	17.9	5.05
4	4.34	2.244	0.634	3.18	16.4	4.62
5	4.95	2.402	0.678	2.97	14.8	4.17
6	4.85	2.846	0.804	3.62	15.2	4.29
7	4.86	2.386	0.674	3.04	17.2	4.85
8	4.80	2.852	0.808	3.68	16.8	4.74

^aAverage of two or more determinations per sample with the range for relative standard deviation of 0.5–2.6%.

^bMilliliters of KOH per 10 g of fat.

then increased to 81% at 10 min, 83% at 15 min, 88.5% at 20 min, and 94% at 22 min. Solvent B was returned to 77%, and the column was re-equilibrated for at least 6 min before the next injection.

RESULTS AND DISCUSSION

The integrator was calibrated for using the internal standard method for reporting fatty acid results. The response factors (sample amount per integrator area counts) for each of the fatty acid derivatives were very similar. The mean response factor was 2.8341×10^{-6} with a relative standard deviation of 5.4%. This was determined from triplicate injections of the standard mixture containing six fatty acids. The detector response at 258 nm, which is due to the *p*-bromophenacyl chromophore of the lipid derivative (Durst et al 1975), was linear for the concentration range of 0.012–2.50 μ eq/ml. The minimum detectable quantity (MDQ) for oleic acid was 2.5 neq/ml. The MDQ is defined as an acid peak height that is 10 times the average noise level.

The results of spiking a cooking oil and two different triglyceride mixtures with palmitic acid at two different concentration levels (1:1 and 10:1 of spiking) is shown in Table I. Although the quantity of free acid as determined by HPLC agrees with calculated values at the higher concentration level of free acid, values determined at the lower free acid level were slightly higher than calculated levels. No stearic or oleic acid was found when free palmitic acid was determined in the presence of tristearin or triolein, respectively, which is evidence that transesterification did not occur during the derivatization process. The HPLC results showed that the relative ratio of individual free fatty acids in the cooking oil sample was the same at both spiked concentration levels.

The data in Table II show that free palmitic acid (FPA) was derivatized in the presence of the corresponding triglyceride. The found ratio of derivatized palmitic acid (PA) to internal standard, margaric acid (MA) as determined by HPLC compared very well with the calculated ratio of FPA/MA. Those results indicate that the derivatization procedure caused no hydrolysis of the tripalmitin. Hydrolysis of the tripalmitin would have resulted in a higher value of the ratio (PA/MA) than expected ratio of FPA/MA.

Figure 1 is a representative chromatogram showing the separation of free fatty acids in oat lipid extracts. Eight different oat lipid samples were analyzed for total free fatty acids by HPLC and titration (Table III). Because of the selectivity of the HPLC method, individual fatty acids were quantitatively determined (not

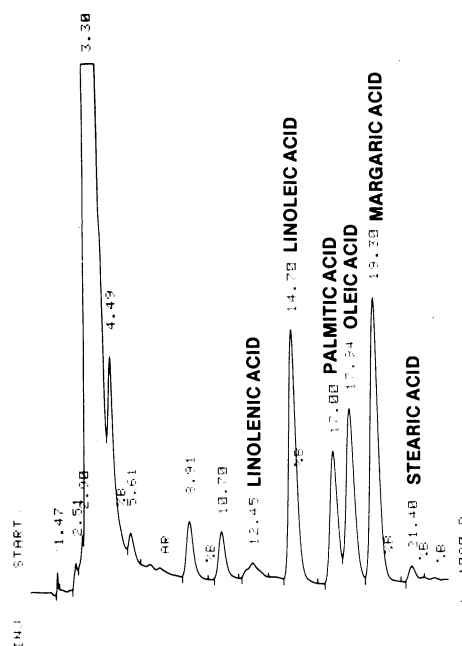


Fig. 1. High performance liquid chromatography determination of free fatty acids in the lipid extract from an oat sample.

shown). Total μeq of free fatty acid in each sample was calculated by adding up individual values. The mass (milligrams) of free fatty acid was obtained by multiplying the μeq of free fatty acid by the equivalent weight (g/equ wt) of oleic acid. Lower total fatty acid contents by HPLC than those by a titration method might be anticipated because the HPLC procedure was specific for free fatty acids, whereas all acidic compounds contributed to titrated acid values. Lower fatty acid values were previously reported (Phillips and Singleton 1978) when comparing results from a gas chromatographic procedure to titration results.

The proposed HPLC method for measuring free fatty acids in evaluating stored grain quality has many advantages over the existing titration method. By controlling the pH of the lipid extract, only free fatty acids in the lipid extract were derivatized in the presence of the other lipid classes, such as glycerides. The derivatization was reproducible and better than 95% efficient. Both individual and total free fatty acid content could be determined. The detector response was independent of fatty acid chain length and unsaturation. Detectability of low free fatty acid levels in the presence of triglycerides was excellent. Precision was good over a wide linear dynamic range. The HPLC analysis can be completely automated for unattended analysis.

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