High Performance Liquid Chromatographic Analysis of Fatty Acid Derivatives from Grain and Feed Extracts[1,2]

T. N. TWEETEN and D. L. WETZEL,[3] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506

Cereal Chem. 56(5):398-402

ABSTRACT

Extracts of fat from a number of grains and feedstuffs were chromatographed as their *p*-bromophenacyl esters in a methanol/water solution with a 10-μm octadecyl silane bonded phase column, an ultraviolet detector, and an internal standard. The lipid extract was converted readily to the derivative without isolation of the intermediate fatty acid salts or necessity of anhydrous conditions. Relative quantities of fatty acids of the various extracts were determined. Parallel analysis of the fat extracts by gas chromatography of the corresponding methyl esters showed that the high performance liquid chromatographic analysis described was a comparable alternative, except that palmitic and oleic acid were not resolved using the isocratic system.

High performance liquid chromatography (HPLC) of phenacyl ester derivatives of carboxylic acids has aided both selectivity and detector sensitivity. Adaptation of this procedure for quantitative work, direct application to triglycerides by optimization of reaction conditions, and a head-on comparison with gas chromatographic analysis of a number of lipid extracts were the purposes of the research reported here.

With a crown ether catalyst, the phenacyl ester derivatization reaction was greatly accelerated and the reaction efficiency was improved to greater than 95% (Durst et al 1975). The crown ether, a large heterocyclic molecule (Pederson 1967), formed metal ion complexes and aided in the dissolution of these complex salts into aprotic solvents (Durst 1974; Liotta et al 1974, 1975). Several authors have reported using the method for analysis of dicarboxylic acids (Grushka et al 1975) and isomers of long-chain fatty acids (Pei et al 1976).

Fatty acid derivatives of phenacyl halides were first prepared by Rather and Reid (1919). Separation was based on differences in solubility. Preparation of substituted phenacyl ester derivatives has been an accepted procedure in organic qualitative analysis of carboxylic acids (Shriner et al 1957). Phenacyl derivatives of carboxylic acids have been prepared and analyzed by open column chromatography (Kibrick and Skupp 1959, Moreland 1956) and gas chromatography (Umeh 1971) but with limited success. A commercially available instrument has separated short-chain carboxylic acids chromatographically and then used a postcolumn reaction to produce the chromophore for colorimetric detection (Nakajima et al 1976). Benzyl (Politzer et al 1973), *p*-nitrobenzyl (Knapp and Kruger 1975), 2-naphthacyl (Cooper and Anders 1974), and phenacyl (Borch 1975, Fitzpatrick 1976, Hendrickson and Kandall 1970, Jordi and Rusilko 1976) derivatives of carboxylic acids have been prepared and analyzed by HPLC. These methods required a separate saponification of the triglyceride and relatively long derivatization reaction times, and did not give

[1]Presented at the AACC 62nd Annual Meeting, San Francisco, October 1977.
[2]Contribution 78-156-J, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.
[3]Graduate research assistant and associate professor, respectively.

0009-0352/79/000062$3.00/0

complete derivatization of the reactants.

A simple quantitative means of analyzing for both free and esterified fatty acids was developed. The practical use of the method was explored for the analysis of fat extracts from both grains and feeds. The fatty acid composition of the various extracts was tabulated. A comparison was made of results obtained by the HPLC analysis of the phenacyl ester derivatives and the gas chromatographic analysis of the methyl ester derivatives.

MATERIALS AND METHODS

Materials

The feed and grain samples used for analysis were obtained from the Kansas State University Experimental Milling Laboratory.

The fatty acids and triglycerides were obtained from Applied Science Laboratories, Inc., State College, PA, and from Analabs, Inc., North Haven, CT. The *p*-bromophenacyl bromide and 18-crown-6 were from Aldrich Chemical Company, Inc., Milwaukee, WI. The derivatizing agent was recrystallized from absolute ethanol and stored in a desiccator. Organic solvents were analytical reagent grade. Skellysolve F (Skelly F) was purified by washing with concentrated sulfuric acid and concentrated potassium permanganate in 10% sulfuric acid, rinsing with water and dilute sodium carbonate solution, and drying over anhydrous calcium chloride, followed by distillation (Vogel 1956).

Procedure for Sample Extraction

The feed and grain were first ground into a fine powder, which was then dried in a vacuum oven at 75°C for 4 hr. Lipid in the samples was extracted for 4 hr on a Goldfisch extractor with Skelly F. Once the extraction was completed, the extract was cooled and adjusted to volume. A portion of the extract was placed in a reaction container from which the Skelly F was removed under a stream of nitrogen.

Analytical Procedure

The lipid sample was taken up in chloroform/methanol (2:1). The concentration of lipid was adjusted so that the potential content of fatty acid was about 0.5 to 2.5 μmol per 2 ml of solution. Conversion factors for calculating fatty acid content of lipids have been tabulated (Weihrauch et al 1977). Potassium hydroxide (0.05*N* in methanol) was added so that the mole ratio of base to potential acid was at least 9:1. One drop of phenolphthalein solution (0.1%) was added. The sample was placed on the rotary evaporator and mixed for 2 min. The sample vial was then lowered (6 to 8 mm) into an oil bath (45 to 55°C) and rapidly rotated while the solvent was removed. The sample turned from pink to colorless when the solvent was removed. The *p*-bromophenacyl bromide/crown ether in acetonitrile was added at levels at least 25 times the concentration of potential fatty acid content. The sample was derivatized in 30 min at 75–80°C in the aluminum digestion block; it was then cooled and analyzed by HPLC. The reactions for the derivatization are summarized in Durst et al (1975).

Derivatization Equipment

A rotary evaporator was used for evaporation to dryness. The reaction vials were the 5 ml size equipped with a microconnector (13 to 20 mm) and a standard taper connecting adapter (13 mm to 24/40).

The digestion block was made to hold the 5-ml reaction vials by boring eight holes into a 12.5 × 12.5 × 4-cm aluminum block. A hole was drilled in the center of the block to hold a Weston thermometer (0–110°C). The block was placed on a hot plate equipped with a stirring mechanism (TekPro Heat Stir 36, Scientific Products). The 5-ml reaction vials, magnetic stirrers, microconnectors, and connecting adaptors can be purchased commercially (Reliance Glass Works, Inc., Bensenville, IL).

High Performance Liquid Chromatography

A Model ALC-202 UV detector (254 nm) and a model M-6000 pump (Waters Associates, Milford, MA) were used with a stop flow injector. The column (25 cm × 4.1 mm, 316 stainless steel) was packed in our laboratory by a modified balanced density slurry packing technique.[4] The packing material was dispersed in a volume of tetrachloroethane and 1,4-dioxane (3:1) by an ultrasonic bath. The slurry was rapidly extruded into the column with hexane at 6,000 psi. The packing was 10-μm diameter Spherisorb octadecyl silane (ODS) (Spectra-Physics, Santa Clara, CA). The results were calculated and recorded on a 3385A Automation System (Hewlett-Packard, Avondale, PA). The eluting solvent system was methanol/water (90:10) with a flow rate of 1 ml/min. Ambient temperature was used.

Gas-Liquid Chromatography

A model 5750 gas chromatograph (Hewlett-Packard) was used. It was equipped with a flame ionization detector. A 1.8 m × 2 mm i.d. stainless-steel column was packed with 10% SP-2330 on Chromosorb WAW 100/120. The liquid coating was applied to the solid support in our laboratory. The liquid coating and solid support were obtained from Supelco, Inc., Bellefonte, PA. Gas chromatograms were run at 180°C with nitrogen carrier gas of 20 ml/min. The fatty acids for gas-liquid chromatography (GLC) were derivatized by the standard BF_3/methanol procedure (AOAC 1970).

DISCUSSION

Method

We found that under optimum conditions of derivatization a mole ratio of base to fatty acid of at least 9:1 was needed to give quantitative conversion of triglycerides to fatty acid derivatives (Table I). The values in the second column give the ratio of derivatized palmitic acid to that of derivatized margaric acid. The margaric acid was used as an internal standard since values in the literature have shown that over 95% of the fatty acid was derivatized in the reaction (Durst et al 1975).

Table I also shows the effect of digestion time. A digestion time of 30 min or more was required to obtain quantitative derivatization of palmitic acid from tripalmitin. Once again, margaric acid was used as an internal standard to measure relative reaction efficiency and injection variation.

To test reproducibility of the procedure, a sample of tripalmitin and margaric acid in the fatty acid mole ratio of 1:1 was used (Table II). For nine analyses, a rather high standard deviation was

[4]D. R. Gere, private communication.

TABLE I
Effect of Base Concentration and Digestion Time on the Derivatization Reaction

	Peak Area Ratio (PA/MA)[a]
Mole ratio of base/fatty acid	
6:1	0.825
9:1	0.923
12:1	0.945
Digestion time (min)	
15	0.740
30	0.949
45	0.981
60	0.956

[a]Palmitic acid (PA) from tripalmitin and margaric acid (MA) as free fatty acid. Mole ratio of PA to MA = 1:1.

TABLE II
Reproducibility for the Derivatization Reaction of Tripalmitin and Margaric Acid[a]

	Peak Area		Peak Area Ratio
	TP	MA	PA/MA
Mean peak area (nine analyses)	8,130	8,608	0.944
Standard deviation	585	533	0.030
Relative standard deviation	0.072	0.062	0.032

[a]Palmitic acid (PA) from tripalmitin (TP) and margaric acid (MA) as a free fatty acid. Mole ratio PA/MA = 1:1.

observed when the fatty acid peak areas from margaric acid and palmitic acid were considered individually. When margaric acid was used as an internal standard, however, the precision for the ratio of area responses improved considerably; the relative standard deviation for nine analyses was 0.032.

The method seemed to show no selective derivatization of free fatty acids in the presence of fatty acids derived from triglycerides (Table III). With the total amount of potential fatty acid held constant, the mole ratio of tripalmitin-derived palmitic acid to free margaric acid decreased linearly (Fig. 1). The peak area response did not depend on the inital form of fatty acid. The peak area ratios of derivatives of palmitic acid to margaric acid agreed well with the mole ratios of these two components.

To determine if the reaction efficiency was dependent on the fatty acid composition of the triglycerides, several different triglycerides using trimargarin as the internal standard were analyzed. Three triglycerides were used: trimyristin, tripalmitin, and tristearin. The results showed that the peak area response relative to the margaric acid derivative was larger for shorter chain fatty acids than for longer chain fatty acids (Table III).

Free fatty acids were analyzed by the same procedure to determine whether this effect was due to the relative chain length of fatty acids or to the derivatization procedure.From the data in Table III, the peak area response seemed to be independent of chain length. In the analysis, the precision, as indicated by relative percent error, was less than 3.8. Therefore, the area response ratios for triglycerides were used to normalize data from analysis of feed and grain samples, which will be discussed later.

TABLE III
Area Response Ratios for Free and Esterfied Fatty Acids

	Area Response Ratios of Fatty Acid Derivative (× 100)[a]		
	Mean	Standard Deviation	Relative Standard Deviation
Triglycerides[b]			
TMy MyA / TM MA	109.2	4.0	0.036
TP PA / TM MA	109.7	2.9	0.026
TS SA / TM MA	95.2	2.6	0.027
Fatty acids[b]			
MyA MyA / MA MA	101.4	3.9	0.038
PA PA / MA MA	104.5	2.8	0.027
SA SA / MA MA	100.3	1.4	0.014

[a] Average of four or more analyses.
[b] TMy = Trimyristin; TP = tripalmitin; MyA = myristic acid; PA = palmitic acid; TM = trimargaric; TS = tristearin; MA = margaric acid; SA = stearic acid.

Comparison of HPLC and GLC Methods

Good efficiency was obtained using both chromatographic techniques. When the HPLC method was used with an isocratic solvent system, however, palmitic and oleic acids were not resolved. Borch (1975) and Jordi and Rusilko (1976) reported that these two fatty acids can be resolved if gradient elution is used. Since a rapid and competitive means of quantitating fatty acid content was the goal of this study, gradient elution was not used.

Analysis of Fatty Acid in Feed and Grain Samples by HPLC and GLC

Analyses of fatty acids by HPLC and GLC in a Skelly F lipid extract of oats are compared in Fig. 2. Both methods demonstrated good resolution of the fatty acids in this extract.

The quantitative results of the feed and grain samples analyzed by the high performance liquid chromatographic method and the gas chromatographic method are summarized in Table IV. Individual peak areas were normalized for determination of fatty acid content by HPLC. Trimargarin was used as an internal standard.

TABLE IV
Comparison of Fatty Acid Content of Feed and Grains Analyzed by High Performance Liquid Chromatography (HPLC) and Gas-Liquid Chromatography (GLC)

	C14		C16	C18:1	(C16+C18:1)		C18		C18:2		C18:3	
	GLC	HPLC	GLC		GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC
Grain:												
Yellow corn	...	...	14.9	40.9	(55.8)	56.3	1.7	2.5	42.5	41.2	...	...
White corn	...	...	12.3	27.9	(40.2)	40.4	1.2	1.5	58.6	58.1	...	...
White sorghum	...	...	12.4	29.4	(41.8)	43.0	0.9	1.9	56.1	53.0	1.2	2.1
Rice (brown)	...	...	17.7	48.4	(66.1)	66.0	1.8	3.6	32.1	30.4	...	...
Oat (groats)	...	...	24.3	19.5	(43.8)	41.3	1.6	2.2	53.1	54.1	1.5	2.4
Barley (dehulled)	...	...	22.8	18.1	(40.9)	41.0	1.9	2.7	54.8	53.8	2.4	2.5
Rye	...	...	24.3	37.7	(35.0)	34.8	1.0	2.9	59.3	57.1	4.7	5.2
Triticale	...	...	24.0	18.2	(42.2)	41.6	1.8	4.1	53.3	50.7	2.7	3.6
Hard red winter wheat	...	...	17.5	19.6	(37.1)	36.3	1.4	2.0	58.1	59.6	3.4	2.1
Western white club wheat	...	...	23.2	17.2	(40.4)	38.2	1.1	2.1	56.9	56.6	1.6	3.1
Durum	...	...	19.4	19.8	(39.2)	37.2	1.4	2.3	57.5	57.6	1.9	2.9
Millet	...	...	8.5	24.6	(33.1)	34.9	2.0	2.9	64.9	62.2	...	...
Flax	...	...	5.1	17.0	(22.1)	17.1	4.4	4.6	17.4	18.5	56.1	59.8
Soybean	...	...	10.7	18.9	(29.6)	30.7	3.1	4.5	62.0	58.3	5.2	6.5
Safflower	...	...	6.6	15.3	(21.9)	22.8	2.1	2.8	76.0	74.4	...	...
Feeds:												
Dog food	...	...	22.2	35.1	(57.3)	57.0	8.4	10.4	34.3	32.6	...	...
Catfish food	8.1	6.4	35.4	38.7	(74.1)	70.7	10.0	12.6	9.5	8.6	...	...
Air-dried corn silage	...	...	22.7	25.4	(48.1)	47.3	3.8	4.0	45.5	45.2	2.6	3.5
Cattle feed ration	...	...	18.6	32.1	(50.7)	49.4	4.9	4.6	44.4	46.0	...	...
Pig feed starter	...	...	18.4	30.9	(49.3)	49.8	4.0	6.4	45.9	42.0	0.8	1.8
Meat and bone meal	5.0	7.4	32.0	41.8	(73.8)	68.4	16.9	17.7	3.2	5.1	1.1	1.4
Dehydrated alfalfa meal	...	...	27.6	18.4	(46.0)	42.2	7.4	7.8	18.2	16.9	28.4	33.1

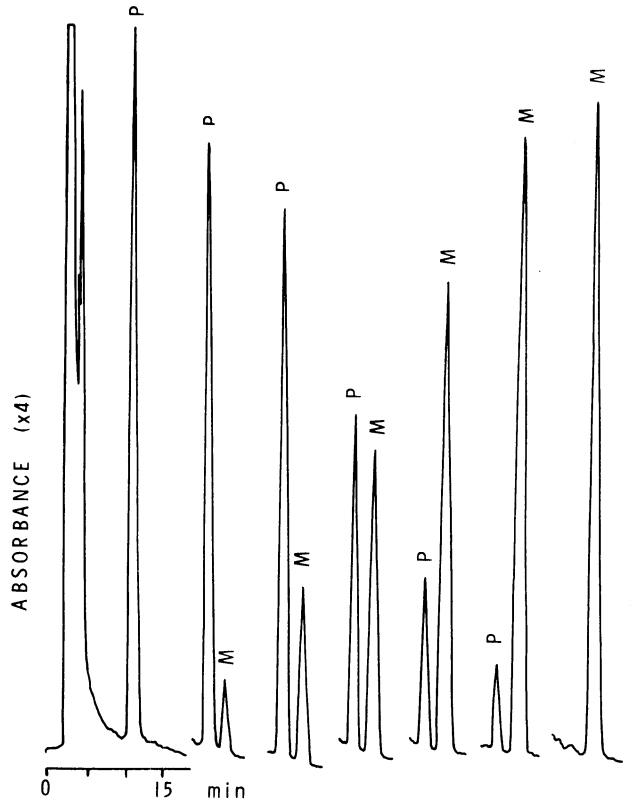

Fig. 1. A high performance liquid chromatographic comparison of the *p*-bromophenacyl derivatives of tripalmitin (P) and margaric acid (M) mixtures. The fatty acid mole ratios from the left, respectively, are 10:0, 9:1, 7.5:2.5, 5:5, 2.5:7.5, 1:9, and 0:10. The total number of moles of acid was held constant.

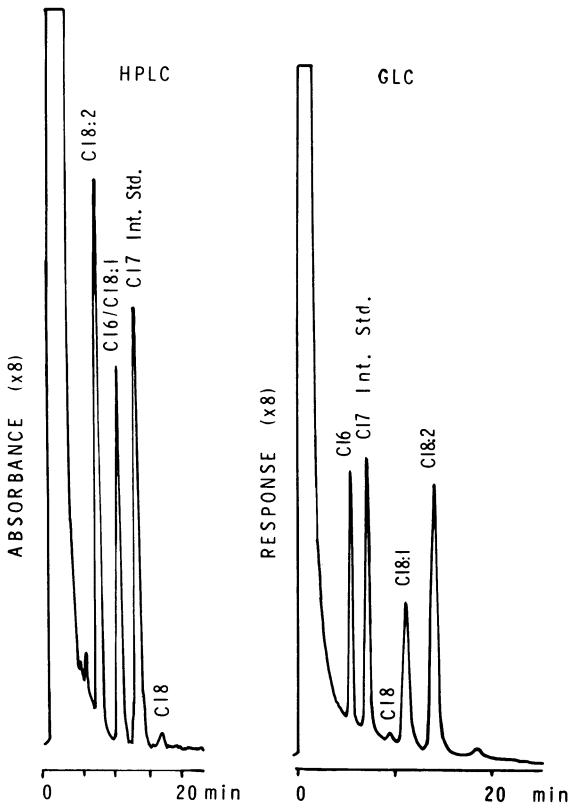

Fig. 2. Comparison of chromatograms of a Skelly F lipid extract of oats run by high performance liquid and gas-liquid chromatography.

CONCLUSIONS

A procedure in which two-step derivatization was followed by quantitative analysis using HPLC on a nonpolar bonded phase column was shown. The derivatization method by HPLC was effective for analyzing both free fatty acids and fatty acids derived from triglycerides. Successful quantitation depends on effective pH adjustment, excess derivatizing agent, and temperature control during evaporation to dryness.

The saponification product did not need to be isolated. Once the sample was extracted, the entire derivatization was done in the same reaction vial. The sample did not have to be completely anhydrous before derivatization or chromatographic analysis. The minimum detectable quantity was comparable to results obtained by gas chromatography. Analysis by HPLC was unable to resolve derivatives of palmitic and oleic acids using an isocratic solvent system. Finally, the practical use of the method was demonstrated. Fat extracts from both grains and feeds were analyzed. The quantitative results using HPLC compared well with results obtained by the gas chromatographic method.

LITERATURE CITED

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. 1970. Official methods of analysis (11th ed.), p. 454. The Association: Washington, DC

BORCH, R. F. 1975. Separation of long chain fatty acids as phenacyl esters by high pressure liquid chromatography. Anal. Chem. 47:2437.

COOPER, M. J. and ANDERS, M. W. 1974. Determination of long chain fatty acids as 2-naphthacyl esters by high pressure liquid chromatography and mass spectrometry. Anal. Chem. 46:1849.

DURST, H. D. 1974. Crown ether catalysis: "Naked" anions as reactive intermediates in synthesis of phenacyl esters. Tetrahedron Lett. 2421.

DURST, H. D., MILANO, M., KIKTA, E. J., Jr., CONNELLY, S. A., and GRUSHKA, E. 1975. Phenacyl esters of fatty acids via crown ether catalysts for enhanced ultraviolet detection in liquid chromatography. Anal. Chem. 47:1797.

FITZPATRICK, F. A. 1976. High performance liquid chromatographic determination of prostglandins $F_{2\alpha}$, F_2, and D_2 from in vitro enzyme incubations. Anal. Chem. 48:499.

GRUSHKA, E., DURST, H. D., and KIKTA, E. J., Jr. 1975. Liquid chromatographic separation and detection of nanogram quantities of biologically important dicarboxylic acids. J. Chromatogr. 112:673.

HENDRICKSON, J. B., and KANDALL, C. 1970. The phenacyl protecting group for acids and phenols. Tetrahedron Lett. 343.

JORDI, H. C., and RUSILKO, D. 1976. Separation of long and short chain fatty acids as naphthacyl and substituted phenacyl esters by high performance liquid chromatography. U.S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, DC

KIBRICK, A. C., and SKUPP, S. J. 1959. Chromatographic separation of fatty acids based on chlorophenacyl esters. Anal. Chem. 31:2057.

KNAPP, D. R., and KRUGER, S. 1975. Use of *o-p*-nitrobenzyl-N, N-diisopropylisourea as a chromagenic reagent for liquid chromatographic analysis of carboxylic acid. Anal. Lett. 8:603.

LIOTTA, C. L., GRISDALE, E. E., and HOPKINS, H. P., Jr. 1975. Relative nucleophilicities of "naked" anions. Tetrahedron Lett. 4205.

LIOTTA, C. L., HARRIS, H. P., McDERMOTT, M., GONZALEY, T., AND SMITH, K. 1974. Chemistry of "naked" anions. II. Reaction of the 18-crown-6 complex of potassium acetate with organic substrates in aprotic organic solvents. Tetrahedron Lett. 2417.

MORELAND, W.T., Jr. 1956. Triethylamine salts in the preparation of esters from active halogen compounds. J. Org. Chem. 21:820.

NAKAJIMA, M., OZAWA, Y., TANIMURA, T., and TAMURA, Z. 1976. A highly efficient carboxylic acid analyzer and its application. J. Chromatogr. 123:129.

PEDERSON, C. J. 1967. Cyclic polyethers and their metal salts. J. Am. Chem. Soc. 89:7017.

PEI, P. T. S., KOSSA, W. C., RAMACHANDRAN, S., and HENLY, R. S. 1976. High pressure reverse phase liquid chromatography of fatty acid *p*-bromophenacyl esters. Lipids 11:814.

POLITZER, I. R., GRIFFIN, G. W., DOWTY, B. J., and LESETER, J. L. 1973. Enhancement of U. V. detectability of fatty acids for the purposes of LC-MS analyses. Anal. Lett. 6:539.

RATHER, J. B., and REID, E. E. 1919. Identification of acids. IV. Phenacyl esters. J. Am. Chem. Soc. 41:75.
SHRINER, R. L., FUSON, R. C., and CURTIN, D. Y. 1957. The Systematic Identification of Organic Compounds (4th ed.), p. 200. Wiley: New York.
UMEH, E. 1971. The separation and determination of low molecular weight (C_2–C_{10}) straight-chain carboxylic acids in dilute aqueous solution by gas chromatography of their *p*-bromophenacyl and *p*-phencylphenacyl esters. J. Chromatogr. 56:29.
VOGEL, A. I. 1956. Small scale preparation, p. 162. In: Elementary Practical Organic Chemistry (2nd ed.), Part 1. Wiley: New York.
WEIHRAUCH, J. L., POSATI, L. P., ANDERSON, B. A., and EXLER, J. 1977. Lipid conversion factors for calculating fatty acid contents of foods. J. Am. Oil Chem. Soc. 54:36.

[Received December 12, 1977. Accepted November 3, 1978]