paper on methodology

Analysis of positional isomers of monounsaturated fatty acids by high performance liquid chromatography of 2,4-dinitrophenylhydrazones of reduced ozonides

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Abstract A method is described for quantifying the positional isomers in monounsaturated fatty acid methyl ester (FAME) fractions. The procedure involves the preparation of 2,4-dinitrophenylhydrazones (DNPH) of the fragments generated during reductive ozonolysis of FAME, class isolation of the aldehyde and aldehyde ester DNPH, and separation of the aldehyde ester derivatives by high performance liquid chromatography (HPLC). The high extinction coefficient of the DNPH provides for a sensitive assay which is linear for a large range of components over a concentration range of 0.075-5 nmol/component, and the stability of the DNPH permits the independent analysis of the aldehyde and aldehyde ester fragments generated during reductive ozonolysis. The reductive ozonolysis-DNPH-HPLC method developed is as sensitive, reproducible, and accurate as reductive ozonolysis-gas-liquid chromatography and does not suffer from some of the drawbacks of the classical procedure. —**Caughman, C. R., L. C. Boyd, M. Keeney, and J. Sampugna.** Analysis of positional isomers of monounsaturated fatty acids by high performance liquid chromatography of 2,4-dinitrophenylhydrazones of reduced ozonides. *J. Lipid Res.* 1987. **28:** 338–342.

Supplementary key words fatty acid methyl ester • ozonolysis

We have been interested in the dietary sources and metabolic fate of *trans* fatty acids. The research requires sensitive and reproducible procedures to quantify the positional isomer distribution in *trans*-octadecenoate fractions of fatty acid methyl esters (FAME) derived from partially hydrogenated fats and tissue lipids of animals fed these fats. Detailed information on the wide range of positional isomers expected in these fractions (1, 2) is usually obtained by gas-liquid chromatography (GLC) of the aldehyde and aldehyde esters resulting from the reductive ozonolysis of isolated monoenes.

When appropriate precautions are taken, reductive ozonolysis-GLC is a rapid, sensitive, and reproducible method. Nevertheless, the method has drawbacks which can lead to possible misinterpretation of the resulting fragmentation data. The aldehyde and aldehyde ester fragments are unstable and non-aldehydic components may be present. Also, individual components may not be resolved when fragments arising from complex mixtures are analyzed on common liquid phases routinely used in GLC. Although modifications have been introduced to minimize limitations in the original procedure, these have focused largely on conditions used to generate the fragments (3, 4) or on the GLC methods used to separate and quantify the fragments (5–7).

We have developed an alternative approach to the traditional reductive ozonolysis-GLC method. Our procedure involves the preparation of stable dinitrophenylhydrazone derivatives (DNPH) of the fragments generated during reductive ozonolysis and a simple class separation of the aldehyde and aldehyde ester-DNPH followed by high performance liquid chromatography (HPLC) to separate the aldehyde ester derivatives. Details of the reductive ozonolysis-DNPH-HPLC method are described herein.

Abbreviations: FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; DNPH, 2,4-dinitrophenylhydrazones; HPLC, high performance liquid chromatography; TPP, triphenylphosphine; 2,4-DNP, 2,4-dinitrophenylhydrazine; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Materials

The margarine and shortening used as a source of *trans*-octadecenoates (*trans*-18:1) were purchased locally and have been characterized with respect to fatty acid composition (See Table 2 in reference (8); the margarine used in this study was sample #122 and the shortening was sample #205). Unless otherwise noted, all solvents used were HPLC-grade or better. Water used in HPLC was high purity reagent purchased from Burdick and Jackson Labs (Muskegon, MI) and that used in the extraction steps was doubly distilled and deionized. Triphenylphosphine (TPP) from M & T Chemicals (Rahway, NJ) and FAME standards (99% purity) from Nu-Chek-Prep (Elysian, MN) were used as purchased. Acetonitrile, hexane, methylene chloride, and ultra-pure sulfuric acid as well as reagent grade 2,4-dinitrophenylhydrazine (2,4-DNP) were purchased from J. T. Baker (Phillipsburg, NJ). The 2,4-DNP was purified as described by Kuwata et al. (9) and the hexane and acetonitrile used in the ozonolysis, derivatization, and extraction steps were purified as described below.

Purification of solvents

Acetonitrile and hexane were made essentially olefin- and carbonyl-free as follows: The solvents were cooled to −70°C and saturated with ozone for 15 min. After removing excess ozone under nitrogen at room temperature and adding (to a final concentration) TPP (7.5 mM), 2,4-DNP (20 mM), and sulfuric acid (0.5 N), the solvents were refluxed for 3–4 hr and then directly distilled in an all-glass apparatus. The purified solvents were degassed and stored in tightly stoppered amber bottles under a blanket of nitrogen.

Ozonolysis

Ozonolysis was performed essentially as described by Privett and Nickell (3). Hexane, saturated with ozone at −70°C, was added to the FAME dissolved in hexane. After 2 min, excess ozone was removed under a stream of nitrogen and aliquots were taken for analyses by GLC and HPLC as described below.

GLC analysis

In a septum-sealed tube, FAME ozonides were reduced overnight at 4°C using TPP essentially as described by Beroza and Bierl (10). The products were analyzed by GLC as described previously (11) and the area counts obtained were converted to mole percent values using appropriate response factors (7).

DNPH-HPLC analysis

Aliquots of FAME ozonides were concentrated under nitrogen and 0.5 ml derivatizing solution (7.5 mM TPP, 20 mM 2,4-DNP, and 0.5 N sulfuric acid in acetonitrile) was added. After 2 min at 45°C, 2 ml of water was added and the DNPH were extracted into hexane using 3 × 3 ml volumes.

The pooled hexane layers were washed three times using 3 ml of water and applied to a silica cartridge (Water Associates; Milford, MA) to separate the DNPH into the aldehyde and aldehyde ester fractions. The silica was previously equilibrated with 20 ml of hexane under pressure to achieve a flow rate of 25 ml/min. The fractions were eluted at ambient pressure using 50 ml of hexane–methylene chloride 2:1 (v/v) to obtain the aldehyde DNPH fraction followed by 20 ml of methylene chloride to elute the aldehyde ester derivatives. Solvents were removed with the aid of a rotary flash evaporator and the derivatives were dissolved in acetonitrile for HPLC.

The individual components in each fraction were separated on a 5-μ ODS column (250 × 4.6 mm) purchased from Beckman Instruments, Inc. (Berkeley, CA). The main column was protected by a 50 × 4.6 mm guard column containing 40 μ ODS pellicular packing (Supelco Inc., Bellefonte, PA), and was installed in a Beckman model 342 HPLC equipped with two model 114 analytical pumps, a model 420 controller, an Altex injector fitted with a 20 μl sample loop, and a model 164 variable wavelength detector.

For routine analyses, distribution patterns in either fraction were obtained using a mobile phase of acetonitrile–water 60:40 for 1 min followed by a linear gradient to 100% acetonitrile over the next 19 min at a flow rate of 1.5 ml/min. The absorbance of eluted components was monitored at 358 nm and quantified by peak area with the aid of a model 3390A integrator (Hewlett-Packard; Avondale, PA).

Trans-octadecenoates

Margarine or shortening fat was transesterified in HCL-methanol and argentation–TLC was used to isolate the *trans*-monoene fraction. Details on these procedures have been described (11). The *trans*-18:1 fraction was further purified by reverse phase HPLC using the apparatus described above, except that methanol was used as the mobile phase and an Altex model 156 refractive index detector (Beckman Instruments; Berkeley, CA) was used to monitor chromatographic separation. Purity of the isolated *trans*-18:1 fraction was verified by glass capillary GLC (11).

Statistical methods

Multiple *t*-tests were used to test for differences among means and/or differences from theoretical values, depending on the experiment. Critical values (α') were calculated using the formula:

$$\alpha' = 1 - (1-\alpha)^{1/k}$$

where α (set equal to 0.05) is the experiment-wise type I error allowed and k is the number of comparisons tested (12).

RESULTS AND DISCUSSION

A typical HPLC trace of aldehyde ester-DNPH is shown in **Fig. 1.** These results were obtained when the procedure was conducted on the *trans*-octadecenoate FAME fraction isolated from a commercially available shortening. The peaks correspond to the fragments expected from a mixture of isomeric octadecenoates with double bonds in positions $\triangle^5$-$\triangle^{16}$. These results were confirmed by analysis of standard unsaturated FAME, by comparison with data obtained by traditional reductive ozonolysis-GLC as well as by information obtained from the complementary aldehyde-DNPH fraction.

Each peak in the chromatogram shown actually consists of two stereoisomers, the *syn* and *anti* forms of the derivatives (13). The stereoisomers are present in constant proportions regardless of the chain length and can be partially resolved by substituting methanol for acetonitrile in the mobile phase (data not shown).

Both the aldeyde and aldehyde ester-DNPH have similar molar extinction coefficients ($\epsilon = 2.25 \times 10^4$ l mol^{-1} cm^{-1}) and absorption maxima ($\lambda = 358$ nm). The detection limit was ca. 50 pmol of DNPH (twice the noise level using the HPLC apparatus described) and the detector response was linear between 0.075–5 nmol of DNPH. The wide linear range and high extinction coefficient allow for a sensitive assay and reliable quantitation of trace components. The DNPH can also be monitored at 254 nm, but the increased baseline noise and lower extinction coefficient (1.29×10^4 l mol^{-1} cm^{-1}) result in decreased sensitivity.

As can be seen in **Table 1,** the reductive ozonolysis-HPLC procedure yields quantitative data that are comparable to that obtained by classical ozonolysis-GLC. The results shown are for a standard mixture of unsaturated FAME which were subjected to both procedures. The mole percent values obtained by GLC and HPLC were not statistically different from each other or from theoretical values calculated for the isomers in the standard mixture. These results demonstrate that the reactions and procedures involved in the reductive ozonolysis-DNPH-HPLC method do not complicate the quantitation of isomer distributions.

The quantitative analysis of complex mixtures encountered in our studies currently requires separation of DNPH into the aldehyde and aldehyde ester fractions prior to HPLC. This is because the reverse phase column and mobile phase employed do not completely resolve some of the aldehyde-DNPH from certain of the aldehyde

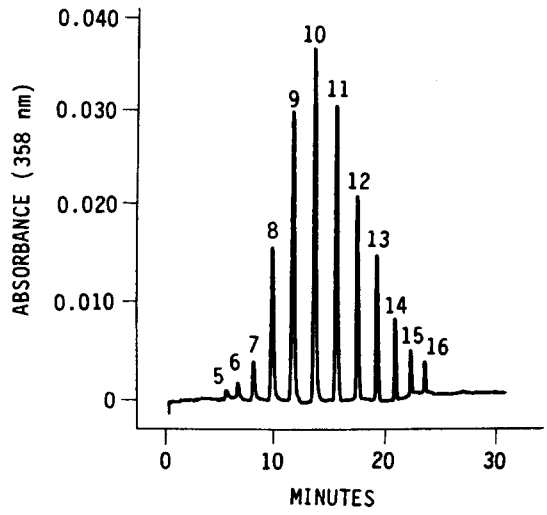

Fig. 1. HPLC chromatogram of the aldehyde ester-DNPH prepared from a *trans*-octadecenoate fraction isolated from a partially hydrogenated shortening. Approximately 20 nmol of DNPH was chromatographed using the apparatus and conditions described in the text. The number above each peak represents the position of the double bond in the parent FAME.

ester-DNPH. Fortunately, the stability of the DNPH permits a facile separation of aldehyde-DNPH from aldehyde ester-DNPH. Routinely, a commercial silica cartridge is employed for this purpose. However, the separation into DNPH classes can also be accomplished using alumina as the adsorbent (14). We prefer the silica gel cartridge; in addition to being convenient, the cartridge can be used at least three times without loss of resolution.

The stability and easy separation of DNPH into classes prior to HPLC is advantageous, for it permits the independent analysis of complementary fragments generated during ozonolysis. As can be seen in **Table 2,** there was excellent correspondence between the complementary derivatives expected for octadecenoates where the aldehyde could be unequivocally quantified. These results demonstrate that independent analysis of DNPH derivatives can be used to confirm or clarify quantitative data.

Unfortunately, at the low concentrations employed in our studies, the aldehyde-DNPH pattern is often complicated by background material. The background absorbance is virtually absent from the aldehyde ester-DNPH fraction but can affect interpretation of the early portion of the aldehyde-DNPH chromatogram where derivatives of aldehydes with chain lengths corresponding to 2–7 carbon atoms are expected. In practice this is not a problem, as the aldehyde ester-DNPH fraction is sufficient for the quantitative analysis of monounsaturated FAME. Nevertheless, this potential limitation to the method should be considered when polyunsaturated FAME are analyzed by the procedure. Although blank analyses car-

ried through the entire procedure could be used to extract quantitative data from the early portion of the aldehyde-DNPH chromatogram, it would be preferable if the source of the background material(s) were eliminated.

As reported by Kuwata et al. (9), it was absolutely necessary to use only recrystallized 2,4-DNP as a derivatizing agent. In addition, the solvents employed in the derivatization and extraction steps may contribute background peaks unless they are purified as described in the Methods section. With these precautions, blank runs of the aldehyde fraction contained background absorbance that corresponded to less than 20 nmol of DNPH. The source of the remaining absorbance is not known, but may arise from components in the 2,4-DNP, itself.

Depending on the complexity of the sample, the procedure requires 10–100 μg of FAME as starting material for routine analyses. A well-characterized margarine fat is used as a source of *cis* and *trans*-octadecenoate FAME to monitor the overall procedure and to check column performance inasmuch as factors such as column age may tend to affect the retention time identification of components. We have used the same reverse phase column for over 1 year without any resolution problems.

Compared to the ozonolysis-GLC procedure, the reductive ozonolysis-DNPH-HPLC method has several advantages. The formation of stable derivatives of the reduced ozonides permits the separation of aldehyde and aldehyde ester fragments using a simple chromatographic step and provides for the independent analysis of two types of fragments. This facilitates the identification of individual components and provides unambiguous information on the original distribution pattern of complex mixtures of monounsaturated FAME isomers. The derivatives have high extinction coefficients and their absorbance directly yields mole percent values, obviating the requirement for response factors often used in GLC methods. In addition, the HPLC method permits the easy collection of components, as may be required when radiotracers are employed to study the metabolism of unsaturated FAME isomers.

For the complex mixtures of monounsaturated FAME typically studied, the reductive ozonolysis-DNPH-HPLC procedure is at least as sensitive, reproducible, and accurate as the reductive ozonolysis-GLC method. Although it was designed as an alternative to the reductive ozonolysis-GLC method for the analysis of monounsaturated FAME, the HPLC method may also be useful in the study of polyunsaturated fatty acids.

TABLE 1. Comparison of expected and observed aldehyde ester fragments after reductive ozonolysis of a standard mixture of monounsaturated FAME[a]

Fatty Acid	Expected Aldehyde Ester	Expected[b]	GLC Analysis[c]	HPLC Analysis[c]
	number of C atoms[d]		*mol/100 mol*	
16:1 Δ^9 18:1 Δ^9	9 AE	46.0	44.5 (2.4)	44.0 (1.3)
20:1 Δ^{11}	11 AE	16.6	16.8 (0.2)	16.8 (0.3)
22:1 Δ^{13}	13 AE	20.6	21.3 (1.1)	21.8 (0.6)
24:1 Δ^{15}	15 AE	16.8	17.5 (1.3)	17.5 (0.5)

[a]Standard FAME mixture was ozonized and aliquots were analyzed as the reduced fragments by GLC (7, 10, 11) or by the DNPH-HPLC method described in the text.
[b]Expected values for the standard mixture were verified by glass capillary GLC of the FAME (11).
[c]Values are the means (± SEM) for four individual determinations after ozonolysis of the standard FAME mixture and subsequent analyses.
[d]Denotes the number of carbon atoms expected in the aldehyde ester (AE) fragment after reductive ozonolysis of the standard FAME.

TABLE 2. Positional isomers in the *trans*-octadecenoate fraction isolated from a margarine fat[a]

Position of Double Bond in *Trans* 18:1 FAME	Aldehyde Esters[b]	Aldehydes[b,c]
	mol/100 mol	
18:1 Δ^6	1.6 (0.1)	1.6 (0.4)
18:1 Δ^7	3.3 (0.3)	3.0 (0.2)
18:1 Δ^8	11.1 (0.1)	11.1 (0.1)
18:1 Δ^9	16.7 (0.1)	16.7
18:1 Δ^{10}	21.4 (0.1)	19.7 (0.5)
18:1 Δ^{11}	17.9 (0.1)	N.D.[d]
18:1 Δ^{12}	12.3 (0.2)	N.D.
18:1 Δ^{13}	8.3 (0.1)	N.D.
18:1 Δ^{14}	4.2 (0.3)	N.D.
18:1 Δ^{15}	2.0 (0.1)	N.D.
18:1 Δ^{16}	1.3 (0.3)	N.D.

[a]Analyses conducted on 100 μg-aliquots of *trans*-18:1 isolated by a combination of argentation-TLC and reverse phase HPLC and subjected to the reductive ozonolysis-DNPH-HPLC method (see text for further details).
[b]Values are the means (± SEM) of four samples subjected to the complete analytical procedure.
[c]Values were normalized to the 18:1 Δ^9 isomer with the assumption that this isomer yielded equivalent amounts of the expected fragments.
[d]N.D., not determined.

This work was supported in part by the Lipid Nutrition Laboratory, Beltsville Human Nutrition Research Center, ARS-USDA, and by a MARC Faculty Fellowship from the NIH for Leon C. Boyd. We thank Anne R. Bresnick and Michael Wong for their expert technical assistance.

Manuscript received 27 June 1986 and in revised form 29 October 1986.

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