Double Bond Analysis of Dienoic Fatty Acids in Mixtures

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ABSTRACT

Mixtures of dienoic fatty acids such as occur in edible hydrogenated fat products cannot be analyzed by current methodology. A method of ozonization, reduction to alcohol fragments by sodium borohydride, gas chromatographic analysis for alcohol, alcohol ester, and internal dialcohol fragments, and computer resolution of a matrix of linear simultaneous equations, is described that gives the analysis of the diene isomers.

INTRODUCTION

The analytical methodology of determining chain length, double bond position, and geometric configuration in mixtures of the monoenoic series of naturally occurring fatty acids may be conceded to be generally available and adequate (1).

In the dienoic series comparable methodology is generally absent. Given the information that the dienoic double bonds are all methylene interrupted or that they are all conjugated, the positions may be deduced after reductive ozonolysis by analysis either of the fragments from the alkyl end of the molecule or from the carboxyl portion. Methods for ozonization and splitting of the resultant ozonides have been well summarized in a recent review (2). However, in the mixtures of dienoic fatty acids that occur in products manufactured by hydrogenation, adequate methodology is generally lacking (1). At the present time, linoleic acid, *cis,cis*-9,12-octadecadienoic acid, may be measured in mixtures by an enzymatic (lipoxygenase) procedure which is specific for *cis,cis*-pentadiene structures and assumes them to be linoleic acid (3) and by capillary gas chromatography (4). The present paper proposes a new procedure of reductive ozonolysis with sodium borohydride to alcohols for obtaining alcohol (O), alcohol ester (OE), and internal dialcohol (OO) fragments. This procedure, which is an alternate to earlier methods of reducing ozonides to aldehydes with triphenyl phosphine, avoids interference of triphenyl phosphine with long chain aldehyde esters and gives a sharper peak for the three carbon monofunctional fragment. Two computer-assisted procedures for solving the linear simultaneous equations that account for these fragments and give the analysis of the dienoic fatty acids are compared.

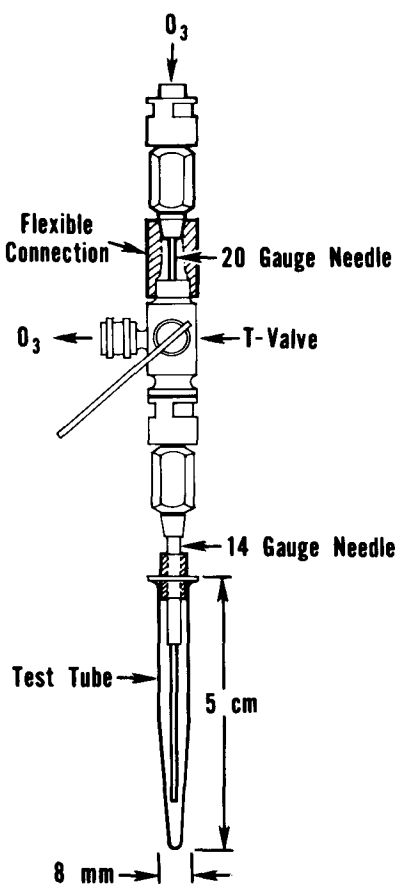

FIG. 1. Microozonization apparatus.

EXPERIMENTAL PROCEDURES

Ozonization

Ozonization was carried out in a 1-ml centrifuge tube cut to a length of 5 cm which was connected to the ozone generator of an integrated microreactor apparatus (MRA) (5,6). Through a system of Luer hypodermic needles (Fig. 1) and valves, ozone was introduced to the sample, and ozone concentration was measured in the entering and exiting streams. The microtest tube for ozonization was surrounded with dry ice to prevent solvent evaporation. Ozone in oxygen (3%) flow for ozonization is adjusted to 50 cc/min. One μl of the methyl ester sample (diene) is introduced into the microtest tube, followed by 10 μl ethanol. A decrease in concentration of ozone is indicated on the MRA meter and a parallel strip chart recorder while the sample is being ozonized. After complete ozonization is indicated by the meter/recorder, flow is continued for an additional 4 min. Sample and chamber are then flushed with oxygen. The microtest tube containing the ozonized sample is removed from the apparatus, 1 μl of water and 1 μl bromothylmol blue indicator solution are added, the tube is closed with a septum, and sufficient $NaBH_4$ solution (50 mg $NaBH_4$ dissolved in 100 μl water) is added to reduce the ozonides to alcohols (7) and just turn the indicator blue. The sample solution is then stabilized with 0.4 μl saturated buffer solution (commercial pH 7.41 dry buffer).

TABLE I

Analysis of Methyl 9,15-Octadecadienoate Compound (Mole % and Standard Deviation)[a]

Run	C_3-O	C_6-O	C_5-OO	C_6-OO	C_8-OE	C_9-OE
1	97.93	2.07	3.32	96.68	1.36	98.64
2	99.04	0.96	4.19	95.81	1.58	98.42
3	100.0		5.75	94.25	1.41	98.57
4	100.0		2.31	97.69	1.90	98.10
5	99.77	0.23	3.67	96.33	2.24	97.76
Ave.	99.35 ± 0.88	0.65 ± 0.88	3.85 ± 1.26	96.15 ± 1.26	1.70 ± 0.37	98.30 ± 0.36

[a]Alcohol (O), Dialcohol (OO), and alcohol ester (OE).

TABLE II

Analysis of Binary Mixture Methyl 9,12- and 12,-15-Octadecadienoates
Compound (Mole %)

Run	C_3-O	C_4-O	C_5-O	C_6-O	C_7-O	C_3-OO	C_5-OO	C_6-OO	C_8-OE	C_9-OE	C_{10}-OE	C_{11}-OE	C_{12}-OE	C_{14}-OE	C_{16}-OE
1	33.65	0.14	0.65	65.49	0.08	92.57	2.75	4.68	1.49	71.46	0.29	0.89	25.47	0.32	0.07
2	31.71	0.23	0.40	67.55	0.11	96.88	1.18	1.93	0.66	71.23	0.30	0.51	26.90	0.29	0.11
Ave.	32.68	0.18	0.52	66.52	0.10	94.73	1.97	3.30	1.08	71.35	0.30	0.70	26.18	0.30	0.09

Chromatography

Use of the MRA (6) for sample injection (8) was found to greatly lengthen column life. First, the MRA needle is inserted into the heated inlet of the gas chromatograph (GC). Helium is the carrier gas for both GC and the MRA system, and the helium gas flow through the MRA in injection position is adjusted to 40-50 cc/min so that MRA pressure is greater than in the GC injection port. Temperature in the Luer joint of the MRA is raised to 300 C, and the sample is injected through the MRA; temperature of MRA is held at 300 C for 2 min, then raised to 400 C for 2 min; this procedure transfers quantitatively all the cleavage products to the GC. The MRA needle is then withdrawn from GC septum.

Because of the great difference in peak sizes, it was impossible to get sufficient area for accurate measurement of small peaks without exceeding the linear range of the equipment for large peaks. Therefore, two chromatographic runs were made on each ozonization sample: a 1 μl sample for major peaks and the remainder of sample (about 7 μl) for minor peaks. Data from the chromatograms were sent to the computer in real time. After receiving area percentages and identifying peaks to the computer, the areas were converted to molar percentages by dividing each by its ionizable carbon number (total number of carbon atoms in molecule minus number of oxygen atoms) and renormalizing (9). Those peaks of suitable size which appeared on both chromatograms were used to adjust the two chromatograms and to normalize alcohols, dialcohols, and alcohol esters each to 100 mole percent.

A Beckman GC-5 gas chromatograph with flame ionization detector was used with helium carrier gas flow at 38 cc/min; attenuation at 2 x 10^4, detector temperature 325 C, and temperature programmed from -40 C to 275 C at 4.4 C/min. The low temperature was reached by adding dry ice to the column oven. As discussed below, best separations were obtained with 5 ft x 1/8 in. columns packed with a mixture of 6.7% of 10% OV 17 on 80/100 Chromosorb WHP and 93.3% of 10% OV 225 on 80/100 Chromosorb WHP.

Because recovery of trimethylene glycol is variable in this procedure, it was usually estimated from alkali isomerization spectrophotometry (10) data.

Analytical results from samples analyzed by this procedure are shown in Tables I-V.

Calculation of diene isomers from this data is carried out by two alternate procedures of setting up linear simultaneous equations as discussed below.

TABLE III

Analysis of Ternary Mixture of Methyl 9,12-, 12,15-, and 9,15-Octadecadienoates
Compound (Mole % and Standard Deviation)

Run	C_3-O	C_6-O	C_3-OO[a]	C_6-OO	C_9-OE	C_{12}-OE
1	57.95	42.05	69.6	30.4	77.26	22.74
2	56.91	43.09	69.6	30.4	78.61	21.39
3	55.68	44.32	69.6	30.4	78.95	21.05
4	58.84	41.16	69.6	30.4	77.10	22.90
Ave.	57.35 ± 1.36	42.65 ± 1.36	69.6	30.4	77.98 ± 0.94	22.02 ± 0.94

[a]Alkali isomerization.

The isomeric composition of some samples was also estimated with a 0.02 in. ID x 150 ft polyphenylether column at 190 C.

DISCUSSION

The reductive splitting of ozonides with sodium borohydride to alcohols, reported by Sousa and Bluhm (7), is a useful alternate to the reduction to aldehydes with triphenyl phosphine. Both aldehydes and ethoxy hydroperoxides as formed by the Criegee mechanism (2) are apparently completely reduced to alcohols by sodium borohydride. The alcohol procedure has the advantage of giving a sharper peak for propyl alcohol, whereas propionaldehyde gives an unsatisfactory tailing peak. Furthermore, triphenyl phosphine on most GC support phases overlaps long chain aldehyde esters and elutes unpredictably. Neither method gives quantitative recovery of 1,3 propanediol, and independent measurement by alkali isomerization is necessary. The following sections discuss some of the problems of solvent choice, gas chromatographic separations, and handling of data to give percentages of positional isomers in a sample.

Solvents

Various solvents have been proposed for $NaBH_4$ reductions, but in general, $NaBH_4$ is soluble in polar compounds containing a hydroxyl or amine group. Further, a solvent was desired that would not mask components eluted on the GC. It would seem that methanol, because of its early elution, would be the solvent of choice, but it presents many difficulties. Although it reacts with $NaBH_4$ (ethanol

TABLE IV

Analysis of Dienes from Shortening
Compound (Mole % and Standard Deviation)

Run	C_3-O	C_4-O	C_5-O	C_6-O	C_7-O	C_8-O	C_3-OO[a]	C_4-OO	C_5-OO	C_6-OO	C_8-OE	C_9-OE	C_{10}-OE	C_{11}-OE	C_{12}-OE
1	2.93	1.46	3.56	91.29	0.48	0.28	82.04	9.42	4.83	3.71	2.06	96.79	0.64	0.34	0.16
2	2.55	1.08	3.13	89.81	0.49	2.93	82.04	10.31	4.04	3.61	3.43	95.56	0.39	0.48	0.14
3	3.29	1.03	3.34	89.82	0.56	1.96	82.04	9.87	4.16	3.93	2.57	96.68	0.38	0.19	0.17
Ave.	2.92 ± 0.37	1.19 ± 0.23	3.34 ± 0.21	90.31 ± 0.85	0.51 ± 0.04	1.72 ± 1.34	82.04	9.86 ± 0.44	4.34 ± 0.43	3.75 ± 0.16	2.69 ± 0.69	96.34 ± 0.68	0.47 ± 0.15	0.34 ± 0.14	0.16 ± 0.01

[a]Alkali isomerization.

TABLE V

Analysis of Dienes from Copper Catalyst Hydrogenated Methyl Linolenate Compound (Mole % and Standard Deviation)

Run	C_3-O	C_4-O	C_5-O	C_6-O	C_7-O	C_8-O	C_9-O	C_{10}-O	C_{11}-O	C_3-OO[a]	C_5-OO	C_6-OO	C_7-OO	C_8-OO	C_9-OO	C_{10}-OO	C_6-OE	C_7-OE	C_8-OE	C_9-OE	C_{10}-OE	C_{11}-OE	C_{12}-OE	C_{16}OE
1	63.51	11.76	1.31	21.13	0.39	0.36	0.26	0.07	1.22	27.2	37.4	20.2	5.1	3.9	2.9	3.3	1.22	2.42	5.82	67.15	13.69	0.63	8.93	0.13
2	60.86	20.26	1.07	15.92	0.37	0.32	0.06	0.04	1.10	27.2	46.1	17.3	4.4	1.0	2.7	1.3	0.83	1.41	5.93	64.93	17.23	00.48	9.11	0.07
3	61.43	16.25	1.53	18.51	0.54	0.42	0.09	0.07	1.16	27.2	44.6	15.5	7.3	1.0	1.7	2.7	1.00	1.87	5.74	64.31	17.56	0.59	8.85	0.07
4	60.75	18.76	1.30	16.16	0.44	0.41	0.33	0.14	1.71	27.2	42.6	17.6	6.4	0.8	2.2	3.2	1.16	1.92	5.56	63.82	18.19	0.59	8.76	—
Ave.	61.64 ±	16.76 ±	1.30 ±	17.93 ±	0.44 ±	0.38 ±	0.18 ±	0.08 ±	1.30 ±	27.2	42.68 ±	17.65 ±	5.80 ±	1.67 ±	2.38 ±	2.63 ±	1.05 ±	1.90 ±	5.76 ±	65.05 ±	16.67 ±	0.57 ±	8.91 ±	0.07 ±
	1.28	3.72	0.19	2.43	0.07	0.05	0.13	0.04	0.28		3.80	1.94	1.30	1.49	0.54	0.92	0.17	0.41	0.15	1.47	2.02	0.06	0.15	0.05

[a]Alkali isomerization.

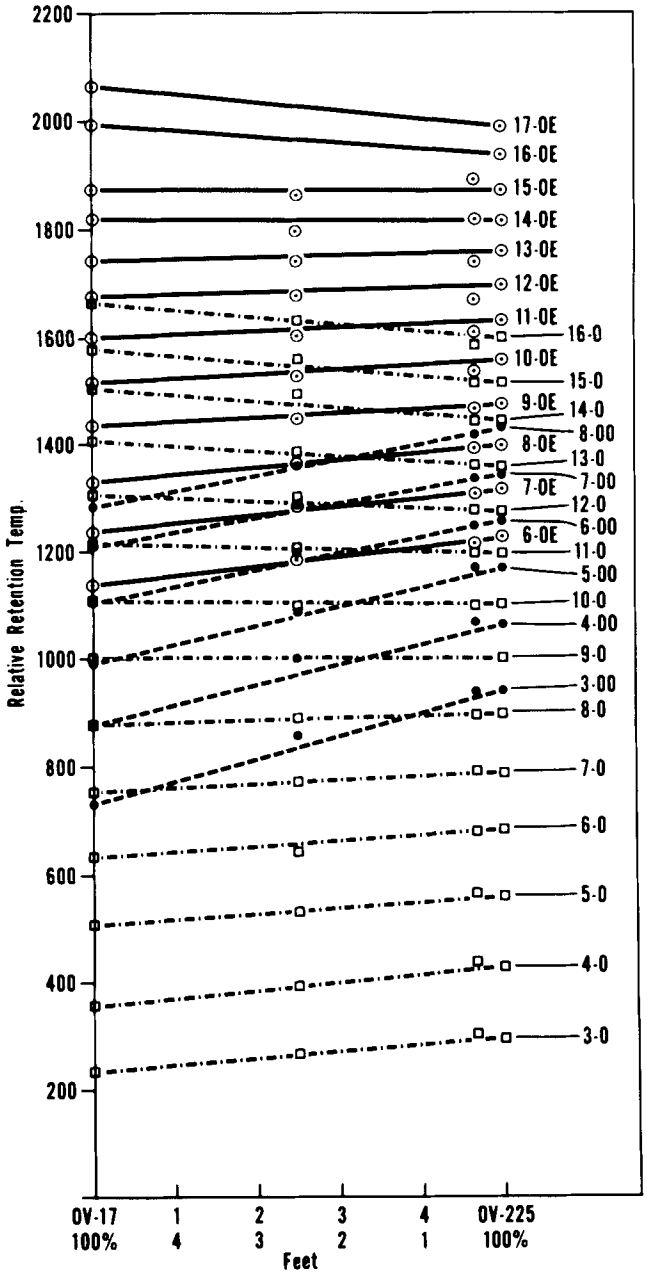

FIG. 2. Modified Rohrschneider representation of substrate (OV 17 and OV 225) polarity. Relative retention temperatures of alcohols (O) open circles, alcohol esters (OE) solid circles, and dialcohols (OO) open squares.

reacts slowly), the reaction can be slowed by using a 50% aqueous solution. More importantly, when methanol was used as solvent, the GC column rapidly became "poisoned," as indicated by small, broad tailing peaks with loss of resolution. This is believed to be caused by carry-in of $NaBH_4$ residual material. Successive microliter injections of methanol would eventually restore the column. However, this is inconvenient. Ethanol is a good solvent for the methyl esters to be analyzed and supplies a polar solvent necessary to this ozonization (nonpolar solvents produce 50-100% aldehydes); no more than a very small amount of ethanol is usually found as a component of the ozonized mixture. Ethanol also eventually causes peak broadening, but many samples could be run before poisoning appeared. In order to eliminate the ethanol carry-in poisoning, the MRA (8) was used; this system permits complete washing of Luer tube and needle after each injection. MRA protection gave normal column life.

Choice of Substrate

The multiplicity and range of ozonolysis products from diunsaturated fats, whose double bonds vary in position from the second or third carbon atom to the 17th carbon atom, pose stringent requirements upon any liquid substrate used for quantitative resolution. Previous work (11) indicated that a mixture of OV 17 and OV 225 gave good separations of aldehydes and aldehyde esters. A mixture of the same two compounds gave good separations of alcohols, dialcohols, and alcohol esters. Based on Figure 2 – a modified Rohrschneider (12) representation of relative retention temperatures (13) of these two substrates – a mixture of 6.7% OV 17 and 93.3% OV 225 was chosen as giving the best separations.

Methods of Calculation

Linear simultaneous equations for analyzing the complex mixtures of diene isomers with double bonds ranging in position from 3 to 15 are given in Figure 3. They account for the experimentally observed percentages of alcohol (O), alcohol ester (OE), and dialcohol (OO) fragments in terms of the mole fractions (MF) for 55 possible nonconjugated isomers. Amounts of conjugated isomers in edible hydrogenated fats are very low, and they are not considered. However, since only 30 equations relate the 55 variables, the set cannot be solved. The area not included within dotted lines in this table of equations includes dienes that may be expected in hydrogenated vegetable oils. Because of implicit relations, the 15 equations and 15 unknowns in the unenclosed area still cannot be solved rigorously. However, two alternate procedures have been used. The one (single skip procedure) assumes that residual double bonds either do not move from their naturally occurring positions or that one of the two bonds moves only once. This assumption is consistent with mechanisms of hydrogenation and isomerization in which double bonds move one position at a time (14). Also, all except a few small fragments from the analysis in Tables I, V are accounted for under this assumption. Those diene isomers enclosed in rectangles satisfy these single skip requirements.

The second (empirical procedure) involves inspection of the data. Fragments – usually very small – which cannot be combined with other fragments to form an octadecadienoate are eliminated. If there are more unknowns than equations, peaks smaller than a fixed value (usually 0.5 to 1.5%) and the isomers associated with them are eliminated to give a set of equations which can be solved.

Since redundancy usually exists in both procedures, the optimal solution is calculated by a modified computer program called ORTHO (15). This program minimizes the summed squared error of all possible solutions by an iterative process. In this process, when a negative value for an isomer is found, the program automatically puts this isomer at zero and the set is then recalculated.

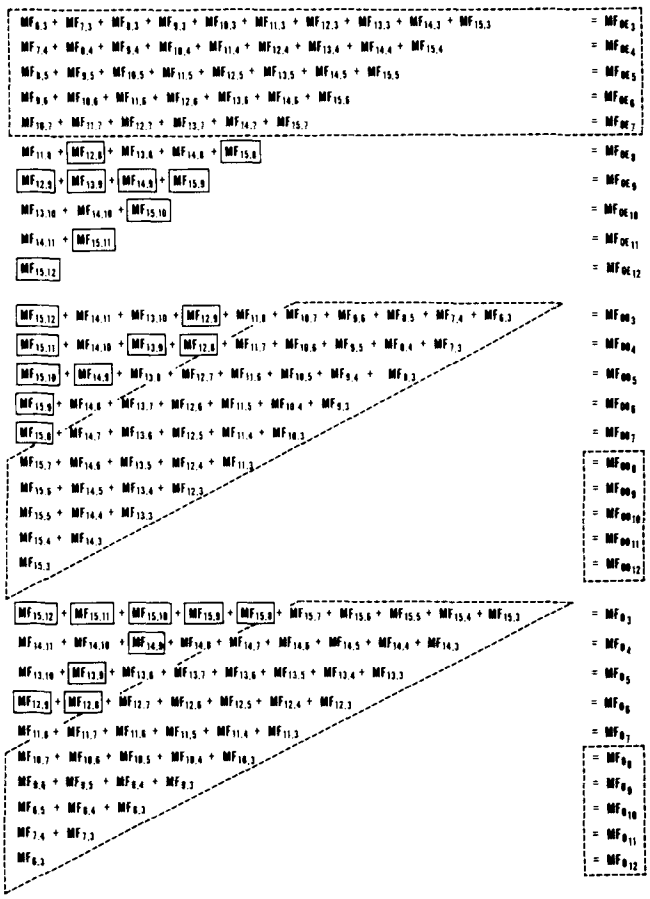

FIG. 3. Linear simultaneous equations relating composition of octadecadienoate isomer mixture to composition of alcohol, alcohol ester, and dialcohol fragments from ozonolysis. The area not included within dotted lines includes dienes that may be expected in hydrogenated oils. The diene isomers enclosed in rectangles assume residual double bonds either do not move from their naturally occurring positions or that one of the two bonds moves only once.

Analytical Results

Data for four methyl ester samples chosen for their wide diversity of fatty acid compositions are presented in Tables I-V. Values for individual runs are given as an indication of reproducibility of the method. Samples in Tables I, II, and III were obtained from hydrazine-reduced linolenic acid for which independently determined capillary gas chromatographic values were measured; that in Table IV is the diene

TABLE VI

Calculation of Isomeric Octadecadienoates by Procedures 1 and 2[a]

Sample	Isomer												
	7,15	8,11	8,12	8,13	8,15	9,12	9,13	9,14	9,15	10,15	11,14	11,15	12,15
A1						67.31	0.11	0.67	3.58	0.90		0.39	27.04
A2		0.26		0.63		66.71		0.19	4.10	0.92	0.33		26.87
B1[b]													
B2						43.79			31.64				24.57
C1			5.12			83.79	4.32	2.83	3.45	0.50			
C2			4.57			84.07	4.62	3.08	3.66				
D1					6.63	18.28	1.35	20.81	21.59	20.60		0.41	9.79
D2	4.32				8.25	16.49		18.36	18.33	22.28			11.97

[a]Sample A is the 9,12-12,15 mixture in Table II; B is the 9,12-9,15-12,15 mixture in Table III; C is shortening diene from Table IV; D is from hydrogenated linolenate in Table V. Numbers 1 and 2 refer to procedure of calculation–1. Single skin, 2. Empirical.

[b]For this sample, methods 1 and 2 give same set of linear equations and the same composition.

fraction from a commercial shortening; and that in Table V is the diene fraction from methyl linolenate hydrogenated with a copper catalyst.

Isomeric composition calculated by solution of the linear equations is shown in Table VI. For comparison, capillary gas chromatography indicated 70% 9,12 isomer and 30% 12,15 for sample A and 44%, 9,12, 29% 9,15, and 27% 12,15 for sample B. Inspection of the data indicates agreement for major diene components by either single skip or empirical procedures when optimized by the ORTHO program. As might be expected, the empirical procedure permits minor dienes to be calculated that are not permitted by the single skip process; the single skip hypothesis calculates other minor constituents not calculated under the empirical approach. Whereas the empirical approach has the advantage of including dienes outside the 8,15 position range but requires human inspection and evaluation, the single skip procedure is applied without inspection and adjustment of the data. Regardless of the procedure employed, it would appear that a new approach to analyzing diene isomers in commercially hydrogenated fat products is now at hand.

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