Studies on the Concentration of Oxidized Components of Abused Fats and the Application of HPLC to Their Separation

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The efficiency of five separation techniques at removing unaltered compounds from the methyl esters of oxidized fats was compared, as were concentrations of oxidized products. A batch type distribution method using acetonitrile/hexane was the most effective in concentrating the polar products and removal of palmitate and stearate from abused fats which had been hydrogenated.

The level of high molecular weight material present in samples was measured indirectly by determination of the percent elutable material via gas chromatography.

A high performance liquid chromatography system developed to partially separate or "profile" the oxidized products used an octadecyl bonded phase column and a linear gradient from 50% aqueous acetonitrile to 85% aqueous acetonitrile, at a rate of 5%/min. The resultant chromatograms can be useful in assessing the quality of used fats.

Much of the fat consumed in an average American diet has been exposed to heat and air during processing or in the preparation of foods during cooking. Extensive research to determine the nature of the changes which occur during such heating has been carried out. A complex mixture of organic compounds, including various nonvolatile oxygen-containing compounds, are among those formed (1-3).

No one analytical technique can separate all the compounds present in a mixture as complex as that of oxidized oils. Investigations have involved the use of one or the combined use of several classical methods for lipid separation. Low temperature crystallization has successfully separated unsaturated fatty acids (4) and concentrated cyclic acids (5-6). Counter-current distribution has been used to separate fatty acids with oxygen-containing functional groups (7-9). Adsorption chromatography readily separates complex lipid mixtures including oxygenated fatty acids from non-oxygenated fatty acids (1,2,10-12). Liquid partition chromatography also has been applied to the preliminary fractionation of oxidized oils. One common system uses a stationary phase of methanol on silicic acid and a mobile phase of benzene (13-15).

High performance liquid chromatography (HPLC) has successfully separated components of autoxidized lipids, but its application to the separation of a complex mixture of oxidation products of wide polarity values has not appeared. Neff et al. analyzed the secondary products of lipid autoxidation, using aqueous acetonitrile for reduced-oxidized linoleate (16) and linolenate (17). The coupling of gas chromatography with mass spectrometry has been applied extensively to the analysis of oxidized oils and has allowed for the structure elucidation of components in complex mixtures (18-20).

The present study reports results obtained in comparison of several methods for the removal of unaltered fatty acids and concentration of oxidized material in heated fats. The application of HPLC to the generation of a profile of lipid oxidation products present in a complex mixture is also shown.

EXPERIMENTAL

Preparation of used oil by deep fat frying. Fresh potato slices were fried in 2.8 l of partially hydrogenated soybean oil (IV 104, Anderson Clayton Foods, Sherman, Texas), using a household appliance deep fat fryer. Every 30 min, a 300-g portion of 1/4" thick unpeeled potato slices was fried at 180-185 C. At the end of each eight-hr period, 100 ml of the oil from the fryer was filtered into a screw-cap vial and stored under nitrogen at -20 C. At the end of each heating period, the oil level was replenished (about 400 ml) in order to compensate for that absorbed by the product and that removed for testing. The frying took place over nine consecutive days.

Fatty acid methyl ester preparation. Fatty acid methyl esters were prepared according to A.O.C.S. Method Ce 2-66 (21).

Hydrogenation of methyl esters. The methyl esters were catalytically hydrogenated in a Parr Apparatus (Parr Instrument Co., Moline, Illinois). For each gram of sample, 20 ml of ethyl acetate (A.C.S. grade, Mallinckrodt, St. Louis, Missouri) was used as solvent. The amount of platinum oxide (Englehard Industries Inc., Newark, New Jersey) catalyst used was 100 mg, regardless of the sample size (1.5-5.5 g). The hydrogenation was conducted at 50 psi and allowed to proceed 2.5 hr. Reduced platinum was removed from the sample by suction filtration using a fritted glass filter. Ethyl acetate was removed under vacuum with a rotary evaporator.

Low temperature crystallization. Sample (0.5 g) was weighed into a test tube and dissolved in 20 ml of acetone (A.C.S. grade, CMS Manufacturing Chemists Inc., Cincinnati, Ohio). The solution was cooled to -74 C and filtered at 2 C using Whatman #1 filter paper. The crystallization was repeated on the filtrate three more times in succession.

Distribution extraction procedure. The procedure of Frankel et al. (8) was used to prepare fractions by batch distribution. Two different sets of immiscible phases were used: 80% aqueous ethanol/hexane and acetonitrile/hexane.

Adsorption and Partition Chromatography. A modification of Gardner's method (22) was used for adsorption chromatography. The column was eluted and the corresponding fractions collected with: 200 ml of 5% anhydrous ethyl ether in hexane, 100 ml of 100% anhydrous diethyl ether (glass distilled, Burdick & Jackson Laboratories, Inc., Muskegan, Michigan) and 100 ml of 100% methanol.

The column packing used for partition chromatography was composed of 50 g silicic acid coated with 10 ml 80% aqueous ethanol. The same elution scheme was used as for adsorption chromatography.

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Trimethylsilyl ether (TMSO) derivative procedure. TMSO derivatives were prepared from hydrogenated methyl esters intended for GC and/or GC/MS analysis. To a known amount of sample (1.0–5.0 mg) in a 1.0-ml reaction vial, 100 μl of anhydrous pyridine (J.T. Baker Chemical Co., Phillipsburg, New Jersey) and 100 μl of N-O-bis-(trimethylsilyl)-trifluoroacetamide (Pierce Chemical Co., Rockford, Illinois) were added. The vial was capped and the contents heated at 60 C for 20 min.

Determination of fatty acid and estimation of polymer content. Hydrogenated methyl esters were diluted with iso-octane to a concentration of 10.0–25.0 mg/ml. A known amount of methyl pentadecanoate was added as internal standard. The samples (underivatized and TMSO derivatives) were analyzed by Gas Chromatography with flame detection on a 6' × 2 mm i.d. glass column packed with 10% SP2250 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania). Quantitation was achieved with an electronic integrator and peak identification with standard mixtures of methyl esters.

The amounts of methyl palmitate, stearate and percent eluted material (as an estimate of polymer content) were calculated from the response obtained from the internal standard.

HPLC system. Reverse phase HPLC analyses were accomplished with a Supelcosil LC-18 column (Supelco, Inc., Bellefonte, Pennsylvania), 25 cm × 4.6 mm i.d., packed with five micron spherical silica support bonded with octadecylsilane. A Tracor 995 Isochromatographic Pump with a Tracor Model 980A Solvent Programmer (Tracor, Inc., Austin, Texas) was used to deliver the mobile phase. Samples were injected onto the column through a Rheodyne 7120 syringe loading sample injector, with a 20-μl loop (Rheodyne, Berkley, California). The detector was a Perkin-Elmer LC-55 Spectrophotometer (Perkin-Elmer, Norwalk, Connecticut) set at 210 nm. The recorder was a HP 3385 A Recording Integrator (Hewlett-Packard, Avondale, Pennsylvania).

A linear gradient was used: Solvent A was a 1:1 acetonitrile:water mixture, and Solvent B was a 17:3 acetonitrile:water mixture. The acetonitrile was spectrograde (Ominosolve, UV cutoff 190 nm, MCB Manufacturing Chemists, Inc., Norwood, Ohio), and the water was deionized and glass distilled. The mobile phases were degassed by sonification before use. The mobile phase concentration of Solvent B increased 0% to 100% at 3.3%/min with a 45-min hold at 100% B. The flow rate was 2 ml/min.

Methyl esters of the hydrogenated samples, unconcentrated, and concentrated by each of the five methods, were injected individually into the HPLC. The sample concentrations were about 100 μg/μl in acetonitrile.

Gas Chromatography-Mass Spectrometry system. Sample (methyl esters and TMSO derivatives) was injected onto a 6 × 2 mm i.d. glass column packed with 3% SP-2100 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania). The sample concentration ranged from 10-25 μg/μl.

A Hewlett-Packard Model 5985B GC-MS (Hewlett-Packard, Avondale, Pennsylvania) was used. The operating conditions for the GC were: Column temperature 1, 150 C; rate, 8 C/min; column temperature 2, 280 C; time, 8 min; injector temperature, 300 C; FID temperature, 300 C; nitrogen flow rate, 30 ml/min.

The mass-analyzer (HP 59875B) was set to scan every two seconds from m/e29 to 600 and mass spectra recorded by the data system. An electron impact ion source was set at 70 eV. For chemical ionization, methane was introduced through the column.

RESULTS AND DISCUSSION

Five methods were compared for their effectiveness in removing unaltered fatty acid methyl esters from thermally oxidized soybean oil, and subsequent concentration of the oxidized products. The removal of the unaltered methyl esters prevents them from interfering with the subsequent separation of the oxidized compounds. In addition, the resulting concentration of the oxidized products is a desirable side-effect, facilitating their detection. The hydrogenated sample containing 64% palmitate and stearate was used as a reference point to which the other samples were compared (Table 1).

Low temperature crystallization of the hydrogenated material provided a mother liquor containing 34.4% of palmitate and stearate. However, batch distribution with 80% aqueous ethanol/hexane removed stearate and palmitate as effectively as crystallization. These esters accounted for 27.6% of the material isolated in the ethanol layer and 62.6% of the material in the hexane layer (Table 1).

Batch type distribution extraction with acetonitrile/hexane resulted in a significant reduction of the saturated fatty acid methyl ester level. Less than 1% of the material present in the acetonitrile layer was due to the two methyl esters. In addition, only 18% of the compounds isolated in the hexane layer were other than palmitate or stearate, versus 38% from the aqueous ethanol/hexane system (Table 1). Both solvent crystallization and distribution between acetonitrile/hexane allowed approximately a fivefold concentration of oxidation products (Table 1).

The application of adsorption chromatography to hydrogenated oxidized material resulted in unaltered methyl esters eluting with 5% diethyl ether in hexane. Partition chromatography was investigated and not pursued. In comparison with the results from adsorption chromatography, no improvement was made in reducing the elution of oxidized products by the first nonpolar elution solvent chosen (Table 1).

A gas chromatographic determination of the amount of elutable material (EM) in the methyl esters of oxidized oils gives an approximation of the percent nonpolymeric material present in an oxidized oil sample. The fractions resulting from the five separation methods were analyzed for polymer content. Furthermore, each fraction was derivatized to form a TMS ether of any hydroxyl-containing compounds present in the material. The differences reported indicated the amount of volatile hydroxylated compounds present.

The results from this estimation are presented in Table 2. The unconcentrated sample of thermally oxidized soybean oil methyl esters was once again used as a reference. The amount of sample eluted from the column was 77%. There was no significant change upon silylation, probably due to the low concentration of hydroxy fatty ester.

Silylation did not significantly increase the EM from either the mother liquor or the product resulting from low temperature crystallization.

The aqueous ethanol layer from the distribution extraction contained more polymeric material and more hydroxylated compounds than the mother liquor from the

TABLE 1

Effect of Separation Methods on Percent Palmitate and Stearate Present in Hydrogenated Methyl Esters of Used Oils

Method	Fraction	Weight (g)	% Total wt	% 16:0[a]	% 18:0[a]	Wt percent oxidation products
Unconcentrated	-	-	-	7.8	56.6	35.6
Crystallization	Mother liquor	0.09	14.3	14.0	20.4	65.6
	Crystals	0.55	85.5	8.4	78.3	13.3
CCD[b]	Aqueous Ethanol layer	0.34	7.4	4.8	22.8	72.4
	Hexane layer	4.69	92.5	7.7	54.9	37.4
CCD[b]	Acetonitrile layer	0.30	6.0	0.3	0.5	99.2
	Hexane layer	4.77	93.9	10.5	71.6	17.9
Adsorption chromatography	5% ether	0.22	83.6	9.9	61.0	29.1
	100% ether	0.02	8.1	0.1	0.5	99.4
	100% methanol	0.02	8.1	0.0	0.0	100.0
Partition chromatography	5% ether	0.25	88.9	9.8	61.3	28.9
	100% ether	0.02	9.7	0.2	0.3	99.5
	100% methanol	0.00	1.2	0.1	0.4	99.5

[a]Determined by comparison of GLC areas with that of an internal standard.
[b]Countercurrent distribution.

TABLE 2

Effect of Separation Methods on Percent Eluted Material (EM) Present in Hydrogenated Methyl Esters of Used Oil

		% EM[a]		
Method	Fraction	Unsilylated	Silylated	% Difference
Unconcentrated	-	76.6	77.2	0.5
Crystallization	Mother liquor	60.4	62.2	1.8
	Crystals	74.2	74.8	0.7
CCD[b]	Aqueous ethanol layer	57.9	64.3	6.3
	Hexane layer	73.0	73.3	0.3
CCD[b]	Acetonitrile layer	39.0	52.3	13.3
	Hexane layer	88.5	89.0	0.4
Adsorption chromatography	5% ether	75.9	87.0	11.1
	100% ether	20.7	21.9	1.2
	100% methanol	8.6	9.0	0.3
Partition chromatography	5% ether	77.9	82.6	4.6
	100% ether	20.9	21.7	0.7
	100% methanol	1.5	68.4	6.9

[a]Eluted material.
[b]Countercurrent distribution.

crystallization. The acetonitrile layer resulting from the distribution showed the presence of even more nonelutable material. It also was the most affected by silylation, yielding an increase of 13% elutable material after silylation, presumably due to hydroxylated material.

With adsorption chromatography, the hexane/5% ether eluate fraction contained a greater amount of material eluted after silylation than the other two fractions. A substantial amount of oxygenated products therefore eluted with the first solvent mixture. The 100% methanol solvent was used to wash the column free of any materials which were strongly retained; over 90% of the material from this fraction injected into the GC column did not elute. The material is of sufficiently high molecular weight that it did not elute under the conditions of the separation (> 400).

Partition chromatography was less satisfactory than adsorption chromatography; the EM from the fraction eluted with 5% ether increased less after silylation than the corresponding fraction from adsorption chromatography (4.6% vs 11.1%). However, with partition chromatography the 100% methanol fraction contained a large amount of elutable material which significantly increased upon silylation. The partition chromatography technique did not adequately concentrate the oxidized materials in the 100% ether fraction. These results indicated that the batch type extraction system, especially that using hexane/acetonitrile and adsorption chromatography, was the most useful in both removing unaltered fatty esters and concentrating the oxidation products present in an abused fat.

Development of HPLC system. HPLC has not been applied to the separation or "profiling" of heated fats because a concentration step prior to separation is required and the resultant concentrate is a very complex mixture. The previous separation studies provide a means to prepare fractions containing a high concentration of oxidation products from heated fats which can in turn be used as starting materials for HPLC separations.

The chromatograms representing HPLC separations obtained for the unconcentrated and concentrated samples of oxidized products as the methyl esters are presented in Figures 1 and 2. The lack of resolution is typical of that obtained from a very complex mixture. Some eluting peaks were identified by comparison with standards, gas chromatography and mass spectrometry. Methyl palmitate eluted at ca. 36 min, and methyl stearate eluted at ca. 42 min. The unconcentrated sample showed relatively small amounts of all compounds present, except for methyl palmitate and stearate (Fig. 1A).

Low temperature crystallization has been used to concentrate cyclic monomers for gas chromatography (6). The chromatogram of the mother liquor resulting from crystallization of thermally oxidized soybean oil methyl esters is shown in Figure 1B. Well defined peaks corresponding to oxidized products appear prior to 36 min.

The area around 36 min indicates three unresolved components. Both polar and nonpolar cyclic monomers have a retention time similar to palmitate with this solvent system, and this accounts for peaks found in this area. Palmitate does not contribute significantly to the combined area of the three peaks.

The chromatograms of the samples obtained from both distribution extraction techniques (acetonitrile layer and aqueous ethanol layer, respectively) are shown in

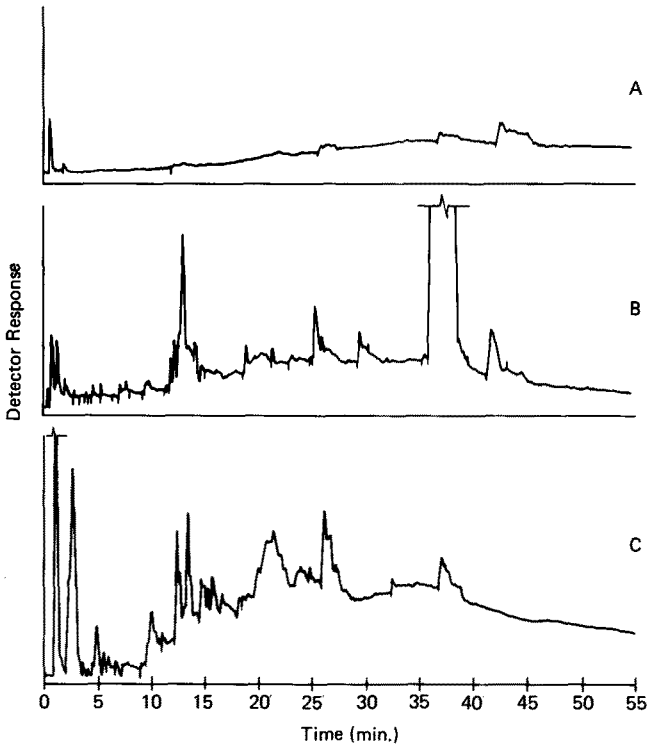

FIG. 1A. HPLC separation of unconcentrated sample. Column, 25 × 0.40 cm Supelcosil LC-18 (5μ); mobile phase, 50–85% acetonitrile/water gradient, with 3%/min increase in linear gradient; flow rate, 2.0 ml/min; UV, 210 nm, sample amount, 1.81 mg; attenuation 4. Fig. 1B. HPLC separation of sample after concentration by low temperature crystallization (mother liquor). Conditions as in 1A; sample amount, 2.41 mg; attenuation 4. Fig. 1C. HPLC separation of sample after concentration in acetonitrile layer by counter-current distribution. Conditions as in 1A; sample amount, 1.90 mg, attenuation 2.

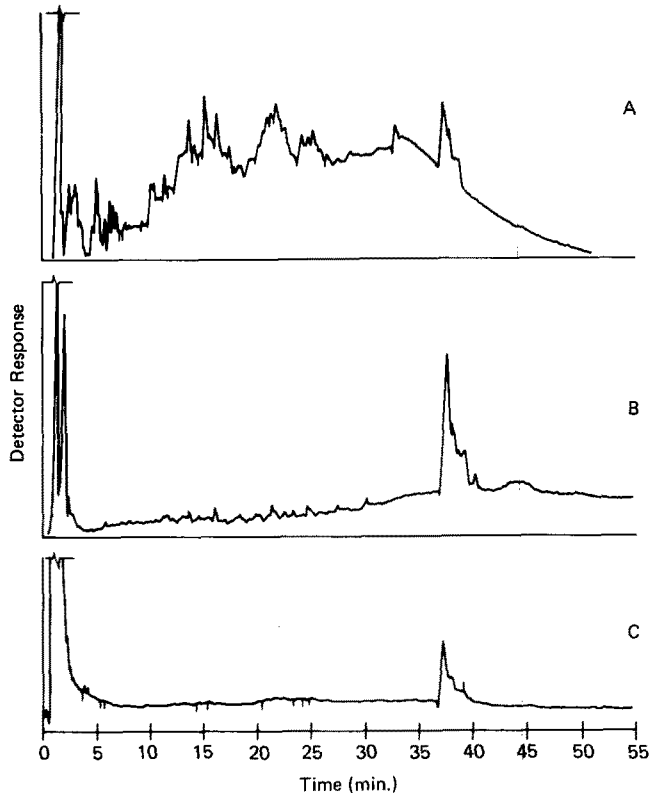

FIG. 2A. HPLC separation of sample after concentration in 80% aqueous ethanol layer by counter-current distribution. Conditions as in 1A; sample amount, 3.12 mg; attenuation 4. Fig. 2B. HPLC separation of sample after concentration in 100% ether fraction by adsorption chromatography. Conditions as in 1A; sample amount, 1.00 mg; attenuation 1. Fig. 2C. HPLC separation of sample after concentration in 100% ether fraction by partition chromatography. Conditions as in 1A; sample amount, 1.60 mg; attenuation 8.

Figures 1C and 2A. The resolution seen in Figure 1B obtained for stearate was not achieved with these two samples because of the increasing complexity of sample. Both show an increased concentration of the oxidized products. Some very polar compounds show little affinity for the column and elute close to the solvent peak. The stearate peak is not present, and palmitate does not contribute significantly to the peaks found at 36 min because it has been removed.

The chromatograms obtained for the 100% ether eluate from adsorption (Fig. 2B) and partition chromatography (Fig. 2C) are similar. Except for a very prominent peak eluting just after the solvent peak, the only other notable peaks are those which upon GC-MS corresponded to methyl palmitate and various cyclic monomers.

The primary purpose of this study was to investigate various methods of extraction and concentration of oxidation products applicable to heated fats. Although the HPLC method presented here is preliminary, the ultimate goal is to develop a method to obtain profiles of oxidation products for comparison purposes to evaluate the quality of used fats. The results obtained indicate that the batch extraction using acetonitrile may be used as a means of sample preparation to produce such profiles easily and quickly. The peaks produced in such profiles can only be indicative of the composition of oxidized fats because they are composed of complex mixtures of components. However, such profiles can be used to evaluate the quality of used fats by comparison with fresh fats to indicate deterioration during use.

Although not the purpose of this study, the methyl esters of the starting material and those from each concentration treatment, as illustrated in Tables 1 and 2, were treated with silylation reagent to convert any hydroxyl groups present to the corresponding trimethylsiloxy-ether derivative. This would result in increased volatility of hydroxylated fatty acids as indicated by the data in Table 2. These fractions were then subjected to GC-MS. The peaks eluted in major concentrations were identified as derivatives of both erythro and threo 9,10-dihydroxy stearic acid. Minor component peaks indicated a mixture of cyclic monomers and the presence of a common laboraory contaminent, di-octyl phthalate.

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