Procedure for Supercritical Fluid Extraction and Gas Chromatographic Determination of Chlorinated Fatty Acid Bleaching Adducts in Flour and Flour-Containing Food Items Utilizing Acid Hydrolysis–Methylation and Florisil Column Cleanup Techniques

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The identification of chlorinated fatty acid adducts formed during the bleaching of flour with chlorine and chlorine dioxide was previously accomplished utilizing chemical ionization mass spectrometry. The current, and subsequent, research has culminated in the formation of a method that has been used to quantify six different classes of isomers of these adducts in flour and flour-containing table-ready food items. The bleaching adducts were extracted with supercritical carbon dioxide and purified via acid hydrolysis–methylation and Florisil column chromatography. Determination was accomplished with gas chromatography using electrolytic conductivity detection (GC-ELCD). The most prominent adducts were the dichlorooctadecenoic acid isomers [18:1 (Cl, Cl)] and isomers of chlorohydroxyoctadecenoic acid [18:1 (Cl, OH)] with maximum levels of each in excess of 40 ppm in cakes, cookies, and biscuits and in excess of 300 ppm in bleached flour. Recoveries of each synthesized compound from sweet rolls ranged from 79.4% to 105%.

INTRODUCTION

Previous research efforts in this laboratory have resulted in the chemical ionization (ethylene oxide) mass spectral identification of several chlorinated bleaching adducts in flour and flour-containing table-ready foods (Heikes, 1992). It was shown that these compounds were present in bleached flour and essentially absent in unbleached flour. Thus, 9,10-dichlorooctadecanoic acid [18:0 (Cl, Cl)], 9,-10-dichloro-12-octadecenoic acid [18:1 (Cl, Cl)], 12,13-dichloro-9-octadecenoic acid [18:1 (Cl, Cl)], 9,10,12,13-tetrachlorooctadecanoic acid [18:0 (Cl, Cl, Cl, Cl)], 9-chloro-10-hydroxyoctadecanoic acid [18:0 (Cl, OH)], 10-chloro-9-hydroxyoctadecanoic acid [18:0 (Cl, OH)], isomers of chlorohydroxyoctadecenoic acid [18:1 (Cl, OH)], and isomers of trichlorohydroxyoctadecanoic acid [18:0 (Cl, Cl, Cl, OH)] were identified and determined in several food items (breads, cakes, muffins, cookies, etc.). The method of analysis was that used to determine chlorophenoxy alkyl acid herbicides as their methyl esters. The bleaching adducts were originally detected as gas chromatographic interferences in the analysis of these herbicides, and as such, their determination represented, at best, a semiquantitative effort.

Several recent publications have described the ease with which fats, both animal and vegetable, can be extracted from natural products using supercritical fluid extraction (SFE) techniques. King et al. (1989) demonstrated the utility of SFE for the selective extraction of fat from a variety of meat matrices. They found that under conditions of dense CO_2 (35–70 MPa, 80 °C) 96% of the theoretical fat content can be removed. King (1990) further described applications of microscale SFE coupled with supercritical fluid chromatography (SFC). Among the applications were the characterization of triglycerides and fatty acids of cottonseed kernels. In another study, the solvating power of supercritical carbon dioxide ($SC-CO_2$) was used for the measurement of extraction yields of lipids, including higher fatty acids, when coupled with Fourier transform infrared spectroscopy (FTIR) (Ikushima et al., 1992). In the current investigation, it was anticipated that the use of SFE techniques would provide the means for a rapid and quantitative extraction of fat from flour, breads, and pastries. Further, it was anticipated that the establishment of an analytical procedure for the rapid and accurate determination of bleaching adducts in flour and flour-containing table-ready food items would provide precise analytical data. This information would allow toxicologists to reliably assess the significance of these chlorinated fatty acids in our nation's food supply on the basis of accurate dietary intake data.

EXPERIMENTAL PROCEDURES

Synthesis of Chloro and Chlorohydroxy Bleaching Adduct Acids. Following the schemes used for the synthesis of the corresponding methyl esters (Heikes, 1992), 9,10-dichlorooctadecanoic acid [18:0 (Cl, Cl)], a mixture of 9,10-dichloro-12-octadecenoic acid [18:1 (Cl, Cl)] and 12,13-dichloro-9-octadecenoic acid [18:1 (Cl, Cl)], 9,10,12,13-tetrachlorooctadecanoic acid [18:0 (Cl, Cl, Cl, Cl)], a mixture of 9-chloro-10-hydroxyoctadecanoic acid [18:0 (Cl, OH)] and 10-chloro-9-hydroxyoctadecanoic acid [18:0 (Cl, OH)], a mixture of chlorohydroxyoctadecenoic acids [18:1 (Cl, OH)], and a mixture of trichlorohydroxyoctadecanoic acids [C18:0 (Cl, Cl, Cl, OH)] were prepared.

Supercritical Fluid Extraction (SFE) Conditions. Extractions were performed using an ISCO Model SFX 2-10 extractor with microprocessor control module, a Model DM 100 syringe pump, and a restrictor heater (ISCO, Inc., Lincoln, NE). Three grams of finely chopped sample was mixed intimately with 1.5 g of Celite 545 (Fisher Scientific Co., Fair Lawn, NJ) and quantitatively transferred to a 10-mL stainless steel extraction vessel (ISCO). Extractions were carried out with SFC/SFE grade carbon dioxide (Air Products and Chemicals, Inc., Allentown, PA) at 680 atm of pressure and 80 °C. Pressure was maintained throughout the system through the use of a 15 cm × 125 μm i.d. stainless steel capillary restrictor with terminal end crimped to obtain a flow of 2.5–3.0 mL/min CO_2 in the supercritical state. Upon filling of the vessel, extraction proceeded dynamically for a total volume of 50 mL. The extracted fat was collected in a 100-mL pear flask held at 60 °C in the restrictor heater oven. The end of the restrictor was positioned so as to be in contact with the inside wall of the collection flask.

Hydrolysis–Methylation Process. SFE-extracted fat was dissolved in 50 mL of ethyl ether. An aliquot equivalent to

Table I. Comparison of Levels of Fat Determined in Several Flour-Containing Food Items Using SFE and an Ether Extraction Procedure

food item	fat, % SFE	fat, % ether extraction
blueberry muffins	10.2	10.0
pancakes	5.6	6.4
chocolate chip cookies	21.7	24.0
sandwich cookies	17.2	15.6
sugar cookies	16.8	16.7

approximately 100 mg of fat was placed in a 50-mL boiling flask containing several boiling beads. After evaporation of solvent (under N_2) and addition of 15 mL of 1.5% H_2SO_4 in methanol, the flask was fitted with an air-cooled condenser and vigorously refluxed for 1 h. The solution of hydrolyzed and esterified acids was quantitatively transferred to a 125-mL separatory funnel with 50 mL of DI water and extracted with two 50-mL portions of methylene chloride. The combined methylene chloride extracts were dried with Na_2SO_4 and concentrated on a steam bath with a Kuderna-Danish concentrator (Kontes, Vineland, NJ). After the volume reached approximately 2 mL, during the concentration, 50 mL of hexane was added through the condenser and the solvent reconcentrated to a final volume of 1 mL.

Florisil Column Cleanup. Eluates from the hydrolysis–methylation process were further purified by eluting from an absorption column (25 cm × 1.0 cm i.d.) containing 3.4 g of Florisil PR (Supelco, Inc., Bellefonte, PA, 60–100 mesh) conditioned by heating at 650 °C for 24 h. The column was prewashed with 30 mL of petroleum ether. Chloro bleaching adducts were eluted from the column with 50 mL of a solution of 12% ethyl ether in petroleum ether. This was followed by a 50-mL solution of 20% ethyl ether in petroleum ether to achieve the elution of the chlorohydroxy adducts. The eluates were individually concentrated to 5 mL with Kuderna-Danish concentrators and analyzed using gas chromatography with electrolytic conductivity detection (GC-ELCD).

GC-ELCD. Quantification was accomplished using a DB-5 (30 m × 0.25 mm i.d.) fused silica column fitted in a Varian 3600 gas chromatograph (Sugar Land, TX) with a halogen-specific electrolytic conductivity (ELCD) detector. Split injections were made with a ratio of 30:1 and a column flow (He) of 1.0 mL/min. The column was initially held at 180 °C for 1 min and then programmed at 10°/min to 220 °C, held for 10 min, ramped to 280 °C at 20 °C/min, and held for an additional 10 min.

RESULTS AND DISCUSSION

During a supercritical fluid extraction, fat can be lost through the formation of an aerosol as the supercritical CO_2 expands to atmospheric pressure at the tip of the restrictor. This is especially true with elevated flow rates. However, in this study it was empirically determined that if precautions are taken, the extracted fat can be recovered quantitatively. First, care was taken to position the tip of the restrictor so as to touch the wall of the collection flask at approximately a right angle. During extractions, the fat initially was frozen (solidified) on the wall of the flask at this point of contact. As the process continued, the flow of the fat was forced from this point, liquefied on the warmed wall of the flask, and the fat flowed down the wall of the flask. A 2-mm spot of solidified fat remained at this point of contact throughout the extraction. This arrangement allowed the fat to be collected with a minimum of aerosol formation. A temperature of 60 °C proved to be optimum for the collection flask. Higher temperatures promoted the formation of aerosols. Additionally, warming the flask to 60 °C coupled with the rapid flow of expanding CO_2 prevented the condensation of any water that might have been extracted from these samples. Consequently, it was unnecessary to dry the fat after collection, and determination of fat levels was accomplished by direct measurement. Finally, all extractions were conducted at relatively low flow rates (between 2.5 and 3.0 mL/min). A comparison of the levels of fat extracted by this technique and that obtained using an ether extraction procedure (AOAC, 1990) is represented in Table I. Values represent the average of duplicate SFE determinations which varied by no greater than 10%. These data suggest that this SFE procedure is a valid approach to rapid fat determination in bakery products. Although complete fat extraction from these food items could be accomplished using SFE conditions with lower temperatures and pressures, the most efficient extraction occurred at 680 atm and 80 °C.

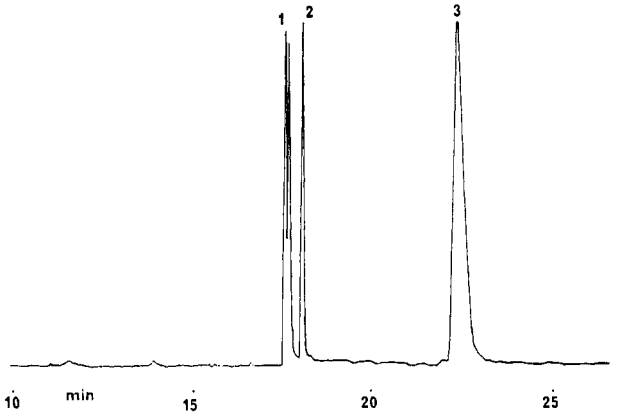

Figure 1. Capillary gas chromatogram (DB-5, 30 m × 0.25 mm) of chloro bleaching adduct standards using an electrolytic conductivity detector (for conditions, see Experimental Procedures): (1) isomers of methyl dichlorooctadecenoate (2.5 ng); (2) methyl 9,10-dichlorooctadecanoate (2.5 ng); (3) methyl 9,10,12,13-tetrachlorooctadecanoate (1.1 ng).

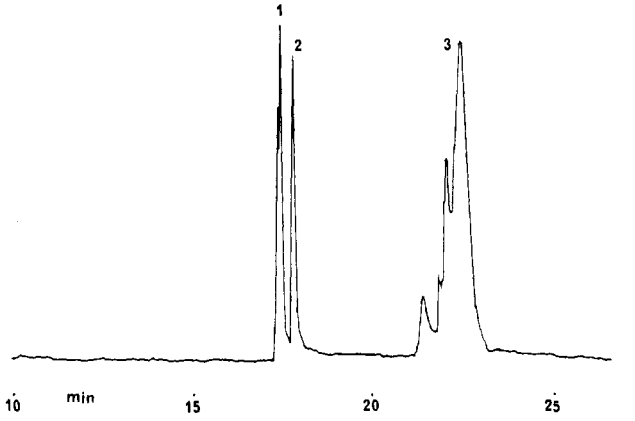

Figure 2. Capillary gas chromatogram (DB-5, 30 m × 0.25 mm) of chlorohydroxy bleaching adduct standards using an electrolytic conductivity detector (for conditions, see Experimental Procedures): (1) isomers of methyl chlorohydroxyoctadecenoate (15 ng); (2) isomers of methyl chlorohydroxyoctadecanoate (9.0 ng); (3) isomers of methyl trichlorohydroxyoctadecanoate (21 ng).

Fat from the supercritical fluid extraction was subjected to acid hydrolysis to release the component acids from the triglyceride complex. Conveniently, this same process served as an acid-catalyzed esterification (methylation) procedure. Thus, both hydrolysis and methylation were efficiently executed in one step.

Although Florisil column chromatography was beneficial for cleanup of the hydrolyzed–methylated fatty acid eluates, the primary purpose of this step was to facilitate the GC-ELCD determination. Even with the use of capillary GC columns, several of these compounds coeluted. Introduction of a Florisil adsorption column provided complete separation of the chloro fatty acid adducts from the chlorohydroxy moieties. Their respective

Table II. Levels of Chloro and Chlorohydroxy Fatty Acid Bleaching Adducts Determined in Flour-Containing Table-Ready Food Items

food item	$C_{18:1}$ (Cl, Cl)	$C_{18:0}$ (Cl, Cl)	$C_{18:0}$ (Cl, Cl, Cl, Cl)	$C_{18:1}$ (Cl, OH)	$C_{18:0}$ (Cl, OH)	$C_{18:0}$ (Cl, Cl, Cl, OH)
sugar cookies	47.7[a]	10.1	5.50	42.7	20.0	38.0
yellow cake	43.1	8.47	nd[b]	37.9	15.2	23.4
biscuits	42.8	8.25	nd	51.3	16.5	19.0
chocolate chip cookies	40.8	8.21	0.510	26.2	15.8	nd
sandwich cookies	20.6	2.87	nd	7.80	4.30	nd
blueberry muffins	20.6	4.38	0.650	10.3	5.80	nd
pancakes	2.70	0.630	nd	3.40	1.20	nd
English muffins	1.20	0.260	nd	2.20	2.60	nd
teething biscuits	0.980	0.130	nd	nd	nd	nd
pretzels	0.760	0.120	nd	nd	nd	nd
saltine crackers	nd	nd	nd	nd	nd	nd
butter crackers	nd	nd	nd	nd	nd	nd
bagel, plain	nd	nd	nd	nd	nd	nd
cake donuts	nd	nd	nd	nd	nd	nd
cheeseburger	nd	nd	nd	nd	nd	nd
sweet roll	nd	nd	nd	nd	nd	nd
white roll	nd	nd	nd	nd	nd	nd
rye bread	nd	nd	nd	nd	nd	nd
white bread	nd	nd	nd	nd	nd	nd
whole wheat bread	nd	nd	nd	nd	nd	nd
cracked wheat bread	nd	nd	nd	nd	nd	nd

[a] Values expressed as ppm. [b] nd, none detected.

Table III. Levels of Chloro and Chlorohydroxy Fatty Acid Bleaching Adducts Determined in Several Types of Flour

flour type	$C_{18:1}$ (Cl, Cl)	$C_{18:0}$ (Cl, Cl)	$C_{18:0}$ (Cl, Cl, Cl, Cl)	$C_{18:1}$ (Cl, OH)	$C_{18:0}$ (Cl, OH)	$C_{18:0}$ (Cl, Cl, Cl, OH)
cake flour A	391[a]	68.2	11.1	328	150	130
cake flour B	287	47.4	7.68	280	78.9	5.59
all-purpose A	247	48.5	1.34	248	86.4	55.7
all-purpose B	169	33.4	0.980	234	62.0	59.0
all-purpose C	101	19.1	0.120	90.3	36.1	16.6
all-purpose D	81.3	14.1	0.500	149	39.7	27.4
self-rising	177	34.9	0.920	240	103	44.3
tortilla	21.0	4.20	0.140	15.0	7.78	7.74
unbleached	1.60	0.320	0.075	1.37	0.55	0.44

[a] Values expressed as ppm.

Table IV. Fortification Levels and Recoveries of Fatty Acid Bleaching Adducts from Fat from Sweet Rolls

bleaching adduct	fortification level, ppm	recovery, %
$C_{18:0}$ (Cl, Cl)	3.9	79.4
$C_{18:1}$ (Cl, Cl)	3.8	105
$C_{18:0}$ (Cl, Cl, Cl, Cl)	1.6	104
$C_{18:0}$ (Cl, OH)	24	94.1
$C_{18:1}$ (Cl, OH)	14	86.2
$C_{18:0}$ (Cl, Cl, Cl, OH)	32	80.8

GC-ELCD chromatograms are presented as Figures 1 and 2. The several isomers of methyl trichlorohydroxyoctadecanoate could not be readily resolved using a 30 m × 0.25 mm DB-5 column. However, it has been demonstrated that with reduced column temperatures the isomers of methyl dichlorooctadecenoate and the isomers of methyl chlorohydroxyoctadecenoate could be completely resolved. Nevertheless, in the interest of reducing the time of analysis, elevated GC temperatures were employed and calculations were based on integrated areas for the combined isomers. The limits of quantitation (LOQ) in table-ready food items ranged from approximately 0.5 ppm for tetrachlorooctadecanoic acid to approximately 10 ppm for trichlorohydroxyoctadecanoic acid. Those for flour fluctuate between approximately 0.03 and 0.5 ppm, respectively.

The Total Diet Study is one of the FDA's oldest residue surveillance programs, having been in continuous operation since 1964. In its current configuration, 265 table-ready food items comprise each market basket sample. Grocery items are purchased from supermarkets in three cities of one of several regions of the country and prepared (made table-ready) individually or as recipes just as they might be in home kitchens. Table-ready food items that have flour as a principal ingredient were selected from a recent market basket sample and analyzed for the presence of bleaching adducts. Of the 21 items examined, crackers, rolls, and breads were found to be free of bleaching adducts. Alternately, the highest concentrations of these compounds were found in cakes and cookies (see Table II). This is consistent with milling industry convention. Baking quality of wheat flours is augmented with the addition of oxidizing–maturing (bleaching) agents such as chlorine and chlorine dioxide. The resulting "high-ratio" flours, because of their ability to absorb elevated levels of sugar and oils, are utilized in the production of cakes and pastries (*Food and Nutrition Encyclopedia*, 1983).

Since flour is the source of these bleaching adducts found in foods, a limited survey of commercial flour samples was conducted. A number of marketed brands of flours, of several different types, were purchased locally and analyzed by this procedure. The results of this survey are given in Table III. The highest levels of adducts were found in cake flours as expected. The range of values within the four "all-purpose" flours might indicate a lack of uniformity within the milling industry. Small amounts of these compounds were found even in flour labeled "unbleached". However, the levels are quite low, being approximately 1% of treated flours. The source of these traces of adducts may have been from carryover from manufacture of batches of bleached flour.

The determination of bleaching adducts was validated through the use of fortified samples. Fat from sweet rolls, which had been shown to be devoid of these bleaching adducts, was fortified with each of the synthesized chloro and chlorohydroxy fatty acid adducts. Recoveries ranged from 79.4% to 105% (see Table IV). Fortification levels varied from 1.6 to 32 ppm for the various bleaching adduct acids and were consistent for the GC-ELCD detection levels for each compound.

This proposed procedure for the determination of chloro and chlorohydroxy fatty acid bleaching adducts has been shown to be both rapid and accurate. The data generated are consistent with flour milling practices; i.e., bleached (high-ratio) flours are commonly utilized in the production of cakes, cookies, and other pastry items. Unbleached flours are used in the manufacture of crackers, rolls, and breads.

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