Cyclic Fatty Acid Monomer: Isolation and Purification with Solid Phase Extraction

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The application of a combined solid phase extraction (SPE)/clean-up procedure to the isolation of a purified fraction containing all the monomeric cyclic fatty acid methyl esters is described. Extraction of the nonpolar lipid components from nonurea-adducting (NUA) filtrates is performed on a reverse phase octadecyl bonded silica minicolumn. Stepwise elution of the SPE-retained materials through silica gel using several solvents allowed the separation of a pure fraction containing the cyclic monomers that can be used for a more reliable quantitative estimation of these compounds in edible fats and oils.

Solid phase extraction (SPE) with bonded silica sorbents offers a new alternative in the isolation of compounds when compared to traditional approaches to sample preparation such as liquid-liquid extraction (LLE). The latter method, although easily accomplished in most laboratories, is time-consuming, labor intensive, and requires large volumes of solvents. The multi-step nature of the sample transfer may result in analyte losses affecting the final precision of the analysis (1). As a part of ongoing research to develop a statistically optimized analytical method for the estimation of monomeric cyclic fatty acids (CFA) in edible fats and oils, the application of SPE combined with silica fractionation for the rapid isolation of hydrogenated CFA methyl esters for GC analysis is reported here.

EXPERIMENTAL

Materials. Standards phenanthrene (99%+) was obtained from Supelco, Inc. (Bellefonte, PA) and 4-cyclohexyl-1-butyl octanoate (99%) was provided by P. R. Bross (Procter & Gamble Co., Cincinnati, OH). GLC reference fatty acid methyl ester (FAME) mixture containing equal amounts of saturated esters for equivalent chain length (ECL) calculation were purchased from Nu-Chek Prep, Inc. (Elysian, MI). Fresh vegetable oil samples (refined, bleached and deodorized) of soybean and sunflower seed were purchased locally. Prepared SPE disposable columns with octadecylsilane bonded silica (Octadecyl-C_{18}, 6 ml, J.T. Baker Chemical Co., Phillipsburg, NJ) were used for reverse phase extraction of lipids from nonurea-adducting (NUA) filtrates. Silica gel (40 μm average particle diameter) and sodium sulfate anhydrous (powder) for preparation of clean-up mini columns were also purhased from J.T. Baker Chemical Company (Phillipsburg, NJ).

Preparation of NUA filtrates. Nonurea-adducting filtrates were obtained from hydrogenated fatty acid methyl esters (HFAME) as described by Rojo and Perkins (2). Prior to hydrogenation, 70–150 mg of FAME, prepared by the official AOCS analytical method (3), were accurately weighed, then 1 ml of an internal standard solution containing 40 μg each of phenanthrene (PHE) and 4-cyclohexyl-1-butyl octanoate (CBO) was added and the solvent evaporated under a stream of nitrogen. Hydrogenation was carried out with platinum oxide as previously described (2), but using 20 psi hydrogen pressure. After filtration of the catalyst, the volume of the sample solution was adjusted to 20 ml with methanol in a 50 ml test tube, and 5 g of urea was added. Urea was dissolved by heating, the tube was capped and thoroughly shaken until some crystals started to form. The suspension was allowed to stand in the dark for 18 hours. The NUA filtrate was separated from the urea crystals using Whatman #2 folded filter paper. The crystals were washed with a small amount of cold methanol saturated with urea and the washings were collected to obtain a total of 20 ml of NUA filtrate.

Solid-phase extraction/clean up (Table 1). All extractions of NUA filtrate samples were carried out using 6 ml-HC Octadecyl SPE-columns and the "Baker"-10 extraction system manifold (J.T. Baker Chemical Co.). Ten ml of deionized water was added to 20 ml of NUA filtrate and thoroughly mixed. For SPE column conditioning a blank solution of urea was prepared by mixing two volumes of urea saturated methanol and one volume of deionized water. Each column was conditioned with 2 ml of methylene chloride followed by 2 ml of methanol and 1 ml of urea blank solution just before sample addition. The diluted NUA filtrate (30 ml) was loaded onto the column using a convenient 75 ml sample reservoir and adaptor attached to the top of the column, and then aspirated using vacuum (ca. 10 psi at a flow rate of 4–5 ml/min. The column was washed with 2 ml of deionized-water and then air dryed under vacuum for six minutes. The clean-up consisted in the stepwise elution of the materials retained in the SPE-column through a small silica gel minicolumn. The minicolumn was prepared by packing 0.7 g of silica gel (40 μm) on the bottom and 0.5 g of anhydrous sodium sulfate on the top of a 3 ml disposable filtration column, which was then attached to the bottom of the SPE column. In order to obtain selective recovery of HCFAM, 0.5–1 ml fractions were collected, concentrated under N_2 and injected directly in the GC. Several combinations of n-hexane, ethyl ether, and methylene chloride as solvent systems were attempted. The best separation was achieved by first eluting a "non-polar fraction" (NPF) with 3 ml of n-hexane and 1 ml of CH_2Cl_2, followed by a second elution with 3 ml of CH_2Cl_2 in which the HCFAM were concentrated. Further elutions with 2 ml of CH_2Cl_2 did not yield any compound, however, two consecutive elutions with 2 ml of methanol each resulted in the separation of two "polar fractions" (PF-1 and PF-2) containing other groups of compounds present in the NUA filtrate.

Capillary gas chromatography. The system used was a Hewlett Packard 5790A series capillary gas chromatograph (Hewlett Packard, Avondale, PA) equipped with inlet splitter system fitted with a Jennings glass liner, flame ionization detector and electronic integrator (HP 3390A). A 30 m × 0.25 mm ID fused silica WCOT

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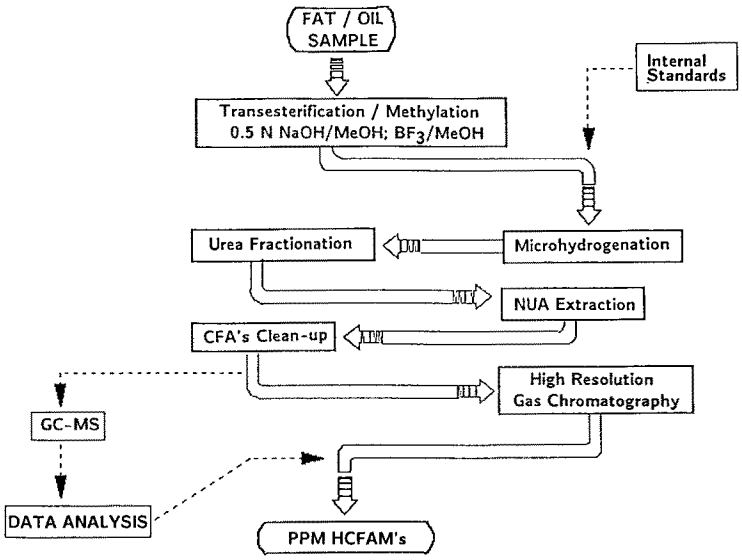

FIG. 1. Analytical scheme for the determination of cyclic fatty acids in edible fats and oils.

capillary column coated with Supelcowax-10® (polyethylene oxides bonded phase), 0.25 μm film thickness (Supelco Inc.) was used. The column was programmed from 175°C (1 min) to 200°C at 1.5°C/min, held for five minutes at 200°C and then raised to 250°C at 5°C/min. The carrier gas was hydrogen at a split ratio (1:100), and an average linear velocity of 50 cm/sec for a column head pressure of 10 psi. The injector port and detector temperatures were 250°C and 270°C, respectively.

RESULTS AND DISCUSSION

The basic analytical scheme for the determination of cyclic fatty acids as hydrogenated methyl esters (HCFAM) is shown in Figure 1. The main differences of this multi-step approach from the approach previously reported by Rojo and Perkins (2) is the addition of two internal standards before hydrogenation and the introduction of a clean-up step to remove interferring substances that coeluted with the analytes during GC analysis. Clean-up of NUA filtrates is particularly important for the determination of low levels of CFA in fresh fats and oils for which the presence of interferences could represent an unacceptable higher value for the error of the estimation.

The isolation of HCFAM involves several individual steps for which the arbitrary selection of the analytical conditions could result in extremely long analysis time and unreliable results. The development of a statistically optimized analytical method requires selection of the most convenient technique to accomplish all the steps included in Figure 1. Thus, the introduction of solid phase extraction for the separation of the lipid compounds contained in the NUA-filtrate offers a significant reduction of analysis time as well as the ability to easily manipulate several samples at a time.

The two alternatives for extraction of the NUA filtrate are compared as a flow diagram (Figure 2). Liquid-liquid extraction (LLE), as described by Rojo and Perkins (2), has obvious disadvantages regarding time and labor when processing more than one sample. On the other hand, SPE appears more convenient and efficient and enables simultaneous multi-sample processing. Optimum conditions for the combined SPE extraction/clean-up of HCFAM are summarized in Table 1.

The selective retention of lipid substances on a properly conditioned C_{18} octadecyl sorbent was accomplished by adjusting the polarity of the NUA filtrate with dionized water. The addition of at least 0.5 ml of water per ml of NUA filtrate was found to be adequate for this purpose. After the diluted filtrate was passed through the SPE column the excess of undesired urea and other matrix components were washed out, and finally the column was air dried under vacuum. The next step was the stepwise elution of the analytes through a small silica gel

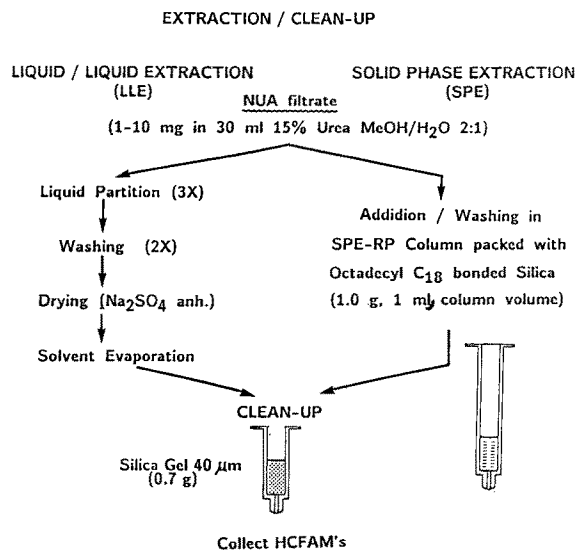

FIG. 2. Comparison between two possible methods for preparation of a purified HCFAM-fraction from NUA filtrates.

TABLE 1

SPE Extraction/Clean-up of HCFAM's Protocol

SPE-extraction column: Octadecyl-C_{18}-, 6 ml (HC)

Clean-up minicolumn: 0.7 g of silica gel (40 μm) and 0.5 g of Na_2SO_4 anh

1. Column conditioning:
   —2 ml of methylene chloride
   —2 ml of methanol
   —2 ml of blank solution (2 volumes of methanol saturated with urea and 1 volume of deionized water)
2. NUA-filtrate addition/washing/drying:
   —20 ml NUA filtrate + 10 ml of deionized water (using sample reserovir)
   —2 ml deionized water
   —air drying under vacuum for 6–10 min
3. Stepwise elution (clean-up):
   —attach clean-up minicolumn to the bottom of SPE column
   —3 ml of n-hexane and 1 ml of methylene chloride ("non-polar fraction," NPF), discard
   —3 ml of methylene chloride; collect HCFAM's
   —2 ml of methylene chloride and 2 ml methanol ("polar fraction" #1, PF-1), optional
   —2 ml of methanol ("polar fraction" #2, PF-2), optional

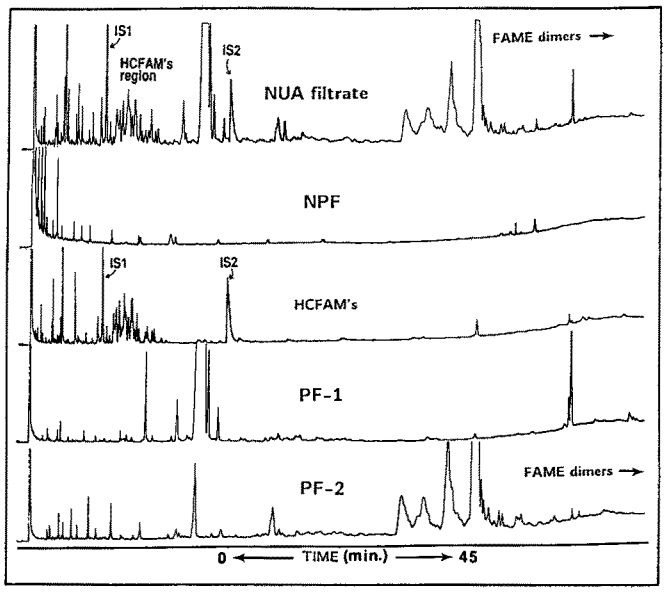

FIG. 3. Chromatographic profiles of several fractions isolated by SPE extraction/clean-up from NUA filtrates [IS1 = 4-cyclohexyl-1-butyl octanoate (CBO); IS2 = phenanthrene (PHE)]. Oil sample: Refined, bleached and deodorized soybean oil.

column (clean-up). Several solvent systems were tried using combinations of n-hexane, ethyl ether, methylene chloride and methanol. However, the most convenient elution system for separation of both HCFAM and the internal standards in a single fraction was that reported in Table 1.

A typical separation of the NUA filtrate into different fractions is illustrated in Figure 3. The isolation of polar fractions (PF-1 and PF-2) was not necessary for the analysis of CFA, but was included here to illustrate the possibility of using this technique to study other groups of important compounds present in the NUA filtrate that are of potential nutritional concern. The PF-1 fraction contains methoxy fatty acids formed either during methylation or by reaction of methanol with hydroxy or epoxy fatty acids present in the fat due to oxidative deterioration. Solvent contaminants, such as phthalates, were also contained in this fraction. Fraction PF-2 contains several dicarboxylic acid esters, fatty acid dimer methyl esters and many nonidentified substances.

Recoveries were evaluated using a relatively pure isomeric mixture of HCFAM and by cross checking recoveries of each internal standard. In all cases recoveries were over 97% for HCFAM. Inconsistent recoveries of phenanthrene (4) were found to be due in part to the adsorption of this substance on the walls of the sample reservoir during NUA filtrate addition on the SPE column. Therefore, the selection of the internal standard merits special consideration in this particular application and, whenever possible, it should be very similar in chemical structure and polarity to the analytes. The substitution of phenanthrene and CBO as the internal standard by either naturally occurring or synthetic cyclic fatty acids is presently under investigation in our laboratory.

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