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One-step SFE-plus-C_{18} selective extraction of low-polarity compounds, with lipid removal, from smoked fish and bovine milk

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Abstract Co-extraction of lipid materials is the major source of interference in determinations of low-polarity compounds in many biological matrixes. "SFE-plus-C_{18}", a recently developed supercritical fluid extraction method employing C_{18} adsorbent in the extraction chamber, can enable selective extraction of low-polarity compounds in lipid-rich biological matrixes without a cleanup step. This study reports the application of the SFE-plus-C_{18} method to the quantification of:

1. polycyclic aromatic hydrocarbons (PAH) in commercially purchased smoked fish; and
2. anti-cancer agents cyclophosphamide (CP) and suberoylanilide hydroxamic acid (SAHA) spiked into homogenized whole bovine milk.

Over the course of SFE-plus-C_{18} extraction, indigenous lipids are preferentially retained on the C_{18} adsorbent. Compared with the conventional method, only 8–15% of the lipids in the smoked fish sample, and only 6–18% of the lipids in the milk sample, were co-extracted by SFE-plus-C_{18}. This reduction in the quantity of background lipids significantly improved chromatographic separations, retarded deterioration of the column, and dramatically improved the ability to quantify PAH present at trace levels in smoked fish by GC–MS. Using the SFE-plus-C_{18} method, ten targeted PAH were detected in the range 9.5–13.5 ng g^{-1} in the smoked fish sample. Compared with these levels, PAH extractions by use of conventional SFE gave values that were lower by 38–86%. Recoveries of CP and SAHA spiked into milk were close to 100% in both SFE-plus-C_{18} and conventional SFE, where the lipid background during the chromatographic elution of CP and SAHA was not so severe.

Keywords SFE lipid removal · Polycyclic aromatic hydrocarbons · GC–MS · Smoked fish · Bovine milk

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Introduction

The problem of detecting trace levels of nonpolar compounds in lipid-rich environments poses a major analytical challenge, because interfering lipids are often difficult to separate completely from all targeted analytes. Moreover, the quantity of lipid molecules present in fat-containing samples is often much higher than that of a targeted trace-level contaminant. Environmentally-persistent nonpolar compounds such as polycyclic aromatic hydrocarbons (PAH) have a strong tendency to accumulate in the lipid material of ingesting organisms. Upon ingestion of contaminated food, bioaccumulation of nonpolar contaminants such as PAH can occur in man. Because of the hazardous nature of PAH, including their suspected role as cancer-causing agents [1, 2, 3], the ability to determine PAH levels accurately in a variety of matrix environments has become an important human health issue. Because the presence of high levels of lipids in samples is often deleterious to the separation and detection systems employed, often causing irreversible damage to chromatography columns, alternative approaches are sought to clean up samples and to improve detection limits.

To determine the quantities of PAH in consumable food products, typically a liquid solvent extraction (e.g. Soxhlet extraction) is performed before a multistep separation procedure. This general approach has been employed for analysis of PAH in meat [4, 5, 6], chicken eggs [7], fish [8], and other seafood [9, 10]. Although different degrees of success have been achieved, the disadvantages of this approach are that it is labor-intensive and it consumes large volumes of solvents (often both costly and toxic, e.g. dichloromethane, benzene). The use of supercritical-fluid extraction (SFE) has received increasing attention as a suitable alternative to classical solvent extraction techniques for environmental sample analyses [11, 12, 13, 14, 15]. Among the clear advantages of employing supercritical CO_2 over liquid solvents for extraction are controllable solvent power, ability to handle smaller sample volumes, reduced analysis times, easy automation, and extremely low toxicity [16, 17, 18].

Despite these advantages of SFE, in studies targeting low-polarity constituents in lipid-containing matrixes, including investigations of polychlorinated biphenyls [19, 20, 21, 22, 23] and pesticides [24, 25, 26], it was inevitable that lipids were co-extracted with the targeted low-polarity analytes. This problem of lipid co-extraction necessitated cleanup steps between extraction and analysis. To remove lipids, obtained SFE extracts were passed through solid-phase adsorbent traps [27, 28] or columns packed with alumina, silica, Florisil, or C_{18} (octadecylsiloxane) beads. An alternative approach to reducing the lipid content was to place basic or neutral alumina in the SF extraction chamber [19, 20]. Bavel and co-workers [21] used basic aluminum oxide in the SF extraction vessel, or activated carbon (i.e. PX-21 or Carbopack C) mixed with octadecylsiloxane as solid-phase trapping materials, packed into an on-line column. This set-up was used to remove lipids from human adipose tissue for the analysis of PCB and pesticides. Barker and coworkers [29, 30] have employed the technique of matrix solid-phase dispersion (MSPD), in which C_{18} is used as a nonpolar adsorbent in a variety of biological matrixes. In these studies, the C_{18} beads can serve to adsorb nonpolar to slightly polar analytes in milk.

In addition to determinations of pollutant contaminants such as the PAH, pharmacokinetics studies of biological fluids are essential to quantifying the levels of drugs and drug metabolites for the development of new drugs. Such studies ensure that the candidate drug concentration is at, or above, the level needed for therapeutic benefit, but not so high that a toxic side effect might result. Suberoylanilide hydroxamic acid (SAHA) is a new-generation anti-cancer drug under clinical trials for treatment of leukemia [31, 32] and human breast cancer [33, 34]. For more than two decades cyclophosphamide (CP) has been incorporated into numerous procedures to treat neoplastic diseases [35, 36, 37]. However, there is evidence that several anti-neoplastic agents are carcinogenic to man, because secondary cancers have been found in cancer patients treated with these drugs, and carcinogenic effects have been observed in non-cancer patients treated with these agents for other purposes [38]. Human exposure to these anti-neoplastic agents during handling (e.g. in a pharmacy or in a hospital) might pose health risks [39, 40, 41, 42]. It is necessary to use a sensitive detection method to measure possible uptake of these drugs by patients and workers.

Several papers report determinations of CP and its metabolites in lipid-rich biological fluids such as human [43, 44] and animal [45, 46] milk. In the current study CP and SAHA were chosen as test analytes of low polarity whose determination in milk might benefit from a reduced lipid background. Conventional determinations involve liquid–liquid extraction, multi-step lipid removal procedures, or evaporation of solvent followed by lipid removal by use of solid phase adsorbent traps or C_{18} cartridges. These approaches are both labor-intensive and time-consuming. A literature review shows that SFE has not undergone development as a sample-preparation tool for analysis of drugs in biological matrixes.

Our group has been engaged in the development and testing of a novel one-step extraction and cleanup method that we call SFE-plus-C_{18} [47, 48, 49]. The method has been shown to enable selective extraction of PAH while largely retaining lipid components from spiked crab tissue [47, 48] and smoked meat [49]; both samples have a high lipid content. This is achieved by placing C_{18} nonpolar adsorbent beads directly inside the SFE chamber during extraction with supercritical CO_2. In the work described in this report, we have extended the realm of application of the SFE-plus-C_{18} method to quantification of PAH in smoked fish, and measurement of the recoveries of the low-polarity antineoplastic agents, CP and SAHA, spiked into whole milk. Quantification was performed by GC–MS, without further cleanup of the SFE-plus-C_{18} extracts.

Experimental

Preparation of PAH, cyclophosphamide (CP), and suberoylanilide hydroxamic acid (SAHA) standards

Analytical grade (purity >99%) solid PAH and analytical grade (purity >98%) solid CP were purchased commercially (Sigma, St Louis, MO, USA). The anti-cancer drug SAHA was synthesized in-house by Dr Blaise LeBlanc. Stock solutions of the individual PAH, CP, and SAHA were prepared and these were subsequently diluted serially with analytical grade dichloromethane (EM Science, Gibbstown, NJ, USA). When not in use, these standards, and mixed standards of these compounds, were stored at –5 °C.

Pretreatment of C_{18} beads

Analytical grade C_{18} nonpolar adsorbent beads (35–75 μm size, 60 Å porosity) were purchased from Alltech Associates (Deerfield, IL, USA). C_{18} beads (approx. 100 g) were washed sequentially with at least two bed volumes of hexane, methanol, and dichloromethane, and then dried and stored at room temperature.

Smoked Fish

Smoked fish packages were purchased from a local New Orleans market. Approximately 100 g of fish was finely ground in a blender, resulting in a smooth solid mixture. The pureed fish was stored at –30 °C before analysis.

Spiked milk and sorbent slurry

Whole milk (carton) was purchased from a local New Orleans store. A mixture of CP and SAHA (100 μg each, obtained from 200 μg mL^{-1} of each analyte in 500 μL solution), each containing an impurity, was spiked into a 0.5-g milk sample in a porcelain mortar and left to stand for 15 min to allow evaporation of the solvent. C_{18} beads (2 g) and pretreated (washed, dried) anhydrous sodium sulfate (2 g) was added and the mixture was stirred. The smooth sample was transferred into an SFE extraction thimble. In another set of experiments, identical procedures were followed with one exception – filter paper (2 g) was used instead of C_{18} beads.

SFE

All SFE was performed with a Hewlett–Packard (Palo Alto, CA, USA) model HP 7680A supercritical-fluid extractor. A mixture of 2.0 g C_{18} beads, or filter paper, and 0.5 g pureed sample was

poured into an extraction thimble in preparation for extraction with supercritical CO_2. SFE was performed at 100 °C and 350 bar utilizing, first, static equilibration for 5 min, then 25 min dynamic extraction at a flow rate of 1.5 mL min^{-1}. Dichloromethane rinsing (3×1.0 mL) of the stainless steel trapping beads (held at 30 °C during extractions) was performed to enable quantitative transfer of the extracted compounds into 1.5-mL glass receiver vials. All three rinses were combined in a 4.0-mL glass vial and then evaporated to dryness to remove dichloromethane with a stream of N_2 gas. The dry product was then re-dissolved in 500 µL dichloromethane and stored at –5 °C before GC–MS analysis. Each smoked fish sample was extracted in triplicate and the three obtained extracts were each injected into the GC–MS instrument three times, so that a total of nine determinations was made for each sample. The same workup procedure was followed for SAHA and CP spiked in milk.

GC–MS

All GC–MS experiments were performed with a Fisons 8000 gas chromatograph coupled to an Autospec tri-sector magnetic mass spectrometer equipped with an Opus data system (Micromass, Manchester, UK). A 30 m×0.32 mm i.d. fused-silica capillary column coated with a 0.25-µm film of BPX-5 5% phenylsiloxane (SGE, Austin, TX, USA) was used throughout. This GC column temperature was held isothermal for 1 min at 70 °C then programmed at 8 ° min^{-1} to 280 °C and lastly held isothermal at 280°C for 15 min. The carrier gas was helium at a flow rate of ~3–4 mL min^{-1}. The injection port was heated to 280 °C and splitless injections were performed. The electron impact (EI) mode of mass spectrometer operation was employed (electron energy 70 eV, source temperature 200 °C), with a magnet scan from m/z 450 to 50 in 1.6 s. Before data acquisition, the instrument was calibrated with perfluorokerosene with a minimum resolution of 1000 (m/Δm) at m/z 219. To quantify unknown PAH in the smoked fish sample, and drugs in spiked milk, three-point GC–MS standard calibration curves were generated each day for all analytes. The instrumental limit of detection for the range of PAH was 3.6–8.0 pg (1.0-µL GC–MS injections); for CP and SAHA it was 1.0 and 3.0 ng, respectively, also for 1.0-µL GC–MS injections. All MS data acquired on the Opus data system were transferred to a Masslynx 3.0 (Micromass) data system where quantitation was performed.

Results and discussion

The goal of this study was to adapt and employ a single-step sample-treatment procedure using the SFE-plus-C_{18} method (no additional cleanup step) for GC–MS analysis and quantification of low-polarity compounds in commercially sold smoked fish, and in a spiked whole-milk sample. Determinations of PAH that are potential carcinogens in smoked fish, or recoveries of the anti-cancer drugs CP and SAHA, might be hampered by interference from co-extraction of nonpolar lipids. Such indigenous lipid materials not only suppress analyte signals but can also degrade the GC column irreversibly. In the procedure employed, before SFE, C_{18} nonpolar adsorbent beads were added directly to the finely ground smoked fish or the homogenized spiked milk sample slurry that was placed inside the extraction chamber.

PAH in smoked fish

To prepare the smoked fish for SFE-plus-C_{18} extraction, three replicate samples of finely-ground smoked fish were mixed with C_{18} adsorbent beads by use of mortar and pestle. These mixtures were then extracted by SFE under previously optimized conditions [48]. Extracts were then injected directly into the GC–MS instrument with no further treatment or clean-up. A control sample was also run under identical conditions except that the C_{18} adsorbent was

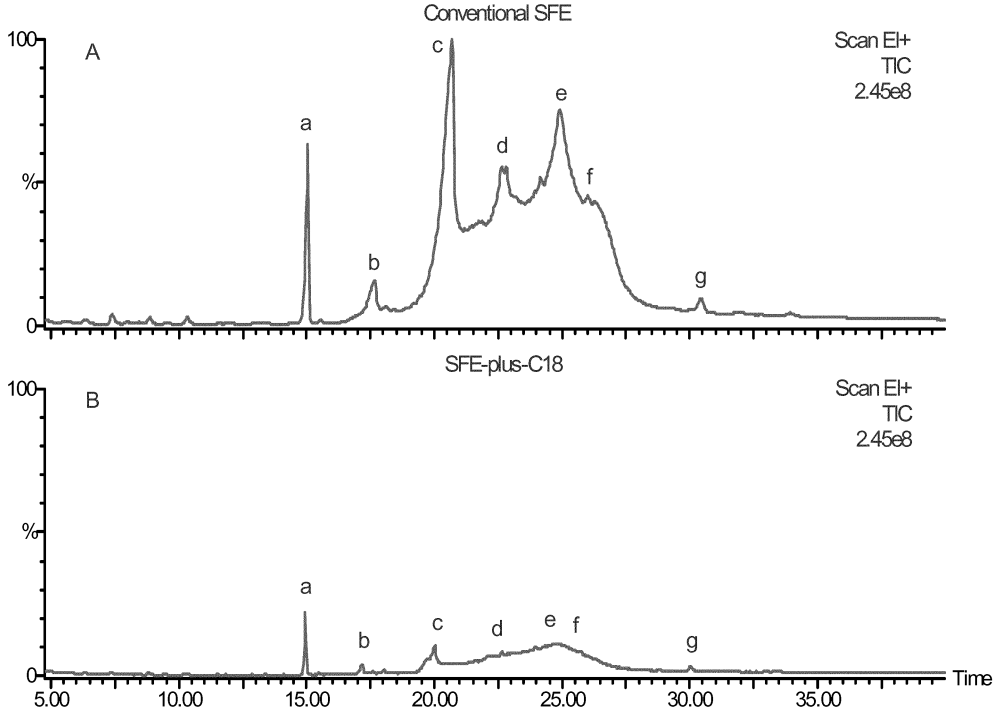

Fig. 1A, B GC–MS total ion chromatograms of extracts from pureed smoked fish. **A** Conventional SFE (using filter paper as an inert sorbent). **B** SFE-plus-C_{18}. Peaks labeled with letters correspond to various indigenous lipids: "*a*" (m/z 268), "*b*" (m/z 228), "*c*" (m/z 256), "*d*" (m/z 320), "*e*" (m/z 320), "*f*" (m/z 320), and "*g*" (m/z 410). Note that the full-scale intensities of the upper and lower chromatograms are identically scaled to enable direct comparison of results

Table 1 Relative signal intensities of lipids in smoked fish by SFE methods

Compound	Signal intensity of lipids (mean±SD)	
	Conventional SFE[a]	SFE-plus-C_{18}[b]
Lipid "a" (m/z 268)	100.0±12.7	14.7±4.2
Lipid "b" (m/z 228)	100.0±13.5	10.2±3.3
Lipid "c" (m/z 256)	100.0±14.2	8.0±2.8
Lipid "d" (m/z 320)	100.0±12.6	11.2±4.5
Lipid "e" (m/z 320)	100.0±11.3	12.5±4.0
Lipid "f" (m/z 320)	100.0±14.2	8.4±3.5
Lipid "g" (m/z 410)	100.0± 9.7	14.3±4.5

[a]Average values are assigned as 100.0. Standard deviation (total procedure, n=9) gives an indication of run-to-run variability
[b]Values are relative to assignment of 100.0 by conventional SFE method for the same sample

replaced by inert filter paper. The molecular structures and the EI mass spectra of these PAH standards have been shown previously [49].

Comparison of the total ion chromatograms (TIC) of the extracts of the smoked fish sample obtained by use of conventional SFE method and the SFE-plus-C_{18} method is shown in Fig. 1. Some indigenous lipids have been identified by use of mass spectral library searches of a compiled database (NIST Libraries and Structure, version VI.0); these peaks have been labeled with letters. Lipids "a" (m/z 268), "b" (m/z 228), and "c" (m/z 256) have been identified, respectively, as a saturated, branched hydrocarbon with a molecular formula of $C_{19}H_{40}$, *n*-tetradecanoic (myristic) acid, and *n*-hexadecanoic (palmitic) acid. PAH cannot easily be seen in the TIC because, compared with the lipids, they are present at very minor levels. On inspection of Figs. 1A and 1B that use identical Y-axis scales to indicate mass spectral response, one can deduce that these same lipids were extracted to a much lesser extent by the SFE-plus-C_{18} method than by the conventional SFE method.

Table 1 shows the signal intensities obtained for indigenous lipids from the smoked fish sample by SFE-plus-C_{18} and by conventional SFE. For the extract obtained by SFE-plus-C_{18}, the signals of lipids "a", "b", and "c" are reduced to 15, 10 and 8%, respectively, of the corresponding signals observed by use of conventional SFE. Peaks "d", "e", and "f" correspond to unresolved mixtures of compounds, presumably lipids, that give ions at m/z 264, 292, and 320. Although ions of these same three m/z ratios elute continuously in the 22 to 27 min range, the relative abundances of the three ions change over time. Using m/z 320 for comparative purposes, in Fig. 1B the areas of peaks "d", "e", and "f" are reduced to 11, 13, and 8%, respectively, compared with Fig. 1A. Similarly, lipid "g" (m/z 410) is reduced to 14%. The data in Table 1 indicate that the lipids were co-extracted to a much lesser extent in the SFE-plus-C_{18} method, only 8–15% compared with conventional SFE. Thus, SFE-plus-C_{18} is clearly quite effective at removing lipids present in the smoked fish extract, because 85–92% of the lipids were retained by addition of C_{18} beads to the extraction chamber.

The summed selected ion chromatograms of m/z 178, 202, 228, and 252 are shown in Fig. 2. The choice of these four m/z values enables visualization of all ten targeted PAH compounds because several are isomeric. The extracts obtained from the conventional SFE method are shown in Fig. 2A, whereas those obtained by use of the SFE-plus-C_{18} method appear in Fig. 2B. It should be noted that each of the ion current scales shown on the Y-axes in

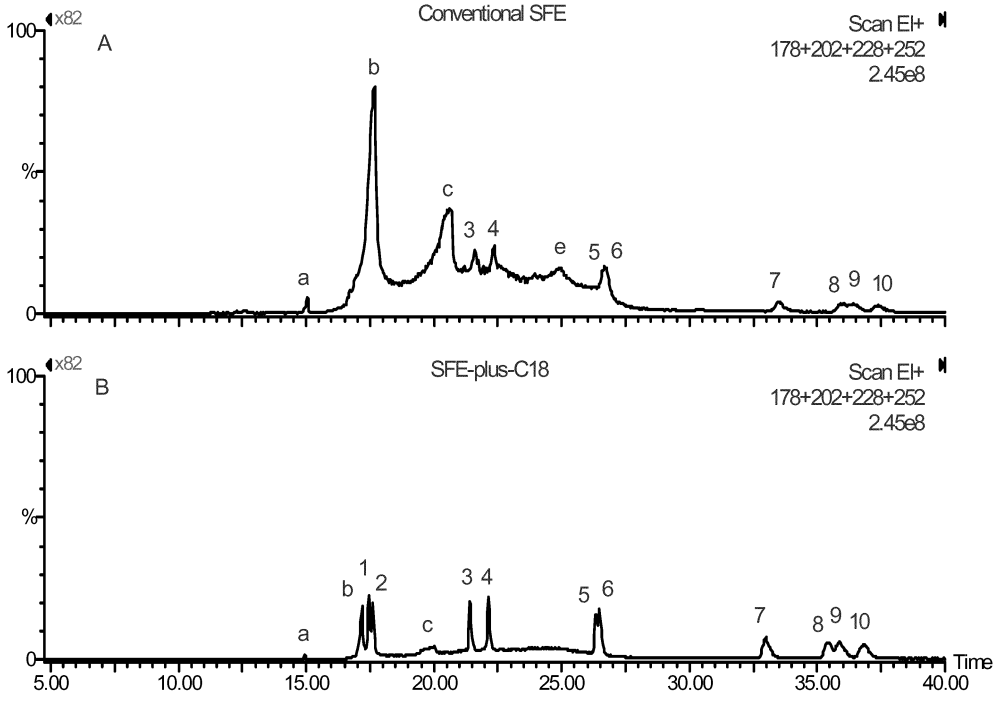

Fig. 2A, B Selected ion chromatograms representing the sum of ion currents from m/z 178, 202, 228, and 252 obtained from extracts of pureed smoked fish. **A** Conventional SFE. **B** SFE-plus-C_{18}. Peaks labeled with numbers correspond to PAH: *1*, phenanthrene; *2*, anthracene; *3*, fluoranthene; *4*, pyrene; *5*, benzo[*a*]anthracene; *6*, chrysene; *7*, benzo[*b*]fluoranthene; *8*, benzo[*e*]pyrene; *9*, benzo[*a*]pyrene; *10*, perylene. Peaks labeled with letters correspond to lipids (labeling is consistent with that in Fig. 1). The full-scale intensities for the upper and lower traces are identical, but each has been magnified by a factor of 82 as compared with Fig. 1

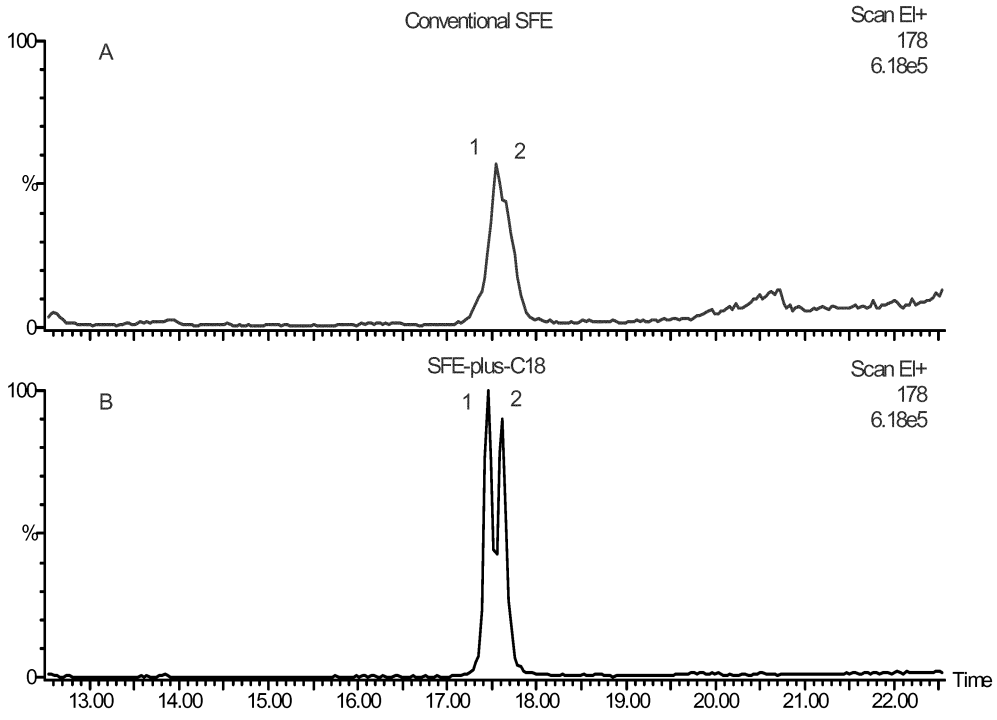

Fig. 3A, B Selected ion chromatogram showing only m/z 178 from extracts of pureed smoked fish. **A** Conventional SFE. **B** SFE-plus-C_{18}. The improved separation in the SFE-plus-C_{18} trace is attributed to the reduced lipid background

Fig. 2 has been magnified by a factor of 82 compared with those in Fig. 1. The selected ion chromatogram corresponding to the conventional SFE extraction (Fig. 2A) reveals overwhelmingly high levels of lipid content (see especially peaks "b", "c", and "e") compared to those of the PAH. This is despite the fact that in reconstructing this chromatogram, only four ions were selected and summed as the minimum number required to encompass the molecular ions of all ten PAH. In contrast, use of SFE-plus-C_{18} (Fig. 2B) results in a substantial reduction in the quantities of lipids present, thereby enabling observation of the PAH peaks, that now dominate.

In Fig. 2A the predominance of lipid "b" that crests between 17 and 18 min completely masks the peaks corresponding to PAH "1" (phenanthrene, m/z 178) and "2" (anthracene, m/z 178). As previously stated, peak "b" originates from myristic acid which gives a molecular ion at m/z 228, i.e. the same as benz[*a*]anthracene and chrysene. To quantify the phenanthrene and anthracene in the conventional SF extract by use of the same set of data, it was necessary to obtain the single ion chromatogram for m/z 178, shown in Fig. 3A. From this figure it is apparent that the presence of the co-eluting myristic acid resulted in degradation of the chromatographic resolution of peaks "1" and "2" (phenanthrene and anthracene, respectively). Although they are still not "baseline resolved", there is, nonetheless, a clear, marked improvement in the separation when "SFE-plus-C_{18}" is used (Fig. 3B). This improvement is attributed directly to reduction of the amount of co-eluting lipid material present.

Another manifestation of lipid interference, in Fig. 2A, is apparent from the fluoranthene and pyrene peaks ("3" and "4", respectively, each at m/z 202); these are significantly hidden by the lipid background corresponding to peak "d" that is centered around 22 to 23 min (Fig. 1). The analogous peaks are much more visible (and are separated at higher resolution) in Fig. 2B, corresponding to the SFE-plus-C_{18} method. Examination of the peaks of isomeric benz[*a*]anthracene and chrysene ("5" and "6" respectively, each at m/z 228) reveals that interference from co-eluting lipid "f" that crests between 26 and 27 min (Fig. 1) heavily degrades resolution and reduces sensitivity in the conventional method. In contrast, after use of SFE-plus-C_{18} (Fig. 2B) the peaks are better separated and appear against a dramatically reduced background. Finally, the peaks corresponding to the last four isomeric PAH (peaks "7", "8", "9", and "10", all of m/z 252) are less sharp and less resolved in Fig. 2A, because of the overall higher lipid background after use of the conventional method, compared with that obtained after use of the SFE-plus-C_{18} method (Fig. 2B).

Table 2 displays quantitative results from determinations of PAH by use of the two SFE methods. By use of SFE-plus-C_{18}, the ten targeted PAH were detected in the smoked fish sample in the range 9.5–13.5 ng g^{-1}, with pyrene present at the highest concentration. The same ten PAH were detectable by use of conventional SF extraction, but the level of each was reduced, yielding a range of values from 4.8 to 8.9 ng g^{-1}. Thus conventional SFE determinations gave values only 38–86% of those obtained by use of SFE-plus-C_{18}. The improvement brought about by use of SFE-plus-C_{18} is most evident in the region of the chromatogram where the lipid background was most severe (corresponding specifically to lipids "c", "d", "e" and "f"). In this range, the SFE-plus-C_{18} determinations of fluoranthene and pyrene (peaks "3" and "4", respectively) gave values that were more than double (265% and 245%, respectively) those obtained by conventional SFE. After

Table 2 Determination of PAH in smoked fish by SFE methods

Compound (peak number, m/z value, instrumental limit of detection in pg)	Amount of PAH (ng g^{-1})		Amount (%) PAH measured by SFE-plus-C_{18} relative to conventional SFE (b/a×100)
	Conventional SFE (mean±SD); a	SFE-plus-C_{18} (mean±SD); b	
Phenanthrene (1, m/z 178, 4.6)	7.9±1.2	11.9±1.4	151
Anthracene (2, m/z 178, 4.9)	7.1±1.3	11.4±1.3	161
Fluoranthene (3, m/z 202, 3.8)	4.8±1.3	12.7±1.0	265
Pyrene (4, m/z 202, 3.6)	5.5±1.4	13.5±1.2	245
Benzo[*a*]anthracene (5, m/z 228, 5.6)	6.6±1.5	12.0±1.3	182
Chrysene (6, m/z 228, 5.1)	8.3±1.4	12.8±1.2	154
Benzo[*b*]fluoranthene (7, m/z 252, 7.3)	8.9±1.3	10.4±1.0	117
Benzo[*e*]pyrene (8, m/z 252, 7.0)	8.4±1.2	9.9±1.2	118
Benzo[*a*]pyrene (9, m/z 252, 7.1)	8.4±1.3	9.9±1.1	118
Perylene (10, m/z 252, 8.0)	8.1±1.0	9.5±0.8	117

SD=Standard deviation for total procedure, n=9

30 min elution time, the lipid background was substantially lower, and recoveries of benzo[*b*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene and perylene (peaks "7", "8", "9", and "10", respectively, all appearing at >30 min) were increased significantly (117, 118, 118 and 117% respectively, compared with conventional SFE). These results attest to the fact that the lipid background, that masks the presence of, and suppresses the detection of, PAH in the conventional method, has been substantially reduced by use of SFE-plus-C_{18}.

Anti-cancer drugs spiked in milk

To test the effectiveness of the SFE-plus-C_{18} method for extraction of low-polarity anti-cancer drugs from milk, cyclophosphamide (CP, m/z 260), and suberoylanilide hydroxamic acid (SAHA, m/z 264) were spiked into whole (bovine) milk. The molecular structures and the EI mass spectra of these drug standards are given in Fig. 4. Comparison of the total-ion chromatograms (TIC) of extracts of the spiked milk obtained after use of conventional SFE and the SFE-plus-C_{18} method appears in Fig. 5. Peaks labeled with numbers arise from the anti-cancer drugs, including their impurities, spiked in the milk sample. The cyclophosphamide (CP, $M^{+\cdot}$= m/z 260) eluted first (peak 1, 19.2 min) and the suberoylanilide hydroxamic acid (SAHA, $M^{+\cdot}$= m/z 264) eluted later (peak 3, 21.8 min). Peaks "2" (m/z 310) and "4" (m/z 410) correspond to the major impurities in CP and SAHA, respectively. Peaks labeled with letters are indigenous lipids in the whole milk sample. Of eleven indigenous lipids, four were identified by means of mass spectral library searches of the compiled database (NIST Libraries and Structure, Version

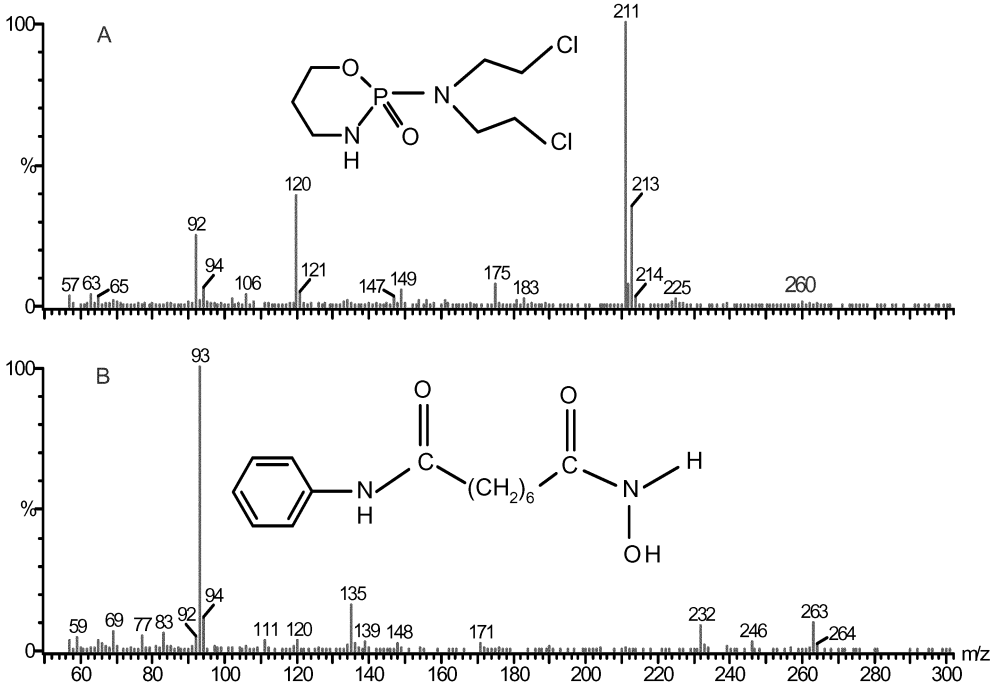

Fig. 4 The molecular structures and electron impact mass spectra of **A** cyclophosphamide (CP, m/z 260) and **B** suberoylanilide hydroxamic acid (SAHA, m/z 264)

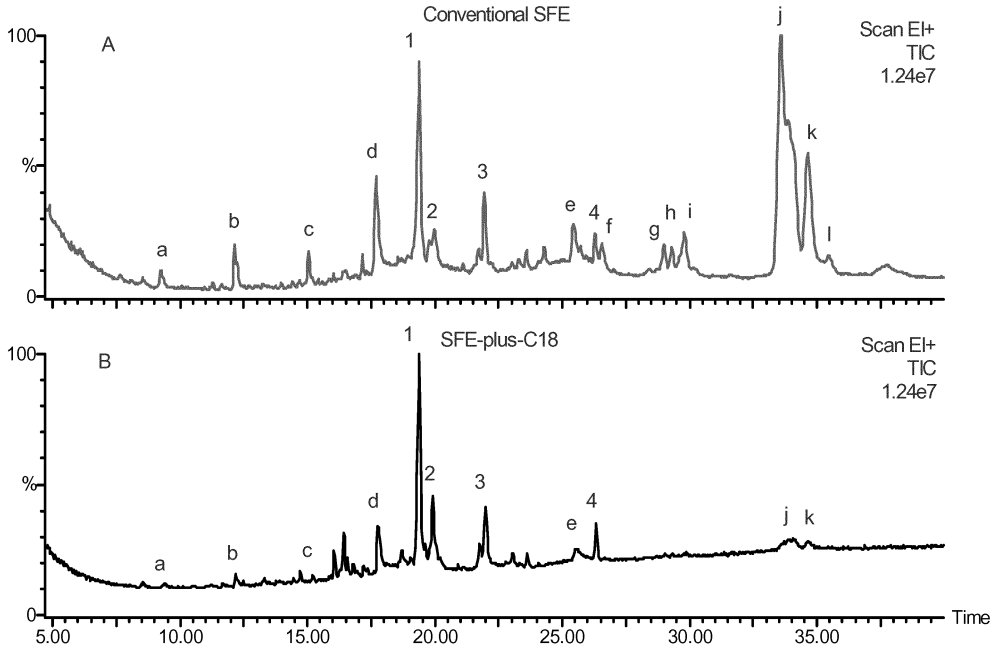

Fig. 5A, B GC–MS total ion chromatograms of extract from whole milk sample spiked with anti-cancer drugs. **A** Conventional SFE (using filter paper as an inert sorbent). **B** SFE-plus-C_{18}. Peaks labeled with letters correspond to indigenous lipids: "*a*" (m/z 172), "*b*" (m/z 222), "*c*" (m/z 228), "*d*" (m/z 256), "*e*" (m/z 248), "*f*" (m/z 382), "*g*" (m/z 382), "*h*" (m/z 382), "*i*" (m/z 382), "*j*" (m/z 386), "*k*" (m/z 410) and "*l*" (m/z 410). Peaks labeled with numbers represent anti-cancer drugs and their impurities: "*1*" (m/z 260, cyclophosphamide, CP), "*2*" (m/z 310, impurity from CP), "*3*" (m/z 264, suberoylanilide hydroxamic acid, SAHA) and "*4*" (m/z 410, impurity from SAHA)

Table 3 Relative signal intensities of lipids in milk by use of SFE methods

Compound	Signal intensity of lipids	
	Conventional SFE (mean±SD)[a]	SFE-plus-C_{18} (mean±SD)[b]
Lipid "a" (m/z 172)	100.0±12.5	12.0±3.1
Lipid "b" (m/z 222)	100.0±11.8	14.5±3.9
Lipid "c" (m/z 228)	100.0±12.2	10.2±3.3
Lipid "d" (m/z 256)	100.0±13.1	18.3±4.7
Lipid "e" (m/z 248)	100.0±12.7	15.5±4.2
Lipid "f" (m/z 382)	100.0±12.7	Not Detected
Lipid "g" (m/z 382)	100.0±10.3	Not Detected
Lipid "h" (m/z 382)	100.0±11.6	Not Detected
Lipid "i" (m/z 382)	100.0±11.5	Not Detected
Lipid "j" (m/z 386)	100.0±10.4	7.4±2.4
Lipid "k" (m/z 410)	100.0±12.4	6.0±2.5
Lipid "l" (m/z 410)	100.0±12.0	Not Detected

[a]Average values are assigned as 100.0. Standard deviation (total procedure, n=9) gives an indication of run-to-run variability
[b]Values are relative to assignment of 100.0 by conventional SFE method for same sample

VI.0). Lipids "a' (m/z 172), "c" (m/z 228), "d" (m/z 256), and "j" (m/z 386) have been identified as decanoic acid, *n*-tetradecanoic (myristic) acid, *n*-hexadecanoic (palmitic) acid, and cholesterol, respectively; peak "b" (m/z 222) is diethyl phthalate. The highest responses were obtained for palmitic acid, cholesterol, and an unidentified compound "k" (Fig. 5A). Comparison with Fig. 5B using an identical absolute signal-intensity scale (Y-axis), indicates that the lipids were extracted to a much lesser extent in the SFE-plus-C_{18} method than in the conventional SFE method.

Table 3 shows a comparison of results indicating the relative signal intensities for indigenous lipids from the spiked milk sample recovered by use of the SFE-plus-C_{18} and conventional SFE methods. For the SFE-plus-C_{18} extract, the signals of lipid "a" (*n*-decanoic acid), "b" (diethyl phthalate), "c" (*n*-tetradecanoic acid), and "d" (*n*-hexadecanoic acid) are reduced to 12, 15, 10, and 18%, respectively, compared with the corresponding signals observed using conventional SFE. Lipid "e" (m/z 248) was reduced to 16% in the SFE-plus-C_{18} method compared with the conventional method. Lipids "f", "g", "h" and "i" are a group of four isomers of m/z 382 that did not appear above the baseline in Fig. 5B, indicating that each of these four lipids was almost completely retained by the C_{18} beads in the SFE-plus-C_{18} experiment. Cholesterol ("j") and lipid "k" were dramatically reduced, to 7 and 6%, respectively, and lipid "l" was virtually eliminated by use of the SFE-plus-C_{18} method. The lipid recovery data in Table 3 indicate that the lipids were co-extracted to a much lesser extent in the SFE-plus-C_{18} method, only 6–18% compared with the conventional method. In other words, addition of C_{18} adsorbent beads resulted in retention of 82–94% of the available lipid during SFE.

Table 4 Recoveries of anti-cancer drugs spiked into milk by use of SFE methods

Compound	Recovery (%) of anti-cancer drugs	
	Conventional SFE (mean±SD)	SFE-plus-C_{18} (mean±SD)
Cyclophosphamide "1" (CP, m/z 260)	99.5± 5.3	97.7± 5.9
Impurity "2" (m/z 310)	99.1±11.7	99.4±11.9
Suberoylanilide hydroxamic acid "3" (SAHA, m/z 264)	99.7± 5.0	97.2± 5.4
Impurity "4" (m/z 410)	99.3±14.9	98.6±12.8

SD=Standard deviation for total procedure, n=9

Table 4 shows the quantitative recovery results obtained for the anti-cancer drugs (including their impurities) spiked in milk samples when analyzed by both SFE methods. The recoveries of CP and SAHA were very close to 100% in the conventional SFE method whereas their recoveries in the SFE-plus-C_{18} method might be slightly less than 100%, possibly because of interaction of the drugs with the non-polar C_{18}. The error associated with the measurements indicates, however, that the differences between mean recovery values obtained by use of the two methods is not significant. Peak "2" (m/z 310) represents an impurity from the CP drug that was recovered quantitatively (~99%) by both methods. Peak "4" (m/z 410) corresponds to an impurity of SAHA which was, likewise, recovered virtually quantitatively (~99%) by both SFE methods. In conventional SFE, despite the higher lipid background, recovery of CP and SAHA did not decrease. It appears that, quite fortuitously, the co-extracted lipids did not co-elute with the drug compounds during gas chromatography. Because separation of lipids was adequate in these instances, suppression of the signals from the analyte molecules (CP, SAHA, and impurities) was not severe. This finding is similar to an earlier crab tissue analysis in which spiked lipid (stearic acid) and cholesterol did not impede PAH detection because they eluted from the GC column sufficiently apart from the analytes [48]. Even though recoveries were not significantly improved by use of SFE-plus-C_{18}, in these instances, the useful life of the GC column was surely prolonged as a result of lipid removal.

Conclusions

In determinations of PAH in smoked fish, or of CP and SAHA spiked in milk, addition of octadecylsiloxane beads (C_{18} adsorbent) to the SFE chamber (i.e. the SFE-plus-C_{18} method) served to preferentially trap interfering lipids while enabling extraction of PAH or CP and SAHA from biological matrixes. The method improved the efficiency of extraction for ten PAH in smoked fish by largely preventing the co-extraction of interfering indigenous lipids that were preferentially retained on the C_{18} adsorbent. This significantly reduced lipid interference in subsequent GC–MS analyses, and improvement in PAH signals was most prominent where co-elution of lipids was most severe. The fact that 85–92% of the fish lipids remained adsorbed to the C_{18} during SFE-plus-C_{18} extraction substantially improved the ability to detect low levels of PAH. The ten targeted PAH were found, by use of the SFE-plus-C_{18} method, to be present in the range 9.5–13.5 ng g^{-1} in the smoked fish product. The SFE-plus-C_{18} method was also successful at recovering nearly 100% of CP and SAHA drugs while simultaneously reducing the co-extraction of indigenous lipids, i.e. 82–94% of the lipids present were adsorbed by the C_{18} beads during the SFE-plus-C_{18} procedure. Although the extracts obtained by use of the conventional SFE method contained a high lipid background, these indigenous lipid materials did not co-elute with the targeted drugs; for these particular anti-cancer compounds, therefore, lipid removal by use of the SFE-plus-C_{18} method was less critical to achieving high recoveries than it was for PAH in fish. Nonetheless, a lower lipid content in extracts serves to extend the life of the GC column used.

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