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Article

Determination of Polycyclic Aromatic Hydrocarbons in Fresh Milk by Hollow Fiber Liquid-Phase Microextraction–Gas Chromatography Mass Spectrometry

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In this work, a two-phase hollow fiber liquid-phase microextraction (HF-LPME) method combined with gas chromatography–mass spectrometry (GC–MS) is developed to provide a rapid, selective and sensitive analytical method to determine polycyclic aromatic hydrocarbons (PAHs) in fresh milk. The standard addition method is used to construct calibration curves and to determine the residue levels for the target analytes, fluorene, phenanthrene, fluoranthene, pyrene and benzo[a]pyrene, thus eliminating sample pre-treatment steps such as pH adjustment. The HF-LPME method shows dynamic linearity from 5 to 500 μg/L for all target analytes with R^2 ranging from 0.9978 to 0.9999. Under optimized conditions, the established detection limits range from 0.07 to 1.4 μg/L based on a signal-to-noise ratio of 3:1. Average relative recoveries for the determination of PAHs studied at 100 μg/L spiking levels are in the range of 85 to 110%. The relative recoveries are slightly higher than those obtained by conventional solvent extraction, which requires saponification steps for fluorene and phenanthrene, which are more volatile and heat sensitive. The HF-LPME method proves to be simple and rapid, and requires minimal amounts of organic solvent that supports green analysis.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds produced as the products of the incomplete combustion of carbon-containing material, such as coal, oil or municipal waste. Interest and attention in the analysis of PAH residues has primarily focused on the 16 PAHs that have been designated by the United States Environmental Protection Agency (USEPA) as mutagenic or carcinogenic pollutants. The first PAH identified as cancer-causing compound was benzo[a]pyrene (1). Therefore, the concentration of benzo[a]pyrene has been used as indicator of total contamination by PAHs and the maximum residue level was set at 1.0 μg/kg of baby foods by European Union (EU) (2).

Fresh milk is one substance possibly containing PAHs because milk contains fat and PAHs are known to be lipophilic compounds. The very low concentration levels set by regulatory bodies and the complex nature of the milk matrix potentially containing PAHs have raised the need to develop simple, sensitive, selective and accurate analytical methods for their routine analysis, to ensure that fresh milk offered to the market is safe to be consumed. For this, both liquid–liquid extraction (LLE) before cleanup with solid-phase extraction or column chromatography and saponification before LLE have been extensively practiced in the determination of PAH residues in fatty food (3–5). Since 2007, solventless headspace and direct immersed solid-phase microextractions (SPME) have been some of the most popular methods to extract PAHs from milk (6–8). Lately, liquid-phase microextraction (LPME) has emerged as a green extraction method because it consumes less organic solvent than LLE. Only few articles have been published related to LPME extracting herbicides, drugs and other food contaminants from milk samples (9–12). In these articles, pH adjustment or ultrasonication of sample solution have been thoroughly discussed as a sample pretreatment to suppress the analyte–milk interaction (9–12). None of the articles reported the comparison of these sample pretreatments with conventional solvent extraction, which requires saponification.

LPME systems that have been reported include single drop microextraction (SDME), hollow fiber protected (HF) LPME and cone-shaped LPME. Among these three LPMEs, SDME is known to have the risk of the acceptor phase dissolving into the donor phase during extraction. Cone-shaped LPME, reported by Sanagi *et al.* (13), possesses more simplified procedures than HF-LPME because it requires less apparatus. However, the cone-shaped LPME requires a few hundreds of microliters of acceptor solvent, which might be not suitable in extraction of trace PAH from complicated milk matrix.

LPME has been extensively studied to extract PAHs from soil (14–15), aqueous samples (16–17) and others (18). However, the extraction of hydrophobic PAHs from food is still not available due to the complex sample matrices. With LPME, sample preparation problems have been overcome, dealing with soil samples such as extensive filtering, sample pretreatment and high sample throughput (15). LPME offers several advantages: it is simple, inexpensive and has high enrichment, which makes this technique a replacement for LLE and solid-phase extraction, which require multi-stage operation and large consumption of organic solvents.

Two-phase LPME of PAHs from fresh milk was studied in this project and compared to saponification before LLE in terms of efficiency, to determine PAH levels in milk that can be used by consumers and manufacturers for monitoring the PAH levels during purchasing and food processing.

Experimental

Chemicals and reagents

Fluorene (FLU), phenanthrene (PHE), fluoranthene (FLA), pyrene (PYR) and benzo[a]pyrene (BaP) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (200 mg/L of each analyte) were prepared by dissolving in acetonitrile (PHE, PYR and BaP) and methanol (FLU and FLA). Working standard solutions were prepared weekly using methanol from stock standard solutions. All standard solutions were stored in the dark at 4°C when not in use. 1-Octanol, methanol (HPLC grade), acetonitrile (HPLC grade), acetone (HPLC grade), sodium chloride, sodium hydroxide, dichloromethane and *n*-hexane were purchased from Merck (Darmstadt, Germany). Fresh milk samples with fat contents ranging from 1 to 4% were purchased from local supermarkets.

Materials

The Accurel Q3/2 polypropylene hollow fiber membrane (600 μm inner diameter, 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana GmbH (Wuppertal, Germany). The hollow fiber was cut into 1.8 cm of length and discarded after a single use. The hollow fiber was sonicated in acetone for 5 min to remove impurities and then air-dried before use. A 10-μL SGE microsyringe with a blunt needle tip was used to introduce the acceptor phase and support the hollow fiber during extraction. The syringe was then clamped to a retort stand. A hot plate stirrer (Favorit, Malaysia) and a stirring bar (12 × 4 mm) were used to stir the milk during extraction.

Chromatographic conditions

All analyses were performed using an HP 6890 Series Plus gas chromatograph (Agilent Technologies, Milan, Italy) equipped with an MSD 5973 mass spectrometer (Agilent Technologies). Helium was used as carrier gas at a constant flow rate of 1 mL/min. Splitless injections were performed at 250°C and the chromatographic separation of PAHs was performed on an HP5 MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent. The oven temperatures began at 150°C for 3 min, and then increased to 250°C at 10°C/min and held for 15 min. The transfer line and ion source temperature were fixed at 250°C, respectively. Data were collected and quantified in the selected ion monitoring (SIM) mode by using the mass values corresponding to the molecular ions of the PAHs. Electron impact mass spectra were recorded at 70 eV and the detector voltage was set at 1.0 kV. Chromatographic data were processed using MSD Chemstation software.

Liquid-phase microextraction

Milk samples were split into six portions of 10 mL for each sample. Five portions were spiked with PAHs to give final levels ranging from 5 to 500 μg/L, whereas the remaining portion was treated as blank. Three replicates were prepared for each level. The spiked milk was stirred at room temperature for 1 h to allow for complete homogenation. A portion of milk (10 mL) was pipetted into a 12-mL sample vial prefilled with 0.25 g of sodium chloride and a magnetic stirrer was placed into the milk. The syringe needle was inserted through a piece of parafilm and then into the hollow fiber segment. Then, 5 μL of 1-octanol was withdrawn into the syringe and the whole assembly was dipped into 1-octanol for 20 s to impregnate the pore of the hollow fiber. The syringe needle-hollow fiber assembly was immersed into the milk and the sample vial was sealed with parafilm (Figure 1). The sample was agitated at 750 rpm. After the extraction (30 min), the magnetic stirrer was switched off and the acceptor phase was quickly withdrawn into the syringe and injected into the GC system. The hollow fiber segment was discarded after analysis.

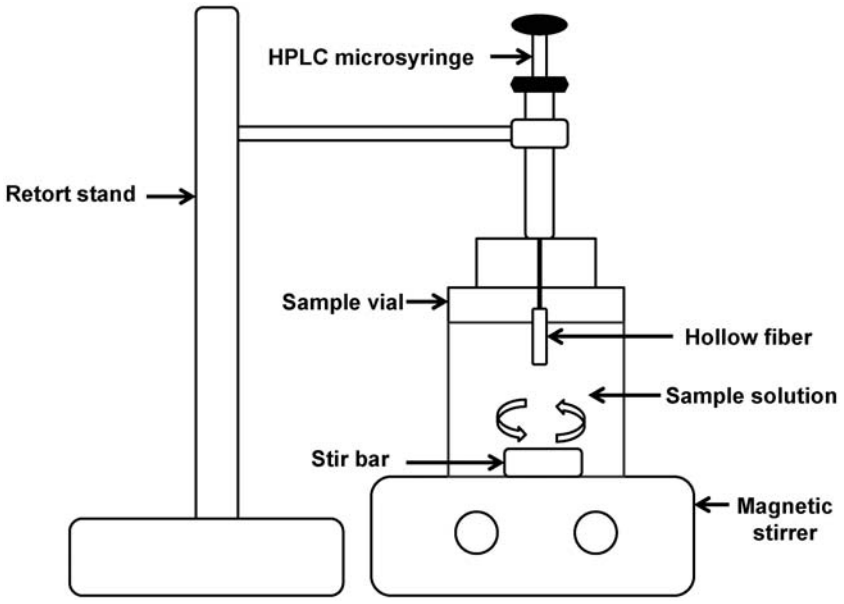

Figure 1. Schematic diagram of HF-LPME.

Saponification prior to solvent extraction

The saponification method was adopted from Kishikawa *et al.* (4) and Chung *et al.* (5). A milk sample (2 mL) was pipetted into a 20-mL sample vial and 4 mL of 0.4M NaOH (prepared in a mixture of methanol and water at ratio 9:1) was added. The sample vial was then incubated at 60°C for 30 min and shaken at 5 min intervals for complete saponification. Then, the sample vial was cooled to room temperature and the content was transferred to a centrifuge tube prior to solvent extraction. Organic solvent (mixture of dichloromethane and n-hexane at ratio 1:1) (2 mL) was added to the tube and the tube was vortexed for 1 min. The sample solution was then centrifuged at 5,000 rpm for 5 min and the organic layer was withdrawn into a 4-mL sample vial. The extraction was repeated with another 2 mL of organic solvent and the purified organic layer was collected into a sample vial. The collected organic layer was then evaporated to dryness and reconstituted into 2 mL of methanol before GC analysis.

Validation of analytical method

The analytical method was assessed for linearity, recovery, repeatability, limit of detection (LOD) and limit of quantification (LOQ) before sample analysis. The LOD was calculated based on a signal-to-noise ratio of 3:1. The LOQ was defined as the analyte concentration giving a signal equal to the blank signal plus 10 times the standard deviation of the blank signal (19).

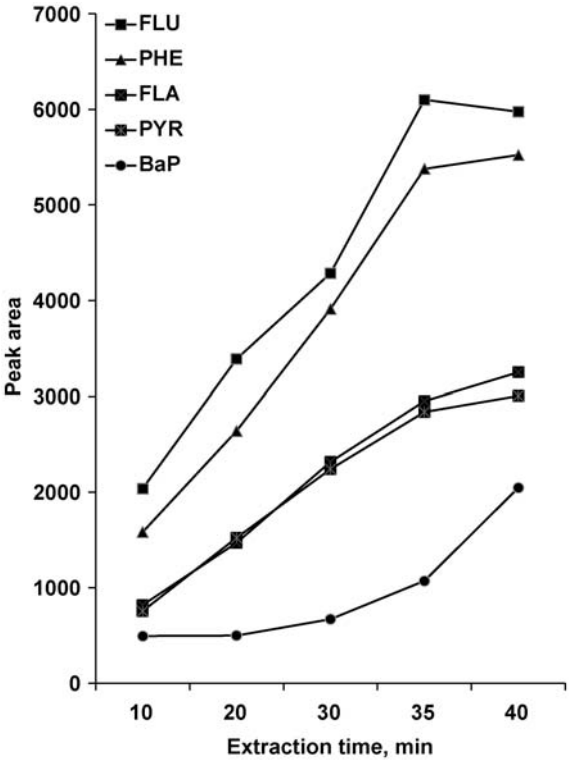

Figure 2. Effect of extraction time on LPME of PAHs in milk.

Results and Discussion

Three parameters that influenced the LPME efficiency were optimized: extraction time, stirring speed and salting-out effect. The optimization was carried out by using fresh milk samples spiked with each PAH to give a concentration of 50 μg/L.

The stirring speeds were studied from 300 to 900 rpm. It was found that higher peak area was always obtained with higher speed. The adsorption of PAHs into the acceptor phase was accelerated with higher speed. However, the volume of the acceptor phase tended to dissipate into the donor phase during extraction at 900 rpm, which resulted in poor repeatability. Therefore, stirring at 750 rpm was selected for further analysis.

The addition of salt or sodium chloride during LPME was intended to reduce the solubility of analytes and enhance their partitioning into the acceptor phase. The addition of salt ranging from 2.5 to 20.0% was studied and compared to LPME without addition of salt. Results showed that the addition of 2.5% salt was better at promoting the partitioning of PAHs into the acceptor phase than the sample without salt addition. Salt addition of more than 2.5% was found to increase the viscosity of the sample solution, and hence, to cause poor kinetics of the partitioning and diffusion rate of analytes from donor to acceptor phases (20). Therefore, the addition of 2.5% or 0.25 g of salt into 10 mL of milk sample was applied to the rest of the experiments.

The effect of extraction time on LPME was investigated in the range of 10 to 40 min. Increases in peak responses with extraction time were observed for all analytes, except for FLU, which showed a decrease in peak response at 40 min (Figure 2). A longer extraction time enhanced the partitioning of the analytes into the acceptor phase. However, due to the greater loss of acceptor phase to sample solution at longer analysis time, which then resulted in poor repeatability, an extraction time of 30 min was selected in this study.

PAHs are known to be hydrophobic and lipophilic analytes, thus it was observed that the extraction efficiency demonstrated with optimized LPME conditions was irregular with different fat contents. Because of the complexity of the milk matrix, the standard addition method was more suitable to quantitate the PAHs in the milk sample with the LPME procedure. One fresh milk sample was used to demonstrate the linearity, LOD and LOQ of the LPME used in this study. Six-point graphs were obtained with concentrations of 5–500 μg/L of PAHs. Calibration graphs were drawn by linear regression of the least-squares method using the peak area of standard as response versus concentration. The R^2 ranged from 0.9978 to 0.9999 for all studied PAHs, which indicated good linearity. The LODs were in the range of 0.07 to 1.4 μg/L, calculated based on a signal-to-noise ratio of 3:1. The LOQs of analytes were in the range of 0.2 to 1.6 μg/L (Table I).

The developed and optimized LPME was then applied to three other brands of fresh milk samples with fat content ranging from 1–4 %. Fresh milk samples with different fat contents revealed different slopes during standard addition calibration and residue determination (Table II). However, linear regression revealed that the LPME is sufficient to produce reproducible residue data with relative standard deviation (RSD) < 10%. PHE, FLA and PYR were detected in three, one and two samples, respectively, analyzed in concentrations

Table I
Validation Data of LPME of PAHs from Milk

PAH	Calibration range (μg/L)	R^2	Slope	LOD (μg/L)	LOQ (μg/L)
FLU	5–500	0.9978	88.245	0.3	0.5
PHE	5–500	0.9979	126.91	1.0	1.2
FLA	5–500	0.9987	33.137	1.4	1.6
PYR	5–500	0.9993	34.157	0.07	0.2
BaP	5–500	0.9999	10.600	0.3	0.5

Table II
Fat Contents and Slopes of Calibration Plots of Different Milk Samples

Sample	Fat content (%)	Calibration range (μg/L)	Slope				
			FLU	PHE	FLA	PYR	BaP
M1	1.3	5–100	60.511	83.192	23.994	27.430	7.368
M2	3.4	5–100	26.701	33.313	12.028	7.000	4.500
M3	2.0	5–100	45.699	74.558	31.804	36.097	16.590

Table III
PAH Residues in Commercial Fresh Milk Products ($n = 3$)

Sample	FLU (μg/L)	PHE (μg/L) (RSD, %)	FLA (μg/L) (RSD, %)	PYR (μg/L) (RSD, %)	BaP (μg/L)
M1	Not detected	2.8 (4.9)	Not detected	Not detected	Not detected
M2	Not detected	6.1 (2.3)	Not detected	33.3 (3.5)	Not detected
M3	Not detected	1.9 (7.2)	6.0 (1.2)	8.7 (2.6)	Not detected

Table IV
Recovery Study*

Method	Average relative recovery (RSD), %				
	FLU	PHE	FLA	PYR	BaP
LPME	104 (3.6)†	100 (3.1)†	109 (4.4)†	110 (5.9)†	85 (6.0)†
Conventional	79 (4.5)‡	83 (8.9)‡	103 (12.2)†	94 (10.3)†	84 (0.8)†

*Note: Values denoted with the same symbols indicate that no significant difference was found between methods for the studied analyte, as determined by t-test ($p > 0.05$).

ranging from 1.9 to 33.3 μg/L (Table III). All of the positive fresh milk samples were found to exceed the EU permitted limit, which is 1 μg/L. The incidence of PAHs in fresh milk samples that exceeded the EU permitted limit have turned it into a significant indicator of PAH intake in case of regular high ingestion of the products.

Relative recovery was studied, because the LPME system is not an exhaustive extraction. The relative recovery is the percent amount of analyte recovered from the matrix with reference to the extracted standard spiked into the same matrix. Triplicate analyte relative recovery studies were conducted using spiked fresh milk at 100 μg/L and the results were compared with conventional solvent extraction method after saponification procedures. The relative recoveries from LPME varied from 81 to 117% with average relative recoveries of 85 to 110% and RSDs of 3.1 to 6.0%. The relative recoveries from the conventional method ranged from 74 to 115%, with slightly lower average relative recoveries of 79 to 103% and RSDs of 0.8 to 12.2% (Table IV). Relative recoveries obtained from both LPME and conventional saponification methods were acceptable according to AOAC International guidelines for method validation (21). However, the relative recoveries for both FLU and PHE using the saponification method were significantly lower than LPME. This was probably because both FLU and PHE were partially lost when they were subjected to heat in the saponification method, because both analytes were semi-volatiles.

Conclusion

The LPME of PAHs from fresh milk was successfully validated for recovery, repeatability and linearity. This method consumes less organic solvent and is more simple than conventional solvent extraction, which requires saponification. The LPME provides clean-up and preconcentration in one step, which reduces analysis times, although standard addition has to be carried out for each milk sample due to the complex matrix effect.

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