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Microwave-assisted extraction and dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry for isolation and determination of polycyclic aromatic hydrocarbons in smoked fish

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ABSTRACT

A simple and efficient method was developed using microwave-assisted extraction (MAE) and dispersive liquid–liquid microextraction (DLLME) coupled with gas chromatography–mass spectrometry (GC–MS) for the extraction and quantification of 16 polycyclic aromatic hydrocarbons (PAHs) in smoked fish. Benzo[a]pyrene, chrysene and pyrene were employed as model compounds and spiked to smoked fish to assess the extraction procedure. Several parameters, including the nature and volume of hydrolysis, extracting and disperser solvents, microwave time and pH, were optimized. In the optimum condition for MAE, 1 g of fish sample was extracted in 12 mL KOH (2 M) and ethanol with a 50:50 ratio in a closed-vessel system. For DLLME, 500 μL of acetone (disperser solvent) containing 100 μL of ethylene tetrachloride (extraction solvent) was rapidly injected by syringe into 12 mL of the sample extract solution (previously adjusted to pH 6.5), thereby forming a cloudy solution. Phase separation was performed by centrifugation and a volume of 1.5 μL of the sedimented phase was analyzed by GC–MS in select ion monitoring (SIM) mode. Satisfactory results were achieved when this method was applied to analyze the PAHs in smoked fish samples. The MAE–DLLME method coupled with GC–MS provided excellent enrichment factors (in the range of 244–373 for 16 PAHs) and good repeatability (with a relative standard deviation between 2.8 and 9%) for spiked smoked fish. The calibration graphs were linear in the range of 1–200 ng g^{-1}, with the square of the correlation coefficient (R^2) > 0.981 and detection limits between 0.11 and 0.43 ng g^{-1}. The recoveries of those compounds in smoked fish were from 82.1% to 105.5%. A comparison of this method with previous methods demonstrated that the proposed method is an accurate, rapid and reliable sample-pretreatment method that gives very good enrichment factors and detection limits for extracting and determining PAHs from smoked fish.

1. Introduction

The smoking of meat and meat products is one of the oldest food-preservation technologies, having been in use for thousands of years and still widely used in fish processing. Smoking is defined as the process of the penetration of volatiles resulting from thermal destruction of organic material such as wood into food products. Potential health hazards associated with smoked fish may be caused by polycyclic aromatic hydrocarbons (PAHs) deposited on the fish. Hundreds of individual PAHs may be formed and released during the incomplete combustion or thermal decomposition (pyrolysis) of the organic material [1,2]. PAHs comprise the largest group of chemical compounds known to be cancer-causing agents. Several PAHs have been classified by the International Agency for Research on Cancer (IARC) as probable or possible human carcinogens and mutagens. However, those PAHs that have not been found to be carcinogenic may act as synergists. As PAHs represent an important class of carcinogens, their presence in food has been intensively studied. Benzo[a]pyrene (BaP) was the first PAH to be identified as a carcinogen, and consequently has received the most attention. According to the EU's Scientific Committee on Food (SCF), benzo[a]pyrene can be used as a marker for the occurrence and impact of carcinogenic PAHs in food [3]. Since August 2011, the EU maximum level for benzo[a]pyrene (BaP) in smoked meat and meat products has been 2 μg kg^{-1} [4].

Exposure of humans to single PAHs does not occur because PAHs are always encountered as complex mixtures. The SCF has identified 15 PAHs as genotoxic carcinogens:

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benzo[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene (BaP), benzo[g,h,i]perylene, chrysene, cyclopenta[c,d]pyrene, dibenz[a,h]anthracene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-cd]pyrene and 5-methylchrysene. In 2008, however, a new scientific opinion adopted by the European Food Safety Authority (EFSA) concluded that BaP alone is not a suitable indicator for the occurrence and toxicity of PAHs in food; the four specific PAHs (BaP, chrysene, benzo[a]anthracene and benzo[b]fluoranthene) are rather more appropriate. Since August 2011, the EFSA maximum level for 4 PAH in smoked meat and meat products has been 12 $\mu g\,kg^{-1}$ [4].

At present, the most frequently reported techniques for analyzing PAHs are chromatographic methods such as GC or HPLC [5–14]. However, direct determination of PAHs in solid food is impossible and sample preparation is needed. The main challenges associated with analysis of PAHs in smoked fish are the extremely low concentration levels ($\mu g\,kg^{-1}$) and the presence of various interfering compounds. Because of the complexity of smoked fish samples and their high lipid content, extraction, sample clean-up and enrichment are necessary. Moreover, PAHs have a tendency to diffuse not only into the non-polar part of the sample but also inside the tissue cells due to the existing concentration gradient [6]. Saponification with potassium hydroxide [15], the Soxhlet extraction method [16,17], sonication [18,19], supercritical fluid extraction [20,21] or pressurized liquid extraction (PLE) [10,13,22] have been used for pre-extraction of PAH compounds from solid matrices to liquid phase. The high consumption of hazardous organic solvents, high costs, prolonged analysis time [22,23], an increased risk of analyte losses, decreased reproducibility, and biases are the main disadvantages of these methods.

Usually, microwave-assisted extraction (MAE) uses polar solvents, such as water, to extract target compounds primarily from solid matrices. When water absorbs the microwave energy, temperature and pressure are increased and target compounds are more rapidly desorbed from the matrix. In recent years, MAE has become a viable alternative to the conventional techniques exhibiting many substantial improvements in the preparation of analytical samples, as it requires much lower volumes of organic solvents, reduces extraction time and increases recovery yield [24–28]. Non-polar solvents do not absorb microwave energy and have poor extraction efficiencies compared to polar solvents or mixtures of solvents in which at least one is polar [29]. Mixtures of non-polar solvents and water solutions improve recoveries of PAHs from a solid sample, as water absorbs the microwave energy, and non-polar solvents enhance the release of PAHs from the sample matrix.

After primary extraction of the PAHs from smoked fish to liquid phase, conventional liquid–liquid extraction (LLE) [30] and solid-phase extraction (SPE) [31,32] have been widely used for pre-concentration and clean-up before analysis. These methods are time consuming and tedious, often require large amounts of potentially toxic solvents and may be relatively expensive [33]. Simplification, speed and miniaturization of this stage are recent trends in analytical processes. One of the techniques attracting special attention is dispersive liquid–liquid microextraction (DLLME), which was introduced in 2006 by Asaadi et al. [34] for the isolation and preconcentration of analytes from aqueous matrices. DLLME is generally based on a ternary component solvent system, in which extraction and disperser solvents are rapidly injected into the aqueous sample to form a cloudy solution. Extraction equilibrium is quickly achieved due to the extensive surface contact between the droplets of the extraction solvent and the sample. After centrifugation, the extraction solvent is normally sedimented at the bottom of the tube (if the density is greater than that of water) and taken with a microsyringe for its later chromatographic analysis [5,35,36].

In this study, for the first time we developed a relatively straightforward procedure using MAE followed by DLLME for extraction, isolation and concentration of 16 PAHs from smoked fish. MAE coupled with DLLME is a useful combination that combines rapid extraction with a simple and quick pre-concentration method. The aqueous alcoholic KOH was used as a solvent for absorbing the microwave energy and accomplishing the fast desorption of PAHs from the smoked fish matrix. After the extract was cleaned up using Carrez solutions, the clear supernatant was directly mixed with the dispersive solvent and extractant. The mixture was then subjected to the DLLME procedure. Final separation and quantification were performed by GC with MS detection. The factors affecting MAE and DLLME efficiency were studied in detail, and the optimal conditions were established. The efficiency of the proposed method for analysis of 16 PAHs in smoked fish was evaluated and compared with previous methods described in the literature.

2. Experimental

2.1. Reagent

PAH reference standards (PAH-Mix 16, QTM, 2000 $\mu g\,mL^{-1}$), as well as BaP (solid 99%) and chrysene (solid 99%), were obtained from Supelco (Bellefonte, PA, USA). Pyrene (solid 99%) was obtained from Sigma–Aldrich Chemie GmBH (Steinheim, Germany). A standard solution (10 $\mu g\,mL^{-1}$) from 16 PAHs was prepared in dichloromethane. Stock solutions of BaP, pyrene and chrysene were prepared separately at a concentration of 1 $mg\,mL^{-1}$ in dichloromethane. These stock standard solutions were diluted with methanol weekly to prepare a mixed working solution with a concentration of 10 $\mu g\,mL^{-1}$ for each compound; this working solution was applied to survey extraction performance under different conditions. Biphenyl (obtained from Merck) was used as an internal standard and prepared in methanol at a concentration of 40 $\mu g\,mL^{-1}$. Stock and working solutions were stored at 4 °C in a refrigerator and were used daily in proper concentrations or directly. Hydrochloric acid, acetone, tetrachloroethylene, 1,1,2,2-tetrachloroethane, carbon tetrachloride, hydroxide potassium (>85%), potassium ferrocyanide (Carrez solution I) and zinc acetates (Carrez solution II) were purchased from Merck (Darmstadt, Germany). Ethyl alcohol (99.6%) was obtained from Bidestan Co. (Qazvin, Iran). All solvents were of analytical reagent grade or HPLC grade.

2.2. Instrumentation

GC–MS analyses were carried out by 7890A GC system from Agilent Technologies (Palo Alto, CA, USA) with a triple-axis detector fitted with a split/splitless injector and coupled with a 5975C inert MSD network mass selective detector. The chemical compounds were separated by using HP-5 MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness). The temperature program commenced at 120 °C for 1 min, and was then raised by 7 °C min^{-1} to 200 °C, held for 1 min, raised by 5 °C min^{-1} to 250 °C and held for 1 min. Finally, the temperature was increased rapidly by 20 °C min^{-1} to 290 °C and kept for 10 min. Other GC terms were as follows: helium as a carrier gas in a constant flow of 1 mL min^{-1}, an injector temperature of 290 °C and a split ratio of 1:50. Approximately 1.5 μL of the sample was injected in a split mode. The auxiliary temperature was set at 280 °C. The compounds were quantified in the selected ion monitoring (SIM) mode, and one qualifier ion was selected for each compound. Centrifuging (Heltich Rotorfix 32A) was applied at 4000 RPM to separate the mixture. A microwave oven (Delonghi type MW 602) was used to accelerate alkaline saponification and primary extraction.

2.3. Procedure

2.3.1. Sample preparation and MAE

The tail, spine and head were removed from the entire fish using a stainless steel knife, and the remaining sample was minced in a meat grinder. The samples were stored at −18 °C in darkness before the analysis. A sample of smoked fish was used for method optimization and 100 g of the ground sample was spiked with 1 mL of mixed working solution containing BaP, chrysene and pyrene (10 μg mL^{-1}). The mixture was stirred mechanically and allowed to dry at room temperature thoroughly for 24 h, and then used to survey extraction variables under different conditions and optimization. Under the finally optimized conditions, 1 g (weighed precisely) of the homogenized sample was placed in a glass container. After addition of 12 mL of mixing solution containing potassium hydroxide (2 M in water), and ethyl alcohol (50:50), the glass container was tightly closed. Microwaving at 500 MHz for 2 min was employed to hydrolyze and saponify the sample. After cooling, the compounds were transferred into the centrifuge tube and centrifuged at 4000 RPM for 5 min. Then the aqueous phase was transferred to another vessel, and pH decreased to 6.5 by adding hydrochloric acid. Finally, to precipitate the proteins, 2 mL of Carrez solutions I and II (50:50) were added to the vessel, which was then centrifuged again at 4000 RPM for 5 min.

2.3.2. DLLME

After primary extraction and centrifugation, the clear phase was separated and the combination of 100 μL tetrachloroethylene as the extracting solvent, 500 μL of acetone as the disperser solvent and 2 μL biphenyl (40 μg mL^{-1}) as the internal standard were added. The mixture was gently shaken and centrifuged at 4000 RPM for 10 min. The dispersed fine particles of the extraction phase were sedimented at the bottom of the vessel. The upper aqueous phase was separated with a syringe and about 1.5 μL of the sedimented phase was injected directly into the GC–MS using a microsyringe.

The validity of the proposed method was established through a study of repeatability, linearity, enrichment factor, limit of detection and limit of quantification and recovery. The spiked fish samples were employed under the obtained optimal condition to investigate the validation parameters.

3. Results and discussion

Analysis of PAHs in the fish sample is problematic because of their extremely low concentrations and their affinity for the fatty fraction of fish. It is very important to extract and eliminate these fats before instrumental analysis without losing the PAHs at any of the steps. Grimmer and Böhnke [37] isolated PAHs from smoked fish with boiling methanol prior to sample hydrolysis with methanolic KOH. It was found that only about 30% BaP and other PAHs was extractable from the samples, whereas an additional alkaline hydrolysis of meat protein yielded another 60% of PAHs. It was concluded that PAHs were linked adsorptively to high molecular structures not destroyed with boiling methanol. Alkaline saponification with aqueous alcoholic KOH is necessary to isolate PAHs quantitatively. Generally, methanolic KOH has been used in previous studies as the primary extraction solvent. Alkaline hydrolysis sample treatment with reflux usually takes 2–4 h depending on the character of the sample [6]. MAE is currently a popular technique and due to its advantages over other conventional techniques has been used for the primary extraction of compounds at trace levels in solid matrices. MAE exhibits many substantial improvements in analytical sample preparations, as it requires much lower volume of extraction solvent, reduces extraction time and increases recovery yield [26]. MAE with low volumes of alcoholic KOH may destroy the fish tissues very quickly leading to rapid release of all PAH compounds from the solid matrix.

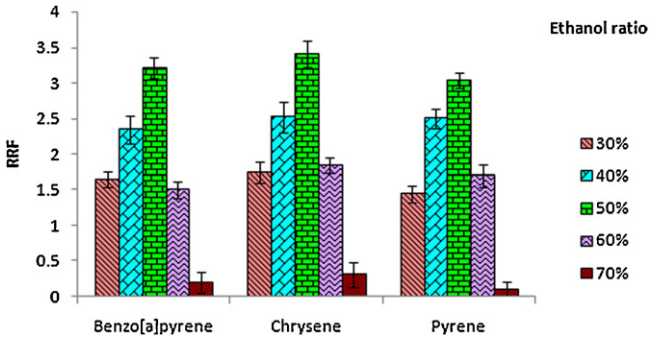

Fig. 1. Effect of the ethanol ratio on the relative response factor ($n = 3$). Experimental conditions: concentration: 100 ng g^{-1}; volume of hydrolyzing solvent: 15 mL; pH: 7; extracting solvent and disperser: 100 and 700, respectively.

The preliminary tests were performed at three different extraction times (0.5, 2 and 3.5 min) with a microwave at 500 MHz. At the lowest time (30 s), the tissue was not effectively destroyed. Therefore, the extraction efficiency was poor. The best results were obtained when the time was set at 2 min; with increased time (3.5 min) the extraction efficiency remained constant or was decreased. In addition, with the increased time of microwave, degradation of PAHs may increase.

The main challenge in the saponification with alcoholic KOH contributes to interference problems in the chromatography analysis because of the formation of methyl esters from fatty acids and methanol, which are then difficult to remove from the PAH fraction [6]. In initial tests, we employed methanol and ethanol as an organic solvent in the hydrolysis process. The results indicated that ethanol provides cleaner chromatography than methanol. The concentration of KOH was studied in the range of 0.5–2 M. The best extraction efficiency was observed with a KOH concentration of 2 M. After the primary experiment, ethanolic KOH as the hydrolysis solvent, KOH concentration at 2 M and microwave energy in 500 MHz for 2 min were determined as optimum.

3.1. Optimization of the MAE–DLLME method

All variables were optimized using the "single-factor-at-time" method, and relative response factor (RRF) was employed as a response to the optimization procedure. RRF was calculated with a ratio of the BaP, chrysene and pyrene peak area to the internal standard peak area. The mixtures of ethanol and water improved the recoveries of PAHs from the solid sample, as water absorbs the microwave energy, and ethanol enhances the release of PAHs from the sample matrix. In the first assay, we optimized the ethanol ratio (30%, 40%, 50%, 60% and 70%) in the hydrolyzing solvent. As seen in Fig. 1, the extraction efficiency increased when the ratio of ethanol in the hydrolyzing solvent increased from 30% to 50%. This may be attributed to the fact that in 30% ethanol, the extraction of PAHs from the smoked fish tissue was not complete. By increasing ethanol, the polarity of the solution decreases, thereby increasing the desorption rate of the compounds from the solid sample to the extraction phase. Furthermore, the extraction efficiency decreased when the ethanol ratio was more than 50%. In DLLME, the polarity of the solution is important to the formation of the sedimented phase. With increases in the ethanol ratio the polarity is decreased, thus reducing the extracting phase. Due to the above mentioned fact, when ethanol 70% was employed to the extraction producers, the sedimented phase was not formed or was negligible.

Another parameter was the volume of the hydrolyzing solution (6, 8, 10, 12, 14 and 15 mL). RRF increased with the increase in the

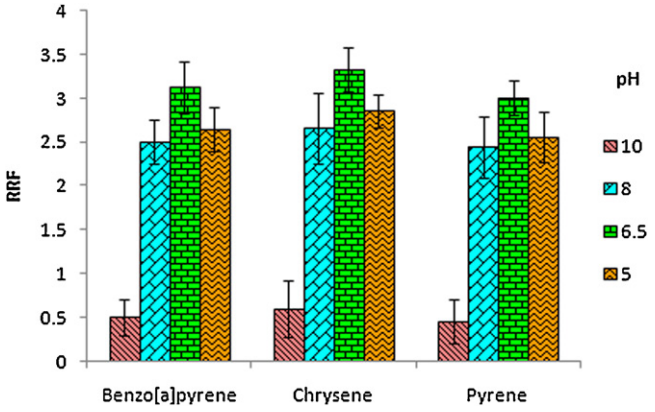

Fig. 2. Effect of the pH on the relative response factor ($n = 3$). Experimental conditions: concentration: 100 ng g^{-1}; ethanol ratio: 50%; hydrolyzing solution: 12 mL; extracting solvent and disperser: 100 and 700, respectively.

hydrolyzing volume, and was highest at 12 mL. RRF remained constant when we employed hydrolyzing solution at 14 mL or 16 mL. Based upon the above considerations, 12 mL was sufficient for complete immersion of the smoked fish sample in the extraction solvent. Therefore, MAE of PAHs is performed very well at 2 min, when 1 gram of smoked fish sample and 12 mL hydrolyzing solvent with 50% ethanol ratio was used.

The DLLME method was optimized with respect to the type of extraction solvent, the volume of extraction and dispersive solvents and sample pH. A high density, low polarity, good chromatographic behavior, high purity and high partitioning coefficient of the PAHs in the solvent are essential factors in selecting a suitable organic solvent for DLLME. Based on the above considerations, three organic solvents; namely, tetrachloroethylene, 1,1,2,2-tetrachloroethane and carbon tetrachloride (at same volumes, 100 μL) were selected as the extraction solvents. The results revealed that tetrachloroethylene had better recoveries for all PAH compounds than the other solvents. This could be related to its higher density and lower water solubility. Based on these results, we decided to use tetrachloroethylene in further experiments.

After primary extraction by microwave, the pH of the extracted solvent was high (pH > 12), and under this condition no sediment phase formed in the bottom of the vessel after centrifuging. Therefore, pH adjustment was necessary. Various volumes of HCl (14 N) were added to the sample solution to study the effect of pH on the extraction efficiency. At a pH of 10, no sedimented phase formed; therefore, DLLME procedure was not possible. When the pH was reduced from 10 to 6.5, the RRF increased considerably (Fig. 2). A cleaner chromatogram was observed at a pH of 5, but the best result was obtained at 6.5; thus, this pH was selected as optimum for DLLME procedure.

Tetrachloroethylene as extracting solvent was applied at six levels (40, 60, 80, 100, 120 and 140 μL) to get the best extraction efficiency. The polarity of the sample solution is reduced due to the presence of ethanol (50%). Therefore, the solubility of tetrachloroethylene increased and the sedimented phase volume was very low when tetrachloroethylene was applied at 40 μL. As shown in Fig. 3, RRF increases noticeably with increases in the tetrachloroethylene volume reaching a maximum at 100 μL. Thus, 100 μL was considered sufficient for good extraction of PAHs during DLLME, since a higher solvent volume considerably decreases analyte enrichment and the pre-concentration factor.

Based on our knowledge, the disperser solvent helps produce the emulsion that is necessary in DLLME for developing the cloudy phase, and for the transfer of analytes from the sample solution to the sedimented phase. We applied five volumes of acetone (300,

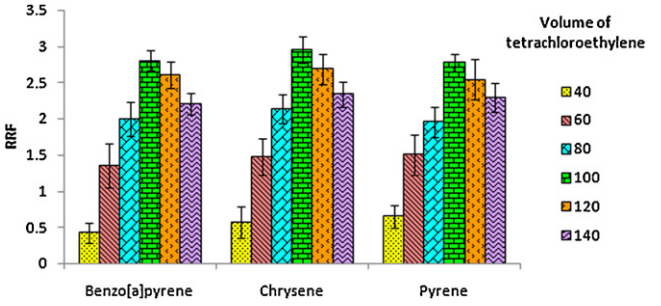

Fig. 3. Effect of the volume of extracting solvent on the relative response factor ($n = 3$). Experimental conditions: concentration: 100 ng g^{-1}; ethanol ratio: 50%; hydrolyzing solution: 12 mL; pH: 6.5; disperser solvent: 500.

500, 700, 800 and 1000 μL) to find the best conditions for extraction. The cloudy phase was not formed very well at 300 μL of acetone; therefore, RRF was low, as shown in Fig. 4. RRF was highest at 500 μL of acetone, and clearly decreased at 700 μL. The extracting phase was not formed or was not significant at 1000 μL of acetone. Based on the above results, 500 μL was chosen as the volume of disperser solvent for the remainder of the study.

3.2. Quantitative analysis of PAHs in fish

The spiked fish samples were employed under the obtained optimal condition to investigate the repeatability, linearity, enrichment factor, recovery, limit of detection and limit of quantification, the results of which are shown in Table 1. The comparative peak area, calculated from seven replicates, was employed to estimate the repeatability and is shown as relative standard deviation percentage (RSD%). As the table shows, the RSD% was between 2.8 and 9 for all PAH compounds. The linearity of the method was tested in the concentration range of 1–200 ng g^{-1} for 16 PAHs. Three replicate extractions and determinations were performed at optimal conditions for each level. This method showed a good linear behavior in the tested range, with correlation coefficients ranging between 0.981 and 0.993. In order to determine the enrichment factors, three replicate extractions were performed at optimal conditions from fish samples containing 5 ng g^{-1} of each analyte. The ratio between the analyte concentration in the sedimented phase following extraction and the primary concentration of the analyte in the hydrolyzing solution was expressed as the enrichment factor (EF). The enrichment factors of the proposed method ranged from 244 to 373. The limit of detection for 16 PAH compounds when using the optimized conditions and GC–MS in SIM mode, based on a signal-to-noise ratio of 3, were at the low parts-per-billion level (ng g^{-1}), well below guidelines established by the European Food Safety Authority for smoked fish samples [4]. The recovery for each analyte was determined for the MA E-DLLME procedure

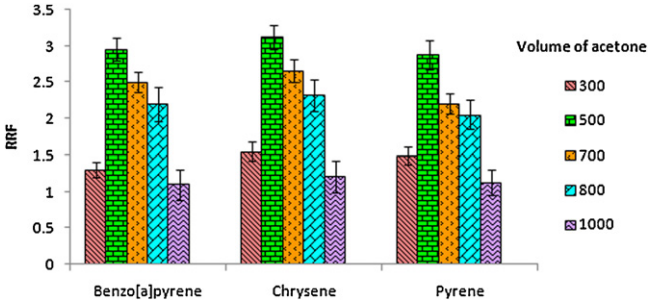

Fig. 4. Effect of the volume of disperser solvent on the relative response factor ($n = 3$). Experimental conditions: concentration: 100 ng g^{-1}; ethanol ratio: 50%; hydrolyzing solution: 12 mL; pH: 6.5; extracting solvent: 100.

Table 1
Linear range, correlation coefficient, enrichment factor, relative standard deviation, limit of quantitation (LOQ), limit of detection (LOD) and recoveries of PAHs obtained with microwave-assisted extraction and dispersive liquid–liquid microextraction followed by GC–MS (DLLME–MAE-GC/MS).

Compound	Linear range ($ng\,g^{-1}$)	R^2	EF	RSD% ($n=5$)	LOQ ($ng\,g^{-1}$)	LOD ($ng\,g^{-1}$)	Recovery
Acenaphthene	1–200	0.992	329	2.87	0.53	0.16	95.4
Acenaphthylene	1–200	0.986	357	2.88	0.36	0.11	101.3
Anthracene	1–200	0.992	309	6.43	0.73	0.22	91.9
Benzo[a]anthracene	2–200	0.986	282	7.93	1.2	0.36	97.8
Benzo[a]pyrene	1–200	0.987	314	8.70	0.70	0.21	102.6
Benzo[b]fluoranthene	2–200	0.989	286	8.20	1.36	0.41	88.1
Benzo[g,h,i]perylene	2–200	0.982	254	6.68	1.6	0.48	84.4
Chrysene	1–200	0.992	332	5.75	0.86	0.26	103.8
Dibenz[a,h]anthracene	2–200	0.989	278	9.09	1.5	0.46	89.5
Fluoranthene	2–200	0.993	373	8.76	0.93	0.28	96.7
Fluorene	1–200	0.978	342	7.51	0.83	0.25	97.2
Indeno[1,2,3-cd]pyrene	2–200	0.983	244	8.92	1.53	0.46	82.1
Naphthalene	1–200	0.987	319	6.64	0.40	0.12	87.5
Phenanthrene	1–200	0.981	358	8.49	0.56	0.17	105.5
Pyrene	2–200	0.984	302	6.64	1.00	0.30	92.4
2-Bromonaphthalene	2–200	0.990	281	5.40	1.3	0.38	86.9

Table 2
The figures of merit for proposed method and comparison with other methods.

Method	Compounds	Linear range ($ng\,g^{-1}$)	R^2	EF	RSD% ($n=5$)	LOQ ($ng\,g^{-1}$)	LOD ($ng\,g^{-1}$)	Recovery
MAE–DLLME-GC/MS (proposed method)	16 PAHs	1–200	0.981–0.993	244–373	2.8–8.9	0.36–1.6	0.11–0.48	82–105
LSE–SPE-GC/MS [32]	5 PAHs	1–60	0.988–0.997	–	11.2–13.7	0.66–1.1	0.2–0.35	73–86
LSE-HPLC [8]	15 PAHs	50–250	–	–	0.6–5.4	4.5–40	1.4–12	84–107
PLE-GC/MS [13]	26 PAHs	–	0.995–1.00	–	4–15	0.9–14.6	0.2–4.4	55–108
MAE–SPE-HPLC/FD [28]	15 PAHs	0.2–40	0.998–1	–	3.7–185	–	0.2–0.6	77–103

by comparing the amount of analyte added to a fish sample with the concentration recovered after the procedure. For fish samples spiked with $10\,ng\,g^{-1}$ per analyte, a line equation of the standard addition graph, obtained individually for 16 PAH compounds, was applied to the calculation of recoveries, which gave results between 82.1 and 105.5 for various analytes (Table 1).

Comparing the results for extraction and determination of PAH compounds using this optimized novel method with literature data using other methods [8,13,28,32] shows that the proposed method is comparable or better for the studied compounds (Table 2).

3.3. Application to real sample

To evaluate the reliability of the proposed method, five smoked fish were purchased from Langroud city market (Rasht, Iran) and tested with the optimized method. The smoked fish samples were isolated from the tail, spine and head, cut with a knife and minced in a meat grinder. The samples were stored at 4 °C until their analysis for PAHs. Some of the compounds included in this study were found at very low levels in the smoked-fish samples, and their concentration was evaluated using the proposed method. The data was confirmed by the standard addition method. The analytical results are summarized in Table 3. BaP and the sum of 4 PAHs as the indicators of PAHs only in sample 2 were more than $2\,ng\,g^{-1}$ and $12\,ng\,g^{-1}$, respectively. These results showed that GC–MS in SIM mode after MAE–DLLME is a powerful method for monitoring PAHs at very low concentration in smoked-fish samples. Fig. 5 shows the chromatograms obtained by MAE–DLLME-GC/MS under SIM mode for a smoked rutilus frisii kutum fish sample when (a) non-spiked and (b) spiked with 16 PAHs at $10\,ng\,g^{-1}$ level. A clean separation and good chromatogram is readily achieved without the presence of sample matrix interference.

Table 3
PAH contents ($ng\,g^{-1}$) obtained in the analysis of smoked fish by MAE–DLLME-GC–MS.

Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Acenaphthene	4.9 ± 0.01	4.5 ± 0.02	2.6 ± 0.02	ND	ND
Acenaphthylene	ND	1.4 ± 0.01	1.2 ± 0.02	1.4 ± 0.03	1.6 ± 0.01
Anthracene	2 ± 0.06	ND	6 ± 0.06	ND	ND
Benzo[a]anthracene	ND	2.4 ± 0.05	ND	ND	ND
Benzo[a]pyrene	1.8 ± 0.04	7.6 ± 0.07	1.4 ± 0.06	1.4 ± 0.05	1.2 ± 0.05
Benzo[b]fluoranthene	2.2 ± 0.06	1.3 ± 0.07	1.4 ± 0.06	1.6 ± 0.05	ND
Benzo[g,h,i]perylene	1.2 ± 0.03	ND	ND	2.1 ± 0.03	ND
Chrysene	1.7 ± 0.05	1.5 ± 0.05	1.8 ± 0.06	1.2 ± 0.04	3.5 ± 0.05
Dibenz[a,h]anthracene	1.1 ± 0.03	ND	ND	ND	ND
Fluoranthene	1.1 ± 0.08	2.1 ± 0.08	8.2 ± 0.06	1.5 ± 0.03	1.6 ± 0.05
Fluorene	ND	6.8 ± 0.05	6 ± 0.03	ND	2 ± 0.06
Indeno[1,2,3-cd]pyrene	1.8 ± 0.04	ND	ND	ND	ND
Naphthalene	3.8 ± 0.03	4.2 ± 0.02	2.4 ± 0.04	4.6 ± 0.02	4.7 ± 0.04
Phenanthrene	3.7 ± 0.08	ND	9.5 ± 0.05	2 ± 0.07	ND
Pyrene	2.1 ± 0.07	ND	3.7 ± 0.08	ND	1.7 ± 0.05
2-Bromonaphthalene	ND	ND	ND	ND	ND

Mean value ± standard deviation ($n=3$). ND: means not detected.

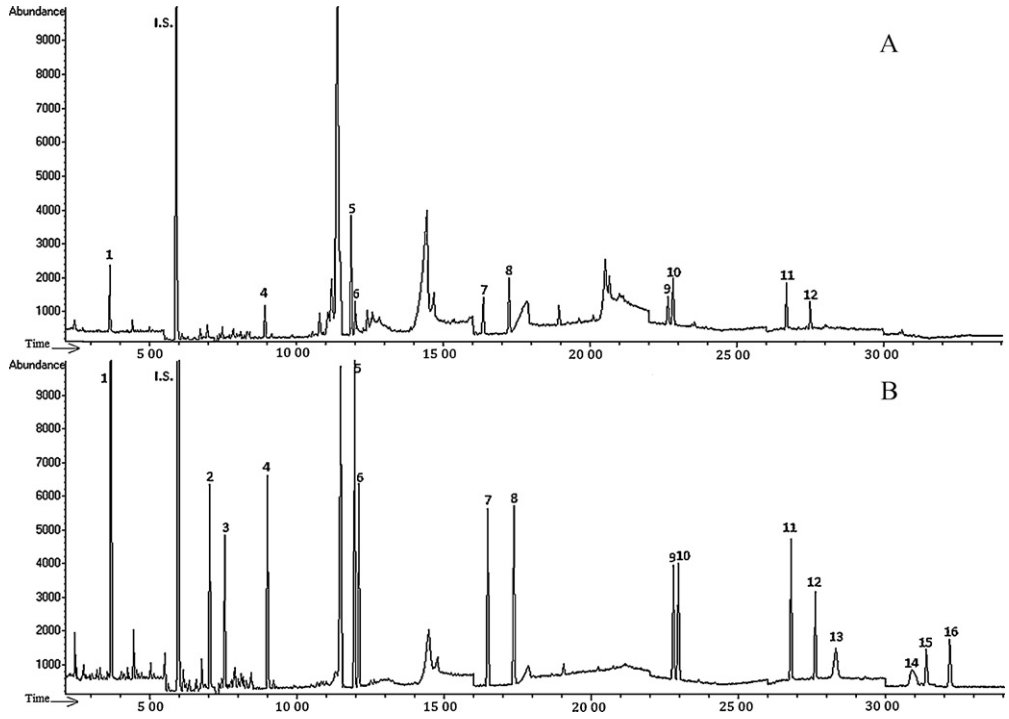

Fig. 5. The select ion monitoring (SIM) obtained by DLLME-GC–MS for a smoked rutilus frisii kutum fish under optimum conditions: (a) non-spiked and (b) spiked with 50 ng g^{-1} of sixteen PAHs. (1) Naphthalene, (2) acenaphthylene, (3) 2-bromonaphthalene, (4) acenaphthene, (5) flurene, (6) phenanthrene, (7) anthracene, (8) fluoranthene, (9) pyrene, (10) benzo[a]anthracene, (11) chrysene, (12) benzo[b]fluoranthene, (13) benzo[a]pyrene, (14) indeno[1,2,3-cd]pyrene, (15) dibenzo[a,h]anthracene and (16) benzo[g,h,i]perylene.

4. Conclusion

In the present study, we successfully developed the MAE–DLLME-GC/MS procedure for rapid extraction and quantification of PAHs at very low levels in smoked fish. The factors affecting MAE and DLLME efficiency were studied in detail, and the optimal conditions were established. The simplicity, facility, low solvent consumption, low cost, high sensitivity, good precision, high enrichment factors and short analysis time are clear advantages of the proposed method for the studied compounds in smoked fish. The figures of merit for analysis of 16 PAHs in smoked fish using the developed method were evaluated and compared with previous methods described in the literature. A comparison of this method with previous methods demonstrated that the proposed method is an accurate, rapid and reliable sample-pretreatment method that gives very good enrichment factors and detection limits for extracting and determining PAHs from smoked fish. A clean separation was readily achieved without the presence of sample matrix interference, and unwanted peaks were negligible in chromatograms when the SIM mode was used to quantify PAHs.

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