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Enrichment of Benzo(a)pyrene in Milk by Saponification-Solvent Microextraction

Khai Wei Lim[1], Zhi Zhong Khor[1], Wan Mohd Afiq Wan Mohd Khalik[1], Sazlinda Kamaruzaman[2] and Saw Hong Loh[1]*

[1]Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

[2]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

*Corresponding author (e-mail: lohsh@umt.edu.my)

Milk carries significant amounts of essential fats and nutrients such as magnesium, calcium and zinc. Hence, they have always been the significant components consumed by all age groups, especially infants and elderly seniors. However, the lipophilic nature of polycyclic aromatic hydrocarbons (PAHs) increases the likelihood of depositing fats within the milk and may result in both acute and chronic adverse health effects for humans. Therefore, a rapid and environmentally friendly saponification-solvent microextraction coupled with high-performance liquid chromatography and fluorescence detection was developed and validated for the determination of benzo(a)pyrene (BAP) in milk samples. BAP was separated by a reversed-phase C18 column with a mobile phase composed of 70% acetonitrile in isocratic mode. The microextraction variables included the type and volume of organic solvent, the effect of salt addition and vortex time were investigated and optimised. Under optimal conditions, the linearity of the method was established in the range of 0.25 to 5 µg L^{-1} with the correlation of determination = 0.9939. The ultra-trace limit of detection and limit of quantification were obtained as 0.02 and 0.07 µg L^{-1}, respectively. The average of the relative recovery ranged from 73.7-116.0% with relative standard deviations ≤ 7.2%. The saponification-solvent microextraction is green considering its microformat in addition to the selectivity and sensitivity enhanced by saponification.

Keywords: Milk; polycyclic aromatic hydrocarbons; saponification; HPLC-FD; microextraction

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Over the last few decades, the environment has been affected by various anthropogenic activities arising from global urbanisation as well as rapid industrialisation which then cause many unnecessary consequences and release hazardous pollutants to the environment. The occurrence of polycyclic aromatic hydrocarbons (PAHs) in the environment is frequently reported. The United States Environmental Protection Agency (USEPA) classified 16 PAHS as significant pollutants in 1983 owing to their high susceptibility, tenacious behaviour, and toxicity [1]. Thereupon, the issue of PAHs has received considerable critical attention.

There is no denying that milk and other dairy products contain significant amounts of essential fats and nutrients, such as magnesium, calcium and zinc. Hence, they have always been the major components in the diet of people, especially infants and senior citizens [2]. It is widely acknowledged that among the most nutrient-dense products available, milk ranks highly. PAHs with a lipophilic nature can develop readily in milk because of the high-fat content. As a result, consuming milk carrying PAHs will contaminate human dietary patterns with these pollutants. Of this, many nations have looked into the levels of cancer-causing PAHs in food samples to restrict and minimise the amounts of PAHs, for instance, the European Union (EU) has recommended the maximum residue of benzo(a)pyrene (BAP) in baby food shall not exceed 1.0 µg kg^{-1} [3].

The occurrence of 16 PAHs was investigated in raw, pasteurised, ultra-high-temperature processing (UHT), semi-skimmed, and UHT whole milk [4]. The findings showed that all types of milk contained 8 out of 16 PAHs namely phenanthrene, anthracene, pyrene, benzo(a)anthracene, chrysene, benzo(K)fluoranthene, BAP and benzo(g,h,i)perylene. Additionally, the study demonstrated that pasteurised samples contained higher PAHs (6.519 ng g^{-1} of milk) as compared to raw milk samples (5.428 ng g^{-1} of milk). Furthermore, UHT whole milk samples (7.753 ng g^{-1} of milk) had more PAHs than UHT semi-skimmed milk (5.941 ng g^{-1} of milk), perhaps because UHT whole milk samples had a greater amount of fat. The fat content, processing methods, as well as heat-treatments, could influence the changes in PAHs content in the analysed milk types (whole, pasteurised, and UHT).

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Considering PAHs have such a broad range of physical and chemical characteristics, measuring them is typically challenging and expensive. In addition, milk has a complex sample matrix, making it tough to get rid of interference-causing matrix elements. What should be noted is that the content of PAHs occurred only in a trace level accompanied by high carcinogenic to human health. The occurrence of PAHs in commercial milk and milk powder samples has been confirmed using commercial QuEChERS tubes as the clean-up tool coupled with gas chromatography (GC)-triple quadrupole (QqQ)-mass spectrometry (MS). The QuEChERS method enhanced the matrix removal and obtained a satisfactory recovery ranging from 63.38−109.17% [5]. The PAHs residues were also detected in milk samples from nine countries using a similar QuECHERS-GC-QqQ-MS method and achieved recovery ranging from 62.81–105.18% [6]. The QuECHERS method was once again proven to possess powerful clean-up characteristics to succeed in the exhaustive extraction of PAHs from fat-rich samples. A tedious solvent extraction followed by saponification and solid-phase extraction clean-up before GC-MS analysis was reported for the determination of selected four PAHs in the infant formula powder. The procedure was lengthy yet attained good recovery of the selected PAHs in the range of 77.3-111.8% [7]. An *in situ* hydrolysis of milk fat to form fatty acids as the precursors to produce a natural deep eutectic solvent for the extraction of selected PAHs in the powdered milk samples has been demonstrated. The technique successfully eliminated the protein and fat and thus attained 70-91% of PAHs recovery [8].

This study aimed to perform a miniaturised sample preparation technique to provide an improvement to replace the conventional saponification-solvent extraction. Consequently, BAP was chosen as the model analyte and treated as a representative of heavy PAHs given its high carcinogenicity among the 16 PAHs present in food. In addition, BAP is frequently used as an indication as well for the existence and impact of carcinogenic PAHs in food [9-10]. The presence and concentration of PAHs in food can also be strictly regulated at a permitted level in terms of food processing and manufacturing. To safeguard against the adverse effects on human health, the general public's consciousness of the consumption of PAHs in foods can be raised.

EXPERIMENTAL

Chemicals and Materials

The BAP reference standard was purchased from Sigma-Aldrich (St. Louis, MO). The methanol (chromatography grade), acetonitrile ((chromatography grade), heptane (analytical grade), hexane (analytical grade), sodium sulphate (analytical grade), and sodium hydroxide (analytical grade) were obtained from Merck (Darmstadt, Germany).

Preparation of Standard and Sample Solutions

A 100 µg L^{-1} of BAP standard stock solution was prepared by dissolving 0.001 g of BAP in 5 mL of volumetric flask and diluted to volume with acetonitrile. A serial working standard solution in the range of 0.25 to 5 µg L^{-1} of BAP was prepared from the stock solution by applying methanol as the diluent. When not being used, all of the standard solutions were kept in a freezer at -10 ˚C. Sterilised full cream milk samples were purchased from local enterprise shops and kept in the refrigerator at 4 ˚C before analysis. Extraction was performed on milk samples without a filtration step.

High-Performance Liquid Chromatography and Fluorescence Detection Chromatographic Conditions

BAP quantitation was performed using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) coupled with fluorescence detection (HPLC-FD) (Shimadzu, Kyoto, Japan). The chromatographic separation of BAP was performed on a reversed-phase C18 column (Shimadzu, Kyoto, Japan) (4.6×150 mm, 5 µm). The elution of targeted analyte applied isocratic mobile phase acetonitrile-water (70:30) (v/v) at a column temperature of 40 ℃ [11]. The flow rate, injection volume and excitation/emission wavelengths were fixed at 1.0 mL min^{-1}, 10 µL and 290/406 nm, respectively [12].

Saponification Procedure

The work by Chung et al. (2010) and Sanagi et al. (2013) served as the basis for saponification [13-14]. A milk sample (2 mL) was pipetted into a 10 mL of centrifuge tube and 4 mL of 0.4 M NaOH (prepared in a mixture of methanol and water at a ratio of (9:1)) was added into the tube. The tube was sealed and incubated for 30 min at 60 °C. For complete saponification, the tube was vortexed to mix at 5 min intervals. Before performing the solvent extraction and solvent microextraction, the tube was cooled down to room temperature. Figure 1 illustrates the procedure of saponification.

Solvent Extraction

After saponification, 2 mL of organic solvent (a mixture of dichloromethane and n-hexane at a ratio of 1:1 (v/v)) was added to the tube and the tube was capped. The tube was vortexed for 1 min to thoroughly mix the content. The tube was then centrifuged at 3,000 rpm for 10 min. Next, the cap was removed, and the mixture was allowed to separate into 2 phases. The organic layer was then withdrawn into a glass test tube. Another 2 mL of an organic solvent was applied for the extraction process and the organic extract was then collected and combined in the same glass test tube.

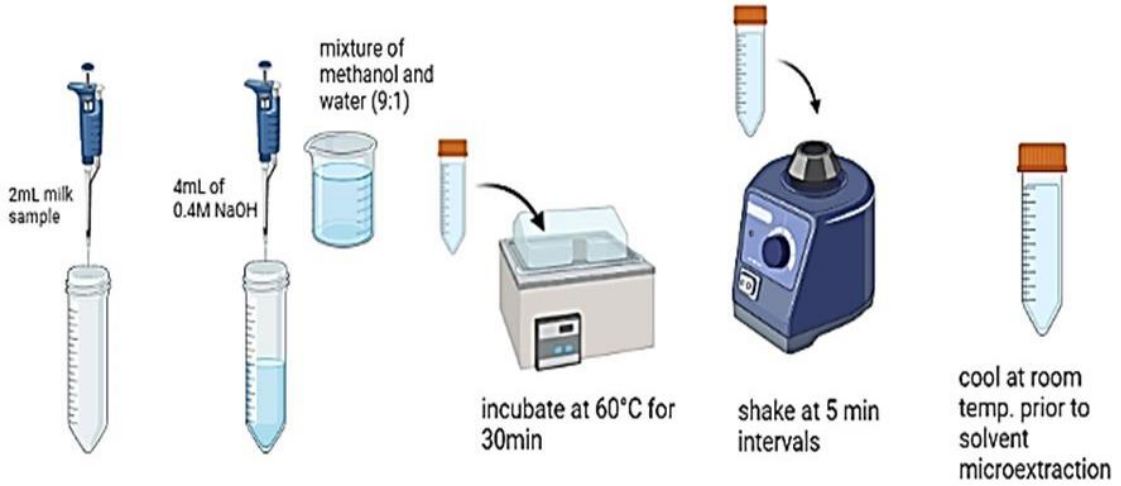

Figure 1. Schematic of saponification procedure.

The collected organic extract was then evaporated to dryness and reconstituted into 2 mL of methanol before filtration using a nylon syringe filter [13]. Finally, the filtered extract was analysed using HPLC-FD.

Solvent Microextraction

After saponification, the centrifuge tube containing the saponified sample was filled with 200 µL of heptane (extraction solvent). The tube was vortexed for 30 s to thoroughly mix the content and form a cloudy solution before being centrifuged at 6,000 rpm for 10 min to separate the layers. The 100 µL of extract (organic layer) that formed on the top of the sample solution was then withdrawn with a micropipette and transferred into an HPLC vial containing 100 µL of methanol. Then, the vial was vortexed to mix well, and the dilute extract was then injected into HPLC-FD. The solvent microextraction technique is shown in Figure 2.

Optimisation of Solvent Microextraction Procedure

The solvent microextraction process was optimised to increase extraction effectiveness. The milk samples with a fat content of 3.3% were chosen as the samples in the optimisation process. The variables included the effect of salt addition (addition of 0, 1, 5 and 7% of sodium sulphate), the type (hexane and heptane) and the volume of organic solvent (200, 300 and 400 µL of heptane) as well as the vortex time (30s, 1 and 2 min).

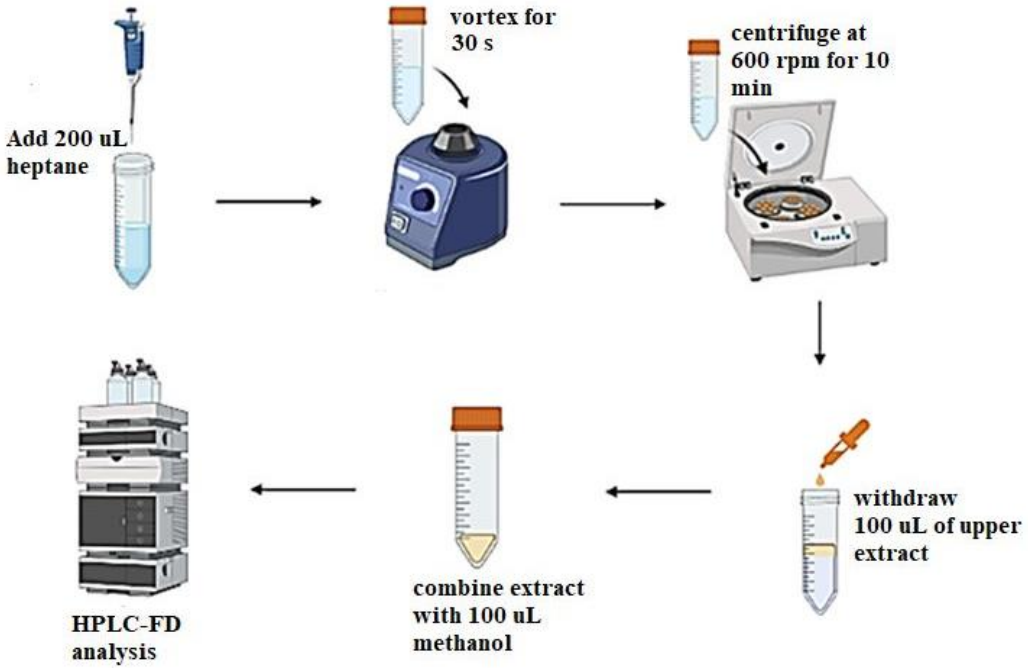

Figure 2. Schematic of solvent microextraction procedure.

Validation of Saponification-Solvent Microextraction-HPLC-FD Method

The linearity, precision, relative recovery, limit of detection (LOD), and limit of quantification (LOQ) of the method for analysing BAP in milk samples were evaluated. 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 10 times the blank signal. The relative recovery was assessed by spiking 2.5 μg L^{-1} of BAP into the sample before extraction. The relative recovery (RR) was then calculated using the following formula (Equation 1).

$$RR = \frac{C_{spiked\ sample} - C_{sample}}{C_{spiked}} \times 100\% \qquad \text{Equation 1}$$

Where, $C_{spiked\ sample}$ is the concentration of BAP in the spiked sample, C_{sample} is the concentration of BAP in the sample, and C_{spiked} is the concentration of BAP spiked.

RESULTS AND DISCUSSION

Optimisation of Solvent Microextraction

The most popular technique for removing lipids to prevent any contamination throughout the analysis is to apply the saponification process with an appropriate alkaline solution, such as sodium hydroxide [15]. The significant process involves the process of hydrolysis which releases free fatty acids by breaking down milk fats. The resulting fatty acids can be extracted with ease using an appropriate solvent [16]. The targeted PAHs in the samples would be substantially bound with fat if the saponification method was not performed.

One variable at a time approach was applied in the optimisation experimental process to identify the microextraction parameters that influenced the efficiency. Several key parameters that would have an impact on the effectiveness of the microextraction for BAP from milk samples, including types of extraction solvent, volume of extraction solvent, salting out effect and vortex time were examined in this study.

Effect of Extraction Solvent Type

Given that the principle of solvent extraction is based on the concept of "like dissolves like", both heptane and hexane are non-polar solvents, with a polarity index of 0.1, which are suitable for extracting the lipophilic BAP from the milk sample. Figure 3a indicates that heptane achieved greater extraction efficiency as compared to hexane. Therefore, heptane was chosen as the extraction solvent for further experiments. Even though these two solvents are non-toxic, low-density, and water-immiscible, aside from being less poisonous and volatile than hexane, heptane contributed to stability throughout the extraction phase to effectively extract the BAP [17]. Heptane is also regarded as a more environmentally friendly alternative. Furthermore, this low-density solvent has demonstrated an easier collection using a micro-syringe because of the larger density difference between the sample and heptane as compared to hexane [18]. This study did not involve a dispersive solvent which served to increase the surface area of the extraction solvent interacting with the targeted analyte as the methanol was already present throughout the saponification process. By doing this, the amount of solvent required during the experiment can also be minimised.

Effect of Extraction Solvent Volume

The volumes of heptane ranging from 200 to 400 μL were investigated in this study. As shown in Figure 3b, the extraction efficiency was at its highest when 200 uL of heptane was applied. This was because the BAP was enriched at a low volume. Thereafter, the extraction efficiency declined due to the dilution effect [19]. Hence, 200 μL was decided upon for the following procedures.

Effect of Salt Addition

The addition of salt can help decrease the solubility of targeted analytes in the aqueous phase and enhance their solubility towards the organic phase [20]. The salt attracts one part of a water molecule to reduce free water molecules in the system, which then increases the distribution ratio. By including different salt concentrations ranging from 0 to 7% of sodium sulphate (Na_2SO_4), the salting-out effect upon the effectiveness of extraction was examined. The saponified milk samples were added with the specified concentrations of sodium sulphate before the solvent microextraction. Figure 3c illustrates that an insignificant difference in peak areas was observed when different salt concentrations were added to the sample. Notably, the results did not reveal this phenomenon. Hence, the research experiments were conducted without using salt as a way to simplify the entire microextraction procedure.

Effect of Vortex Time

The effectiveness of analyte extraction can be influenced by vortex time. The surface area of contact between the extraction solvent and the targeted analyte can be increased by agitation to enhance the diffusion of the analyte into the extraction phase [21]. In this study, the mixing of extraction solvent and saponified sample was conducted from 30 s to 2 min. The mixing using a vortex mixer allowed the immiscible extraction solvent to form tiny droplets in the saponified sample. While at the vortex time of 30 s, the optimal extraction efficiency was attained and accompanied by high precision (Figure 3d). On the other hand, a drop in mass transfer was observed beyond the 30 s. This could be due to the mass transfer disruption induced by the back extraction [22]. Thus, the ideal vortex time for further tests was determined to be 30 s.

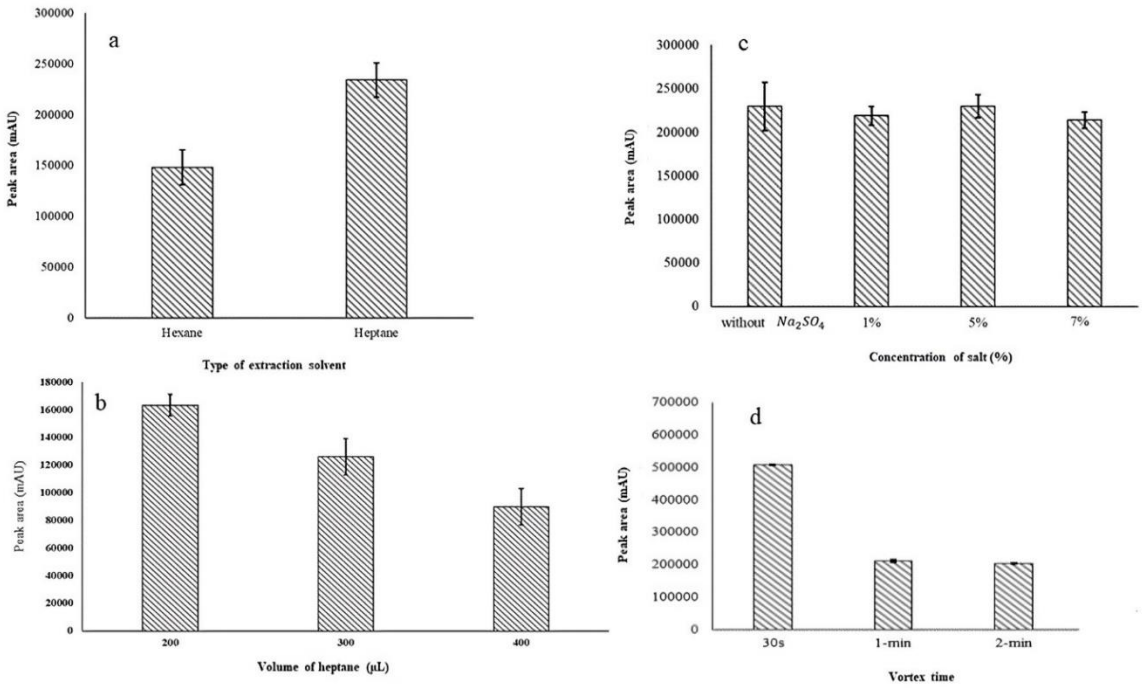

Figure 3. Effect of (a) type of extraction solvent, (b) volume of heptane, (c) concentration of salt, and (d) vortex time on the extraction of BAP from the spiked milk.

Validation of Saponification-Solvent Microextraction-HPLC-FD

As part of the validation process for analytical methods, it is crucial to evaluate the applicability of the method for the extraction of BAP in milk for the proposed application. Therefore, the developed and optimised saponification-solvent microextraction coupled with HPLC-FD was then validated for linearity, LOD and LOQ, precision, as well as relative recovery of BAP in milk samples.

The linearity was assessed with the aid of a mathematical relationship developed from the experimental results with an analyte at various concentrations by the employed range. The milk samples with a fat content of 3.3% were spiked with the BAP at five different concentrations (0.25-5.0 µg L^{-1}). The concentration of the milk sample blank was also determined and deducted from the spiked samples. The calibration curve was obtained by plotting the peak areas against the concentrations of the targeted analytes in the milk. The curve revealed a good linear behaviour of the developed method over the range of 0.25-5.0 µg L^{-1}, with coefficients of determination (R^2) of 0.9939.

LOD is referred to as the lowest concentration of analyte in a sample that is distinguishable from noise background based on the chromatographic signal. LOQ is known as the lowest concentration of analytes in a sample that is capable of being accurately and precisely quantified within experimental factors specified for the analytical method [23]. For determining LOD, a signal-to-noise ratio of 3 was employed, whereas for the determination of LOQ, a signal-to-noise ratio of 10 was applied. The LOD and LOQ values achieved in this research were 0.02 µg L^{-1} and 0.07 µg L^{-1}, respectively as summarised in Table 1. The results revealed that the saponification-solvent microextraction-HPLC-FD is capable of determining ultra-trace of the BAP in milk samples which then allows for routine monitoring of carcinogenic BAP in milk samples.

The relative recovery study was conducted to investigate the systematic error that could be caused by the matrix effects in different milk samples that varied in fat contents. The relative recovery study involved the addition of a known amount of the BAP to the milk samples that varied in fat content (1.0-3.3%) and then determining the amount that could be recovered from the samples by the saponification-solvent microextraction-HPLC-FD. Again, the sample blanks were determined and deducted from the spiked samples. The averages of relative recoveries (RR) in the range of 73.7% to 116.0% for BAP in different milk samples that were spiked with 2.5 µg L^{-1} of BAP were achieved, with acceptable repeatability indicated by relative standard deviations (RSD) values of less than 13.3%. The results suggested the saponification-solvent microextraction of the BAP offers a clean extract before HPLC-FD analysis due to the negligible matrix effects from the milk samples that varied in fat content. This was credited to the saponification procedure which released the lipophilic BAP from the fat to succeed in the accurate extraction and enrichment in the minimal extraction solvent.

Table 1. Validation of saponification-solvent microextraction-HPLC-FD for the determination of BAP in milk.

Analyte	Linearity range, µg L^{-1}	R^2	LOQ, µg L^{-1}	LOD, µg L^{-1}	Average of RR, % (n=3)
BAP	0.25-5.0	0.9939	0.02	0.07	73.7-116.0

Table 2. Comparison of accuracy results between saponification-solvent microextraction-HPLC-FD and saponification-solvent extraction-HPLC-FD.

Method	Average of RR or R, % ± RSD, % (n=3) Milk Fat content, %(w/v)	
	3.3	1.0
Saponification-solvent microextraction-HPLC-FD	73.7 ± 7.2	95.1 ± 5.4
Saponification-solvent extraction-HPLC-FD	110.0 ± 16.1	116.0 ± 2.1

The saponification-solvent microextraction was then compared to saponification-solvent extraction that utilised a total of 6 mL of extraction solvent for the extraction of BAP from milk after the saponification procedure. The comparison was made on their respective accuracy performance for the extraction of spiked milk samples with 2.5 µg L^{-1} of BAP. The accuracy of solvent extraction was expressed in the recovery (R) instead of relative recovery because it is an exhaustive extraction. Table 2 reveals that both methods attained good accuracy, although the saponification-solvent extraction recovered slightly higher BAP. This indicated that the non-exhaustive saponification-solvent microextraction could offer comparable performance as the exhaustive saponification-solvent extraction yet provide a greener alternative due to its minimal usage in organic solvent.

Application of Saponification-Solvent Micro-extraction-HPLC-FD

The proposed validated saponification-solvent micro-extraction-HPLC-FD was performed to quantify the BAP in milk samples. Three different brands with different fat contents ranging from 1.0-3.3% of milk samples were purchased from a local enterprise shop. The milk samples were extracted using the saponification-solvent-microextraction procedure in triplicates and analysed using HPLC-FD. The ultra-trace BAP was detected in the three samples with concentrations ranging from 0.15 to 0.25 µg L^{-1}, as well as the acceptable repeatability that was less than 11.5%. All values were well below the guidelines established by the European Food Safety Authority for milk samples, which is 1 µg L^{-1} [24].

CONCLUSION

Polluted crops and vegetation consumed for livestock feeding might be one of the reasons for the presence of BAP in food, such as dairy products. The fact that BAP has a likelihood of triggering diseases, such as cancer and mutation, is crucial and should be the primary goal to get rid of them along with eliminating trace and also ultra-trace amounts of BAP to keep people away from being exposed to them. The analysis of BAP has mostly been done using conventional sample preparation techniques up to this point in time. These techniques are generally expensive, time-consuming, as well as laborious. As a result, this study proved that saponification-solvent microextraction is capable of offering a sensitive and reliable method for the enrichment of BAP in milk samples. The applied method offers a few major advantages in terms of less poisonous low-density solvent and minimal organic solvent.

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