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Research Article

Headspace micro-solid phase extraction of pyrene in tea infusions and beverages

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Abstract

Tea is a significant beverage in many cultures and often serves a purpose beyond mere consumption. However, pyrene raises concerns regarding lung health and cancer risks for humans. Therefore, a sensitive and environmentally friendly headspace micro-solid phase extraction (Hs-μ-SPE) combined with fluorescence spectrophotometry (Fs) was examined and evaluated for analysing pyrene in tea infusions and beverages. The extraction of pyrene was performed using a commercial adsorbent termed Monotrap^{TM}. Microextraction parameters, such as extraction temperature, extraction time, stirring rate, type of desorption solvent, desorption time, and volume of desorption solvent, were investigated and optimised. The limit of detection (LOD) and limit of quantification (LOQ) of Hs-μ-SPE-Fs for determining pyrene were 12 and 15 μg L^{-1}, respectively, indicating the method's high sensitivity, despite the absence of chromatography instrumentation. A negligible matrix effect was observed, as indicated by the relative recovery of pyrene from spiked tea infusions and beverages, which ranged from 77.9 to 99.9%. Pyrene, at concentrations ranging from 29 to 99 μg L^{-1}, was detected in some commercially available tea infusions and beverages, potentially posing a health risk to regular consumers. The environmental impact of Hs-μ-SPE-Fs was then assessed using the analytical greenness metric for sample preparation (AGREEprep), a tool designed to evaluate the sustainability and environmental friendliness of sample preparation methods. With a total AGREEprep score of 0.61, the proposed Hs-μ-SPE-Fs method demonstrated above-average environmental performance. However, further improvements could be performed in areas such as on-site applicability and the use of renewable materials. Despite these limitations, the method offers a sensitive and accurate approach for detecting pyrene in tea infusions and beverages that is beneficial to tea lovers.

Keywords: AGREEprep, fluorescence, μ-SPE, PAH, tea

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds possessing characteristics of strong toxicity, mutagenicity, and carcinogenicity. Their stable molecular structures and extended half-life make removing them from the environment difficult [1]. PAHs can come from either natural sources or man-made activities. The United States Environmental Protection Agency (USEPA) has identified 16 PAHs as substances that could cause cancer or mutagenesis. This has eventually acquired most research interest, focusing on studying PAHs residue [2]. Open burning, seepage of coal or petroleum deposits, and volcanic activity are some of the natural sources of PAHs in the environment. The manufacturing of coke and aluminium, coal-tar pitch and asphalt, carbon black, home heating, coal gasification and liquefying facilities, catalytic cracking towers and related processes in petroleum refineries, and motor vehicle exhaust are some of the main anthropogenic sources of PAHs [3]. Humans are exposed to PAHs through a variety of routes, with food consumption being the main one for non-smokers. Natural and large man-made environmental sources can contaminate food, including industrial food processing methods, such as smoking, roasting, and drying, and various home cooking techniques like grilling, frying, and barbecuing. With proper control methods and being aware of the processes by which PAHs are created in food, contamination can be avoided.

One of the three drinks that is widely consumed worldwide is tea. China is a big producer, with the largest tea plantation area, production, and exports worldwide. Tea has been dubbed a "healthy beverage" due to credible research suggesting it consists of polyphenols such as catechins, flavonoids, anthocyanins, and phenolic acids [4]. Nevertheless, some recent studies revealed that tea also contains potentially harmful chemical substances such as PAHs [5]. PAHs can be found in the air, soil, and water. Due to the automobile exhaust, industrial emissions, and other forms of pollution, PAHs are present in these environments. These pollutants can be absorbed by tea plants from their environment. Additionally, due to their large surface area, tea leaves have the potential to absorb PAHs from air pollution and/or from burning coal or wood during the drying process [6]. After rain, $PM_{2.5}$ contaminates the soil because it absorbs and concentrates the majority of PAHs. As a result, PAHs will be absorbed by tea plants grown on contaminated soil. Since tea leaves must be dried by burning coal and wood, preparing the tea leaves could also introduce PAHs into the final product. When tea products come into contact with combustion gases, they can absorb PAHs, which are always present in those gases [7].

Pyrene is a type of PAH that is carcinogenic, mutagenic, and teratogenic. It has also been linked to several kinds of cancer in humans and experimental animals [8]. The PAH levels and profile of surrounding vegetation are rising as a result of the increasing average number of road vehicles and industrial activity in developing countries. This is because persistent PAHs will eventually enter the food chain through bioaccumulation and biomagnification [9]. Even though there are no regulatory agencies that have established a specific maximum residue limit for pyrene in tea, it should be monitored because it has various influencing factors on the content of contamination, like methods of processing them, geographic locations, contaminating plants and water, industrial activity and urbanisation near heavily industrialised areas, and atmospheric conditions [10]. Low-molecular-weight PAHs, such as pyrene, are more volatile and easier for tea leaves to absorb from the environment, and they are often found in tea. The combustion of organic materials, like coal or wood, which are frequently used to dry and treat tea leaves, can produce these smaller PAHs. Low-molecular-weight PAHs are also more likely to be found in tea infusions because they are more soluble in water [11].

Sample preparation describes the technique to separate a representative sample from a larger quantity and prepare it for examination. Steps in the sample preparation procedure are extraction and sample extract cleanup. In the traditional sample preparation process, a significant amount of hazardous organic solvents, which could be dangerous to analysts' health and the environment, are utilised, for example, liquid-liquid extraction (LLE) [12-13]. To address these serious flaws, LLE has been substituted with solid-phase extraction (SPE) [12] and quick, easy, cheap, effective, rugged and safe (QuEChERS) techniques, which are conducted with smaller amounts of solvents [14-15]. To date, microextraction [16-18] has emerged as an alternative to both LLE and SPE due to its micro and eco formats contributed by the application of minute amounts of chemicals and organic solvents throughout the microextraction process.

The study focused primarily on demonstrating the feasibility and potential advantages of the proposed approach, termed headspace micro-solid-phase extraction combined with fluorescence spectrophotometry, as an additional option for researchers and practitioners. In this study, the effectiveness of the approach in extracting pyrene from tea infusions and beverages was investigated. A detailed examination was conducted on the extraction variables to improve its extraction efficiency, including extraction temperature, stirring rate, extraction time, desorption solvent, desorption time and desorption solvent volume. The quantification of pyrene was performed using a fluorescence spectrophotometer. This was followed by minor method validation to determine pyrene in tea infusions and beverages to ensure the method's applicability for pyrene analysis. Finally, the greenness of the method was assessed using the analytical greenness metric for sample preparation to identify steps that could be further improved for an eco-friendlier analysis.

Materials and Methods

Chemicals and reagents

Monolithic Material Sorptive Extraction Monotrap™ was purchased from GL Sciences (Selangor, Malaysia). The pyrene reference standard was obtained from Sigma-Aldrich (Germany). Chromatography-grade acetonitrile, ethanol, isopropanol, and methanol were also sourced from Sigma-Aldrich (Germany).

Preparation of standard and sample solutions

The pyrene standard stock solution (500 mg L^{-1}) was prepared by dissolving 0.005 g of pyrene in methanol in a 10 mL volumetric flask and diluting to volume with methanol. The pyrene working standard solutions, ranging from 1 to 100 mg L^{-1}, were prepared by diluting the standard stock solution with methanol. All standard solutions were stored in a refrigerator at 4 °C when not in use.

A variety of tea packets and beverages, namely tea leaves bags and ready-to-drink green tea, were purchased from neighbourhood shopping centres in Kuala Nerus, Terengganu. A commercial packet bag containing red tea leaves (2 g) was immersed in 200 mL of boiling deionised water for five minutes to create a tea infusion [19]. Tea beverages were subjected to extraction without any pre-treatment.

Headspace micro-solid phase extraction

A needle tip was poked into a vial septum and passed through a Monotrap™ in a ring shape. The tip was then poked into a tiny septum to prevent the Monotrap™ from dropping into the sample solution. Next, the Monotrap™ was inserted into the headspace of a sample vial containing 40 mL of sample solution and a stir bar (2 cm × 0.5 cm). The vial cap was then screwed on. The needle was connected to a disposable syringe, and both the syringe and the sample vial were clamped in place with a retort stand to secure their positions. The sample vial was placed in a 500 mL beaker containing pre-heated water at 70 ˚C on a hot plate (IKA, Malaysia). The sample solution was stirred at 900 rpm at 70 ˚C for 20 min. After extraction, the Monotrap™ was removed and placed into a 10 mL glass vial containing 3 mL of acetonitrile. The tube was subjected to sonication for 5 min in an ultrasonic bath (Cole-Parmer, Thermo Fisher Scientific, United States). Finally, the extract was filtered with a 0.2 µm nylon syringe filter before fluorescence spectrophotometry (Fs) measurement. **Figure 1** summarises the headspace micro-solid phase extraction (Hs-µ-SPE) procedure.

Fluorescence spectrophotometry conditions

The standard solutions and extracts were analysed for pyrene using a fluorescence spectrophotometer (Varian Cary Eclipse, SpectraLab, Canada) by monitoring the excitation wavelength at 330 nm and the emission wavelength at 390 nm using a 1 cm quartz cuvette.

Optimisation of Hs-µ-SPE for the extraction of pyrene in spiked tea infusions

To enhance the extraction efficiency of Hs-µ-SPE, several extraction variables were investigated, including extraction temperature (40-80 ℃), extraction time (15-30 min), stirring rate (300-900 rpm), type of desorption solvent (ethanol, isopropanol, methanol and acetonitrile), desorption time (1-9 min), and volume of desorption solvent (3-6 mL). Tea infusion samples spiked with 0.5 mg L^{-1} of pyrene were used in the optimisation studies. The optimisation experiments were performed using a one-variable-at-a-time (OVAT) approach, where only one extraction parameter was modified while keeping other parameters constant.

Validation of Hs-µ-SPE-Fs for the determination of pyrene in tea infusions and beverages

Linearity, relative recovery (RR), limit of detection (LOD), limit of quantification (LOQ), and precision tests were conducted to validate the proposed Hs-µ-SPE-Fs for the determination of pyrene in tea infusions and beverages. Signal-to-noise ratios of 3:1 and 10:1 were used to determine the LOD and LOQ, respectively. RR was assessed using the samples spiked with 0.02 and 0.5 mg L^{-1} of pyrene and was calculated using the following formula.

$$RR\% = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad (1)$$

The initial pyrene concentration in the sample blank is denoted by C_{real}, the amount of pyrene spiked into the original sample is denoted by C_{added}, and the total pyrene found in the spiked sample is shown by C_{found}.

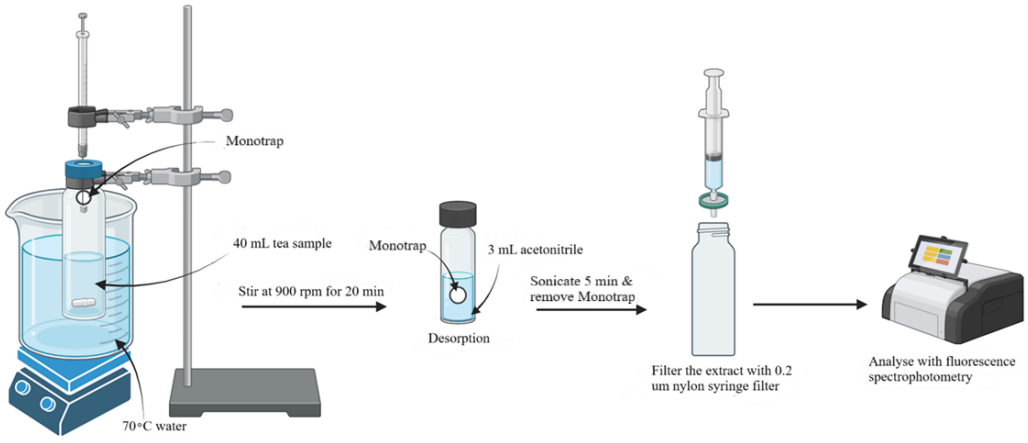

Figure 1. Headspace micro-solid phase extraction procedure using Monotrap™

Analytical greenness metric for sample preparation

The open-access analytical greenness metric for sample preparation (AGREEprep) loaded from https://agreeprep.anvil.app/ was utilised to assess the Hs-μ-SPE-Fs AGREEprep greenness metric score. The assessment criteria were based on the 10 principles of green sample preparation (GSP), which served as the foundation for evaluation. These included preferring the preparation of in situ samples, using safer reagents and solvents, paying attention to renewable, recyclable, and sustainable materials, reducing waste, reducing sample, chemical, and material quantities, increasing throughput for samples, encouraging automation and incorporating processes, reducing the amount of energy used, selecting the most environmentally friendly post-sample preparation setup of analysis, and assuring the operator of safe protocols.

After the evaluation was completed, a circular pictogram was then generated, featuring a central circle displaying the total score and ten trapezoid bars, each representing one of the 10 criteria. The length of each trapezoid bar corresponded to the weight assigned to that criterion. The colour of each element varied depending on the procedure performance, making it easy to identify the strong and weak aspects of the method and their contribution to the final score [20].

Results and Discussion

Optimisation of Hs-μ-SPE for the extraction of pyrene in spiked tea infusions

Optimising a new technique before applying it to real sample analysis is a scientific best practice and a necessary step to ensure credibility, efficiency, and compliance of analytical results. The effects of several extraction variables on the Hs-μ-SPE were investigated to optimise the extraction conditions of Hs-μ-SPE of pyrene from tea infusions and beverages. In each investigation, one variable was modified while the others remained constant. To assess the extraction efficiency of Hs-μ-SPE, 40 mL of tea infusion was spiked with 0.5 mg L^{-1} of pyrene before the extraction process. The experimental parameters that were examined included extraction temperature, extraction time, stirring rate, desorption solvent, desorption time, and desorption solvent volume.

Effect of extraction temperature

Lower temperatures often slow down molecular diffusion, causing the analyte to take a longer time to reach the adsorbent. However, they can also preserve thermally sensitive compounds, resulting in higher yields. Conversely, an increase in temperature typically enhances extraction yield and reduces extraction time by improving the diffusion rate and solubility of the analyte in the adsorbent [21]. Nevertheless, extremely high temperatures may diminish yield. The analyte might evaporate or decompose at excessively high temperatures, which then reduces the extraction yield. Therefore, temperature has a significant impact on the extraction yield [22].

In this study, the water bath temperature was modified and maintained before immersing the sample vial containing the sample solution. Increasing extraction temperature generally improves extraction yield by enhancing diffusion and volatility, but excessively high temperatures can lead to degradation of the extracted compounds [23]. The effect of extraction temperature was examined over a range of 40 to 80 °C. As shown in **Figure 2a**, the fluorescence intensity increased from 40 °C to 80 °C and the highest fluorescence intensity was observed at 80 °C. However, 80 °C was not considered ideal due to the formation of small bubbles in the sample solution and within the beaker, which served as a water bath. This phenomenon may have compromised data accuracy in prolonged analysis. A similar phenomenon was also observed in the report by Gevod and Borisov, whereby air conducts heat less well than water; air bubbles can serve as insulators [24]. The efficient heat transfer of the extraction vessel to the sample was diminished when bubbles formed around it. As a result, the extraction process may slow down or become inefficient in certain localised cooler areas. Therefore, 70 °C was selected as the optimal extraction temperature.

Effect of stirring rate

The equilibrium and extraction time can be shortened by using a higher stirring rate. Mass transfer is generally improved by increasing the stirring rate, which accelerates extraction and may result in higher yields [25]. In this study, stirring of the sample solution was conducted at rates ranging from 300 rpm to 900 rpm during extraction. As indicated in **Figure 2b**, the highest intensity was achieved when the sample was stirred at 900 rpm, suggesting that the mass transfer of the analytes to the headspace and Monotrap™ was significantly enhanced. A stirring rate beyond 900 rpm was not pursued, as the entire extraction system was not designed for such a high rate, causing the stir bar to agitate unstably in the sample vial. Therefore, 900 rpm was selected as the optimal stirring rate and applied in the subsequent experiment. In this experiment, the cause of the decrease in extraction efficiency at 500 rpm, which then increased again at 700 and 900 rpm, was

investigated. The bubble formation or turbulence at 500 rpm disrupted the system and caused a slight desorption of pyrene that was loosely bound [26], especially when the system was still under optimisation. The flow became more chaotic at lower speeds and stabilised as the speed increased, improving efficiency and vapour-phase replenishment.

Effect of extraction time

The amount of time required for analytes to be adsorbed onto the Monotrap™ and to reach equilibrium is crucial in attaining the best possible extraction efficiency and enrichment [23]. In this study, the extraction time was counted when the sample vial was immersed in the water bath and the sample solution was stirred. The extraction time, ranging from 15 to 30 min was examined. The maximum intensity for pyrene was attained after 20 min, as shown in **Figure 2c**. The intensity increased from 15 to 20 min, then started to decline slowly. The drop could be due to the system having reached equilibrium. The net amount of pyrene retained on the adsorbent may also be decreased by partial desorption or redistribution brought by the agitation turbulence. Therefore, 20 min was chosen as the best extraction time to pursue the next experiments.

Effect of desorption solvent

For effective desorption of analytes from the adsorbent, the analytes must exhibit a greater affinity to the desorption solvent rather than to the adsorbent itself. The structure of pyrene is hydrophobic and nonpolar with a log n-octanol/water partition coefficient ($\log_{Kow}$) of 4.88 [27]. Although it has very low solubility in water, it dissolves easily in polar organic solvents, namely acetonitrile, methanol, and ethanol. In this study, four organic solvents, namely ethanol, isopropanol, methanol, and acetonitrile (ACN), with viscosity values of 1.1, 2.4, 0.59 and 0.38 cP, respectively, were evaluated as potential desorption solvents. As shown in **Figure 2d**, acetonitrile yielded the highest fluorescence intensity for pyrene. This superior performance was attributed to the strong elution strength and excellent solubility of ACN for polycyclic aromatic hydrocarbons (PAHs), such as pyrene, which facilitated efficient desorption of pyrene from the reversed-phase C18-coated Monotrap™. As compared to other solvents, ACN more effectively disrupted the hydrophobic interactions between pyrene and the C18 phase, thereby enhancing desorption efficiency. Furthermore, the relatively low viscosity of ACN improved mass transfer, enabling the rapid release of pyrene into the solvent. These combined properties made ACN the most effective desorption solvent amongst those tested.

Effect of desorption time

Desorption time is the time taken for analytes to exit the adsorbent and enter the desorption solvent [28]. In this study, the desorption time was counted when the Monotrap™ was immersed in 3 mL of acetonitrile in a 10 mL glass vial and sonicated simultaneously. Sonication times from 1 to 9 min were investigated. Sonication was employed to enhance the desorption of pyrene from the Monotrap™ adsorbent into acetonitrile. The application of ultrasonic waves generated rapid vibration and cavitation in the solvent, which disrupted interactions between the analyte and sorbent surface. This mechanical agitation promoted efficient mass transfer, thereby accelerating the release of pyrene from the porous structure of Monotrap™ into the surrounding solvent. The desorption efficiency increased linearly as the sonication was extended from 1 to 5 min, eventually reaching the maximum desorption at 5 min (**Figure 2e**). Once the desorption equilibrium was reached, continual sonication may cause the analyte to be re-adsorbed onto the adsorbent, reducing overall recovery [29]. Therefore, a desorption time of 5 min was applied in the subsequent experiments.

Effect of desorption volume

Desorption volumes ranging from 3 to 6 mL of acetonitrile were examined in the current study (**Figure 2f**). The highest intensity was produced by 3 mL of desorption solution. Smaller desorption volume enriched pyrene in the extract, whereas larger desorption volume diluted it [30]. Nevertheless, the volume must be sufficient to ensure the submergence of Monotrap™ for a complete desorption process. Therefore, a minimum of 3 mL was required for the desorption process.

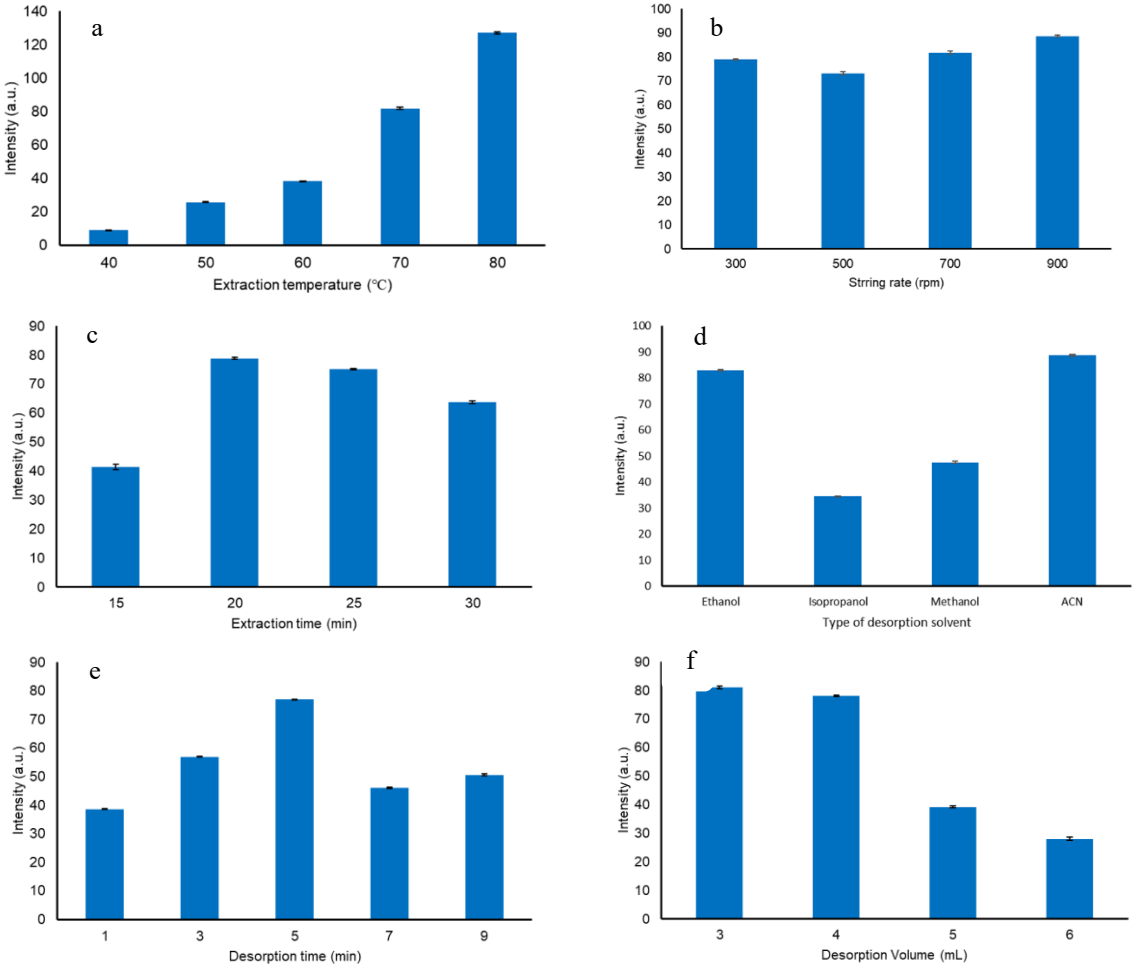

Figure 2. Effect of the extraction temperature (a), stirring rate (b), extraction time (c), desorption solvent (d), desorption time (e), and desorption volume (f) on the extraction efficiency of Hs-μ-SPE of pyrene from spiked tea infusion

Validation of Hs-μ-SPE-Fs for the determination of pyrene in tea infusions and beverages

To ensure precise, accurate, and repeatable results, a comprehensive validation of the proposed optimised Hs-μ-SPE combined with fluorescence spectrophotometry was performed under ideal extraction conditions. The evaluation included linearity, relative recovery, precision, LOQ and LOD. For Hs-μ-SPE to be suitable for the intended analytical applications, it must operate consistently and reliably.

The interaction between the result of a quantitative procedure and the actual analyte concentration is objectively described by linearity [31]. Matrix-matched calibration was conducted in this study to mimic the behaviour of the tea sample and suppress the signal of other organic interferences, as the spectrophotometry was applied rather than a chromatography technique. Pyrene concentrations ranging from 25 to 500 μg L^{-1} were spiked into the tea infusion samples before the Hs-μ-SPE procedure. The coefficient of determination (R^2) for the calibration curve was 0.9945, indicating strong linear behaviour. Linearity assessment confirmed the reliability of Hs-μ-SPE method in delivering consistent linear responses over a broad concentration range in the extract, as determined by fluorescence spectrophotometry.

LOD is the lowest concentration from a sample measurement that contains the component and from which it could be distinguished from the concentration of a blank sample measurement that does not contain the component [32]. LOQ is defined as the minimum amount of a material that can be measured with a reasonable level of accuracy and precision [33]. The LOD and LOQ derived from the investigation were 12 and 15 μg L^{-1}, respectively. The findings showed that the suggested Hs-μ-SPE-Fs could provide trace determination of pyrene in tea infusion and beverage samples.

In the accuracy study, three types of samples, namely red and green tea infusions and green tea ready-to-drink beverages, were spiked with pyrene at concentrations of 50 and 100 µg L^{-1}. The samples included blank controls, and the spiked samples were then analysed using Hs-µ-SPE-Fs.

Table 1 summarises the relative recovery (RR) results. Pyrene was detected in all spiked samples, and the RR was calculated after deducting the blank sample concentration. According to the Association of Official Analytical Collaboration (AOAC) International [34], an acceptable RR ranges from 80 to 110% for samples spiked at 100 ppb and 60 to 115% for samples spiked at 10 ppb, respectively. These ranges served as references to assess the accuracy of the proposed Hs-µ-SPE-Fs. It was found that the observed RR values fell within the acceptable ranges, and the precision, calculated using relative standard deviation (RSD), was within 10% when real samples were analysed. This indicated that the proposed Hs-µ-SPE-Fs could provide an accurate method for determining trace pyrene in tea infusions and beverages, with minimal matrix effects.

Application of Hs-µ-SPE-Fs in the analysis of real tea infusions and beverages

Pyrene residues of three different brands of tea samples, including tea infusions and beverages, were then examined using the proposed method. The amount of pyrene detected in each sample is shown in **Table 2**. Pyrene was present in all samples, which could be due to samples possibly being processed with water from the pipeline that was coated with bitumen or coal tar [35].

Greenness assessment of Hs-µ-SPE-Fs using AGREEprep

Using Monotrap™ as an adsorbent, the proposed Hs-µ-SPE-Fs for the determination of pyrene in tea infusion and tea beverages achieved a total greenness metric score of 0.61 (**Figure 3**). However, the suggested approach could be further improved to reach a perfect score of 1.0, with all displaying the green colour code.

The tea bag and tea beverage were purchased from a nearby store and taken to the laboratory for further analysis, making the sample preparation in this study ex-situ according to principle 1. In this study, none of the chemicals or reagents originated from sustainable or renewable sources, resulting in a low score and a red segment for principle 3. A 40 mL sample was required to ensure analyte enrichment for improved sensitivity when a fluorescence spectrophotometer was applied as a quantification instrument. This allowed for reliable and trace detection of pyrene, which also contributed to a red segment in principle 5. According to principle 7, the Hs-µ-SPE was performed manually in two steps: extraction or adsorption, and desorption. Additionally, the quantification process was also conducted manually, which led to a lower score in principle 7.

Table 1. Relative recovery for the proposed method

Samples	Spiked Concentration (µg L^{-1})	Average of Relative Recovery (%)	RSD (%), n=3
Red tea infusion	50	77.9	1.9
	100	91.2	0.3
Green tea infusion	50	88.5	0.7
	100	82.3	2.8
Green tea beverage	50	78.9	0.7
	100	99.9	0.7

Table 2. Concentration of pyrene residues in different tea samples analysed using optimised Hs-µ-SPE-Fs

Sample	Pyrene Residues (µg L^{-1})	RSD (%), n=3
Tea infusion A	54	1.6
Tea infusion B	29	2.6
Tea beverage A	99	0.7

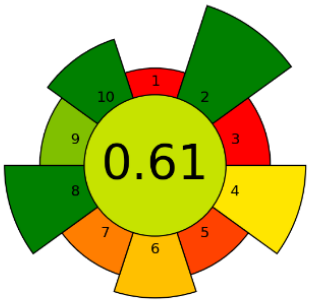

Figure 3. Graphical results of Hs-μ-SPE-FS for the determination of pyrene in tea infusions and beverages analysed using AGREEprep software v.0.9.

Conclusion

This study demonstrated the use of HS-μ-SPE-Fs to determine the amount of pyrene in tea infusions and beverages. This method offers sensitivity and reliability under optimal conditions, showcasing strong analytical qualities including excellent linearity, low detection and quantification limits, and satisfactory accuracy and precision. Additionally, the greenness assessment by the AGREEprep tool indicated that the HS-μ-SPE-FS method is an environmentally friendly approach with room for improvement in a few areas. The application of the proposed HS-μ-SPE-FS in analysing the selected tea infusions and beverages showed that the presence of pyrene residues in the tea samples is common. This highlights the importance of developing a sensitive and eco-friendly microextraction technique to monitor these residues and increase awareness amongst tea enthusiasts.

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