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Trace Determination of Phenanthrene and Fluorene in Beverages Using D-μ-SPE and Liquid Chromatography Analysis

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Dispersive micro solid phase extraction (D-μ-SPE) method using Graphene Oxide (GO) for the extraction and pre-concentration of selected polycyclic aromatic hydrocarbons (PAHs) from beverage samples prior to high-performance liquid chromatography - diode array detector (HPLC - DAD) is reported. Selected PAHs Fluorene (FLU) and Phenanthrene (PHE) were used as targeted analytes. An experimental design using Response Surface Methodology (RSM) and Box–Box–Behnken design (BBD) was performed to evaluate the interactive effects of three significant parameters: mass of sorbent, sample volume and extraction time. Under the optimum conditions, the method revealed good linearity (R^2 = 0.9995 – 0.9998) over a concentration range of 0.5 – 5.0 mg L^{-1}. The limit of detection (LOD) was in the range of 0.03 – 0.24 mg L^{-1} with satisfactory relative recoveries (81 – 99 %) and good relative standard deviation (RSD) of ≤ 3.80 % (n = 3). The method was successfully applied to tea, coffee, and milk samples and proved to be a simple, rapid, and reliable method with good extraction efficiencies for the detection of PAHs in beverage samples.

Keywords: dispersive micro solid phase extraction; response surface method; Box – Behnken design; polycyclic aromatic hydrocarbons; beverage samples

I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are nonpolar organic compounds made up of primarily carbon and hydrogen atoms. PAHs are common environmental contaminants consisting of two or more fused benzene rings in linear, angular or cluster arrangements generated during the incomplete combustion or high-temperature pyrolysis of organic materials such as coal, oil, wood, or other organic foods. PAHs are mostly colourless, white, or pale-yellow solids (Dos *et al.,* 2019; Shi *et al.,* 2016).

PAHs are reported to possess carcinogenic and mutagenic properties and potent immune suppressants (Abdel-Shafy, 2016). The effect of PAHs on human health is determined by their concentration, method of exposure, and relative toxicity. Generally, PAHs can contaminate foods through air, water, and soil exposure, and they are considered ubiquitous in the environment.

Tea, coffee and milk are four of the most widely consumed beverages in the world. It has been reported that the total amount of PAHs in brewed coffee ranges from 0.52 to 1.8 μg/L (Orecchio *et al.,* 2009). PAH levels in tea infusions have been reported to be 10.5 μg/L while milk and milk powders may contain between 20 – 42 μg/L of PAHs (Ciemniak *et al.,* 2019; Rawash *et al.,* 2018).

Various sample preparation techniques have been used in the analysis of PAHs. PAHs are often extracted in various matrices using traditional procedures such as liquid-liquid

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extraction (LLE) and solid phase extraction (SPE) (Loh *et al.,* 2018). However, large amounts of organic solvent are used during extraction, which is costly and harmful. These approaches are not environmentally friendly and are time-consuming (Houessou *et al.,* 2005; Anthemisis *et al.,* 2009).

Dispersive Solid Phase Extraction (DSPE) is an alternative approach developed by Anastassiades and co-workers in 2003 in which the sorbent is mixed with the sample matrix or its extract. The method is also known as QuEChERS, as it is quick, easy, cheap, effective, rugged, and safe. DSPE can retain matrix components while maintaining the analyte in the liquid phase by introducing small amounts of solids. This method also increases the selectivity of the determination by maintaining the analyte in a cleaner liquid phase (Chisvert *et al.,* 2019). Compared to conventional SPE, DSPE reduces sample treatment time, allows more samples to be examined in shorter time, and is simple, versatile, and easy to handle (Islas *et al.,* 2017).

Recently, Dispersive Micro Solid Phase Extraction (D-μ-SPE), a simpler and miniaturised form of DSPE, has been developed (Nascimento *et al.,* 2019). D-μ-SPE can extract and enrich nonsteroidal anti–inflammatory drugs (NSAIDs), pesticides, PAHs, heavy metal ions and bisphenol A (Ferreira *et al.,* 2007). In addition, techniques for micro-scale sample preparation are being actively developed as they promote green chemistry (Loh *et al.,* 2018). Although D-μ-SPE is a selective, sensitive, and rapid method to extract PAHs from food samples as well as minimising solvent usage, the integrated effect of the mass of sorbent, sample volume and extraction time can be better determined using Response Surface Methodology (RSM). This may be achieved by employing a Box–Behnken Design (BBD) to construct three-dimensional (3D) surface and contour plots.

In this study, the RSM and BBD approaches were employed in D-μ-SPE of selected PAHs, namely fluorene (FLU) and phenanthrene (PHE) in beverage samples using High-Performance Liquid Chromatography with diode array detector (HPLC–DAD). This method was expected to extract PAHs (fluorene and phenanthrene) rapidly and efficiently.

II. MATERIALS AND METHOD

A. Chemical Reagents

Selected Polycyclic Aromatic Hydrocarbons standards, namely fluorene (FLU) and phenanthrene (PHE), were purchased from Sigma-Aldrich (Missouri, United States). All of the solvents that were used in the experiment were of HPLC grade and purchased from Kermel Chemicals (Tianjing, China). The extraction method was carried out with dichloromethane and isopropanol as solvents, and the samples were analysed with acetonitrile. Graphene oxide (GO) was used as sorbent and procured from Advance GO company (Germany). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) (37%) were obtained from Merck (Darmstadt, Germany).

B. Preparation of Stock and Standard Solution

The individual stock solutions of FLU and PHE were prepared separately in HPLC-grade methanol to a final concentration of 1000 mg L^{-1}. A standard mixture of 100 mg L^{-1} was prepared by diluting 10 mL of each stock solution with methanol to a final volume of 100 mL. A series of working standard solutions were prepared by dilution in methanol before analysis to prevent the decomposition of analytes. Spiked beverage samples were prepared by adding 1 mL of 10 mg L^{-1} standard solutions into 9 mL of deionised water to obtain a final concentration of 1 mg L^{-1}. All standard solutions were stored at 4°C in glass vials in the refrigerator when not in use.

C. Sample Preparation

Coffee (Sin Sing), tea (BOH) and milk (Farm Fresh) samples were obtained from the local supermarkets. All the samples were stored in the chiller at 4°C prior to analysis.

D. Dispersive Micro Solid Phase Extraction (D-μ-SPE) Procedure

Twenty milligrams of Graphene Oxide (GO) were added to 10 mL of the aqueous mixture (pH=3). The mixture was vigorously stirred with a magnetic stirrer for 15 min to trap the analytes. Subsequently, the GO powder was isolated from the solution by centrifugation at 4000 r min^{-1} for 5 min, and

the supernatant was discarded. 2 mL of desorption solvent (isopropanol) was added to the centrifuge tube and sonicated for another 15 min. The mixture was then centrifuged at a speed of 4000 r min^{-1} for 5 min. The solvent was collected and evaporated to 1 mL under a gentle stream of nitrogen gas. 1 mL of the extracted analyte was transferred into a 1 mL amber glass vial. Finally, 20 µL of the extract was injected into the HPLC system. A schematic illustration of the D-µ-SPE procedure is shown in Figure 1.

F. Experimental Design

To obtain the optimum conditions for the simultaneous extraction of PAHs, a Box–Behnken Design (BBD) of Response Surface Methodology (RSM) was employed to optimise three independent variables, namely the mass of sorbent, sample volume and extraction time. The experimental design was generated using Design Expert version 6.0.4 (Stat-Ease Software) for regression analysis with the coded level of selected factors (-1, 0, +1), as shown in Table 1.

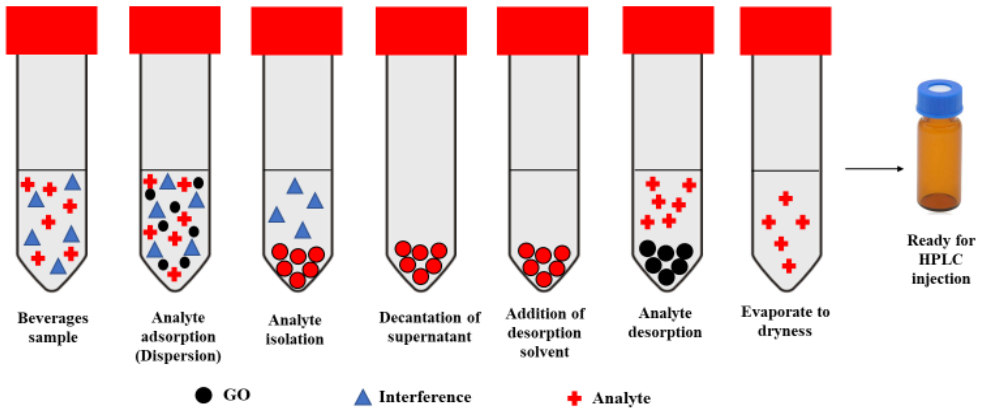


Figure 1. A schematic illustration of the D-µ-SPE procedures.

Table 1. Coded values of variables for the experimental design.

Factor	Parameter	Coded Level of Variables		
		-1	0	+1
A	Mass of sorbent (mg)	70	110	150
B	Sample volume (mL)	1	10.50	20
C	Extraction time (minutes)	5	15	25

E. Chromatographic Conditions

The determination of targeted analytes was performed using a High-Performance Liquid Chromatograph (Agilent G1313A HPLC) equipped with diode array detector (DAD), RPC18 column and 20 µL sample loop. The targeted PAHs were chromatographically separated using the isocratic gradient mobile phase of acetonitrile-water (80:20, v/v) at a flow rate of 1.0 mL min^{-1} and an injection volume of 20 µL. The chromatography data were detected at a wavelength of 254 nm.

G. Validation of Analytical Method

The validation of D-µ-SPE included an assessment of the linearity (R^2), the limit of detection (LOD), the limit of quantification (LOQ), precision and accuracy to ensure that the analytical procedure was reliable and fit for the intended purpose. LOD and LOQ were calculated based on the linear regression of the calibration curve. Precision was expressed in terms of relative standard deviation (RSD %) and accuracy by the percentage relative recovery.

III. RESULT AND DISCUSSION

A. Experimental Design using BBD

BBD is an effective design as it provides the best compromise between the number of experiments and the degrees of freedom for 3 factors, as well as reducing the number of experimental trials (Ferreira *et al.,* 2007). A set of experiments consisting of 17 runs was generated with a design matrix consisting of three levels of the three factors. The experimental results are shown in Table 2.

Optimisation for the extraction of two types of PAHs using D-μ-SPE-LC yielded three optimised conditions, which are 110 mg mass of sorbent, 10.50 mL of sample volume and 15.00 min extraction time. Equation 1 is the regression equation of the fitted model, where Y is the response (total peak area) of target analytes, A is the mass of sorbent, B is sample volume, and C is the extraction time.

$$Y = 19.10 - 0.76A + 3.07B + 0.16C - 1.76A2 - 4.22\ B2 - 1.59C2 - 0.16AB - 0.34AC - 0.33BC \quad \text{(Eq.1)}$$

Table 2. Box Behnken Design (BBD) for the analysis of selected PAHs.

Run	Mass of Sorbent (mg)	Sample Volume (mL)	Extraction Time (min)	Total Peak Area of PAHs (mAu*sec)
1	150.00	10.50	5.00	245.992
2	150.00	10.50	25.00	224.802
3	70.00	20.00	15.00	298.504
4	110.00	10.50	15.00	353.364
5	110.00	10.50	15.00	372.113
6	110.00	1.00	5.00	88.2451
7	70.00	10.50	5.00	250.254
8	110.00	10.50	15.00	359.412
9	150.00	20.00	15.00	216.959
10	110.00	20.00	5.00	273.779
11	70.00	1.00	15.00	125.032
12	70.00	10.50	25.00	272.144
13	110.00	10.50	15.00	376.596
14	110.00	10.50	15.00	362.114
15	110.00	20.00	25.00	273.399
16	110.00	1.00	25.00	114.376
17	150.00	1.00	15.00	86.0032

Table 3. Results of ANOVA for the Regression Models.

Source of Variation	Sum of Squares	DF	Mean Square	F Value	P Value	
Model	188.28	9	20.92	85.64	< 0.0001	Significant
A	4.64	1	4.64	19.01	0.0033	
B	75.30	1	75.30	308.26	< 0.0001	
C	0.20	1	0.20	0.83	0.3917	
A^2	13.08	1	13.08	53.54	0.0002	
B^2	74.93	1	74.93	306.73	< 0.0001	
C^2	10.59	1	10.59	43.33	0.0003	
AB	0.10	1	0.10	0.42	0.5381	
AC	0.47	1	0.47	1.92	0.2089	
BC	0.43	1	0.43	1.76	0.2260	
Residual	1.71	7	0.24			
Lack of Fit	1.46	3	0.49	3.05	0.1369	Not significant
Pure Error	0.25	4	0.062			
Total	189.99	16				

B. Analysis of Variance

Analysis of variance (ANOVA) and regression analysis were used to assess the significance of variables presented (P-values), sum of squares, mean square, lack of fit test (F-values) and degree of freedom (DF). Multi-linear regression was applied to the results of the BBD. The effect of independent variables such as sorbent mass, sample volume, and extraction time was evaluated by second order (quadratic). The data are presented in Table 3.

The results revealed the statistical significance of the second-order equation, and regression was for all analytes. The P-value obtained was <0.0001, which indicates the significance of the regression model. The "Lack of Fit F-value" of 3.05 implies that the Lack of Fit is not significant relative to the pure error. The P-value obtained indicates that there was a 13.69 % statistical probability that the large "Lack of Fit F-value" could be observed. The high F-values and low P-values proved the reliability of the fitted model.

Table 4 presents the summary of the ANOVA regression model for the response quadratic model for FLU and PHE. The value of R^2 shows that there is an acceptable relationship between the predicted and actual values. The R^2 value of a well-fitted model should be at least 0.80. The calculated R^2 value for the extraction of two PAHs was 0.9910, and the adjusted R^2 was 0.9794, indicating a well-fitted model and significance. The coefficient of variation (CV) of 3.18 %, which was less than 10 %, indicates that the model is reproducible. The prediction residual errors sum square (PRESS) value of 0.24, which is low, also supported the model.

The parity plot shows a satisfactory correlation between the actual and predicted values, where the points clustered around the diagonal line indicate a good fit for the model (Figure 2). The differences between the actual or experimental values and the predicted values are very small, with an average difference of less than 1.

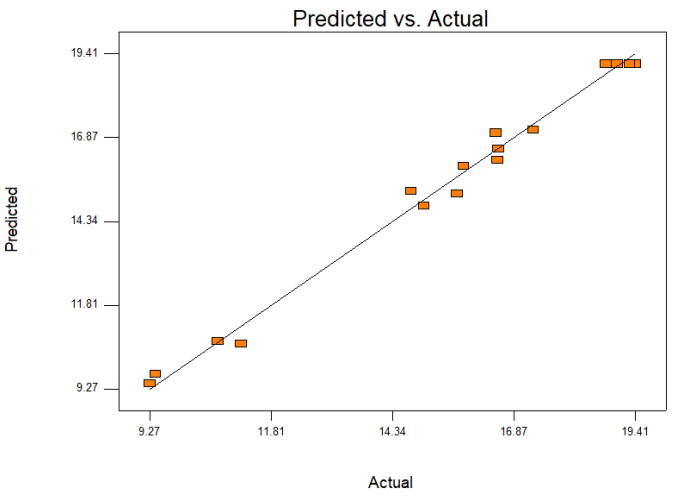


Figure 2. The parity plot between predicted and actual (experimental) values for all analytes.

Table 4. Summary of ANOVA Analysis of PAHs.

Transform	Model	Lack of Fit	DF	R-square	Equation
Square Root	Quadratic Significant	Not Significant	9	0.9910	Sqrt (Total Peak Area) = Y = 19.10 – 0.76A + 3.07B + 0.16C – 1.76A2 – 4.22 B2 – 1.59C2 – 0.16AB – 0.34AC – 0.33BC

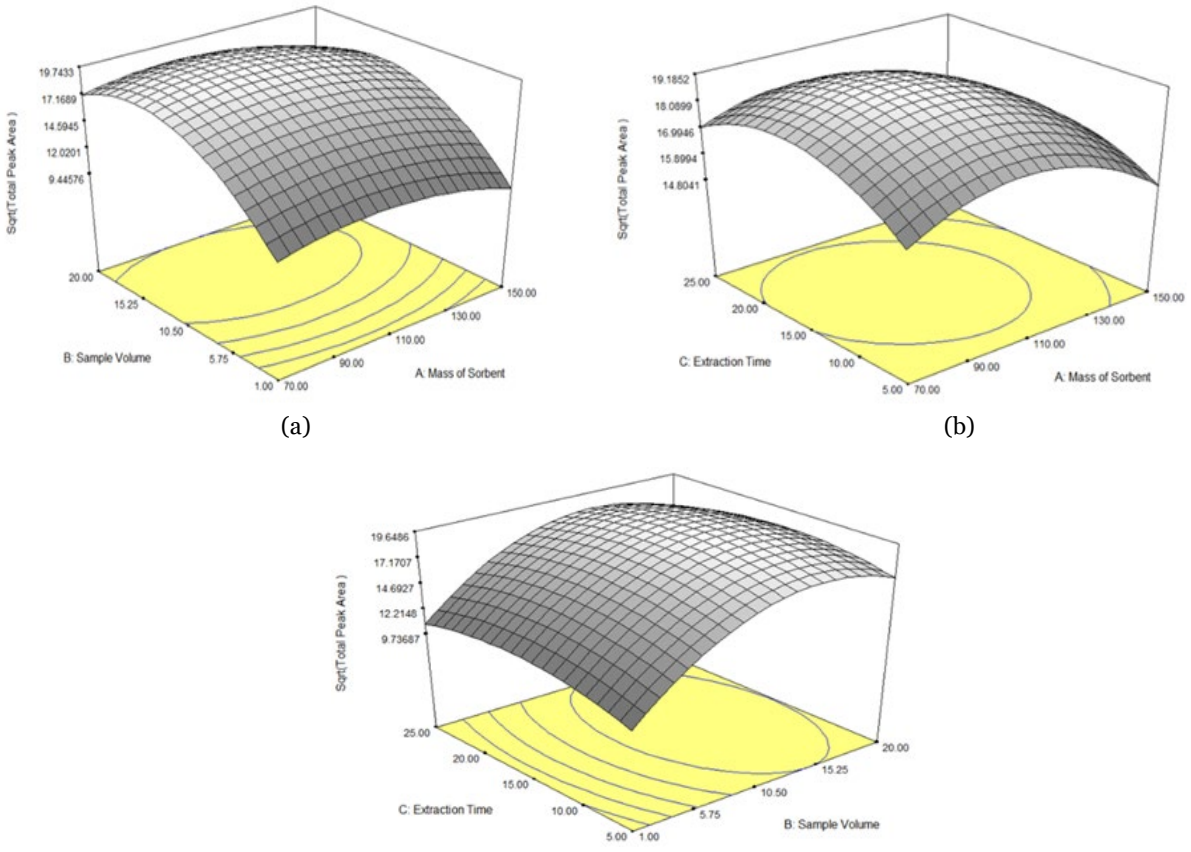


Figure 3. RSM 3-D Contour Plots for GO-D-μ-SPE-LC of (a) mass of sorbent and sample volume, (b) mass of sorbent and extraction time, (c) sample volume and extraction time.

C. Response Contour Plot

The results of the BBD experiments were further visualised in the form of three-dimensional (3D) surface and contour plots. RSM was used to investigate the integrated effect of the mass of sorbent, sample volume and extraction time in the form of 3D plots. As illustrated by Figure 3, the variables acted in parallel, which considerably influenced the response or peak area.

Figure 3 (a) represents the interaction between the mass of sorbent and sample volume at a specific time of extraction (15 min) and their effect on the peak area of two selected analytes. It indicates that at low mass of sorbent and less sample volume, the total peak area of analytes was low and increased gradually when the mass of sorbent and sample volume

increased. The highest peak area of analytes (376.596 mAu*s) was obtained at 110 mg sorbent and a sample volume of 10.50 mL. The increment of GO mass used from 70 – 110 mg resulted in an increase in the peak area. However, the use of more than 110 mg of sorbent and a sample over 10.50 mL led to a decrease in the peak area of analytes.

Figure 3 (b) shows the response for the interactive factors of mass of sorbent and extraction time with the sample volume (10.50 mL) kept constant. At low mass of sorbent and extraction time, the total peak area of analytes was low but increased with increment in sorbent and extraction time. The highest peak area of analytes (376.596 mAu*s) was obtained at 110 mg of sorbent and an extraction time of 15 min. However, as the mass of the sorbent exceeded 110 mg and the extraction time exceeded 15 min, the total peak area of the analytes decreased as the targeted analytes may have been extracted back into the sample matrices from the sorbent.

Figure 3 (c) represents the interaction between the sample volume and extraction time at constant mass of sorbent (110 mg). The lowest peak area of analytes (86.003 mAu*s) was obtained at 1 mL of sample volume and 5 min extraction time. The highest peak area of analytes (376.596 mAu*s) was obtained at 10.50 mL of sample with 15 min of extraction time. However, it is also observed that when the sample volume exceeded 10.50 mL while the extraction time exceeded 15 min, the peak area of analytes decreased. This indicates that efficiency gradually decreased. Therefore, the optimum mass of sorbent, which is 110 mg, 10.50 mL of sample volume and extraction time of 15 minutes, was chosen for subsequent experiments.

D. Method Validation and Analytical Performance of D-μ- SPE-LC

The optimisation of the D-μ-SPE-LC method was then validated for linearity, precision, and relative recoveries. A calibration curve was generated using five (5) concentrations of standard mixture in the range of 2 to 10 mg L^{-1} with three replicates. Table 5 presents validation data of the D-μ-SPE method of PAHs in the coffee, milk and tea samples.

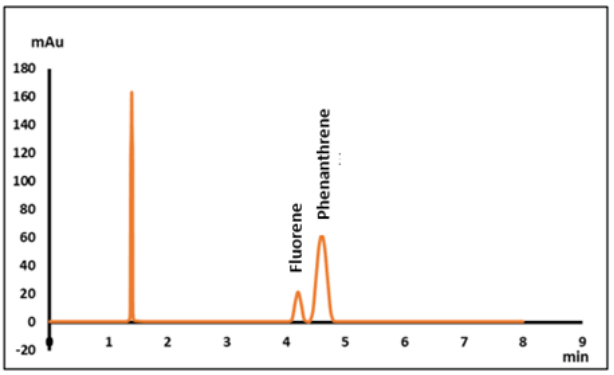
Linear curves for each analyte were obtained with a good correlation coefficient (R^2 = 0.9995-0.9998) and good reproducibility with a relative standard deviation (RSD) of ⩽ 3.4 %. The sensitivity of the method expressed as LOD was calculated using the linear regression method, and the results were in the range of 0.03 - 0.24 mg L^{-1}. As for the LOQ, the results were in the range of 0.08 - 0.72 mg L^{-1}. A percentage recovery study was done by spiking the beverage samples to give a final concentration of 0.5 and 5 mg L^{-1}. Results showed that good percentage recoveries were obtained in the range of 81 % to 99 % with a relative standard deviation (RSD) of ⩽ 3.8 % (Table 6). Thus, the D-μ-SPE method proved to be a simple, sensitive, selective, and green extraction method that could potentially be used in the chemical laboratory for routine analysis of beverage samples.

Table 5. Quantitative results of D- μ-SPE-LC of PAHs in coffee, milk and tea samples.

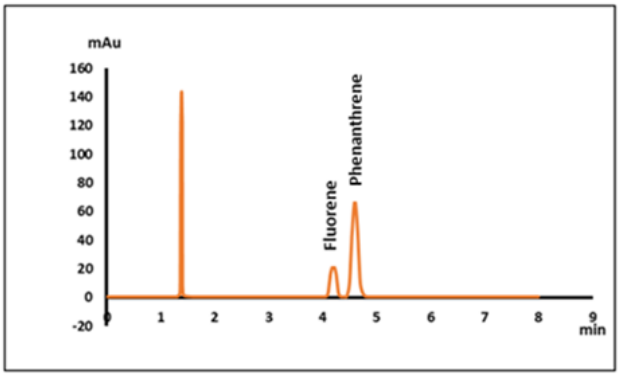
Sample	Analyte	Linear range (mg L^{-1})	Coefficient of determination (R^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})	RSD (%) (n=3)
Coffee	FLU	2-10	0.9997	0.03	0.08	1.4
	PHE	2-10	0.9995	0.07	0.22	2.3
Milk	FLU	2-10	0.9996	0.21	0.63	2.5
	PHE	2-10	0.9998	0.09	0.27	2.0
Tea	FLU	2-10	0.9998	0.12	0.36	3.4
	PHE	2-10	0.9997	0.24	0.72	2.1

Table 6. Relative recovery studies of DSPE-LC of PAHs from different spike beverage samples (n=3).

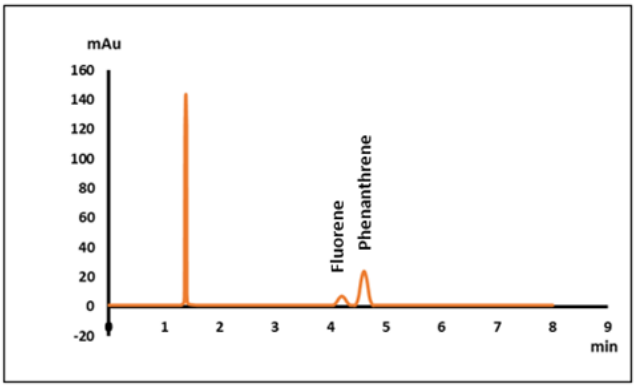
Analyte	Spiked concentration (mg L^{-1})	Coffee		Milk		Tea	
		Relative Recovery (%)	RSD (%)	Relative Recovery (%)	RSD (%)	Relative Recovery (%)	RSD (%)
FLU	0.5	99	3.8	94	1.5	97	2.3
	5	91	1.2	96	0.6	95	2.8
PHE	0.5	82	0.4	85	1.1	85	0.9
	5	90	0.7	81	2.2	88	1.4



(a)



(b)



(c)

Figure 4. LC chromatograms of the selected PAHs in (a) coffee, (b) tea and (c) milk samples.

Table 7. Comparison study of the determination of PAHs by SPE with other published methods.

Analysis method	Sorbent	Type of sample	Linear range (ng/mL)	LOD (ng/mL)	Recoveries (%)	Ref.
D-μ-SPE-GC-FID	β-CD-starch	Water	0.1 – 1000	0.01 – 0.07	84.1 – 94.8	(Yazdanpanah *et al.,* 2021)
D-μ-SPE-GC-FID	MGO/HMDI/β-CD	Soil, tree leaves, water	5.0 – 1000	0.1 – 0.5	73.0 – 97.1	(Majd *et al.,* 2021)
MD-μ-SPE-LC	Fe3O4/Cu: CuO/GO-NC	Vegetable, fruit, water	5.0 – 3200	0.015 – 0.061	95.1 – 106.8	(Asfaram *et al.,* 2020)
HF-SPME-HPLC-UV	MWCNT/ZrO2	Coffee, tea	0.1 – 200	0.033 – 0.16	92.0 – 106.0	(Yazdi *et al.,* 2018)
D-μ-SPE-LC	GO	Tea, coffee, milk	2000 – 10000	30 – 240	81.0 – 99.0	This work

The HPLC-DAD chromatograms of spiked coffee, tea and milk samples concentration in 10 mg L^{-1} of mixed polycyclic aromatic hydrocarbons are shown in Figure 4 (a), Figure 4 (b) and Figure 4 (c), respectively. The chromatograms revealed that all analytes were successfully extracted and separated from the beverage samples. Comparison between the efficiency of the developed D-μ-SPE method for PAHs with previously reported methods in terms of linear range, LODs, and percentage recoveries is summarised in Table 7.

IV. CONCLUSION

In conclusion, this study has shown that D-μ-SPE of selected PAHs in beverage samples using High-Performance Liquid Chromatography with a diode array detector (HPLC–DAD) can be optimised by setting up the mass of sorbent, sample volume and the extraction time using response surface method (RSM). Optimum conditions to achieve the highest peak area were found to be 110 mg of mass of sorbent, 10.50 mL of sample volume and 15 min extraction time. This method was successfully applied for the extraction of fluorene (FLU) and phenanthrene (PHE) with relative recoveries between 81 – 99 %.

V. ACKNOWLEDGEMENT

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