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# Rapid and Sensitive Determination of Polycyclic Aromatic Hydrocarbons in Green Tea Samples by Dispersive Liquid-Liquid Microextraction Coupled to High-Performance Liquid Chromatography-Diode Array Detection

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## Abstract

A rapid and sensitive dispersive liquid-liquid microextraction (DLLME) has been developed for the extraction of selected polycyclic aromatic hydrocarbons (PAHs), namely phenanthrene (PHE) and fluoranthene (FLA) in green tea beverage samples. The extracted PAHs were separated and determined by using high-performance liquid chromatography diode array detection (HPLC-DAD). An experimental design using response surface methodology (RSM) and central composite design (CCD) was performed to evaluate the interactive effects of the 2 most significant parameters, namely extraction time and sample pH. The optimal conditions were sample pH of 6.5 with 1.25 min extraction time. The analysis of variance (ANOVA) showed that the most influential parameter was the extraction time. The predicted values were obtained in good agreement with the actual value ($R^2 \geq 0.9086$). Under the optimal extraction conditions, the method demonstrated acceptable linearity ($R^2 \geq 0.9996$) over a concentration range (10 - 1,000 µg $L^{-1}$) for different PAHs. The limits of detection and quantifications were in the range of (4 - 10 µg $L^{-1}$) and (18 - 50 µg $L^{-1}$), respectively. Good analytes recovery (102 - 110 %) and excellent precision level with relative standard deviations (RSD %, n = 3) were obtained between 1.53 and 4.52 %. The method was successfully applied to the analysis of spiked green tea beverage samples. The proposed method is rapid, reliable, sensitive, and environmentally friendly for the detection of PAHs.

**Keywords:** Dispersive liquid-liquid microextraction, Experimental design, green tea, HPLC, PAHs

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that consist of 2 or more fused aromatic rings [1]. PAHs generally originate from incomplete combustion of organic materials such as coal, meats, tobacco, and wood. Considering their ability to accumulate in living organisms and soils, PAHs can resist biodegradation [2]. PAHs are known mutagens or carcinogens and they have been blamed as one of the leading causes of cancer in humans [3]. Since PAHs compounds exist at very low concentrations and were found at high-level interferences of matrices, a great microextraction technique such as sensitive, reliable, rapid was required before instrumental analysis [4].

Green tea is one of the famous beverages containing antioxidants that have been consumed by hundreds of millions of people worldwide. It has potential health benefits of reducing the occurrence of cardiovascular diseases, anti-microbial activity, regulating blood sugar, and promoting digestion [5]. Nevertheless, studies have shown that tea leaves contain some PAHs organic contaminates that might reveal harmful effects to human health [6]. The primary sources of PAHs contamination in green water are usually soil pollution [7]. It was also reported that the manufacturing process of tea mainly affects the transfer of PAHs to the end of tea products [8].

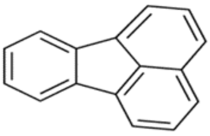
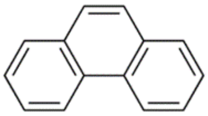
Therefore an efficient sample preparation method is crucial to measure interferences and enrich the target analysis of PAHs residues in green tea. Great efforts have been made to explore and develop green microextraction that focuses on the green microextraction features such as fast extraction time, minute organic consumption, reduced cost, and low energy [9]. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) have been successfully applied for the determination of PAHs in green tea beverages [10]. However, both methods are time-consuming, significant tendency to form emulsions, consumed of large volumes of solvents are needed, and disposal of toxic or flammable chemicals. In fact, this method also requires several successive extractions to recover more than 99 % of the analyte [11].

Aware of the PAHs pollution issue, green analytical chemistry such as solid-phase microextraction (SPME) [12], stir bar sorptive extraction (SBSE) [13], solid-phase membrane tip extraction (SPMTE) [14], magnetic solid phase (MSPE) [15] and dispersive liquid-liquid microextraction (DLLME) [16] have been reported to have been developed and practices into research with the aim to reduce and eliminate the drawbacks conventional extraction.

Dispersive liquid-liquid microextraction (DLLME) was developed to extract organic compounds and inorganic compounds in different matrices such as pharmaceuticals, food, and environmental samples. This method has proven to be an efficient method due to the several advantages: Simple, low cost, high enrichment factor, high recovery factors, and rapidity [17]. In the DLLME method, the extraction procedure is based on the complete dispersion of an extraction solvent into an aqueous sample assisted with disperser solvent. DLLME provides very high-performance extraction efficiencies such as fast extraction time, low cost, simple and low consumption of organic solvent [18].

In this study, dispersive liquid-liquid microextraction (DLLME) coupled with a high-performance liquid chromatography diode array detector (HPLC-DAD) was developed for the analysis of selective PAHs, namely fluoranthene (FLA) and phenanthrene (PHE) in green tea beverage samples. **Table 1** shows the molecular structure of the studied PAHs. Through this work, response surface methodology (RSM) and central composite design (CCD) were used to identify the interaction between the 2 most independent parameters (sample pH and extraction time) to achieve high extraction efficiencies.

**Table 1** Molecular structure of the studied PAHs.

| Compound | Structure | Molecular weight (amu) | Boiling point (°C) | Molecular formula |
|---|---|---|---|---|
| Fluoranthene (FLA) |  | 202 | 384 | $C_{16}H_{10}$ |
| Phenanthrene (PHE) |  | 178 | 340 | $C_{14}H_{10}$ |

## Materials and methods

### Chemical and reagents

Phenanthrene (PHE) and fluoranthene (FLA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and methanol were HPLC grade purchased from Merck (Darmstadt, Germany). Reagent grade 1-octanol was obtained from Sigma-Aldrich (USA). Stock solutions (1,000 mg $L^{-1}$ of each analyte) were prepared by dissolving in acetonitrile (PHE) and methanol (FLA). Working standard solutions were prepared weekly using methanol from stock standard solution. For calibration standards in the extraction procedure, spiked samples (1,000 µg $L^{-1}$) were prepared by adding both standard solutions into deionized water. All standard solutions are store in the dark at 4 °C when not in use.

### Sample analysis

Green tea beverage samples were obtained from local retail shops. The samples were subjected to extraction without any pre-treatment.

### Chromatographic conditions

High-performance liquid chromatography (HPLC) (Agilent Technologies, Milan, Italy) coupled with a diode array detection (DAD) (Agilent Technologies) was used throughout the analysis. A ZORBAX Eclipse Plus C18 column (4.6×250 mm, 5 µm) from Agilent was used to carry out the chromatographic separation of PAHs using isocratic mobile phase ACN-water (80:20) (v/v) at column temperature of 30 °C with fixed flow rate, injection volume, and detection wavelength at 1.5 mL $\text{min}^{-1}$, 10 µL and 254 nm, respectively. Agilent Chem Station software was used to process chromatographic data.

### Procedure dispersive liquid-liquid microextraction

Sample (3 mL) was pipetted into a centrifuge tube with a suitable range of pH and a mixture of 50 µL of 1-octanol (extraction solvent) and 200 µL of ACN (disperser solvent) was rapidly injected into the sample. The resulting cloudy solution was left to stand at room temperature over a suitable period of time to allow the analytes to separate. Then, the sample was centrifuged at 4,000 rpm for 5 min, and the floated extraction solvent droplet was withdrawn. The extraction solvent was diluted with methanol to make it compatible with the HPLC system. **Figure 1** shows the schematic for the DLLME technique.

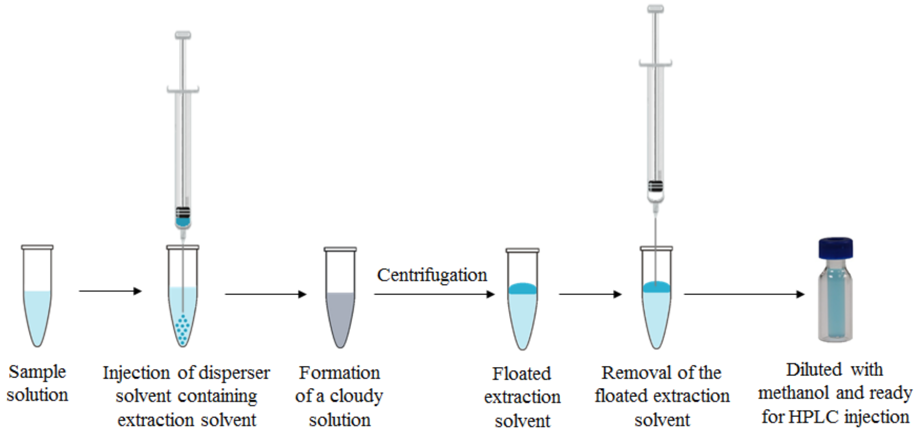


**Figure 1** Schematic of DLLME procedure.

### Optimization of DLLME procedure

Factors that can affect the efficiency of the analyte extraction from the sample, such as the pH of the sample and extraction time, were studied to find optimum conditions for the extraction of analytes, response surface methodology (RSM) based on central composite design (CCD) as a multivariate statistic technique was used. The experiment was designed using Design-Expert version 6.0.4 (Stat-Ease Software) for regression analysis of the experimental data fit the equations. The optimum conditions were used in real sample analysis.

### Validation of an analytical method

In order to ensure the reliability of the method and instrument used are good for the determination of analytes, validation process based on the limit of detection (LOD), the limit of quantification (LOQ), precision, and recovery analytes were conducted.

## Results and discussion

### Experimental design using RSM and CCD

In order to obtain the optimum extraction, the 2 most important parameter extraction time and sample pH were studies. RSM and CCD were applied to optimize both parameters and get a predictive model that adequately represents changes in the response depending on the input variables. RSM is a valuable method for analyzing the relationship between several influencing variables on one or more response variables simultaneously [19]. CCD is used to conduct a second-order mathematical model relating the enrichment factor with significant independent variables. The optimum conditions were predicted using the mathematical model and the 3-dimensional (3D) response surface [20]. The following equation determined the total number of design point needed (N);

$$N = 2f + C_p \tag{1}$$

where f is the number of parameters and $C_p$ is the number of center points. Therefore, 13 experiments had to be carried out for the CCD ($C_p = 5$) of 2 parameters.

Based on the results from the experiments the second order polynomial equation was obtained by the following equation;

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{12} AB + \beta_{22} B^2 \tag{2}$$

For Phenanthrene (PHE) recovery;

$$Y = -14.68 + 6.72A + 8.64B - 0.40A^2 - 0.83AB - 1.16B^2$$

For Fluoranthene (FLA) recovery;

$$Y = -12.71 + 5.48A + 2.56B - 0.43A^2 + 0.05AB - 1.35B^2$$

Where Y is the response (total peak area), $\beta_0$ is the intercept, A (pH), B (extraction time), and $\beta_1, \beta_2, \beta_{11}, \beta_{12}, \beta_{22}$ are terms representing those parameters of the model which are optimized iteratively to fit.

### Analysis of variance (ANOVA)

ANOVA was developed to evaluate the fitness and significance of the model, the precision, the effect of the parameters, and their interactions on the response. The F-values was implied that the model is significant, and the *p*-values (probability of error value) less than 0.05 indicated the significance of the term whereby, in this study, both of the analytes have significant model terms [21]. To express the quality of fit of the polynomial model equation, the coefficients of determination ($R^2$, adjusted $-R^{2,}$ and adequate precision) were applied. $R^2$ was used to measure the variation around the mean explained by the model in which, in this study, $R^2$ for extraction of PHE was 0.9086 and 0.9157 for extraction of FLA. The adjusted $-R^2$ was adjusted for the number of terms in the model in which, in this study, the adjusted $-R^2$ for extraction of PHE was 0.8433 and 0.8555 for extraction of FLA. As the number of terms in the model increases, the adjusted $-R^2$ decreases if those additional terms do not add value to the model. In Eq. (2), the value of the coefficient of the effects shows how the response changes regarding these parameters and the absolute value of a coefficient shows the effectiveness of the related impact. Adequate precision is a signal-to-noise ratio that will compare the range of predicted values at the design points to the average prediction. Ratios greater than 4 indicate the adequate model discrimination, whereby in this study, the adequate precision for extraction for PHE is 5.263 and 8.394 for extraction for FLA.

### Response contour plot

Variables (parameters) giving quadratic and interaction terms with the most significant absolute coefficients in the fitted model (Eq. (2)) were chosen for the axes of response surface plots to account for the curvature of the surfaces. This is used to visualize the interaction between the response and experimental levels of each parameter. Thus, the response was plotted against 2 experimental parameters. **Figures 2(a) - 2(b)** show 3D response surfaces and contour plots of the model in which the responses were mapped against 2 experimental parameters for PHE and FLA, respectively.

Sample pH was one of the most important parameters which affect the extraction efficiency of DLLME. In this study, the sample pH was conducted in the range of 4.38 to 8, as suggested by RSM, to obtain optimum pH of maximum extraction efficiency for DLLME. The pH of the sample was modified by using 0.1 M NaOH and 0.05 M HCl. **Figure 2** shows that the extraction efficiency of DLLME for PAHs metabolites was maximum at pH 6.5. Both PAHs analytes have their pKa in the range of 14 - 15, in which the analytes behave as weak acids due to lower dissociation constant [22]. Thus, it might be the reason for obtaining maximum extraction efficiency of PAHs at slightly acidic pH of the sample.

Extraction time was also an important parameter in this study since DLLME was a non-exhaustive system, thus making it is time-dependent. The extraction time of DLLME can be defined as the time interval between the injection of the mixture of disperser (acetonitrile) and extractant (1-octanol) solvents and their contact time with the sample before centrifugation (4,000 rpm for 5 min). It was studied in the range of 0.19 - 2.31 min based on suggestion data from RSM to determine the effect of the extraction time on the extraction efficiency. The extraction equilibrium was achieved within a minute as the dispersive solvent provided a large contact surface area between the analytes and the extraction solvent. The homogeneously distributed extraction solvent favored the transfer of the analytes by a simple solvent partition which then enhanced the DLLME extraction efficiency. Still, the prolonged extraction time broke the equilibrium since the emulsion solution was unstable and lead to low extraction efficiency [23]. Thus, 1.25 min of extraction time was adopted in the subsequent experiments. The equilibrium state was achieved quickly, and it became the advantage of applying the DLLME technique.

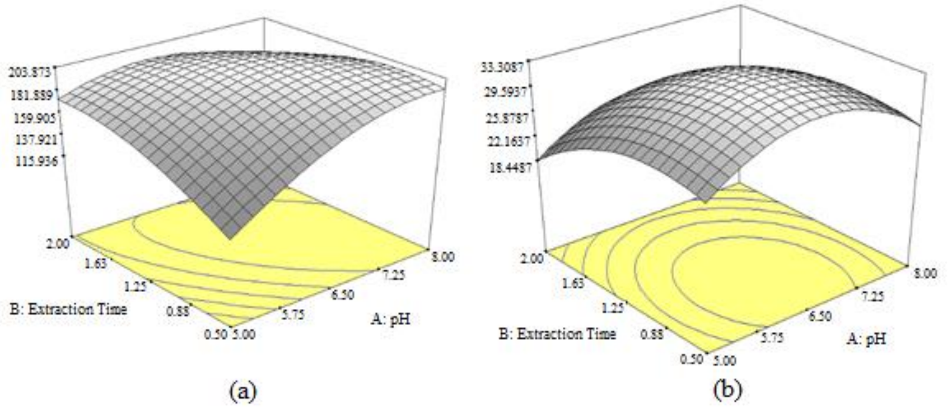


**Figure 2** Estimated response surfaces with related contours by plotting enrichment factor versus pH (A) and extraction time (B) for phenanthrene (a) and fluoranthene (b).

### Method validation and analytical performance of DLLME

To investigate the applicability of the proposed method for determining PAHs, several factors, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and percentage recovery, were evaluated. The linearity test was established using 5 different concentrations for both analytes. The calibration graph was plotted using peak area (mAUs) as a function against the concentration (mg $L^{-1}$) under the optimal conditions. The coefficient of determination ($R^2$) was obtained for both analytes in which were 0.9996 for PHE and 0.9997 for FLA. The LOD (3S a/b) and LOQ (10S a/b) values were determined based on linear regression. The LOD value for extraction of PHE is 4.0 and 10.0 µg $L^{-1}$ for FLA. The LOQ value extraction of PHE is 18.0 and 50.0 µg $L^{-1}$ for FLA. Relative standard deviation (RSD) was used to determine the precision of the method by analyzing 3 spiked deionized water ($n$ = 3) at 2 different concentration levels (0.2 and 1.0 mg $L^{-1}$). **Table 2** shows the validation data of DLLME.

**Table 2** Validation data of DLLME-HPLC-DAD method for selected polycyclic aromatic hydrocarbon in spiked green tea beverage samples ($n$ = 3).

| **Analyte** | **Linearity range, µg $L^{-1}$** | **Linear equation** | **$R^2$** | **LOD, µg $L^{-1}$** | **LOQ, µg $L^{-1}$** |
|---|---|---|---|---|---|
| PHE | 10 - 1,000 | $y = 190.17x + 162.04$ | 0.9996 | 4.0 | 18.0 |
| FLA | 10 - 1,000 | $y = 27.32x + 3.95$ | 0.9997 | 10.0 | 50.0 |

The accuracy of the extraction method from the sample matrices was determined by spiking the sample with PHE and FLA standard solution at a concentration of 10 mg $L^{-1}$. The relative recoveries of PHE and FLA from the analysis of spiked samples were determined in which 110.20 % for extraction of PHE and 102.94 % for FLA. The analysis was replicated ($n$ = 3) and gave % RSD values of 4.52 % for extraction of PHE and 1.53 % for FLA. **Table 3** shows the relative recovery studies of DLLME using spiked green tea beverage samples. **Figure 3** shows the HPLC chromatogram of the analyte.

**Table 3** Relative recoveries studies of DLLME-HPLC-DAD.

| Analyte | Average relative recovery, % | Average RSD, % |
|---|---|---|
| PHE | 110.20 | 4.52 |
| FLA | 102.94 | 1.53 |

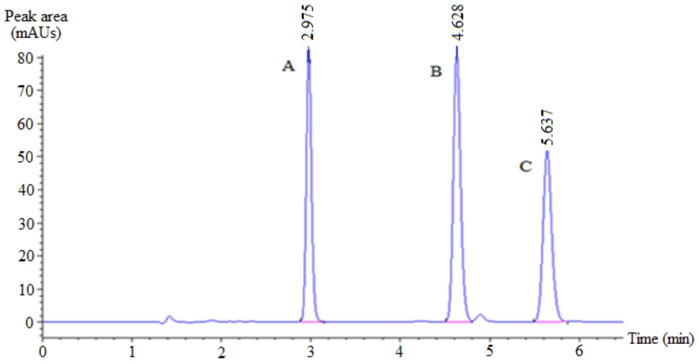


**Figure 3** HPLC-DAD chromatogram of analyte (A), PHE (B) and FLA (C) at 10 mg L$^{-1}$ (composition B:C = 3:1), injected for peak identification. LC conditions: acetonitrile (A) and water (B) were used as mobile phase (composition (A:B):80:20) at a flow rate of 1.5 mL min$^{-1}$, C18 column (4.6×250 mm ×5 µm), column temperature: 30 °C and a diode-array detector (DAD) at 254 nm (sample) and 360 nm (reference).

### Real sample analysis

The developed DLLME-HPLC-DAD method was successfully applied to the tea beverage sample under the optimal conditions to study its capability to extract PHE and FLA from the actual sample. The analysis showed that the average residues of PHE (1.31 mg L$^{-1}$) and FLA (0.73 mg L$^{-1}$) were detected in tea beverages at a much higher concentration than the LOQ. **Table 4** shows the result of the application of DLLME on the green tea beverage sample.

**Table 4** Result on DLLME application on green tea beverage samples (*n* = 3).

| Sample no. | Residue level, mg L$^{-1}$ | |
|---|---|---|
| | PHE | FLA |
| 1 | 1.31 | 0.75 |
| 2 | 1.30 | 0.70 |
| 3 | 1.31 | 0.73 |
| Average Concentration | 1.31 | 0.73 |

### Comparison with other reported methods

The comparisons of the DLLME-HPLC-DAD and other methods published previously for extraction and determination of PAHs from green tea samples are tabulated (**Table 5**). The developed method exhibits high potential as an alternative procedure for PAHs analysis. SPE involves multi-stage operation, which is tedious but achieved exhaustive extraction while HS-SPME and MWCNT-AFME are non-exhaustive systems. Therefore, the extraction is time-dependent. The present method also shows excellent relative recoveries and good precision comparable to MWCNT-AFME, SPE-GC-MS. The LOD achieved by this method can be observed to be comparable to other established methods such as HS-SPME, MWCNT-AFME, SPE-GC-MS, and Agaraose-Chitosan-C18 Film-Micro-SPE-HPLC. DLLME-HPLC-DAD, offers fast extraction time compared to other methods, reducing energy consumption and increasing analytical productivity.

**Table 5** Comparison of results of this study with those other published methods for determining PAHs in green tea samples.

| **Analysis methods** | **LOD,** (**μg L$^{-1}$**) | **Extraction time, min** | **Precision,** (RSD), (%) | **Recovery,** (%) | **References** |
|---|---|---|---|---|---|
| HS$^a$-SPME$^b$ -HPLC$^c$ -FLD$^d$ | 0.004 - 0.145 | 60 | 4.0 - 16.0 | - | [24] |
| MWCNT$^e$-AFME$^f$-μ-HPLC-UV | 0.0001 - 0.05 | 40 | 0.8 - 4.5 | 91.1 - 107.2 | [25] |
| MSPE-GCMS | 0.02 - 14.3 | 5 | 0.1 - 9.5 | 75.6 - 112.4 | [26] |
| Agarose-Chitosan-C$_{18}$ Film -Micro-SPE-HPLC | 0.549 - 0.673 | 20 | $\leq$ 13.53 | 100.8 - 105.99 | [27] |
| DLLME-HPLC-DAD | 4 - 10 | 1.25 | 1.35 - 4.52 | 102 - 110 | Present study |

## Conclusions

The current work demonstrates the application of the DLLME method coupled with HPLC-DAD to determine PAHs in green tea beverages, and it was successfully developed and applied. RSM optimized DLLME parameters based on CCD, and the optimal conditions were used during the analysis of PHE and FLA in the tea beverage samples. This method was proven green and extracted the non-polar PHE and FLA with good sensitivity. In addition, the procedure was simple, rapid, sensitive, and minimized organic solvent consumption.

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