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## Determination of Polycyclic Aromatic Hydrocarbons in Beverage by Low Density Solvent Based-Dispersive Liquid-Liquid Microextraction-High Performance Liquid Chromatography-Fluorescence Detection

(Penentuan Hidrokarbon Polisiklik Aromatik dalam Minuman dengan Pengekstrakan Cecair-Cecair Serakan Berdasarkan Pelarut Berketumpatan Rendah-Kromatografi Cecair Prestasi Tinggi-Pengesanan Pendarfluor)

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### ABSTRACT

A simple and fast dispersive liquid-liquid microextraction (DLLME) technique using organic solvent lighter than water has been developed for the extraction of selected polycyclic aromatic hydrocarbons in green tea, chrysanthemum tea and coffee beverage. The 1-octanol and acetonitrile were pre-mixed and injected into the sample solution. After dispersing, the cloudy solution was subjected to centrifugation to separate the solution into 2 phases, where the micro-droplet of 1-octanol was clearly floated on the top layer. The 1-octanol extract was then diluted and injected into high performance liquid chromatography-fluorescence for the quantitative analysis. The technique offered rapid analysis as the 1-octanol was homogeneously dispersed in the sample solution thus speeding the analytes diffusion. Under the optimized extraction conditions, the technique achieved trace detection limits in the range of 0.001 to 0.3  $\mu\text{gL}^{-1}$  for the targeted analytes, namely phenanthrene, fluoranthene and benzo[a]pyrene. The method was successfully applied to the spiked green tea, chrysanthemum tea and coffee beverage samples with good average relative recoveries obtained in the range of 86.7 to 103.0%. The utilization of low density organic solvent as extraction solvent has allowed for easier operation and eliminated the use of hazardous halogenated solvent that is commonly applied in DLLME.

**Keywords:** Beverage; dispersive liquid-liquid microextraction; high performance liquid chromatography-fluorescence detection; low density solvent; polycyclic aromatic hydrocarbons

### ABSTRAK

Suatu teknik pengekstrakan cecair-cecair serakan (DLLME) yang mudah dan cepat menggunakan pelarut organik berketumpatan lebih rendah daripada air telah dibangunkan untuk mengekstrak hidrokarbon polisiklik aromatik terpilih dalam minuman teh hijau, teh kekwa dan kopi. 1-oktanol dan asetone nitril dipracampur dan disuntik ke dalam larutan sampel. Selepas penyerakan, larutan yang kabur tertakluk kepada pengempuran untuk mengasingkan larutan kepada 2 fasa, dengan titisan mikro 1-oktanol terapung dengan jelas pada lapisan atas. Ekstrak 1-oktanol seterusnya dicairkan dan disuntik ke dalam kromatografi cecair prestasi tinggi-pendarfluor untuk analisis kuantitatif. Teknik ini menawarkan analisis yang cepat kerana 1-oktanol diserakkan dengan homogen dalam larutan sampel yang seterusnya mempercepatkan resapan analit. Dalam keadaan pengekstrakan yang optima, teknik ini mencapai had pengesanan dalam lingkungan 0.001 to 0.3  $\mu\text{gL}^{-1}$  untuk analit sasaran, iaitu fenantrena, fluorantena dan benzo[a]pirena. Kaedah ini berjaya diaplikasikan kepada sampel minuman teh hijau, teh kekwa dan kopi yang dipakukan dengan analit sasaran dengan perolehan semula purata yang baik dalam lingkungan 86.7 to 103.0%. Penggunaan pelarut organik berketumpatan rendah telah membolehkan operasi yang lebih mudah dan menyingkirkan penggunaan pelarut berhalogen yang berbahaya dan biasa digunakan dalam DLLME.

**Kata kunci:** Hidrokarbon polisiklik aromatik; kromatografi cecair prestasi tinggi-pengesanan pendarfluor; minuman; pelarut berketumpatan rendah; pengekstrakan cecair-cecair serakan

### INTRODUCTION

Determination of trace pollutants or contaminants in biological, food or other complex samples always requires excessive sample preparation to release the target compounds from unnecessary binding prior to instruments analysis. A survey conducted by LCGC North America in 2013 showed that 95% of the respondents deal with the liquid samples and these samples are seldom analyzed

without any pretreatment (Majors 2013). A clean sample is always a must to produce better chromatography, low detection level and less instrument maintenance. The green microextraction technique such as liquid phase microextraction (LPME) has been increasingly developed, modified and in-house validated for the analysis of food samples (Asensio-Ramos et al. 2011; Ratola et al. 2008; Sanagi et al. 2013; Wu et al. 2015; Yamini et al. 2015) due

to the markedly reduced amounts of organic solvent used and simplified procedures as compared to liquid-liquid extraction (LLE).

Single drop microextraction (SDME) was among the first miniaturized LLE introduced in 1996 (Liu & Dasgupta 1996). The basic principles of SDME and LLE are identical where an organic solvent immiscible with water is used as the extractant. Dispersive liquid-liquid microextraction (DLLME) was first described by Rezaee et al. (2006). The basic principle is quite similar to cloud point extraction. The technique involves three phases where the extraction solvent with a high density and immiscible with the sample and the dispersion solvent that is miscible with both sample and extraction solvent are injected into an aqueous sample. The homogeneously distributed extraction solvent drops favor the transfer of the analytes by simple solvent partition (Zang et al. 2009). The extraction solvent is separated by centrifugation and then analyzed. The main advantages attributed to DLLME are simplicity of operation, rapidity, inexpensive, powerful enrichment and being environmentally benign (Liu et al. 2009). Dispersive liquid-liquid phase microextraction based on solidification of floating organic (DLLME-SFO) has been first demonstrated in 2008 (Leong & Huang 2008) to simplify the withdrawal of extraction solvent by applying organic solvent that is less toxic and solidifies at low temperature.

Polycyclic aromatic hydrocarbons (PAHs) are organic environmental pollutants identified as mutagen or carcinogen in 1976 (Bjorseth & Ramdahl 1985). Their presence in vegetation (Meharg et al. 1998; Simonich & Hites 1995; Tuteja et al. 2011) from atmospheric deposition and food processing at elevated temperature was frequently reported and these included tea leaves (Fledler et al. 2002; Lin et al. 2006, 2005) and coffee bean (Garcia-Falcon et al. 2005; Houessou et al. 2007). The transfer of PAHs to the tea and coffee beverage may be attributed to the factors such as tea variety, tea/water ratio, tea brewing time and coffee bean roasting steps during manufacturing process (Houessou et al. 2007; Lin et al. 2006). The benzo[a]pyrene has been used as a marker to indicate the occurrence of PAHs and its maximum residue level has been set at 1.0  $\mu\text{g}/\text{kg}$  of baby foods (including infant formulae) by European Union (European Union 2006). To the best of our knowledge, none of the regulations recommends on the maximum allowable PAHs in any beverage.

Solid phase extraction (SPE) (Garcia-Falcon et al. 2005; Kayali-Sayadi et al. 1998; Lai et al. 2004), dispersive SPE followed by LLE (Sadowska-Rociek et al. 2014) and solvent extraction followed by column chromatography clean-up (Bishnoi et al. 2005; Lin et al. 2005) have been extensively applied to extract PAHs in tea and coffee samples. Both SPE and LLE techniques are among the most popular sample pretreatment techniques mainly because they are selective and do not require expensive equipment. However, both techniques are tedious and consume large amount of organic solvents and chemicals, which are less supportive towards green analysis. Microextraction techniques such as headspace solid phase microextraction

(Viñas et al. 2007), stir bar sorptive extraction (Zuin et al. 2005) and multi-walled carbon nanotubes-impregnated agarose film microextraction (Loh et al. 2013) have been demonstrated to overcome the shortcoming. The microextraction techniques enhanced the analytes enrichment, which indirectly offered ultra-trace detection limits. However, the application of micro-scale sample preparation techniques to extract PAHs from tea and coffee is still limited.

In this work, 1-octanol was applied as the extraction solvent in DLLME for the analysis of PAHs in some common beverage samples. The method shortens the routine analysis time and its microextraction format contributes to minimize the use of chemicals and organic disposal.

## MATERIALS AND METHODS

### REAGENTS AND CHEMICALS

Acetone, acetonitrile and methanol (HPLC grade) and 1-octanol (AR grade) were purchased from Merck (Darmstadt, Germany). Selected polycyclic aromatic hydrocarbons (PAHs) namely phenanthrene (PHE), fluoranthene (FLUO) and benzo[a]pyrene (BaP) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Standard stock solutions of PHE, FLUO and BaP each at 500  $\text{mgL}^{-1}$  were prepared individually by dissolving 0.005 g of each analyte in methanol (FLUO) and acetonitrile (PHE and BaP). The stock solution mixture was prepared from 500  $\text{mgL}^{-1}$  standard stock solutions. Serial working solutions were prepared daily by diluting the stock solution mixture with methanol prior to use. The solutions were stored at 0°C in darkness when not in use.

### CHROMATOGRAPHIC CONDITIONS

All analyses were performed using high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) coupled with a fluorescence detection (FD) (Shimadzu, Kyoto, Japan). The chromatographic separation of PAHs was carried out on a reversed phase C18 column (4.6  $\times$  250 mm, 5  $\mu\text{m}$ ) purchased from Agilent. The separation was performed using isocratic mobile phase acetonitrile-water (80:20) (v/v) at column temperature of 30°C. The flowrate, injection volume and detection wavelengths were fixed at 1.0 mL/min, 10  $\mu\text{L}$  and 250/400 nm of excitation/emission wavelengths, respectively.

### DISPERSIVE LIQUID-LIQUID MICROEXTRACTION (DLLME)

The sample (3 mL) was placed in a 5 mL centrifuge tube. A mixture of 50  $\mu\text{L}$  of 1-octanol (extraction solvent) and 200  $\mu\text{L}$  of acetonitrile (disperser solvent) was rapidly injected into the sample with a 1 mL disposable syringe. The resulting cloudy solution was left to stand at room temperature for 1 min to allow for the analytes partitioning. The tube was then centrifuged at 4000 rpm for 5 min and the extraction solvent droplet (10  $\mu\text{L}$ ) that floated

on the top layer was withdrawn with a GC microsyringe. The extraction solvent was diluted with another 10  $\mu\text{L}$  of methanol because the 1-octanol extract was too viscous to be injected directly into the HPLC-FD system for the quantification.

#### PREPARATION OF SAMPLES

Different brands of green tea, chrysanthemum tea and coffee beverage samples were obtained from local enterprise shops. The samples were extracted using optimized DLLME procedure without pre-filtration steps. Triplicate extractions were conducted for each sample.

#### VALIDATION OF ANALYTICAL METHOD

The extraction method was assessed for linearity, relative recovery, repeatability, limit of detection (LOD) and limit of quantification (LOQ) before sample analysis.

### RESULTS AND DISCUSSION

#### OPTIMIZATION OF DLLME

Three parameters that influenced the DLLME efficiency were optimized, these included types of disperser solvent, volume of extraction solvent and extraction time. The optimization was carried out using deionized water samples spiked with 50  $\mu\text{gL}^{-1}$  of each PAH.

#### EFFECT OF DISPERSER SOLVENT ON DLLME

A disperser solvent in DLLME must be miscible with both organic extraction solvent and aqueous sample solution (Rezaee et al. 2006) to increase the contact surface

area between the analytes and extraction solvent. Three disperser solvents, namely acetonitrile (ACTN), methanol (MeOH) and acetone (ACE) were investigated in this study. Figure 1 shows that ACTN showed the highest extraction efficiency as compared to MeOH and ACE. The ACTN posed higher polarity index that enabled it to well mix with the aqueous sample solution, as well as dissolving the organic extraction solvent. Its low viscosity also allowed for the rapid dispersion, which was an important criterion to succeed the DLLME. Therefore, ACTN was applied for the subsequent experiments.

#### EFFECT OF EXTRACTION SOLVENT VOLUME ON DLLME

The volume of the extraction solvent determines the enrichment of the extracted analytes. A smaller volume ensures higher analyte enrichment. In this study, 1-octanol was used as the extraction solvent because of its smaller density as compared to the water and high affinity for the non-polar compounds. Extraction solvent in the range of 50 to 100  $\mu\text{L}$  was investigated and the results as shown in Figure 2 prove that the smaller volume enhances the analyte enrichment. Acceptor volume with 50  $\mu\text{L}$  was chosen as this was the lowest volume practical for the retrieval of the extraction solvent using a microsyringe for HPLC analysis.

#### EFFECT OF EXTRACTION TIME ON DLLME

The DLLME is a non-exhaustive system and thus it is time-dependent. This is because the minute amount of the extraction solvent applied in the system is not capable to completely remove the analytes. The extraction time of DLLME is defined as the time interval between the injection of the mixture containing both disperser and

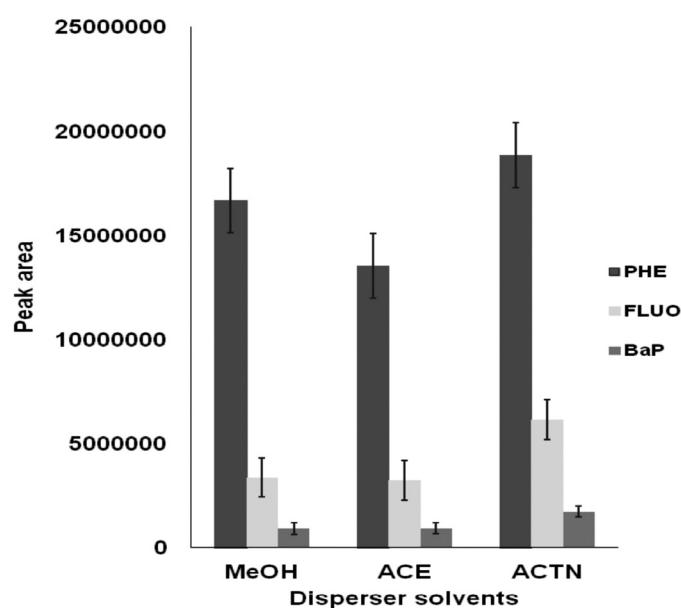


FIGURE 1. Effect of disperser solvents on DLLME of PAHs from spiked deionized water sample ( $n=3$  in each case). Error bars represent the standard deviation

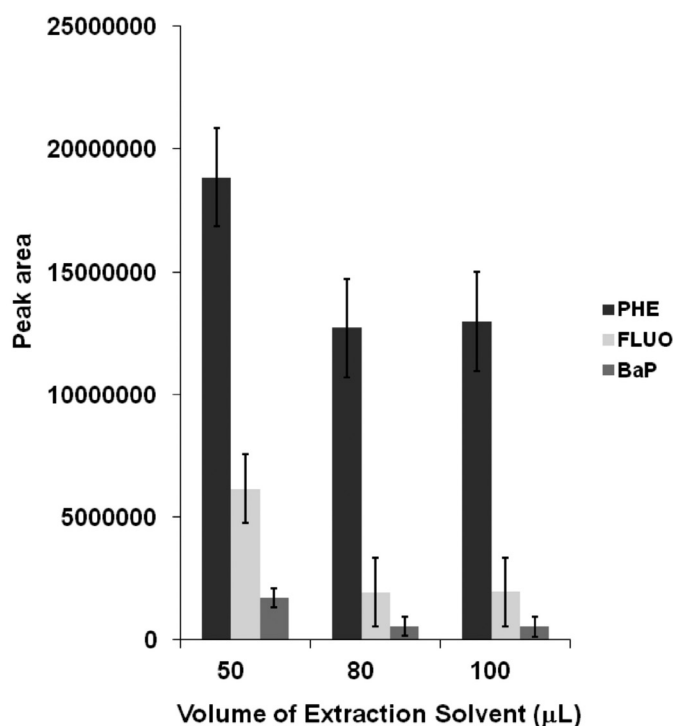


FIGURE 2. Effect of extraction solvents volume on DLLME of PAHs from spiked deionized water sample ( $n=3$  in each case). Error bars represent the standard deviation

extraction solvents and the time before centrifugation (Jahromi et al. 2007). The extraction time ranging from 1 to 5 min was investigated in this study. The extraction equilibrium was achieved within a minute as the dispersive concept provided large contact surface area between the analytes and the extraction solvent (Yan et al. 2011). The homogeneously distributed extraction solvent favored the transfer of the PAHs by simple solvent partition which then increased the DLLME extraction efficiency. However, the prolong extraction time broke the equilibrium as the emulsion solution was unstable and thus resulting in lower extraction efficiency (Yan et al. 2011), as indicated in Figure 3. Therefore, 1 min of extraction time was adopted in the subsequent experiments. The equilibrium state was achieved quickly and this was also the advantage of applying DLLME technique.

#### VALIDATION OF DLLME-HPLC-FD

Experiments were carried out to validate the applicability of the optimized DLLME technique in green tea, chrysanthemum tea and coffee beverage samples. Matrix matched calibrations were performed by spiking different amounts of the selected PAHs into these samples, which were free from the PAHs' contamination. It was found that the matrix matched calibrations were required when the calibrations showed different slopes, as summarized in Table 1. This approach eliminated the matrix effect that may cause signal suppression. The validation results showed that good linearities were obtained in the specified

concentration ranges with correlation coefficients,  $r \geq 0.9952$ . The trace LODs in the range of  $0.001$  to  $0.3 \mu\text{gL}^{-1}$  established based on the signal to noise ratio of 3:1 showed that the DLLME technique was sensitive and sufficient to support trace PAHs analysis in the beverage samples.

Relative recovery was conducted by spiking the samples to give final concentration of  $2.0 \mu\text{gL}^{-1}$ . Relative recovery was performed as the DLLME is a non-exhaustive system. The results showed that the good average relative recoveries ranging from 86.7 to 103.0% were obtained in this study. This showed that matrix effect was negligible among the similar matrices.

DLLME has been proven to be a cost- and time-effective extraction technique resulting in high recovery and enrichment. The 1-octanol with smaller density has replaced the halogenated solvents that were commonly applied as the extraction solvents in the DLLME (Farajzadeh et al. 2011; Han et al. 2010; Wen et al. 2011; Zarei et al. 2012). The analytes were enriched in the fine droplets floated on the sample solution and this approach has simplified the withdrawal of the extraction solvents. In addition, the microextraction format has minimized the consumption and disposal of the solvents and chemicals in the analysis, which succeeding the green analysis.

#### CONCLUSION

The study showed that DLLME-HPLC-FD is suitable for trace analysis of environmental pollutants in beverage samples.

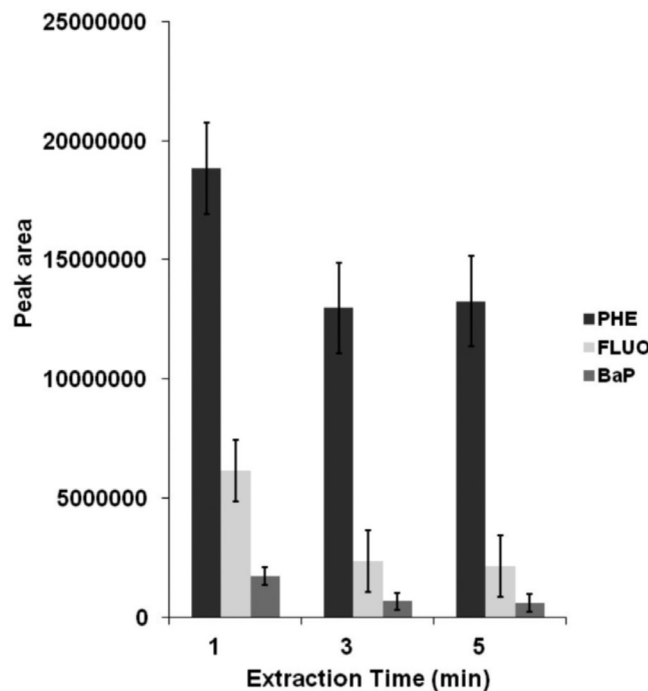


FIGURE 3. Effect of extraction time on DLLME of PAHs from spiked deionized water sample ( $n=3$  in each case). Error bars represent the standard deviation

TABLE 1. Validation data of the DLLME-HPLC-FD for the determination of selected PAHs in green tea, chrysanthemum tea and coffee beverage samples

Sample	Analyte	Linearity range, $\mu\text{gL}^{-1}$	Regression equation	r	LOD, $\mu\text{gL}^{-1}$	Relative recovery, % $\pm$ RSD, % ( $n=3$ )
Green tea	PHE	0.005-50	$y = 39440x + 78024$	0.9952	0.001	$86.7 \pm 7.2$
	FLUO	0.02-50	$y = 13784x + 11957$	0.9981	0.004	$95.6 \pm 11.7$
	BaP	0.06-50	$y = 2779x + 6365$	0.9970	0.02	$100.9 \pm 11.7$
Chrysanthemum tea	PHE	0.05-50	$y = 99430x + 12169$	0.9996	0.03	$103.0 \pm 7.3$
	FLUO	0.3-50	$y = 16721x + 1321$	0.9986	0.2	$89.3 \pm 3.1$
	BaP	0.4-50	$y = 4788x + 5478$	0.9967	0.3	$89.6 \pm 6.3$
Coffee	PHE	0.01-50	$y = 144880x + 52824$	0.9999	0.008	$95.3 \pm 10.3$
	FLUO	0.05-50	$y = 74760x + 4547$	0.9999	0.03	$99.4 \pm 9.8$
	BaP	0.01-50	$y = 381580x + 422618$	0.9999	0.004	$95.3 \pm 3.3$

This simple approach utilized minimal amount of solvents that are evenly distributed in the sample solution to enhance the analyte diffusion. The method has greatly reduced the analysis time and demonstrated high sensitivity and selectivity in analyzing the environmental pollutants in complicated beverage samples.

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