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DISPERSIVE LIQUID-LIQUID MICROEXTRACTION FOR THE EXTRACTION OF POLYCYCLIC AROMATIC HYDROCARBONS IN APPLE JUICE

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are hazardous and persistent organic pollutants that usually exist at low concentrations in the environment. In this study, dispersive liquid-liquid microextraction (DLLME) technique coupled with high performance liquid chromatography-fluorescence detection (HPLC-FD) was optimized for the analysis of selected PAHs, namely phenanthrene (PHE), fluoranthene (FLA) and benzo[a]pyrene (BaP) in apple juice. Under the optimal extraction conditions (the mixture of 200 µL of acetone and 50 µL of 1-octanol was applied to extract the selected PAHs for 1 min), the DLLME-HPLC-FD showed excellent linearity over the concentration range of 5 to 200 µg/L for both PHE and FLA, and 0.01 to 5 µg/L for BaP with correlation coefficients, $r \geq 0.9956$. The method offered ultra-trace detection of selected PAHs in the range of 0.002 to 0.5 µg/L, and negligible matrix effects in determining selected PAHs with relative recovery average within the range of 92.6 to 109.6% in apple juice. The advantages of applying this method for the extraction of PAHs include rapidity, simple operation, as well as small consumption of organic extraction solvent, which is beneficial for routine analysis.

Keywords: Apple juice, DLLME, fluorescence detection, HPLC, polycyclic aromatic hydrocarbons

Introduction

Polycyclic aromatics hydrocarbons (PAHs) are two- to eight-ring semi-volatile organic compounds (Singh *et al*., 2011). PAHs are originated from either natural sources or man-made activities. For instance, decomposition of organic materials through forest fires, volcanic eruptions, incomplete combustion of wastes and smoking. All these human activities have contributed to a huge impact on the accumulation of PAHs into our environment. Hence, when these PAHs are adsorbed to the soil particles, all the plants or vegetables will be contaminated by the PAHs as the plant uptake their nutrients from the contaminated soil. The PAHs acquire a higher potential to cause gene mutation in DNA and consequently induce the development of cancer in the body parts of living things (Fetzer, 2000). Due to their persistence properties, the PAHs will undergo bioaccumulation and bio-magnification and finally end its transportation routes into the food chain (Madhavan & Naidu, 1995). People of different ages, gender, or religion consume apples every day. Instead of consuming a whole apple, people nowadays tend to drink apple juice for the sake of convenience. However, the contamination of PAHs in vegetation are common and therefore, determination of PAHs residues in apple juice is important to prevent the adverse effect of drinking apple juice.

Determination and measurement of trace amounts of the targeted analytes need to be carried out in a series of operations. Extraction of analytes from different matrices, followed by the purification are the two important sample preparation steps before quantification of analytes (Moret & Conte, 2000). In past researches, classic sample preparation techniques, for instances, solid phase extraction

(SPE) (Moja & Mtunzi, 2013) and liquid-liquid extraction (LLE) (Lee *et al*., 2003) have been demonstrated to extract PAHs in aqueous samples. However, both techniques consume large amounts of organic solvents which are costly and harmful, and they are both time-consuming and tedious (Houessou *et al*., 2005; Anthemisis & Miro, 2009).

Alternative microextraction techniques have been demonstrated in the analysis of organic compounds since the past two decades. These include the utilization of micro-solid phase extraction (Loh *et al*., 2013a; 2013b; 2018a; 2018b; Aow Yong *et al*., 2019; Zulkipli *et al*., 2019), liquid phase microextraction (Sanagi *et al*., 2012) and dispersive liquid-liquid microextraction (Zhao *et al*., 2009; Loh *et al*., 2016; 2017) for the extraction of organic pollutants in aqueous and beverage samples. These techniques offer high sensitivity and high analyte enrichments, as well as require only trace amounts of organic solvent.

This study provided rapid extraction of selected PAHs in apple juice samples using dispersive liquid-liquid microextraction (DLLME). The dispersive concept enables massive contacts between the analytes and the extraction solvent disperse in droplets and thus results in accelerating the mass transfer of the analytes into the extraction solvent.

Materials and Methods

Chemicals and reagents

Chemical reagents like acetonitrile and methanol (HPLC grade), acetone and 1-octanol (analytical grade) were obtained from Merck. Selected polycyclic aromatic hydrocarbons (PAHs), namely phenanthrene (PHE), fluoranthene (FLA) and benzo[a]pyrene (BaP) were purchased from Sigma-Aldrich. Eppendorf 5702 centrifuge (Hamburg, Germany) was used to centrifuge the cloudy solution formed during extraction step.

Individual standard stock solution of 500 mg/L of PHE, FLA and BaP were prepared by weighing 0.005 g of each analyte in 10 mL of volumetric flask and diluting to volume with acetonitrile (PHE and BaP) and methanol (FLA), respectively. A series of mixture working standard solutions were prepared by diluting the stock solutions using methanol. The solutions were then stored at 0^{o}C when not in use.

Apple juice samples were purchased from an enterprise shop in Kuala Terengganu. Three samples with same producer and brand were mixed well for relative recovery study. It was stored at 0 ^{o}C prior to analysis. PAHs (PHE, FLA, BaP) in apple juice were extracted through optimized DLLME procedure without pre-filtration step.

Dispersive liquid-liquid microextraction procedure

Apple juice sample (10 mL) was accurately measured and placed in a 10 mL centrifuge tube. A mixture containing 200 µL of acetonitrile (dispersive solvent) and 50 µL of 1-octanol (extraction solvent) was then injected into the sample using 1.00 mL of disposable syringe. Next, the sample solution was left at room temperature for 1 min in order to form a cloudy solution consisting of a very fine droplets of 1-octanol well dispersing in the sample solution. The sample solution was then centrifuged at 4000 revolution per minute (rpm) for 10 min. The clear droplets of organic solvent (10 µL) was transferred into a safe-lock tube using a GC micro syringe and diluted with 50 µL of methanol prior to high performance liquid chromatography-fluorescence detection.

High performance liquid chromatography chromatographic conditions

All analyses were performed using high performance liquid chromatography (HPLC) coupled with fluorescence detection (Shimazu, Kyoto, Japan). The chromatographic separation of selected PAHs was carried out on a C_{18} column (4.6 x 250 mm, 5 µm) purchased from Agilent. The separation was performed with isocratic mobile phase, acetonitrile-water (80:20) (v/v) at column temperature of 30 ºC. The flow rate,

injection volume and detection wavelength were fixed at 1.0 mL/min, 10 µL and 250/400 nm of excitation/emission wavelengths, respectively.

Optimization and validation of DLLME-HPLC-FD

The DLLME analytical technique was optimized through modifying different types of disperser solvents, volume of disperser solvent, volume of extraction solvent and extraction time to achieve high analyte enrichment. The DLLME-HPLC-FD was then assessed for linearity range, relative recovery, limit of detection (LOD) and limit of quantification (LOQ) before analysing apple juice samples. The LOD and LOQ were calculated based on signal to noise ratio of 3:1 and 10:1, respectively.

Results and Discussion

Optimization of DLLME

Four parameters, namely types of disperser solvent, volume of disperser solvent, volume of extraction solvent and extraction time, which influenced the extraction efficiency of DLLME were thoroughly investigated and optimized in this study. The optimization was carried out using deionized water samples spiked with 10 µg/L of PHE, 100 µg/L of FLA and 5 µg/L of BaP. Optimization of DLLME technique was carried out in order to obtain high analyte enrichment and sensitive analysis.

Types of disperser solvent

The most important criteria for selection of disperser solvent is the capability for it to dissolve both organic phase (extraction solvent) and aqueous phase (sample solution) (Rezaee *et al*., 2006). In this research work, five types of disperser solvents that are frequently applied in DLLME, such as acetonitrile, acetone, methanol, ethanol as well as isopropanol were studied. As indicated in Figure 1, it was found that acetone showed the highest extraction efficiency as compared to the rest of the disperser solvents. This was because the viscosity of acetone was the lowest among the disperser solvents and therefore it was capable to completely disperse the extraction solvent, 1-octanol. This enabled it to be highly miscible between the organic 1-octanol and aqueous sample solution (Mudiam & Ratnasekhar, 2013) and thus allowing the extraction solvent to be homogenously distributed in the sample solution and extract the PAHs with simple solvent partition. Besides, it is less toxic and cheaper than other disperser solvents.

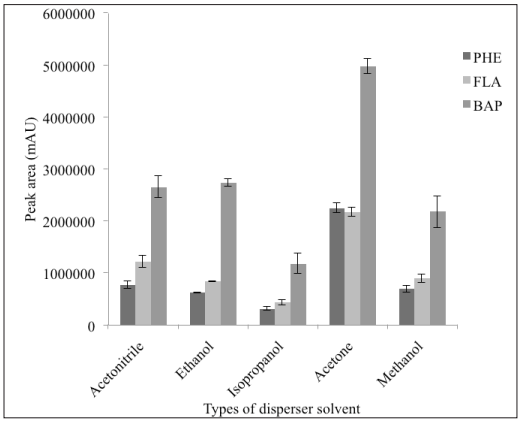

Figure 1: Effect of disperser solvents on the extraction efficiencies of DLLME for the extraction of selected PAHs. Error bars represent the standard deviations of triplicate extractions

Volume of disperser solvent

The volume of disperser solvent will directly influence the possibility for the mixed solution to turn cloudy completely, followed by its ability to disperse the extraction solvent into the aqueous sample solution, and finally the extraction efficiency (Saidi & Emara, 2014). Insufficient of disperser solvent will not disperse the extraction solvent properly, and therefore causing the difficulty to form a cloudy solution thoroughly. On the contrary, the solubility of analytes in the disperser solvent increases when the volume of disperser solvent increases. Hence, a sufficient volume of disperser solvent is vital to achieve high extraction efficiency. In this study, the volume of acetone in the range of 0 to 300 µL was investigated. As shown in Figure 2, 200 µL of acetone showed the highest peak area while

0 µL showed the lowest peak area. It was clear that the acetone with lower volume caused the cloudy state did not form properly, therefore low recovery was obtained (Pashazanousi *et al*., 2012). Hence, 200 µL of acetone was chosen as the optimum volume because 250 µL and 300 µL decreased the extraction efficiency in overall due to the analytes dilution effect.

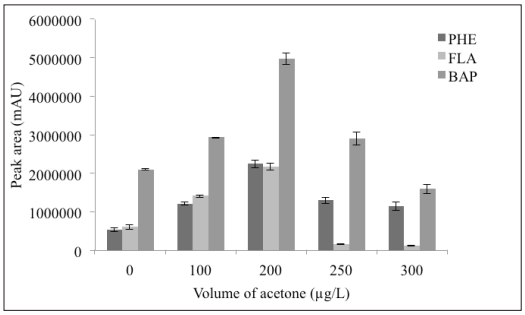

Figure 2: Effect of disperser solvent volumes on the extraction efficiencies of DLLME for the extraction of selected PAHs. Error bars represent the standard deviations of triplicate extractions

Volume of extraction solvent

The 1-octanol was chosen as the extraction solvent because it has high affinity towards PAHs, low solubility in water and low density compared to water. A series of extraction solvents with different volumes ranging from 40 µL to 70 µL were studied and the results of the experimental analysis were shown in Figure 3. Basically, the smaller the volume, the higher the concentration of the analytes as well as the enrichment factor. In this study, 50 µL of 1-octanol was chosen as this was the lowest volume which was suitable for the practical retrieval of the extraction solvent using a micro syringe for HPLC analysis. Besides, the tiny fine droplets using 40 µL was difficult to be seen with naked eyes.

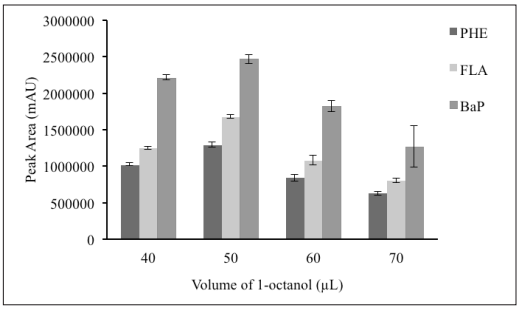

Figure 3: Effect of extraction solvent volumes on the extraction efficiencies of DLLME for the extraction of selected PAHs. Error bars represent the standard deviations of triplicate extractions.

Extraction time

Extraction time is defined as the time interval between the injection of both disperser and extraction solvents into sample solution and the time when the centrifuge button is pressed on (Jahromi *et al*., 2007). In this study, extraction time ranging from 1 min to 5 min were studied with all other experimental conditions remained fixed. Extraction time of 1 min was chosen and applied throughout the analysis. This was because within a minute of extraction time, a large surface of contact area was formed between the extraction solvent and the sample solution in the emulsion system and this enabled the extraction equilibrium was achieved easily (Yan *et al*., 2011). Conversely, Figure 4 shows that when the extraction time was prolonged, the equilibrium broke and subsequently led to lower extraction efficiency since the emulsion solution was unstable and it would delaminate.

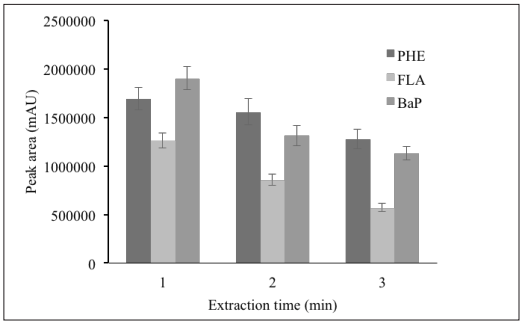

Figure 4: Effect of extraction time on the extraction efficiencies of DLLME for the extraction of selected PAH. Error bars represent the standard deviations of triplicate extractions

Validation of DLLME-HPLC-FD

A few experiments were carried out to validate the applicability of the DLLME extraction technique using the optimal conditions. The deionized water samples were spiked with each of the analyte to give five concentration levels in the range of 5 to 200 µg/L for both PHE and FLA, and 0.01 µg/L to 5 µg/L for BaP, respectively. The results (Table 1) showed good linearity within the specified concentration range with correlation coefficients, $r \geq 0.9956$.

The international union of pure and applied chemistry (IUPAC) defines LOD as the smallest concentration or absolute amount of analyte that has a signal significantly larger than the signal arising from the reagent blank (Harvey, 2000). Whilst LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. In this study, LOD and LOQ were established based on signal to noise ratio of 3:1 and 10:1, respectively. Table 1 shows that the trace LODs in the range of 0.002 to 0.5 µg/L and LOQs in the range of 0.01 to 5 µg/L were obtained in this study. These revealed that the DLLME technique was sensitive and sufficient to support trace PAHs analysis in the apple juice samples.

Relative recovery is defined as the percentage number of analytes recovered from the apple juice sample with reference to the extracted standard. Relative recovery was studied as DLLME was not an exhaustive extraction. The apple juice blank was performed to ensure the samples were free from PAHs before the spiking. Relative recovery study was conducted by spiking the apple juice samples with a final concentration of 10 µg/L for both PHE and FLA, and 2 µg/L for BaP, respectively. PAHs are usually present in low concentration ranges in the natural water bodies as they are insoluble in the aqueous solution. Hence, it is impractical or not rational to spike too high concentration of PAHs. Table 1 shows that the excellent average of relative recoveries ranging from 92.6 to 109.6% and good repeatability with relative standard deviations (RSDs) of < 10% were obtained in this study. This revealed that matrix effects would not influence the sample analysis and DLLME can be applied in apple juice samples analysis.

Table 1: Validation data of DLLME-HPLC-FD for the analysis of selected PAHs in spiked deionized water and apple juice samples

PAH	Linearity range, µg/L	Regression equation	r	LOD, µg/L	Average of relative recovery ± RSD (n=3), %
PHE	5 - 200	y = 65115x + 755478	0.9956	0.5	92.6 ± 7.1
FLA	5 - 200	y = 11726x + 22887	0.9986	0.9	93.1 ± 8.9
BaP	0.01 - 5	y = 537277x + 204539	0.9980	0.002	109.6 ± 8.1

Conclusion

In this research study, the low density of 1-octanol extraction solvent has been successfully applied for the extraction of selected PAHs in apple juice samples. The use of this low-density solvent has solved the solvent hazardous problem encountered in the previous study because the low-density extraction solvent is much safer than the high-density halogenated solvents. DLLME is a technique that incorporates trace amounts and less hazardous solvent in the achievement of green analytical chemistry for pre-concentration of the PAHs present in the complicated sample matrices.

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