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Lower Density Solvent-Based Dispersive Liquid-Liquid Microextraction for the Determination of Benzoic Acid

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Abstract

A lower density solvent-based dispersive liquid-liquid microextraction (DLLME) was optimized for the determination of benzoic acid in fruit juices by HPLC-UV method. The type and volume of lower density of extraction and dispersive solvents were investigated. A 0.5 mL octanol was found to be an optimal extraction solvent, and a 2.0 mL acetonitrile was suitable as dispersive solvent. An extraction procedure was applied for extraction of benzoic acid from three orange juice samples, which was purchased from the local market. An optimal HPLC condition was achieved under a mixture condition of methanol and 1 % acetic acid (97 and 3) was found as an optimal mobile phase. The method was validated under the optimized conditions of the extraction and the determination. The good linearity with R^2 0.9939 was obtained in the concentration range of 25 - 1000 mg/L. The relative standard deviations (%RSD) of retention time and peak area were acceptable. The LOD was 2.2 mg/L and the recovery was satisfied (104±7 %). The results show that the extraction and the determination were efficient for determining of benzoic acid.

Keywords: DLLME; Benzoic Acid; Fruit Juices; HPLC

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Introduction

Benzoic acid, a chemical preservative, has always been of great importance for inhibiting various bacteria, yeasts and fungi growths. It is widely used as a food preservative where it is most active in foods or drinks of low pH value. For humans, the WHO's International Programme on Chemical Safety (IPCS) suggests that a provisional tolerable intake would be 5 mg/kg body weight per day. Even though the acute toxicity of benzoic acid is low, the monitoring of benzoic acid in beverages has great importance with respect to estimation of the risks of customers. Benzoic acid derivatives are known to cause non-immunological contact reactions (pseudoallergy). Moreover, the maximum concentrations reported for preservation purposes have been limited in the range of 2000 mg/kg of food [1].

There are several methods available for the determination of benzoic acid in foods and beverages. Spectroscopic methods have been performed for determining, however, the methods are not specific and need extensive extraction process. Chromatographic methods (GC and HPLC) are sensitive and specific; they are frequently applied to determine benzoic acid [2] - [5]. The determinations of benzoic acid in various foods have been commonly employed by HPLC technique because it offers high specificity with minimal preparation and does not require derivatization as same as GC method. Many extraction procedures of benzoic acid from various samples have been presented with minimal preparation. Liquid-liquid extraction (LLE) methods have been mostly reported with ethanol or/and methanol as optimal extraction solvents [6] - [7]. Using ethanol or/and methanol for extraction, an emulsion was however obtained in some food stuff samples (fats or sauces) [8] which affects to extraction efficiency. The defatting processes can be applied to remove oil from liquid-containing samples using hexane [9]. An ultrasonic extraction was also presented for extraction of benzoic acid in soft drinks [10], an it also helps to reduce the consumption of reagents compared to LLE. However, the efficiency of an ultrasonic extraction is depended on many parameters such as solvent composition, extraction time, or sample load. A very sensitive and effective method as solid phase extraction (SPE) was presented for separation of benzoic acid in wines and distillates as Ref. 3.

One of the attractive extraction methods is dispersive liquid-liquid microextraction (DLLME), which was explored in 2006 by Rezaee and co-workers [11]. An extraction is a simple and fast microextraction method, which is based on the use of a few volumes of reagents. A method is obtained under two systems of organic solvent; one is extraction solvent and the other is dispersive solvent. The extraction of organic solvents with high density such as chloroform, dichloromethane or carbontetrachloride have been

commonly used but their toxicity must be realized. The lower density of extraction solvent was studied in this work. The dispersive solvents with high miscibility in both organic and aqueous phases such as methanol or acetonitrile have been mostly obtained. The mixture of extraction and dispersive solvents is rapidly injected into sample solution, the very small droplets as cloudy are obtained. A cloudy solution appears on the surface area between the organic phase and aqueous sample, which become large and achieve the equilibrium state in short period. A solution is then centrifuged, and a phase separation later occurs. An organic phase is collected for determination. The advantages of DLLME include simplicity of preparation, rapidity, low cost, high recovery, high enrichment factor and environmental benignity [12]. Following the advantages of DLLME method, it is achieved for extraction of benzoic acid in milk using Carrez solutions [13] and in beverage samples using ethanol and chloroform conditions, and they were later quantified by GC-FID [14]. In water samples, a mixture of acetonitrile and 1-butyl-3-methylimidazolium hexafluorophosphate $[C_4MIM][PF_6]$ is optimal for extracting benzoic acid via ionic liquid cold-induced aggregation dispersive LLME (IL-CIA-DLLME) procedure [15].

To explore our knowledge, we decided to take the advantages of DLLME method combined with HPLC methods for determination of some preservatives. We optimized the extraction parameters using the lower density and lower toxicity than water and chlorinated solvent, respectively. A method was then applied for determination of benzoic acid in aqueous media of fruit juices.

Materials and Methods

1. Chemicals and Reagents

Most of the chemicals were of analytical grade. Chloroform, acetone and sodium chloride were purchased from Ajax Finechem (Australia). Diethylether was from BDO Laboratory supplies (United Kingdom). Ethanol, carbon tetrachloride and acetic acid were obtained from Merck (Germany). Octanol was from APS Chemical Limited, Laboratory (Australia). The HPLC grade of methanol and acetonitrile were purchased from RCL Laboratory Limited (Thailand).

2. DLLME procedure

DLLME was performed under the optimal conditions as follows: 5.0 ml of fruit juice sample was pipetted into a 10 mL centrifuge tube. A mixture of 0.5 mL octanol as an extraction solvent and 2.0 mL acetonitrile as a dispersive solvent was rapidly injected into the tube of sample. The cloudy solution from tiny dispersed droplets of solvent (water/

octanol/acetonitrile) was formed after injection. The solution was shaken and then centrifuged for 6 min. The phase separation between aqueous and organic was obtained. Octanol is lighter than aqueous sample, the top layer of organic solvent was presented. The upper layer of organic solvent was later collected before injecting to HPLC system. A DLLME steps is shown in Figure 1.

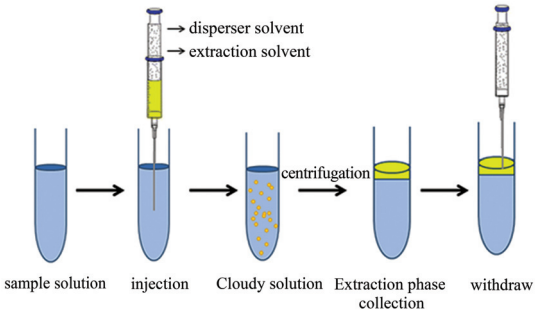

Figure 1 DLLME steps

3. Instrumentation

Chromatographic determination was performed by HPLC system (Waters w6007172424) equipped with UV-V is detection (Waters 2489) at 254 nm. The HPLC conditions were carried out under proper conditions using RP-HPLC column (C18 Waters, 150 mm × 4.6 mm I.D., 5 µm). An optimal mobile phase of methanol : 1 % acetic acid (97 : 3) was run using an isocratic elution with a 1.0 mL/min flow rate. All standard solutions and juice samples were injected to HPLC system at 20 µL. The retention time of benzoic acid was less than 4 min, presented under these conditions.

Results and Discussion

1. DLLME optimization

1.1 Effect of extraction solvent

The efficiency of DLLME is involves various parameters, including type and volume of both extraction solvents and dispersive solvents, while the extraction time is also impacted by the separation capability. Selection of a suitable optimal extraction solvent is significance for optimizing DLLME method. For the extraction solvent, it should have high extraction capability of analyzed compounds, different density to water and low solubility in aqueous sample [5]. Using the simple solvents, the less toxic solvents and lighter density in aqueous sample as methanol, diethylether and octanol were mainly

studied, and the common extraction solvents with higher density in aqueous sample such as carbontetrachloride and chloroform were also tested. An acetonitrile (2.0 mL) was used as dispersive solvent for this study. After the extraction process, the 3 layers were represented; the upper one was the organic phase, the middle one was the suspended phase (orange color), and the bottom one was the aqueous phase. The upper layer was considered for further determination. From our result, methanol and diethylether were eliminated because phase separations between aqueous and organic were both poor, as shown in Figure 2. It was due to high solubility of those organic solvents. Then, organic phase could not be collected for these two solvents. Carbon tetrachloride, chloroform and octanol were better than those two where phase separation cloud was observed and collected. According to the result, octanol is a lighter density solvent than aqueous sample, we found the upper layer of organic phase. Moreover, it is less toxic and inexpensive than carbon tetrachloride and chloroform. Where the toxicity must be realized, we considered to use octanol as a proper extraction solvent in further study. Compare the peak area.

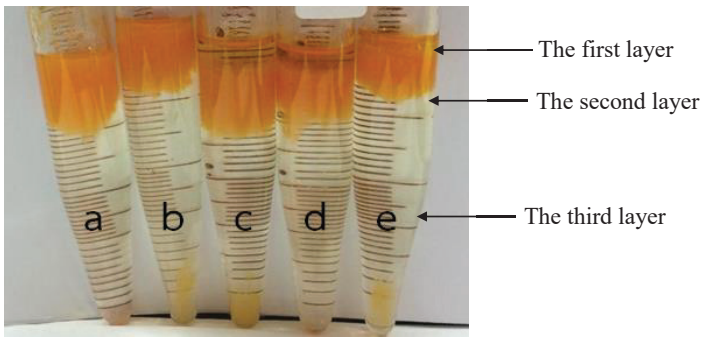

Figure 2 Effect of the extraction solvent types (a) methanol (b) diethyl ether (c) carbontetrachloride (d) chloroform (e) octanol

1.2 Effect of dispersive solvent

The key point for selection of disperser miscibility in both the extraction solvent and the aqueous sample is that the dispersive solvent functions to take extraction solvent through the aqueous sample phase. Thus, the density and solubility of extraction and dispersive solvents were considered. Dispersive solvent is soluble in extraction solvent and should be miscible in aqueous sample, thus enabling the extraction solvent to be dispersed as fine particles in aqueous phase to form a cloudy solution. The surface area between extraction solvent and aqueous sample can be large, thus increasing the extraction efficiency [16]. The dispersive solvents miscible in water (acetone, ethanol and acetonitrile)

were studied, where the densities of these dispersive solvents were lighter than octanol (density of octanol is 0.824 g/mL). Thus, the solubility of extraction and dispersive solvents could be possible where a 0.5 mL octanol was used as an extraction solvent in this study. We found the good phase separation between a mixture of octanol and acetonitrile solvents as shown in Figure 3. The good phase separation provides a facile collection of sample for injection, and a large volume of sample mostly gave the high efficiency separation. However, acetonitrile was chosen as an optimal dispersive solvent.

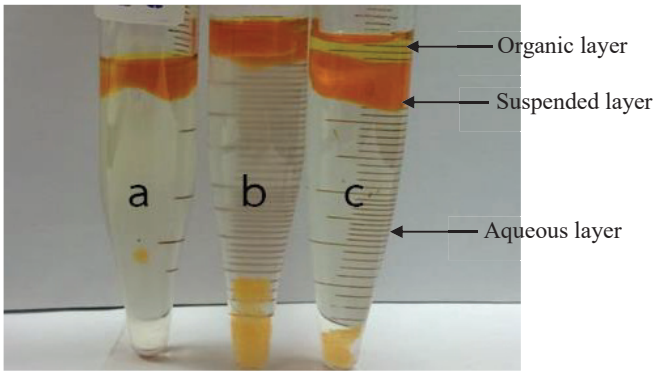

Figure 3 Effect of the dispersive solvent types (a) acetone (b) ethanol (c) acetonitrile

1.3 Effect of volume of dispersive solvent

The different volumes of dispersive solvent were compared with change in the collected phase. To determine the best extraction conditions, the peak area of interest was used to evaluate the extraction efficiency. The results under different dispersive solvent are in Table 1. A 2.0 mL of dispersive solvent (acetonitrile), giving the highest peak area (Figure 4), was chosen in the subsequent experiments

Table 1 Effect of volumes of dispersive solvent

Volume of dispersive solvent (mL)	Volume of extraction solvent (mL)	Ratio of dispersive solvent : extraction solvent	Peak area (AU)
0.4	2.0	2:1	45745
0.8	2.0	4:1	54364
2.0	2.0	10:1	131265
4.0	2.0	20:1	50850

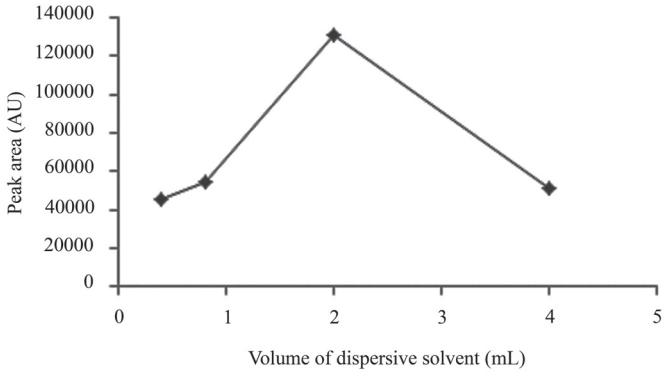

Figure 4 Effect of the dispersive solvent volumes (mL)

1.4 Effect of volume of extraction solvent

The volume of extraction solvent is also a parameter which may affect the detection of the method. A series of sample solutions were performed by using octanol and acetonitrile (2.0 mL). The volumes of octanol were changed in the range of 0.05 - 0.50 mL. The highest of peak area was obtained with octanol volume for 0.50 mL (compared to other volume) where the ratio of dispersive and extraction solvents was 4 : 1 (The results are shown in Table 2 and Figure 5). The increasing of the extraction solvent volume provided the increasing of the final organic phase obtained after centrifugation. Thus, a 0.5 mL of extraction solvent (octanol) was chosen as a proper volume in further experiments.

Table 2 Effect of volumes of extraction solvent

Volume of extraction solvent (mL)	Volume of dispersive solvent (mL)	Ratio of dispersive solvent : extraction solvent	Peak area (AU)
0.05	2.0	40:1	54997
0.10	2.0	20:1	44866
0.20	2.0	10:1	45702
0.50	2.0	4:1	55181

2. HPLC optimization

The reversed phase HPLC condition was investigated with the different ratios of methanol: 1 % acetic acid (97:3, 95:5, 90:10 and 85:15). We accomplished the determination using methanol : 1 % acetic acid at a ratio of 97:3 at pH 3.0, giving the highest peak and area than others, at a flow rate of 1 mL/min and UV detection at 254 nm. The retention time of benzoic acid was less than 4 min.

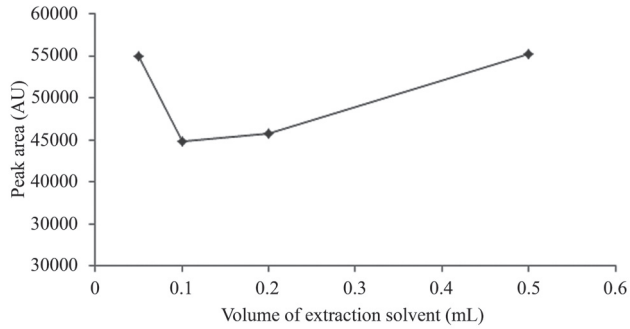

Figure 5 Effect of the extraction solvent volumes (mL)

3. Method validation

A good linearity between the concentrations of benzoic was obtained over the various concentrations in the wide range of 25 - 1000 mg/L (n = 3). The regression equation was y = 51.596x + 4388.7 with the correlation coefficient 0.9939 as shown in Figure 6. The LOD, was 3 times the standard deviation (SD) of reagent blank (n = 7). Under the operation, a high value of SD of reagent blank was obtained. Then, the calculated LOD was 2.2 mg/L. The LOQ, calculated from 10 times standard deviation to slope was 7.5 mg/L. Precision of method was presented as within day (n = 7) and day to day (2 days) variations in term of %RSD. The variations were calculated for 3 concentrations (50, 300 and 1000 mg/L) of benzoic acid. The "within day" variations of retention time and peak area were less than 1.0 % and 3.0 %, respectively. The reproducibility (day to day variation) of retention time and peak area were less than 5 % and 15 %, respectively. Under the optimal extraction condition, the enrichment factor, the ratio of concentration of benzoic acid in the upper layer of organic phase to that in the initial sample, was 25 fold. The method was successfully applied for determination of benzoic acid in orange juice samples.

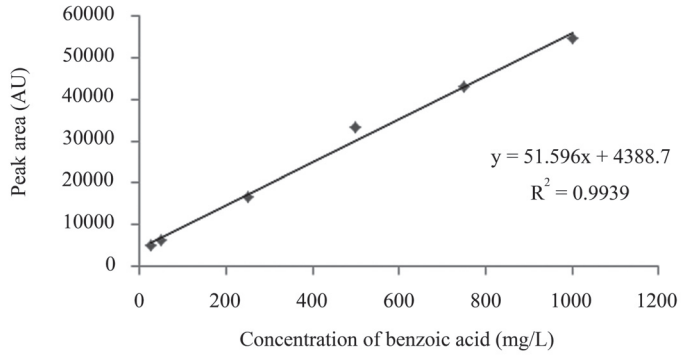

Figure 6 Standard calibration curve of benzoic acid 25 - 1000 mg/L

4. Analysis of orange juice samples

Under the extraction conditions, the 0.5 mL of extraction solvent (octanol) and 2.0 mL of dispersive solvent (acetonitrile) were employed for sample determination. The three commercial available orange juices were purchased from a local supermarket. The obtained results are shown in Table 3. We found the amounts of benzoic in samples are less than the permitted level from the General Standard for Food Additives: GSFA 2014 (FDA 2014) [17]; chromatogram is shown in Figure 7. In order to verify the accuracy of the method, the recovery study was carried out by spiking selected sample with standard. The % recovery was calculated from the difference between spiked and un-spiked samples. The result is shown in Table 4 where the recovery was satisfied.

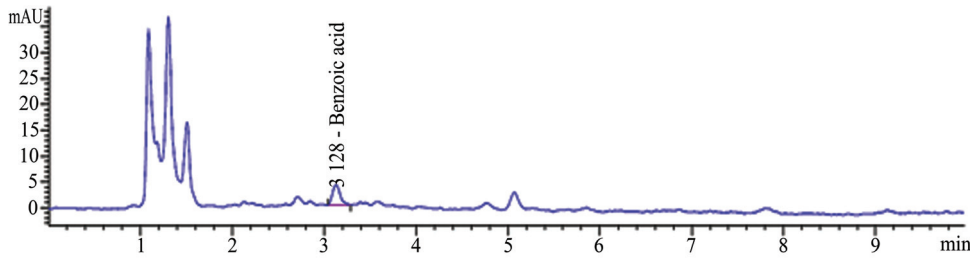

Figure 7 Chromatogram of sample 1

Table 3 Benzoic acid levels in fruit juice samples (n = 3)

Sample	Maximum permitted level in fruit juice (mg/L)	Benzoic acid level (mg/L)
1	1000	985±50
2		1145±49
3		1089±81

Table 4 Percentage recovery result (n = 3)

Sample	Founded in sample (mg/L)	Spiked concentration (mg/L)	Determined amount (mg/L)	% Recovery
1	985	50	1037	104±7

Conclusion

A method based on the DLLME coupled with HPLC-UV was optimized for determination of benzoic acid in fruit juices. We achieved the extraction of benzoic under an optimal mixture of octanol and acetonitrile as extraction and dispersive solvents. The good linearity

was obtained. The LOD was 2.2 mg/L. The accuracy of method in terms of percentage recovery was in the range 104±7 %, which was acceptable. The precision as percentage relative standard deviations were satisfied. The DLLME method provides a simple, low cost, short time and high efficiency for determining preservatives in various aqueous samples.

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