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# Hollow Fiber Liquid-Phase Microextraction with in Situ Derivatization Combined with Gas Chromatography–Mass Spectrometry for the Determination of Root Exudate Phenylamine Compounds in Hot Pepper (*Capsicum annuum* L.)

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**ABSTRACT:** Hollow fiber liquid-phase microextraction (HF-LPME) with derivatization was developed for the determination of three root exudate phenylamine compounds in hot pepper (*Capsicum annuum* L.) by gas chromatography–mass spectrometry (GC-MS). The performance and applicability of the proposed procedure were evaluated through the extraction of 1-naphthylamine (1-NA), diphenylamine (DPA), and *N*-phenyl-2- naphthaleneamine (N-P-2-NA) in a recirculating hydroponic solution of hot pepper. Parameters affecting the extraction efficiency were investigated. The calibration curves showed a good linearity in the range of 0.1–10 $\mu$g mL$^{-1}$. The limits of detection (S/N = 3) for the three compounds were 0.096, 0.074, and 0.057 $\mu$g mL$^{-1}$, respectively. The enrichment factors reached 174, 196, and 230 at the concentration of 5 $\mu$g mL$^{-1}$, and relative standard deviations (RSD) of 9.5, 8.6, and 7.8% and 8.4, 7.6, and 6.2% were obtained at concentrations of 2 and 5 $\mu$g mL$^{-1}$ for 1-NA, DPA, and N-P-2-NA, respectively. Recoveries ranging from 90.2 to 96.1% and RSDs below 9.1% were obtained when HF-LPME with in situ derivatization was applied to determine root exudate 1-NA, DPA, and N-P-2-NA after 15 and 30 days of culture solution, respectively.

**KEYWORDS:** *hollow fiber liquid-phase microextraction, derivatization, phenylamine, gas chromatography–mass spectrometry*

## INTRODUCTION

Plants not only absorb nutrients and moisture from the environment but continuously secrete inorganic ions and organic compounds to the media by root during the growth period. The composition and content of root secretions are not only the most direct adaptive responses of plants to the environmental stress but also an adaptive mechanism of different ecotype plants for living environment.[1] In the research of root exudates, the methods of collection, extraction, and identification for root exudates always are on the forefront and represent a difficulty in the research field. 1-Naphthylamine (1-NA), diphenylamine (DPA), and *N*-phenyl-2-naphthaleneamine (N-P-2-NA) are representative root exudate phenylamine substances with high polarity in hot pepper.

Some sample preparation techniques have been reported for the pretreatment of aromatic amines in aqueous samples based on liquid–liquid extraction (LLE)[2,3] and solid-phase extraction (SPE).[4−6] Both methods are time-consuming and require large amounts of high-purity organic solvents, which are expensive and toxic.[7] In recent years, a miniaturized technique, that is, solid-phase microextraction (SPME), has been developed as a solvent-free extraction method for the analysis of amine compounds.[8−13] However, this method has several disadvantages. First, SPME fibers are still comparatively expensive and have a limited lifetime. Second, they are susceptible to carryover from one extraction to the next.[14,15] As a modification of classic liquid–liquid extraction, an alternative miniaturized "green" sample preparation approach, hollow fiber liquid-phase microextraction (HF-LPME), has been considered as an excellent invention for the sample preparation.[16−19] It is mainly because hollow fibers are not only expendable and disposable but can also protect the acceptor phase from the interference of matrices and widely connected with the available apparatus.[20] Therefore, HF-LPME is generally complementary to SPME and has found wide applications.

Chromatographic separation and quantification of amine compounds are hampered by their polarity, which can cause tailing and incomplete adsorption. In addition, the mass spectrometry of amines is not often ideal for their characterization, especially when these compounds exist at low concentration.[21] Therefore, derivatization is required to increase extraction efficiency and improve chromatographic performance.[2,21] Derivatization can be mainly divided into three modes. The first mode is pre-extraction derivatization.[22] The second mode is postextraction derivatization, and injection port[23−27] and on-column derivatization [28] are the two main approaches. The third model is in situ derivatization; that is, derivatization and extraction of analytes take place simultaneously in donor phase or acceptor phase. The mixture of extraction and derivatization reagent has been held within a hollow fiber of LPME for the simultaneous purification, derivatization, and extraction in many research studies.[29−36] This approach not only decreases solvent consumption but also simplifies sample preparation steps. In addition, the hollow fiber can exclude biomacromolecules and protect water-

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sensitive derivatization reagents. Furthermore, extracted analytes would be exhausted in the hollow fiber, which enhances extraction velocity and quantities due to in situ derivatization. Therefore, this technique is simple, inexpensive, time-saving, and environmentally friendly. To the best of our knowledge, HF-LPME with in situ derivatization combined with GC-MS has not been reported for analyzing phenylamine compounds. Commonly used derivatization reactions include esterification, silylation, acetylation, and alkylation. Derivatization of amine compounds for transformation to less polar amide compounds by acylation is most often adopted.[37,38]

In this work, three derivatization methods with HF-LPME have been attempted to improve the sample preparation of 1-NA, DPA, and N-P-2-NA in hydroponic solution, and the hollow fiber liquid-phase microextraction with in situ derivatization method was proved to be the most suitable. The derivatization reagent acetic anhydride (AA) was protected within the hollow fiber, which allowed the derivatization and extraction of the analytes prior to GC-MS analysis. Derivatization and extraction conditions including the proportion of mixed extraction/derivatization reagent, extraction time, extraction temperature, salt addition, stirring rate, and solution pH were discussed and optimized. The extraction performance between HF-LPME with in situ derivatization and LLE was compared at the same concentration level, and the proposed method was applied to determine the concentrations of 1-NA, DPA, and N-P-2-NA in real samples after 15 and 30 days of culture.

## MATERIALS AND METHODS

**Reagents and Materials.** 1-Naphthylamine (1-NA, 99.5%), diphenylamine (DPA, 99.5%), and derivatization reagent acetic anhydride (AA) were obtained from Anpel Scientific Instrument Co., Ltd. (Shanghai, China), and *N*-phenyl-2-naphthalene amine (N-P-2-NA, 99.5%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The individual stock solutions of 1-NA, DPA, and N-P-2-NA (1 g $L^{-1}$) were prepared by dissolving each compound in methanol (Merck, Darmstadt, Germany). The standard solutions for 1-NA, DPA, and N-P-2-NA were prepared by diluting stock solutions with ultrapure water to the required concentration, respectively. Other reagents were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China).

Two 50 $\mu$L microsyringes (10F, SGE Australia) with blunt tips were used for the HF-LPME. The Accurel Q 3/2 polypropylene hollow fiber membrane (600 $\mu$m inner diameter, 200 $\mu$m wall thickness, 0.2 $\mu$m pore size, 75% porosity) was purchased from Membrana GmbH (Wuppertal, Germany).

**Plant Cultivation and Root Exudates Collection.** Hot pepper seeds were obtained from the Academy of Agricultural Science, Daqing, China. Hot pepper seeds were sterilized by 1% $KMnO_4$ treatment for 30 min before germination, then washed several times with distilled water, and finally placed in a Petri dish with two sheets of moist filter paper. Seeds were cultivated for 15 days and transplanted to a recirculating hydroponic culture system. The number of seedlings and the solution volume were constant in every container, and carbon dioxide and sunlight were regularly supplemented every day. The hydroponic solution was prepared with distilled water. Real sample solution was obtained from recirculating hydroponic solution of hot pepper after 15 and 30 days of cultivation. The collected solutions were maintained in brown glass bottles. Blank tests were performed using distilled water and hydroponic solution without plants to eliminate the effect of external factors, respectively. All of the experiments were performed in three replicates.

**GC-MS Analysis.** The GC-MS experiments were performed using a GC 2010 gas chromatograph coupled to a QP mass spectrometer (Shimadzu, Japan) operating in full scan in the $m/z$ range of 50−500 and selected ion monitoring (SIM) mode for analytes. The column used was an Rxi-5 ms (5% diphenyl/95% dimethylpolysiloxane) fused silica capillary column (30 m long × 0.25 mm i.d × 0.25 $\mu$m film thickness). The initial temperature of the column was set at 50 °C and held for 1 min, then increased to 250 °C at a rate of 15 °C $min^{-1}$ and held for 2 min, and finally increased to 270 °C at a rate of 20 °C $min^{-1}$ and held for 6 min. The injector temperature was set at 250 °C. The MS operating conditions were as follows: Helium (99.999%) was employed as carrier gas, and its rate was adjusted to 3 mL $min^{-1}$ in constant flow mode. The ion source and transfer line temperatures were set at 200 and 280 °C, respectively. The electron energy (70 eV) was operated in the EI mode. SIM mode was used to perform all quantification experiments for three target analytes. The ions used for the confirmation of $m/z$ values of 1-NA, DPA, and N-P-2-NA were 143, 115, and 116; 169, 115, and 84; and 219, 218, and 115, respectively.

**HF-LPME with in Situ Derivatization and LLE Procedure.** The hollow fibers were cut into 5 cm length pieces. Each piece was discarded after each extraction to avoid the memory effect. Hollow fiber was sonicated for 5 min in acetone to remove any possible contaminants prior to extraction. Then, they were removed from the acetone, and the solvent was naturally evaporated at room temperature. Ten milliliters of standard solution with 5 $\mu$g $mL^{-1}$ 1-NA, DPA, and N-P-2-NA after adjustment of the pH with an FE20 pH meter (Mettler, China) was added into a 15 mL sample vial (Supelco, America) containing a 2 mm × 12 mm magnetic stirring bar. The sample was stirred during the extraction with a 6798-420D (Corning, America) magnetic heated stirrer. The mixture of extraction and derivatization reagent was prepared by mixing toluene with AA (80:20, v/v%) in proportion. The fiber was immersed in the organic solvent for a few seconds to impregnate the membrane pores of the hollow fiber wall. A 50 $\mu$L microsyringe filled with 15 $\mu$L of mixture was inserted into the hollow fiber to fill it with the acceptor phase, and the other microsyringe was used to seal the end and withdraw organic solvent. Sample solution was extracted at 30 min of extraction time, 50 °C extraction temperature, and 800 rpm stirring rate. After extraction, the solvent mixture with analyte was withdrawn into the syringe and the hollow fiber was discarded. Then, 1.0 $\mu$L of solvent mixture was injected into the injection port of the GC-MS.

The LLE method was used for the isolation organic compounds to compare extraction performance between LLE and the HF-LPME with the in situ derivatization method in this paper. Four different organic solvents (toluene, dichloromethane, ethyl acetate, and *n*-hexane) were used to extract the hydroponic solution of hot pepper. The organic phase was evaporated to dryness at 40 °C by a rotary evaporator after dehydration by $CaSO_4$ and brought to a volume of 1 mL by extractant. Then, 1 $\mu$L of organic solvent containing the target analytes was used for analysis after filtration through a 0.45 $\mu$m organic membrane.

## RESULTS AND DISCUSSION

**Selection of Derivative Methods.** The response of phenylamine with high polarity was poor in GC-MS without derivatization, so derivative methods were first selected. In the first method, 15 $\mu$L of toluene was used for LPME, and 4 $\mu$L of extractant was combined with 1 $\mu$L of AA in the injection port. In the second method, 12 $\mu$L of toluene was held in the hollow fiber for the extraction of the analytes. The extraction was performed at an 800 rpm stirring rate for 30 min without NaCl. The used toluene was retrieved into a derivative bottle, and 3 $\mu$L of AA was added into the bottle for derivatization at 60 °C for 20 min. Then 1.0 $\mu$L of extractant was injected. In the third method, 15 $\mu$L of a mixed solution of toluene and AA (v/v = 80:20, v/v %) was held in the hollow fiber for simultaneous extraction and derivatization, followed by injection of 1.0 $\mu$L of extractant.

Experimental results showed that the first method might lead to incomplete derivatization and that the derivatization reagent


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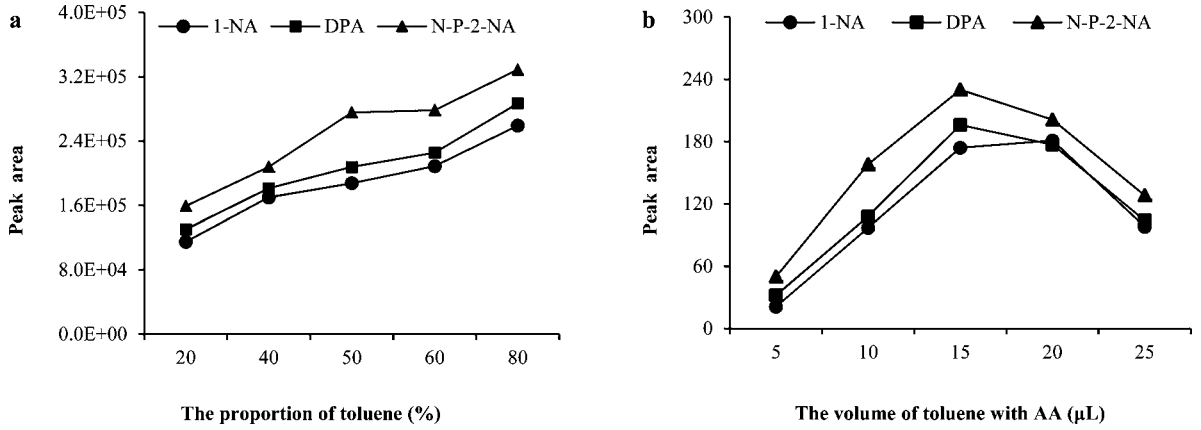


**Figure 1.** Optimization of toluene/AA: (a) optimization of toluene/AA proportion; (b) optimization of volume of toluene/AA. Conditions of HF-LPME with in situ derivatization: extraction time, 30 min; extraction temperature, 50 °C; no NaCl; stirring rate, 800 rpm; pH, 12.0. The concentrations of target analytes were 5 $\mu$g mL$^{-1}$.

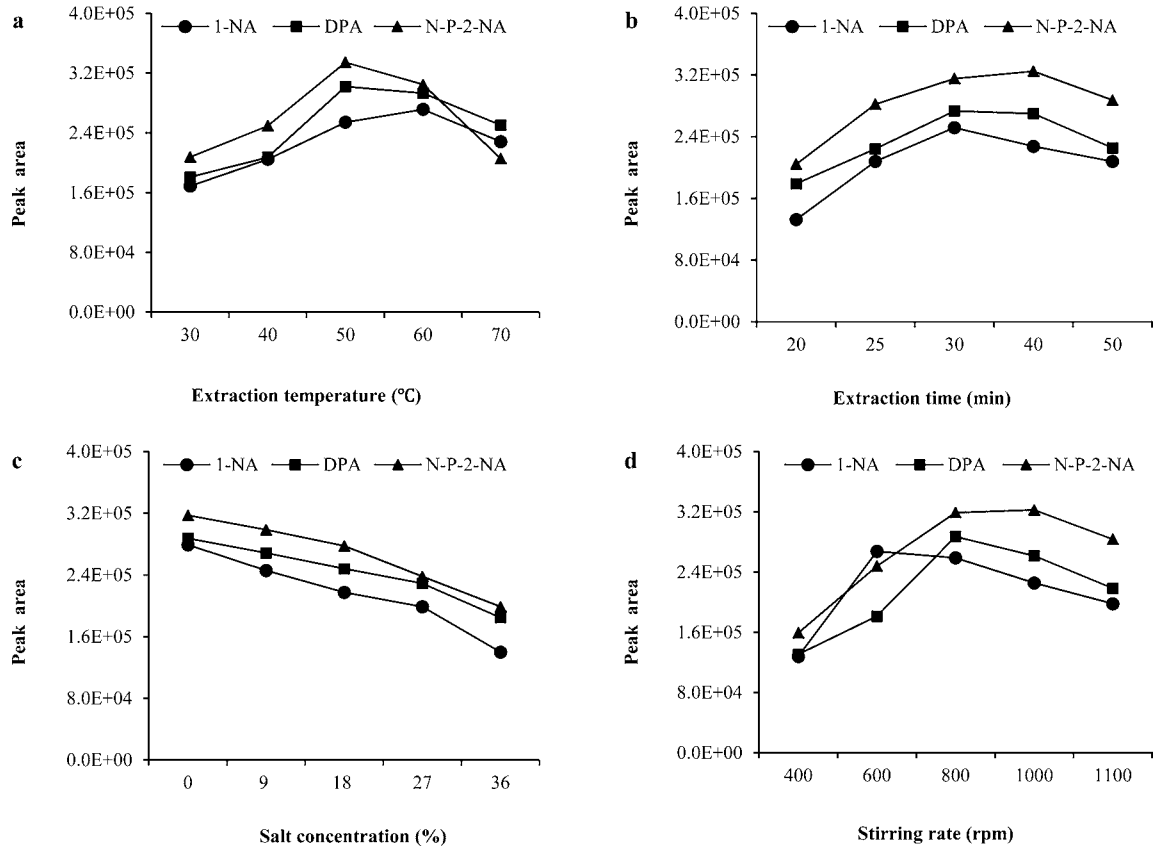


**Figure 2.** Optimization of the HF-LPME parameters: (a) extraction temperature; (b) extraction time; (c) salt concentration; (d) stirring rate. Conditions of HF-LPME with in situ derivatization: toluene/AA, (v/v = 80:20, v/v %), 15 $\mu$L. The concentrations of target analytes were 5 $\mu$g mL$^{-1}$.

might damage the capillary column. The second method needed complex operation; it easily caused a high limit of detection and low precision. The third method, in situ derivatization, integrated derivatization into extraction, which was simple and rapid. The comparison result suggested that the third method was suitable for three target analytes using a mixed solution of toluene and AA (v/v = 80:20, v/v %).

**Optimization of HF-LPME with in Situ Derivatization.** *Effect of Organic Solvent.* The selection of organic solvent is important for HF-LPME to gain maximum enrichment factor (EF). The extractant should have the following properties. First, it should be immiscible with water. Second, the polarity of the organic solvent should match that of the derived product. Third, it must be stable during the extraction process.[39] As analytes were derivatized at amino groups, solvents without amino groups were chosen. For the selection of the optimum extraction solvents, benzene, cyclohexane, isooctane, toluene, and chloroform were evaluated. The peak areas of the derivative product using HF-LPME with in situ derivatization were determined by GC-MS ($n$ = 5) in 10 mL of working solution. Each of the extraction solvents was mixed with AA (v/v = 80:20, v/v %), and 15 $\mu$L of the mixture was held in the hollow fiber for extraction. The largest peak area was obtained with chloroform, but it is volatile and led to poor precision. Thus,


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**Table 1. Analytical Performance of Developed Method for Target Analytes**

| analyte | linearity range ($\mu$g mL$^{-1}$) | $r^2$ | LOD[b] ($\mu$g mL$^{-1}$) | RSD[a] (%, $n$ = 5) 2 $\mu$g mL$^{-1}$ | RSD[a] (%, $n$ = 5) 5 $\mu$g mL$^{-1}$ | EF[c] |
|---|---|---|---|---|---|---|
| 1-NA | 0.1−10 | 0.9964 | 0.096 | 9.5 | 8.4 | 174 |
| DPA | 0.1−10 | 0.9978 | 0.074 | 8.6 | 7.6 | 196 |
| N-P-2-NA | 0.1−10 | 0.9989 | 0.057 | 7.8 | 6.2 | 230 |

[a]Relative standard deviation. [b]Limit of detection for S/N = 3. [c]Target analyte concentration: 5 $\mu$g mL$^{-1}$.

toluene was selected as the extraction solvent on the basis of comprehensive consideration.

*Effect of Toluene/AA.* The amount of AA required for derivatization was investigated by mixing AA with 20, 40, 50, 60, and 80% (v/v) of toluene (Figure 1a). It was found that a ratio of toluene/AA of 80:20 (v/v %) produced the optimal result. Perhaps it is because the appropriate amount of organic solvent assists the mass of analytes diffusing and transferring from the organic solvent in the pore of the hollow fiber wall into the mixed solution. Hence, 20% of AA was used in the following experiments.

The effect of mixed toluene/AA solution volume was evaluated by calculating EF according to the following formula:[28]

$$\mathrm{EF} = 1/(V_o/V_a + 1/K) \quad (1)$$

$K$ is the distribution coefficient, $V_o$ is the volume of organic solvent, and $V_a$ is the volume of aqueous sample. $K$ is calculated on the basis of the two-phase equilibrium condition

$$K = C_{o,eq}/C_{a,aq} \quad (2)$$

where $C_{o,eq}$ is the concentration of analyte in the organic phase and $C_{a,aq}$ is the concentration of analyte in the aqueous phase.

The experiment was performed using mixed toluene/AA (v/v = 80:20, v/v %) solution volumes from 5 to 25 $\mu$L, and the results were evaluated by comparison of EF (Figure 1b). It was found that the optimum volume of mixed toluene/AA (4:1) was 15 $\mu$L and the EF of 1-NA, DPA, and N-P-2-NA reached 174, 196, and 230, respectively.

*Effect of Extraction Temperature and Extraction Time.* Temperature not only affects derivatization efficiency but also affects the kinetics and thermodynamics of the diffusion process of analytes during the extraction.[40] In this experiment, the extraction temperature, ranging from 30 to 70 °C, was examined, and the amount of extracted analytes increased with increasing temperature up to 50 °C (Figure 2a). It can be explained that higher temperature may increase derivatization efficiency and improve the amounts of analytes in the hollow fiber, which results in the increase of 1-NA and DPA; however, N-P-2-NA showed a slightly increasing trend with increasing extraction temperature above 50 °C. It was possibly caused by high vapor pressure of toluene that could volatilize out of the hollow fiber and dissolve into the water solution above 50 °C. With comprehensive consideration, 50 °C was chosen as the extraction temperature in the following experiments.

The optimal extraction efficiency is obtained when equilibrium time is established. Equilibrium time is another key parameter influencing the extraction efficiency. However, equilibrium time depends on the mass transfer of the analytes from aqueous solution to organic solvent. Therefore, the extraction time plays an important role in the whole process. The extraction efficiency was evaluated by extraction time in the range of 20−50 min in this experiment (Figure 2b). 1-NA, DPA, and N-P-2-NA reached equilibrium at 30 min, and no further increase was observed after 30 min. Therefore, 30 min of extraction time was selected in the subsequent studies.

*Effect of Salt Concentration.* The viscocity of sample solution may increase with increasing salt concentration. In addition, salt addition can decrease the solubility of analytes in the sample solution and improve the partition coefficient into the organic phase. Sample solutions with various concentrations of NaCl in the range of 0−36% (w/v) were investigated (Figure 2c) to investigate the effect of salt addition. It was observed that extraction efficiency showed a slightly decreasing trend with increasing salt concentration. This is most likely due to the increased viscosity of the sample solution, which limited the movement of the analyte from the aqueous phase to the organic phase. Therefore, no salt was added in the following experiments.

*Effect of pH.* The pH of the aqueous phase should be adjusted to ensure that the analyte is electrically neutral so that it can be efficiently transferred into the organic phase.[32] The p$K_a$ values of the three analytes were all <5; therefore, the aqueous phase should be adjusted to basic condition to maintain the neutrality and reduce the solubility of analytes within the aqueous phase. A comparison of extraction of the analytes was performed at pH 9.0, 10.0, 11.0, 12.0, and 13.0. This showed that pH 12.0 was optimum to obtain the highest extraction efficiency.

*Effect of Stirring Rate.* Mass transfer of target analytes through the organic solvent in the hollow fiber can be improved by agitation. The interface between the aqueous phase and the organic phase will be continuously exposed with agitation.[41] In the experiment, the effect of stirring rate on the extraction efficiency was investigated in the range of 400−1100 rpm (Figure 2d). It was found that the extraction efficiency of 1-NA and DPA increased with increasing stirring rate up to 800 rpm; after that, the extraction efficiency slightly decreased with increasing stirring rate. However, N-P-2-NA showed a slightly increasing trend with increasing stirring rate 800 rpm. Perhaps, their dissolution gradually increased with the increase of stirring rate. On the basis of comprehensive consideration, the stirring rate was set at 800 rpm in the following experiments.

**Method Evaluation.** *Validation of HF-LPME with in Situ Derivatization and LLE.* Analytical performances for the extraction of 1-NA, DPA, and N-P-2-NA including their linearity, repeatability, limits of detection (LODs), and recoveries of HF-LPME with in situ derivatization were evaluated. The extraction results obtained by extracting standard solutions over a concentration range of 0.1−10 $\mu$g mL$^{-1}$ are shown in Table 1. For HF-LPME with in situ derivatization, good linearity was obtained with acceptable correlation coefficients ($r^2$) of 0.9964 (1-NA), 0.9978 (DPA), and 0.9989 (N-P-2-NA); limits of detection (S/N = 3) of 0.096, 0.074, and 0.057 $\mu$g mL$^{-1}$, EF up to 174, 196, and 230 at 5 $\mu$g mL$^{-1}$, and relative standard deviations (RSD) below 9.5% were obtained at concentrations of 2 and 5 $\mu$g mL$^{-1}$ for 1-NA,


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DPA, and N-P-2-NA, respectively. In LLE, dichloromethane had better extraction efficiency than other solvents. Analytical performances for the extraction of 1-NA, DPA, and N-P-2-NA including their linearity, repeatability, LODs, and recoveries were validated. Poor repeatability, low recoveries, and high LODs for target analytes were obtained at 5 $\mu$g mL$^{-1}$ concentration level (Table 2). The chromatograms of 5 $\mu$g mL$^{-1}$ standard solution by HF-LPME with in situ derivatization and LLE are shown in Figure 3. The result showed that higher EF, better repeatability, lower LOD, and satisfactory recovery were obtained by HF-LPME with in situ derivatization under optimum conditions for target analytes compared with LLE.

**Table 2. Analytical Performance of the LLE Method for Target Analytes**

| analyte | linear range ($\mu$g mL$^{-1}$) | $r$ | LOD ($\mu$g mL$^{-1}$, $n$ = 5) | RSD[a] (%, $n$ = 5) | recovery[b] (%, $n$ = 5) |
|---|---|---|---|---|---|
| 1-NA | 0.1−10 | 0.9165 | 0.184 | 18.7 | 62.1 |
| DPA | 0.1−10 | 0.9287 | 0.157 | 15.4 | 65.2 |
| N-P-2-NA | 0.1−10 | 0.9496 | 0.125 | 12.5 | 71.2 |

[a]Relative standard deviation. [b]Target analyte concentration: 5 $\mu$g mL$^{-1}$.

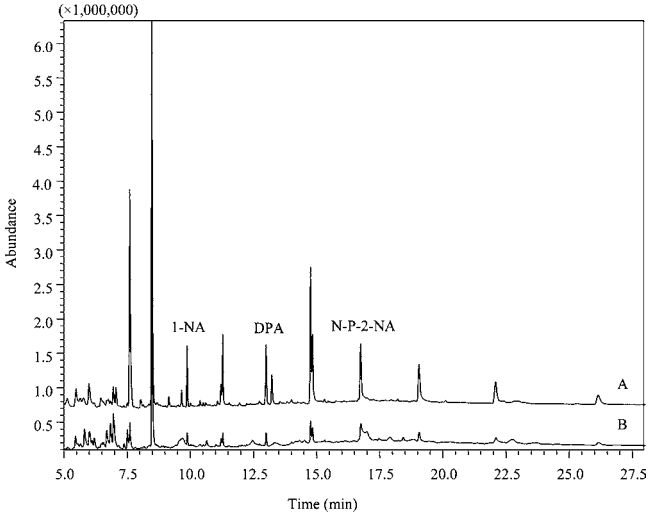


**Figure 3.** Chromatogram of 5 $\mu$g mL$^{-1}$ standard solution by two extraction methods: (A) HF-LPME with in situ derivatization; (B) LLE.

*Application in Real Sample Analysis.* The developed method has been applied to determine original concentrations of 1-NA, DPA, and N-P-2-NA for 15 and 30 days of root culture solution under optimum conditions. Meanwhile, RSD and recovery were evaluated at concentrations of 2.0 and 5.0 $\mu$g mL$^{-1}$ spike levels, respectively. The determination results are shown in Table 3. Recoveries, ranging from 90.2 to 96.1%, and RSDs below 9.1% were obtained for the root exudates 1-NA, DPA, and N-P-2-NA. This proposed method is simple, inexpensive, and environmentally friendly, and only small amounts of organic solvent and derivatization reagent are required. Moreover, HF-LPME with the in situ derivative method can be applied for simultaneous extraction and derivatization of the target compounds in real sample solutions and also be an excellent alternative method for phenylamine compound analysis in aqueous samples.

**Table 3. Concentrations and Recoveries of Target Analytes Determined in Sample Solution by HF-LPME with in Situ Derivatization**

| | original ($\mu$g mL$^{-1}$) | | spiked ($\mu$g mL$^{-1}$) | | RSD (%, $n$ = 5) | | recovery (%, $n$ = 5) | |
|---|---|---|---|---|---|---|---|---|
| analyte | $a$ | $b$ | $a$ | $b$ | $a$ | $b$ | $a$ | $b$ |
| 1-NA | 2.87 | 3.89 | 2 | 5 | 9.1 | 7.9 | 90.2 | 91.7 |
| DPA | 4.17 | 4.88 | 2 | 5 | 7.4 | 6.4 | 91.8 | 92.4 |
| N-P-2-NA | 4.05 | 5.12 | 2 | 5 | 7.7 | 5.9 | 93.8 | 96.1 |

[a]Extraction for 15 day root culture solution. [b]Extraction for 30 day root culture solution.

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### Notes

The authors declare no competing financial interest.

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