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Continuous-Flow Microextration Coupled with HPLC for the Determination of 4-Chloroaniline in *Chlamydomonas reinhardtii*

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Aromatic amines are widely used as intermediates in the dye, photographic, pharmaceutical and pesticide industries, and as antioxidants in the polymer industry. Because of their toxicity (Shonali and Richard 1990), several of them have been listed on the priority blacklist released by US Environmental Protection Agency in 1970s. Among several types of methods for degrading aromatic amines, biodegradation is one of the most important methods. So far, many species of bacteria have been isolated to biodegrade aromatic amines (Schnell et al. 1989). Accordingly, it is necessary that a simple and sensitive method be founded to determine the residue of aromatic amines in biological samples.

HPLC with UV detection is often applied for the analysis of aromatic amines (Lewin et al. 1997). It is essential that an enrichment step be conducted to achieve low detection limit prior to analysis. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are conventional pretreatment methods (Smith et al. 2003). However, the procedure of LLE is time-consuming and requires large quantity of toxic solvent that can be harmful to the environment. SPE requires less solvent, but the presence of particulate matter in the sample often results in plugging of the cartridge. Solid-phase microextraction (SPME), which is a rapid and solvent-free extraction, has been widely used for detecting aromatic amines (Wang et al. 2004; Yan and Jen 2003). However, the main drawback of SPME is that the fiber is expensive yet with a limited lifetime.

Liquid-phase microextraction (LPME) is an extremely simple, low-cost and virtually solvent-free sample-preparation technique. Now LPME has developed several different forms, for example static and dynamic LPME, hollow-fiber membrane LPME, headspace LPME, and continuous-flow microextraction (CFME). Among these, CFME is a relatively novel LPME method, first reported by Liu and Lee (2000). It possesses such advantages as low cost, simplicity of pretreatment process, little usage of organic solvent and amity to environment.

Much research has been carried out so far on microextraction of aromatic amines from water samples (Zhao et al. 2002). However, little information is available on it from *Chlamydomonas reinhardtii* samples. Therefore, the objective of this research was to use CFME-HPLC technique for analysis of 4-chloroaniline (4-CA) in *C. reinhardtii* cells. The effects of different factors on CFME efficiencies were also investigated and these factors included the kind of extraction solvent, solvent drop volume, sample flow rate, extraction time and addition amount of salt. To evaluate its real application effect, the optimal method was used to determine 4-CA in *C. reinhardtii* cells and tap water samples.

Materials and Methods

4-CA (99.0% purity) was purchased from Fluka (USA). HPLC-grade methanol was obtained from Tedia (Fair lawn, New Jersey, USA). Deionized water was purified with a Millipore Mill-Q plus System. All of the used reagents in this experiment (isooctane, *n*-hexane, carbon

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tetrachloride, benzene, toluene, xylene and dimethylsulfoxide) were of analytical grade and redistilled before use. The stock solution (100 μg mL^{-1} in methanol) of 4-CA was prepared freshly every 2 weeks and stored at 4°C until use. Working standard solutions of 4-CA were prepared by appropriate dilution of the former stock solution using deionized water.

An axenic *C. reinhardtii* culture was obtained from Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). *C. reinhardtii* was cultured in CHU-11 medium (http://www. Index of Cyanobacteria Medium Recipes for Modern Browsers) in 250 ml Erlenmeyer flask at 25°C under illumination of 45 μ$Em^{-2}s^{-1}$ with a 12:12 h light and dark shift cycle in an environmental chamber. The pH of the medium varied between 7 and 8, and was not adjusted over the whole experimental period (8 days).

Chlamydomonas reinhardtii cells at exponential growth phase were diluted to a density of 1×10^5 cell mL^{-1} by the sterile CHU-11 medium. The final volume was 500 mL. An appropriate amount of the 4-CA stock solution was added to the former medium to obtain a final concentration of 10 μg mL^{-1}. The preceding sample was incubated as described above for 8 days, and centrifuged at 3,500 rpm for 15 min. The precipitated algal cells were weighted (0.032 g) and ground with quartz sand until no intact cell could be observed under a microscope. Dimethylsulfoxide (DMSO, 1 mL) was used to extract the residue of 4-CA in algal cells, which was then centrifuged at 3,500 rpm for 15 min. The supernatant was pipetted and diluted with deionized water to 100 mL, i.e., 4-CA in algal cells was dissolved in 100-mL aqueous solution. This aqueous solution was prepared for further CFME procedure.

The continuous-flow liquid-phase microextraction device is shown in Fig. 1, and the extraction procedure is described as follows: a HL-2 model microperspex peristaltic pump (Shanghai, China) delivers the aqueous sample in the glass bulb extraction chamber (about 0.2 mL) via the connecting PTFE tubing at a constant rate; a 10-μL LC microsyringe introduces the appropriate volume of organic solvent into the extraction chamber when the chamber is full of sample solution and the organic solvent forms a drop which remains at the tip of the microsyringe above the PTFE tube outlet in the extraction chamber; the organic solvent extracts the analytes from the sample solution which flows through the chamber continuously; the organic solvent is retracted into the microsyringe and injected directly for HPLC analysis after extraction for an appropriate period of time (Guo et al. 2005).

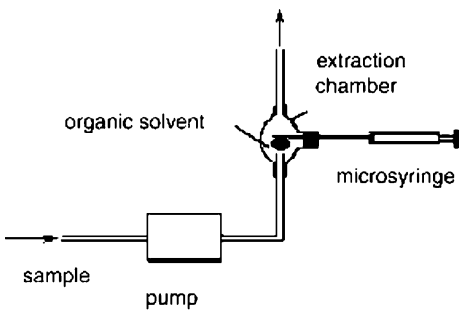

Fig. 1 Schematic diagram of the continuous-flow microextraction device

The residual analysis of 4-CA was carried out using an Agilent 1100 HPLC system equipped with a manual injector and variable wavelength detector (VWD). The column used was a Venusil XBP-C_{18} column (4.6 × 250 mm, 5-μm particle size). The operating conditions were as follows: mobile phase, methanol–water (70:30 by volume); flow rate, 0.6 mL min^{-1}; column temperature, 25 ± 1°C; the wavelength of detector, 254 nm; and the size of loop, 5 μL. Under the above conditions, the retention time of 4-CA was about 8.0 min.

Results and Discussion

To determine the optimal extraction conditions, the peak area of the analyte was used to evaluate extraction efficiency under different conditions. Selection of a proper extraction solvent should consider two factors, one is that the solvent must be immiscible with water and the other is that it should possess excellent chromatographic behavior. On the basis of these considerations, isooctane, *n*-hexane, carbon tetrachloride, benzene, toluene and xylene were tested in this work. Isooctane, benzene, toluene and xylene could be used to extract the analyte, but their solvent peaks were high enough to interfere with the analyte peak. In contrast, not only did the peak of *n*-hexane and carbon tetrachloride not interfere with the analyte peak but also they could be used for effective extraction of the analyte. Extraction efficiency with carbon tetrachloride was about 3.5 times over that with *n*-hexane. As a result, carbon tetrachloride was selected as the extraction solvent in this experiment.

In CFME, the volume of extraction solvent is an important factor in extraction efficiency. Under the following constant conditions (extraction time, 10 min and flow rate of sample solution, 1.0 mL min^{-1}), the effect of different solvent drop volumes on extraction efficiencies was tested and the result is shown in Fig. 2. Obviously, an increase in the volume of the solvent drops in the range of 1.5–3.5 μL resulted in the substantial enhancement of the extraction efficiency. However, when the microdrop volume exceeded 4 μL, the drop was too large to suspend stably at the needle tip. Consequently, a microdrop volume of 3.5 μL was selected for subsequent experiments.

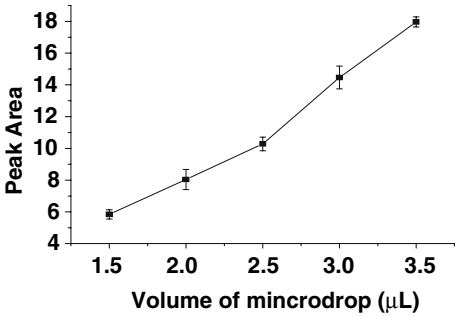

Fig. 2 Effect of carbon tetrachloride micro-drop volume on extraction of 4-CA [*Each point* represents the mean of triplicate measurements and error bar indicates standard deviation of triplicate (n = 3). The same replicates in the following figures unless otherwise stated]

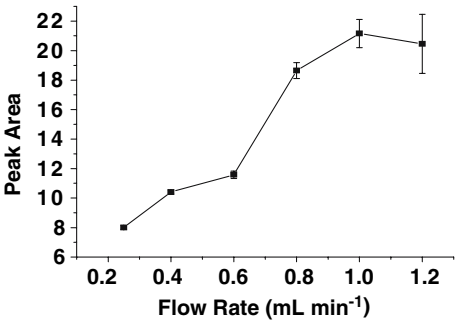

Fig. 3 Effect of flow rate of the sample solution on extraction of 4-CA

In CFME, the extraction process is an important step to transfer the analytes in both phases. This step can be influenced by the interfacial thickness, which is further affected by the flow rate concurrently. Therefore, the flow rate can affect the extraction efficiency in CFME. The extraction efficiencies with different flow rates (0.2–1.2 mL min^{-1}) were determined under the following conditions (extraction time, 10 min and solvent-drop volume, 3.5 μL) and the result is demonstrated in Fig. 3. The increase of the sample flow rate could lead to the increase of analyte peak area when the flow rate was below 1.0 mL min^{-1}. On the contrary, analyte peak area decreased when the flow rate exceeded 1.0 mL min^{-1}. Maybe the extraction equation in both phases was difficult to establish commendably at higher flow rate. Consequently, a flow rate of 1.0 mL min^{-1} was used for subsequent experiments.

When CFME was carried out at a constant flow rate of 1.0 mL min^{-1} and the volume of the microdrop was 3.5 μL, the longer extraction time (2–20 min) could lead to higher extraction efficiency. However, the longer extraction time was always concomitant with larger volume loss of microdrop, which reduced the experimental precision. The relationship between the loss of solvent microdrop volume and the extraction time was tested, and is shown in Fig. 4. If we adopted the short extraction time, the satisfactory extraction efficiency could not be obtained. After taking into account both cases, 10 min was selected for subsequent experiments.

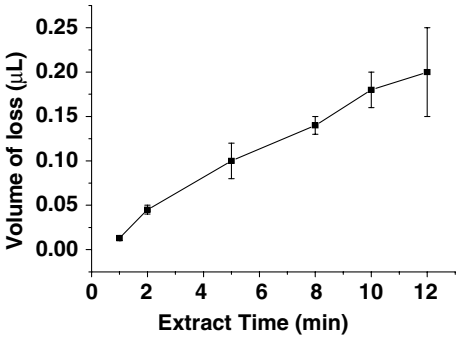

Fig. 4 Relationship between extraction time and loss in volume of the micro-drop

The concentration of salt in aqueous solution may have several effects on extraction efficiency. The presence of salt can increase the ionic strength of the solution and affect the solubility of organic analyte. Some researchers (Penalver et al. 2000; Sarrion et al. 2002) have reported that addition of NaCl to samples could enhance the efficiency of extraction because of the salting-out effect. However, the contrary results to the former conclusions also have been pointed out by other investigators (Guo et al. 2005; Psillakis and Kalogerakis 2001; Zhao et al. 2004) and the possible explanation may be that NaCl dissolved in aqueous solution may have changed physical properties of Nernst diffusion film and reduced rate of diffusion of the target analyte into the drop (Psillakis and Kalogerakis 2001). In this study, the effects of different NaCl concentrations ranged from 0 to 20% (w/v) on extraction efficiencies are illustrated in Fig. 5. The results showed that peak areas decreased apparently even at low NaCl concentrations. Consequently, no addition of salt was selected in subsequent experiments.

In total, the optimal conditions for this CFME method was as follows: carbon tetrachloride as the extraction solvent, the flow rate of 1.0 mL min^{-1}, extraction time of 10 min, drop volume of 3.5 μL and no addition of salt in the extraction process. The former optimal method was further used for determination of real *C. reinhardtii* samples.

Chromatograms obtained from a 100-ng mL^{-1} standard solution before and after CFME under the optimal conditions are shown in Fig. 6a and b, respectively. It was obvious that the peak area of 4-CA substantially increased when the sample was extracted by CFME. Additionally, no interference with 4-CA peak (RT, 8.0) was observed.

The linearity for the determination of 4-CA by CFME-HPLC method was evaluated under the optimal conditions. The calibration plot was obtained by plotting peak area against six levels of 4-CA (0.01–10 μg mL^{-1}). The corre-

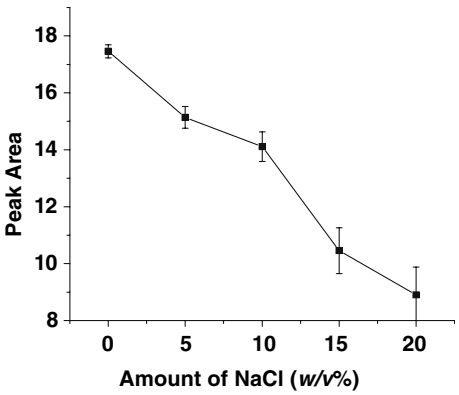

Fig. 5 Effect of the concentrations of salt on extraction efficiency

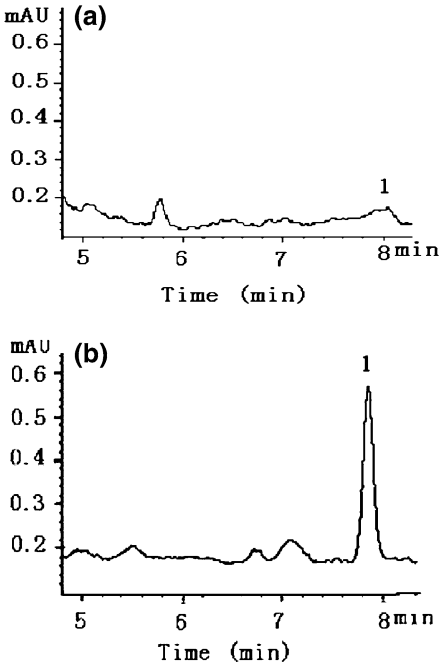

Fig. 6 Chromatograms obtained from a 100 ng mL^{-1} standard solution before (**a**) and after (**b**) CFME of 4-CA under the optimal conditions

lation coefficient (r) was 0.9997, and the limit of detection (LOD) was 6.6 ng mL^{-1} based on the signal-to-noise ratio of 3. The precision of the method was tested by successive five time analyses of a 100 ng mL^{-1} standard solution of 4-CA and the results showed that the relative standard deviation (RSD) was 5.9%. By the ratio of the peak areas of the analyte obtained after and before CFME, the estimated concentration factor was about 11.9. Therefore, this method could be applied for analysis of 4-CA in *C. reinhardtii* and tap water samples.

C. reinhardtii and tap water samples from our laboratory were used as the real samples to test the applicability of CFME-HPLC method. The detection result showed that 4-CA concentration in tap water was below detectable level (<6.6 ng mL^{-1}), which showed that the investigated tap water was nearly free from 4-CA contamination. As compared to tap water, 4-CA residue in 100-mL aqueous solution (see section of 4-CA extraction procedure in *C. reinhardtii* cells) was found to be 22 ng mL^{-1}. As a result, the total mass of 4-CA in C. *reinhardtii* cells was 2.2 μg. *C. reinhardtii* and tap water samples were spiked with 4-CA at 20 ng mL^{-1}, respectively, to evaluate extraction recoveries by CFME method. The results showed that recoveries from *C. reinhardtii* and tap water samples were 113.5% and 96.4%, and RSD were 6.7% and 5.2%, respectively. The preceding results demonstrated the different samples had little effect on CFME extraction of 4-CA. Therefore; the former method could be extensively used in 4-CA analysis of contaminated water.

The mean recovery for CFME-HPLC method to determine 4-CA in *C. reinhardtii* cells was 113.5% and RSD was 6.7%, which proved that CFME was an efficient sample-preparation technique for the determination of 4-CA in *C. reinhardtii* cells. In addition, the procedure of CFME pretreatment is relatively simple, low cost and amity to environment. As a result, this method has tremendous potential in trace analysis of 4-CA in polluted waters.

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