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Liquid–liquid–liquid microextraction of aromatic amines from water samples combined with high-performance liquid chromatography

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Abstract

A simple preconcentration and clean-up liquid–liquid–liquid microextraction of aromatic amines is described in this paper. The compounds were extracted from 2.0 ml aqueous samples (donor phase) into an organic phase, layered on the donor phase, and then back extracted to a microdrop of aqueous receiving phase, suspended in the organic phase. After extraction, the microdrop was injected into the HPLC system directly for analysis. Optimal conditions of the extraction were donor phase (a_1): 2 ml of water sample adjusted to pH 13 with NaOH–NaCl; organic phase (o), 150 μl ethyl acetate; and receiving phase (a_2) of 2 μl aqueous solution at pH 2.1. The $a_1 \rightarrow o$ extraction time was 15 min and for $o \rightarrow a_2$, 30 s. 18-Crown-6 ether, which can complex with amine, was added to the aqueous receiving phase to improve the extraction performance. Enrichment factors ranged from 218 (for 4-nitroaniline) to 378 (for 4-chloro-2-aniline). The calibration curve for these anilines was linear within the range 2.5 ng/ml–2.5 μg/ml (r^2=0.998). Detection limits ranged from 0.85 to 1.80 ng/ml (at S/N=3). This procedure can be a selective preconcentration method for aromatic amines present in water samples.
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Keywords: Liquid–liquid–liquid microextraction; Extraction methods; Water analysis; Environmental analysis; Amines, aromatic

1. Introduction

To obtain preconcentration and effect sample cleanup prior to high-performance liquid chromatographic (HPLC) and capillary electrophoretic (CE) analysis, two of the most common techniques used are liquid–liquid extraction (LLE) [1] and solid-phase extraction (SPE) [2,3]. However, both techniques require the evaporation of solvent to dryness and the reconstitution of the dry residue in a suitable solvent for HPLC or CE. These manual procedures are often tedious and very prone to loss of analytes through evaporation and adsorption. Also, in order to achieve high preconcentration, a large volume of sample is needed against a much smaller volume of organic phase.

The solvent evaporation step can be eliminated by performing SPE on-line using a short precolumn. To eliminate both the solvent evaporation step and large sample volume consumption, we can perform solid-phase microextraction (SPME) which needs only a small volume of sample to achieve high preconcentration. However, when coupled to HPLC or CE, a

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solvent desorption step is required to recover all the sorbed analytes and to avoid carry-over in the experiment. The coatings currently available for SPME are either nonpolar or slightly polar, hence SPME cannot be satisfactorily used for highly polar analytes.

Liquid-phase microextraction (LPME) [4–8] is an alternative to SPME. Only one drop of organic solvent is used to extract compounds from water samples. However, this method is usually more suitable for GC, and it is almost always used for nonpolar compounds.

Based upon the principles of LPME, Ma and Cantwell [9,10] developed a solvent microextraction technique recently, which we term here as liquid–liquid–liquid microextraction (LLLME), to achieve preconcentration and purification for polar analytes without the need for both solvent evaporation and analyte desorption. In this method, three liquid phases were used, a_1 is the water sample where pH is adjusted to deionize the compounds; the organic liquid membrane phase (o), consisting of 40 or 80 μl of *n*-octane, is layered over the donor phase; then the receiving aqueous phase (a_2), the pH of which is adjusted to ionize the sample, is layered over the organic phase. With the help of stirring, polar compounds are extracted to the organic solvent and then back-extracted to the receiving phase, which can be directly analysed.

Similar to this method, the supported liquid membrane (SLM) technique [11–14] is another technique to extract basic compounds from water or human plasma. An organic solvent film was immobilized in a porous membrane or hollow fiber and subsequently back-extracted into a stagnant aqueous receiving phase on the other side of the membrane. Compared to the SLM, LLLME uses an unsupported microliter-sized liquid membrane. The thickness of the organic film is easier to control, and because this organic membrane is changed for every extraction, there are no memory effects and long-term instability in LLLME as compared to the SLM. Compared to the bulk liquid membrane (BLM) technique, LLLME uses a far smaller volume of organic phase (50–150 μl). The BLM uses a volume of a few milliliters to over 100 ml and in transport experiments, is usually very much more time consuming than LLLME.

Aromatic amines are widespread environmental pollutants. They are present in the aquatic environment as a result of industrial discharges from pharmaceutical companies or from other factories using them as reagents for the synthesis of chemicals such as dyes or some pesticides. So it is important to monitor their levels in environmental samples, particularly aqueous matrices.

In this paper, LLLME combined with HPLC is described to extract and analyse aromatic amines from water samples. Various aspects of the LLLME of these analytes including the effect of organic solvent, extraction time and extraction-enhancing additives were investigated.

2. Experimental

2.1. Chemicals and reagents

Aromatic amine compounds were used as obtained from AccuStandards (New Haven, CT, USA), which included 4-nitroaniline, 2-nitroaniline, 4-chloroaniline, 4-bromoaniline, 2-chloro-4,6-dinitroaniline, 4-chloro-2-nitroaniline and 2,6-dichloro-4-nitroaniline. The 18-crown-6 ether ($C_{12}H_{24}O_6$) was bought from Fluka (Buchs, Switzerland). Isooctane (J.T. Baker, Phillipsburg, NJ, USA), *n*-hexane (J.T. Baker), cyclohexane (Fisher, Fair Lawn, NJ, USA), butyl acetate (Lab-Scan, Dublin, Ireland) and ethyl acetate (J.T. Baker) were all HPLC-grade solvents. The HPLC-grade methanol was bought from Mallinckrodt (Paris, KY, USA) and the ultra pure water was prepared on a Nanopure system (Barnsted Thermolyne, Dubuque, IA, USA).

Stock solutions of the analytes (2.5 mg/ml) were prepared separately in methanol. Subsequent working solutions were prepared by diluting the stock solutions with a pH 13 NaOH–NaCl buffer solution at known concentrations. The surface sea water sample was collected using glass bottles from Desaru (Malaysia). These samples were filtered through a 0.45-μm filter membrane prior to use. Different concentrations of NaOH–NaCl and 50 m*M* Na_2HPO_4–50 m*M* H_3PO_4 were used to prepare the buffer solutions. A standard solution containing all seven aromatic amines (at 2.5 μg/ml) was prepared in 50 m*M* Na_2HPO_4–50 m*M* H_3PO_4 (pH 2.1).

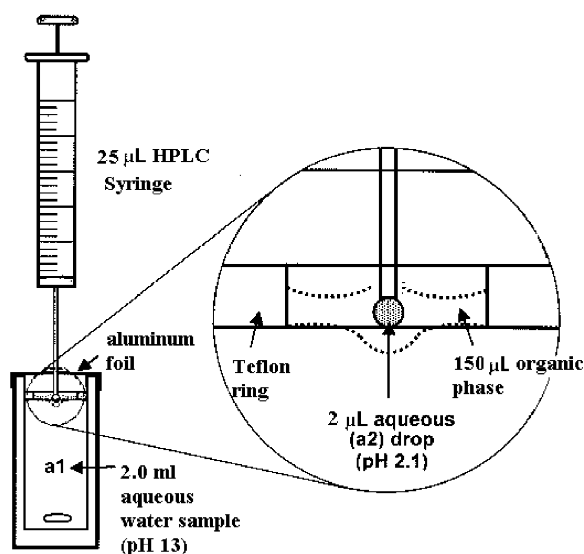


Fig. 1. Illustration of the microextraction apparatus for LLLME.

2.2. Extraction apparatus

The microextraction device is shown in Fig. 1. A 4-ml glass sample vial (Supelco, Bellafonte, PA, USA) measuring 44 mm×14 mm O.D.×13 mm I.D., was used as the receptable for the three phases. A PTFE ring measuring 3 mm×13 mm O.D.×10 mm I.D., was manually cut from a PTFE tube and set in the vial so that the surface of the 2 ml aqueous sample solution would just touch the bottom of the ring. Once set in place, the ring remained there throughout multiple extractions. A stirring bar, measuring 10 mm×3 mm was used to facilitate the mass transfer process. A Heidolph (Kelheim, Germany) MR3001 K magnetic stirrer was used to stir the extraction mixture and also to control the temperature of the water bath when an elevated temperature was required. Aluminum foil was used to cover the glass vial during extraction to prevent the evaporation of the organic phase. A 25-μl flat-cut HPLC syringe was used to suspend the microdrop of receiving phase during extraction and also for injection into the HPLC system after extraction.

2.3. Extraction procedure

The PTFE ring was first inserted into a clean glass vial, followed by the magnetic stirrer. A 2-ml volume of aqueous sample solution, a_1, containing all seven aromatic amines compounds in known trace concentrations dissolved in NaOH–NaCl buffer solution (pH 13), was then added to the vial. A 150-μl volume of the organic phase, o, was carefully added within the PTFE ring which was placed with its bottom touching the surface of the aqueous sample solution. The vial was covered with aluminum foil, placed upon the motor controller and clamped in place with a retort stand. Experiments were carried out at room temperature (set at 22 °C). Stirring speed ranged from 300 to 500 rpm. Also, the stirring for the first two phases can last from 5 to 15 min to facilitate the extraction of the analytes from the aqueous sample phase to the organic membrane.

The syringe filled with 2 μl of the aqueous receiving phase, a_2 (50 m*M* Na_2HPO_4–50 m*M* H_3PO_4 at pH 2.1), penetrating the aluminum foil, was positioned above the sample vial. The tip of the syringe needle was placed slightly below the surface of the organic phase. The tip must be in the middle of the vortex of the organic phase, which was very important for the back extraction, created by the stirring of the contents of the vial.

After the extraction time set for the transfer of analytes from the buffered aqueous sample, a_1, into the organic membrane, the aqueous receiving phase in the syringe was slowly pushed out to form a single microdrop in the organic phase and retained there for a short period of time. At the end of the extraction, the aqueous microdrop was withdrawn back into the syringe and injected into the HPLC system for analysis.

2.4. HPLC system

The HPLC system consisted of a Shimadzu (Tokyo, Japan) LC-6A pump, a Rheodyne (Cotati, CA, USA) 7010 injector equipped with a 10 μl loop, a Shimadzu SPD-GVA UV detector and a Shimadzu C-R6A integrator. Separation was accomplished using a 100 mm×4.6 mm I.D. Whatman (Maidstone, UK) Partisil 5 μm C_{18} column and a mobile phase of methanol–water (45:55, v/v). The flow-rate of the mobile phase was 1 ml/min and the detection wavelength was set at 254 nm.

3. Results and discussion

3.1. Basic principle

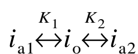
Prior to extraction, the sample solutions were adjusted to strongly basic condition (pH 13), so that the aromatic amine compounds were deionized, and their solubility in the sample solution reduced. The amines were first extracted into the organic membrane phase because of their affinity to the organic phase. The mass transfer was enhanced with stirring. Aromatic amines protonate very easily in strong acidic aqueous solution, and the protonated species have very slight affinity for the organic phase, so at the interface of $o \leftrightarrow a_2$, they enter rapidly into the receiving aqueous phase. Since the volume of acceptor solution (2 μl) is very small compared to the initial water sample solution, the target compounds are preconcentrated in the aqueous receiving water phase (a_2).

3.2. Effect of organic solvent

During the course of the experiment, several organic solvents were tested to investigate their effect on the extraction efficiency. Out of the five organic solvents tested, isooctane (polarity index: 0), *n*-hexane (0) and cyclohexane (0.2) were only able to extract 4-chloroaniline and 4-bromoaniline. On the other hand, butyl acetate (4.0) and ethyl acetate (4.4) could extract all seven analytes of interest and transfer them into the aqueous receiving phase. Hence, organic solvents with higher polarity are more suitable for extraction of aromatic amines. However, in the case of butyl acetate, because of some interferences encountered that rendered it difficult to distinguish the target sample peaks, ethyl acetate was selected as the most suitable organic solvent for subsequent extractions.

3.3. Equilibration and kinetic considerations

As described by Ma and Cantwell [10], there are two equilibria in the system, and the extraction equations for analyte i can be written as:



$$i_{a1} \overset{K_1}{\leftrightarrow} i_o \overset{K_2}{\leftrightarrow} i_{a2}$$

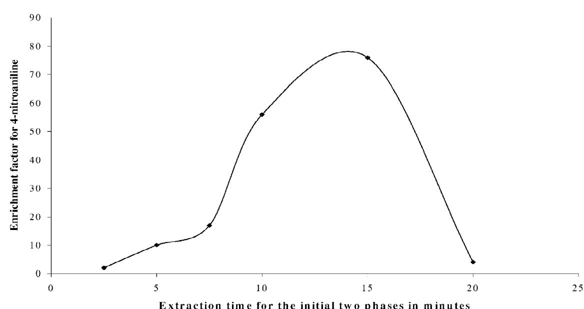


Fig. 2. Graph of enrichment factor versus extraction time for the initial two phases of a_1 and the organic membrane, 4-nitroaniline was used for an example. a_1, 2 ml, pH 13 (NaOH–NaCl), o, 150 μl ethyl acetate; a_2, 2 μl, pH 2.1 receiving aqueous phase, no addition of 18-crown-6 ether.

where the subscript a1 represents the donor aqueous, o, the organic membrane phase and a2 the receiving aqueous phase. The constants, K_1 and K_2 are equilibrium constants.

For the first equilibrium, i.e., $i_{a1} \overset{K_1}{\leftrightarrow} i_o$, the mass transfer is a slow procedure. This can be shown in Fig. 2. The extraction efficiency increases with the extraction time of the first step. For the back extraction process because the analytes are ionized to a species that have very slight affinity for the organic membrane phase, the distribution ratio, $K_2 = C_{o,eq}/C_{a2,eq}$ should be very small, where $C_{o,eq}$ and $C_{a2,eq}$ are the analyte concentration in organic phase and receiving phase at equilibrium, respectively. According to Ma and Cantwell [9], the ionization of analytes at the $o–a_2$ interface is a significantly faster process than mass transfer through the diffusion film. Thus, once the analytes enter the acceptor phase, it is difficult for them to return to the organic phase. The back extraction is therefore very fast and the extraction efficiency is very high.

3.4. Effect of extraction time of the initial two phases (T1)

The extraction of the aromatic amines from the water sample into the organic phase can be described as a slow equilibrium process. Hence, the extraction time is expected to play an important role in the extraction efficiency of the process. As shown in Fig. 2, the enrichment factor (EF, defined as the ratio

between the final analyte concentration in the acceptor phase and the initial concentration of analyte within the sample) increased with increasing exposure time from 5 to 15 min and reached a maximum when the initial two phases were stirred for as long as 15 min. But after 15 min, the efficiency showed a decline conceivably because of the loss of the ethyl acetate due to its evaporation. On the basis of these results then, the optimized extraction time for the first step is 15 min.

3.5. Effect of extraction time of the back extraction (T2)

As described before, the extraction is very fast for the back extraction from the organic solvent to the aqueous receiving phase, and the rate constant is large. It took only 30 s for the back extraction to attain equilibrium. The EFs did not increase significantly after 30 s. Thus, the back extraction was performed for no more than this length of time.

3.6. Addition of 18-crown-6 ether to a_2

Crown ether can form complexes with ammonium salts selectively [15]. This fundamental characteristic of crown ether was exploited in this work by adding 18-crown-6 ether into the aqueous receiving phase to increase the extraction efficiency of of the procedure with respect to extraction from the organic phase into the second aqueous phase a_2. The addition appears to help in facilitating the back extraction and stabilizing the compounds in the aqueous phase. Different concentrations (0–400 m*M*) of 18-crown-6 ether were used in the aqueous receiving phase, and the results of the effect of this compound on the extraction of the second step are shown in Fig. 3. The EF increased with the concentration of 18-crown-6 ether and achieves maximum preconcentration when 300 m*M* 18-crown-6 ether was present in a_2. As reported in some studies [16], the adsorption of surface active species at the liquid–liquid interface can produce an interfacial resistance which reduces the mass transfer rates of other compounds across the interface. Possibly due to this reason, the EF decreased when 18-crown-6 ether concentration was >300 m*M*.

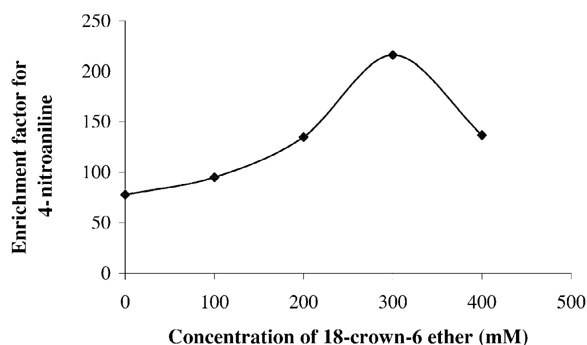


Fig. 3. The effect of concentration of 18-crown-6-ether in the aqueous receiving phase on the extraction efficiency, the 4-nitroaniline was used as an example; a_1, 2 ml of pH 13 water sample; o, 150 μl ethyl acetate; a_2, 2 μl, pH 2.1 receiving aqueous phase containing 18-crown-6-ether in different concentration. Time for the a_1–o extraction (T1) is 15 min; for o–a_2 (T2), 30 s.

3.7. Extraction from water samples

Based on the preliminary investigations and optimization work reported here, the following conditions were optimal for LLLME in extracting aromatic amines from water samples: 2 ml 0.3 *M* NaOH–saturated NaCl (pH 13) as the donor phase (a_1); 150 μl ethyl acetate as the organic phase (o) and 2 μl of 300 m*M* 18-crown-6 ether in 50 m*M* Na_2HPO_4–50 m*M* H_3PO_4 (pH 2.1) as the aqueous receiving phase, a_2. The extraction time with respect to the a_1–o phases is 15 min, and for o–a_2, 30 s.

3.8. Quantitative consideration

To evaluate the practical applicability of the proposed LLLME technique, repeatability, linearity, detection limit and limit of quantification were investigated by utilizing standard solutions of aromatic amines in water. Calibration curves for the seven compounds were obtained by plotting peak areas vs. the original sample concentrations (Ca_1). The linearity of all the compounds were in the range of 2.5 ng/ml–2.5 mg/ml, and their correlation coefficients (r^2) were 0.995–0.998. Table 1 shows other relevant data that indicate that the limits of detection (LODs) (0.85–1.80 ng/l) and EFs (218–378) are highly satisfactory. The RSD values are generally <10%.

A seawater sample spiked with 5 ng/ml each of

Table 1
Performance of LLLME

	Enrichment factor	Limit of detection (ng/ml)	RSD (%, $n=5$)	Relative recovery* (%)
4-Nitroaniline	218	0.90	4.16	99.2
2-Nitroaniline	313	0.85	2.07	98.0
4-Chloroaniline	299	1.50	8.55	90.8
4-Bromoaniline	306	1.50	8.32	90.6
2-Chloro-4,6-dinitroaniline	347	1.20	9.90	91.1
4-Chloro-2-nitroaniline	378	1.10	6.84	89.8
2,6-Dichloro-4-nitroaniline	263	1.80	10.90	88.5

*Relative recovery values of spiked seawater sample at 5 ng/ml compared to that of spiked pure water.

the analytes was extracted using LLLME with this set of optimal conditions, the results of which are shown in Fig. 4. The matrix had an effect on the pH adjustment, more concentrated NaOH (6 *M*) was needed to adjust the pH of seawater to 13. We had to use a spiked sample to test the procedure because no aromatic amines were detected in real water samples (Fig. 4a). There were two potential interfering peaks but fortunately both had retention times that did not coincide with those of the amines. The recoveries of the analytes from this seawater were higher than 88% compared with that of spiked pure water. This implies that the matrix of the seawater did not have a significant effect on the extraction efficiency.

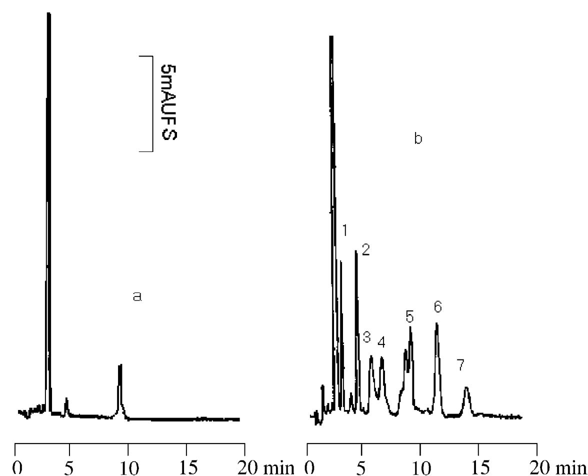


Fig. 4. Chromatogram generated after LLLME of a sea water sample, (a) 2 ml seawater, (b) 2 ml seawater spiked with 5 ng/ml of the seven aniline samples, the pH of the water sample (a_1) was adjusted to 13 using 6 *M* NaOH. o, 150 μl ethyl acetate; a_2, 2 ml, pH 2.1 receiving aqueous phase containing 300 m*M* 18-crown-6 ether. Time for the a_1–o extraction (T1) is 15 min; for o–a_2 (T2), 30 s. Peak identification: 1, 4-nitroaniline; 2, 2-nitroaniline; 3, 4-chloroaniline; 4, 4-bromoaniline; 5, 2-chloro-4,6-dinitroaniline; 6, 4-chloro-2-nitroaniline; 7, 2,6-dichloro-4-nitroaniline.

4. Conclusion

The present work presents the possibility of using LLLME in extracting aromatic amines from water samples prior to HPLC by utilizing a simple, cheap and disposable extraction device. With this method, the analytes were extracted from water sample quantitatively. Compared to most conventional extraction procedures, this extraction technique requires very little aqueous sample solution, organic extractant and the final aqueous receiving solvent. Moreover, no complicated solvent evaporation and reconstitution steps were involved. 18-Crown-6 ether was added to the aqueous receiving phase to increase the extraction performance, enabling enrichment factors ranging from 218 to 378 to be achieved.

Acknowledgements

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