Separation of Xylidine Isomers by Micellar Electrokinetic Chromatography

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Abstract

Xylidines are important precursors for the production of dyes, drugs, and various other products. Because of the high carcinogenic nature of some xylidine isomers it becomes very essential and relevant to develop suitable analytical procedures to separate isomers as well as enhance detection at very low concentrations. Micellar electrokinetic chromatography has been standardized at various influencing parameters such as pH, ionic strength, and micelle modifiers, and the optimum conditions have been ascertained for the best separation and sensitivity of standard mixtures. The applicability of the procedure in environmental samples is studied.

Introduction

Xylidines are important industrial compounds found in applications such as dyes, pesticides, and pharmaceuticals. The identity of the isomer is very important because the property of the target substance is strongly dependent on the structure of the precursor in deciding vital properties such as shade, stability of the dye, insecticidal activity, or the therapeutic efficacy of a drug molecule (as the case may be). Environmental monitoring, remedial measures, and toxicological studies depend on the exact confirmation of the structure. It is required to identify the right xylidine isomer by a reliable protocol because this isomer decides the end product and collecting impurity profiles becomes an important necessity in the chemical analysis. The undesirable and objectionable categories of impurities in some important syntheses can be from two possibilities: (*a*) direct presence of the banned isomer and (*b*) toxic impurity generated during the course of the reaction. It is therefore very crucial that the analytical procedure guarantees separation and detection of isomers at extremely low concentrations. The method also warrants identification and detection in different matrices.

Separation science plays a very vital role in isolating chemical species, and it offers tougher assignment when the chemistry of separating species is very similar. Without the separation of the species, identification or quantitation is never reliable. Although chromatography dominates the separating techniques, capillary electrophoresis is quickly becoming a method to serve just as equally. The micellar electrokinetic chromatography (MEKC) technique of the capillary electrophoresis mode helps to a very great extent in developing newer analytical methods to deal with such tough chemical separations. The technique described in this study is simple, fast, selective, stretchable for wider matrices, and ensures good recovery and reproducibility for environmental samples. There are six xylidine isomers: 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-xylidine. The 2,4- and 2,6-xylidines are suspected carcinogens (1), and the others are highly toxic (2).

There are not many analytical publications for the separation and determination of xylidines. Even available information is not satisfactory in regards to the resolution of isomers (3,4). Therefore, a reliable procedure that assures high resolution and suitability to environmental samples becomes essential.

MEKC (5,6)—the most popular among various capillary electrophoretic analyses, which serves very effectively for the separation of neutral molecules (7,8,11), the hydrophobic compound (9–12), and ionic solutes (13) as well—was chosen for the present attempt. In order to achieve the best separation of xylidines, the conditions of the analysis such as pH, ionic strength of the buffers, sodium dodecyl sulfate (SDS) concentration, and micelle modifiers (14,15) were investigated in detail and the results discussed.

Experimental

Equipment

A Beckman P/ACE system 5510 (Beckman Instruments, Fullerton, CA) equipped with a photo diode-array detector and P/ACE Station Version 1.0 software was used for all experiments. An untreated fused-silica capillary with a 50-cm length and 75-µm i.d. (eCAP, Beckman) was used. The capillary was thermostatted at 25°C for all experiments, and the analytes were monitored at a 214-nm wavelength with a bandwidth of 10-nm.

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Sample injection was done through pressure mode for 5 s. A voltage of +10 kV was set for all separations.

Chemicals

The standards 2,3-, 2,4-, 2,5-, 2,6-, and 3,5-xylidine were all of analytical-reagent grade procured from SIGMA (St. Louis, MO) and used without further purification. Standard solutions were prepared in high-performance liquid chromatographic (HPLC)-grade methanol. All reagents used were of analytical-reagent grade. A buffer solution was prepared with HPLC-grade water using an ELGA (Bucks, U.K.) water purifier (Model ELGASTAT MAXIMA HPLC).

Table I. Influence of SDS Concentrations on Selectivities of Xylidines

Analytes	100mM SDS	200mM SDS	300mM SDS
2,6-Xylidine	1.084	1.065	1.063
2,3-Xylidine	1.056	1.052	1.060
2,5-Xylidine	1.096	1.079	1.081
2,4-Xylidine	1.046	1.040	1.047
3,5-Xylidine	–	–	–

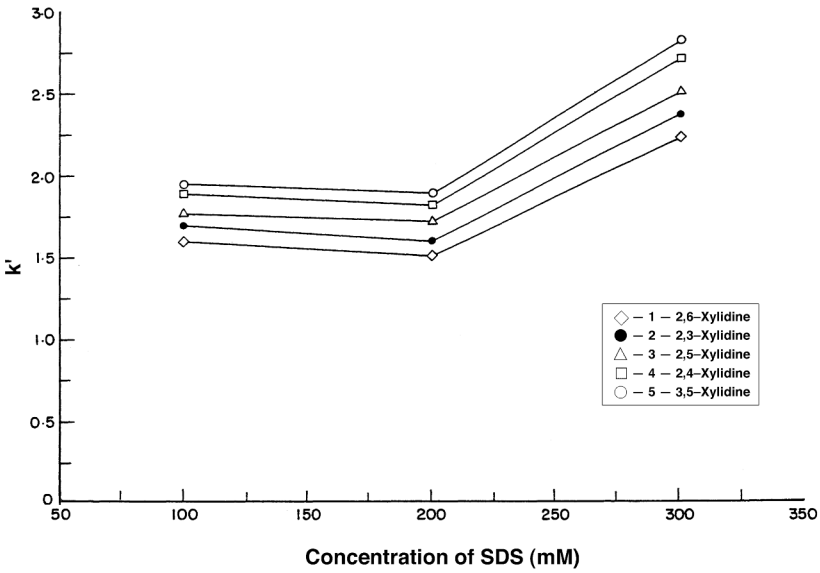

Figure 1. Influence of SDS on the retention of xylidines.

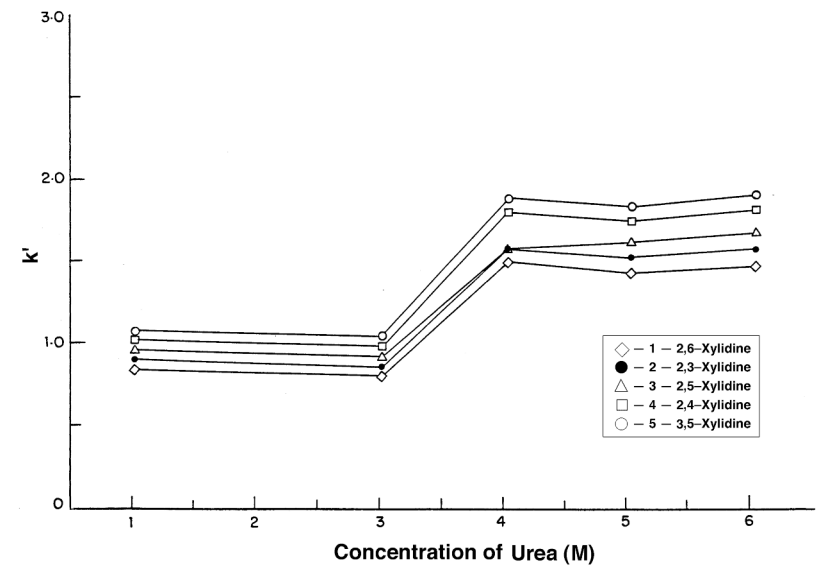

Figure 2. Influence of various concentrations of urea on the retention of xylidines.

Preparation of buffer

A 50mM phosphate solution containing 300mM SDS and 6M urea was prepared and adjusted to pH 8.0 with 30mM borate. Buffers were freshly prepared just prior to analyses. Buffer solutions were filtered through a Supor 0.22-µm filter membrane from Gelman (Ann Arbor, MI). Between runs the capillary was rinsed for 2 min with running buffer. After each run rinsing was done for 2 min with 0.1N NaOH, 2 min with water, and 2 min with running buffer. In order to avoid buffer depletion resulting from electrolysis, the buffer in the vials was replaced after each run.

Preparation of standard xylidines

A mixture of xylidines was prepared comprising of five constituents (2,3-, 2,4-, 2,5-, 2,6-, and 3,5-xylidine) in HPLC-grade methanol, and the final solutions for the run were diluted with water. Sudan (IV) was chosen as a neutral marker and was added at 15 µg/mL to the mixture solution of xylidines diluted to 15 µg/mL. The standard mixture contained all the isomers at an equal concentration of 15 µg/mL.

Safety considerations

The xylidine isomers are toxic and should be handled and disposed in a safe manner. Gloves and goggles should be used when handling these compounds, because xylidines may be absorbed through the skin. A detailed safety procedure has to be provided in the work place in the event of spillage or contact by the analyst.

Results and Discussion

MEKC

MEKC possesses both the advantage of capillary zone electrophoresis in the separation of charged species and the selectivity for the separation of neutral molecules.

We investigated the use of buffers containing borate, phosphate, and SDS at various concentrations and pH values. The influence of ionic strength and SDS concentrations is very significant in this separation.

The buffer that was found to yield optimum results consisted of 50mM phosphate and 300mM SDS adjusted to pH 8.0 with 100mM borate (resulting in the final concentration of 30mM).

Buffer concentrations lower than this did not favor separation. An SDS concentration lower than 150mM decreased the analysis time, but the separation was not achieved.

Influence of SDS

Good separation was observed at a 100mM SDS concentration, and with a further increase in concentration the separation improved continuously but with an increase in migration time. The reason for the increased migration times was that the increase in SDS concentration affected the solubilization of the solutes in micelles more and finally led to the migration time of the micelles (16). This observation was also a result of the ion-pair formation between the positively charged solutes and the negatively charged groups on the micelles (17). The best separation was achieved at a concentration of 300mM SDS with a buffer concentration of 50mM phosphate and 30mM borate at pH 8.0 (Figure 1 shows the influence of SDS on the retention of xylidines and Table I for separation). The capacity factors (k') were calculated using the formula:

$$k' = t_r - t_0 / t_0(1 - t_r/t_{mc}) \qquad \text{Eq. 1}$$

where t_r, t_0, and t_{mc} are the migration times of the analyte, the solute that does not interact with the micelle (solvent peak), and the micelle, respectively, and $t_{mc} = \infty$ is based on the retention of the neutral marker.

Table II. Influence of Urea Concentrations on the Selectivity of Xylidines

Analytes	1M Urea	3M Urea	4M Urea	5M Urea	6M Urea
2,6-Xylidine	1.063	1.061	1.062	1.061	1.064
2,3-Xylidine	1.057	1.068	1.056	1.065	1.057
2,5-Xylidine	1.076	1.074	1.078	1.079	1.081
2,4-Xylidine	1.045	1.053	1.044	1.049	1.043
3,5-Xylidine	–	–	–	–	–

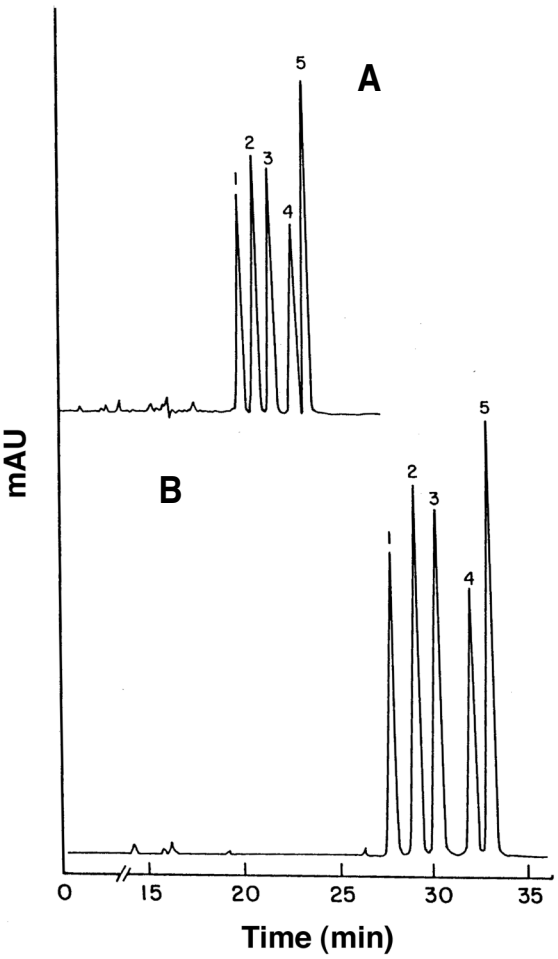

Figure 3. Separation of xylidine isomers (A) with and (B) without the addition of urea: 2,6-xylidine, 1; 2,3-xylidine, 2; 2,5-xylidine, 3; 2,4-xylidine, 4; and 3,5-xylidine, 5. The current setting was (A) 57.6 µA and (B) 72.1 µA.

MEKC with urea

In this study, we describe the addition of urea to micellar solutions for the separation of xylidine isomers. Urea has been proven to be a useful additive for aqueous-phase modification in MEKC (14) and it influences the retention of the xylidines in MEKC. Because urea had been cited to serve MEKC separations, the effect of its addition in the concentration range of 1M to 6M was studied.

Urea was found to appreciably decrease the analysis time by decreasing the retention. When used at a concentration level of 1M, urea decreased the migration time from 33.1 to 15.5 min, and there was no change up to a concentration of 3M. With a further increase from 3M to 6M, this caused an increase in migration time (Figure 2 shows the influence of various concentrations of urea on the retention of xylidines). The k' value data were calculated using equation 1. The t_{mc} value was that of a neutral marker, which was assumed to be infinity in this run from the observation of a 100-min retention time. The noticeable difference found above 3M was that urea caused some improvement in the separation, especially in the case of 2,4- and 3,5-xylidine. The species 2,3-, 2,4-, and 2,6-xylidine were satisfactorily separated only in the concentration range of 4M to 6M, and the best peak shapes were observed at a 6M concentration. Table II shows the selectivity data for various concentrations of urea. This is because of the fact that 2,4- and 3,5-xylidine are more hydrophobic and added urea helps in the separation with an increased hydrophobic character of the micelles. Although the exact interaction of the urea with micelles in MEKC separation is not clear, the effect of urea probably alters the water structure in the micelle by diminishing surrounding water molecules around the micelles and improving hydrophobicity (18).

This trend is contrary to the observation made by S. Terabe et al. (14) in which they found that the addition of urea to hydrophobic analytes leads to increased migration times. The exact role of urea in MEKC separations is not understood thoroughly and a detailed study will be reported at a later time.

Comparison of MEKC with MEKC–urea

MEKC separation with urea at a concentration of 6M provided a better baseline separation of xylidines. A comparison with MEKC (without urea) is considered when the advantage of lowering migration times becomes evident. The analysis time of MEKC with the urea addition was 23.7 min compared with 33.1

min without it. The electrokinetic chromatograms for the separation of xylidine isomers are shown in Figure 3.

In Figure 4, the k' values for an MEKC run of xylidine solutes had higher k' values than MEKC–urea (6M), which provided support for the previous observation.

Influence of pH

The pH of the buffer containing 50mM phosphate, 300mM SDS, and 6M urea was varied in the pH range of 5 to 9, with the borate concentration ranging from 5mM in the case of pH 5.0 to 40mM in the case of pH 9.0. When experiments were done at lower than pH 5.0, the migration times were observed to be longer because of the lowering of electro-osmotic flow (EOF). The other possibility is that the charged form of solutes at pH 5.0 forms ion pairs with the anionic micelles, leading to a retardation of the migration of the solutes (17). At pH 7.0 better separation was observed (the best was seen at pH 8.0). When the pH was further increased to pH 9.0 with 40mM borate, the ionic strength of the buffer crossed the critical barrier, thus lowering the EOF and zeta potential and leading to a peak distortion and increased migration time. Accordingly, pH 8.0 proved to be the best condition with an optimum analysis time and good separation. Because the neutral structure of the solutes (refer to Table III for the pK_a values) was retained at higher pH conditions, the micelle solubilization was affected completely, which served the best for the partitioning mechanism of MEKC separation for this kind of solute. The influence of pH was rather specific on the migration of the xylidines, as is shown by the k' values provided in Figure 5.

Table III. The pK_a Values for Xylidines

Peak number	Xylidine	pK_a
1	2,6-xylidine	3.95
2	2,3-xylidine	4.70
3	2,5-xylidine	4.53
4	2,4-xylidine	4.89
5	3,5-xylidine	4.76

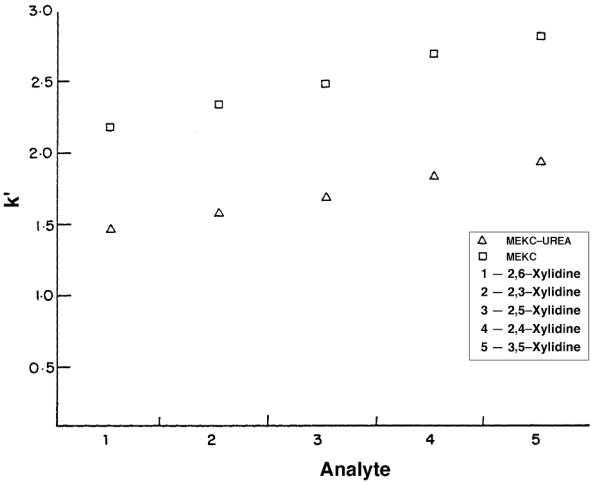

Figure 4. The k' values for MEKC and MEKC–urea.

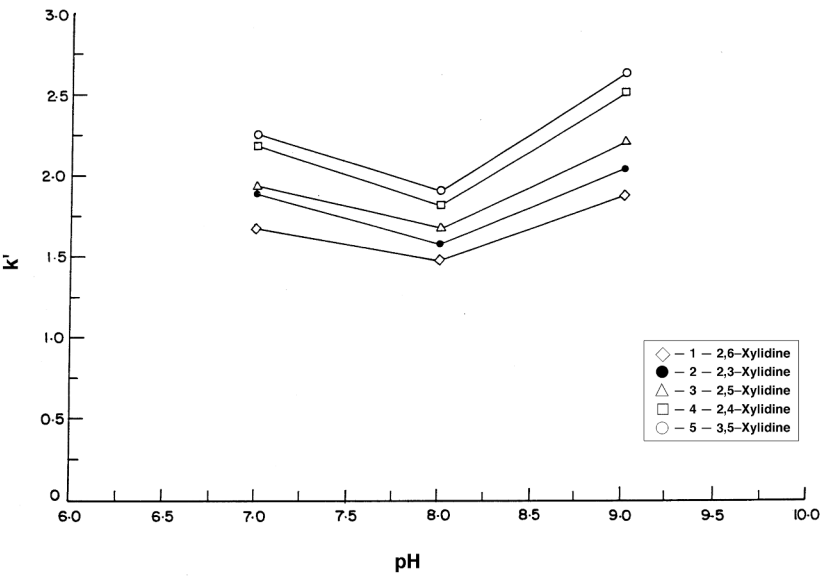

Figure 5. Influence of pH on the retention of xylidines.

Influence of voltage

A variation of voltage caused significant changes in the retention of xylidines. At a high SDS concentration (300mM), more solubilization of solutes in micelles happen when the solutes ultimately migrate at the rate of micelles (the increased voltage at 15 kV) The voltage of 20 kV decreased migration time, but with poor separations. Only at 10 kV were an optimum retention and resolution achieved.

Application to environmental samples

The efficacy of the procedure was tested with environmental samples such as a polluted lake and seawater. In the case of such samples, the matrix effect (which is the origin of innumerable unknown organics and inorganics) poses a challenge to the procedure. Analyses were done with samples of seawater spiked with xylidines at 15-µg/mL levels. When seawater samples clarified by filtration to remove solid particles were run directly without any cleanup, it led to a serious distortion of peaks because of strong electrolytes such as chlorides affecting micellation (see Figure 6A). In order to check the influence of ions in seawater on the peak shapes and migration times of xylidines, we studied the case of xylidines spiked to a 7% sodium chloride solution. The results from this confirmed that the electrolyte (chloride) really distorted peaks and separation (see Figure 6B).

Because there was a shift in the migration with a distortion of xylidine peak shapes in the case of water samples with electrolytes, sample cleanup became necessary for which a solid-phase extraction (SPE) procedure was adopted.

Seawater

Seawater spiked with xylidine isomers at 15 µg/mL each was transferred to an SPE cartridge column that was approximately 15-cm in length and prepared using 20 g extruletNT (Merck, Darmstadt, Germany) material. The extruletNT

was preconditioned with 1 mL of sodium methoxide (20%). The column was eluted first with 20 mL methyl *tert*-butyl ether and subsequently with 15-mL volumes. The combined ether layers were evaporated to near dryness following a rotary evaporation technique, and the final few drops of ether were eliminated in a current of nitrogen. The residue was dissolved in water purified by ELGA to the purity of 18.0 MΩ and diluted to 5 mL. A duplicate was done for the same sample, then MEKC with urea was run with an injection by pressure mode for 5 s. Migration times observed from this extract matched perfectly with the xylidine isomers in the standard mixture. The resolution of the isomers was intact (see Figure 7A).

Lake water

Lake water samples were gathered from a lake near an industrial belt consisting of several dyeing and chemical industries. Water samples were analyzed on the day of collection. The samples were adjusted to pH 8.0 in the same way as that of the run buffer and initially tested without any cleanup. HPLC-grade water that was free of any organic pollutant was chosen for evaluating the validity of the procedure by way of spiking the reference mixture of xylidines at a 15-µg/mL concentration adjusted to pH 8.0 with 50mM disodium orthophosphate and run directly.

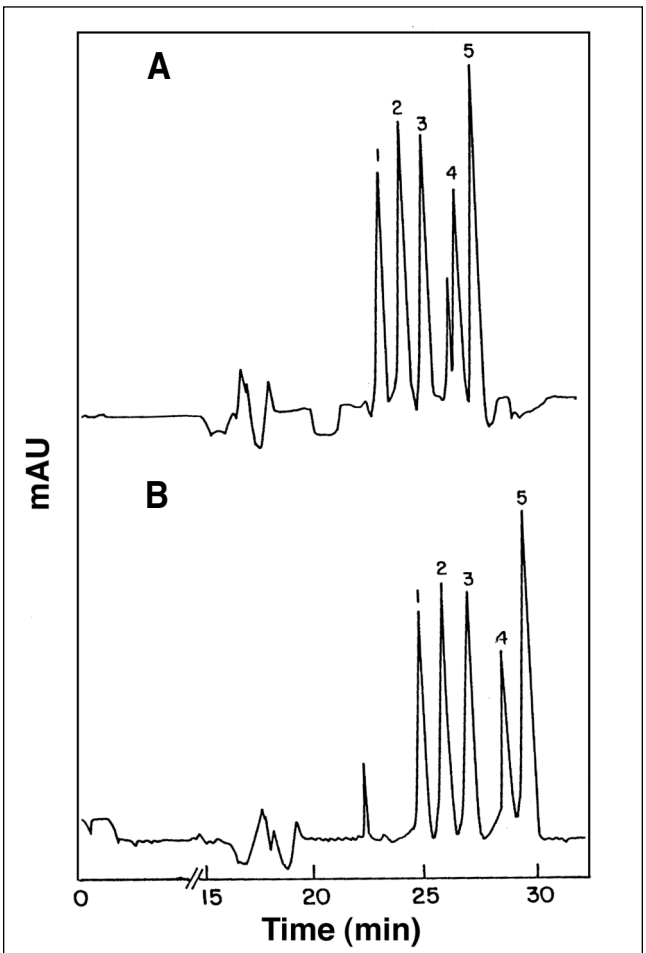

Figure 6. Seawater, A, and 7% NaCl, B, spiked with 15 µg/mL xylidine isomers: 2,6-xylidine, 1; 2,3-xylidine, 2; 2,5-xylidine, 3; 2,4-xylidine, 4; and 3,5-xylidine, 5.

The direct runs were found to produce xylidine peaks with migration times slightly inconsistent. These were then subjected to SPE cleanup (as detailed previously for seawater samples) in which the reproducibility of the migration times was found to be in good agreement with the reference mixture of xylidines (see Figure 7B). The sample collected from the lake (which had signs of pollution from dyes) responded for the presence of 2,6-xylidine when analyzed after SPE cleanup and run immediately by MEKC (see Figure 7C). The confirmation of the presence of 2,6-xylidine was accomplished by the spectral matching of the solute with that of the pure reference 2,6-xylidine and also beside that of a perfect match of their migration times (see Figure 8). In order to confirm that the response was only from xylidine and not because of other organics, the extract was also analyzed by a gas

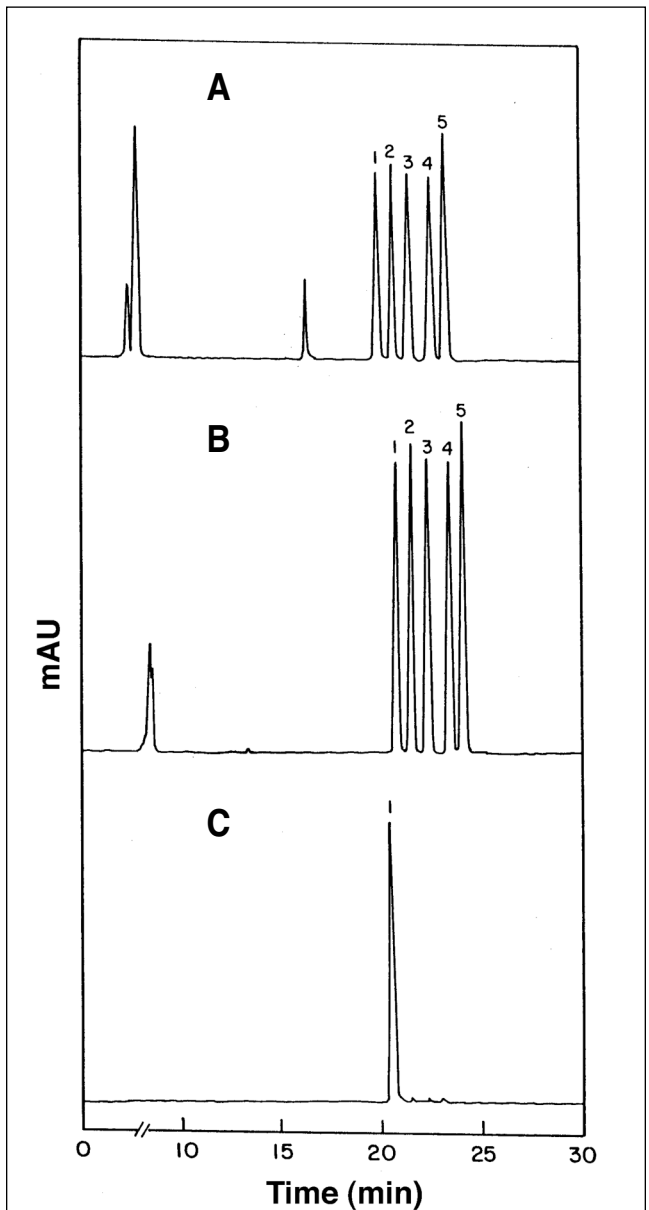

Figure 7. Sample cleanup of seawater spiked with 15 µg/mL xylidine isomers, A; HPLC-grade water spiked with 15 µg/mL xylidine isomers, B; and a lake water sample found to contain 2,6-xylidine, C. The following numbered peaks are: 2,6-xylidine, 1; 2,3-xylidine, 2; 2,5-xylidine, 3; 2,4-xylidine, 4; and 3,5-xylidine, 5.

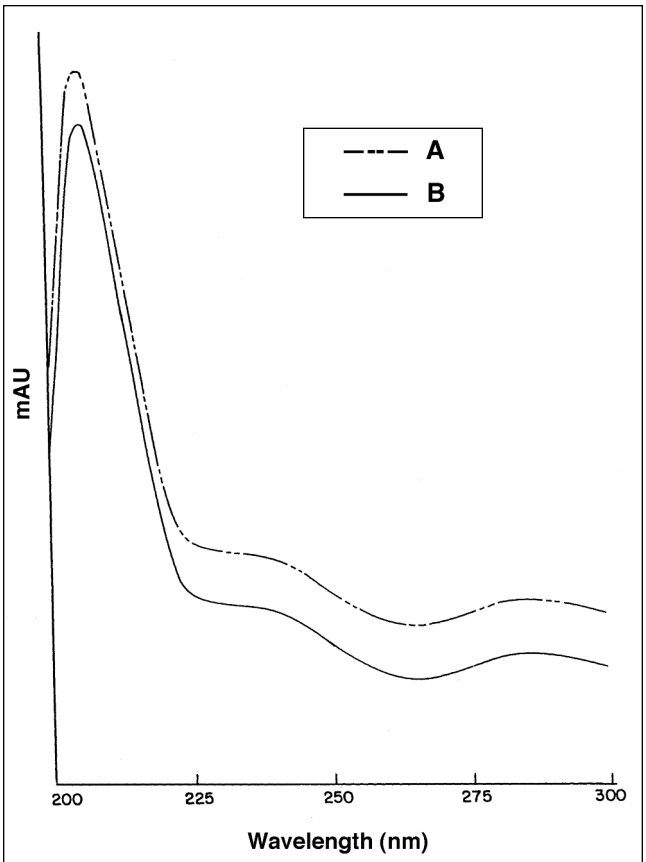

Figure 8. The spectrum of (A) 2,6-xylidine in lake water and (B) the reference 2,6-xylidine.

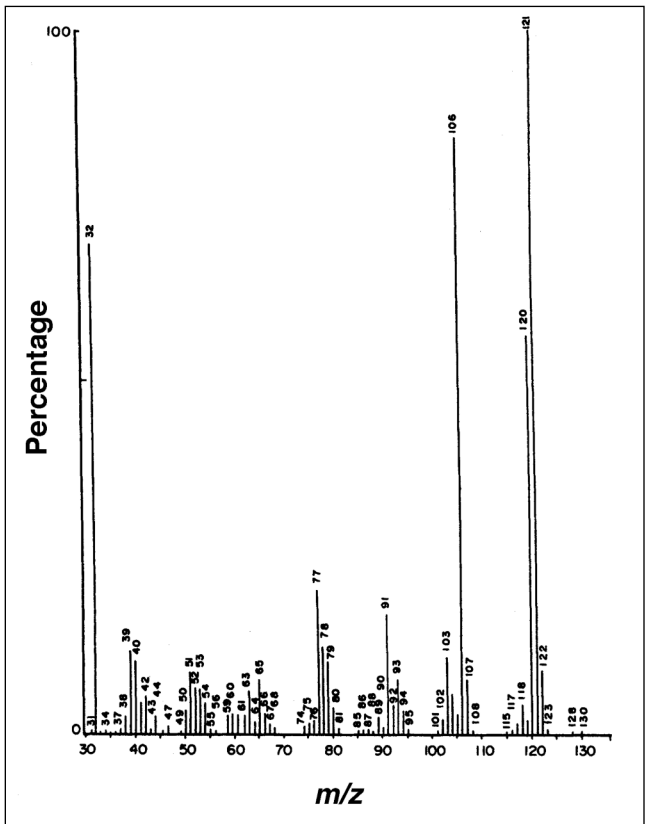

Figure 9. Mass spectrum of the real sample.

chromatograph (GC)–mass spectrometer (GC 8000-MD Model 800 in total ion current mode, Fisons Instruments, Rodano-Milan, Italy), which confirmed the presence of xylidine (see Figure 9).

Recovery studies performed by spiking xylidine isomers at 15 µg/mL with deionized water showed 98.5–99.6% for each of the isomers. The limit of detection was found to be 0.08 µg/mL with a signal-to-noise ratio of 5:1.

Conclusion

The analytical procedure was targeted to find a versatile method for various isomers of xylidines. The results indicated that the high-performance capillary electrophoresis method is well-suited and, following the MEKC–urea method, is quite effective for the separation of xylidine isomers. The separation technique serves well in the case of real samples such as polluted waters, seawater, and lake water. Spiked samples were run because they represented the true matrix elements. The analysis of polluted lake water revealed very well the validity of this analytical method for the environmental samples.

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