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Fast separation and sensitive detection of carcinogenic aromatic amines by reversed-phase μ-liquid chromatography coupled with electrochemical detection

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Abstract

A μ-LC method was developed for the fast and sensitive analysis of aromatic amines by electrochemical detection. The chromatographic separation of nine carcinogenic aromatic amines was performed on an ABZ + PLUS column with detection limits up to pM L^{-1} levels. Mobile phase comprised of methanol-acetate buffer of pH 5 (45:55, v/v) used at a flow rate of 0.2 ml min^{-1}. The detection was performed with a 6 mm glassy carbon electrode at an applied potential of 0.8 V versus Ag/AgCl. An intraday RSD for retention time and peak area were between 0.22% and 0.73% and 1.86% and 4.03%, respectively. The interdays RSD for retention time and peak area were between 0.47% and 1.35% and 2.04% and 4.42%, respectively. The applicability of the assay has been demonstrated by analyzing these aromatic amines in lake water and synthetic food colour additives. A comparison is given between electrochemical and UV detection.

Keywords: μ-Liquid chromatography; Aromatic amines; Electrochemical detection; UV detection

1. Introduction

Aromatic amines and their *N*-nitroso derivatives are potential carcinogenic agents [1–4]. Aromatic amines are widely used as raw material or at an intermediate stage in the manufacturing of industrial chemicals such as pesticides, medicines, dyestuffs, polymers, surfactants, cosmetics and corrosion inhibitors [5,6]. As these amines are discharged into the atmosphere and water, they constitute an important class of environmental pollutants. This has increased attention for the development of reliable, sensitive and rapid analytical methods. Several analytical methods have been reported for the determination of aromatic amines. Among them, GC methods are most commonly employed [7–11]. However, it is difficult to analyze aromatic amines by GC due to their polar nature. To overcome these difficulties, it is usually necessary to derivatize them before GC. Flow injection coupled with voltammetry has been employed with diazotization [12] or bromination [13] reactions. These methods involve tedious and time consuming sample preparation. Capillary electrophoresis (CE) has also emerged as fast and efficient tool for chemical analysis, and a few methods for the analysis of aromatic amines using CE are reported in the literature [14–16]. Nevertheless, liquid chromatography (LC) is known as the most convenient technique for aromatic amines. Variety of separation and detection methods for the analysis of anilines has been reported [17–22].

According to the EU regulations the limit of detection of analytical methods should be as low as 0.1 μg L^{-1} for the study of environmental pollutants in water [23]. It is difficult to obtain such a low detection limit with path length dependent UV and fluorescence detectors. Although, better sensitivities could be achieved using laser induced fluorescence detector (LIF), expensive instrumentation makes its routine use difficult. Moreover, LIF detection of aromatic amines requires adding a fluorescent tag to them. Mass spec-

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trometer can be used with LC for qualitative and quantitative analysis of aromatic amines but mass spectra of aromatic amines are often not ideal if these compounds are present in low concentrations [24]. Aromatic amines are well known to show good electrochemical behaviour due the presence of amino group that permits their sensitive electrochemical detection (ECD) without making derivatization a prerequisite. There are reports on the analysis of aromatic amines by LC-ECD [25–31] but they involve tedious procedures leading to analysis period longer than 1 h. In an ion-exchange method with ECD and gradient elution [32] the analysis time for nine anilines was 30 min. In spite of all the success of LC-ECD, this technique remains less exploited for the analysis of environmental and food samples.

The high sensitivity and selectivity of μ-LC-ECD, as reported here, offer considerable advantages in the analysis of aromatic amines. This paper presents a simple method for the separation of nine carcinogenic aromatic amines that are listed among 20 priority anilines by the EU. The separation was complete within 9 min using LC-ECD. This detection method has been compared with UV detection. To the best of our knowledge, the proposed method is the fastest chromatographic method for the analysis of carcinogenic aromatic amines with smallest injection volume and minimal solvent consumption. The method has also been applied to the analysis of aromatic amines in lake water and synthetic food colourants.

2. Experimental

2.1. Instrumentation

μ-Liquid chromatography was carried on HPLC system equipped with a Hitachi L-7110 pump (Merck, Germany), a Rheodyne 9725 injector and a Supelcosil ABZ + PLUS (100 mm × 2.1 mm, 5 μm) column (Supelco, USA). A Discovery C_{18} (150 mm × 2.1 mm, 5 μm) column (Supelco, USA) was used for comparison. An Unijet Radial Cell (Bioanalytical Systems, USA) was used for electrochemical detection in conjunction with LC-3D battery operated potentiostat (Bioanalytical Systems, USA). Electrochemical cell consisted of a three electrode system with 6 mm glassy carbon working electrode and an Ag/AgCl reference electrode housed in a peek block; the stainless steel half of the cell served as the auxiliary electrode. Spectra 100 UV–vis (Thermo Separation Products, USA) was used for UV detection. The acquisition and analysis of chromatographic data were performed using DAx version 7.1 data acquisition software (Prince Technologies, The Netherlands). The aromatic amines were extracted from food colourants by using a C_{18} SEP-PAK Light solid phase extraction cartridges (Waters, USA). The eluent solutions were filtered through 0.45 μm nylon membrane filters (Filtech Pharma Lab, India) and sample solutions through 0.45 μm PVDF syringe filters (Whatman Inc., USA).

2.2. Chemicals and reagents

Aniline, 4-chloroaniline (BDH, UK), 2-toluidine, benzidine, 3,3′-dimethoxybenzidine, 2,6-dimethylaniline (Sigma, USA) and 2-naphthylamine, 1,2-phenylenediamine, *N*,*N*-diethylaniline (Merck, Germany) were used as supplied. Their 10 mM stock solutions were prepared in HPLC grade methanol (Merck, India). Working standards were prepared by sequential dilution of the stock solution with mobile phase. The stock and working solutions were stored in dark at 4 °C when not in use. Acetate buffer, pH 5, was prepared by adding with stirring 5.02 ml of 100 mM acetic acid to 10 ml of 100 mM sodium acetate and adjusting to the desired pH using a pH meter. HPLC grade methanol (Merck, India) was used in the mobile phase with acetate buffer of pH 5 in 45:55, v/v, ratio. A portion of 3 ml of 200 mM potassium chloride was added to 100 ml of mobile phase to stabilize flow in the reference electrode. All other solutions were prepared using double distilled water and filtered through a 0.45 μm nylon membrane filter.

2.3. Procedures

Each day before and after analysis, the column was rinsed with doubly distilled and filtered water, and before analysis, conditioned with methanol both at a flow rate 0.5 ml min^{-1} for 30 min. For separations, the flow rate of mobile phase was kept at 0.2 ml min^{-1} at an ambient temperature and injection loop of 2 μL was used. Electrochemical detection was performed at an applied potential of 0.8 V unless otherwise mentioned.

For the analysis of aromatic amines in food colourants, about 100 mg of sample was dissolved in 5 ml of borate buffer of pH 9 and 1 ml of this solution was loaded on to a C_{18} SPE cartridge that was previously washed by passing 5 ml of methanol and then conditioned by passing in sequence 5 ml of water and 2 ml of borate buffer. The cartridge was washed with 2 ml of borate buffer, drained by passing air for 5 min and the amines were eluted with 1 ml of methanol. Recovery experiments were done on samples spiked with known amounts of amines.

3. Results and discussion

3.1. Optimization of separation

The aim of this study was to develop a fast analytical method for carcinogenic aromatic amines with high resolution and low detection limit. Two different reversed phase columns were evaluated in the preliminary experiments, viz., deactivated C_{18} column (150 mm × 2.1 mm, 5 μm) and ABZ + PLUS column (100 mm × 2.1 mm, 5 μm). Resolution, efficiency and selectivity was found to be better on ABZ + PLUS column (Table 1). It is because of the presence of amide group within the bonded phase molecules

Table 1
Comparison of chromatographic features in the separation of aromatic amines on ABZ + PLUS column, 100 mm × 2.1 mm; 5 μm, and on C_{18} column, 150 mm × 2.1 mm; 5 μm, (results given in parentheses)[a]

Aromatic amine	t_R	R_s	k'
1,2-Phenylenediamine	1.58 (2.24)		0.89 (0.79)
Aniline	2.24 (2.92)	1.19 (0.53)	1.69 (1.33)
Benzidine	2.74 (2.72)	1.10 (1.22)	2.29 (1.17)
2-Toluidine	3.18 (4.13)	0.86 (1.81)	2.81 (2.30)
3,3′-Dimethoxybenzidine	4.56 (5.24)	2.56 (1.29)	4.47 (3.22)
2,6-Dimethylaniline	4.97 (8.01)	0.76 (2.35)	4.96 (5.40)
4-Chloroaniline[b]	5.65	1.12	5.77
N,*N*-Diethylaniline	7.35 (6.62)	2.43 (1.31)	7.82 (4.29)
2-Naphthylamine	8.86 (13.69)	1.65 (2.50)	9.63 (9.88)

[a] t_R = retention time (min), R_s = resolution, and k' = retention factor; all results are average of four determinations.
[b] Strongly retained on C_{18} column.

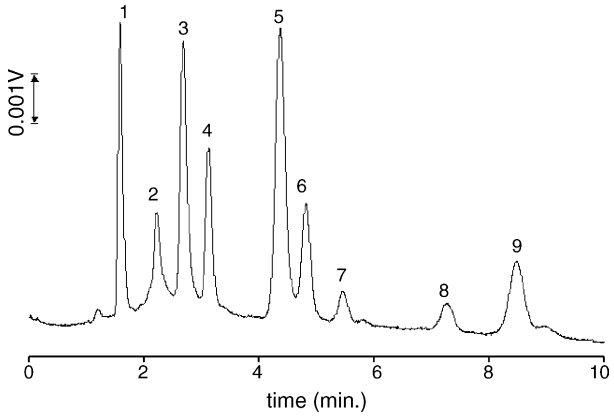

Fig. 1. Standard chromatogram for the separation of aromatic amines with UV detection. Column ABZ + PLUS (100 mm × 2.1 mm, 5 μm), mobile phase methanol–acetate buffer (pH 5), 45:55 (v/v), flow rate 0.2 ml min^{-1}, detection at 210 nm. Peaks identification, 1 = 1,2-phenylenediamine; 2 = aniline; 3 = benzidine; 4 = 2-toluidine; 5 = 3,3′-dimethoxybenzidine; 6 = 2,6 = dimethylaniline; 7 = 4-chloroaniline; 8 = *N*,*N*-diethylaniline; and 9 = 2-naphthylamine, each at 1 μM L^{-1}.

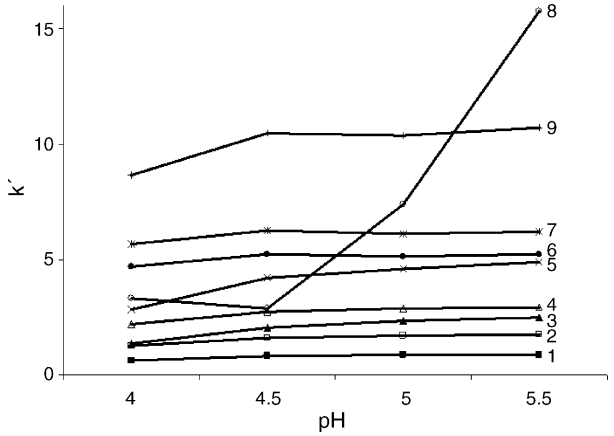

Fig. 2. Influence of mobile phase pH on the retention factor (k') of aromatic amines. Conditions (excepting pH of the mobile phase) and peaks identification as in Fig. 1.

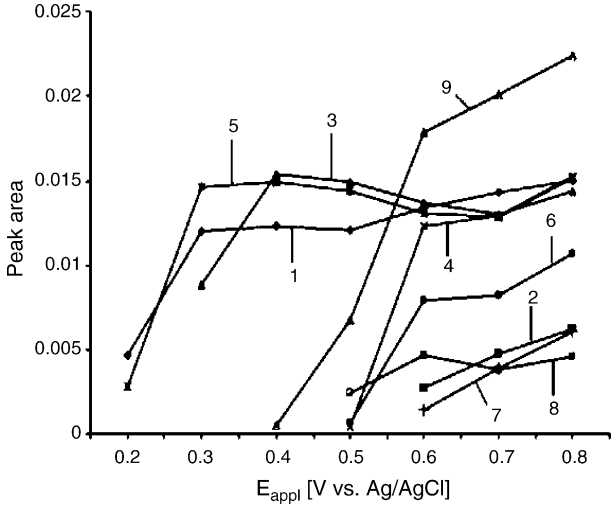

Fig. 3. Hydrodynamic voltammograms of aromatic amines, 1 μM L^{-1} each, with respect to their corresponding peak areas. Conditions (excepting detection) and peaks identification as in Fig. 1.

in ABZ + PLUS column; this has also enabled the use of methanol–water mobile phase comprising of low ionic strength buffer. ABZ + PLUS column was considered to be highly efficient for polar analytes and was chosen for further studies.

Most of the aromatic amines used in the present work have pK_a values in the range 4–5.3. Therefore, acetate buffer was chosen as an aqueous phase in combination with methanol for separation. Fig. 1 shows the typical chromatogram obtained for the separation of nine anilines with UV detection. Different ratios of methanol and acetate buffer of pH 5 were tried to study the effect on separation behaviour of anilines, however, methanol and acetate buffer, 45:55 (v/v), gave the best separation at a flow rate of 0.2 ml min^{-1}. Fig. 2 shows the effect of buffer pH on the retention factor (k') of aromatic amines. The influence of mobile phase buffer pH, range 4–5.5, on the separation was studied and buffer pH 5 was found to

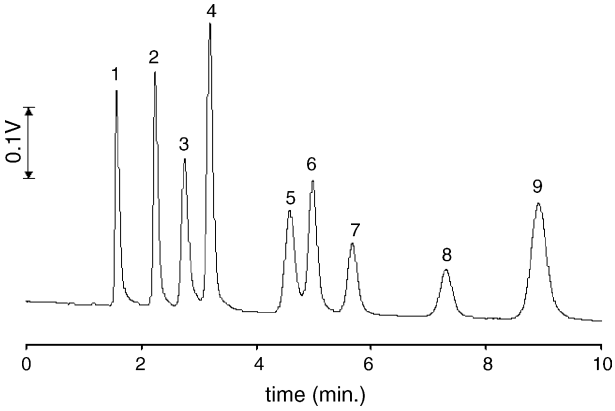

Fig. 4. Standard chromatogram for the separation of aromatic amines with EC detection. Detection potential 0.8 V vs. Ag/AgCl. Amines 1 μM L^{-1} each. Conditions (excepting detection) and peaks identification as in Fig. 1.

Table 2
Analytical performance of μ-LC-ECD

Aromatic amine	RSD (%), intraday[a] (interday[b])		LOD[c], ECD (UVD)	Regression analysis[d]		r^2
	Area	t_R		Slope ± SD (standard error)	Intercept ± SD (standard error)	
1,2-Phenylenediamine	4.03 (4.42)	0.28 (0.83)	24 (10)	18035.8 ± 3809 (885.3)	0.0083 ± 0.009 (0.00226)	0.9952
Aniline	1.86 (2.04)	0.22 (0.59)	105 (31)	16156.6 ± 3888 (903.7)	0.0108 ± 0.01 (0.00231)	0.9938
Benzidine	2.35 (2.57)	0.35 (1.00)	36 (11)	11735.1 ± 3496 (812.5)	0.013 ± 0.0089 (0.00208)	0.9905
2-Toluidine	3.35 (3.67)	0.26 (1.16)	48 (18)	19848.7 ± 5193 (1206.9)	0.023 ± 0.0133 (0.00309)	0.9924
3,3′-Dimethoxybenzidine	2.49 (2.73)	0.54 (1.11)	59 (10)	9033.3 ± 2639 (613.2)	0.011 ± 0.0067 (0.00157)	0.9909
2,6-Dimethylaniline	3.24 (3.55)	0.35 (1.02)	143 (26)	16224.6 ± 4178 (971.1)	0.0093 ± 0.010 (0.00248)	0.9929
4-Chloroaniline	3.52 (3.85)	0.48 (0.47)	191 (97)	10659.7 ± 3209 (745.8)	0.006 ± 0.0082 (0.00191)	0.9903
N,*N*-Diethylaniline	2.40 (2.99)	0.73 (0.87)	288 (113)	10095.7 ± 2092 (486.2)	0.0053 ± 0.005 (0.00124)	0.9954
2-Naphthylamine	3.01 (3.30)	0.70 (1.35)	89 (45)	16194.7 ± 4504 (1046.8)	0.0176 ± 0.011 (0.00268)	0.9917

[a] $n = 7$.
[b] $n = 6$.
[c] LOD: limit of detection (S/N = 3); ECD: electrochemical detection, pM L^{-1}; UVD: ultraviolet detection, nM L^{-1}.
[d] $n = 4$.

be optimum. At this pH the analysis was complete within 10 min with adequate resolution of all amines. Major shifts in k' value relative to buffer pH were observed for the internal standard *N*,*N*-diethylaniline, and this observation was related to its pK_a 6.56 that was relatively a higher value than for other amines.

3.2. Electrochemical detection

The influence of detection potential over the range of 0.2–0.8 V on peak areas of aromatic amines was investigated. The variation in peak areas of the nine anilines with detection potential is shown in Fig. 3. All amines showed good

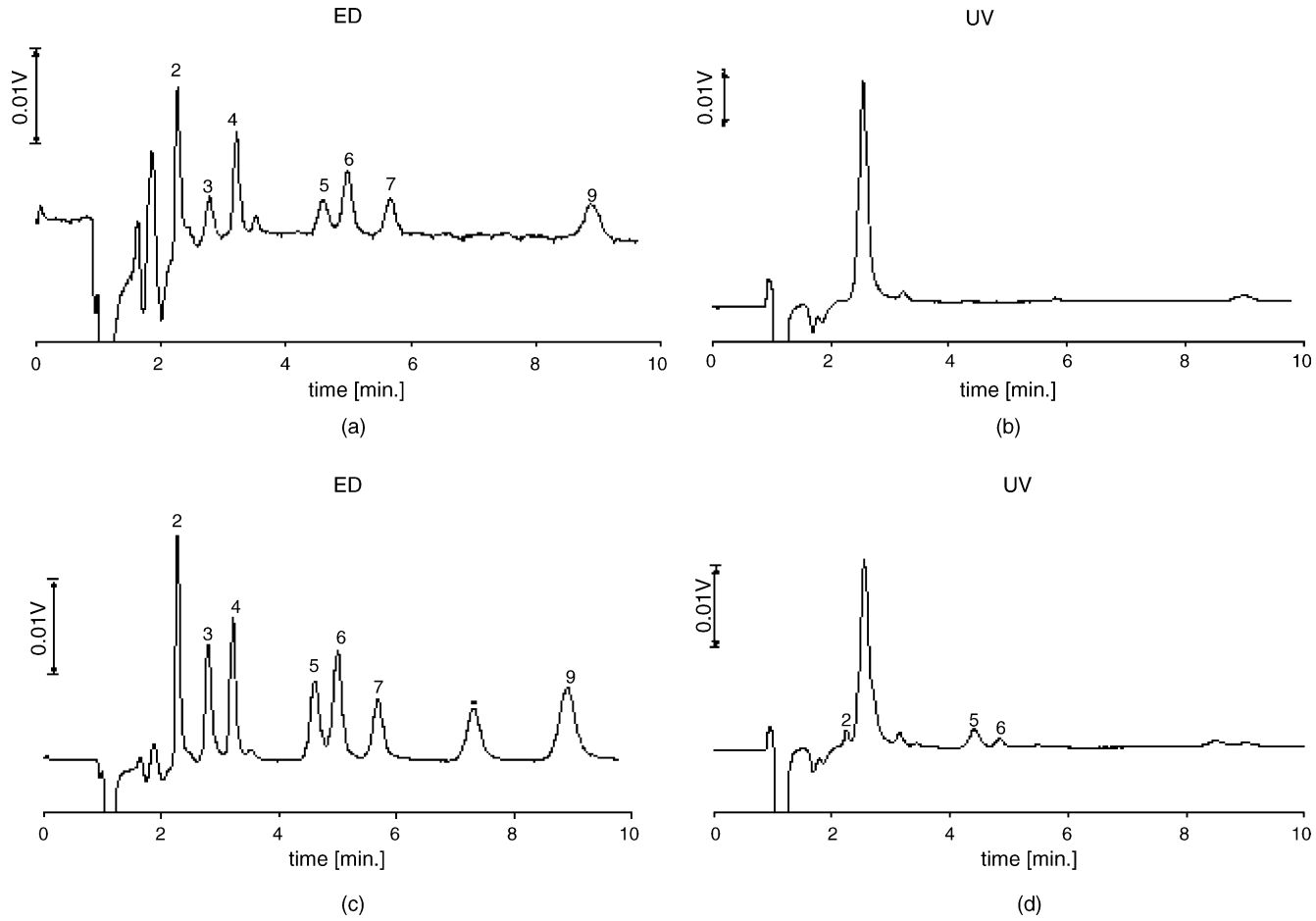

Fig. 5. Chromatograms of lake water samples (a) unspiked and (b) spiked with 0.1 μM L^{-1} of each amine by EC and UV detection. Conditions (EC detection potential 0.8 V vs. Ag/AgCl) and peaks identification as in Fig. 1. (*) Internal standard *N*,*N*-diethyl aniline peak; unmarked peaks have not been identified.

response at 600 mV and above. There was marked increase in the response up to 0.8 V, however, further increase resulted into increased baseline noise. Therefore, 0.8 V was chosen as optimum detection potential. Typical chromatogram for the separation of nine aromatic amines with electrochemical detection shown in Fig. 4.

3.3. Analytical performance

3.3.1. Validation

The analytical performance of the proposed method was assessed for its linearity, precision, detection limits, specificity, accuracy, robustness and ruggedness. The results are summarized in Table 2. Calibration graphs were found to be rectilinear in the range 0.1–5 μM of amines using the proposed experimental conditions. The repeatability of the method was estimated from seven consecutive injections of a standard mixture of nine amines at 1 μM level. The same mixture of amines was injected for six consecutive days to determine reproducibility. The relative standard deviation (RSD) of retention time and peak areas of amines were calculated from these intraday and interday measurements. For intraday measurements the average RSD of the retention time and peak area is 0.43% and 2.58%, respectively and for interdays measurements 0.93% and 3.23%, respectively.

The ruggedness of the method is indicated by the interday reproducibility as it is influenced by any changes in chemicals, reagents, solvents and to some extent in temperature. Stability of solutions is often considered as a part of ruggedness of the method. The response factors of standard stock solutions were found to be unchanged for up to 25 days. Less than 5% difference in peak areas was found between standards that were freshly prepared and of 25 days old. The stock solutions can therefore be stored (4 °C) up to 25 days after their preparation without affecting the results. The robustness of the method was accessed by applying small changes in experimental parameters. When the methanol–acetate buffer mobile phase composition 45:55 (v/v) was changed to 43:57 and 47:53, whilst keeping other parameters the same, RSD for the retention times of nine aromatic amines were found to be in the range 0.24–1.08%. These values are comparable with the RSD of method itself. However, when the flow rate was changed to 0.18 or 0.22 ml min^{-1}, changes in retention time up to 8.28% were noted. Additionally, the method has selective EC detection because few compounds can oxidize at the potential used in detection.

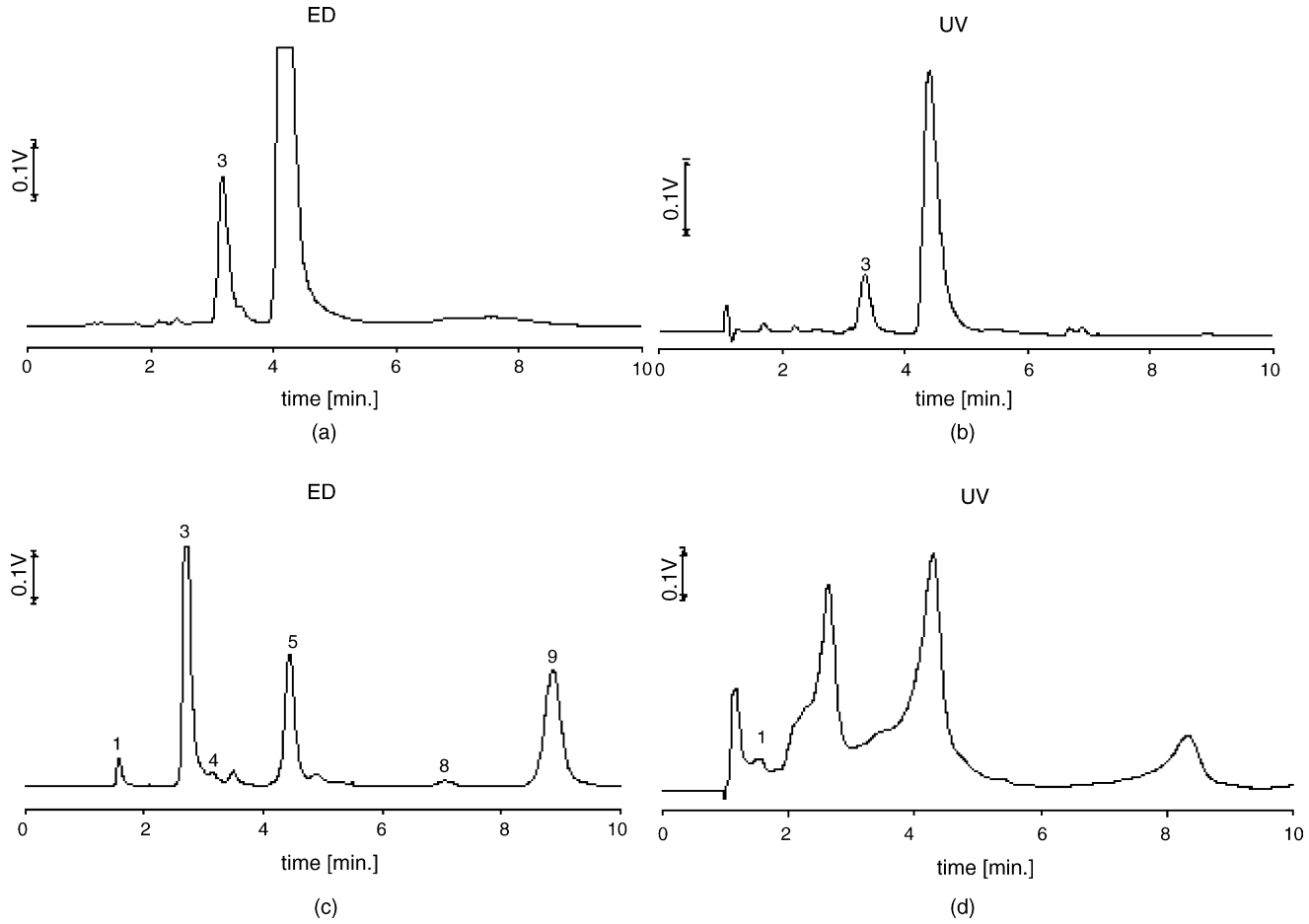

Fig. 6. Chromatograms of synthetic food colourants (a) Tartrazine and (b) Sunset Yellow FCF by EC and UV detection. Conditions (EC detection potential 0.8 V vs. Ag/AgCl; and UV detection at 210 nm) and peaks identification as in Fig. 1. Unmarked peaks have not been identified.

3.3.2. Limit of detection

The limit of detection (LOD) for all nine amines were determined at a signal-to-noise ratio of 3. Under the given separation conditions, the LODs with UV detection were in the range 10–113 nM L^{-1}. These were three orders of magnitude higher than those obtained with ECD, range 24–288 pM L^{-1} (Table 2). It showed that ECD is more suitable detection technique for trace levels of aromatic amines. It should be noted that the 2 μL injection volume in the present method was approximately two orders of magnitude lower than used in the previously published LC-ECD procedures for aromatic amines [26,27], and yet LODs were better.

3.4. Application

3.4.1. Water samples

Water samples were collected from a lake nearby Bhopal, Madhya Pradesh, India, and filtered through a 0.45 μm syringe filter. These samples were subjected to chromatography without any further treatment. Comparative chromatograms of unspiked lake water samples analyzed by the presented method with UV and electrochemical detection are given in Fig. 5a. Aniline, benzidine, 2-toluidine, 3,3′-dimethoxybenzidine, 2,6-dimethylaniline, 4-chloroaniline and 2-naphthylamine were identified in the lake water sample when ECD was applied; their native concentrations are given in Table 3. *N*,*N*-Diethylaniline, which was not detected in water samples, was used as an internal standard. Fig. 5b shows the chromatograms of spiked lake water samples. Recoveries of analytes were found in the range 94.1–105.3% with RSD 2.47–6.41% (Table 3). Precision studies of four replicate runs gave RSD in the range 1.03–3.71% for retention time and 0.25–7.65% for peak area.

3.4.2. Synthetic food colourants

Proposed method was applied for the determination of free aromatic amines in two synthetic food colorants, Sunset Yellow FCF and Tartrazine. Synthetic food colorants obtained in powder form from the local market. The samples without any cleanup produced giant peak of parent dyes and it was difficult to locate aromatic amines in the chromatogram. Therefore, solid phase extraction method was used in order to remove parent dyes which all have strong retention on C_{18} and aromatic amines could be eluted easily; chromatograms obtained by EC and UV detection are given in Fig. 6. 1,2-Phenylenediamine, benzidine, 2-toluidine, 3,3′-dimethoxybenzidine, *N*,*N*-diethylaniline and 2-naphthylamine were identified in Sunset Yellow FCF using EC detection (Fig. 6b). On the contrary, UV detection gave many broad peaks owing to the presence of many UV absorbing materials in real world sample that co-eluted with amines. Thus, EC detection was selective for aromatic amines. Recovery studies were done by adding known amount of amines to the Sunset Yellow FCF samples and analyzing them by the same procedure after solid phase extraction; the results are given in Table 4. RSD in peak area and retention time repeatability were in the range 0.25–4.04% and 0.22–0.73%, respectively ($n = 3$). Recoveries of amines in the range of 87.2–111.9% were obtained with RSD 0.31–6.0%. Only 2-toluidine was found in the Tartrazine sample. RSD for peak area and retention time of 2-toluidine were found to be 0.32% and 0.14%, respectively. Recovery of spiked 2-toluidine was found in range 91.5–95.2% with RSD 1.61–4.65%.

Table 3
Analysis of amines in lake water[a]

Aromatic amine deviation[b]	Found (nM L^{-1} ± SD)	Recovery (%) ± SD
Aniline	4.46 ± 0.053	104.6 ± 6.4
Benzidine	0.479 ± 0.0044	95.5 ± 3.4
2-Toluidine	1.27 ± 0.0257	96.6 ± 2.5
3,3′-Dimethoxybenzidine	0.445 ± 0.001	96.3 ± 4.0
2,6-Dimethylaniline	3.12 ± 0.016	101.6 ± 5.5
4-Chloroaniline	2.27 ± 0.01	105.3 ± 6.1
2-Naphthylamine	0.965 ± 0.0066	94.1 ± 5.5

[a] All results are average of four determinations.
[b] Recovery (%) of 0.1 μM L^{-1} spike.

Table 4
Analysis of amines in synthetic food colours[a]

Aromatic amine	Found (nM L^{-1} ± SD)	Spike (μM L^{-1})	Recovery (%)
Sunset Yellow FCF			
1,2-Phenylenediamine	0.49 ± 0.0062	10	94.2
		1	92.5
		0.1	97.9
Benzidine	0.538 ± 0.37	5	87.2
		1	89.2
		0.5	89.4
2-Toluidine	4.7 ± 0.188	10	109.6
		1	111.9
		0.1	106.5
3,3′-Dimethoxybenzidine	0.379 ± 0.33	5	94.1
		1	91.5
		0.5	92.7
N,*N*-Diethylaniline	8.69 ± 0.20	5	99.1
		1	95.1
		0.5	99.8
2-Naphthylamine	0.695 ± 0.177	5	100.7
		1	100.9
		0.5	102.8
Tartrazine			
2-Toluidine	46.0 ± 0.14	1	91.5
		0.5	95.2
		0.1	94.4

[a] All results are average of three determinations.

4. Conclusions

A fast precise, specific, very sensitive and robust method has been developed for the determination of carcinogenic aromatic amines. The method is very simple and practicable in routine analysis. Excellent detection limits (down to 24 pM L^{-1}) enable the application of proposed method in

environmental and food analysis. However, there is still a scope to improve the detection limits by using smaller volume detection cell. Similarly, peak area repeatability can be further improved by using pulsed amperometry.

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