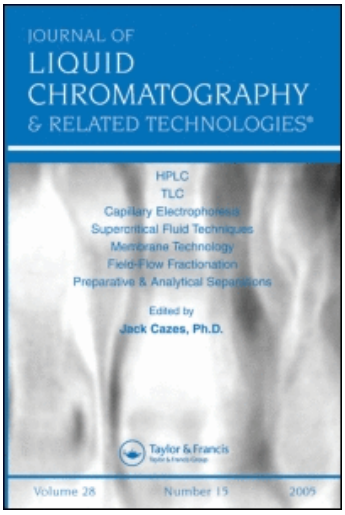

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M. S. Narvekar[a]; A. K. Srivastava[a]
[a] Department of Chemistry, University of Mumbai, Mumbai, India

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Ion-Pair Reversed-Phase HPLC Determination of Aromatic Amine Isomers

M. S. Narvekar and A. K. Srivastava*

Department of Chemistry, University of Mumbai,
Mumbai, India

ABSTRACT

High performance liquid chromatography (HPLC) with isocratic ion-pair reversed-phase separation and UV detection at 220 nm is proposed for the analysis of six amine azo dye isomers prohibited under a German ban. The analysis is achieved on a C_{18} polaris column (5 μm, 250 mm × 4.6 mm ID) using a mixture of 20 mM phosphate buffer pH = 2.5, containing 5 mM 1-hexanesulfonic acid, sodium salt and methanol (65 : 35). A flow rate of 1.0 mL min^{-1} and a column temperature of 25°C were employed. A total of 16 amines have been studied for limits of detection, relative standard deviations (RSD%) and linearity. The method gave excellent separation between isomers.

*Correspondence: A. K. Srivastava, Department of Chemistry, University of Mumbai, Vidyanagari, Santacruz (E) Mumbai 400 098, India; E-mail: aks@chem.mu.ac.in.

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Key Words: HPLC; Ion-pair; Azo dyes; Aromatic amines.

INTRODUCTION

Azo dyes form the largest and the most versatile group of synthetic dyes. They are adaptable to a wider variety of applications then any other group of dyes. Though these dyes are stable, they can undergo degradation, primarily due to reduction of the N═N bond. The reduction of the azo group can release the original amine. This resulted in the legislation known as the German ban[1] which is effective from 1st April, 1996 and stipulates that articles regularly coming in contact with human body must not be dyed with azo dyestuffs, which release harmful amines on reductive cleavage under conditions of wear. There are 20 amines in the banned list.[2] Of the amines in the banned list, six, namely *p*-chloroaniline, *o*-toluidine, 2-naphthyl amine, 4-chloro-*o*-toluidine, 2,4-diamino toluene, and 2-amino-4-nitrotoluene occur in isomeric forms. The isomers commonly encountered in technical products are *o*-chloroaniline and *m*-chloroaniline (isomer of *p*-chloroaniline), *p*-toluidine (isomers of *o*-toluidine), 1-naphthyl amine (isomer of 2-naphthyl amine), 3-chloro-*o*-toluidine and 5-chloro-*o*-toluidine (isomer of 4-chloro-*o*-toluidine), 2,6-diaminotoluene and 2,3-diaminotoluene (isomer of 2,4-diaminotoluene), and 2-amino-5-nitrotoluene and 2-amino-6-nitrotoluene (isomer of 2-amino-4-nitrotoluene). It has been pointed out, that isomer separation has not been given adequate consideration[3,4] and this can result in a dye or fabric being banned, even though it contains the isomer which is not banned. Prohibited amines have also been detected in some dyestuffs, even though such amines were not employed in their manufacture.[5] Thus, it is essential to develop methods which can specifically resolve the isomers. Even though HPLC has been used to separate aromatic amines resulting from the degradation of azo dyes,[6–12] quantitative isomer separation has not been reported. Separation of *o*, *m*, and *p*-toluidine by TLC[13] and separation of 4-chloro-*o*-toluidine and 5-chloro-*o*-toluidine by GCMS[14] have been reported. Recently, HPTLC separation and quantification on silica plates,[15] modified silica plates,[16] and GCMS, after derivatization with pentafluoropropionic anhydride[17] of some isomers of the above mentioned amines, have been reported. This communication describes an HPLC method involving isocratic ion-pair reverse-phase separation with UV detection of the above mentioned amines. The procedure is simple, rapid, and excellent separation between isomers is achieved. A total of 16 amines have been studied, limits of detection and relative standard deviations (RSD%) and linearity have been reported.

EXPERIMENTAL

Chemicals and Reagents

The aromatic amines reference compound, 95 to 99% purity from Aldrich, Fluka, Riedel-de Haen and Merck were used without further purification. Pentane, hexane, heptane, octanesulfonic acid (C5,C6,C7,C8), sodium salts, were obtained from CDH Chemicals India. Extrulet® 20 column, diameter 24 mm and length 90 mm (Merck art. No. 1,11737), methanol HPLC grade and methyl *tert*-butyl ether (GR) were from E. Merck, India. Potassium dihydrogen phosphate and orthophosphoric acid were obtained from E. Merck, India.

Equipment

Separation of the amines was carried out with a dual pump HPLC system of Shimadzu, with LC10ATVP pumps and SPD-M10AVP Diode Array detector, 20 µL Rheodyne loop injector and Polaris C_{18} column (250 mm × 4.6 mm I.D., 5 µm particle size) from Metachem Technologies Inc. The detector output was integrated and quantified on a Class-M10A LC workstation.

Standard Solutions

The standard amines solutions (1 mg mL^{-1}) were prepared by dissolving in HPLC grade methanol and stored at less than 4°C. These solutions were suitably diluted for determining limits of detection (LOD) and calibration range. This was computed on basis of peak areas.

Sample Preparation

Commercial dye samples (0.1 g) or fabric sample (1.0 g) were decomposed using the standard test method[18] (Test no. 35 LMBG Method no. B82.02-2 of September, 1996), which involves treating the sample with 17 mL of 0.05 molar aqueous citrate buffered at pH 6 for 30 min at 70°C followed by addition of 3.0 mL 20% sodium dithionate for 30 min at 70°C. After cooling, the extract was passed over an extrelut 20 column and eluted

with 80 mL methyl *tert*-butyl ether. The ether solution is evaporated to dryness and dissolved in 2 mL methanol.

Synthetic mixtures were prepared as follows: Synthetic stock solution A: Stock solution ($100\,\mu g\,mL^{-1}$) of *p*-chloroaniline (a), *o*-chloroaniline (b), *m*-chloroaniline (c), 2,3-diaminotoluene (d) 2,4-diaminotoluene (e) and 2,6-diamino toluene (f) were prepared in methanol. Synthetic mix A1: 1 mL (a) +1 mL (b) +1 mL (c) +1 mL (d) +1 mL (e) +1 mL (f) diluted to 100 mL with methanol. Synthetic mix A2: 2 mL (a) +2 mL (b) +2 mL (c) +2 mL (d) +2 mL (e) +2 mL (f) diluted to 100 mL with methanol. Synthetic mix A3: 5 mL (a) +5 mL (b) +5 mL (c) +5 mL (d) +5 mL (e) +5 mL (f) diluted to 100 mL with methanol.

Synthetic stock solution B: Stock solution ($100\,\mu g\,mL^{-1}$) of 3-chloro-*o*-toluidine (g), 4-chloro-*o*-toluidine (h), 5-chloro-*o*-toluidine (i), 2-amino-4-nitrotoluene(j), 2-amino-5-nitrotoluene (k), 2-amino-6-nitrotoluene (l). Synthetic mix B1: 1 mL (g) +1 mL (h) +1 mL (i) +1 mL (j) +1 mL (k) +1 mL (l) diluted to 100 mL with methanol. Synthetic mix B2: 2 mL (g) +2 mL (h) +2 mL (i) +2 mL (j) +2 mL (k) +2 mL (l) diluted to 100 mL with methanol. Synthetic mix B3: 5 mL (g) +5 mL (h) +5 mL (i) +5 mL (j) +5 mL (k) +5 mL (l) diluted to 100 mL with methanol.

HPLC Analysis

The mobile phase was a mixture of 20 mM phosphate buffer pH 2.5, containing 5 mM 1-hexanesulfonic acid, sodium salt and methanol (65 : 35). The flow rate was $1.0\,mL\,min^{-1}$.

Prior to sample injection, the column was flushed with at least 20 times its column volume of the mobile phase.

RESULTS

Optimization of Chromatographic Conditions

As ion-pair chromatographic separations are more complicated to develop than reverse phase separations,[19] different variables were studied to optimize the separation between the isomers. Table 1 shows the variation of retention time with pairing-ion alkylchain length (C5, C6, C7, C8). It was observed, that the retention times of the amines decreased with the chain length of the ion-pairing molecule from C8 to C5. This decrease is not identical for all the amines, it is more pronounced for the late eluting

Table 1. Variation of retention time of amines with alkyl chain length (C5, C6, C7, C8). The mobile phase contained aqueous component buffered at pH = 2.5 with orthophosphoric acid, 20 mM phosphate buffer, 5 mM pairing ion concentration and methanol (65 : 35).

	Retention time (min)			
Amines	Pentane	Hexane	Heptane	Octane
o-Chloroaniline	17.208	18.139	26.124	26.894
m-Chloroaniline	12.205	13.704	23.415	24.889
p-Chloroaniline[a]	9.360	11.297	19.210	19.337
2,3-Diaminotoluene	4.653	5.970	8.563	13.254
2,4-Diaminotoluene[a]	3.911	4.570	7.093	11.166
2,6-Diaminotoluene	3.596	4.018	6.081	10.126
3-Chloro-*o*-toluidine	21.742	25.138	36.502	58.550
4-Chloro-*o*-toluidine[a]	17.311	21.124	34.078	50.392
5-Chloro-*o*-toluidine	24.448	27.063	36.815	60.505
2-Amino-4-nitrotoluene[a]	19.715	19.579	20.251	21.202
2-Amino-5-nitrotoluene	18.366	18.246	18.499	18.606
2-Amino-6-nitrotoluene	15.496	15.639	16.422	17.634
o-Toluidine[a]	4.677	5.996	8.105	19.290
p-Toluidine	4.852	6.310	8.412	19.756
1-Naphthylamine	13.344	16.470	29.629	59.891
2-Naphthylamine[a]	13.713	18.139	36.973	72.540

[a]Banned amine.

components, mainly naphthylamines and chloro-*o*-toluidines. From the table, it is evident that pentanesulfonic acid and hexanesulfonic acid are better suited for the separation of amines as heptane and octane sulfonic acid give longer retention times. Hexanesulfonic acid is preferred over pentanesulfonic acid, mainly due to its suitable retention time for diaminotoluenes and better resolution between naphthylamines. The concentration of the buffer was varied from 10 mM to 50 mM. It was observed, that at 10 mM buffer concentration, inadequate buffering results in poor reproducibility. However, at 20 mM there is good reproducibility, further increase does not influence the retention time.

Table 2 shows the relationship between retention time and pH, which is varied from 2.3 to 3.6. It was observed, that lowering the pH reduces the retention time. As pH is increased beyond 2.5, there is a gradual decrease in resolution of 3-chloro-*o*-toluidine and 4-chloro-*o*-toluidine and also an increase in analysis time. At pH 2.3 and 2.5 isomers are well resolved,

Table 2. Variation of retention time with pH. The mobile phase contained aqueous component 20 mM phosphate buffer, 5 mM 1-Hexanesulfonic acid, pH was adjusted with orthophosporic acid and methanol (65 : 35).

	Retention time (min)				
Amines	pH = 2.3	pH = 2.5	pH = 2.8	pH = 3.2	pH = 3.6
o-Chloroaniline	17.572	18.139	18.815	21.045	21.505
m-Chloroaniline	12.762	13.704	14.338	19.209	20.581
p-Chloroaniline[a]	11.157	11.297	12.033	15.712	17.390
2,3-Diaminotoluene	5.910	5.970	6.155	6.176	6.289
2,4-Diaminotoluene[a]	4.552	4.572	4.741	4.751	4.788
2,6-Diaminotoluene	4.010	4.018	4.134	4.186	4.201
3-Chloro-*o*-toluidine	23.108	25.138	26.845	38.166	41.102
4-Chloro-*o*-toluidine[a]	20.120	21.124	22.315	32.380	35.798
5-Chloro-*o*-toluidine	24.616	27.063	27.187	38.899	41.208
2-Aamino-4-nitrotoluene[a]	19.573	19.579	20.084	21.250	21.619
2-Amino-5-nitrotoluene	18.248	18.246	18.508	18.733	20.581
2-Amino-6-nitrotoluene	15.593	15.639	15.963	17.013	17.390
o-Toluidine[a]	5.991	5.996	6.108	6.508	8.447
p-Toluidine	6.285	6.310	6.385	6.950	8.880
1-Naphthylamine	16.269	16.470	17.743	23.977	26.967
2-Naphthylamine[a]	18.058	18.139	19.570	27.828	28.220

[a]Banned amine.

however as low pH values reduce column life, pH 2.5 is preferred. As methanol–water mobile phases provide greater solubility than acetonitrile-water or tetrahydrofuran–water solution, and also as methanol is less toxic, it was used as the organic solvent. Reduction of the organic phase component in the mobile phase to 30%, results in considerable increase in the retention time (>60 min.) for most of the amines, without any improvement in resolution, whereas increase to 40% results in complete loss of resolution between the isomers.

Analytical Variables

Table 3 lists the retention time of the individual amines, limit of detection (LOD) and standard deviation at LOD. Replicates analysis ($n = 7$) were used for computing RSD%. Linear regression least squares fit data from the measurements are presented in Table 4, their respective intercepts 'a', slope 'b', and the standard deviations (computed for 6 data points) of intercept 'S_a'

Table 3. Retention time in minutes, Limit of detection (X), and percentage standard deviation at X.

Amines	Retention time (min)	Limit of detection (X)	RSD % at X
o-Chloroaniline	18.139	0.4	2.29
m-Chloroaniline	13.704	0.5	1.32
p-Chloroaniline[a]	11.297	0.3	1.76
2,3-Diaminotoluene	5.970	0.4	2.20
2,4-Diaminotoluene[a]	4.572	0.3	1.94
2,6-Diaminotoluene	4.018	0.3	1.60
3-Chloro-*o*-toluidine	25.138	0.5	2.13
4-Chloro-*o*-toluidine[a]	21.124	0.6	1.39
5-Chloro-*o*-toluidine	27.063	0.4	0.99
2-Amino-4-nitrotoluene[a]	19.579	0.2	1.33
2-Amino-5-nitrotoluene	18.246	0.5	1.77
2-Amino-6-nitrotoluene	15.639	0.3	1.12
o-Toluidine[a]	5.996	1.2	1.12
p-Toluidine	6.310	0.6	1.29
1-Naphthylamine	16.470	0.04	1.47
2-Naphthylamine[a]	18.139	0.04	1.62

[a]Banned amine.

Table 4. Linear regression (Least squares fit) data for calibration curves for six data points.

Amines	Concentration range ($\mu g\,mL^{-1}$)	Intercept 'a'	Slope 'b'	'S_a'	'S_b'	'r'
o-Chloroaniline	0.4–8.0	−388.98	58283.92	3056.79	683.54	0.99973
m-Chloroaniline	0.5–10.0	1000.43	56183.25	4211.05	753.33	0.99964
p-Chloroaniline[a]	0.3–6.0	−4033.53	116419.10	7302.21	2177.26	0.99921
2,3-Diaminotoluene	0.4–8.0	−779.54	43387.42	694.77	155.36	0.99997
2,4-Diaminotoluene[a]	0.3–6.0	−19.53	95900.63	2209.48	658.8	0.99991
2,6-Diaminotoluene	0.3–6.0	2163.23	83455.75	2060.90	614.52	0.99989
3-Chloro-*o*-toluidine	0.5–10.0	720.93	42710.52	2001.76	358.10	0.99986
4-Chloro-*o*-toluidine[a]	0.6–12.0	−578.07	41917.26	873.48	130.22	0.99998
5-Chloro-*o*-toluidine	0.4–8.0	−998.86	113912.81	2015.10	450.61	0.99997
2-Amino-4-nitrotoluene[a]	0.2–4.0	−3158.17	152430.42	3777.96	1689.58	0.99975
2-Amino-5-nitrotoluene	0.5–10.0	−390.22	48715.82	649.90	116.27	0.99999
2-Amino-6-nitrotoluene	0.3–6.0	−224.19	105368.51	465.10	138.68	0.99999
o-Toluidine[a]	1.2–24.0	1042.59	20418.04	829.10	52.97	0.99998
p-Toluidine	0.6–12.0	2749.72	66281.98	4652.35	693.58	0.99978
1-Naphthylamine	0.04–0.8	2535.77	910129.15	4753.81	10630.12	0.99978
2-Naphthylamine[a]	0.04–0.8	1997.14	811178.32	3232.55	7228.17	0.99987

[a]Banned amine.

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Table 5. Results of analysis of synthetic mixture of chloroanilines, diaminotoluenes and commercial dye samples.

	Concentration ($\mu g\,mL^{-1}$)				
Amines	Synthetic mix A1	Synthetic mix A2	Synthetic mix A3	Test sample1 brown dye	Test sample 2 orange dye
o-Chloroaniline	0.98 (1.0)	2.02 (2.0)	5.08 (5.0)	Nil	Nil
m-Chloroaniline	1.02 (1.0)	2.03 (2.0)	5.10 (5.0)	2.60 (52.0)	13.51 (270.2)
p-Chloroaniline	0.98 (1.0)	1.98 (2.0)	5.05 (5.0)	3.21 (64.2)	Nil
2,3-Diaminotoluene	1.10 (1.0)	1.98 (2.0)	4.97 (5.0)	Nil	Nil
2,4-Diaminotoluene	0.98 (1.0)	1.97 (2.0)	4.98 (5.0)	Nil	Nil
2,6-Diaminotoluene	0.98 (1.0)	2.02 (2.0)	5.05 (5.0)	Nil	Nil

Note: Amounts in brackets indicate injected concentration.

Table 6. Results of analysis of synthetic mixture of chloro-*o*-toluidines and aminonitrotoluenes.

	Concentration ($\mu g\,mL^{-1}$)		
Amines	Synthetic mix B1	Synthetic mix B2	Synthetic mix B3
3-Chloro-*o*-toluidine	0.98 (1.0)	2.04 (2.0)	5.05 (5.0)
4-Chloro-*o*-toluidine	0.98 (1.0)	2.05 (2.0)	5.07 (5.0)
5-Chloro-*o*-toluidine	1.02 (1.0)	2.02 (2.0)	5.05 (5.0)
2-Amino-4-nitrotoluene	1.01 (1.0)	2.0 (2.0)	4.95 (5.0)
2-Amino-5-nitrotoluene	1.0 (1.0)	2.01 (2.0)	5.03 (5.0)
2-Amino-6-nitrotoluene	0.98 (1.0)	1.98 (2.0)	5.02 (5.0)

Note: Amounts in brackets indicates injected concentration.

and slope 'S_b' are reported, along with the concentration range studied. All these values have been calculated as given by Miller.[20] The correlation coefficient (r) was found to be greater than 0.999 for all the amines.

Tables 5 and 6 show the analysis of synthetic mixtures with the actual amine contents in brackets. Amines have been quantified by triplicate analysis. The

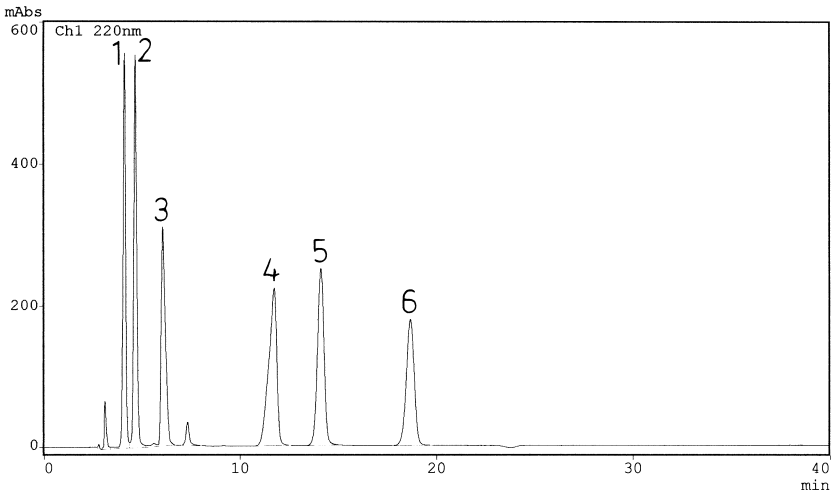

Figure 1. Typical chromatogram of amine mixture. Peaks 1 = 2,6-diaminotoluene, 2 = 2,4-diaminotoluene, 3 = 2,3-diaminotoluene, 4 = *p*-chloroaniline, 5 = *m*-chloroaniline, 6 = *o*-chloroaniline.

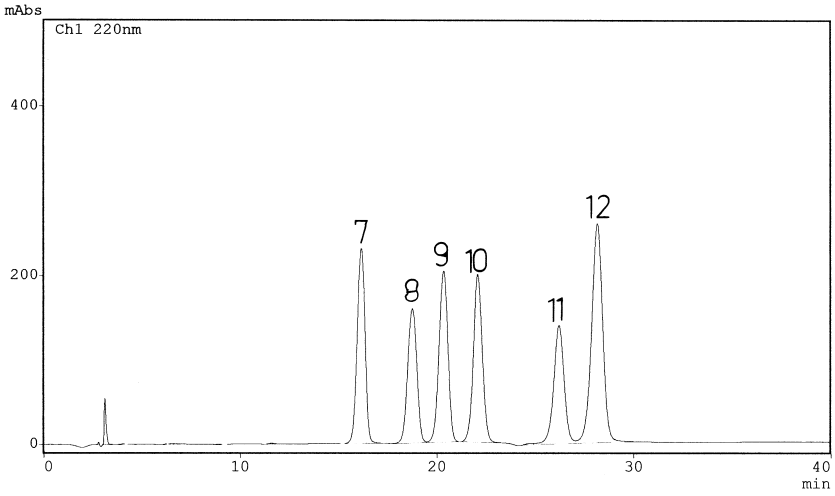

Figure 2. Typical chromatogram of amine mixture. Peaks 7 = 2 amino-6-nitrotoluene, 8 = 2-amino-5-nitrotoluene, 9 = amino-4-nitrotoluene, 10 = 4-chloro-*o*-toluidine, 11 = 3-chloro-*o*-toluidine, 12 = 5-chloro-*o*-toluidine.

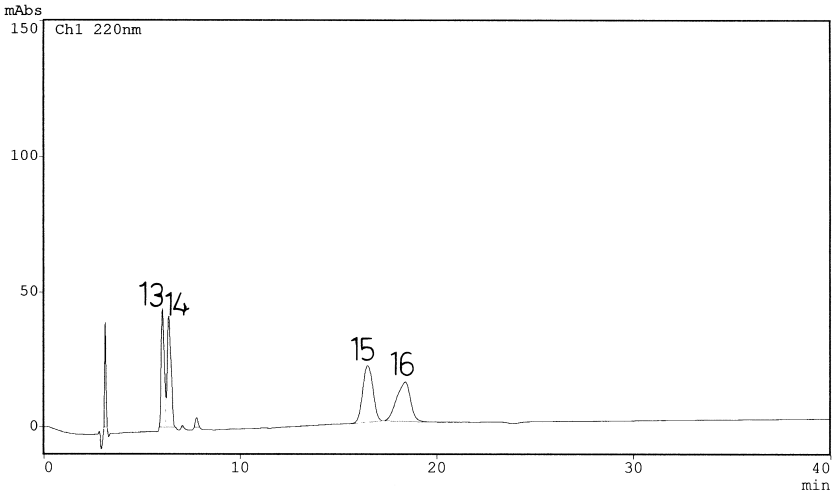

Figure 3. Typical chromatogram of amine mixture. Peaks 13 = *o*-toluidine, 14 = *p*-toluidine, 15 = 1-naphthylamine, 16 = 2-naphthylamine.

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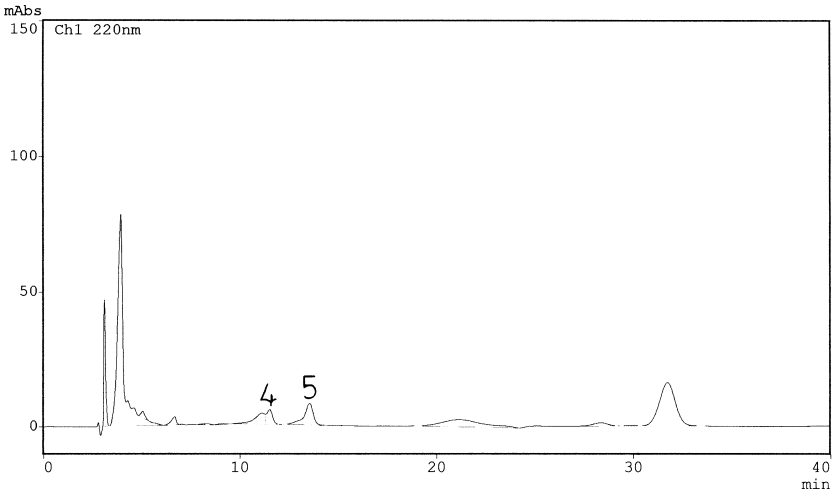

Figure 4. Chromatogram of commercial brown dye sample. Peak 4 = *p*-chloroaniline, peak 5 = *m*-chloroaniline.

typical chromatograms of synthetic mixtures are portrayed in Figs. 1–3. Two chloroaniline based commercial dye samples (brown and orange, both 2 : 1 metal complex dyes for wool) have been analysed in triplicate by the present method and the chromatograms are given in Figs. 4 and 5 respectively. It is clear from Fig. 4 that the brown dye sample contains *m*-chloroaniline (peak 5) and *p*-chloroaniline (peak 4), where as Fig. 5 shows the presence of only *m*-chloroaniline (peak 5) in the orange dye sample. These peaks were identified on the basis of their retention time and confirmed by spiking. Figures 4 and 5 show some additional peaks, which are due to other components present in dye sample. The brown dye sample was found to contain 2.60 and 3.21 $\mu g\,mL^{-1}$ of *m*-chloroaniline and *p*-chloroaniline, respectively, which corresponded to 52.0 and 64.2 $\mu g\,mL^{-1}$, respectively, in the dye. Orange dye was found to contain 13.51 $\mu g\,mL^{-1}$ *m*-chloroaniline, which corresponded to 270.2 $\mu g\,mL^{-1}$ in the dye. Thus, *p*-chloroaniline prohibited amine could be separated and quantified in the presence of its isomer *m*-chloroaniline.

From the retention data, it is clear that retention times of *o*-chloro aniline and α-naphthylamines, *o*-toluidine and 2,3-diaminotoluene are very close. In commercial dye samples, however, this is unlikely to be a problem because dyes are manufactured from a particular amine and would, therefore be, for e.g., naphthylamine based or toluidine based and on reduction would release naphthylamine or toluidine, respectively, and not a mixture of two.

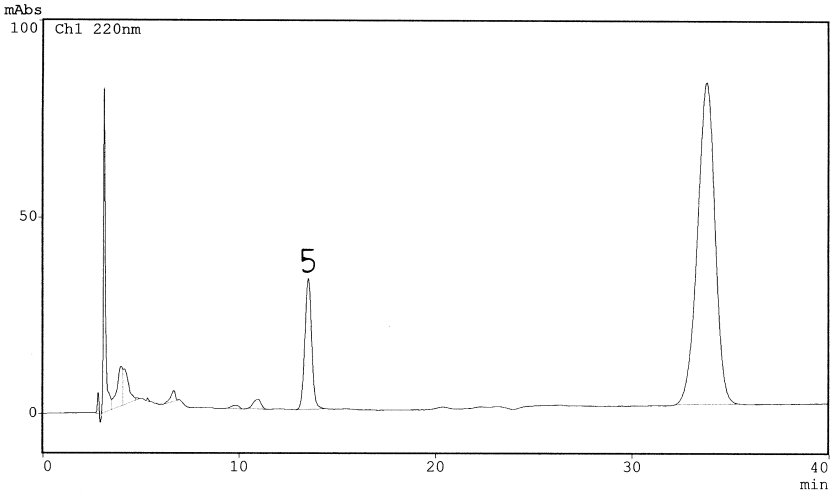

Figure 5. Chromatogram of commercial orange dye sample. Peak 5 = *m*-chloroaniline.

CONCLUSION

Various aromatic amines can be resolved from their isomers on C_{18} column by using hexanesulfonic acid as an ion pairing agent. This method is found to be accurate, precise, and rectilinear in detector response in the concentration range studied, hence, can be used for the separation and quantification of the aromatic amine isomers.

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