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Resolving the benign and the malign isomers of aryl amines by HPLC

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ABSTRACT

The present work is related to an improved analysis for aryl amine isomers thereby its more reliable identification. Over 24 aromatic amines were declared as carcinogenic and hence banned for usage in consumer articles based on textile, leather or toys etc. There is a specific official method for screening these aryl amines in consumer articles. But the official protocol could not facilitate the confirmation of exact isomer of aryl amines due to poor chromatographic resolution. This study had targeted this lacuna and involved HPLC exploiting various RP columns and mobile phase conditions to achieve the improved detection of isomers of arylamines. The method successfully separated isomers of about 8 different aryl amines with selectivity of 1 or better and the method is applied to real cases of commercial dye samples.

KEYWORDS

Resolving carcinogenic aryl amines;separation of isomers of aryl amines;
HPLC separation of aryl amines;
Reverse phase LC separation for aryl amines;
Influence of C18 column on elution of polar and non polar aryl amines.

INTRODUCTION

Aryl amines are widely used as precursors in the manufacturing of dyes, medicines, pesticides, adhesives, polymers and hence gain significance in their analysis and exact identification[1]. In fact the large usage of aryl amines is for making dyes. Azo dyes are extensively used in industrial application since 19th century, for their colorfastness and being cost-effective. Azo dyes are used for coloring many consumer goods like toys, leather, clothes, cosmetics and plastics[2, 3] and also used in foods[4].

They undergo reduction biologically and release aromatic amines. This reduction is done by the azo reductases present in sweat, intestinal bacteria, skin and liver cells as reported[2, 3, 5-7]. Some of the aromatic amines are found to be carcinogen[8, 9]. Occupational exposure to aromatic amines used in dyes industries increases the risk of developing cancer[10, 11, 12].

Azo dyes and their degraded products in consumer goods come into contact with human beings through dermal absorption or oral ingestion. The skin absorption can result from skin contact of the clothes with azo dyes released due to sweat and friction. The oral ingestion especially with children can be when they suck these dyed clothes or toys as saliva can also release the dyes which may under appropriate conditions generate aromatic amines. On this concern, the German government brought a ban on these dyes and pigments in their consumer goods and now the entire European block followed it up[13].

This ban list encompassed 24 aryl amines as of now. Accordingly, the dyed articles when directly comes into contact with human skin should not produce any of those amines in the list and the current method detection limit is 30 $mgkg^{-1}$ which is notified as the tolerance limit[14].

The IUC/IULTCS official procedure for determination of aryl amines in leather is mainly by using HPLC and it included the alternative methods like capillary electrophoresis, TLC and GCMS[14]. All the aryl amines notified as carcinogens have one or more isomers which are all not in ban list as the ban is for only one isomeric form except the case of xylidines which has 2 of its isomers in ban list. The structural activity relationship of aryl amines plays an important role in deciding the carcinogenity of aryl amines[15]. Hence, we need to study the isomeric form of these analytes that is facilitated mainly by good separation of these aryl amines. For which, neither does the official method[14] nor does any simple reported procedure serve us. There are no many studies for the isomer separation except that some using capillary electrophoresis (CE) and reported as mixed miceller chromatography[16, 17] the major disadvantage of that is that CE suffers from poor reproducibility. In another study, a few of pairs of aryl amine isomers were analyzed through derivatization and followed by GC-MS[18]. But in this proposed study, the method developed is based on HPLC-DAD that is a popular technique with good reducibility and the method is a simple, reliable for deciding the isomeric form of these aryl amines and without any derivatization.

EXPERIMENTAL

Reagents

Arylamines isomer standards chosen for separation are: 2,4-Diaminotoluene(24DAT), 2,5-Diaminotoluene(25DAT),2,3-Diaminotoluene (23DAT),2,6-Diaminotoluene (26DAT),3,4-Diaminotoluene (34DAT);2-chloroaniline(2CA),4-chloroaniline (4CA);1-napthylamine(1NAP),2-napthylamine (2NAP); 2-aminobiphenyl(2ABP), 4-aminobiphenyl (4ABP);2,6 xylidine (26XD),2,4 xylidine (24XD),2,5 xylidine (25XD),2,3 xylidine (23XD),3,4 xylidine (34XD), 3,5-xylidine (35XD) ;2,4trimethylaniline(24TMA), 5, 2trimethylaniline (52TMA), 4,6trimethylaniline (46TMA);2-toluidine(2TD), 4- toluidine(4TD). These arylamines will be referred in their addrevaition in the further discussion as given in the bracket. All these arylamines were purchased from Sigma chemicals (St Louis, MO, USA). Extrulet(trade mark of Merck for a diatomaceous earth offered by Merck, Darmstadt, Germany),Sodium dithionite, HPLC grade Acetonitrile were purchased from Merck (Darmstadt, Germany). Hexane was purchased from Qualigens,Mumbai,India. Methyl tert-butyl ether was purchased from J.T.Baker. Water for mobile phase was prepared using Millipore; All other reagents used were analytical grade reagents. Water for chromatography was prepared by using Millipore model Milli-Q Reference which was procured from Merck-EMD Millipore Corporation, Billerica, MA, USA.

Apparatus

High Performance Liquid Chromatography (HPLC) was purchased from Waters Instruments Corporation, Milford, USA. That consisted of Alliance pump model 2695 incorporating an automated injector and Photo Diode Array detector of model 2996. The operation and data processing was done by Empower software from Water's Instruments Corpn.

Lichrocart -Puroshpher RP-18e (5µm) 250x4mm column was purchased from Merck, Darmstadt, Germany; Brownlee spheri-5, RP-18, (5µm), 220x2.1 mm column was purchased from Perkin Elmer,Norwalk,CT,USA and Kromasil ODS (C18) (5µm) 150x3.2 mm column was purchased from Phenomenex,Hyderabad,India.

Aryl amines in the banned (suspected carcinogens)list

2,4-Diaminotoluene(24DAT), 4-chloroaniline (4CA), 2-napthylamine (2NAP), 4-aminobiphenyl (4ABP);2,6 xylidine (26XD),2,4 xylidine (24XD),2,4trimethylaniline(24TMA), 2- toluidine (2TD).

Sample preparation

Stock solutions for 8 carcinogenic aryl amines

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TABLE 1 : Gradient conditions for mobile phase chosen for separation of isomers of Arylamines

Time	CAN (%)	Water (%)	Curve
0	15	85	*6
20	17	83	6
21	17	83	6
30	20	80	6
34	22	78	6
37	25	75	6
40	75	25	6
43	90	10	6
50	15	85	6
60	15	85	6

***linear gradient programme**

and their corresponding isomers were prepared individually in 1000 parts per million (ppm) concentration using HPLC grade acetonitrile; from this stock solution the lower concentration of 15 ppm solution was prepared afresh daily and that was injected into HPLC.

Application to real sample

The official procedure adopted for the analysis of the dyes is described in brief as follows:

About 0.1g of the dye sample was weighed in a 25ml capacity amber colored reagent bottle fitted with silicone rubber self–sealing septum. To this, 16ml of the sodium citrate buffer of pH 6 and 1ml of the octa fluoro benzidine (OFB) (as ISD) were added. The contents was kept in an oven set at 70ÚC. Once the temperature reached, about 0.5g of the sodium dithionite dissolved in 2.5ml of water was injected. A similar second injection of dithionite was done after 15 minutes, which was then cooled. The contents were loaded onto a Solid phase extraction column packed with a diatomaceous material called Extralute to rid of the many organic contaminants from the sample that are hydrophobic. Aryl amines were eluted from the column using methyl tert-butyl ether for 3 times. The ethereal extracts were combined and concentrated using rotor vapour to near dryness. The final complete evaporation was achieved using gentle current of nitrogen. Then, the residue was dissolved in acetonitrile, made up to 2 ml, filtered using syringe filter 0.2µ and injected to HPLC.

HPLC conditions

A gradient mobile phase composition based on acetonitrile and water was used.The details of the gradient composition is given in TABLE 1.The flow rate was kept at 0.7ml/min for this set of isomers.The PDAdetector was set at wavelengths of 240nm,280nm and 305nm along with spectrum collection.Sample of 10µl was injected.

Safety Measures

Many of these aryl amines are declared as carcinogenic substances and hence these should be handled with all safety measures and precaution; and disposed as per the local regulatory procedures. Detailed safety guidelines should be provided in the work place in the event of any spillage or contact by the analyst.

RESULTS AND DISCUSSION

Purosphere column

Separation of isomers of aryl amines was done by employing "LiChrocart Purospher RP-18e". It was observed that not all isomers were separated using this column. In this attempt, diaminotoluene, which is of 5 isomers was separated along with 2 isomers of toludines and naphthylamines were separated. The remaining cases of aminobiphenyls, trimethylamines and xylidines were not separated by this column. The separation of isomers of polar to moderately non-polar aryl amines was achieved by this column bearing a carbon load of 18% (that is

C_{18}derivatization through silanization of silica) while isomers of relatively higher nonpolar arylamines were not resolved. The gradient program detailed in TABLE.1 was used for the separation and when further attempts involving number of variations of gradient compositions done the desired results were not achieved.

Brownlee Spheri RP18 column

The carbon loading of this column is claimed to be 14%. When the separation was tried using Brownlee Column "Spheri-5 RP-18",diamino toluenes, napthylamines and toludines were separated. But, xylidines,aminobiphenyls and trimethylamine remained unsuccessfully. Even any number of variations mainly with the gradient compositions could not serve.

Kromasil column

Kromasil is with a carbon load of 19% was highest among three columns tried in this study. When separation was tried with this column, using the same gradient composition, all the isomers taken in this study was successfully separated. Even the xylidines which has 6 isomers and not separated in any of the earlier attempts with other columns was successfully separated. All other isomers of chloroaniline, aminobiphenyl, trimethylaniline, napthylamine, and toludine were separated by this column as shown in Figures 1-7.

The highly polar isomers of diaminotoluene are also observed with good separation. Selectivity was calculated for this Kromasil column and found to be greater than or equal to one as shown in TABLE.2.

Significance of carbon load and end capping of RP for arylamines

Although the C_{18} columns in general is found widely used for aryl amine analysis, when the demand is for the separation of various isomers of basic analytes like aryl amines, only a few stand fast to the demand. In this study, the column Kromasil ODS provided a satisfactory separation which can be attributed to its high carbon loading of 19% as claimed by the manufacturer; also in C_{18} columns the silencing of the residual hydroxyl groups by base deactivation that is also known as "end capping "(achieved by silanization of those polar reactive hydroxyls by small alkyl like methyl silanes) is also important. This later is revealed by the separation of highly polar amines like diamino toluenes while the separation of aryl amine isomers of the more non polar ones like trimethyl amines,amino biphenyls revealed that this high carbon loading helped these analytes. In short, the carbon loading and base deactivation of residual hydroxyl groups are achieved suitably for these basic analytes to a desirable extent by Kromasil C_{18} and hence that provided the most satisfactory separation of these

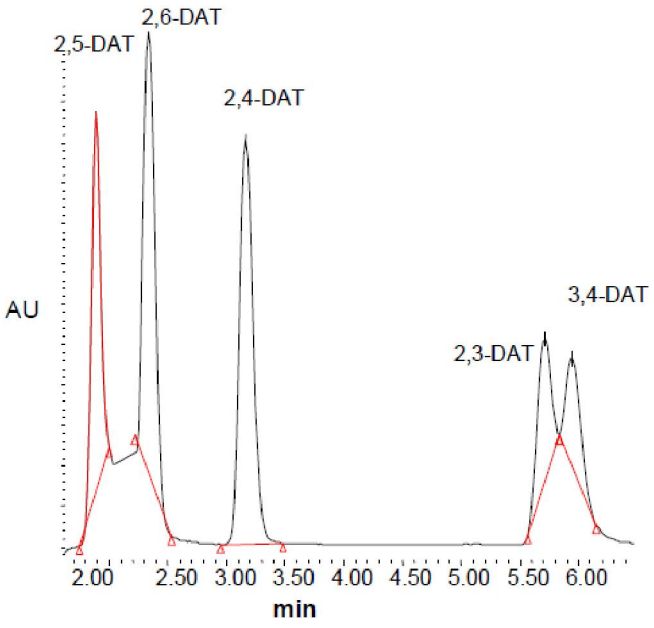

Figure 1 : Separation of isomers of DAT by KROMASIL ODS

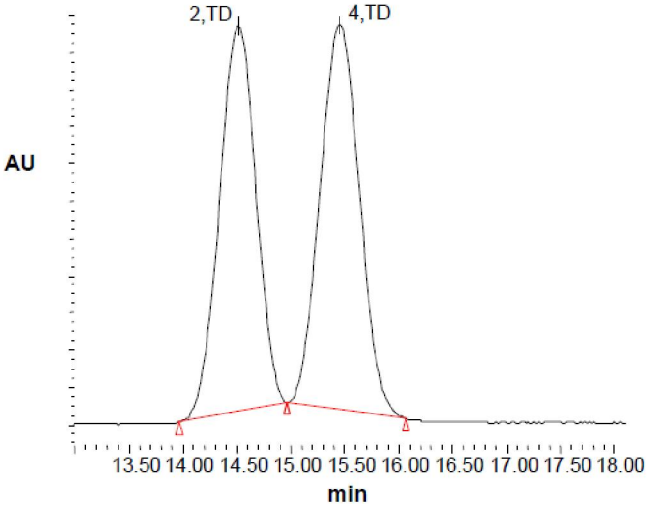

Figure 2 : Separation of isomers of TD by KROMASIL ODS

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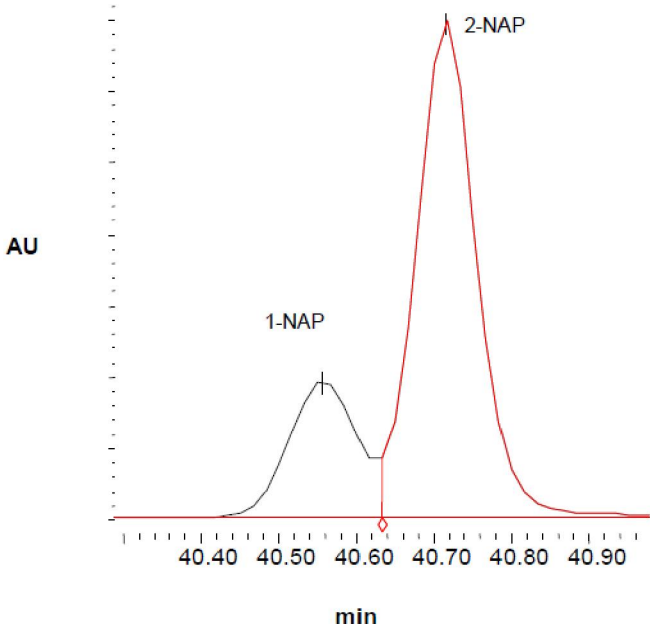

Figure 3 : Separation of isomers of NAP by KROMASIL ODS

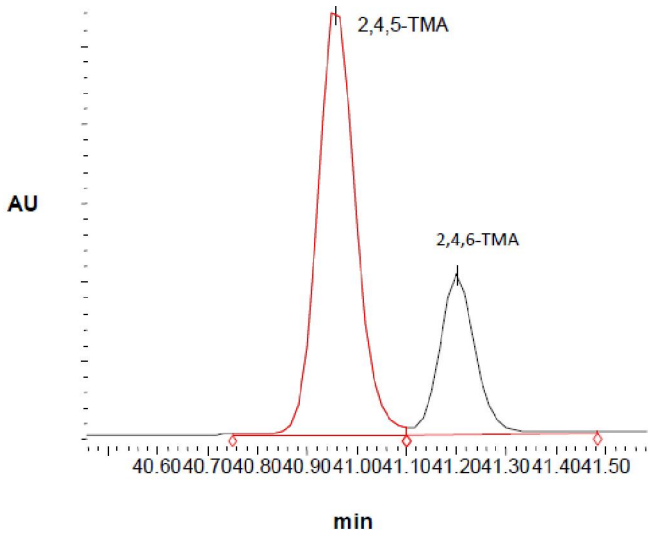

Figure 4 : Separation of isomers of TMA by KROMASIL ODS

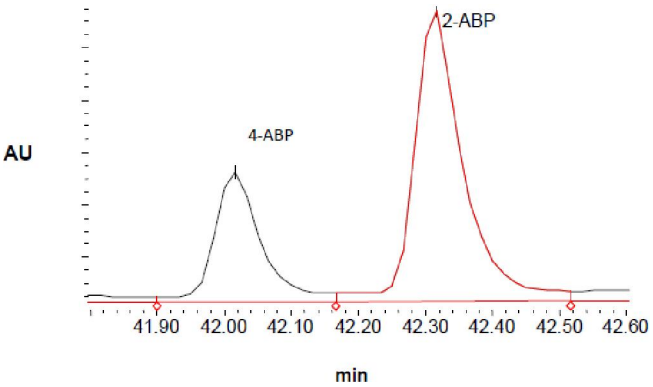

Figure 5 : Separation of isomers of ABP by KROMASIL ODS

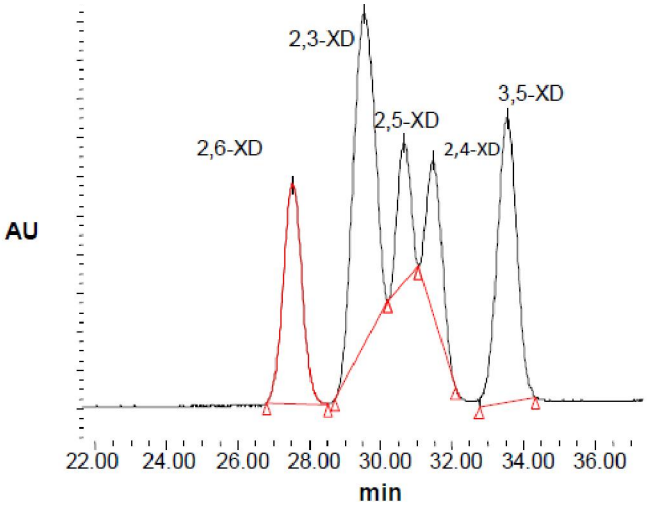

Figure 6 : Separation of isomers of XD by KROMASIL ODS

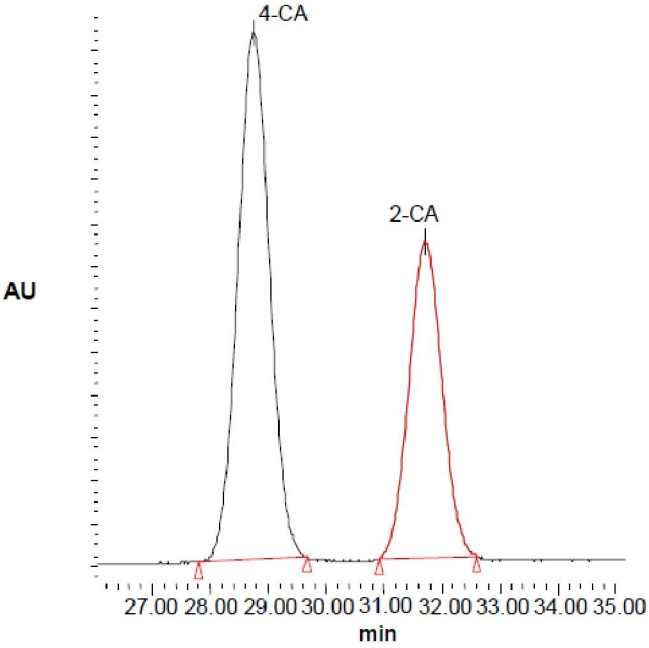

Figure 7 : Separation of isomers of CA by KROMASIL ODS

analytes.

Analysis of real samples

Two commercial dye samples were found to be positive of toludines when analysed by official European protocol and toludine found was compared to the standard 2-toludine that was in ban list and always run parallely as standard. The retention time was found to differ by 0.1min with 4-toludine while the spectrum option by the PDA detector revealed that the two peaks namely 230nm and 280nm differed by 2nm between the two isomers of toludines.

TABLE 2 : Selectivity data for aryl amine isomeric pairs achieved by kromasil ODS

S. No.	Analyte	Selectivity
1	Xylidine	1.077
2	Chloroaniline	1.109
3	Aminobiphenyl	1.007
4	Trimethylamine	1.006
5	Napthylamine	1.004
6	Toludine	1.072

In all probability establishing its identity as 2-toludine was with element of doubt. Hence those two dyes were taken for isomeric confirmation by the proposed method by using Kromasil ODS column which resolved this pair of amines by close to 1min and helped us to establish the identity that it is nothing but 4-toludine in the cases of both these samples.

CONCLUSIONS

The separation of isomers of aryl amines plays an important role in the analysis. In this study we screened different reverse phase columns and also tried different gradient elution programs for mobile phases. The analysis carried out using Kromasil ODS column showed a better and complete separation of all the isomers taken for this study. The extent of separation was revealed by selectivity data shown that also supported these findings.

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