A Fast and Robust Analytical Method Based on QuEChERS Technique using UPLC- PDA for Quantification of Carcinogenic Arylamines in Consumer Goods inclusive of Leather

by

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Abstract

Arylamines present in leather and textile products are reduced in vivo by cleavage of azo groups to form highly mutagenic and carcinogenic products that pose consumer's health risk. The major textile and leather products that are offered to consumers need to be safe. In order to ensure safety as per the global regulations, the allowed limit of carcinogenic amines in textile and leather products cannot be more than 20 mg/kg. There are several methods that have been used for extracting the azo dyes and analyzing them in textile and leather products. Most of these methods are polluting from high solvent usage, time consuming, laborious, and have lower recovery rates. In the present study QuEChERS method, (dispersive solid phase extraction-dSPE) method was utilized for extraction of carcinogenic arylamines from dyed leather and textile products. The released arylamines are extracted into acetonitrile using QuEChERS salt and analyzed by Ultra Performance Liquid Chromatography-Photo Diode Array (UPLC-PDA). The extracted product was further confirmed by Gas Chromatography-Mass Spectrometer (GC-MS). The method proposed in this study is novel as it eliminates all significant concerns associated with the official ISO-17234-1 &14362-1 test methods, such as use of solvents like tert-butyl methyl ether (MTBE) and the long duration of analysis. The sample preparation time is minimized from 120 min to 20 min. The developed method is easy to adopt with efficient recovery rate of arylamines in comparison to the official ISO method.

Introduction

Azo dyes are the primary class of dyes, which find extensive usage in various products like food, garments, cosmetics, drugs, leather and toys. Arylamines are chemically synthesized and find extensive usage in pesticides, fungicides, explosives, adhesives and hair dyes. Arylamines are generated from azo dyes by azo reduction (bioreduction) in skin, intestine, environment by many of the microbes. Azo reductase present in human skin converts the azo dyes.[1-4] Most of the arylamines are considered carcinogenic, mutagenic or known to induce malformations in the human body. The European Union regulation has identified 24 different arylamines, which are extremely dangerous for human health. These are banned by EU regulation authority.[5-8] The hazardous nature of these arylamines has already been reported.[1-4]

Various studies have been carried out for determining arylamines in leather and textiles,[9-15] dyes,[16] toys,[17] water[18] and food.[19-20] The separation and estimation of arylamines is routinely performed by using High Performance Liquid Chromatography (HPLC) with a wide variety of detectors which include UV,[15,18] DAD,[13,16,17,21] electrochemical detectors[22] and mass spectroscopic techniques used in both HPLC and GC.[14,19,23-25] Apart from these techniques, Capillary Electrophoresis,[20,21] UV-VIS Spectrophotometer [26] and TLC [27-29] are also used to identify the arylamines.

Several methods have been reported for the release of arylamines from dyes by the use of chemical [13,14,28] biological[16] and microwave reduction.[13] The released arylamines have been extracted by several techniques such as Liquid-liquid Extraction (LLE),[22,28,30] Supercritical Fluid Extraction (SFE),[13] Microwave-assisted Extraction (MAE)[13] and Solid-phase Extraction (SPE).[14,21-23,31,32] The standard testing procedure [33-34] for detection of arylamines is based on chemical reduction by heating the samples followed by column purification.

The dispersive SPE also known as QuEChERS[35-38] technique is commonly used in food testing for screening pesticides, antibiotics, polynuclear aromatic hydrocarbon (PAH), etc. Commonly used test methods are the unbuffered method, EN 15662, and AOAC 2007.07. The same approach has been used to extract arylamines from leather and textile matrix in the present study.

The QuEChERS method is an effective method for removal of matrix constituents. The QuEChERS method allows for preparation of samples for analysis from a wide range of compounds, as it is not analyte specific. The QuEChERS method achieves a clean matrix by eliminating numerous matrix components while ensuring the analyte enrichment. The method is highly suitable for analysis of solids, semi-solids, viscous liquid mixtures, and liquid samples. The QuEChERS method reduces the usage of hazardous solvents and thus cuts down the cost. At the same time, analyte enrichment is attained with a much better clean-up of the samples. Magnesium

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Manuscript received July 22, 2022, accepted for publication October 7, 2022.

sulphate ($MgSO_4$) and sodium acetate (CH3COONa) salts increase the ionic strength of the aqueous mixture and induces phase separation with acetonitrile this is called the salting out effect. The salting out effect enriches analyte during the extraction procedure. The mixture is shaken in order to provide better extraction. The phase separation of the organic and aqueous phase is well achieved by centrifugation thus allowing the easy sub-sampling of the extract.

The EN ISO 17234-1[33-34] method is the standard method for analysis of azo dyes. The method utilizes MTBE, which is a hazardous solvent and a significant polluter of the environment. The methods also involve heating of arylamines to 70°C. This could result in generation of artifacts. The recovery of arylamines by official method does not exceed 25% for most of the arylamines. To address these snags a simple, rapid and effective technique for estimating arylamines from consumer goods like leather and textile matrices using dispersive SPE technique was developed. This technique was widely used for extraction of pesticides from fruits and vegetables. The present study adopts a novel approach for reduction of azo dyes. In the present method, use of MTBE is eliminated and the heating step of arylamines to 70°C is avoided. Thus, the present method avoids the formation of any artifacts. This study has reduced the extraction process time to 20 minutes. The developed method thus helps in ensuring the integrity and recovery of the analytes and eliminating any matrix interference during extraction. This is a much easier method than the previously proposed SPE method.[39]

Experimental

Chemicals and reagents

The various arylamine compounds, which were used as reference for the study, were obtained from Sigma - Aldrich Corporation, St. Louis, MO, USA. The list of arylamine reference compounds obtained include 4-aminobiphenyl, benzidine, 4-chloro-o-Toluidine, 2-naphthylamine, p-chloraniline, 2,4-diaminoanisol, 4,4'–diaminodiphenylmethane, 3,3'-dichlorbenzidine,3,3'-dimethoxybenzidine,3,3'-dimethylbenzidine, 3,3'-dimethyl-4,4'- diaminodiphenylmethane, p-cresidine, 4,4'-methylen-bis-(2-chloraniline), 4,4'-oxydianiline, 4,4'-o-toluidine, 2,4-toluylendiamine, 2,4,5-trimethylaniline, o-anisidine, 2,4-xylidine, 2,6-xylidine and p-phenylenediamine and clean-up materials of Supelclean LC-18, particle size-45 µm (C18 SPE). Another reference material 4,4'-thiodianiline was procured from Acros organics, Geel, Belgium. Analytical grade sodium dithionite was procured from Merck KGaA, Frankfurter Str, Darmstadt, Germany. Analytical grade magnesium sulphate, sodium acetate, sodium chloride (NaCl), sodium hydroxide, citric acid, ammonia and HPLC grade acetonitrile were procured from Avantor Performance Materials, Thane, Maharashtra, India. Tert-butyl methyl ether was procured from Merck Life science, Vikholi (East), Mumbai, India

Instrumentation

Ultra-Pressure Liquid Chromatography- Photo Diode Array (UPLC-PDA) used for analysis was procured from Waters Corporation, Milford, Massachusetts, USA. The instrument was equipped with AQUITY 'H'class quaternary solvent manager, AQUITY – FTN sample manager and an AQUITY eλPDA detector. Raw data from the instrument was analyzed using the Empower 3 software version 3.0 from Waters Corporation, Milford, Massachusetts, USA. Purospher STAR RP18e column was used to separate arylamines. The column included an integrated guard column with dimensions of 125 mm x 3 mm, particle-size of 5.0-micron. The column was purchased from Merck KGaA, Frankfurter Str, Darmstadt, Germany. Water used for Mobile-phase and other applications was produced in the laboratory using Type I grade water purifier. The water purifier was a Flex 3 model purchased from ELGA, Lane End, High Wycombe, U.K. The Gas chromatography-Mass spectrometric (GC-MS) operations were performed using Agilent Technologies, Stevens Creek Blvd, Santa Clara, United States. The GC model used was 7890A equipped with 7823B auto sampler and MS model used was 5975 C. DB -5MS analytical column was used for various operations. The dimensions of the column were 30 m × 0.25 mm with a film thickness of 0.25 µm. The carrier gas used for the study was helium which was 99.99% pure and pumped at a rate of 1 mL min^{-1}. Ultrasonication was performed using Ultrasonicator Model LBS2-10 purchased form FALC Instruments, G. M. Compagnoni, Treviglio BG, Italy. The operating temperature of the instrument was up to 50˚C with operating frequencies of 40 Hz and 60 Hz. Centrifuge model Z366 from Hermle Labortechnik, Siemensstraße, Wehingen, Germany was used for various centrifugation operations. Sample grinding was done using a mill obtained from Fritsch, Idar-Oberstein, Germany. PTFE filter was procured from Pall Corporation Port Washington, NY, USA.

List of banned arylamines

A complete list of banned arylamines includes 4-aminobiphenyl (ABP), benzidine(BEN), 4-chloro-o-toluidine(COT), 2-naphthylamine(NAP), 2,4-toluylendiamine (DAT), O-aminoazotoluene and 2-amino-4-nitrotoluene are further reduced to OTD or DAT, p-chloraniline (PCA), 2,4-diaminoanisol (DAA), 4,4-diaminodiphenylmethane (DDM), 3,3'-dichlorbenzidine (DCB), 3,3'-dimethoxybenzidine (DOB), 3,3'-dimethylbenzidine (DMB), 3,3'-dimethyl-4,4'-diaminodiphenylmethane (DDD), p-cresidine (KRE), 4,4'-methylen-bis-(2-chloraniline) (MOCA), 4,4'-oxydianiline (DDE), 4,4'-thiodianiline (TDA), o-toluidine (OTD), 2,4,5-trimethylaniline (TMA), o-anisidine (MOA), 2,4-xylidine (24XD), 2,6-xylidine (26XD), 4-aminoazobenzene is converted to p-phenylenediamine (PPDA) and aniline(ANI).

Preparation of reference standard for analysis

The various banned arylamines were used as reference standards. Stock of 1000 µg/ml was prepared for each amine in acetonitrile. The twenty-four banned arylamines were grouped as four different mixes using acetonitrile. The concentration of each arylamine in the mix was 15 µg/ml. These mixes were used as reference standards in UPLC-PDA. However, for GC-MS 5 µg/ml of reference standard was injected to confirm arylamine detected in the sample.

The official procedure, ISO 17234-1 and ISO 14362-1 for extraction of arylamines

Leather and natural textile samples were ground using a laboratory mill such that the final particle size was 100 to 200 microns. For samples that could not be ground to such a small size, they were cut into small pieces of approx. 1 to 2 mm. One gram of leather or textile samples were weighed and were degreased with about 20 ml of hexane using an ultrasonic bath at 40˚C for 20 min with frequency of 40 Hz. After repeating the degreasing step once again, the sample was left to dry in air overnight. Textile samples are directly taken for extraction. The sample was then transferred to a 50 ml amber-colored glass bottle with a tight closure bearing a silicone septum at the top. A quantity of 17 ml of 0.06 M Citric acid buffer (pH 6.0) was added to the bottle and the contents were heated to 70°C over a sand bath in a hot air oven. Once the temperature was stable, 1.5 ml of aqueous sodium dithionite (200 mg/ml) was added to the bottle using a syringe and kept at 70°C for 10 min. Another 1.5 ml was added similarly and maintained at 70°C temperature for 10 more minutes. Then the contents were cooled to room temperature within two min using running water.

The reaction solution was decanted on an Extrelut column. The column was made of diatomaceous earth. The absorption step was performed for 15 min. To the residual mixture in the bottle 5 ml of MTBE and 1ml of 20% methanolic NaOH was added. The mixture was shaken vigorously, and the solution was transferred to the Extrelut column. The residual mixture in the reaction bottle was washed using 15 ml of MTBE following an additional wash with 20 ml of MTBE. The contents were transferred to the Extrelut column to start eluting the amines. Afterward, 40 ml of MTBE was flushed on the column directly. The eluate was collected in a 100 ml rotary evaporator flask. The ether extract was concentrated to 1 ml by rotary vacuum evaporator using low vacuum at a temperature not more than 50°C. The remaining ether was evaporated to dryness by an inert gas like Nitrogen. Immediately the residue was dissolved with methanol and volume made up in a 2 ml volumetric flask. This solution was filtered through a 0.2 µm PTFE filter for further chromatography analysis.

In case of man-made textile due to the presence of disperse dye, the arylamines were extracted with xylene. After extraction, xylene was evaporated, and the residue was further processed as explained above.

Chromatographic Condition

UPLC-PDA condition followed for the arylamine separation

Water (pH 8, adjusted with ammonia) and acetonitrile were used as mobile phases to separate the arylamines with the following gradient program: The system's total flow rate was 0.5 ml per minute. Gradient at 60% of water and minimised down to 20% in 5 min, further minimised to 0% at 5.5 min, then increased to 60% in 6 min, held for 8 min equilibration. The injection volume of the sample was 1 µl. The detector›s wavelength range used for collecting the spectrum of analytes was 220 nm to 400 nm. The chromatograms collected at 230 nm, 280 nm and 305 nm.

GC-MS condition followed for the arylamine confirmation

The temperature programme for GC was as follows: initial temperature, 70°C held for 2 min, linearity to 280°C at 10°C/min, held for 7 min. The injector temperature was 250°C, and injection of 1 µL sample was performed in split less mode. The interphase between GC and MS was maintained at 280°C. The quadrupole and source temperature were maintained at 230°C and 250°C respectively. The analyte was analyzed on the GC-MS in the full scan mode; quantification was carried out by Selective Ion Monitoring (SIM) mode using specific ions as shown in Table I. Mass spectra were obtained in electron impact mode (70eV) in the range between 45 and 300 m/z. The total GC-MS analysis time was about 30 min.

Table I
The list of specific mass ions (SIM) of arylamines for GCMS analysis

Arylamines	SIM (amu)	Arylamines	SIM (amu)
4-aminobiphenyl	169	p-cresidine	122
Benzidine	184	4,4'-methylene-bis-(2-chloroaniline)	231
4-chloro-toluidine	141	4,4'-oxydianiline	200
2-naphthylamine	143	4,4'-thiodianiline	216
o-aminoazotoluene[a]	225	o-toluidine	106
4-nitro-toluidine[a]	152	4-methyl-m-phenylenediamine	121
4-chloroaniline	127	2,4,5-trimethylanilne	120
4-methoxy-m-phenylenediamine	123	o-anisidine	108
4,4'-diaminodiphenylmethane	198	4-aminoazobenzene	92
3,3'-dichlorobenzidine	252	2,4-xylidine	121
3,3'-dimethoxybenzidine	244	2,6-xylidine	121
3,3'-dimethylbenzidine	212	1,4-phenylenediamine	108
4,4'-methylenedi-o-toluidine	226	Aniline	93

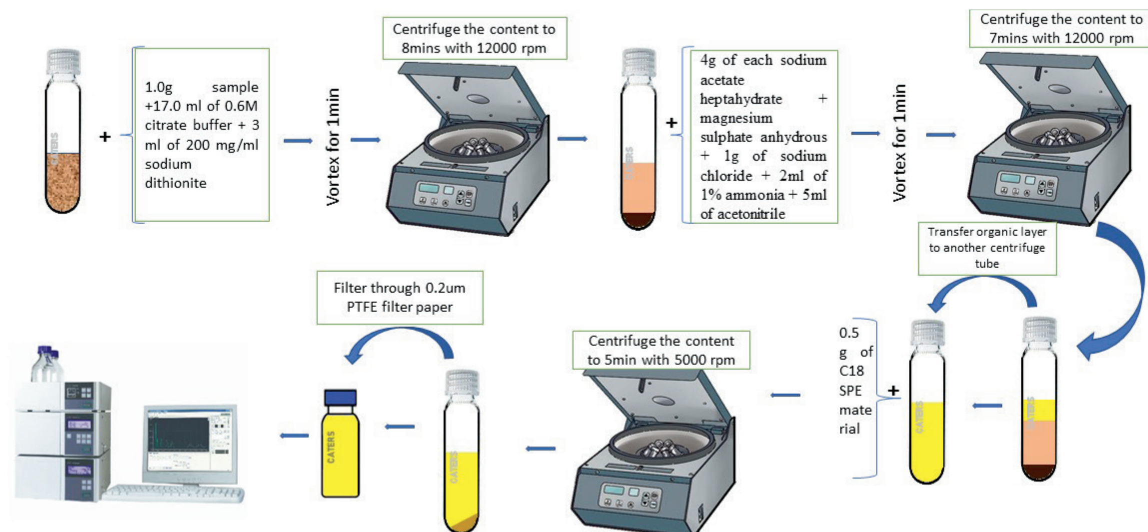

Figure 1. Schematic diagram of proposed method

Sample preparation of the proposed QuEChERS method

The initial sample was cut into small size of about 2 to 3 square mm. As directed in the official method, leather samples were degreased while textile sample were proceeded with as such. One gram of sample was weighed into a 50ml centrifuge tube with a lid. To the tube 17.0 ml of 0.6 M citrate buffer, 600 mg of sodium dithionite was added. The mixture was vortexed for 1 min and centrifuged for 8 min at 12,000 rpm. Then 4 g each of CH3COONa and $MgSO_4$, 1 g of NaCl, 2 ml of 1% ammonia, along with 5 ml of acetonitrile were added and lid closed. The tube with contents was vortexed for 1 min. The contents were centrifuged at 12,000 rpm for 7 min. Then, the top acetonitrile layer was transferred to another centrifuge tube with 0.5 g of $MgSO_4$ and 0.5 g of clean-up C18 SPE materials. Centrifugation was done at 5000 rpm for 5 min, and then the organic layer (acetonitrile) was collected and filtered through a 0.2 µm PTFE filter. The filtrate was subjected to chromatography analysis. For man-made textiles, the sample was extracted with xylene and after evaporation of the residue it was further processed using the proposed method (Figure 1).

Results and Discussion

In-situ reduction

It is the first ever study for the reduction of dyes by chemical means under room temperature conditions with help of centrifugal force. When hot condition reduction of dyes is done the release of unrealistic, and artifacts of chemical compounds are suspected. Whereas the ambient reduction is close to realistic release.

QuEChERS for analysis of arylamines

Magnesium sulphate concentration when varied from 4g to 6g, it was observed that the recovery of arylamines varied from 45% to 60%. In case of highly polar analytes such as PPDA and DAA resulted in a lowered recovery of 19.9% and 24.8%. Hence, it was planned to increase the ionic strength by adding CH3COONa at varying concentrations from 1 g to 4 g, to increase recovery of arylamines. The recovery of arylamines improved in the range of 90% to 106% (Figure 2) when 4 g of MgSO4 and 4 g of CH3COONa were used at pH 8.0 to 9.0. Magnesium sulphate and sodium acetate were used for liquid-liquid extraction of arylamines in the solvent system of acetonitrile and water. Sodium chloride was added to increase the ionic strength further and mainly to serve as emulsion breaker if any. The C18 SPE materials was added in the system to remove fatty compounds, sterols, and other non-polar interferences like hydrophobic compounds. In order to further refine the study, the effect pH on the extraction of analytes was assessed.

Effect of pH on recovery of arylamines

For further study, eight arylamines were identified in the range of highly polar to nonpolar. These include PPDA, DAT, BEN, DDM, PCA, DDD, 4ABP and DCB. At pH below 6.0, which is acidic in nature, arylamines form ionic ammonium salts. The ammonium salts have a low solubility in organic solvents. The phase transfer of ionic forms of these analytes to acetonitrile from an aqueous solution becomes difficult. This affects the recovery rate of arylamines as recoveries were found to be less than 60%. At pH 8-9 which is alkaline in nature, the arylamines are neutral. This facilitates the transfer of arylamines from the aqueous phase to the organic phase. The recovery of all the analytes improved to be more than 80% at this alkaline pH. (Figure 3).

Optimization of Centrifugal speed for extraction and recovery of arylamines

The centrifugal force plays a vital role in transferring arylamines from the aqueous layer to the organic layer. An intricate study to

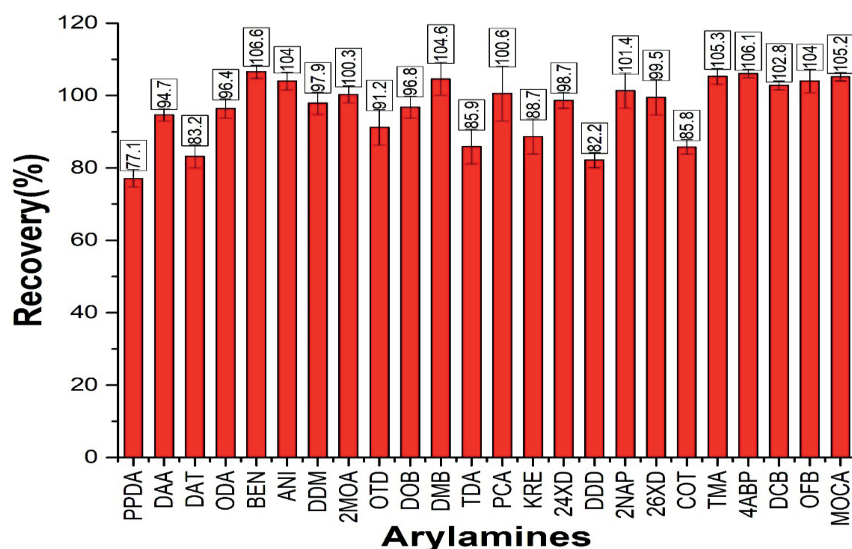

Figure 2. Recovery of complete arylamines

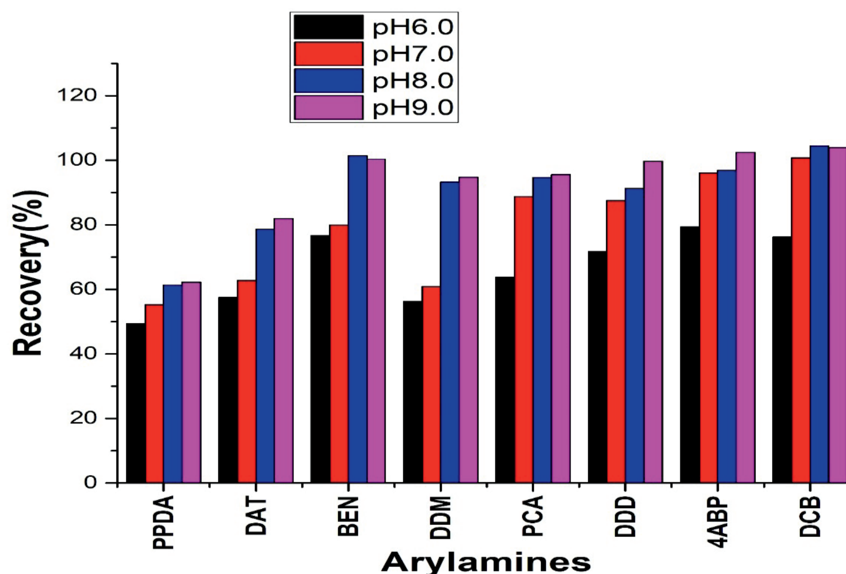

Figure 3. Recovery of arylamines by various pH

monitor its influence on the recovery rate at varying speeds was done. Centrifugal speed was varied from 5000 rpm to 12,000 rpm. At 5000 rpm, the effective transfer of arylamines was less because the recoveries of many arylamines were less than 60%. But at 12,000 rpm, the recoveries were more than 90%. When the speed of centrifugation was increased above 12,000 rpm it did not show any considerable further change in the recovery of arylamines. (Figure 4). The enhanced recovery at higher speeds is mainly associated with effective separation of the organic layer which helps in better recovery of the analyte.

Optimization of centrifugal time for extraction and recovery of arylamines

This study focused on the optimal time required for the centrifugal force to accelerate the transfer of arylamines from the aqueous to organic layer at 12,000 rpm. Centrifugation for shorter durations such as 5 minutes resulted in lower recovery rates of 14% to 68%. However, when centrifugation was done for 15 to 20 minutes the recovery rates were found to be 80% to 104%. (Figure 5). Longer periods of centrifugation enhanced the extraction of arylamines into the acetonitrile phase resulting in better recoveries of all the analytes.

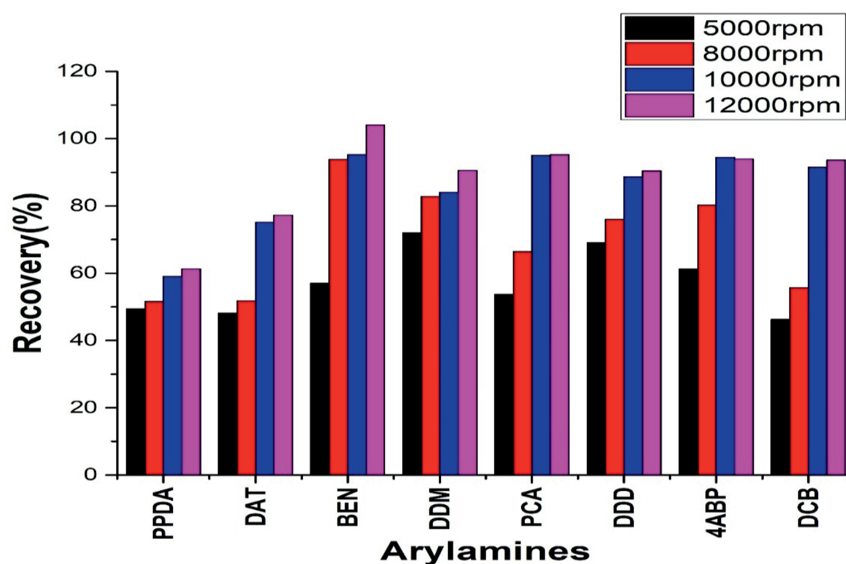

Figure 4. Recovery of arylamines by various centrifugal speed

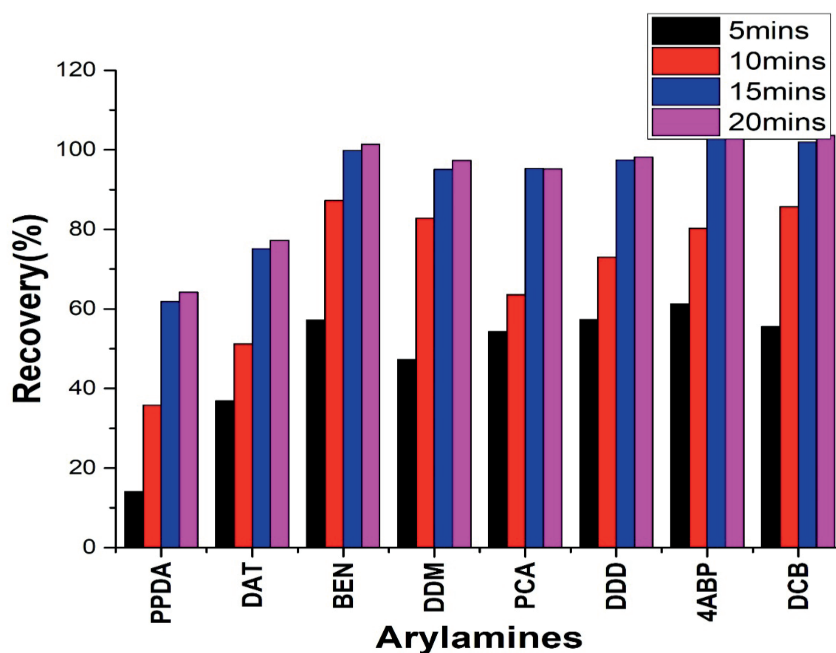

Figure 5. Recovery of arylamines by various centrifugal time

Validation of the method

The performance characteristics of the method such as Limit of Detection (LOD), Limit of Quantification (LOQ), Linearity, Accuracy, Method precision, Reproducibility and Robustness were studied. LOD of the proposed method was found to be between 1.2 to 2.0 ppm for all arylamines. LOD was determined by injecting the analyte at 10 times lower concentration than normal. Based on the values obtained from the experiment the standard deviation was calculated. LOD was determined by multiplying the standard deviation by 3. LOQ was determined by multiplying the standard deviation by 5. LOQ of the method for all arylamines varied from 2.0 to 3.4 ppm. Linearity ranging from 1 to 15 ppm for 24 arylamines mixture was performed. The experiment showed a correlation coefficient (R^2) ranging from 0.9914 to 0.9997. Accuracy of the method was measured with the matrix blanks or test samples un-spiked and spiked with the analyte of interest over a range of concentrations. Each level of concentration (i.e. 30% of LOQ, 100% of LOQ and 150% of LOQ) was injected three times. The difference between mean spiked value X and mean value XI with the added concentration X spike was compared. The relative spike recoveries for accuracy and precision at various concentrations i.e., 30% LOQ, 100% LOQ and 150% of LOQ were calculated.

Acceptance criterion for accuracy was within the range of 80-120% and precision was within range of ≤ 20.0%. The robustness of the method was within range of ≤ 20.0% (Table II).

Table II

Method validation data for proposed method

Parameter		Arylamines	Acceptance criteria
LOD	ppm	0.4 – 0.6	—
LOQ	ppm	0.7 – 0.9	—
LINEARITY	R^2	0.9914 -0.9997	≤ 0.990
ACCURACY 30% LESS LOQ	Recovery (%)	81.1 – 101.1	80 – 120%
ACCURACY 100% LOQ		92.0 – 101.3	80 - 120%
ACCURACY 150% LOQ		91.6 – 100.7	80 – 120%
PRECISION 30% LESS LOQ	RSD (%)	4.3 – 9.6	≤ 20.0%
PRECISION 100% LOQ		4.3 – 8.5	≤ 20.0%
PRECISION 150% LOQ		3.9 – 10.6	≤ 20.0%
METHOD PRECISION		3.7 – 8.9	≤ 20.0%
ROBUSTNESS		3.7 – 8.9	≤ 20.0%

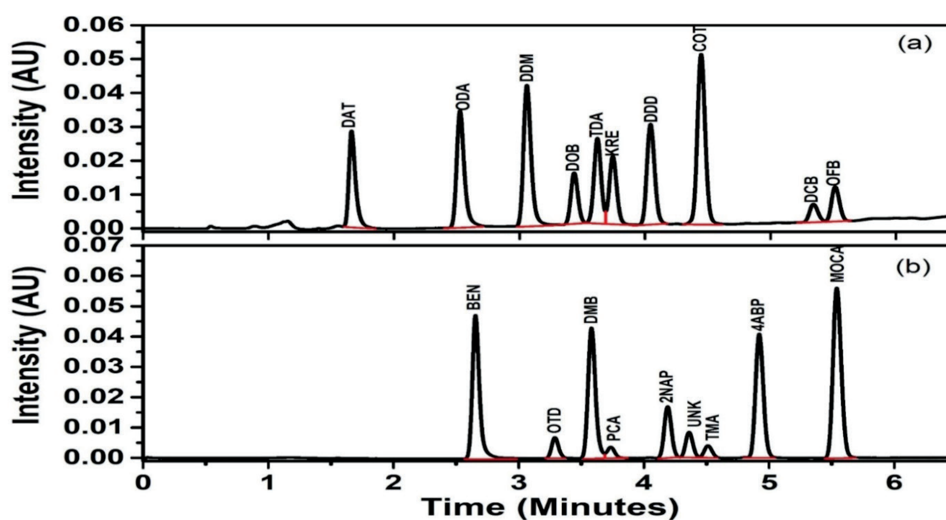

Figure 6. Chromatogram for arylamines reference standards **(a)** MIX-1 **(b)** MIX-2

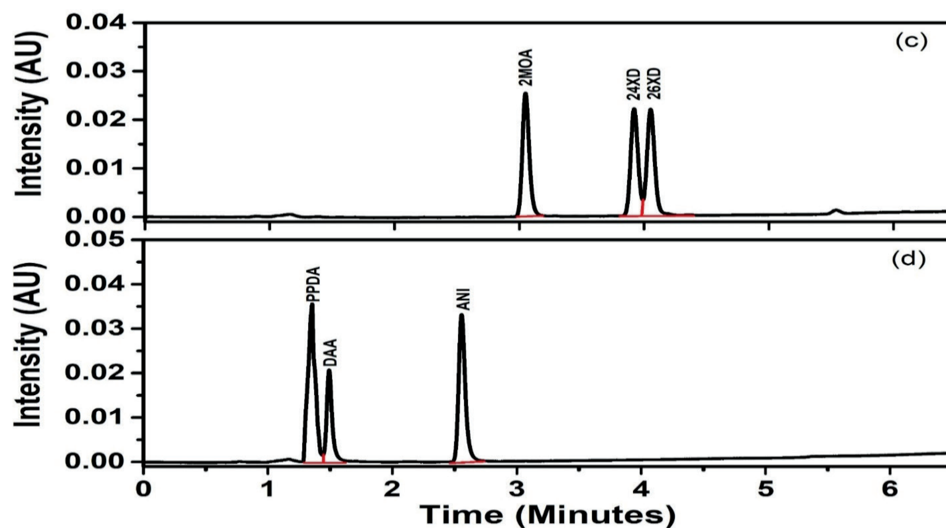

Figure 7. Chromatogram for arylamines reference standards **(c)** MIX-3 **(d)** MIX-4

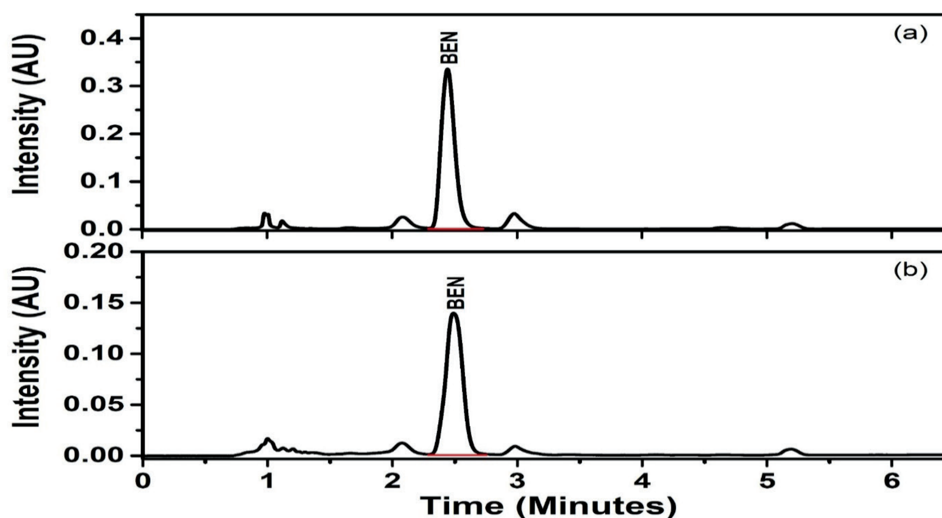

Figure 8. Chromatogram for real samples analysed in both **(a)** Official method **(b)** Proposed method for comparison.

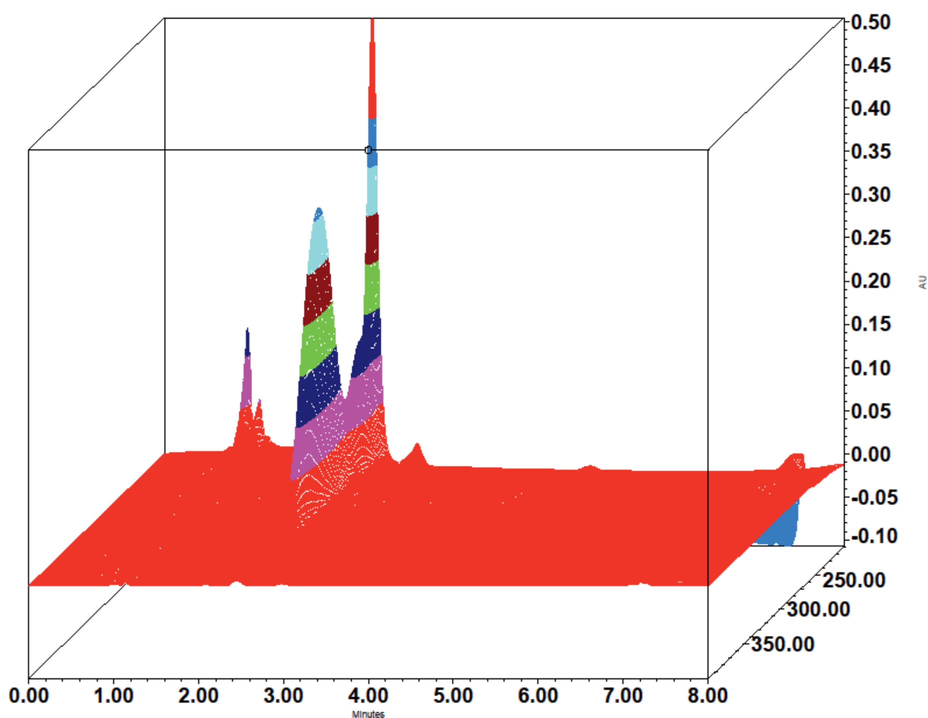

Figure 9. 3D spectral view of the real time sample analysis in PDA

Analysis of Real samples

Several leather, textile and dye samples were screened by the proposed method UPLC-PDA (Figure 6, 7, 8 & 9) and further confirmed by GC-MS technique (Figure 10). Samples of leather, textile and dye, which were originally found to contain the banned arylamines benzidine (by the official protocol), were chosen for the comparison with the proposed method. Benzidine %RSD found in leather, textile and dye in the proposed method is shown in Table III. The %RSD obtained from benzidine by the proposed method was found to be well within the acceptable limits.

Discussion

QuEChERS method was primarily developed for detection of pesticides in food materials. The method is simple and easy to adapt. This proposed method has minimised solvent usage and is a green analytical method for arylamines. Although earlier, a SPE method was reported[39] with much improved recoveries for most of the arylamines in the banned list, that method claimed to be successful and used the same reduction of azo dyes at hot condition as that of the official method. Compared to that study, present approach

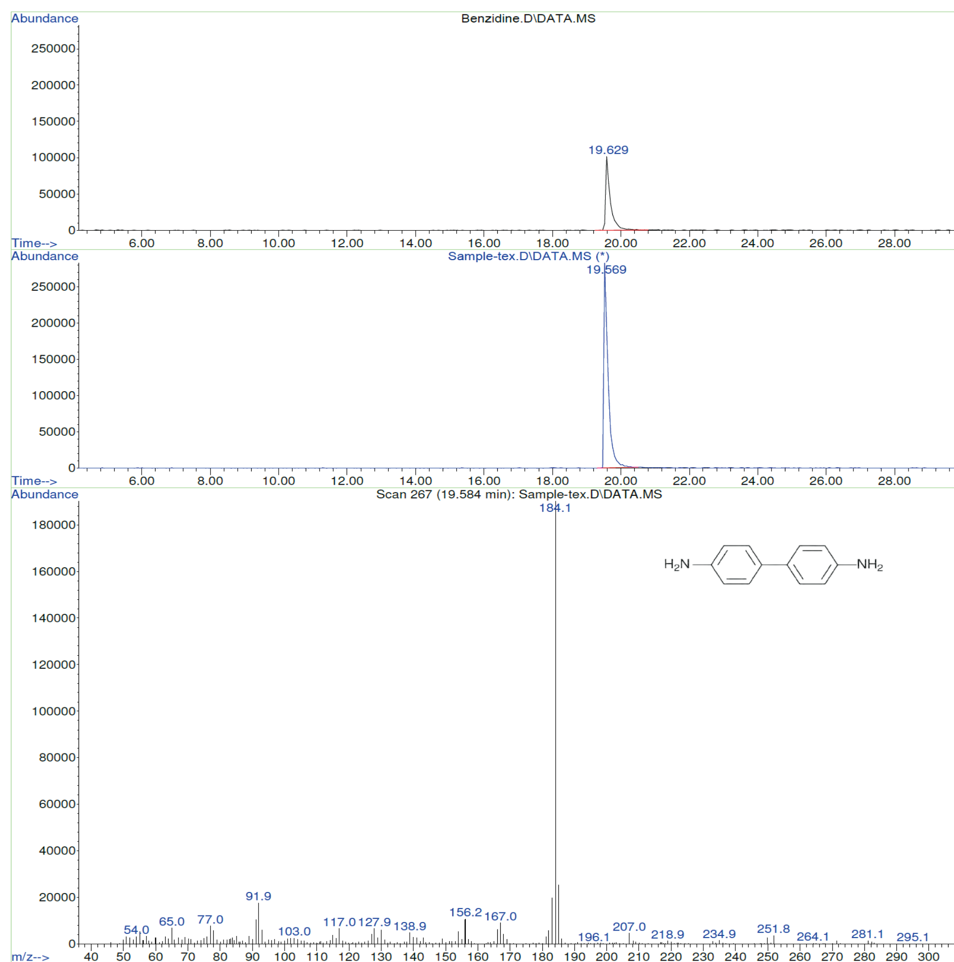

Figure 10. Chromatogram and mass spectrum for real time sample analysis in GCMS

Table III

Real sample analysis data for leather, textile and dyes by the proposed method with RSD

Material	Benzidine (mg/kg)		RSD (%)
	Official method	Proposed method	
Leather	126.4	128.4	5.3
Textiles	613.3	642.6	3.8
Dye	2161.4	2160.7	2.7

followed a reduction step in ambient condition and secondly the solvent extraction is almost simultaneous along with the release of the arylamines. Many of these arylamines are suspected to be unstable and lost especially when the procedure steps are long and spaced with long intervals for successive steps. The proposed work is also greener approach as use of MTBE from 80 ml in the official method is completely eliminated. This proposed procedure is very speedy, requiring only 20 minutes versus 180 minutes for the official method. More importantly, all the analyte recoveries improved in the range 80% to 108% while the official method claimed recoveries from 20% to 70%.

Conclusion

This proposed method is considered to substitute all tedious parts of the analysis namely the exchange of the analytes into a solvent layer. A great enrichment of the analytes by introducing the QuEChERS method was observed with matrices of samples found to be cleaner as observed from those cases of long official procedures involving multiple steps in its approach. The solvent reduction happened from 80 ml to 5 ml. Multiple steps were consolidated into two short simple steps, which helped to gain time in sample preparation alongside minimization of transfer loss from

considerable change over the vessels. This proposed procedure for arylamines can determine the range of 7-2500 mg/kg of consumer goods comprising leather, textile and dye /pigments. This proposed method ensured a greener approach, ensures good recoveries (80% to 108%), ambient reduction for releasing arylamines, relatively speedy approach for sample preparation (from 180 minutes to 20 minutes) along with advantage of cutting out the transfer loss as proceeded with only 2 vessels.

Acknowledgement

This research work was financially supported by CSIR-Central Leather Research Institute (CSIR-CLRI- MLP-06). CLRI communication number: 1603.

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