ECO-FRIENDLY, RAPID AND EFFICIENT ANALYTICAL PROCEDURE FOR CARCINOGENIC ARYL AMINES IN DYES AND CONSUMER PRODUCTS

by
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ABSTRACT

This study addressed the analysis of aromatic amines from azo dyes and dyed consumer articles mainly based on leather as they are subjected to screening for release of any of the 24 hazardous aryl amines listed by the European agency. This study involves a reverse phase SPE approach for enriching, and cleaning-up of the matrices to facilitate further analysis by UPLC. The proposed study does away with tertiary butyl ether, other solvents and subsequent evaporation steps thereby cutting down the solvent usage to a major extent (from close to 80ml into 2ml) in sample preparation and the further analysis developed by UPLC, cut short the gradient analysis from 40 min to 6 min enabling additionally the reduction of solvent for mobile phase. Thus the procedure is improvised to serve as Green analytical procedure. The present study offers the scope to complete the sample preparation in 45 min in comparison to 180min by the official procedure (excluding the degreasing step in both these cases). The proposed procedure is simple to adopt with more efficient recovery values in the range 52-104% for all the analytes. The LOD and LOQ of the analysis were 2μg g $^{-1}$ and 9μg g $^{-1}$.

INTRODUCTION

Aryl amines are precursors of azo dyes which constitute the major class of dyes extensively used in various products including foods, drugs, cosmetics, consumer wear consisting of natural and synthetic textiles, leather and toys and this is aside, aryl amines are used directly in adhesives. Some scores of aryl amines were classified as hazardous and carcinogenic[1-4] and about 24 different aryl amines were listed by EU regulation as banned.[5-7] The various hazardous nature and exposure routes were reported.[3-4] Studies led to the findings that enzymes like azo reductase that is abundant in our skin, intestine etc. splits the azo dyes into its basic aromatic amine constituents and such bio release sets up serious health threats including cancer.[1-4] Several studies were reported for the analysis of aryl amines in water,[8] dyes,[9] food,[10,20] toys[11] and consumer goods of textiles and leather.[12-18] HPLC was extensively used for the determination of aryl amines by employing different types of detectors including UV,[8,18] DAD,[9,11,16,30] electrochemical[19] and in recent trend mass spectrometry;[10,17] however, usage of other types of analytical techniques like CE,[20,30] TLC,[21-23] UV-VIS spectrophotometry,[24] GC-MS[25-27] were also reported. The analysis of solid samples involves extraction of dyes followed by release of aryl amines by chemical reduction and collection of the analytes into a solvent layer.

Studies reported on release of aryl amine from dyes included chemical reduction,[16,17,22] biological reduction[9] and even microwave reduction[16] and the official protocol for azo dyes testing is based on chemical reduction by conventional heating of samples. Then for the subsequent extraction of aryl amines into a solvent phase, techniques like LLE[19, 22, 28] (liquid-liquid extraction), SPE [17,19,25,29,30](solid-phase extraction), SFE[16] (super-critical fluid extraction), MAE[16] (microwave-assisted extraction) were all attempted but found to fall short in one or more aspects like recoveries of the aryl amines or the considerable use of solvents or simplicity in approach. LLE is a solvent-intensive technique with subsequent concentration steps necessitated.

In an attempt to develop a green analytical method, Eskillson *et al.* studied sample preparations based on Microwave and SFE[16] still with significant use of solvents for only a marginal improvement in recoveries. In that pursuit to do a method without using any solvent, pyrolysis[27] was also reported. But one major concern for heating aryl amines is the integrity of the analytes. SPE and SPME (solid –phase micro extraction) were found to be green analytical techniques as they help to cut down solvents significantly while also achieving enrichment and clean up of analytes. The main issue in developing a SPE based method is its varying break-through volumes for different nature of analytes and there is a need to study before hand for a particular chemistry of SPE its break through volume (The breakthrough volume is defined as the maximum volume of sample which can be passed through a

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Manuscript received May 13,2015, accepted for publication August 29, 2015.

SPE cartridge ensuring 100% recovery of an analyte) for an analyte for that liquid medium, its pH and other conditions as well. But it serves as a green technique and also can avoid concentration steps normally followed further, while SPME is good for only very traces as it attains saturation for higher concentration and even in the case of SPME there are different chemistries and also SPME, for its cost invariably the fiber is recycled and that may run into a risk of memory effects apart from the impact of progressive deterioration of the polymeric fiber. This is apart thermally desorbing some analytes may harm the integrity of the analytes like aryl amines of low molecular weights. SPE technique is employed for aryl amines and those claimed with almost low or free of solvent usage in many studies reported earlier were the reverse phase SPE chemistries (RP-SPE) of C_{18}[19] or polymeric SPE[25, 29] and cation exchange.[17,30] SPME techniques were also reported[8,26] for aryl amines although only for water samples.

The official protocol DIN 53316[31] or the subsequently brought ISO 17234-1 or IUC /IULTCS 20-1[32] all are the same procedure. The official method of EU makes use of good amount of tert-butyl methyl ether and other solvents and also involves multi steps consuming time, chance for draining solvent as only a fraction is used for analysis and with steps like heating and nitrogen blow-down for concentration that might be deleterious to analytes and solid-waste generation through bulk use (in tens of grams) of SPE based on diatomaceous earth etc. and the nature of the official procedure involving large use of organic solvents was already highlighted.[12,16,17]

In order to improvise the method towards greener by minimizing the solvent driven steps, the present study was attempted. This study involved a RP-SPE (C_{18}) approach for sample preparation and concluding it by developing a fast LC analysis leading to the minimization of solvents to the extent of 90%. An earlier fast LC analysis was reported by M. Shelke et al.[19] for aryl amines but not all the banned aryl amines in this were included. Earlier, some studies have been carried out with same objective (of developing a greener procedure) by following SPE technique for aryl amines. In that pursuit, one study reported by Lavanderia was for consumer products of leather and textiles.[17] In that study, the requirement of 2 SPE materials used in sequence and also special instruction to invert the strongly non-polar SPE to elute analyte is a major limitation of the work. In contrast, a simple approach is made in this study with good recovery values for all the analytes with just one RP-SPE (C_{18}) that accomplished the recovery of aryl amines from aqueous medium.

In this proposed study we retained the same steps from degreasing to chemical reduction as followed in the official IULTCS method and the amines released after the chemical reduction were dealt by a modified approach that is an eco-friendly approach as it consumes only 2ml of acetonitrile solvent (as solvent is used only for the elution) while the official method required solvents of 5ml sodium methoxide, 75ml tert. butyl ether and 2ml methanol in various steps alongside a concentration step like rotary evaporation and nitrogen-blow down. All these solvents and a few procedural steps like evaporation or concentration are done away with, making this approach greener (excluding degreasing step as that step of the official IUC protocol is retained that involves hexane for the purpose) and also cutting down the sample preparation time from 180 min to 30 min (excluding degreasing and grinding steps). Also, the harsh exposure to high temperature of these sensitive analytes is dispensed with in this proposed method. Thus, this study is a Green and rapid yet effective procedure.

EXPERIMENTAL

Chemicals and Reagents

All aryl amine reference compounds of the following list: 4-Aminobiphenyl,Benzidine, 4- Chloro- o- Toluidine, 2- Naphtylamine, 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, p-Phenylenediamine; RP SPE bulk materials of Supelco LC-18 ,ENVI-18,ENVICHROME P (A polymeric RP) were obtained from Sigma-Aldrich Corporation, St. Louis, MO, USA. 4,4' Thiodianiline was procured from Across organics, Geel, Belgium. HPLC grade solvents of acetonitrile, methanol, methyl tertiary butyl ether, Extralut- a SPE material, analytical grade reagents of sodium dithionite were from Merck Kga, Darmstadt, Germany. All other reagents used were of analytical grade.

Instrumentation

The fast LC analysis was done by Ultra Pressure Liquid Chromatography of Waters with the following details: model AQUITY 'H'class quaternary, sample manager with PDA eλ detector driven by the software Empower Pro version were procured from Waters Instrument Corporation, Milford, USA. For HPLC analyses, a column cartridge of 125 x 3mm with 5.0-micron particles packing, Purospher STAR RP18e with integrated guard column of the same type procured from Merck, Darmstadt, Germany was used. Water for chromatography was prepared by using Millipore model Milli-Q Reference which was procured from Merck- EMD Millipore Corporation, Billerica, MA, USA. Ultrasonicator Model 275 was procured from CREST Ultrasonics, Trenton, NJ, USA. The model has provision to operate up to 50°C with thermostat and has fixed sonicating power of 90W on an average. "Fritsch" make Laboratory mill was from Fritsch, Idar-Oberstein, Germany.

UPLC Condition Followed for the Aryl Amine Separation:
A gradient mobile phase system consists of acetonitrile and water starting from 40% acetonitrile, linearly increased to 80% in 5 min as detailed in Table I. The total gradient program for separation was 5.5 min. Flow rate was kept at 0.5 ml min^{-1}. 1µl sample was injected for LC. Wavelengths of 240nm, 280nm and 305nm along with spectral collection were opted to study the analytes.

List of Banned Aryl Amines
4-Aminobiphenyl(ABP),Benzidine (BEN), 4- Chloro- o-Toluidine(COT), 2- Naphtylamine(NAP), 2,4-Toluylendiamine(DAT), o-Aminoazotoluene * is converted into DAT, 2-Amino -4- nitrotoluene* is converted into DAT, p-chloraniline (PCA), 2,4-Diaminoanisol(DAA), 4,4-Diamino diphenyl methane(DDM), 3,3 ' -Dichlorbenzidine (DCB), 3,3 '- Dimethoxybenzidine(DOB), 3,3'- Dimethyl benzidine(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).

Release of Aryl Amine from Dyed Articles
The first step was preparing the samples for test and that involved the extraction of dyes and releasing the aryl amines through chemical reduction step. A prior crucial preparatory test is micronising the sample and removing the grease so that aqueous extractant can penetrate the material. Until this stage the official procedure (ISO17234-1) was simply followed and without deviation. Hence, leather samples were reduced to approx. 100 to 200 microns using a laboratory mill (samples that could not be ground were cut into small pieces of approx. 1 to 2 mm^2). About 0.5g of leather/textile samples were weighed and at first subjected to degreasing with about 20ml of hexane using an ultra sonic bath at 40°C for 20min and after repeating the degreasing step once again the sample was left to dry in air overnight. The sample was transferred to a 50ml amber colored glass bottle with a tight closer bearing a silicone septum at the top. To this, citric acid buffer of 0.06 M at pH6.0 of about 17ml was added and the contents were heated to 70°C over a sand bath. Once the temperature was stable, 1.5 ml of aqueous sodium dithionite 20% was added with a syringe by piercing through silicone septum of the bottle. Then another 1.5 ml was added in a similar way and the contents were maintained at that temperature for 10 more minutes.

TABLE I
HPLC Gradient program for Arylamines separation.

Time	Water	Acetonitrile
Initial	60	40
5.0	20	80
5.5	0.0	100
6.0	60	40
8.0	60	40

SPE Extraction of Aryl Amines
This Step is the Beginning of the New Approach:
In continuation, the citric buffer solution in which the reduction of sample carried out with dithionite solution was cooled to room temperature and then adjusted to pH 9.0 with 10% ammonia or 10% sodium hydroxide aqueous solution. Then, the contents were transferred to RP-SPE column prepared using 0.5 g of ENVI-18 (RP-SPE) material in a syringe barrel with supports of Glass fiber filters of 1µm placed at the bottom and top of the packing. Earlier, the RP-SPE was conditioned with methanol then with 5 ml of 10% aqueous ammonia and then the RP-SPE was used immediately without allowing it turning dry. The contents were transferred to RP-SPE at a rate of 1.0 -1.5 ml min^{-1}. Once this total volume of 20ml of the contents passed through the RP-SPE, elution was done with acetonitrile of total 2ml in such way as 0.5ml × 4 times. The eluted solvent portions were collected in a 2ml volumetric flask and the volume reconstituted if required with acetonitrile. About 1µl of this is used for fast LC analysis

RESULTS AND DISCUSSION

Evaluation of Different SPE Materials for Recovery of Aryl Amines
ENVI-18, SUPELCO LC-18, ENVICHROME-P were all studied for the recovery of aryl amines. Of which, ENVI-18 produced the best recoveries for all the aryl amines (in the ban list taken for study). RP-SPE recovery of an analyte is dependent on the volume of aqueous sample taken for recovery of analyte apart from the compatible pH. For basic analytes like amines the pH should be alkaline so that the analytes are in neutral state to get enriched by C_{18} RP-SPE .To evaluate the RP-SPE materials, mixture of 22 aryl amines was added at 15µg (by adding 100µl of 150mgl^{-1} solution in methanol) to an aqueous solution of citric acid (0.06m) and dithionite (equivalent to 3ml of 20% solution) at the concentrations used in extraction and reduction stage and adjusting the solution to pH 9.0 with ammonia and the total volume of the contents was kept up at 20ml for carrying out SPE studies. This is to simulate the total aqueous volume of 17ml at the extraction

and reduction stage of the analysis which with washings gets amounted to 19ml and hence break-through studies for volume of 20ml was considered relevant for ENVI-18 RP-SPE for aryl amines. RP-SPE materials of SUPELCO LC-18, ENVICHROME-P could not obtain recoveries exceeding 60% for most of the aryl amines and mainly the polar aryl amines like PPDA, DAA suffered very badly as they could achieve only 20% with these SPE materials. Where as ENVI-18 RP-SPE gave recoveries at 80-100% for most of the amines and at around 75% in 2 cases and at 50-60% for PPDA and Aniline. Even the poor recoveries observed with PPDA and Aniline is not a set back as they are not in banned amines list and the presence of them only indicated the probable presence of Amino azo benzene that had to be done separately in alkaline reduction condition. The reason for using 15µg (for the sample mass of 0.5g of this proposed method) of analytes concentration was to check the recovery, matching the permissible /detection limit of aryl amines by the official procedure, that is 30mgkg^{-1}. The recovery data for the analytes from this study is furnished in Figure 1, which reveals the choice of ENVI-18 obviously.

Breakthrough Studies for Aryl Amines for SPE by ENVI-18

Mixture of reference substances of Aryl amines at appropriate volume equivalent to concentration of 15µg of each of the aryl amines was added to the aqueous matrix of citric acid (0.06m) and 3ml of 20% sodium dithionite and adjusted to pH 9.0 with aqueous ammonia was used for this study and such aqueous preparations of aryl amines in various volumes viz. 10ml, 15ml, 20ml and 25ml were used to determine the break through volume. Each of these volumes was separately passed through a fresh ENVI-18 cartridge prepared and conditioned as detailed earlier. The solutions of these analytes were transferred to SPE at a flow rate of 1-1.5ml. As mentioned earlier the elution was done with acetonitrile of total 2ml (0.5ml x 4). The recoveries from different volumes were plotted which revealed that even up to 25ml, recoveries were acceptable for even highly polar molecules like DAT, DAA while for relatively non polar aryl amines the break through volumes are not at all a threat as expected. This study confirmed that the total volume of 17 ml of the sample at chemical reduction step is quite a comfortable volume to achieve recovery of all the aryl amines. Figure 2 provided the trend observed with different aryl amines when passed through ENVI-18 SPE cartridge from varying volumes of aqueous contents. The case of DAT represented the high polar type, BEN represented mid way non-polar and ABP gave idea about the highly non-polar category of these analytes. From these studies, ENVI-18 is found to be a good chemistry for all these aryl amines.

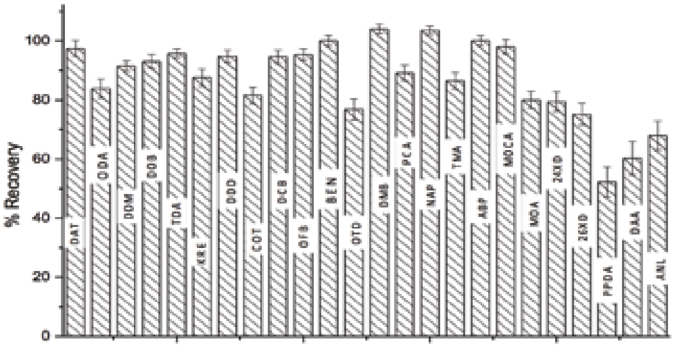

Figure 1. Recovery data for all the banned aryl amines taken for study by way of spiking the analytes (15μg/20ml) in the sample matrix and passing through ENVI-18 SPE.

Capacity of ENVI-18 SPE for Aryl Amines

The mixture of aryl amine standards spiked into an aqueous solution of citric acid (0.06m) and dithionite (equivalent to 3ml of 20% solution) and turned to pH 9.0 with ammonia constituted a total volume of 20ml. The spikes were done at different concentrations ranging from 5 to 250µg in 20ml of such aqueous solutions in each separate case were studied to ascertain the capacity of ENVI-18 SPE. This study led to the finding that 150µg /20ml could be recovered satisfactorily using ENVI-18 and the sample size of 0.5g. The total volume 20ml is inclusive of washings that is passed through the ENVI-18 SPE and this total 20ml carries the analytes derived from 0.5g of the experimental sample and hence this indicates that aryl amines could be quantified easily up to 300mgkg^{-1} of leather if 0.5g is used as sample mass. In case of results found higher than 300mgkg^{-1} then to ascertain the exact value, sample mass of lower side like 0.1-0.25g can be employed.

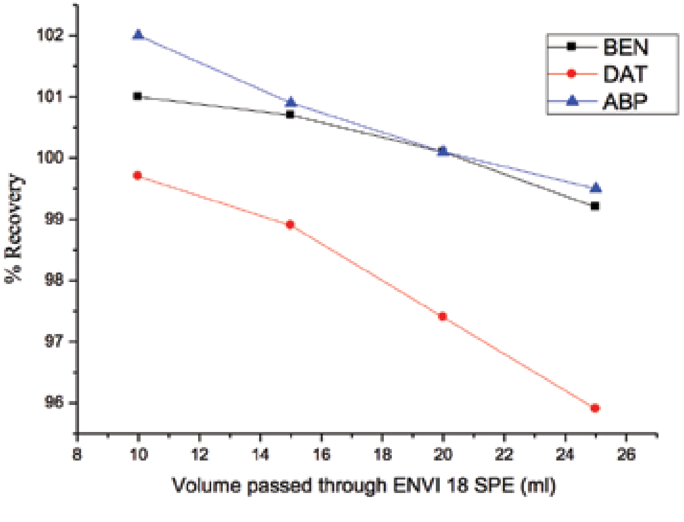

Figure 2. Study of the recovery of aryl amines from different volumes of sample matrices through ENVI-18 SPE. The most polar DAT, next mid way non-polar BEN and highly non-polar ABP were shown to prove the effective recovery of wide nature of aryl amines.

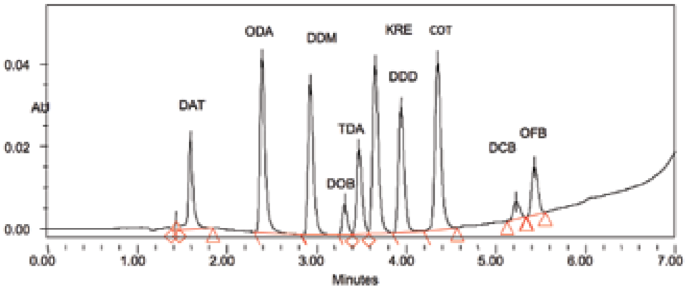

Figure 3. HPLC chromatogram of standard mixture of aryl amines at 240nm.

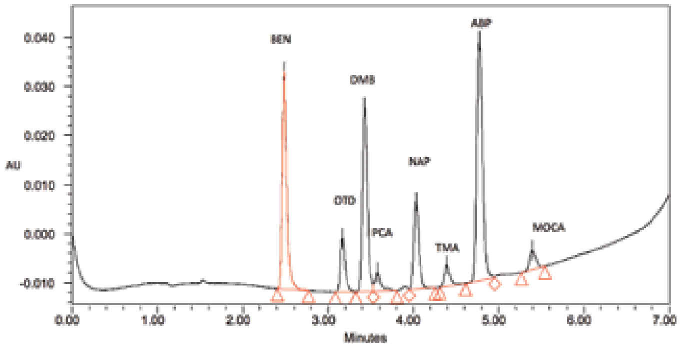

Figure 4. HPLC chromatogram of standard mixture of aryl amines at 280nm.

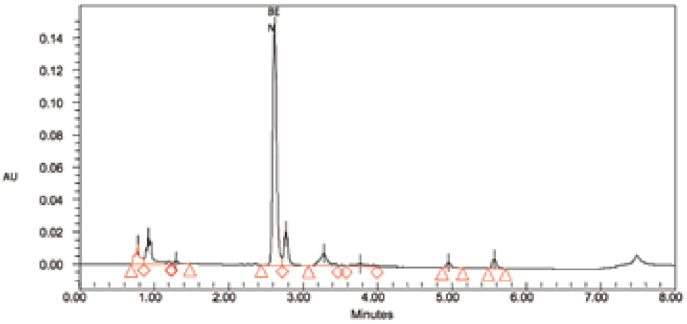

Figure 5. LC chromatogram of sample prepared by ISO/IUC protocol showing the presence of benzidine at 280nm.

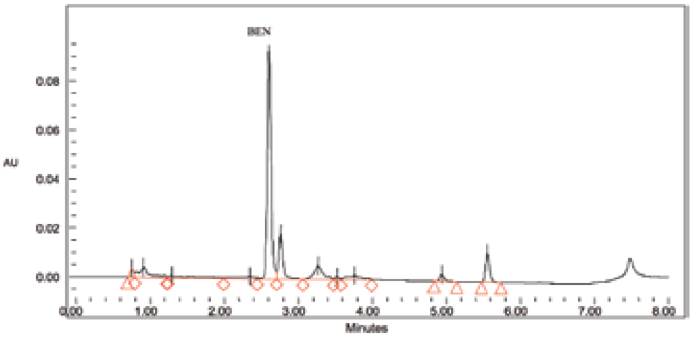

Figure 6. LC chromatogram of sample prepared by the proposed method (ENVI-18SPE) showing the presence of benzidine at 280nm.

TABLE II
Recovery data for the selective Aryl amines from leather on spiking and subsequently analyzed by the proposed procedure.

Aryl amine	**Recovery (%)**	**%RSD (from 3 replicates)**
DAT	93.5	3.9
OTD	81.9	4.4
TMA	93.7	2.4
DOB	89.2	3.5
BEN	97.3	2.6
NAP	94.9	1.9
PCA	88.6	3.8
ABP	99.0	2.1
DDM	90.4	2.5
DCB	91.3	1.7

Validation of Method

The finished leather sample which was found not to release any aryl amines was chosen and 10 randomly chosen aryl amines was spiked at 15µg by transferring 100µl of 150ppm solution (in acetonitrile) by a Hamilton syringe to 0.5g weighed sample. The sample was allowed to dry for about 10 min and then taken for analysis by the proposed method. The results are tabulated in Table II.

The results reveal the complete reliability of the present work as the recoveries of almost all the aryl amines spiked were above 70-102% with good RSD values. The LOD (limit of detection) at 3 times the averaged baseline noise an LOQ (limit that quantitation) at 6 times that noise were calculated. The LOD was 2.0µg/g (2 mgkg^{-1}) and LOQ was 9.0µg/g (9mgkg^{-1}).

Analysis of Real Samples

Several leather samples were screened and a leather sample, which was found to release, a banned amine (by the official protocol) only was chosen for comparison by official ISO/IUC method and the proposed method. That sample was found to release benzidine by the proposed method and the official ISO method and benzidine was found to be 118.7mgkg^{-1} (%RSD: 7.3) by the official method while by the proposed method it was 122.2 mgkg^{-1} (%RSD: 4.6). Figure 3 & 4 are provided to show the separation of standard mixture of aryl amines at 15µgml $^{-1}$ concentration at 240nm and 280nm respectively; Figure 5 is produced for showing benzidine found in the case of leather when analyzed by the official protocol and Figure 6 is produced for showing the benzidine in the same sample when analyzed by the proposed procedure. The reproducibility of the procedure from five replicates was calculated to be 4.1% and the repeatability of the procedure was also studied and found to be 5.3%.

CONCLUSIONS

This proposed procedure which replaces the non-benign part of the analysis (excepting degreasing stage) namely the exchange of the analytes into a solvent layer along with an enrichment of the analytes is achieved by the introduction of RP natured SPE .The succeeding step by LC analysis was accelerated by implementation of UPLC that helped with saving on solvent and time of analysis. In the holistic approach the solvent reduction happened from 80ml into 2ml with the reduction of multiple steps, which also helped reduce sample preparation time alongside minimization of transfer loss from the use of multiple vessels. This proposed procedure for aryl amines can serve for determination in the range of 9-300 mgkg^{-1} of consumer goods mainly of leather. Lately most of the dyed consumer articles are being tested for the presence of any hazardous aryl amines and this procedure serves us with the advantage over the current official one by being much quicker, green as drastic reduction of use of solvent and ensuring better recoveries of arylamines.

ACKNOWLEDGEMENT

Authors thank The Director, CSIR-CLRI for their kind consent to do this project. This article bears CSIR-CLRI Communication No. 1155.

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