Micellar Enhanced Chromatographic Separation of Selected Hazardous Chemical Present in Hair Dye and their Detection in Formulations, Swab Including Assessment of Damage Caused to Cuticle of Hair Shaft

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Abstract

Hydroquinone (HQ), resorcinol (RS), m-aminophenol (m-AMP) and p-phenylenediamine (p-PPD) are aromatic compounds which are generally used in hair dyes to provide different colours to hair. In European Union the concentrations of HQ, RS, m-AMP and p-PPD is regulated in hair dyes and other cosmetic products by EU commission regulation EU/2019/831. This legislation is generally exercised because all these compounds are toxic and may cause severe allergies when used regularly. However in India no such regulations exist to monitor these toxic compounds in hair dyes therefore in this study a simple, rapid, economical and ecofriendly micellar liquid chromatographic (MLC) technique has been developed which can monitor all the selected toxic compounds simultaneously. HQ and RS are positional isomers and are difficult to be separated by HPLC whereas with the developed MLC method it was well separated and detected. The developed MLC technique has been applied to detect and quantify selected analytes in oxidative and non-oxidative hair dyes and swab samples from the scalp. The simultaneous separation of selected analytes was performed in mobile phase 0.09 M SDS, 0.01 M NaH_2PO_4-2% v/v 1-butanol at pH 7 running through C_{18} column under isocratic mode at 1 mL/min. flow rate. All the analytes were eluted within 6 min. The present method has been validated following the EURCHEM Guideline, 2014 in terms of calibration range (0.08-15 µg/mL), limit of detection (0.01-0.08 µg/mL), limit of quantification (0.08-0.18 µg/mL), accuracy (<5.6%), precision (91-105%) and robustness (<5.8%). The selected compounds in hair dye formulation were found in the range of 0.09-253 µg/mL. Hair dyes persistence study was conducted up to 10 days from the day of application on the scalp, suggesting that the dyes were not completely washed off and were retained on the scalp for more than one week. SEM analysis of dyed hair revealed that hairs are severely damaged due to use of dyes. The advantage of the developed method is that it could easily be adopted by quality control and cosmetic laboratories for quality control and check for the simultaneous separation of positional isomers together with two other aromatic compounds.

Keywords: Hair, Hair Dyes, Micellar Liquid Chromatography, Scanning Electron Microscope

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1. Introduction

Irrespective of age and gender, changing the natural colour of hair is quite prevalent all over the world. In the past few years, the global demand for hair dyes has increased at an exponential rate. As per the CAGR (compound annual growth rate) report, the hair dyes market could reach up to 42 billion dollars by 2025 [1]. The dyes used for colouring hairs are either oxidative (permanent) or non-oxidative (semipermanent, temporary) in nature [2]. Chemically speaking hair dyes use primary intermediates [m-aminophenol (m-AMP), p-aminophenol (p-AMP), p-phenylenediamine (p-PPD), m-phenylenediamine (m-PPD), resorcinol (RS)), couplers [hydroquinone (HQ), 1-naphthol (1NpOH)] and oxidizing agents (hydrogen peroxide, ammonia) which react together to form a coloured dye molecule.

Primary intermediates generally used in hair dyes are derivatives of aromatic rings like diamines, aminophenol, phenols, benzene and naphthol while couplers are the compounds that provide a chemical bond between oxidizer and primary intermediate. In case of oxidative hair dyes, hair colour persists for a longer duration due to penetration of small molecules of the dye into hair cortex and thus are known as permanent hair dyes [3]. On the other hand, non-oxidative hair dyes last only for few weeks as the dye molecule is big and cannot penetrate the cortex which generally gets trapped in the cuticle. As the dye molecules are attached to the hair shaft, they get washed away and are thus termed as temporary hair dyes. During the hair dyeing process, the hair dyes also get absorbed through the scalp and enter the systemic circulation. This is possible due to the fact that the hair are attached to the dermis of the scalp and the hair follicles are in contact with blood vessels [4]. Talking about the harmful constituents present in hair dyes the primary intermediate used in hair dyes have been reported as allergen,

carcinogen and mutagen in several in-vitro and in-vivo studies [5]. Although different allergic and sensitive compounds are also present in hair dyes but allergy patch tests have been done only for p-PPD [6]. As per European Union regulation (EU/2019/831) the hair dye preparations containing p-PPD must mention about its allergic nature on the product wrapper or packing [7]. However, in order to increase the popularity of their products, some manufacturers do not indicate these allergens on product wrappers [8]. Regulatory authorities worldwide have established strict cosmetics regulations due to the potential health risk of exposure to toxic hair dye components [9]. Therefore in the present work, only those compounds were selected which are most commonly used and classified as non-permitted dyes by EU/2019/831 i.e., HQ (pKa, 4.3; log P, 1.32), RS (pKa, 9.9; log P, 0.6), p-PPD (pKa, 5.4; log P, -0.25) and m-AMP (pKa, 5.4; log P, 0.04) [7]. Many analytical methods have been reported for the determination of non-permitted and prohibited compounds in hair dyes formulation [5], wastewater [10], urine and serum [11] using different chromatographic techniques like thin layer chromatography (TLC) [12], gas chromatography (GC) [13], high-performance thin layer chromatography (HPTLC) [14] and high-performance liquid chromatography (HPLC) coupled with UV-visible detector, electrochemical, fluorescence [15] photodiode array (PDA), amperometric detector [16] or mass spectrometry detection [17]. Among all these techniques, HPLC-PDA is a method of choice by cosmetic industries and regulatory laboratories for routine analysis of selected compounds but the main problem with this method is that it is not suitable for simultaneous determination and separation of positional isomers [18]. In order to overcome this problem micellar liquid chromatography coupled to a photodiode array detector (MLC-PDA) could be a good choice which uses a hybrid mobile phase of water, surfactants and short-chain alcohols. Separation in MLC is slightly different from the HPLC due to the interaction of analyte with sodium dodecyl sulfate (SDS) modified stationary phase, micelles, hydro-organic bulk mobile phase, electrostatic, dipole-dipole and proton donor-acceptor interactions [19]. The advantages

of MLC are its eco-friendly nature, reduction of sample preparation steps other than filtration, low concentration of organic solvent used, thus reducing the time and cost of analysis [20].

Therefore in the present work MLC method has been developed for the simultaneous determination of HQ, RS, p-PPD and m-m-AMP in hair dye formulations and swab obtained from scalp obtained from volunteers who dyed their hair. It has also been studied that the hair treated with different colouring agents also damages the cuticle scales and root of the hair. In the present work apart from analyzing the selected compounds by chromatographic technique the hair shaft and root were physically examined using a scanning electron microscope (SEM) to assess the damage caused by the chemicals present in hair dyes.

2. Material and methods

2.1. Standard and reagents

HQ, RS, p-PPD and m-AMP was procured from Sigma Aldrich (Mumbai, India). Oxidative and non-oxidative hair dyes were purchased from the local market of Sagar (Madhya Pradesh, India). Sodium dodecyl sulfate (SDS >99%) and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Himedia Laboratories Private Limited (Mumbai, India). HPLC grade 1-propanol, 1-butanol and 1-pentanol were provided by Rankem, RFCL Limited (New Delhi, India).

2.2. Instrumentation and software

The MLC system consists of a Shimadzu Prominence HPLC (Kyoto, Japan), an isocratic system coupled with a PDA (SPD-M20 A; 190-800 nm) detector and the data analysis was carried out using LC Solution software version 1.22 SP1. For chromatographic separation, a C_{18} column of 250 mm length, 4.6 mm internal diameter, 5 µm particle size from Phenomenex HyperClone™ (California, USA) was used. Mobile phase was pumped through the column at

a flow rate at 1 mL/min. and the injection volume of 20 µL was used for all chromatographic analyses.

An analytical balance Mettler-Toledo ME204 (Pocklington, United Kingdom) was used for weighing standard and real samples. The pH measurements were performed using a Contech LAB pH meter, Model pH-103 (Mumbai, India) equipped with a combined Ag/AgCl/glass electrode. The magnetic stirrer and the ultrasonic bath were purchased from PCI Analytics (Mumbai, India). Type-I ultrapure water obtained using Indion LAB Q Ultra system (Mumbai, India) was used to prepare mobile phase, standard and real samples.

The MLC system was equipped with LC-solution software version 1.22 SP1 used for data acquisition. The chromatographic data were treated using Microsoft Excel (version, 2010) and Origin Pro (version 8.1).

2.3. Scanning electron microscope and condition

A SEM of FIE consisting of vacuum system, sample navigation, electron optics, scanning system and optics was used to find out the damage caused to the hair shaft due to the use of hair dyes. Morphological characterization of hair shaft was carried out by SEM (NOVA NANO SEM450, FEI) at 2000X under continuous scanning voltage (15 kV). Moisture-free hair samples were coated with silver and spread uniformly on conductive adhesive tape for SEM analysis.

2.4. Chromatographic solution preparation

2.4.1. Preparation of standard solution

The mobile phases for chromatographic analysis were prepared by accurately weighing surfactant (SDS) and buffer salt (NaH_2PO_4) which were then dissolved in Type-I ultrapure water with the help of sonication. Depending on the composition of mobile phase a small portion of organic solvent was added and the mobile phase was made up to the desired volume

using Type-I ultrapure water. The mobile phase was ultrasonicated for 5 min. and then filtered through a 0.45 µm nylon membrane filter (Axiva, New Delhi, India) with the help of a vacuum pump.

Individual stock solutions containing 100 mg/L of HQ, RS, p-PPD and m-AMP were prepared by accurately weighing the analytes and dissolving them in Type-I ultrapure water.

2.4.2 Sample collection and preparation of marketed hair dyes formulation

Hair dyes (oxidative and non-oxidative) were purchased from general stores at Sagar (Madhya Pradesh, India). 200 mg of oxidative (the base and oxidizer/coupler were mixed in prescribed proportion before weighing) or non-oxidative formulation was diluted with 5 mL of mobile phase. After that, it was filtered through 0.45 µm nylon membrane filter and directly injected into the chromatographic system.

2.4.3. Hair sample collection and preparation for SEM

Five volunteers named in roman from I-V belonging to the research group were selected for the present study. Volunteers 'I' (oxidative), 'II' and 'III' (non-oxidative) regularly used hair dye, while volunteers 'IV' and 'V' never used them. Questionnaires were filled by volunteers regarding age, gender, hair dye, hair oil, shampoo used and the frequency of their application and use etc. Hair samples were collected from the middle part of the scalp. All collected samples were packed into zip-lock polybags and stored at room temperature. The collected samples were further analyzed by SEM to know the damage caused by the selected analytes on hair shaft morphology. Before SEM analysis hair shaft was washed with Type-I ultrapure water and shampoo to remove adhered dust and dirt. After washing, the hair samples were dried at room temperature and 1 cm long hair strand and hair root with bulb were used for SEM analysis. The hair strand and root were mounted onto a stub before silver coating under a

vacuum. A silver layer of 10 nm was deposited on each hair strand and root. Secondary electron images were obtained along the length of each sample at an acceleration voltage of 15 kV.

2.4.4. Dyed hair sample preparation

Dyed hair was examined to check the persistence of hair dyes on the scalp as well as hair shaft. The first step of this study was dyeing hair with oxidative and non-oxidative hair dyes. For this purpose two volunteers (I and II) from the research group applied the selected hair dyes on their hair. Volunteer 'I' applied oxidative while 'II' used non-oxidative hair dye. Samples from both volunteers were collected daily for a period of ten days from the day of hair dye application. As volunteer 'III' was applying non-oxidative hair dye therefore, it was not included in this study.

For the purpose of sample collection, the scalp region i.e., frontal, parietal, temporal, vertex and occipital was divided into 10 zones and marked as 'i-x' in which 'i' was the frontal region and 'x' was the occipital region of the scalp **(Figure 1)**. The 10 zones correspond to 10 day of sampling i.e., the swab was taken from 'i' on day 1 and 'x' on day 10. Each zone of the scalp was approximately measured in 4×4 cm using a measurement tape for sample collection. Hair that comes in this area was exactly clipped at the centre of the hair lengthwise. For sample collection, swab technique was employed where cotton balls of 100 mg were socked in 5 mL of Type-I ultrapure water. Swab samples were collected from the designated zone and its corresponding hair. Swab from hair corresponding to the zone marked on the scalp was collected from approximately 8 cm above the scalp, which was taken approximately from 4×4 cm area, i.e. 8-12 cm of hair strand. Samples collected from scalp and hair strands were marked as 'S-1' and 'S-2', respectively and the cotton ball was squeezed with forceps. The cotton ball was finally washed with 5 mL methanol and both washes were mixed and filtered using 0.45 µm nylon membrane filter and chromatographed individually.

3. Result and discussion

3.1. Optimization of the detection conditions

The first part associated with chromatographic method development is the selection of a sensitive detector. The selection of detection mode is based on the properties of selected analytes. For the present study, the selected analytes i.e., HQ, RS, p-PPD andm-AMP were found to be UV active due to the presence of conjugated, saturated and unsaturated bonds. They also contain heteroatoms such as nitrogen and oxygen, which helps to increase their sensitivity in the UV region. Based on UV spectroscopic analysis the λ_{max} recorded for HQ was 222 and 290 **(Figure 1Sa)**, RS was 220 and 275 **(Figure 1Sb)**, m-AMP was 203 and 283 **(Figure 1Sc)** and p-PPD was 203 and 240 **(Figure 1Sd)**. As all the compounds showed two λ_{max} i.e., below 220 and below 290. It was decided to carry out the further study by selecting the wavelengths 290, 275, 240, 283 for HQ, RS, p-PPD and m-AMP, respectively.

3.2. Optimization of pH of the mobile phase

In MLC charges on the analyte plays a significant role in the separation behaviour of analyte. This is simply achieved by adjusting the pH of mobile phase with pKa of analyte [21]. The pKa value provides an idea about ionic and non-ionic characteristics of the analyte. The pKa value of HQ and RS are 9.9 and 9.3 and it is due to the fact that both possess OH^- charge. In the working pH range of column (3-7) [22] both the compounds act as neutral at pH 7 due to their pKa values. On the other hand, m-AMP (pKa; 4.3) and p-PPD (pKa; 6.2) have NH_2 groups that are protonated (NH_3^+) at pH 3 and pH 5 and neutral (NH_2) at pH 7. To study the behaviour of compounds under the effect of pH the selected analytes were chromatographed at pH 3, 5 and 7. At pH 3 these compounds have more positive charge in comparison to pH 5 which is responsible for increased retention time (Rt). However, using pH 5 the Rt decreased but it was not as remarkable as compared to pH 7. The possible explanation to this could be that at pH 7

analytes are more likely to behave as a neutral compound, so the retention time of analytes drastically decreased compared to pH 3 or pH 5. Based on the above facts pH 7 was selected as the optimum pH for further study.

3.3. Optimization of SDS concentration

Based on common surfactants a SDS was selected due to its novelty and working with this technique. Critical micellar concentration (CMC) plays an important role because at this concentration maximum surfactant forms micelles and to achieve the best result the range of SDS should be between 0.05 to 0.15 M. For the selected compounds when the concentration of SDS was fixed at 0.05 M, p-PPD and m-AMP did not elute until 8 min. Talking about HQ it did not show marked variation in Rt upon varying SDS concentration from 0.05 - 0.15 M. When the concentration of SDS was raised to 0.15 M both the analytes eluted out within 4 min. but using high strength of SDS the peak resolution was poor. Based on the above results, it was decided to conduct further chromatographic analysis using 0.09 M SDS where the retention time clocked by the separating analytes was below 6 min.

3.4. Optimization of type and volume of organic modifiers

In the present work the analytes were slightly non-polar and did get eluted at acceptable chromatographic time using pure micellar mobile phase. Therefore, to enhance chromatographic parameters i.e. efficiency (N) and asymmetry factor (B/A) a small amount of organic modifier i.e. 2% 1-butanol was added to the micellar mobile phase. Using pure mobile phase the chromatographic analysis generally shows poor chromatographic parameters like. Rt, N and B/A. To improve both parameters a short-chain alcohol i.e., 1-propanol, 1-butanol and 1-pentanol were added (**Figure 2Sa-d**). The selection of organic modifiers also depends on log P value of the analyte to be chromatographed.

3.5. Method validation

Validation is one of the important step in any analytical method development. For the present work the method was validated based on the guidelines described in Eurachem Method validation guidelines (EURCHEM Guide, 2014) [23,24]. The studied validation parameters were linearity, the limit of detection and quantification, intraday and interday accuracy as well as precision and robustness.

3.5.1. Calibration range, linearity and sensitivity

Calibration study was performed using seven different concentrations of selected analytes in the range of 0.08-10 µg/mL prepared using a micellar medium **(Figure 2a-d)**. Calibration curves were constructed using the average peak area versus concentration. The slope, intercept, coefficient of determination (r^2) and residual standard deviation (RSD) were calculated by the least square linear regression method.

The limit of detection was calculated with a minimum concentration of selected analytes which generates a response significantly different from the noise of the baseline [25]. Calculation of LOD for HQ, RS, p-PPD and m-AMP were based on 3s criteria. LOQ was calculated using 10s criteria which is the lowest concentration of analytes that could be quantified with acceptable accuracy and precision. The obtained values of LOD and LOQ for selected analytes were in the range of 0.04-0.09 µg/mL and 0.08-0.30 µg/mL respectively for all samples (**Table 1**).

To study the selectivity of the developed method real matrix (blank hair dye formulations and hair swab sample) were injected into the chromatographic system. The purpose of the study was to see the effect of matrix on the elution time of the selected compounds. As observed from the chromatograms of blank samples peaks corresponding to the endogenous compounds eluted before 3.0 min. and no peaks were detected thereafter. The

chromatograms corresponding to blank hair dye formulations **(Figure 3a)** and hair swab **(Figure 3b)** have been shown in **Figure 3**. Chromatograms obtained from the analysis of spiked samples of hair dye formulation **(Figure 4a)** and hair swab **(Figure 4b)** are shown in **Figure 4**.

3.5.2. Precision and accuracy

The intra-day and inter-day precision of the method was studied by injecting the analytes at three different concentrations i.e. near to quantification limit, middle of the calculated values and upper quantification limit **(Table 2)** which were injected on the same day. Similarly, the intra-day analysis was determined based on six replicates analyzed over two months by different analysts in random order. The precision of the developed method shown as a percentage of relative standard deviation (% RSD) was < 5.6 %. The accuracy of the method was measured by % recovery which was in the range of 91-105% for all the selected compounds **(Table 2)**. The results showed that the proposed method is suitable for analyzing selected compounds in hair dye formulation and hair swab samples.

3.5.3. Robustness of the method

The robustness of the developed method was evaluated by performing small changes in mobile phase composition i.e., SDS; 0.08-0.1 M, 1-butanol; 1.9-2.1%, pH; 6.5-7.5 and flow rate; 0.9-1.1 mL/min. for all the analytes. The RSD% was obtained for changes in the area of the peak and resolution. The retention times of analytes were calculated and the observed difference was less than < 5.8%. Based on RSD% values, it could be observed that the studied parameters had no significant effect on the resolution, peak area and Rt of analytes. Therefore the method was robust enough to withstand any minor changes made in the chromatographic parameters.

3.6. Analytical results

3.6.1. Analysis of marketed hair dyes formulations

. In order to detect the presence of different toxic compounds in hair colour the developed method was applied to twenty-one (21) hair dyes marketed under different brand names which were either non-oxidative or oxidative in nature. Out of 21 samples, **fourteen** (14) hair dyes were of non-oxidative nature and **seven** (7) were oxidative.

Out of fourteen (14) non-oxidative hair dyes, five black and six brown (**Figure 5a**) hair dyes were found to be positive for p-PPD. It is pertinent to mention here that only two samples coded as NNH BL and PKM had mentioned p-PPD as one of the contents in the ingredient list. The positive hair dye samples contained p-PPD in the range of 253 µg/mL (further diluted to obtain the concentration of 15 µg/mL) to 134 µg/mL (further diluted to obtain the concentration of 12 µg/mL) (**Figure 6c**). The EU regulation (EC No 1223/2009) of cosmetic products (amended in 2013) has specified that the concentration of p-PPD applied to hair shafts should not exceed 2% (calculated as free base) after mixing under oxidative conditions [7].

m-AMP was detected in three black and one brown (**Figure 5a**) hair dyes in the range of 2.3-3.5 µg/mL. Regarding RS it was detected in two black hair dye samples with the highest concentration of 5.6 µg/mL. Two black non-oxidative hair dyes were also found to be positive for HQ in the range of 0.09 to 2.34 µg/mL. As shown in **Table 3**, most oxidative hair dye products correctly labelled the ingredients.

The selected oxidative hair dye sample included four black and three brown **(Figure 5b)** colour dyes and all of them were positive for p-PPD. The ingredients mentioned on packing of all hair dyes mentioned p-PPD as one of the ingredients (1% of weight) except for IBN. Out of seven samples, three samples abbreviated as GBN (93 µg/mL), IEDBR (307 µg/mL), GEXBL (228 µg/mL) **(Figure 6a)** have shown high concentration of p-PPD against the quantity claimed on the wrapper of packing. All the analyzed samples which have shown the

presence of p-PPD out of the calibration range were further diluted to 12 µg/mL concentration to make them plausible. Regarding HQ many dye manufacturers had not claimed the presence of HQ in the ingredient list, but they were positive for HQ in the range of 0.06- 23 µg/mL. Talking about RS only one brown hair dye sample was found to be positive for it (labelled on five hair dye samples). Three brown and two black samples tested positive for m-AMP. However, m-AMP was mentioned only on one sample without its concentration (**Table 3**). All positive samples contain m-AMP in the range of 4.1 - 24 µg/mL (further diluted to obtain the concentration of 10 µg/mL). In most of the hair dye formulations two to three selected analytes were detected simultaneously **(Figure 6b)**.

3.6.2. Analysis of hair swab

Five volunteers from the research group were selected for the present study but the hair swab was collected only from volunteers and 'I' and 'II'. As per ***section 2.4.4.*** hair swab collected from a volunteer 'I' (applied oxidative hair dye) was injected into the chromatographic system. The collected swab from both parts named as 'S-1' and 'S-2' of the hair was found positive only for p-PPD. **Figure 7 a and b** showed that 'S-1' has a low concentration of p-PPD in comparison to 'S-2' (oxidative hair dye). The study was conducted for ten days, but p-PPD was only detected on the first and second day. It is worth mentioning that hair was not washed during the sample collection period. Therefore only excess dye molecules which adhered to the cuticle leached out. After two days, no p-PPD was detected in the swab.

The analytical data obtained from hair swabs 'S-1' and 'S-2' (dyed with non-oxidative dye) of volunteer 'II' was positive for p-PPD and m-AMP both. After the application of dye, hair was only washed under running water and no shampoo, soap and hair oil were used. From day one to five, the amount of p-PPD and m-AMP decreased almost at a constant rate. However, on the sixth day, when hair was washed with shampoo using mild hot water the highest concentration for p-PPD and m-AMP was reported. Because hot water opens the

cuticles of hair and dye molecules which were adhered escape from there this is the reason for increased concentration. Then on successive days concentration of both analytes decreased. The obtained results have been shown for p-PPD in **Figure 8 a and c** and m-AMP in **Figure 8 b and d**.

3.6.3. Scanning electron microscope analysis of dyed hair

Apart from chemical analysis in the present work it was also decided to study the physical damage caused by the regular use of dyes for the purpose of colouring of hair. In the present study hair shaft and root of five volunteers (I-V) aged between 25-35 were examined by SEM to assess the damage caused to the hair shaft due to the application of hair dyes [26, 27]. Volunteers 'I-III' frequently use dyes to colour their hair. Based on the questionnaire filled by the volunteers, it was observed that volunteers 'I-III' were dyeing, bleaching and not nourishing hair with any kind of hair oil. However, volunteers 'IV' and 'V' were using hair oil as well as were also dyeing their hair **(Table 4)**. Many researchers have reported that dyeing of hair roughens the hair surfaces as well as the cuticle scales are damaged and inclined outwards. Volunteers who were frequently dying their hair were more vulnerable to damage.

In the present study different hair shafts were observed under SEM and microphotographed to study the hair shaft damage. Volunteers 'IV' and 'V' acted as our control as the volunteer never used any dye and nourished the hair with hair oil regularly so healthy cuticles were observed during SEM analysis and no splitting was revealed **(Figure 9a and b)** in the hair sample obtained from Volunteer 'IV' and 'V'. The surface of the hair shaft appeared smooth and the root was healthy [27].

The SEM examination of the hair sample from the volunteer 'II' and 'III' who applied non-oxidative hair dye but also used regular hair oil showed that cuticle scales were outward inclined and irregular with moderate damage and cortex was not damaged in this case **(Figure**

9c and d). In case of volunteer 'I' who applied oxidative hair dyes, bleached hair and never applied hair oil showed that hair root and shaft were severely damaged and no cuticle scales were observed **(Figure 9e and f)**. During the study, microphotographs from SEM revealed that hair and root damage was moderate for those who were applying hair dyes but also nourishing their hair with hair oil.

4. Conclusion

The micellar liquid chromatographic method has been successfully optimized and validated for simultaneous determination of HQ, RS, p-PPD and m-AMP in oxidative and non-oxidative hair dyes formulations and hair swab samples obtained from volunteers. The advantage of the developed method is the possibility of direct injection of hair dyes samples into the chromatographic system after centrifugation and filtration, avoiding long and tedious extraction procedures. All four analytes were separated and detected in different sample matrix within 7 min. with satisfactory precision and accuracy (< 5.6 %). The method validation was accomplished using Eurachem method validation guidelines with satisfactory results in linearity, limit of detection, limit of quantitation, precision, accuracy and robustness. The linearity range and LOQs were sufficient to detect all selected compounds in hair dye formulation.

The developed method meets the criteria of 'green chemistry' as a low amount of organic solvents has been used. Some of the other noteworthy advantage of the method is that it is relatively inexpensive compared to other chromatographic methods (HPLC, LC-MS or GC-MS). Apart from detecting selected analytes in hair dye formulation, the study also included analysis of hair swab samples from volunteer who used oxidative and non-oxidative hair dye which were collected for up to ten days from the day of application of dye on the hair. This study was conducted to determine the persistence of hair dye molecules on scalp and hair. It has been found that oxidative hair dye was detected only on the first day of analysis while

non-oxidative hair dyes persisted till ten days with measurable quantity. Study was also conducted to see the damage caused on the hair surface due to dye treatment given to hair. Scanning electron microscopic results showed that the hair damage was severe for those who frequently applied hair dyes. Therefore it could be concluded that there is a need to create awareness among hair dye users about the presence of toxic compounds which are being used in hair dye formulations and which may pose severe health issues to its users. Due to the lack of strict regulation in India, there is also a need to regularly monitor the concentration of toxic compounds in hair dye formulation. It should also be noted that formulations of strict laws is highly needed for the monitorization of hair dyes in Asia.

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Figures

Figure 1. Different regions of the scalp

Figure 2 (a-d). Calibration curve and chemical structures of selected analytes: (a) HQ calibration curve; (b) RS calibration curve; (c) m-AMP calibration curve; (d) p-PPD calibration curve in the optimized chromatographic conditions: mobile phase 0.09 M SDS-2% 1-butanol (v/v) pH 7, flow rate 1 mL/min., injection volume 20 µL

Figure 3 (a-b) Chromatograms of blank matrix (a) Chromatogram of blank oxidative hair dye; (b) Chromatogram of blank non-oxidative hair dye; in the optimized chromatographic conditions: mobile phase 0.09 M SDS-2% 1-butanol (v/v) pH 7, flow rate 1 mL/min., injection volume 20 µL

Figure 4 (a-b) Chromatograms obtained by spiking standard solution of analyte (a) Chromatogram of spiked oxidative hair dye; (b) Chromatogram of spiked non-oxidative hair dye; in the optimized chromatographic conditions: mobile phase 0.09 M SDS-2% 1-butanol (v/v) pH 7, flow rate 1 mL/min., injection volume 20 µL, detection at 254 nm

Figure 5 (a-b) Graph showing black and brown hair dyes found positive for selected analytes: (a) Non-oxidative hair dye; (b) Oxidative hair dye

Figure 6 (a-c) Chromatograms of the real hair dye formulations (a) Chromatogram of sample number 20 (oxidative); (b) Chromatogram of sample number 18 (oxidative); (c) Chromatogram of Sample number 7 (non-oxidative) in the optimized chromatographic conditions: mobile phase 0.09 M SDS-2% 1-butanol (v/v) pH 7, flow rate 1 mL/min., injection volume 20 µL

Figure 7 (a-b) The concentration of p-PPD in the dyed hair (oxidative hair dye) swab: (a) Swab from root part of hair; (b) Swab from 8 cm above hair root

Figure 8 (**a-d**) The concentration of p-PPD and m-AMP (a) Swab from hair scalp (p-PPD); (b) Swab from hair scalp (m-AMP); (c) Swab from 8 cm above scalp (p-PPD); (d) Swab from 8 cm above scalp (m-AMP)in the dyed hair (non-oxidative hair dye) swab

Figure 9 (a-f) Scanning electron photomicrographs of studied hair: (a) Hair shaft of normal hair; (b) Root of normal hair; (c) Shaft of moderately damaged hair; (d) Root of moderately damaged hair; (e) Shaft of damaged hair; (f) Root of damaged hair hair

Figure 1S (a-d) UV spectra of selected analytes: (a) UV spectra of hydroquinone; (b) UV spectra of resorcinol; (c) UV spectra of p-phenylenediamine; (d) UV spectra of m-aminophenol

Figure 2S (a-d) Chromatograms of standard compounds: (a) Chromatogram of hydroquinone; (b) Chromatogram of resorcinol; (c) Chromatogram of p-phenylenediamine; (d) Chromatogram of m-aminophenol in the optimized chromatographic conditions: mobile phase 0.09 M SDS-2% 1-butanol (v/v) pH 7, flow rate 1 mL/min., injection volume 20 µL