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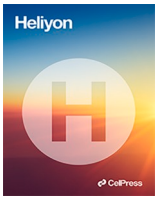

Research article

Method to analyse and quantify the propensity of hair dyes to desorb from human hair fibre

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A B S T R A C T

Background: The use of hair dyes to alter the aesthetics of human hair is a very popular consumer habit. Dyes and their precursors used in hair coloration have been investigated for safety both in terms of exposure of consumers during the dyeing process, and afterwards in rinse water. However, few analytical methodologies exist that provide understanding of the dye desorption process and are able to relate this to visible colour change on the hair. There are also limited quantitative methods for evaluating morphologically different hair types, and the colour changes that occur during the life of the colorant on the user.

Results: Herein, we describe a method of back-extraction of a two-component dye system from morphologically different hair types. Through screening of common back-extraction methods used in analysis of dyed textile artefacts, a new back-extraction methodology has been developed and adapted for application on dyed hair using a 2:1 (*v/v*) water:pyridine solvent system. By separation of extracted compounds with high liquid pressure chromatography, quantitative data on the amount of dye removed are obtained, representing the first application of such methodology in analysing hair dyes.

Significance and novelty: For the first time, an analytical method is provided that can directly relate dye molecule desorption from hair with visual colour loss quantified by spectrophotometry. Moreover, it has been demonstrated that these techniques are applicable to morphologically different hair types, including those with significant underlying natural pigmentation.

1. Introduction

The use of hair dyes in order to alter the appearance of human hair is a commonplace procedure, both in professional salon environments and at home through consumer self-application [1–4]. Hair colour alteration is a trend of adornment that has been historically documented [5] and grown into a multibillion-dollar industry [6]; in 2022, the global hair colour market generated a value of about $25 billion and is forecast to reach about $42 billion by 2029 [7]. More recently, due to the Covid-19 outbreak and subsequent lockdowns, a survey done by Garnier using a sample size of 2000 women showed that one in three women altered their hair colour using self-application kits for the first time with more than 60 % indicating intention to continue home application [8].

Traditionally, hair dye systems are split into three categories based on colour stability: **temporary** dyes involve deposition of high molecular mass compounds onto the hair surface that may be easily removed in one or two washes [2]; **semi-permanent** dyes achieve

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some penetration into the hair with colour lasting several washes [9]; and **permanent** dyes penetrate into the cortical layer and are most effective in covering grey hairs with substantially improved resistance to multiple shampoo washes [2,6,10,11]. Colour formation in permanent hair dyes relies on complex chemical reactions of relatively small organic molecules. An alkaline medium promotes the hair cuticle opening, and dye precursors penetrate and diffuse into the hair cortex wherein colorants are generated *in-situ* [3,11,12]. Generally, *in-situ* generated permanent hair colour is due to the reaction of a primary intermediate (generally *p*-diamines or *p*-aminophenols) and a coupler (generally *m*-diamines, *m*-aminophenols, and mono or polyhydric phenols) in the presence of hydrogen peroxide [13] that promotes the complex oxidative reactions [14–16].

The first stage in the oxidative formation of coloured compounds is the conversion of the primary intermediate in aqueous alkaline solutions of hydrogen peroxide to reactive electrophilic quinonediiminium (QDI) and quinonemonoiminium (QMI) species. This is exemplified in Scheme 1, showing the process of oxidation of *p*-phenylenediamine (PPD) to a QDI that then couples to *m*-aminophenol (MAP) to form both bright magenta dimer and dull brown trimer reaction products; it is proposed that the trimer forms *via* reaction of the dimer dye with unoxidised PPD and subsequent oxidation [17,18].

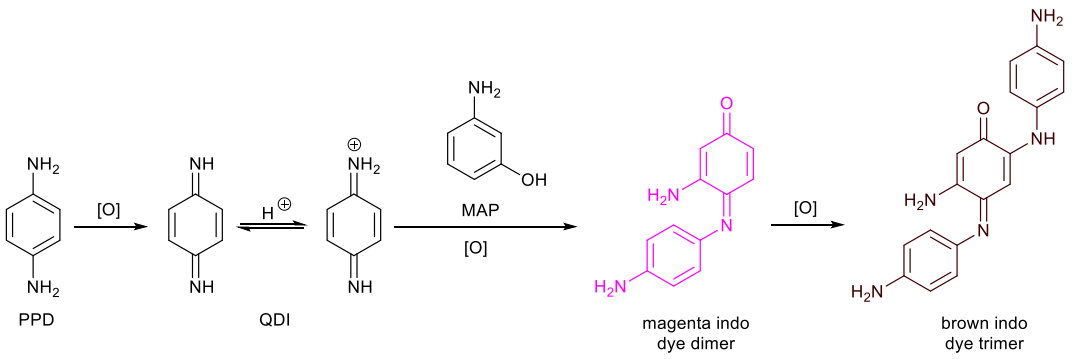
If the structure of the coupler prevents further reaction of the dimer, the resultant colorant formed tends to by brighter and purer in tone. This is exemplified in Scheme 2, showing the process of oxidation of *p*-aminophenol (PAP) to a QMI that then couples to 2-methyl-5-aminophenol (2M5AP) to produce a bright orange dimer reaction product. The presence and location of the blocking methyl group means that PAP can couple only once with 2M5AP, preventing formation of duller trimer or any other oligomeric species [17,18]. Some unblocked systems may also form dark coloured oligomeric species. It has been shown [19] that during oxidative coupling between PPD and resorcinol, formation of oligomeric species occurs *via* combination of dye dimers as shown in Scheme 3; as evidenced by mass spectrometry, the unstable dye dimer can be further oxidised to the brown octameric oligomer, but if the more stable green trimer forms, no further reaction occurs, other than aggregates of this form.

Permanent oxidative hair dye products have a larger molecular size and lower water solubility than their pre-cursors, so effectively become mechanically entrapped within hair through this *in-situ* reaction and are not easily removed during hair washing. This effect is enhanced by the cortical layer swelling in the presence of ammonia, which aids diffusion into the hair, and then shrinking back when pH is reduced to normal physiological levels, reducing the propensity of the dyes to diffuse out of the hair [17]. However, this is not consistent across all colours; notably, red permanent hair dyes have poorer wash fastness in comparison with other permanent dyes [10,20], in particular those based on pyrazole chemistry, *e.g.* the dimer reaction product of oxidative coupling of 4,5-diamino-1-(2--hydroxyethyl)pyrazole and *m*-aminophenol (Fig. 1) [18], display greatest loss in colour on hair over 10 shampoo cycles [20].

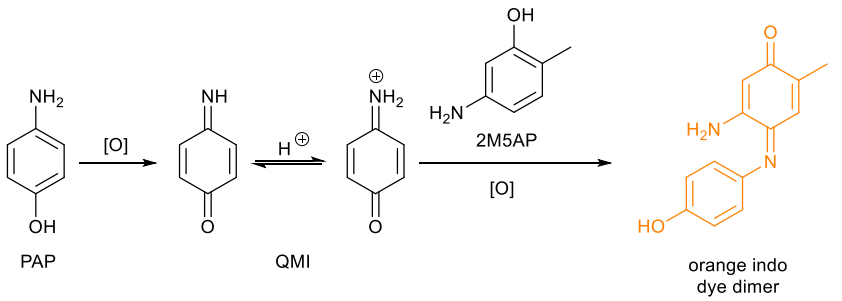
Robbins [21] states that most of the peoples of Europe, Asia, South America, and Australia can fit into one of three groups: Caucasians, Ethiopians, and Mongolians; the latter two groups also being referred to as African and Asian, respectively. Hair fibre characteristics of these three racial groups is shown in Table 1. Different fibre shapes influence both the physical (tenacity and abrasion resistance) and chemical properties of the hair. For example, irregular shapes within the hair structure create topographical peaks and ridges along the fibre axis, and it has been demonstrated that greater chemical damage occurs in ridged areas in comparison with flat areas on the fibre, making high spots more susceptible to water penetration and further damage by chemical treatments and abrasion.

However, this area has received only limited research and what work has been done provides contradictory or inconclusive findings. In relation to hair dyeing, water sorption and resistance to chemical damage are two key factors in both dye uptake and wash fastness of dyed hair. Studies show that Caucasian hair contains the highest amount of water, followed by African, and the lowest in the Asian hair, inferring that Asian hair is more resistant to hydration changes. However, African hair has the highest diffusion coefficient, and Caucasian the lowest, thus implying that water content is not related to diffusion capacity [22]. This makes relating water swelling and dye sorption to hair classification difficult. Studies examining oxidative treatments also offer inconsistent results, suggesting that the chemical treatment itself and not the hair type is the principal factor in relation to damage [23,24]. As such, in researching any hair dye related system, it is important to include an examination of different hair types within each individual study to ascertain any potential hair type related differences.

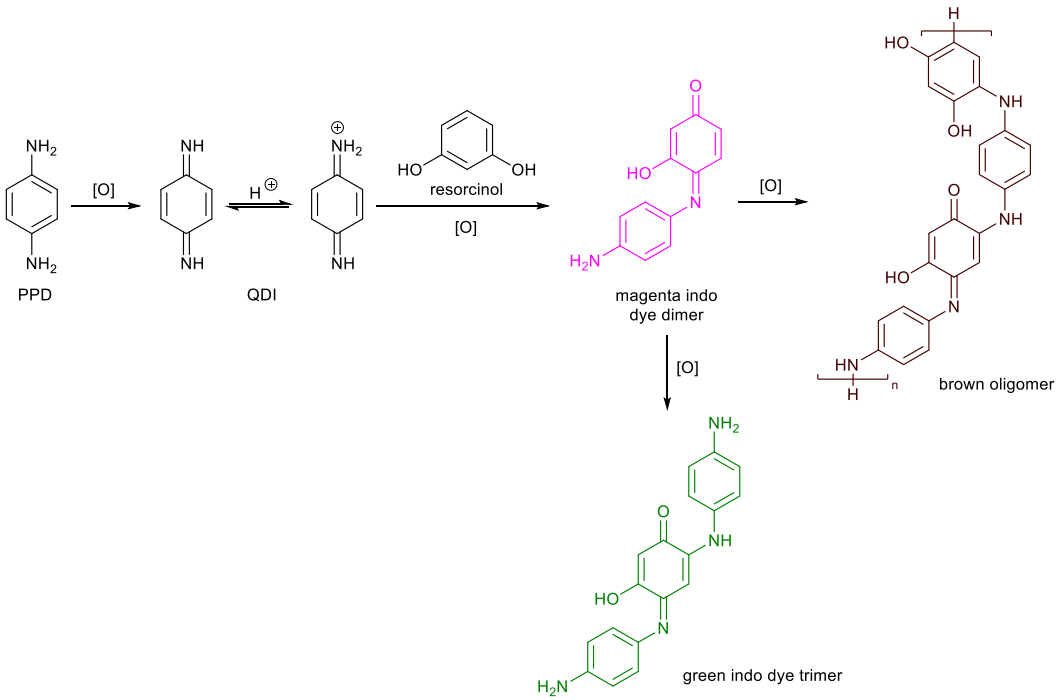
Permanent dyes have been subjected to rigorous research with two main objectives: 1) determine safety; and 2) develop colour lasting results. Safety concerns regarding exposure to ingredients for both salon specialists (due to repeated daily applications) and self-applying consumers, as well as environmental hazard from wastewater, have shaped the hair dye formulation landscape with strict



Scheme 1. Conversion of PPD to a QDI intermediate followed by oxidative coupling with MAP.



Scheme 2. Conversion of PAP to a QDI intermediate followed by oxidative coupling with 2M5AP.



Scheme 3. Oxidative chemistry of the coupling of PPD with resorcinol.

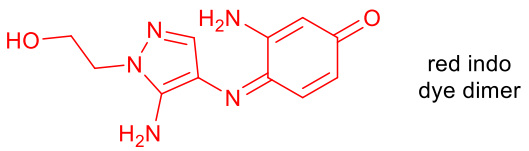


Fig. 1. Red dimer reaction product of oxidative coupling of 4,5-diamino-1-(2-hydroxyethyl)pyrazole and MAP.

Table 1
Hair fibre characteristics by race.

Race	Thickness	Curvature	Cross-sectional shape	Natural Color
Caucasian	Fine	Straight to curly	Nearly round to slightly oval	Blond to dark brown
Ethiopian (African)	Coarse	Wavy to woolly	Slightly oval to elliptical	Brown-black to black
Mongolian (Asian)	Coarse	Straight to wavy	Nearly round to slightly oval	Dark brown to brown-black

regulation for manufacturers in place [25–28]. Due to toxicological and sensitizing potential of certain organic compounds that produce colour, some have either been prohibited or have restrictions around concentrations in use. As a result, regulation has reduced the selection of hair colour actives employed in dye formulations [29], such that innovation in performance and safety has become increasingly difficult [6].

A variety of analytical methods have been established to test and determine the concentration of hair dye ingredients in commercial formulations [11,30], what ingredients end up in salon wastewater following application [9], and the penetration of dyes into the hair [31–34]. The majority of these analytical methods rely on separation of the mixture of dye ingredients using liquid chromatography coupled to an absorbance detector, such as high-performance liquid chromatography with photodiode-array detection (HPLC-DAD), or liquid chromatography coupled to mass spectrometry (LC-MS); desorption electrospray ionization coupled to mass spectrometry (DESI-MS) has also been used [35].

Since safety concerns have been widely assessed and methodologies developed, colour fastness remains a chief driver in formulation as well as a stringent necessity from a consumer viewpoint. Moreover, although colour fastness protocols have been used to determine colour loss in shampooing [10,36–38], these vary in conventions and often assess colour components found in a 'naïve' wash system following dyeing. In general, the hair colour industry assesses and quantifies colour loss by colour measurement, usually using a spectrophotometer, and colour loss is expressed as ΔE according to the CIELab colour space model. However, ΔE is not currently relatable to loss of colorant as it considers changes in hue, chroma and lightness that may not change linearly with removal of dye components, or indeed may change *in situ*, without any colorant being removed [39]. Accordingly, there is an unmet need in the hair colour industry for analytical techniques that can not only quantify colour loss relative to removal of dye components but also relate this colour loss to changes in the observed colour of the hair with washing. These new analytical techniques should be developed with rapidity and applicability in mind, which could be used by most hair colour industry researchers, and not in the exclusive space of only those with the highest specification analytical equipment.

Current methods probing wash fastness of hair dyes are time-consuming and lack robustness; this is due to a constricted analysis of the first wash post-coloration with no consideration of dye leeching in subsequent washes. In addition, the translation of these methods to morphologically different hair types has not been examined. As such, there is significant need for protocols that can push colour fading to answer questions regarding colour retention and complete our understanding of dye desorption from such different hair types.

In this current work, an analytical back-extraction methodology has been tailored for use in hair colour fading studies of four morphologically different hair types, unlike existing methodologies that are indiscriminate of hair type. Although hair has some commonality in its structure, such as presence of keratin proteins, lipids and water, the difference in occurrence of amino acid constituents result in different structural properties of proteins and thus in the hair. This fact, alongside the difference in relative functional groups present in different regions of the hair fibre, results in morphologically different hair types [3]. Understanding differences in hair structure informs design of dye penetration and components that has been a driving force in the development in the methodology described herein.

Back-extraction is a frequent practice in microanalysis of natural dyes in historical textile artefacts in efforts for conservation and restoration [40]; it is possible that methodological development for extracting dyes from hair can draw from the many years of research in this field. Rapid analytical procedures for textile artefacts often involve solvent extraction with a 37 % hydrochloric acid: methanol: water (2:1:1, *v/v/v*) mixture [41]. In this method H^+ ions in the strong acid displace Al^{3+} ions in the dye-mordant-fibre complex (typical of traditionally dyed textiles) and release the dye into solution, which is then subsequently analysed by separation and detection techniques. As there is (in theory) no metal-complexation in hair dyeing, this technique may not be required for hair analysis as, despite providing a high recovery of extracted dye, significant dye degradation may occur with acid-sensitive dyes under such harsh conditions, and also may cause significant damage to (or even digestion of) the hair, which would be problematic when evaluating multiple washing cycles. However, the possibility exists that there may be some dye complexation with residual metals in hair where such breakdown of the metal complexes could be required.

'Softer' methods of extraction may be more appropriate to use in order to preserve as much dye integrity as possible. Simple, but effective, methods of extraction using organic acids in either water or organic solvents have proved successful, including formic acid: methanol (5:95, *v/v*) at 40 °C for 30 min [42], pyridine: water: 1.0 M (aq.) oxalic acid (95:95:10, *v/v/v*) [43], 0.5 M aqueous solution of citric acid [43], and 2 M aqueous trifluoroacetic acid (TFA) solution [44]. EDTA-based methods have also been used where metal complexes are still an issue; EDTA is a very strong chelating ligand with metals such as aluminium (commonly used as a mordant in textile dyeing), and it complexes to the aluminium with greater affinity than the dye and subsequently releases the dye from its insoluble complex into solution – techniques used successfully include Na_2EDTA: acetonitrile: methanol (2:10:88, *v/v/v*) at 60 °C for 30 min, 0.1 % (aq.) Na_2EDTA: DMF (1:1, *v/v*) [45], and 15 % $NH_3.H_2O$ (aq.): 1 mM Na_2EDTA (1:1, *v/v*) [46]. These softer techniques could be particularly useful for analysis of dyes in hair due to their ability to remove colorants from substrates but also preserve both dye and fibre integrity. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) have been employed to remove dyes from textile artefacts [47], but neither solvent has been demonstrated as able to remove dyes complexed to a metal mordant without an additional acid extraction step.

What is of significant interest in hair colour research is the ability to understand how much dye is likely to desorb from the substrate over the lifetime of the dyeing, the primary cause of this removal being washing with shampoo. As such, and by using knowledge from back-extraction of dyed historical textile artefacts, the purpose of this research is to develop a new analytical technique including: a) human hair tresses dyed with oxidative hair dyes and analysed using spectrophotometry before and after dyeing and before and after extraction; b) post-extraction solutions analysed using HPLC with a UV–Vis spectroscopy detector. The combination of these two orthogonal tools is used to provide an understanding of.

- How much dye in hair after application is labile with the potential to be removed (quality of the dyeing);
- How much dye is possible to be removed during washing with shampoo;
- How this analytical technique can be related to visual colour change;
- How this technique can be applied to understand behaviour of the dyes in different hair types.

2. Experimental section

2.1. Materials

The following chemicals used for the study were supplied from Sigma-Aldrich, UK: *p*-aminophenol (PAP), 2-methyl-5-aminophenol (2M5AP); hydrogen peroxide solution 34.5–36.5 %; acetonitrile for HPLC $\geq$99.9 %, 1-heptane sulfonic acid sodium salt, potassium phosphate monobasic, (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), and ammonia 25 % solution ($NH_3.H_2O$). Commercial hair dye and hair care products *Aveda Color Catalyst Conditioner Cream Developer*, *Clairol Professional BW2 powder*, *Clairol 20V Developer* and *Aveda Color Conserve Shampoo* were supplied by Aveda (Blaine, MN, USA); details of the ingredients of these commercial products are provided in Supplementary Information (SI). All other chemicals were purchased from Sigma-Aldrich, UK.

All hair tresses were purchased from International Hair Importers and Products, Glendale, NY, USA. Out of 12 different types of hair (in 4 categories: Type 1 = straight, Type 2 = wavy, Type 3 = curly and Type 4 = coily), 4 different hair types were selected for this study: Natural White Hair (NWH) corresponding to Caucasian Type 1 white hair from mixed source; Medium Brown Hair from mixed source of Caucasian Type 1 and bleached to Bleached Blonde Hair (BBH); Natural Grey Hair (NGH) corresponding to Caucasian Type 1 grey hair from mixed source; and Curly Hair (CH) Caucasian type 3 black curly hair from mixed source. Images of the four undyed hair tresses are provided in SI (S1).

2.2. Instrumentation

HPLC analysis was conducted using a ThermoFisher Ultimate 3000 with a UV–Vis absorption spectroscopy detector with detection at 280 nm, 465 nm, 475 nm, and 505 nm. UV–Vis spectroscopy analysis was conducted using a Jasco V-530 spectrophotometer connected to a computer. Data were collected and processed using Jasco Spectra Manager Software version 1.54.03. Colour measurement analysis was conducted using a Datacolor 500 spectrophotometer connected to a computer. Data were collected and processed using Datacolor software version 2.3.3. pH measurement was conducted using a Thermoscientific Orion Star A111 pH meter connected to a Sentek P11 rod glass combination pH electrode.

2.3. Preparation of bleached blonde hair (BBH)

Caucasian type 1 medium brown hair tresses were bleached using a mixture of *Clairol Professional BW2 powder* and *Clairol 20V Developer*, used according to professional instructions. Bleaching formulation was applied to the hair with a brush until thoroughly coated, wrapped in aluminium foil, and kept in an oven for 30 min at 37 °C. After this time, the tresses were rinsed with warm water and washed using 5 % sodium lauryl sulfate (SLS) aqueous solution applied at 10 % by weight to the hair. The tresses were then combed, and blow dried with a hair dryer with a diffuser for 5 min on the highest setting. All experiments where BBH was mentioned were done in triplicates unless otherwise stated in the text.

2.4. Aqueous dye application

0.16 g *p*-aminophenol (PAP; **1**) (1.50 mmol) and 0.18 g 2-methyl-5-aminophenol (2M5AP; **2**) (1.50 mmol) were slowly added to water (20 ml) and adjusted to pH 10.5 with ammonia ($NH_3.H_2O$). Once ingredients were fully dissolved, hydrogen peroxide solution (30 % in water; 7.50 mmol) was added, this concentration being equivalent to concentrations of hydrogen peroxide used with developers used with commercial hair dye products. pH was monitored and maintained at pH 9 using $NH_3.H_2O$ addition if necessary. The solution containing the hair tress was heated to 37 °C and maintained at temperature for 30 min. Following dye application, the hair tresses (3 replicates) were removed and washed using 1:1 ratio of *Aveda Color Conserve Shampoo* to hair weight ($\sim$0.60 g), where the shampoo was massaged into the hair tresses for 30 s, followed by rinsing with warm tap water for 1 min. The tresses were then combed, and blow dried with a hair dryer with a diffuser for 5 min on the highest setting. All aqueous dyeing was done in triplicates unless otherwise stated in the text.

2.5. Formulated dye application

Formulated dye (80 g) was prepared by mixing 1.36 % *p*-aminophenol (PAP; **1**) and 1.54 % 2-methyl-5-aminophenol (2M5AP; **2**) into a formulation base (details of ingredients in provided in SI). Formulated dye was then mixed in a 1:1 ratio with *Aveda Color Catalyst Conditioner Cream Developer*. The mixture was applied to the hair with a brush until thoroughly coated, wrapped in aluminium foil, and kept in an oven for 30 min at 37 °C. Following dye application, the hair tresses were washed, rinsed, and dried as described above. All formulated dyeing was done in triplicates unless otherwise stated in the text.

2.6. Colour measurement

Hair tresses were measured using the spectrophotometer before and after dyeing and dyed hair tresses were measured before and after back-extraction. Four measurements were taken on each side of the hair tress and the mean value calculated.

The colour strength (K/S) of dyed hair tresses was calculated from reflection spectra by the Kubelka-Munk function (equation (1)):

$$\frac{K}{S} = \frac{(1 - R_\lambda)^2}{2R_\lambda} \tag{1}$$

where K is the absorption coefficient, S is the scattering coefficient, and R_λ is the reflectance any given wavelength (λ).

For dyed hair tresses, especially for dark and dull shades, it is often preferable to express the total colour strength of the sample through a summation of all K/S values across the visible spectrum. Total colour strength (f_k) is calculated using Equation (2), which is summation of all K/S values from 400 to 700 nm at 20 nm intervals:

$$f_k = \sum_{400}^{700} \frac{K}{S} \tag{2}$$

The tone of the colour of the hair dyeings was quantified in terms of L^*, a^* and b^* values within the CIELab system. Colour difference (ΔE) between dyed hair tresses before (L^*_1, a^*_1, b^*_1) and after (L^*_2, a^*_2, b^*_2) back-extraction was quantified by Equation (3):

$$\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2} \tag{3}$$

The human eye can perceive difference in colour when the ΔE value between two analysed samples in greater than 2 units.

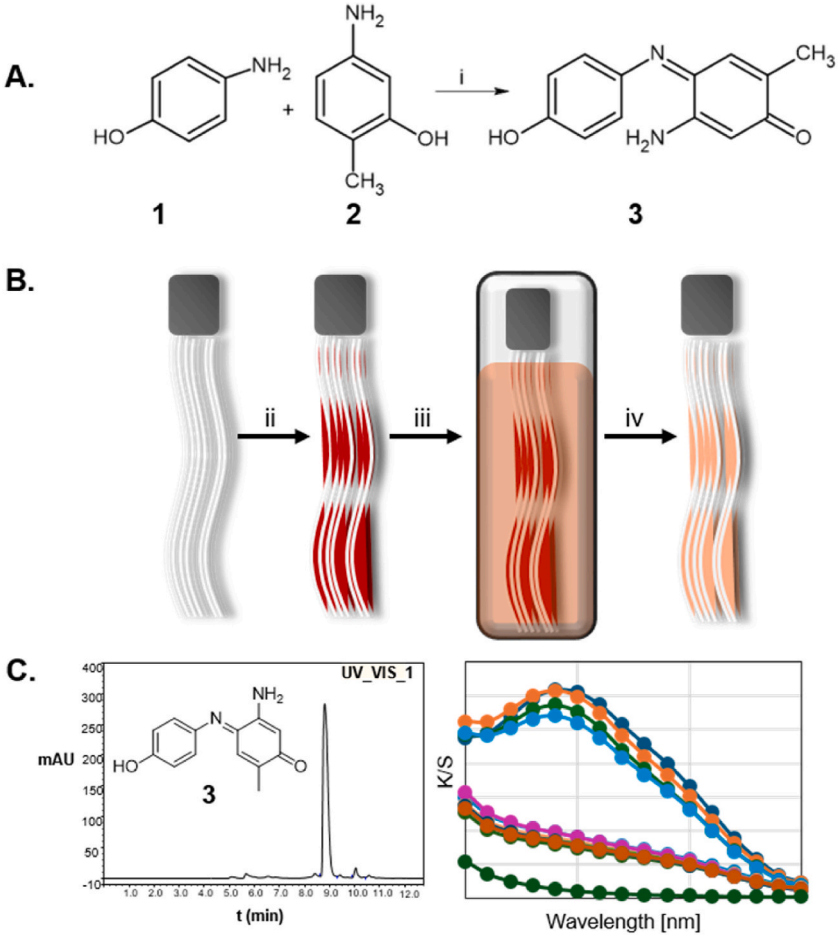


Fig. 2. Back-extraction methodology: **A.** Two-component dye system (PAP (**1**) + 2M5AP (**2**)) and the resultant coloured product (**3**) with reaction conditions (i) pH 9 with $NH_3.H_2O$ and H_2O_2; **B.** Hair tress journey through back-extraction methodology: (ii) hair undergoes dyeing with dye system in A, (iii) dyed hair sample is submerged in the back-extraction solvent for 2 h at 40 °C, (iv) back-extracted hair sample is rinsed; **C.** Analysis of back-extraction solution using HPLC-UV-Vis, and analysis of hair tress before and after extraction using spectrophotometry.

2.7. Back-extraction method development: screening

The ideal back-extraction technique for application to hair dyed with oxidative dyes is one that could remove the maximum concentration of dye possible, but without changing the chemistry of the extracted dye from its form inside the hair; not all dye is necessarily able to be extracted, especially considering oligomeric dye that forms *in situ* and has relatively very large molecular size that might 'entrap' it within the hair structure. As such, the optimal solvent extraction system may not be one typically encountered during the lifetime of dyed hair. Once a technique could be established that could extract all removable dye, the stability hair dyes to single or multiple washing with shampoo could be evaluated and related back to the maximum concentration of removable dye.

Development of a back-extraction technique for dyed hair fibres was based on similar techniques for extracting dyes from historical textile artefacts for chromatographic separation and analysis. Herein, twelve solvent combinations were evaluated in duplicate at 40 °C based on previous literature procedures that were compatible with both the fibre and the dye structures present, with the capability to be adapted to dyed hair fibres [42,43,46–53].

- Methanol (MeOH)
- MeOH + 1 % (*v/v*) formic acid
- 85:15 (v/v) MeOH:$NH_3.H_2O$
- Toluene
- 85:15 (*v/v*) Toluene:$NH_3.H_2O$
- 0.5 M (aq.) Citric acid
- 1:1 (*v/v*) Water:pyridine
- 1:1 (*v/v*) 0.1 M (aq.) Oxalic acid:pyridine
- 55:45 (*v/v*) 22 % (aq.) $NH_3.H_2O$:pyridine
- 15 % (aq.) $NH_3.H_2O$
- 15 % (aq.) $NH_3.H_2O$ + 1 mM Na_2EDTA
- DMSO

NWH was chosen as the hair type for the solvent screen optimization step of the back-extraction methodology. The structural health of the hair was important in developing the basis of this methodology and NWH had the advantage of having little structural damage due to the lack of previous chemical treatments [3], as well as virtually no inherent pigmentation that may complicate quantification of additive colour.

Dyed NWH tresses (12 tresses) were submerged in 20 mL of the back-extraction solution, and stirred for 2 h at 40 °C. The hair tresses were then removed from the back-extraction solution and rinsed using tap water for 30 s. The tresses were then combed, and blow dried with a hair dryer with a diffuser for 5 min on the highest setting.

A summary of the analytical methodology developed is presented in Fig. 2. A two-component dye system comprising the precursors PAP (**1**) + 2M5AP (**2**) to produce the resultant coloured product (**3**), was used to colour human hair (Fig. 2A). Subsequently, dyed hair tresses were back-extracted with a particular solvent system to leave residual solvent and hair with any dye not removed remaining on the hair tress (Fig. 2B). Finally, post-extraction solutions were analysed using UV–Vis and HPLC, and hair tresses were analysed using spectrophotometry before and after extraction (Fig. 2C).

The aim of the study is to quantitatively monitor dye precursors and the formation of the dye product on hair using a solvent system that can remove all labile components to trace levels. With this approach, the risk of error when measuring and considering background colour left on hair after back-extraction are considerably reduced. The solvent systems were chosen for compatibility with the hair structure, avoiding hair damage or digestion; and compatibility with the dye precursors and dye product molecules, avoiding triggering additional dye product formation or side reactions. It is also critical for the study that both tools (HPLC and spectrophotometry) provided coherent information about this complex process and ascertain that the colour change observed and measured corresponds to the dye product extracted and quantified by HPLC.

2.8. HPLC analysis

The Agilent Zorbax Eclipse Plus C18 column was used with the mobile phase consisting of: A) 25 mM L^{-1} phosphate buffer pH 6 + 0.1 % heptane sulfonic acid sodium salt; B) acetonitrile; and C) water at 30 °C, using a flow rate of 1.0 mL min^{-1}. Mobile phase A was prepared in a 2 L volumetric flask, 6.12 g KH_2PO_4 (44.8 mmol), 0.76 g Na_2HPO_4 (5.35 mmol) and 2.00 g sodium salt of 1-heptane sulfonic acid (9.87 mmol) dissolved by shaking in water (1 L), then filled up to the mark with water and mixed. The pH of the buffer should be 6.0, otherwise adjusted to pH 6.0 by the addition of 1 M NaOH or 2 M H_3PO_4. Gradient elution of the mobile phase: 0 min 10 % B, 0 % C; 2 min 10 % B, 0 % C; 6 min 40 % B, 0 % C; 10 min 60 % B, 25 % C; 12 min 75 % B, 25 % C; 14 min 95 % B, 5 % C; 18 min 95 % B, 5 % C; 22 min 95 % B, 5 % C; 24 min 10 % B, 90 % C.

Sample preparation for HPLC analysis was done as follows: 500 μl of back-extraction solution was added to 500 μl of 1 % formic acid containing methanol in order to halt any further reaction of starting components to product which would otherwise be encouraged in the basic conditions of the back-extraction material (Section 3.4). The solutions were then submitted to HPLC analysis.

2.9. UV–Vis spectroscopy analysis

Dye solution (200 μL) was diluted in 2.8 mL of either distilled water or back-extraction solvent mixture, and diluted solutions transferred to a rectangular quartz cell 10 × 10 mm and the UV–Vis spectrum (200–800 nm) measured at 1 nm intervals.

3. Results and discussion

3.1. Dye selection and analysis

A two-component dye formulation was chosen for this study containing the primary intermediate PAP (**1**) and the coupler 2M5AP (**2**). PAP (**1**) was previously studied [54,55] and is currently used as a replacement for *p*-toluenediamine (PTD) and *p*-phenylenediamine (PPD), both of which formed products that presented mutagenic properties in *in vitro* studies [14,15]. The combination of PAP (**1**) and 2M5AP (**2**) affords a vibrant orange-red colour on hair and most notably gives the heterodimeric dye product (**3**); as discussed earlier (shown in Schemes 1–3), although other reaction products are possible from this oxidative reaction, the heterodimer is the main product formed. The reaction can be monitored with ease using a variety of analytical techniques such as UV–Vis, HPLC and TLC. The synthesis of the heterodimer (**3**) was necessary to ensure analytical assessment and was completed using the method described by Kalopissis et al. [56] and successfully assigned (see SI NMR and LCMS protocols, assignment data and Fig. S2).

HPLC is a method of choice of fine quantitative analysis of multi-component mixtures and in this study, provides quantitative information about the dyes (precursors and products) extracted from dyed hair. Thus, the HPLC conditions have been optimized for the quantification of the more polar hair dye precursors but also intermediates and dye products in formulations. While studies reported use of amide-bonded C16 silica columns for the separation of oxidative hair dyes, good results were obtained with a more conventional Zorbax-C18 column. The mobile phase was fine tuned to allow sufficient retention, good separation, and good peak shape of the components. The optimal conditions were met when using an ion-pairing reagent (sodium salt of 1-heptane sulfonic acid) in phosphate buffer pH 6 – acetonitrile – water system. Quantification of each dye component was imperative; thus, calibration curves were obtained [57–59]. HPLC references were prepared for each compound (**1**, **2** and **3**) at four different concentrations: 0.2, 0.1, 0.05, and 0.0025 mg mL^{-1}. Using reverse-phase chromatography conditions described above and UV diode array detection at two different wavelengths (280 nm used for quantification of all compounds and 460 nm as a secondary verification due to heterodimeric product having two maxima in UV see UV trace in SI) calibration curves were generated by plotting the area ratio against sample concentration. All calibration curves were linear ($R^2 > 0.99$) in the investigated concentration range (S3).

Control experiments provided additional confidence on the HPLC tool. The dye in formulation was extracted and analysed, the concentration of dye product (detected at 280 nm) was calculated based on absorbance values from UV detection relative to calibration curves. While the amount of hair dye used for each experiment is known, and the dye:hair ratio is kept constant, the total dye adsorbed by the hair after dyeing and before back-extraction is problematic to measure. To gain a better understanding on how much dye is on the hair during dyeing, the dye (and any residual precursors) was carefully weighed from all points during the application process in triplicates, which comprises: hair dye formulation on the weighing-mixing container; hair dye formulation on the application brush; hair dye formulation on the application gloves; hair dye formulation on the aluminium foil; and hair dye formulation on the hair tress (Table 2). Through this experiment we accounted for 97–99 % of dye, and more importantly how much dye was present on the hair, and thus the theoretic yield of product potentially developed within each type of hair shaft.

The presence of unreacted dye precursors as well as excess dye product molecules are found in rinse water after dyeing and rinse water following the first shampooing of hair [60] while subsequent washing of dyed hair encourages formed dye to desorb from the hair substrate depending on their solubility in water and how labile the dye is [10,61]. The dye precursors and dye product washed off at the first rinse and first shampoo were quantified by HPLC. The initial rinse water carries an average of 10.2 % of the total dye applied and the first shampooing carries an average 1.3 % of the total dye initially applied.

While HPLC allows analysis of the molecular composition of mixtures, it does not allow monitoring and quantification of visible colour of the hair. Measured colour strength (f_k based on K/S data) of hair compared to the reference hair is a better tool for the quantitative analysis of dyed hair. Herein we demonstrate the complementarity of these two tools when analysing back-extracted hair.

Table 2
% dye product recovered from each contact point in the dye application process ±standard deviation.

Dye formulation contact point	% dye recovered			
	NWH	BBH	NGH	CH
Aluminium foil	47.7 ± 1.3	44.6 ± 1.2	41.2 ± 0.5	45.9 ± 2.8
Hair tress	12.7 ± 1.1	8.9 ± 0.7	9.6 ± 1.1	5.9 ± 1.2
Gloves	6.1 ± 1.5	9.6 ± 1.8	5.7 ± 0.6	9.7 ± 1.0
Weighing-mixing container	12.4 ± 1.1	9.1 ± 0.4	13.5 ± 0.2	9.1 ± 1.2
Application brush	20.3 ± 2.2	26.4 ± 0.7	27.3 ± 0.4	26.7 ± 2.5
Total	99.1 ± 2.7	98.6 ± 1.5	97.2 ± 2.3	97.4 ± 3.8

3.2. Back-extraction: screening and optimization

Following back-extraction with different solvent systems, varying degrees of hair dye removal were qualitatively observed on the NWH tresses when compared to the dyed NWH that was not subjected to back-extraction. On visual inspection of the hair dyeings and the back-extracted tresses (Fig. 3), it was noted that greatest colour removal was from tresses back-extracted with either 1:1 (*v/v*) water:pyridine or with MeOH + 1 % (*v/v*) formic acid. Fig. 4A shows measured colour strength (*K/S*) of hair tresses following back-extraction, compared to the reference dyed hair, where lower *K/S* values than the reference indicate that the solvent system was able to remove dye from the hair following treatment. It is observed that 1:1 (*v/v*) water:pyridine solvent and MeOH + 1 % (*v/v*) formic acid were both able to remove the greatest colour from the hair based on this colorimetric data. Surprisingly, dyed hair tresses back-extracted with toluene, toluene + $NH_3.H_2O$, and DMSO had higher *K/S* values, compared to the reference, following treatment; this indicates that treatment with these solvent systems generates further colour on the hair, perhaps by some mechanism where further colour development was enabled (DMSO can act as an oxidant over time) or some form of solvent adduct was formed resulting in the greater colour strength observed. This observation is worthy of further investigation, but for the purposes of this analytical research use of these solvents for this work was discontinued. This is not a suggestion that DMSO should be used on human heads, only that it is worthy of scientific investigation into the mechanism of action.

Fig. 4B shows colour change (ΔE) for hair tresses following back-extraction relative to the reference dyed hair, where higher ΔE values indicate greater colour change, relative to the untreated dyed reference, following treatment. Again, it is observed that greatest

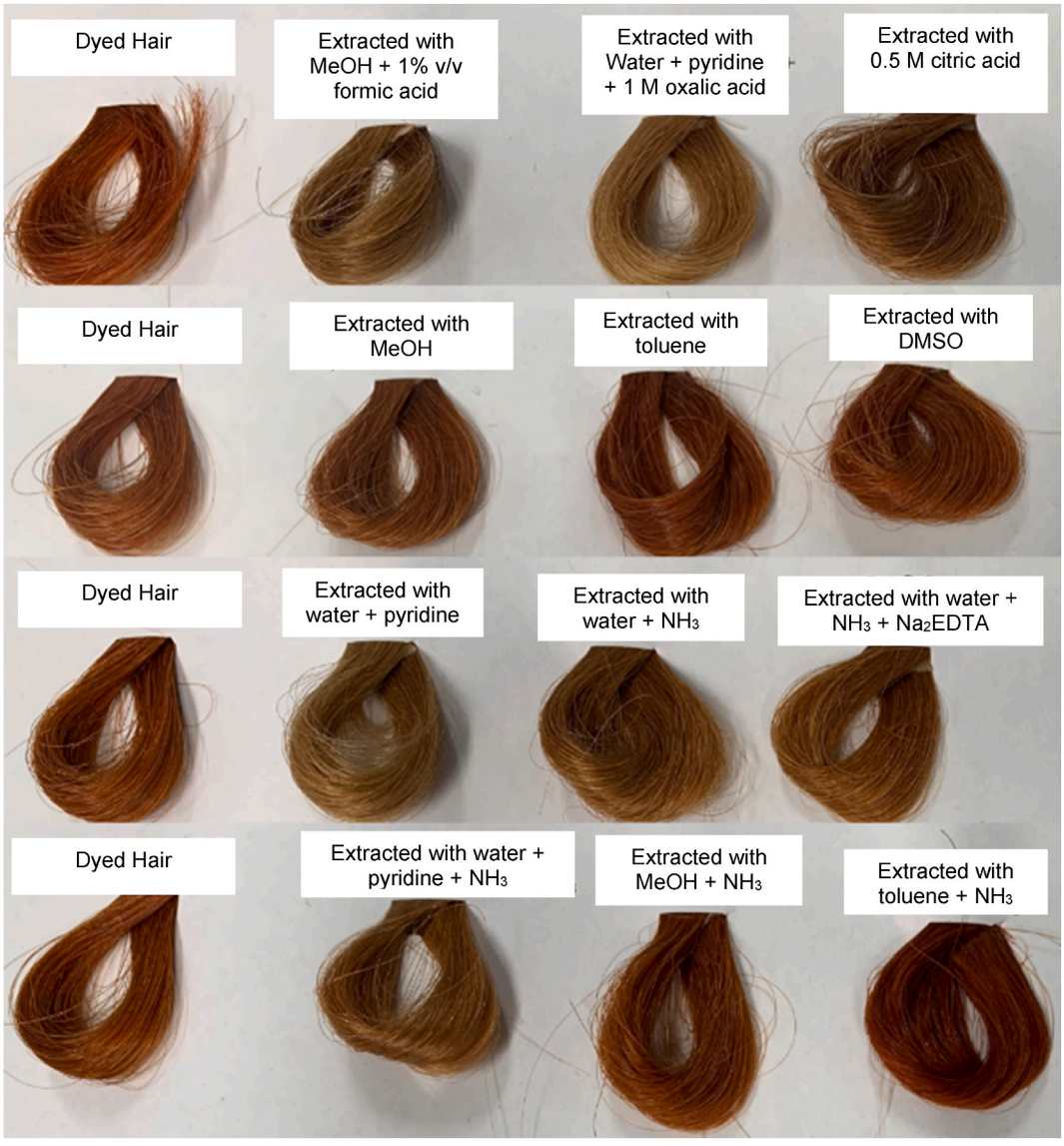
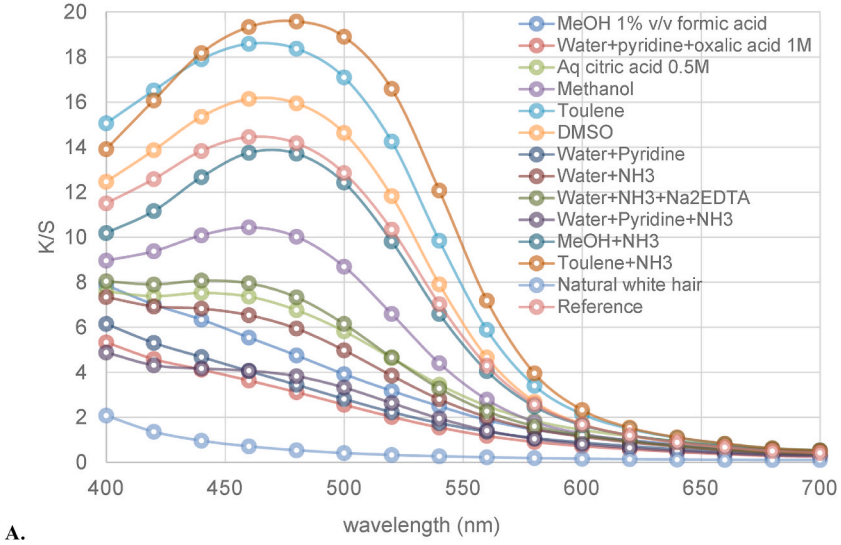
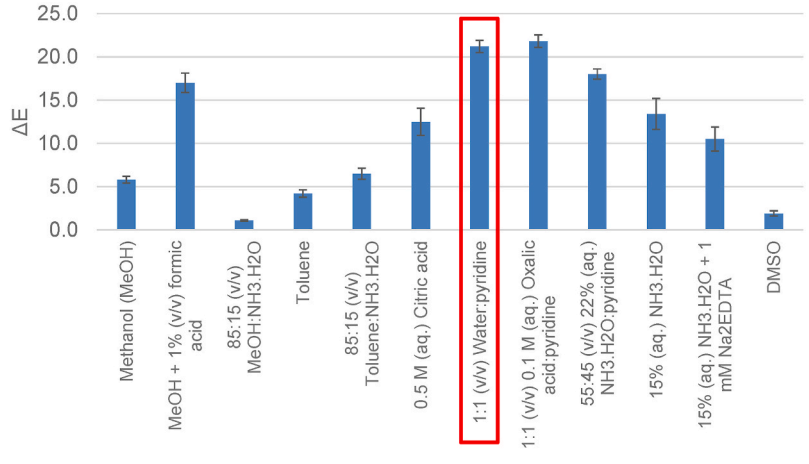


Fig. 3. Dyed NWH tresses following back-extraction with different solvent system compared to reference dyed hair (PAP+2M5AP).



A.



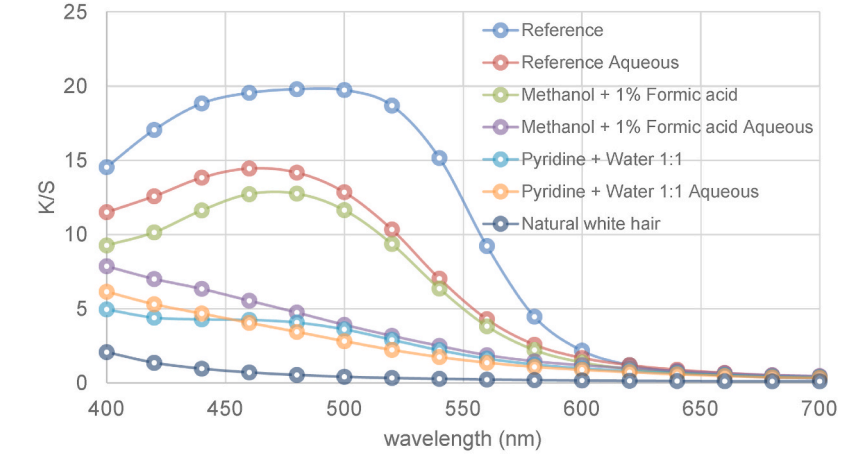
B.

Fig. 4. Solvent screen results: **A.** Colour strength (*K/S*) of dyed NWH tresses (over 400–700 nm) following back-extraction, compared to reference dyed hair; **B.** Colour change (ΔE) of NWH tresses following back-extraction relative to reference dyed hair values.

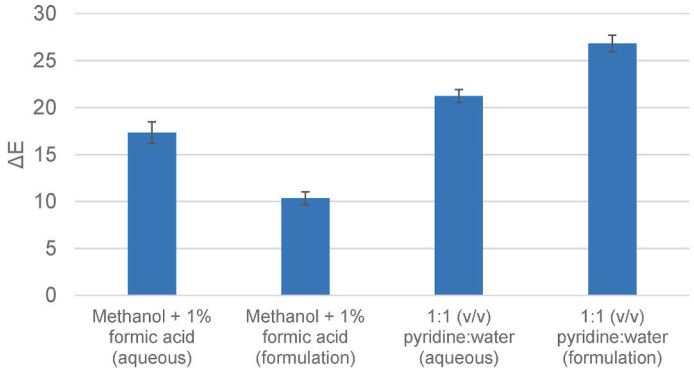
colour difference was observed for the 1:1 (*v/v*) water:pyridine and MeOH + 1 % (*v/v*) formic acid solvent systems because of their ability to remove more colour from the hair than other solvent systems. It is noted that ternary solvent combinations of water and pyridine with either oxalic acid or $NH_3.H_2O$ also provided high levels of colour removal, but the additional benefit was minimal, so for simplicity the binary combination of water and pyridine was taken forward. MeOH +1 % (*v/v*) formic acid was also selected for further development of the methodology.

3.3. Back-extraction: aqueous vs. formulated dye application

The next step was to ascertain if the initial back-extraction results obtained from work using hair dyed with the PAP+2M5AP two-component system in water would give comparable results when applied to hair dyed in a commercial hair dyeing formulation. NWH tresses were dyed using the PAP+2M5AP two-component dye system, half using the aqueous methodology and half using PAP+2M5AP in formulation (S4). These dyed tresses were then subjected to back-extraction using the MeOH +1 % (*v/v*) formic acid and 1:1 (*v/v*) water:pyridine solvent systems and compared to the untreated dyed hair. From Fig. 5A, it is observed that hair dyed from a commercial formulation deposited more colour on the hair tress compared to hair dyed in water. Although both the MeOH +1 % (*v/v*) formic acid and 1:1 (*v/v*) water:pyridine solvent systems were effective at removing colour, it was notable that the latter system gave the same final back-extraction results in terms of colour removed, irrespective of the dyeing process used or the total colour deposited. This result suggests an ability of the 1:1 (*v/v*) water:pyridine solvent system to remove all labile colour present and supported earlier findings that this solvent system was the most optimal tested thus far for this back-extraction methodology. The observations were reinforced from colour change data (Fig. 5B), where it is observed that back-extraction with 1:1 (*v/v*) water:pyridine gives the highest Δ*E* values for



A.



B.

Fig. 5. A. Colour strength (*K/S*) of NWH tresses (over 400–700 nm) dyed in aqueous conditions and in formulation, then back-extracted with selected solvent systems; **B.** Δ*E* values of the NWH tresses dyes using aqueous *vs.* formulated dye applications after back-extraction.

both NWH dyed in formulation and in water.

BBH was subsequently investigated with the back-extraction methodology (S5). Bleaching treatment is known to cause damage to hair fibres, with protein degradation taking place from cuticle to cortex [62–64], hence, the damaged nature of BBH is advantageous in further testing the MeOH + 1 % (*v/v*) formic acid and 1:1 (*v/v*) water:pyridine solvent systems in analysing dye desorption from hair. BBH tresses were dyed in formulation and subjected to colour analysis before and after back-extraction was conducted, offering comparable results as were observed with NWH (S6), insofar as 1:1 (*v/v*) water:pyridine offered the optimal back-extraction solvent system tested thus far.

3.4. Back-extraction: water:pyridine ratio optimization

Back-extraction with water:pyridine was further investigated in triplicate by sequentially lowering the relative amount of pyridine in the solvent combination in order to determine if the same positive results could be obtained, but by using less pyridine to reduce cost and potential hazards. This was conducted on NWH dyed in formulation that was back-extracted using 50:1, 10:1, 4:1, 2:1 and 1:1 (*v/v*)

Table 3
Influence of water:pyridine ratio on yield of dye product back-extracted from NWH after 120 min and colour loss calculated from spectrophotometric data (before and after normalization) ± standard deviation.

(*v/v*) water: pyridine	Calc. from HPLC data	Calc. from spectrophotometric data	
	Yield of dye extracted (%)	Δ*E*	% colour loss (f_k)
50:1	38.4 ± 2.5	5.0 ± 0.3	13.7 ± 0.9
10:1	53.4 ± 2.8	10.7 ± 0.6	32.5 ± 1.7
4:1	60.1 ± 1.5	20.2 ± 0.5	67.1 ± 1.6
2:1	75.6 ± 0.5	18.5 ± 0.1	60.9 ± 0.4
1:1	73.7 ± 1.0	20.7 ± 0.3	67.7 ± 0.9

water:pyridine. Qualitative observations of the hair following back-extraction showed colour removal from dyed hair tresses reduced when higher levels of water were used in the solvent system. However, hair tresses back-extracted using 4:1, 2:1 and 1:1 (*v/v*) water:pyridine were very close in appearance (S7). Confirmation of this qualitative observation was acquired by the HPLC quantification and *K/S* colour data of each hair tress. Dye product (**3**) that was effectively back-extracted using the solvent systems was quantified using HPLC and calculated based on absorbance values from UV detection relative to calibration curve (Table 3, column 2). Based on HPLC the yield of dye extracted from the hair tresses after a 2 h back-extraction, it became apparent that increased water content in the solution resulted in less dye back-extracted with 50:1 (*v/v*) water:pyridine affording only 38 % when compared to the 2:1 (*v/v*) at 75 % or 1:1 (*v/v*) at 74 % water:pyridine solutions where there was virtually double the amount of dye back-extracted. Optimal results were obtained by using the 2:1 (*v/v*) water:pyridine back-extraction solution, affording a total of 76 % dye back-extracted as quantified by HPLC.

The % colour loss (calculated from f_k values) from spectrophotometry analysis is also shown in Table 3 (column 4) for each water:pyridine ratio. It is noted that colour data mirrors HPLC quantification results, in general, % dye extracted increases proportionally with % colour loss from f_k values; ΔE values (Table 3, column 3) also show a similar proportional change. The % colour loss values for hair tresses back-extracted using 4:1, 2:1 and 1:1 (*v/v*) water:pyridine were in close proximity, reinforcing the observation that these solvent combinations are optimal for extracting dye. The results of the colour data in combination with the quantification by HPLC guided the methodology towards adopting the 2:1 (*v/v*) water:pyridine as the most accessible solvent in further application to morphologically different hair types.

Consequently PAP (**1**) and 2M5AP (**2**) were studied in the back-extraction solution of 2:1 (*v/v*) separately and in combination. It was theorised that the basic medium of back-extraction could encourage further reaction of the starting materials thus, all HPLC samples were preventatively quenched in methanol +1 % formic acid. The study of each individual starting components in the back-extraction solution showed complete recovery of starting materials after 2 h (Table S1), while the study of the components mixed in the back-extraction solution showed some conversion to product (Table S2). This did not present any future issue as quenching was done before the analysis of each HPLC sample.

3.5. Back-extraction of different hair types

Four hair types (NWH, BBH, NGH and CH) dyed with PAP+2M5AP dye formulation were back-extracted with 2:1 (*v/v*) water:pyridine. Adsorption of dye onto hair is influenced by physical morphology and prior chemical treatments such as bleaching. It was anticipated that the more damaged and 'open' structure of bleached hair [13,62–64] would allow both greater dye uptake, but also greater desorption from subsequent washing (and herein from back-extraction methods). In contrast, undamaged NWH should not present the effects as bleached hair, but research articles describing work with NWH are limited, as white yak hair has been historically used as the model hair system [65]. Melanin pigment is not present in white hair, and sparingly present in grey hair [3], which can influence dye permeation in and out of the hair shaft.

Following back-extraction of each hair type with 2:1 (*v/v*) water:pyridine, aliquots of the solutions were taken after 30 min and after 2 h and analysed using HPLC. The colour measurement of the hair was also recorded after 2 h (Table 4). NWH and BBH both showed similar mass of dye back-extracted following the 2-h protocol, however BBH leached more dye (65 %) in the first 30 min, in comparison with NWH (58 %). BBH has been subjected to chemical processing (bleaching) that alters chemical and physical bonds and the physical structure of hair, including the pattern and spacing of hair cuticles. Consequently, BBH is more porous than untreated natural blonde hair, hence dye adsorption and desorption occur at a higher rate initially, then plateaus to a similar amount observed for NWH (81 %). The extent of damage of BBH is highly dependent on the bleaching protocol; BBH used in this study underwent one round of bleaching where damage would not be as great in comparison with hair that undergoes bleaching on a more frequent basis. *K/S* data (Table 4) shows that despite NWH (Fig. 6A) and BBH (Fig. 6B) leaching similar amounts of dye after the 2 h back-extraction, more colour is retained on NWH. This suggests that more extensive dye interactions are possible with the structural and chemical components of NWH, enabling greater colour retention, despite the effective back-extraction method.

For NGH, a total of 41 % of dye was quantified at the 30-min mark during the back-extraction, with overall 68 % dye removed after 2 h. NGH is a more complex hair type to study, it is a combination of pigmented hair and white hair completely devoid of melanin

Table 4
Influence of hair type on yield of dye product back-extracted with 2:1 (*v/v*) water:pyridine after 30 and 120 min, and colour loss calculated from spectrophotometric data (before and after normalization) ± standard deviation.

Hair Type	Time (min)	Calc. from HPLC data	Calc. from spectrophotometric data		
		Yield of dye extracted (%)	ΔE	% colour loss (f_k)	% colour loss (f_k) (norm.)
NWH	30	58.4 ± 0.4	N.M.	N.M.	N.M.
	120	81.4 ± 0.6	17.0 ± 0.1	59.7 ± 0.4	62.5 ± 0.4
BBH	30	64.9 ± 0.3	N.M.	N.M.	N.M.
	120	81.2 ± 0.4	18.2 ± 0.1	52.8 ± 0.3	59.1 ± 0.3
NGH	30	40.9 ± 0.5	N.M.	N.M.	N.M.
	120	68.0 ± 0.9	8.1 ± 0.1	27.0 ± 0.4	37.2 ± 0.5
CH	30	64.8 ± 1.2	N.M.	N.M.	N.M.
	120	95.4 ± 1.7	1.6 ± 0.0	7.3 ± 0.1	78.2 ± 1.4

N.M. = not measured.

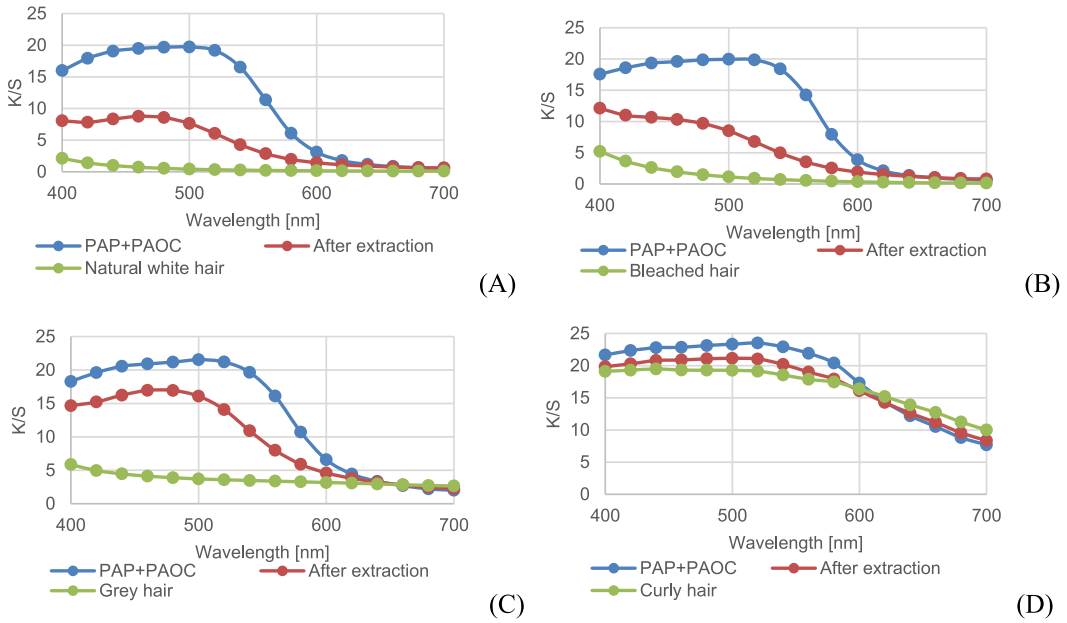


Fig. 6. Colour strength (*K/S*) of dyeings before back extraction (PAP + POAC), after back-extraction using 2:1 (*v/v*) water:pyridine, and original undyed hair colour for: (A) NWH; (B) BBH; (C) NGH; and (D) CH.

pigmentation and other oils which would render this hair type with less opportunities amenable for electrostatic interactions between the dye and structural components within the shaft. This heterogeneous composition could also affect the mechanical entrapment of dyes following opening and closing of hair shaft. *K/S* data (Fig. 6C and Table 4) also shows the lowest levels of dye leaching after the 2 h back-extraction of all four hair types evaluated.

CH was identified as the hair type that demonstrated greatest desorption dye following back-extraction, compared to the other hair types tested with 65 % dye removed after 30 min and 95 % after 2 h; this was not an unexpected outcome as CH is known to be naturally more porous [66]. *K/S* data (Fig. 6D and Table 4) is more complex to understand for CH because both ΔE and % colour loss from f_k values (not normalized) demonstrate only minimal visual colour change despite greatest measured dye removal.

The hair coloration industry primarily assesses colour change on hair using ΔE values, but this is not an accurate representation of dye loss, more a change in colour that could be brought about by hue change *in situ* on the fibre; ΔE represents a change in CIELab coordinates (L^*, a^*, b^*) rather than a linear representation of colour strength loss. The use of *K/S* values introduces a colour measurement system that is linear and representative of changes in colour strength because as dye is lost *K/S* values decrease proportionally [67]; the derivation of % colour loss from f_k values ensures that complex coloration systems (such as those observed for hair dyes) with significant *K/S* values over wide regions of the visible spectrum are accounted for.

A key objective of this research was to develop an analytical method that could relate HPLC quantification of dye molecule removal to colour change quantified by spectrophotometry. This was achieved in initial research on NWH as both % colour loss from f_k values and ΔE values showed a similar proportional change to % dye extracted (from HPLC data). However, when using hair types with significant underlying colour (NGH, CH) the relationship did not hold. To account for the background colour of the underlying hair, normalized % colour loss values were calculated by subtraction of f_k values measured for hair where no dye was applied (background hair colour), which revealed a proportional change to % dye extracted (Table 4).

This is further exemplified when plotting the relationship between % dye extracted to % colour loss (f_k) and colour change (ΔE) using the data obtained for the four different hair types (Fig. 7), where it is observed that there is no correlation between % dye extracted and non-normalized % colour loss (f_k) or ΔE. However, there is evidence of strong correlation ($R^2 = 0.9783$) between % dye extracted and normalized % colour loss (f_k); **this for the first time provides an analytical method that can directly relate dye molecule desorption from hair with visual colour loss, irrespective of hair type or background hair colour.**

4. Conclusions

A new methodology has been developed and optimized for the back-extraction of a two-component dye from hair. Inspired by robust methods applied to textile artefacts, the methodology uses orthogonal analysis techniques: colour analysis and HPLC analysis. The aim of the subsequent study was to track down the dye precursors and dye product molecules during and after a hair dyeing process. The methodology has been successfully applied on four morphologically different hair types, providing insights into hair properties and dye uptake and retention capacity. The back-extraction approach is key to this methodology and by combining it with HPLC analysis allows quantification and significant step towards better understanding of dye/hair interaction and behaviour. The

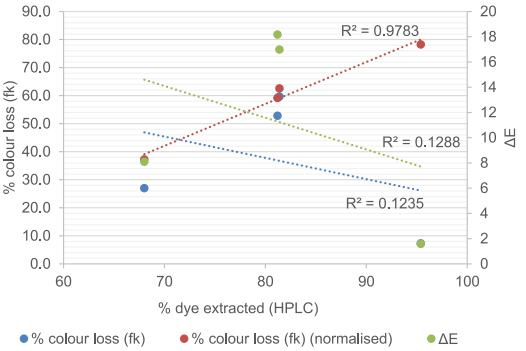


Fig. 7. Relationship between % dye extracted (quantified by HPLC) to % colour loss (f_k) and colour change (ΔE) (quantified by spectrophotometry) for the four different hair types examined.

robustness of the methodology is demonstrated when applied on different hair types. Indeed, the objectives of this study was attained when the HPLC quantification and the colour data aligned with rational behind the percentage of dye back-extracted dependent on hair physical differences based on hair type. Currently, no methodologies for hair dye back-extraction have been applied in such a context. Additionally, for the first time an analytical method is provided that can directly relate dye molecule desorption from hair with visual colour loss, irrespective of hair type or background hair colour. Future development of this method can ultimately help serve in better understanding of dye/hair interactions, can aid in quantification of maximum potential amount of dye lost in customer use, and as a technique to inform design of dyes with better colour retention for different hair types.

CRediT authorship contribution statement

Kristina Hetherington: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alenka Tidder:** Methodology, Investigation, Formal analysis. **Bethany J. Tack:** Methodology, Investigation, Formal analysis. **Meryem Benohoud:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Dan Nowlan:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Anwar Zahar:** Writing – review & editing, Validation, Methodology, Formal analysis. **Xiaoguang Li:** Writing – review & editing, Validation, Methodology, Formal analysis. **Darcy Prater:** Writing – review & editing, Validation, Methodology, Formal analysis. **Jeanna C. Zguris:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Tanu Tokle:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Formal analysis. **Christopher M. Rayner:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Richard S. Blackburn:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data availability

The data that support the findings of this study are available upon reasonable request from the authors.

Ethics declaration

Review and/or approval by an ethics committee was not needed for this study because the human hair used in this study was ethically sourced and purchased from International Hair Importers & Products, Inc. from willing participants who are paid for their tress.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e43528.

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