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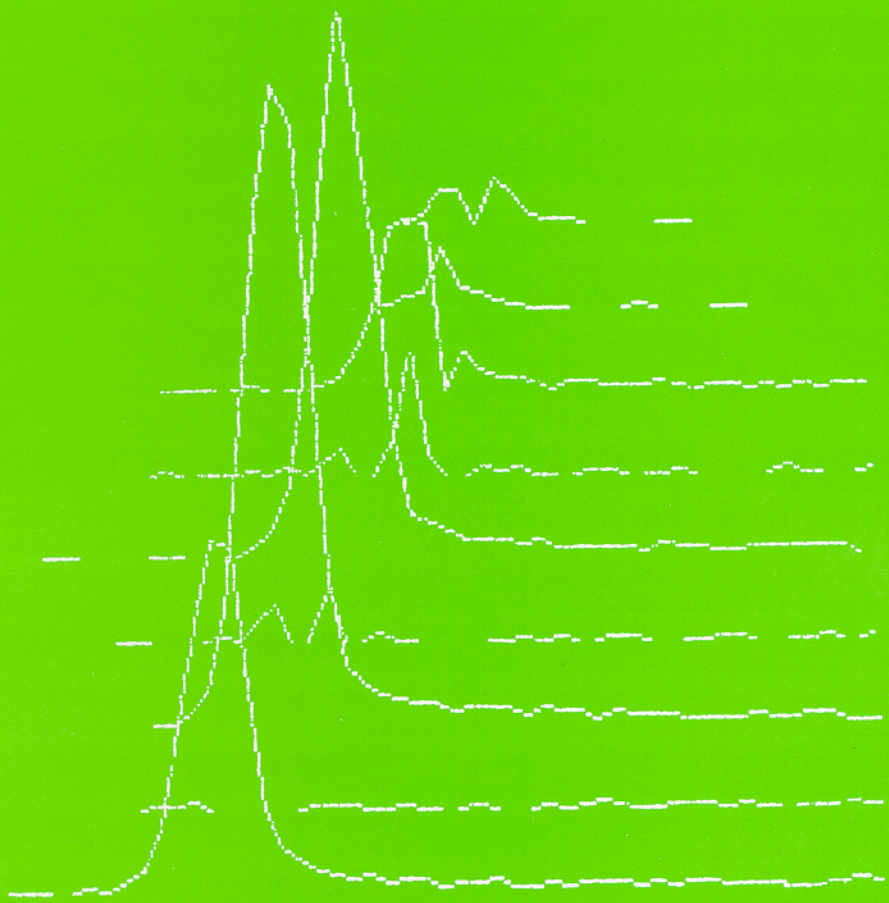

Miljøkemi

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Subtitle: Analytical control of chemical substances and chemical preparations

Tema: Environmental Chemistry

Miljø- og Energiministeriet, Danmarks Miljøundersøgelser

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Miljøkemi – Environmental Chemistry

A method for the measurement of intermediates of oxidative hair dyes in cosmetic products

Analytical control of chemical substances and chemical preparations

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Department of Environmental Chemistry

Data sheet

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Abstract: The intermediates of oxidative hair dyes in hair coloring formulations are regulated by EU Cosmetic Directive. A method for the measurement of contents of some commonly used intermediates of oxidative hair dyes in cosmetic products has been developed and evaluated in the present investigation. The samples are extracted in acetonitrile-phosphate buffer containing an ion-pairing reagent and an antioxidant, followed by HPLC analysis employing a column packed with amide bonded C_{16} silica and detection at wavelength range 200 nm-400 nm. The target substances were identified by comparing their HPLC retention times and 220 nm-400 nm spectra with the retention times and spectra of the standard compounds. The quantification of the identified substances is performed using the calibration curves of the respective standard compounds.

Keywords:	Cosmetics, oxidative hair dyes, HPLC, aminophenols, phenylene diamines, toulene, diamines, resorcinol, 1-naphthol and hydroquinone, Cosmetic Directive
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Resumé

Indholdet af oxidationshårfarver i kosmetiske produkter er reguleret ifølge Miljø- og Energiministeriets bekendtgørelse om kosmetiske produkter. Der er i nærværende undersøgelse udviklet og evalueret en metode til identifikation og kvantitiv bestemmelse af almindeligt anvendte oxidationshårfarver (phenylendiaminer, toluendiaminer, aminophenoler, resorcinol, 1-naphthol og hydroquinon) i hårfarve produkter. Prøverne ekstraheres i acetonitril-phosphat buffer indeholdende ion-par reagensen 1-heptan-sulfonsyre og antioxidanten ascorbinsyre. Prøveekstraktet analyseres ved højtryksvæske-kromatografi (HPLC) med anvendelse af en analytisk HPLC kolonne pakket med amid-bonded C_{16} silica. Gradient elueringen ved HPLC udføres med anvendelse af mobilfase sammensat af acetonitril, phosphatbuffer og ion-par reagensen. Stofferne detekteres ved UV i bølgelængde området 220 nm-400 nm med anvendelse af en photodiode array detektor. De udvalgte stoffer i en prøve identificeres ved sammenligning af deres HPLC-retentionstider (t_R) og 220 nm-400 nm UV-spektrum med t_R og 220 nm-400 nm UV-spektra af standard stoffer.

Kvantitativ bestemmelse af de identificerede stoffer udføres ved anvendelse af kaliberingskurve for de respektive standard stoffer. Detektionsgrænsen forf alle stofferne var < 5 µg/g, kalibreringskurverne var lineære (r^2 >0.999), og repeterbarhed af bestemmelsen af stofferne var inden for 5%. Genfindingen af stofferne fra prøver, spikede med de udvalgte stoffer, var 89-101%. Med nærværende metode er der udført en ”screeningsundersøgelse” for at bestemme indholdet af udvalgte oxidationshårfarver i 56 kosmetiske produkter.

Arbejdet er udført som faglig støtte til Miljøstyrelsen.

Abstract

The contents of intermediates of oxidative hair dyes in cosmetic products are regulated according to the EU Cosmetic Directive. In the present investigation, a method has been developed and evaluated for the identification and determination of some commonly used intermediates of oxidative hair dyes (aminophenols, phenylene diamines, toulene diamines, resorcinol, 1-naphthol and hydroquinone) in hair coloring products. The samples were extracted in acetonitrile-phosphate buffer containing ion-pairing reagent 1-heptane sulfonic acid, and ascorbic acid as an antioxidant. The sample extracts were analysed by reverse phase high performance liquid chromatography (HPLC) employing an analytical HPLC column packed with amide bonded C_{16} silica. A gradient elution of the compounds was performed by HPLC using mobile phase composed of acetonitrile, phosphate buffer and the ion-pairing reagent. The compounds were detected using a photodiode array detector at the wavelength range 220 nm-400 nm. The identification of the intermediates of oxidative hair dyes in a sample was performed by comparing their HPLC-retention times (t_R) and 220 nm-400 nm spectra with the t_R and spectra of the standard compounds.

The quantification of the identified substances was performed by the use of calibration curves of the respective standard compounds. The detection limit of all of the target substances was <5 µg/g, the calibration curves of the substances were linear (r^2 >0.999) in the investigated concentration range and repeatability of the determination of all of the target substances was within 5%. The recoveries of the target compounds, from the samples spiked with these substances, were found to be 89-101%. Employing the present method, a ‘screening analysis’ has been performed for the determination of the contents of target compounds in 56 cosmetic products.

The present work has been performed as technical support to Danish Environmental Protection Agency.

1 Introduction

Hair dyeing formulations belong to 3 categories, i.e. for temporary, semi-permanent and permanent coloring of hairs. The products for temporary dyeing of hairs comprise generally of acid dyes, which are deposited on the surface of hairs to give a coloring effect. These dyes are removable by a single effective shampooing. The formulations for semi-permanent dyeing of hairs consist mainly of various amino-nitrobenzenes and/or hydroxy-nitrobenzenes. These molecules diffuse into the hairs giving coloring effect. During shampooing these molecules diffuse out of the hair. The coloring effect of these dyes thus vanishes during 5-10 shampooing. The formulations for permanent dyeing of hairs, also called oxidative hair dyes, consist essentially of aromatic diamines, aminophenols, and polyhydroxy benzenes (such as resorcinol) and naphthol[1]. The aromatic amines and aminophenols serve as primary intermediates of oxidative hair dyes, while polyhydroxy benzenes/1-naphthol serve as secondary intermediates for the functions such as colour modifier, stabiliser etc. The dye formation involves slow oxidation of a primary intermediate (diffused into the hair) by hydrogen peroxide producing a diimine, which reacts with the couplers (for example, m-diamines, m-aminophenols, resorcinol, 1- naphthol) resulting in dinuclear, trinuclear or polynuclear structures[2]. These molecules are too large to escape from hair structure.

The contents of intermediates of oxidative hair dyes in hair coloring formulations are restricted according to Annex III of the EU Cosmetic Directive[3]/Kosmetik bekendtgørelse[4]. Moreover, certain substances are banned in these formulations according to Annex II of the Directive. In 1998, NERI analysed on behalf of Danish Environmental Protection Agency (DEPA) the content of colorants in a series of hair coloring formulations. Attempts were also made to analyse semi-permanet dyes as well as intermediates of oxidative hair dyes in these formulations. However, semi-permanent dyes and intermediates of oxidative hair dyes could not be analysed due to non-suitability of the method selected for the analysis. The project was continued this year with the aim to establish a method for the identification and determination of intermediates of oxidative hair dyes in cosmetic products for hair coloration.

Several chromatographic methods have been reported for the analysis of intermediates of oxidative hair dyes[5-15]. Some of these methods may be suitable only for checking the purity of the raw materials, while others may also be used for the determination of the contents of intermediates of oxidative hair dyes in cosmetic formulations. However, only the method reported by Weyland et al.[15] may be considered as a routine method for the determination of the contents intermediates of oxidative hair dyes in commercial preparations. Earlier, this HPLC (high performance liquid chromatography) method did not work satisfactorily at NERI, because the HPLC peaks were broad, non-symmetrical and they were tailing. In 1998, Pel et al.[16] reported a modified version of the method of Weyland et al.[15]. However, the modified method[16] also did not work satisfactorily in our laboratory due to asymmetric peaks and peak tailing. We have also attempted to modify the method of Pel et al.[15] in several ways, but it was impossible to get good peak shape of intermediates of hair dyes. In the mean time, we have developed a new method in which ion-pairing of in-

termediates of oxidative hair dyes is followed by HPLC analysis employing a reverse phase column packed with a newly developed amide bonded C_{16} silica. This method and its application for the determination of contents of some commonly used intermediates of oxidative hair dyes (Table 1) are described in the present report. While our work was in progress, a new HPLC column material based on hypercross-linked polystyrene has also been reported to be suitable for the analysis of intermediates of oxidative hair dyes[17]. However, the chromatograms described in this paper do not convince that this method may be better than the methods reported earlier.

The present work has been performed as technical support to Danish EPA.

***Table 1**:* Intermediates of oxidative hair dyes investigated.

Compound	Short name referred in the text
1, 2-phenylene diamine	1,2-DPA
1, 3-phenylene diamine	1,3-PDA
1, 4-phenylene diamine	1,4-PDA
2,4-toluene diamine	2,4-TDA
2,5-toluene diamine	2,5-TDA
2,6-toluene diamine	2,6-TDA
3,-toluene diamine	3,6-TDA
2-aminophenol	2-AP
3-aminophenol	3-AP
Resorcinol	Resorcinol
1-naphthol	1-naphthol
Hydroquinone	Hydroquinone

2 Products

56 cosmetic products for hair coloration, both for professional and non-professional use, were analysed in the present study. These products were from various cosmetics manufacturing companies in several EU Member States and USA. The products were obtained through the manufactures/importers of cosmetic products in Denmark, in August-September 1998. The colorants, semi-permanent dyes and intermediates of oxidative hair dyes labelled on these products are described in Table 2.

Table 2: Labelled colorants, semi-permanent dyes, intermediates of oxidative hair dyes and related compounds on the investigated products

2-amino-6-chloro-4 nitrophenolenol 4-aminocresol/p-aminocresol 6-aminocresol 2-amino-3-hydroxy pyridine 4-amino-2-hydroxytoluene 2-amino-4-hydroxyethylamino anisole sulfate Aminomethylpropanol 2-amino-3-nitrophenol 4-amino-3-nitrophenol m-aminophenol p-aminophenol Ammonia Ammonium acrylate copolymers Ammonium polyacrlates Basic blue 26, Basic Blue 99, Basic Brown 16, Basic Red 76, Basic violet7, Basic Violet14, Basic Yellow 57 N^4,N^4-bishydroxyethyl-p-phenylene diamine sulfate *t*-butylhydroquinone 2-chloro-5-nitro-N-hydroxyethyl-p-phenylene diamine 6-chloro-2-nitro-2-aminophenol. HCl 4-chlororesorcinol CI 15850, CI 15985, CI 16035, CI 19140, CI 42090, CI 42510, CI 60725,CI 76060, CI 76505, CI 76545, CI 76605, CI 76510, CI 76520, CI 77007, CI 77499, CI 77891, DC Violet 2 2,4-diaminophenoxyethanol. HCl Diaminophenols Diaminotoluenes /2,5-toluenediamine sulfate/-phenylenediamines Ethanolamine 2,6-dimethoxy-3,5-pyridine diamine Disperse Black 9, Disperse Blue 3, Disperse Violet 1	HC Blue 2, HC Blue 8, HC Orange 2, HC Red No. 3, HC Red 7, HC Red 10, HC Red 11, HC Red 12, HC Red 13, HC Violet 1, HC Violet 2, HC Yellow 2, HC Yellow 6, HC Yellow 7, HC Yellow 9, HC yellow 10, HC Yellow 11, HC Yellow 12 2-hydroxyaminoethyl-5-nitroanisole Hydroxyanthraquinone aminopropyl methylmorpholinium methosulfate Hydroxybenzomorpholine Hydroxyethyl-3,4-methylene dioxyaniline.HCl Hydroxyethyl-2nitro-p-toluidine 6-hydroxyindole Hydroxymethyl aminophenol 2-hydroxyethyl picramic acid 4-hydroxypropylamino-2-nitrophenol 4-hydroxypropylamino-3-nitrophenol Hydroxypropyltrimonium hydrolysed wheat protein p-methylaminophenol 1-methoxy-2-amino-1-beta-hydroxyethyl aminobenzene 3-methylamino-4-nitrophenoxyethanol 4-methylenedihydroxy aniline. HCl 2-methyl-5-hydroxymethyl aminophenol 2-methyl resorcicnol 1,5-naphthaleindiol 2,7-naphthaleindiol 1-naphthol 2-nitro-5-glyceryl methylaniline 3-nitro-p-hydroxymethyl aminophenol 4-nitrophenyl aminoethylurea 6-nitro-o-toluidine Phenylene diamines/Benzene diamines 1-phenyl-3-methyl-5-pyrazolone N-phenyl-p-phenylene diamine sulfate Picramic acid Resorcinol 2,4,5,6-tetraminopyridine

3 Experimental

The details of the method used for the identification and determination of contents of intermediates of oxidative hair dyes are described in Annex of the present report. The method employs ion-pairing of intermediates of oxidative hair dyes followed by HPLC analysis with gradient elution. The mobile phase for HPLC was comprised of phosphate buffer (pH 6)-acetonitrile, and a reverse phase HPLC column packed with amide-bonded silica was used for the analysis. The detection was performed using a photodiode array detector.

10 µl of solution(s) of a target compound(s) was injected into the HPLC column and the data was acquired continuously in the wavelength range 220 nm-400 nm. The chromatographic data was processed to obtain a max-plot chromatogram of the substances, where a max-plot chromatogram of a substance is defined as the chromatographic peak of the substance at its λ_{max}. With the use of the chromatographic data of target substances, a spectral library 'Hair colors' consisting of 220 nm-400 nm spectra of the target compounds and their HPLC-retention times (t_R) was build for the identification of unknown substances. The spectra included in the spectral library were from the analyses in which UV-absorbance at the HPLC peak-apex of the target substances were 0.05-0.80 AU.

A solution/homogeneous suspension of a cosmetic formulation is prepared in phosphate buffer-sodium ascorbate–acetonitrile and filtered through a 0.45 µ membrane filter. 10 µl of the solution is injected into the HPLC column and data acquired as for the standard substances. The identification of intermediates of oxidative hair dyes in the product is performed by comparing the spectra and the retention times (t_R) of the unknown substances with the spectra and t_R of the standard substances in the spectrum library 'Hair colors'.

The determination of the contents of target compounds was performed by the use of calibration curves of the standard compounds. To check the suitability of the method for the quantification, the linearity of calibration curves of the target compounds were evaluated, where calibration standard solutions containing 40-800 mg/L of all compounds, except 1-naphthol (6-130 mg/L) and 1,3-PDA (21-410 mg/L) were analysed. In addition, the stability of the solutions of all of the substances stored at 4°C and at room temperature (22°) was also evaluated. The repeatability of the method was determined by 10 repeated analysis of calibration standard solutions (Cal A III and Cal B III, cf. Annex). Furthermore, the suitability of the method was also evaluated by determining the recovery of target compounds from two commercial hair coloring formulations spiked with these substances. One of the samples used for recovery studies did not contain any of the target substances, and the other contained 3 of the target substances.

4 Results and Discussion

A method is developed for the identification and determination of contents of some commonly used primary and secondary intermediates of oxidative hair dyes in cosmetic formulations. The method is based on ion-pairing of the target substances, followed by reverse phase HPLC with gradient elution employing an amide bonded silica column. The compounds eluted from the column are detected using a photodiode array detector. A spectral library consisting of 220 nm– 400 nm spectra and HPLC-t_R of the standard compounds, analysed under the same conditions as samples, has been build for the identification of oxidative hair dye intermediates in cosmetic formulations. The chromatography of single target compounds revealed that 2 of the target compounds (1,2-PDA and 3,4 -TDA, purity >90% of both compounds) showed several peaks. The spectra and of t_R of maximum 3 major peaks of these compounds were included in the spectrum library. As it was not possible to ascertain the 'real' peak of 1,2-PDA and 3,4 –TDA, it was decided to perform only identification of these compounds in hair care formulations.

The chromatographic separation of the target standard compounds is shown in Figure 1, and their t_R are described in Table 3. Day-to-day variation of t_R of all of the target substances, observed for 3 months, was within 2%. The identification is performed by comparing t_R as well as the 220 nm-400 nm spectrum of an unknown substance with the t_R and spectra of standard compounds in the spectral library. An example of identification of target compounds (by t_R and spectrum match) in a hair color formulation is shown in Figure 2. The parameters that may influence the identification, especially the concentration of an unknown substance compared to the concentration of standard compound for spectrum match, have earlier been described in detail (18).

To establish the detection limits of the target substances, serial dilutions of the calibration standards were analysed. It was found that 5 µg/g or less of these substances could be identified as sharp peaks without significant base line noise (Figure 3). Thus, the detection limit of all of the target substances, except 1,2-PDA and 3,4 –TDA, is below 5 µg/g (0,005%). It may not be necessary to analyse <5 µg/g (corresponding to approximately 100 µg/g in the products) solutions of standard compounds, as the dye intermediates may not be used at concentration <100 µg/g in the cosmetic products. However, still lower contents (<100 µg/g) of the target compounds in a product can be detected by increasing the HPLC-injection volume from 10 µl to 50 µl for the HPLC analysis.

The quantification of target compounds was performed by the use of calibration curves of the standard compounds. The peak areas in max-plot chromatograms were used for determination of the contents of the identified substances. The calibration curves of the compounds determined in the present investigation are shown in Figure 4. All calibration curves were linear (r^2 >0.999) in the investigated concentration range: 40-800 mg/L of all of the target substances, except 1-naphthol (6-130 mg/L) and 1,3-PDA (21-410 mg/L). The relative standard deviation of the determination, measured within a day, for all of the target substances was within

5%. The recoveries of the target substances evaluated by the analysis of a sample (not containing any of the target compounds) spiked at two concentration levels of the target substances, were found to be between 89-101% (Table 4). The **r**ecoveries of 2,5-TDA, 3-AP and resorcinol were also determined using another sample (spike level III of Cal A and Cal B, cf. Annex), which contained these 3 substances. The recoveries were found to be similar to those found in the first case, i.e. 95.4%, 93.3%, and 93.9% respectively for 2,5-TDA, 3-AP and resorcinol.

The stability of the calibration solutions (Cal A III and Cal B III. cf, Annex), stored in dark bottles at room temperature (22°C) and at 4°C, were checked on day 2, day 3 and day 7. It was found that the concentrations of target compounds decreased with the increasing storage time (Figure 5). The concentration of target compounds (amino compounds specifically) decreased 2-4%, when their solutions were stored 1 day at 22°C. However, when the respective solutions were stored at 4°C, a 2-4% decrease in the concentration of target compounds was observed after 2 days. As the relative standard deviation of the method was within 5%, it was acceptable that the stock solutions of the standards (stored at 4°C) could be used up to 2 days; and the sample extracts were analysed within 24 hours after sample preparation.

The content of target compounds in 56 products for hair coloration, which were analysed 2 years back for the contents of colorants, was investigated employing the present method. As the composition of some of the products (with respect to the contents of intermediates of oxidative hair dyes) might have changed during the 2 years storage, the investigation has been considered as 'screening analysis' of target compounds in hair-coloring formulations. Results of the screening analysis of the hair-coloring products investigated in the present study are described in Table 6. Among the target compounds, 1,4-PDA, 2,5-TDA, 3-AP, resorcinol and 1-naphthol were frequently present in the products investigated; while 1,2-PDA, 2,4 –TDA, 2,6- TDA, 3,4-TDA were not found in any of the products.

The method developed in the present study was also evaluated for the suitability of analysis of some non-target intermediates of oxidative hair dyes. Thus, qualitative analysis of relevant compounds (available in NERI's stock) was performed. As shown in Figure 6, a number of important intermediates of oxidative hair dyes could nicely be separated from each other, by the present method. These substances have been included in the spectral library 'Hair colour'. The validation of the method for the determination of these substances will be undertaken later.

In conclusion, a method has been developed and evaluated for the analysis of some commonly used intermediates of oxidative hair dyes in cosmetic products. The method has been found to be suitable for the identification and determination of the contents of the target compounds in a series of cosmetic formulations. The method developed in the present investigation may also be suitable for the identification and determination of the contents of several other intermediates of oxidative hair dyes.

Table 3: HPLC-retention times (t_R) of the target intermediates of oxidation hair dyes.

Compound	***t_R (min)***
1,4-PDA	4.98
1,3-PDA	5.29
2,6-TDA	6.06
2,5-TDA	6.23
3-AP	6.77
Hydroquinone	7.42
2,4-TDA	8.20
2-AP	9.29
Resorcinol	11,15
1-naphthol	30.01
1,2-PDA*	7.10, 7.40, 13.22
3,6-TDA*	3.86, 13.74, 20.22

* t_R of the 3 major peaks of the compound

Table 4: Recovery of intermediates of oxidation hair dyes from the sample 8-0431 spiked with two concentrations of these substances.

Compound	***Spike level µg/g***	***% Recovery***	***Spike level µg/g***	***% Recovery***
1,3-PDA	105	96.6	53	97.1
1,4-PDA	208	92.4	104	89.3
2,4-TDA	50	96.0	25	96.0
2,5-TDA	204	94.5	102	93.4
2-AP	202	95.6	101	95.0
3-AP	205	93.6	103	90.2
1-naphthol	6.5	95.3	3.3	95.4
Resorcinol	51	94.6	26	93.6
Hydroquinone	201	100.9	101	96.4

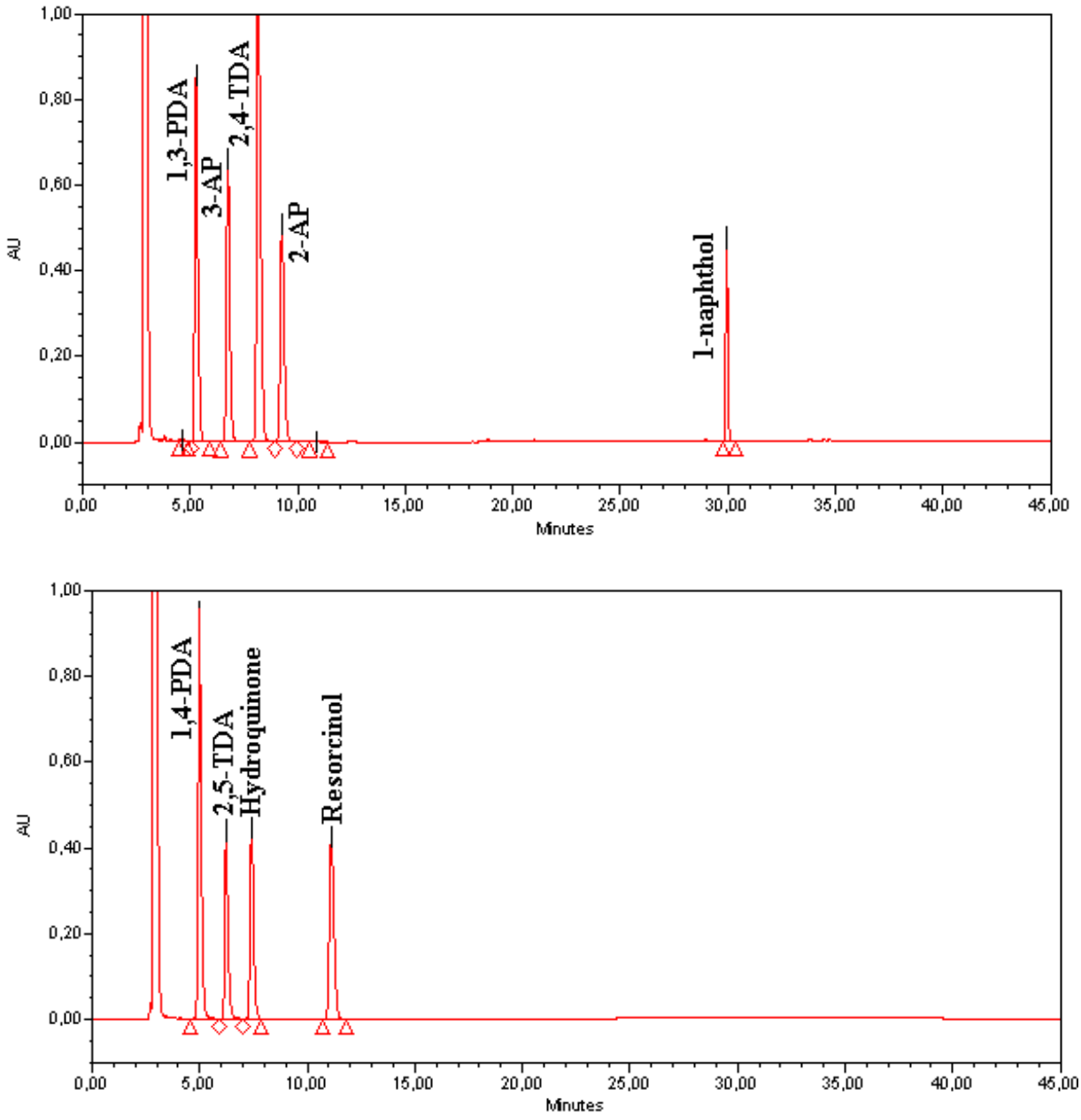

***Figure 1*:** HPLC separation of intermediates of oxidative hair dyes. Top: 1,3-PDA (108.8 mg/L), 3-AP (206.9 mg/L), 2,4-TDA (207.1 mg/L), 2-AP (204.8 mg/L), 1-naphthol (21.3 mg/L). Bottom: 1,4-PDA (206.6 mg/L), 2,5-TDA (211.3 mg/L), hydroquinone (202.28 mg/L), resorcinol (208.4 mg/L). Injection volume: 10 µl.

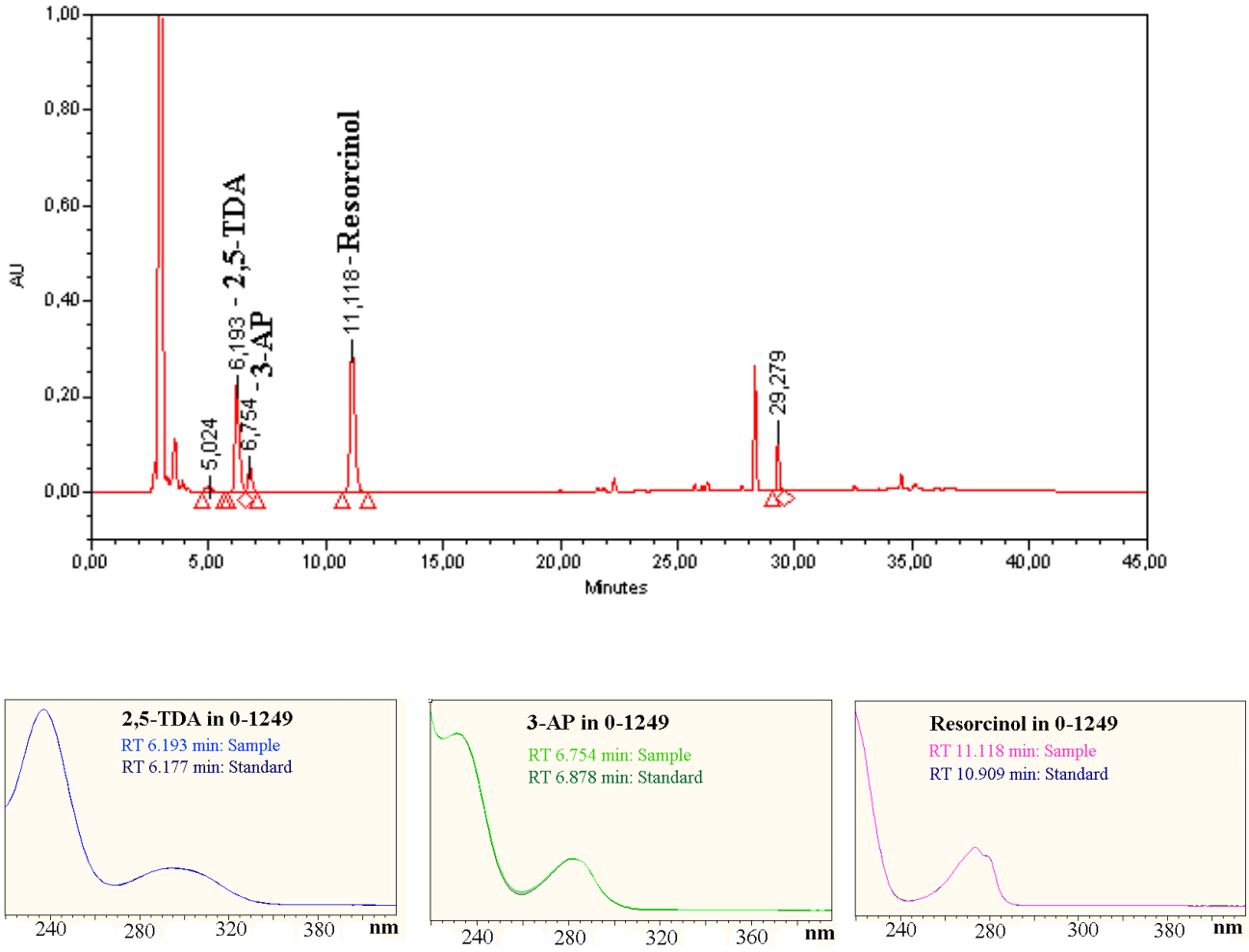

Figure 2: Identification of intermediates of oxidative hair dyes in the Sample No. 0-1249.

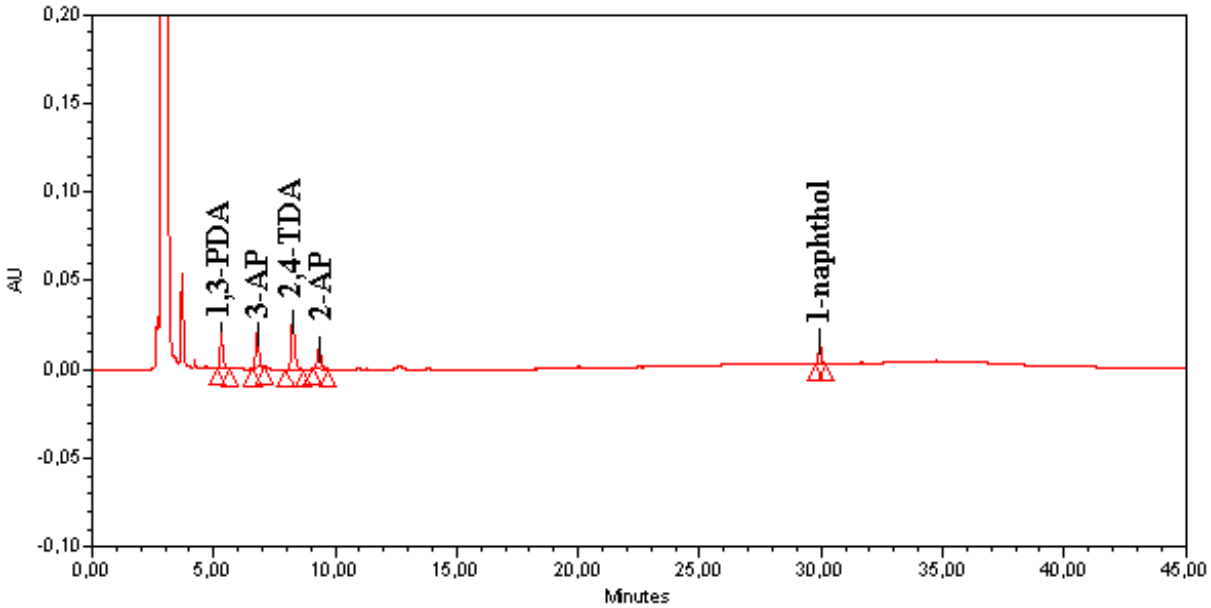

Figure 3: HPLC chromatogram of lowest concentration of some of the target compounds analysed. 1,3-PDA: 2.5 µg/g, 3-AP: 5.3 µg/g, 2,4-TDA: 5.2 µg/g , 2-AP: 5.1 µg/g, 1-naphthol: 0.7 µg/g

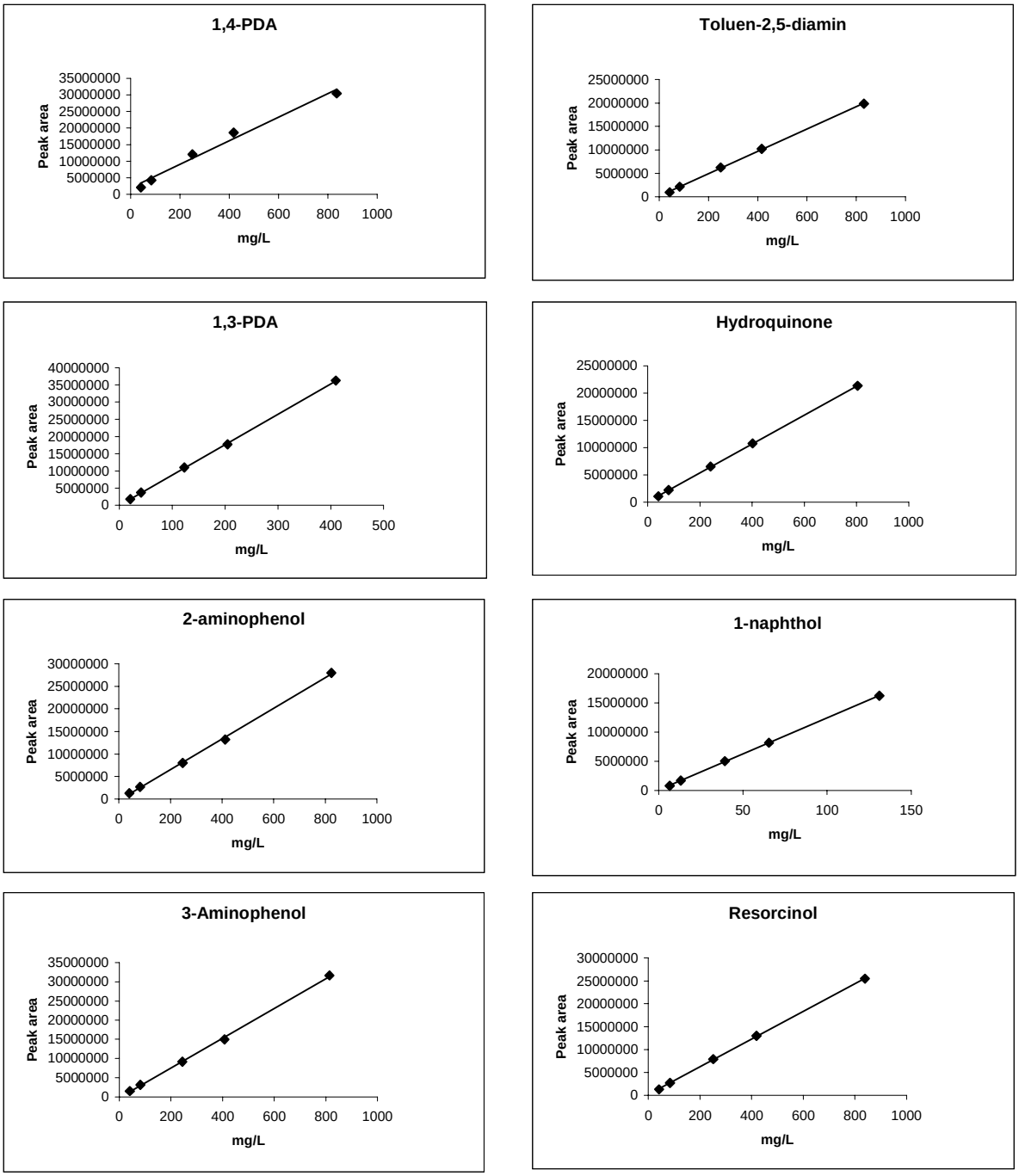

Figure 4: Calibration curves of the target intermediates of oxidative hair dyes.

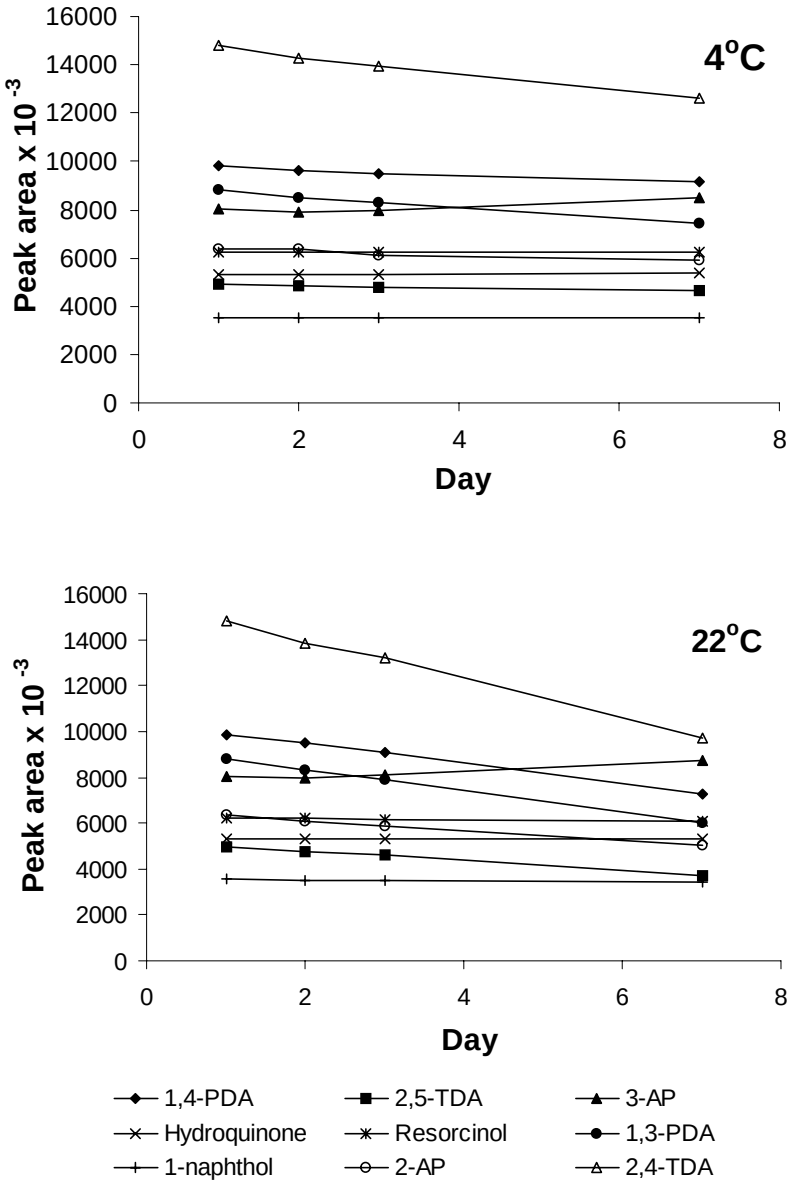

Figure 5: Stability of intermediates of oxidative hair dyes in solution. 1,4-PDA (212 mg/L), 2,5-TDA (203 mg/L), 3-AP (212 mg/L), Hydroquinone (202 mg/L), Resorcinol (203 mg/L), 1,3-PDA (99 mg/L), 1-naphthol (28 mg/L), 2-AP (202 mg/L), 2,4-TDA (207 mg/L).

Table 5: Contents (% m/m) of target substances identified in the cosmetic products investigated.

NERI No.	*DEPA No.*	*1,3-PDA*	*1,4-PDA*	*2,5-TDA*	*2-AP*	*3-AP*	*Resorci-nol*	*1-naphthol*	*Hydro-quinone*
8-0390	740	n.d.	n.d.	0.058	n.d.	n.d.	0.001	0.005	n.d.
8-0391	742	n.d.	n.d.	0.393	n.d.	0.040	0.339	0.028	n.d.
8-0392	745	n.d	n.d.	0.555	n.d.	n.d.	0.090	n.d.	n.d.
8-0395	753	n.d.	n.d.	0.382	n.d.	0.026	0.206	n.d.	n.d.
8-0396	754	n.d.	n.d.	0.056	n.d.	0.005	0.0144	n.d.	n.d.
8-0397	760	n.d.	0.161	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0399	765	0.002	n.d.	n.d.	n.d.	n.d.	n.d.	0.314	n.d.
8-0400	766	0.011	n.d.	0.108	n.d.	n.d.	n.d.	n.d.	n.d.
8-0401	767	n.d.	n.d.	0.596	n.d.	n.d.	n.d.	n.d.	n.d.
8-0402	768	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0403	769	n.d.	n.d.	0.022	n.d.	n.d.	n.d.	0.275	n.d.
8-0404	770	n.d.	0.432	n.d.	0.029	0.025	0.006	n.d.	n.d.
8-0405	771	n.d.	n.d.	0.291	n.d.	0.055	0.385	n.d.	n.d.
8-0408	774	n.d.	0.244	n.d.	n.d.	n.d.	0.159	n.d.	n.d.
8-0409	775	n.d.	0.043	n.d.	n.d.	0.006	n.d.	0.044	n.d.
8-0411	777	n.d.	n.d.	0.497	n.d.	n.d.	0.050	0.004	n.d.
8-0413	779	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0414	780	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0415	781	n.d.	n.d.	0.053	n.d.	0.002	0.029.	n.d.	n.d.
8-0416	782	0.005	n.d.	0.105	n.d.	n.d.	0.006	n.d.	n.d.
8-0417	783	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0421	787	n.d.	0.129	n.d.	n.d.	0.002	0.004	n.d.	n.d.
8-0422	788	n.d.	0.287	n.d.	n.d.	n.d.	0.008	n.d.	n.d.
8-0427	793	n.d.	0.266	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0428	794	n.d.	0.009	n.d.	n.d.	n.d.	0.074	0.014	n.d.
8-0429	795	n.d.	0.014	n.d.	n.d.	n.d.	0.019	0.014	n.d.
8-0430	811	n.d.	0.303	n.d.	n.d.	n.d.	0.002	n.d.	n.d.
8-0431	812	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0432	814	n.d.	0.128	n.d.	n.d.	n.d.	0.005	n.d.	n.d.
8-0433	815	n.d.	0.228	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0434	818	n.d.	0.199	n.d.	n.d.	0.030	0.082	n.d.	0.031
8-0435	819	n.d.	n.d.	0.366	n.d.	n.d.	n.d.	n.d.	n.d.
8-0436	820	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0437	822	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0440	826	n.d.	n.d.	0.393	n.d.	0.037	0.149	n.d.	n.d.
8-0441	827	n.d.	n.d.	0.209	n.d.	n.d.	n.d.	n.d.	n.d.
8-0442	828	n.d.	n.d.	n.d.	n.d.	n.d.	0.0002	n.d.	n.d.
8-0443	838	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	n.d.
8-0444	839	n.d.	n.d.	0.274	n.d.	n.d.	0.023	0.001	n.d.
8-0445	840	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-0446	841	n.d.	n.d.	n.d.	n.d.	n.d.	0.002	n.d.	n.d.
8-0448	843	n.d.	n.d.	0.498	n.d.	0.037	0.127	n.d.	n.d.
8-0449	844	n.d.	n.d.	0.567	n.d.	n.d.	0.008	n.d.	n.d.
8-0451	846	n.d.	n.d.	0.141	n.d.	n.d.	0.009	n.d.	n.d.
8-0452	847	n.d.	n.d.	0.542	n.d.	n.d.	0.009	n.d.	n.d.
8-0453	848	n.d.	n.d.	0.746	n.d.	n.d.	0.134	n.d.	n.d.
8-0454	849	n.d.	n.d.	0.528	0.024	0.192	0.440	n.d.	n.d.
8-0455	850	n.d.	n.d.	0.275	0.054	0.064	0.455	n.d.	n.d.
8-0461	831	n.d.	n.d.	n.d.	n.d.	n.d.	0.003	0.261	n.d.
8-0462	832	0.045	0.408	n.d.	n.d.	0.094	n.d.	n.d.	n.d.
8-0463	833	n.d.	0.092	n.d.	n.d.	n.d.	0.038	0.166	n.d.
8-0464	834	n.d.	0.002	0.036	n.d.	0.029	0.190	0.031	0.003
8-0465	835	n.d.	n.d.	0.041	n.d.	n.d.	0.290	n.d.	n.d.
8-0466	836	n.d.	n.d.	0.047	n.d.	0.084	0.073	0.055	n.d.
8-0467	837	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

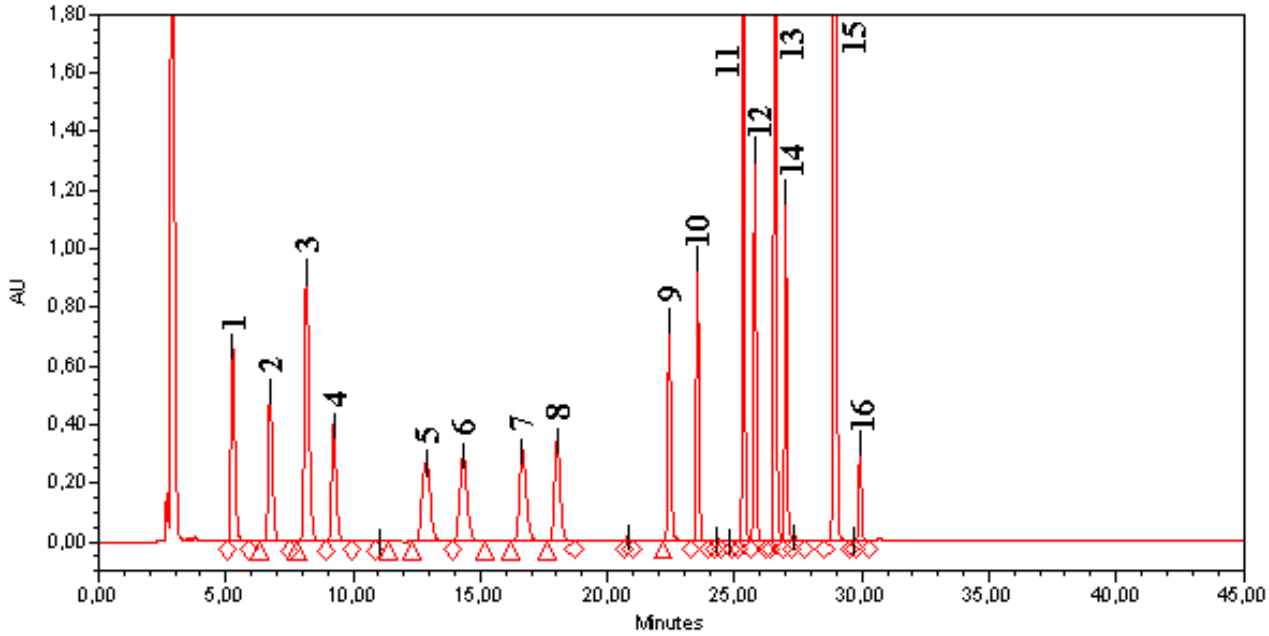

Figure 6: HPLC separation of intermediates of oxidative hair dyes.**1**: 1,3-PDA, **2**: 3-AP, **3**: 2,4-TDA, **4**: 2-AP, **5**: p-anisidine, **6**: 5-amino-o-cresol, **7**: 2-methyl resorcinol, **8**: 2-amino-p-cresol, **9**: 2-amino-4-nitrophenol, **10**: 2-amino-5-nitrophenol, **11**: 2-nitroaniline, **12**: 2-chloroaniline, **13**: 1,6-dihydroxy naphthalene, **14**: 4-aminodiphenylamine, **15**: 2-naphthol, **16**: 1-naphthol.

5 References

1. M.G. deNavarre, The chemistry and manufacture of cosmetics, Vol. IV, Continental Press, Orlando, Florida, 1975, p. 864.

2. J.F. Corbet, Chemistry of hair colorant processes- Science as an aid to formulation and development. J. Soc. Cosmet. Chem. **35**, 297-310 (1984).

3. Council Directive *76/768/EC* of 27th July on the approximation of the laws of Member States relating to cosmetic products. EC Offical J., No. L262, 27.9.1976, p.169.

4. Miljø- og Energiministeriets bekendtgørelse om kosmetiske produkter . Bekendtgørelse nr. 594 af 6. juni 2000

5. H. Gottschalck, R. Machens, Identifizierung und quantitative bestimmung von oxidationsfarbestoffen in haarfärbemitteln und haartönungsmitteln. J. Soc. Cosmet. Chem. **33**, 97-114 (1982)

6. N. Goetz, P.Lasserre, P. Boré, G. Kalopissis, Precutaneous absorption of p-phenylene diamine during an actual hair dyeing procedure. Int. J. Cosmet. Sci. **10**, 63-73 (1988).

7. S. Fanali, Host-guest complexion in capillary isotachophoresis. II. Determination of aminophenol and diaminobenzene isomers in permanent hair colorants by using capillary isotachphoresis J. Chromatogr., **470**, 123-129 (1989)

8. M. C. Gennaro, P.L. Bertolo, E. Marengo, J. Chromatogr., Determination of aromatic amines at trace levels by ion-interaction reagent reversed-phase high performance liquid chromatography. Analysis of hair dyes and other water soluble dyes. **518**, 149–156 (1990).

9. L. Gagliardi, M. Ambroso, J. Mavro, F. Furno, G. Discalzi, Exposure to paraphenylene diamine in hair dressing parlours. Int. J. Cosmet. Sci., **14**, 19-31 (1992).

10. C. J. Dowle and, A. P. Malyan, A.M. Matheson, Separation of the *ortho*, *meta* and *para* isomers of aminophenol by high-performance liquid chromatographyAnalyst, **115**, 105-107 (1990)

11. H. Tokuda, Y. Kimura, S. Takano, Determination of dye intermediates in oxidative hair dyes by fused-silica capillary gas chromatography, J. Chromatogr. **367**, 345-356 (1986)

12. N. Goetz, J. Mavro, L. Bouleau, A. de Labbey, “Analysis of oxidative hair dyes using high performance liquid chromatography” in Cosmetic Analysis, P. Boré (Editor), Marcel Dekker, New York, 1985, pp. 245-287.

13. V. Andrisano, R. Gotti, A.M. Di Pietra, V. Cavrini, HPLC analysis of oxidation hair dyes in permanent hair colorants. J. Liquid Chromatogr. **17**, 2919-2937 (1994)

14. V. Andrisano, R. Gotti, A.M. Di Pietra, V. Cavrini, Analysis of basic hair dyes by HPLC with on-line post-column photochemical derivatization. Chromatographia **39**, 138-145 (1994)

15. J. Weyland and J. Rooselaar HPLC of oxidative hair dyes - chromatographical behaviour and method development, Ispectie Gezondheidsbescherming, Keuringsdienst van Waren, Enschede, Netherlands. Cosmetica Rapport 50, 1991.

16. E. Pel, G. Bordin, A. R. Rodriguez, HPLC candidate reference method for oxidative hair dye analysis. I. Separation and stability testing. J. Liq. Chromatogr. & Rel. Technol. **21**,883-901 (1998).

17. Natalia A. Penner and Pavel N. Nesterenko, Simultaneous determination of dihydroxybenzenes, aminophenols and phenylenediamine in hair dyes by high-performance liquid chromatography on hypercross-linked polystyrene. Analyst **125**, 1249-1254 (2000).

18. Rastogi S.C., Jensen GH, Jensen C.D. Frausig A.H. Identification of organic colourants in cosmetics by HPLC-photodiode array detection Chemical substances and chemical preparations. National Environmental Research Institute, Roskilde, Denmark. NERI Technical Report No. **183**, 1997. pp 233.

Annex I
Method for the analysis of oxidative hair dyes in cosmetic products

1. Scope and field of application

This method is suitable for the analysis of intermediates of oxidative hair dyes: phenelyene diamines, toluene diamines, aminophenols, resorcinol, 1-naphthol and hydroquinone in cosmetic formulations.

2. Principle

The target substances are extracted in acetonitrile-phosphate buffer containing an antioxidant and an ion-pairing reagent. The extract is analysed by reverse phase HPLC (high performance liquid chromatography) with gradient elution, followed by detection employing a photodiode array detector. Quantification of the target substances is performed by the use of calibration curves of the respective compounds.

Safety: Some of the analytes are allergenic and they may be harmful. The skin contact with the analytes must be avoided.

3. Chemicals and Reagents

3.1 All the chemicals should be of analytical grade, and HPLC grade where necessary. The water should be Milli Q grade or equivalent.
3.2 Potassium dihydrogen phosphate, KH_2PO_4
3.3 Disodium hydrogen phosphate, Na_2HPO_4
3.4 1-heptanesulfonic acid, sodium salt
3.5 Sodium ascorbate
3.6 Acetonitrile
3.7 1,3-pheneylene diamine (1,3-PDA)
3.8 1,2-pheneylene diamine (1,2-PDA)
3.9 1,4-pheneylene diamine (1,4-PDA)
3.10 2,4-toluene diamine (2,4-TDA)
3.11 2,5-toluene diamine (2,5-TDA).2HCl
3.12 2,6-toluene diamine (2,6-TDA)
3.13 3,4-toluene diamine (3,4-TDA)
3.14 2-aminophenol (2-AP**)**
3.15 3-aminophenol (3-AP**)**
3.16 Resorcinol
3.17 Hydroquinone
3.18 -naphthol
3.19 25 mM/L phosphate buffer-0.1% heptane sulphonic acid: In a 2 L volumetric flask, dissolve by shaking 6.12 g KH_2PO_4 (3.2), 0.76 g Na_2HPO_4 (3.3) and 2 g sodium salt of 1-heptane sulfonic acid (3.4) in 1 L water. Fill up to the mark and mix. *The pH of the buffer should be 6.0, otherwise adjust pH 6.0 by the addition of 1 M NaOH or 2 M H_3PO_4.*

3.20 0.5% sodium ascorbate solution: Dissolve 2.5 g sodium ascorbate (3.5) in 500 ml of phosphate buffer (3.19). *The solution is stable for 2 weeks at 4°C.*

3.21 Stock solutions of standard substances: Weigh accurately approximately 0.025 g of the compound 3.7, 0.05 g of the compounds 3.8-3.17 and 0.005 g of the compound 3.18 in separate 10 ml volumetric flasks, dissolve by shaking in 4 ml sodium ascorbate solution (3.20), fill up to mark with acetonitrile and mix.

3.22 Calibration standard solutions: Dilute stock solution to prepare 2 separate calibration standards, Cal A (I-V) and Cal B (I-V), as described in Table 1. The calibration solutions were stored for maximum 2 days.

Note 1*: Concentrations of some of the compounds in calibration solutions decreased 2-4%, when these were stored in dark containers for 2 days at 4°C. A similar decrease in the concentration of the respective compounds was found, when the solutions were stored at 22°C for 1 day.*

4. Apparatus, glass- and palsticware

4.1 Normal laboratory equipmment, glass- and plasticware

4.2 0.45 µ membrane filters, PTFE (Sartorius Minsart)

4.3 HPLC system: Waters HPLC pump 616 with controller 600S, autosampler 717 plus, photodiode array detector 996 and chromatography software Millenium 3.2

4.4 HPLC column for the analysis was Discovery™ RP-amide C_{16}, 250mm x 4.6 mm, particle size 5µ from Supelco; and the gaurd coulm used was C18 Security gauard from Phenomenex.

Table 1: Preparation of calibration standard solutions

Cal A	Cal B
I Transfer by pipette 4 ml stock solutions (3.21) of each of the compounds 3.7, 3.10, 3.14, 3.15 and 3.18 in a 25 ml volumetric flask, fill up to the mark with acetonitrile and mix.	I Transfer by pipette 4 ml stock solutions (3.21) of each of the compounds 3.9, 3.11, 3.16 and 3.17 in a 25 ml volumetric flask, fill up to the mark with acetonitrile and mix.
II Transfer by pipette 5.0 ml of Cal A-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.	II Transfer by pipette 5.0 ml of Cal B-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.
III Transfer by pipette 3.0 ml of Cal A-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.	III Transfer by pipette 3.0 ml of Cal B-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.
IV Transfer by pipette 1.0 ml of Cal A-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.	IV Transfer by pipette 1.0 ml of Cal B-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.
V Transfer by pipette 0.5 ml of Cal A-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.	V Transfer by pipette 0.5 ml of Cal B-I and 4 ml sodium ascorbate solution in a 10 ml volumetric flask, fill up to the mark with acetonitrile and mix.

Note 2*: The compounds 3.8, and 3.13 were not included in calibration standard solutions beacuse they appeared to be mixture of several substances. These substances were analysed individually.*

5. Chromatographic procedure

5.1 HPLC system (4.3) and HPLC column (4.4)
5.2 Column temperature 25±1°C
5.3 Injection volume: 10 µl
5.4 Gradient elution as described in Table 2, Analysis time: 50 min.
5.4 Detection was performed at wavelength range 200 nm-400 nm: collect data as 1 spectrum/s, using spectrum resolution 1.2 nm.

Table 2: Mobile phase for gradient elution. Analysis time: 50 min

Time (min)	***Flow (ml/min)***	***% A***	***% B***	***% C***	***Curve***
0.0	1.0	90.0	10.0		Linear
10.0	1.0	90.0	10.0		Linear
20.0	1.0	50.0	50.0		Linear
30.0	1.0	20.0	80.0		Linear
40.0	1.0	90.0	50.0		Linear
55.0	1.0	90.0	10.0		Linear
70.0	1.0	0.0	50.0	50.0	Linear
80.0	1.0	0.0	50.0	50.0	Linear
90.0	1.0	0.0	100.0		Linear
150.0	0.5	0.0	100.0	0.0	Linear
155.0	0.0	0.0	100.0	0.0	Linear

A: phosphate buffer-1-heptane sulfonic acid (3.19), B: acetonitrile and C: water.

Note 3*: The conditions described above also take care of safely switching over of the column packing in acetonitrile, for resting period. For safely switching over of column packing material to HPLC conditions, mobile phase program described in Table 3 should be adapted.*

Table 3: *Mobile phase program for restoring the column for analysis*

Time (min)	***Flow (ml/min)***	***% A***	***% B***	***% C***	***Curve***
0.0	1.0	90.0	10.0		Linear
10.0	1.0	90.0	10.0		Linear
20.0	1.0	50.0	50.0		Linear
30.0	1.0	20.0	80.0		Linear
40.0	1.0	90.0	50.0		Linear
55.0	1.0	90.0	10.0		Linear
70.0	1.0	0.0	50.0	50.0	Linear
80.0	1.0	0.0	50.0	50.0	Linear
90.0	1.0	0.0	100.0		Linear
150.0	0.5	0.0	100.0	0.0	Linear
155.0	0.0	0.0	100.0	0.0	Linear

6. Spectral library

6.1 Perform HPLC analysis of 10 µl of individual standard solutions (3.21) at concentration levels corresponding to Cal III (diluted as described in 3.22) and record the HPLC-retention time (t_R) and 220 nm-400 nm spectrum of each compound.

6.2 With the use of Millenium software, process the data to create a max-plot chromatogram, and build a spectral library 'Hair colors'consisting of the t_R and spectrum (at peak-apex) of the compounds. Ideally the absorbance at λ_{max} for each compound in spectral library should be 0.05-0.8 AU. If the absorbance is below 0.05 AU, a concentrated solution of the respective compound should be analysed, or up to 50 µl of the solution may be analysed. In case of absorbance is higher than 0.8 AU, the solution should be diluted appropriately.

7. Sample analysis

7.1 Sample preparation

Weigh accurately approximately 1 g sample in a 50 ml dark glass bottle with screw cap. Transfer by pipette 10 ml sodium ascorbate solution (3.20) and 15 ml acetonitrile in the glass bottle containing sample, mix and heat the contents at 60±1°C for 5 min. Shake the bottle gently for 1 min and replace in an ultrasonic bath. After ultrasonic treatment for 5 min, cool to room temperature and filter the sample solution/suspension through a 0.45 µ membrane filter. Transfer the filtrate in dark HPLC vials and analyse within 24 hours.

7.2 Identification

7.2.1 Analyse 10 µl of the sample extract (7.1) and collect the chromatographic data.

7.2.2 Process the chromatographic data to obtain a max-plot chromatogram.

7.2.3 Compare the t_R and spectrum of each chromatographic peak (in the sample chromatogram) with the t_R and spectra of the standard compounds in the spectral library. The library match parameter for the automatic library search should be: wavelength 2.4 nm and retention time 5 %.

Note 4: *It was observed that automatic library search in some cases did not identify the substance(s) present in a sample. Therefore, a manual library search is recommended to check the results obtained by automatic library search. Some intermediates of oxidative hair dyes may have very similar spectra, but different retention times. Therefore, it is important that a maximum of 5% tolerance in the retention time is allowed for the identification by manual library search.*

7.3 Determination

7.3.1 Analyse 10 µl of each calibration standard solution (3.22 , Table 1) and collect the chromatographic data.

7.3.2 Prepare calibration curves of each of the target substance by plotting peak-area against the concentration of the respective sub-

stance. The coefficient of correlation (r^2) of all of the calibration curves should be ≥0.999. *The calibration curves were not forced to pass through Zero.*

7.3.3 With the use of calibration curve, calculate the concentration of the identified substances (7.3).

Note 5: *As some of the target compounds in solution are not stable for long time, one point* calibration may be used for routine analysis. In such a case, duplicate analysis of two sample extracts (prepared from two separate weighing) should be performed, and a suitable calibration solution (normally Cal III) must be analysed (in duplicate) before and after every second sample. The content C (%, m/m) of a compound in a product is then determined as described below:*

$$\%C = \frac{C_i \cdot A}{A_i \cdot m} \cdot 25 \cdot 10^{-4}$$

where
C_i: Content (mg/L) of the compound in the calibration solution
A_i: Peak area of the compound in the calibration solution
A: Peak area of the compound in the sample sample solution
m: Weight (g) of the sample used for the analysis

****If the contents of the target substances are close to the maximum allowed concentration, 5 point calibration curves of the respective substances must be used for the quantification.***

8. Report

Report the substances identified and their contents in mass percent (%, m/m) in a sample.

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