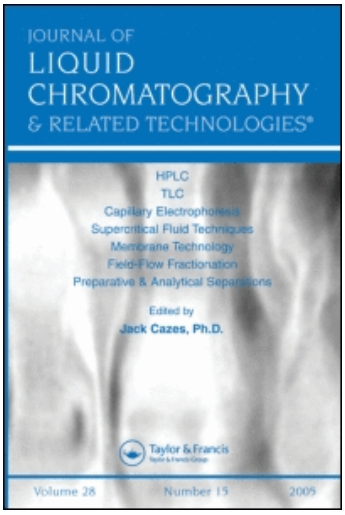

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HPLC CANDIDATE REFERENCE METHOD FOR OXIDATIVE HAIR DYE ANALYSIS. I. SEPARATION AND STABILITY TESTING

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ABSTRACT

Among the different methods of changing the color of human hair, oxidative hair dyeing plays an important role. The formulations consist of a broad spectrum of organic compounds among which most of the dye forming substances, i. e. the dye intermediates, are of aromatic nature. With the aim of establishing an analytical method for the separation and quantification of the dye intermediates, the chromatographic behavior of a selection of these products was investigated. The separation was done by HPLC, using reversed-phase chromatography. UV and diode array detection were employed. A quantitative separation procedure was established with which it is possible to separate and identify intermediates according to their retention times and their UV spectra.

In order to test the influence of light, temperature, and antioxidants on the sample solutions, changes in the chromatographic behavior of the investigated substances were recorded as a function of these parameters. It was shown that

their influence on the stability of the different substances varied. The consequences of these results are that the conditions for the analytical separation of hair dye intermediates must be very narrowly defined in order to obtain reproducible results.

INTRODUCTION

Today a choice between different organic hair dye formulations can be made if one wishes to change the color of one's hair. The great variety of products can be roughly classified in temporary, semipermanent, and permanent dyes. The difference in these products has not only to do with how long the color remains in the hair, but also with the dye forming mechanism. The oxidative dyes, which are the subject of this paper, belong to the group of permanent dyes. In the case of oxidative hair dyeing, a new hair color is the result of different chemical reactions which have taken place between the dye forming compounds. These reactions occur in the inner part of the hair fiber.

According to deNavarre,[1] the dye forming intermediates used in oxidative dyes are essentially aromatic diamines, aminophenols, and their derivatives. Secondary intermediates, such as polyhydric phenols and 1-naphthol are also essential for the dye forming process, they work as color modifiers, stabilizers, antioxidants, or retardants. In contrast to most of the secondary intermediates, the primary intermediates form dyes upon oxidation by themselves. Nitro dyes (nitrobenzenediamines and nitroaminophenols), as direct dyes, form an extra group because they do not require oxidation to color the hair. They are also used in oxidative dye formulations. Examples of some important intermediates and direct dyes are given in Table 1.

The mechanism of oxidative hair dyeing can be described as follows: small aromatic molecules enter the inner part of the hair fiber and once located there, an oxidizing agent induces a chemical reaction which leads to coupling of the molecules, which are then too large to escape out of the hair structure. During this coupling, chromophoric systems are formed which absorb light from the visible part of the spectrum. A description of the chemical reactions involved in the process of dye forming is described in an overview article by Corbett.[2]

A very important step in this process is the formation of a diimine from a 1,4-diamine. This diimine can be coupled with, for example, 3-diamine, 3-aminophenol, or 1-naphthol under oxidative conditions, resulting in dimeric and polymeric structures.

Table 1

Some Examples of Primary and Secondary Intermediates and Direct Dyes used in Oxidative Hair Dye Formulations

Primary Intermediates	Secondary Intermediates	Direct Dyes
1,4-phenylenediamine	1,3-phenylenediamine	2-nitro-1,4-phenylenediamine
2-aminophenol	3-aminophenol	4-nitro-1,2-phenylenediamine
4-aminophenol	resorcinol	

In most cases hydrogen peroxide is used as an oxidizing reagent, the oxidizing properties of which also lead to the decoloration of the melanin, the substance which formerly was responsible for the natural color of hair. So bleaching the natural hair color and dyeing the hair with the desired color can take place in one step.

Chromatographic methods are the most commonly used methods for identification and quantification of substances in oxidative hair dyes. In earlier times, thin layer chromatography (TLC) and gas chromatography (GC) were used.[3-6] Nowadays, high performance liquid chromatography (HPLC) gives an excellent opportunity for fast and precise analyses. With this separation method, quantification is easy and no problems with volatility occur. Theoretically all substances which are soluble in the used solvent can be separated.

For the determination of components which might appear in oxidative hair dyes, several HPLC methods have been proposed.[7-14] The differences between the described methods consist of variations of the chromatographic parameters and of the detection method. However, most of these works concern only a limited number of compounds.

Due to the many possibilities of synthesizing organic compounds with dyeing abilities, the number of substances used for hair dye formulations can not be determined exactly: up to now only a few of the hair dye producing companies list the ingredients contained in their products and, additionally, a range of organic dyes and dye forming compounds are protected by patents.

Some of the compounds, which could be used for the design of hair dye formulations, have a toxicological or sensitizing potential and are prohibited or restricted in concentration by the 6th Amendment of the European Union Council Directive 93/35/EEC. To identify and quantify the substances which could be used for the formulation of hair dyes, there is a need for a reliable analytical method. The aim of our studies, therefore, is to develop a reversed-phase method which enables the identification and quantification of a broad spectrum of possible dye forming compounds present in commercial oxidative hair dye products, in order to provide the European Commission with a candidate reference method.

In this first part, after optimization of the method, the chromatographic behavior and the stability of the active compounds (hair dye intermediates) prepared in standard solutions have been investigated.

MATERIALS AND METHODS

Instrumentation

All chromatographic separations were done using the following equipment: a two piston HPLC pump with a low pressure ternary gradient system module (System 325 from Kontron Instruments S.P.A., Milano, Italy), an autosampler 360 with a loop of 20 µL (Kontron Instruments S.P.A., Milano, Italy), a diode array detector 440 (Kontron Instruments S.P.A., Milano, Italy), and a vacuum degassing system Degasys DG 1300 (Uniflows, Japan). The column temperature was kept constant by means of a thermostat. Data processing was done with the Data System 450-MT2/DAD Series (Kontron Instruments S.P.A., Milano, Italy). The column was a Merck LiChrospher RP Select B, 250mm x 4mm, 5 µm particle size.

For UV irradiation tests a UV lamp from digestor equipment was used (Metrohm UV-Digestor 705). UV spectra of sample solutions not subjected to HPLC were obtained by a spectrophotometer Lambda 7 UV/VIS (Perkin Elmer).

Chemicals

4-aminodiphenylamine, 2-amino-4-nitrophenol, 2-amino-5-nitrophenol, 2-aminophenol, 3-aminophenol, 4-aminophenol, p-anisidine, 2-chloro-1,4-phenylenediamine sulfate, 4-chloroaniline, 2,5-diaminoanisole sulfate, 4,4'-

diaminodiphenylamine, 2,4-diaminophenol dihydrochloride, 2,6-diaminopyridine, 3,4-diaminotoluene, 2,4-diaminotoluene, 2,5-diaminotoluene sulfate, hexylresorcinol, hydroquinone, metol, 1-naphthol, 2-naphthol, 2-nitro-1,4-phenylenediamine, 4-nitro,1,2-phenylenediamine, 1,3-phenylenediamine, and 1,4-phenylenediamine were obtained from Fluka. 2-amino-3-hydroxypyridine, 4-amino-3-methyl-phenol, 2-amino-4-methyl-phenol, and 4-chlororesorcinol were obtained from Merck. 3,4-diaminophenol dihydrochloride was obtained from Sigma. 4-amino-2-hydroxytoluene, 2-amino-4-hydroxyethylaminoanisol sulfate hydrate, 1-hydroxy-2-amino-4,6-dinitrobenzene sodium salt, 2-amino-4-methyl- amino anthraquinone, N1,N4,N4-tris-(2-hydroxyethyl)-2-nitro-1,4-phenylene- diamine, N1-(2-hydroxyethyl)-2-nitro-1,4-phenylene-diamine, N1,N4-bis-(2-hydroxyethyl)-2-nitro-1,4-phenylenediamine,1-(2'-hydroxyethyl)-amino-2-nitrobenzene and 4-(3-hydroxypropylamin)-3-nitrophenol were kindly provided by COLIPA (Comité de Liaison Européen de l'Industrie de la Parfumerie, des Produits Cosmétiques et de Toilette, Brussels).

L-ascorbic acid sodium salt was purchased from Fluka, sodium tetraborate decahydrate (p.a.), acetic acid 95% (suprapure), ammonia 25% (suprapure) and hydrochloric acid (0.1M) were obtained from Merck. Methanol (HPLC quality) was obtained from Janssen Chimica. The water used for the preparation of solutions was filtered by a Barnstead NANOpure II system and then doubly distilled.

Preparation of Reagents

Buffer solution pH 8 (Soerensen buffer): to 560 mL 0.05 M sodium tetraborate solution, add 440 mL 0.1 M hydrochloric acid, adjust to a pH of 8.

Acetic acid eluent: adjust 0.05 M acetic acid to a pH of 5.9 with ammonia 10%, filter through a 0.45 μm filter. When not in use, store this solution at a temperature of 4°C to prevent microbiological growth.

Procedures

20 μL of a freshly prepared solution containing 1 - 100 μg/mL of one or more hair dye intermediates, was subjected to separation by chromatography. For all of the following investigations the basic chromatographic conditions such as column length and packing, eluent composition, detection mode etc. were kept constant. The diode array detector and the connected computer software offer an excellent opportunity to record chromatograms and spectra of the eluting substances in one step.

Table 2

HPLC Conditions

Column:	Merck LiChrospher RP Select B, 250 mm x 4mm, 5 μm particle size
Detection:	Diode array + fixed wavelength 235 nm
Column temperature:	48°C
Injection volume:	20 μL
Mobile phase A:	0.05 M acetic acid, adjusted to pH 5.9 with ammonia
Mobile phase B:	methanol
Flow:	1 mL per minute

Gradient:	**Time in min.**	**%A**	**%B**
	0	100	0
	20	75	25
	30	20	80
	35	20	80
	37	100	0
	45	100	0

After each single run, UV-spectra of unknown substances can be compared with stored spectra of standard substances. In this way, besides the retention time, a second characteristic to aid the identification of an unknown analyte can be obtained. Sample preparation was also subject of investigation and will be described below.

The basic optimization of the chromatographic method has been previously done by Weijland and Rooselaar.[15] These separation conditions are marked in Table 2, and where not otherwise stated, the separating conditions marked in this Table were applied.

RESULTS AND DISCUSSION

Optimization of the Sample Preparation

As it is not possible to cover all possible forbidden and allowed dye intermediates by the investigations described, a selection had to be made. Eight compounds were chosen, seven of which are considered as being precursors

frequently used in commercial oxidative hair dye formulations.[15] They are all substituted benzenes. In order to have a slightly broader spectrum of chemical compounds, a pyridine derivative was added for investigation. The selected compounds are: 1,4-phenylenediamine, 4-aminophenol, 3-aminophenol, 2,5-diaminotoluene sulfate, 2,6-diaminopyridine, resorcinol, 2-nitro-1,4-phenylenediamine and 4-chlororesorcinol.

Methanol content in the sample solution

To achieve optimum column efficiency, samples should be dissolved in a medium containing the lowest possible amount of methanol. However, in a sample with an unknown composition it is not possible to predict the minimum amount of methanol which will be needed for its solubilization. Still, the results obtained for sample solutions comprising 60% of methanol are satisfying.

To ensure good reproducibility and reliability of the method, sample solutions have to remain stable during preparation, storage and analysis. Therefore, factors which might affect the stability (redox conditions, storage time, light and temperature) had to be studied.

Role of antioxidant during sample preparation

Because of the known instability of dye forming compounds towards oxidation, the sample solutions were stabilized by sodium ascorbate. An amount of 2 mg of this antioxidant was added per mL of sample solution. The sample medium consisted of 60% methanol. To determine the influence under different light and temperature circumstances and to show the antioxidative properties, a series of tests was carried out. For each chosen substance a sample solution was prepared and divided into five portions, each of these portions being subjected to different experimental conditions. Two of them were stored in clear flasks in the daylight with the conditions being indicated by the letters RTL and RTAL, denoting **R**oom **T**emperature, **L**ight and **R**oom **T**emperature, **A**ntioxidant, **L**ight, respectively. Three of them were stored in the dark with an indication of the conditions given by: RTD, RTAD, and 4TD, denoting **R**oom **T**emperature, **D**ark; **R**oom **T**emperature, **A**ntioxidant, **D**ark, and **T**emperature of 4°C, **D**ark, respectively. To the samples labelled RTAL and RTAD, sodium ascorbate was added. The sample labelled 4TD was stored in the refrigerator. Subsamples of these solutions were taken and subjected to chromatography. Besides the chromatographic separation, the dye forming compounds were checked for changes in their UV spectra.

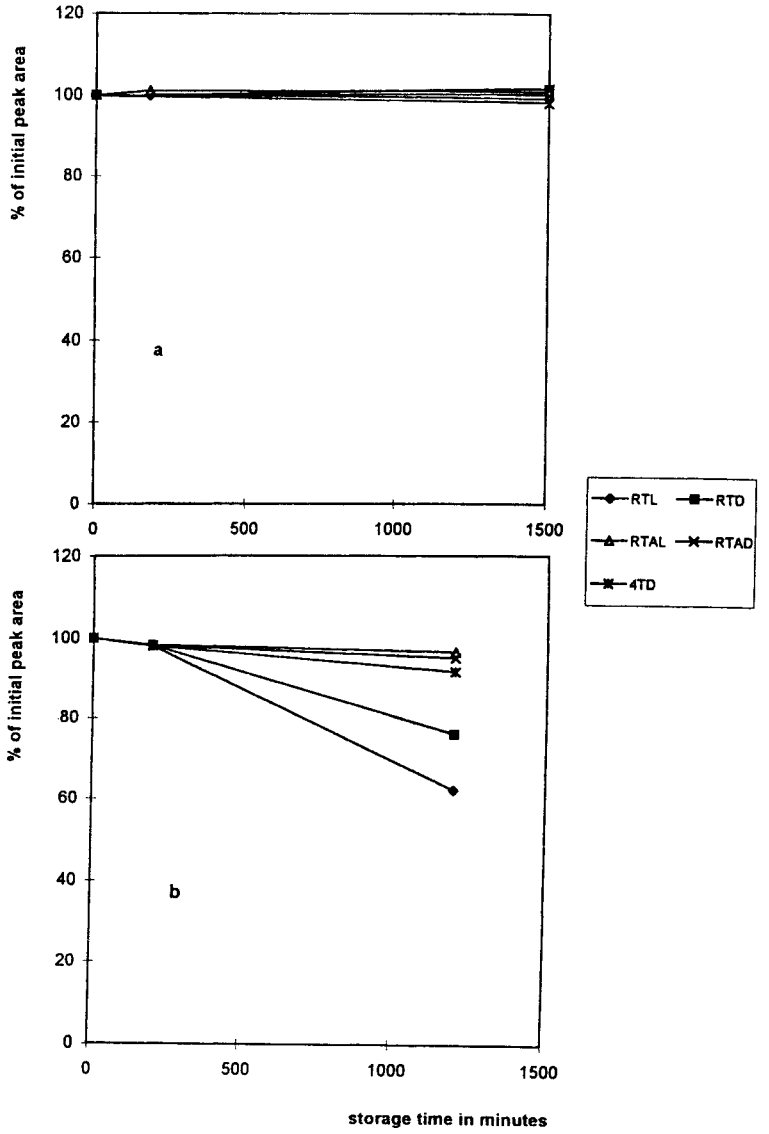

Figure 1. Influence of antioxidant sodium ascorbate (in light or dark, room or low temperature) on the peak area of 2,6-diaminopyridine (a) and 2,5-diaminotoluene sulfate (b) (see text for abbreviations).

It was shown, that for all eight tested products, no change in peak area and in UV spectra took place during a storage period of 20 hours, when sodium ascorbate was added (RTAL, RTAD experiments). Also, samples stored at refrigerator temperatures did not show any changes during this period (4TD experiments). From the sample solutions prepared without the addition of sodium ascorbate and stored at room temperature (RTL, RTD experiments), compounds behaved differently. For 2,6-diaminopyridine, 4-chlororesorcinol, 3-aminophenol, resorcinol, and 2-nitro-1,4-phenylenediamine the peak area in the chromatograms remains unchanged under all test conditions. An example of this behavior is shown for 2,6-diaminopyridine in Figure 1a. On the contrary, for 2,5-diaminotoluene, 1,4-phenylenediamine and 4-aminophenol, a significant decrease of peak area units with increasing storage time is observed. The behavior of 2,5-diaminotoluene sulfate is shown in Figure 1b.

As a consequence of these experiments, sodium ascorbate was used in all the following tests.

Time dependence

The next series of tests was carried out on the time dependence of the sample stability. Samples were prepared and stored at 20°C in the dark for time periods of 0 - 100 - 300 - 1000 - 3000 and 10000 minutes, before acquisition of chromatograms. Even after a storage time of up to several days, peak areas remain unchanged, so samples prepared and eluted on the same day should not result in any problems.

Influence of light

In an other series of tests the influence of UV-light was studied. Sample solutions were irradiated by a UV-lamp. The irradiation times were 1 - 2 - 5 - 10 and 20 minutes. Chromatograms of the solutions were recorded and the resulting peak areas were plotted as a function of irradiation time. A severe change in peak area is obtained by irradiation of sample solutions of all compounds. It must be noted that after an irradiation time of only 10 minutes the sample solutions had become heated up to a temperature of 60 °C. Therefore, the irradiation procedure was performed in intervals not longer than 10 minutes. To ensure that the heating of the solutions was not responsible for the variations in peak area, the influence of temperature on the sample preparation was tested as well. Two examples of disintegration are shown in Figure 2. It can be seen, that after only 5 minutes of irradiation 3-aminophenol is not detectable any more and that the peak area of 1,4-phenylenediamine decreases to 70 % of its initial value during a 20 minute irradiation. The two examples shown are extremes. The other tested products show a decrease in

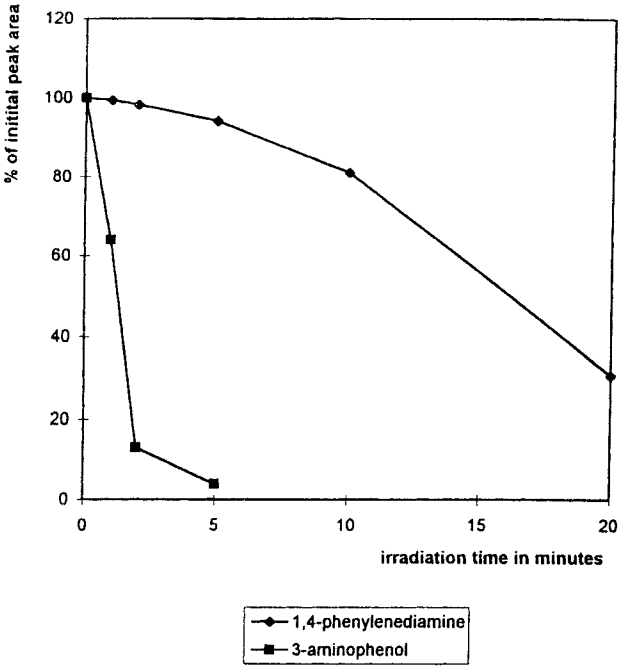

Figure 2. Influence of irradiation time on the peak area of 1,4-phenylenediamine and 3-aminophenol.

peak area after 20 minutes to 31 % (1,4-phenylenediamine), 30% (4-aminophenol), 24% (2-nitro-1,4-phenylenediamine), and 14% (resorcinol). Under the tested conditions 3-aminophenol is no longer detectable after 10 minutes of irradiation and 4-chlororesorcinol after 20 minutes.

Influence of the temperature of the sample solution

As a last influencing factor the storage temperature was examined. Sample solutions were kept for 20 minutes at temperatures of 20, 30, 45, and 60°C in the dark and chromatograms were recorded. The peak areas remain almost constant over the whole range of temperatures. In the first place, this means, that the treatment temperature does not play an important role in the separation characteristics of hair dye intermediates. In the second place, this result shows, referring to the section above, that the UV-irradiation is the only factor responsible for the decrease of stability.

Table 3

Retention Times for 40 Dye Intermediates*

Compound	Retention Time, Min
sodiumpicramate	5.25
1,4-phenylenediamine	7.16
4-aminophenol	8.04
pyrogallol	8.28
2-amino-3-hydroxypyridine	8.77
hydroquinone	8.78
1,3-phenylenediamine	9.06
3-aminophenol	9.99
2,5-diaminotoluene sulfate	10.11
2,6-diaminopyridine	10.29
resorcinol	11.54
2,5-diaminoanisole sulfate	11.76
2-aminophenol	12.61
4-amino-3-methyl-phenol	12.99
metol	13.36
1-(2'-hydroxyethyl)amino-2-nitrobenzene	14.57
2,4-diaminotoluene	14.70
2-chloro-1,4-phenylenediamine sulfate	14.95
2-nitro-1,4-phenylenediamine	15.63
4-nitro-1,2-phenylenediamine	17.94
4-amino-2-hydroxytoluene	18.31
2-amino-4-hydroxyanisole sulfate hyddrate	18.70
2-amino-4-nitrophenol	19.54
3,4-diaminotoluene	20.01
p-anisidine	20.66
N1-(2-hydroxyethyl)-2-nitro-1,4-phenylenediamine	20.96
2-amino-4-methylphenol	21.14
2-amino-5-methylphenol	21.29
2-amino-5-nitrophenol	21.57
4,4'-diaminodiphenylamine sulfate	23.18
4-chlororesorcinol	23.63
N1,N2-bis-(2-hydroxyethyl)-2-nitro-1,4-phenylenediamine	24.79
4-(3-hydroxypropylamino)-3-nitrophenol	25.78
N1,N4,N4-tris-(2-hydroxyethyl)-2-nitro-1,4-phenylenediamine	27.18

(continued)

Table 3 (continued)

Retention Times for 40 Dye Intermediates*

Compound	Retention Time, Min
4-chloroaniline	28.91
2-amino-4-methylaminoanthraquinone	29.70
4-aminodiphenylamine	30.71
2-naphthol (β)	31.39
1-naphthol (α)	31.67
4-hexylresorcinol	33.31

* The separation conditions used are those marked in Table 2

Chromatography of dye forming compounds

Forty different compounds were subjected to chromatography and retention times and UV spectra were recorded. This work confirms the fact that some compounds have very similar retention times (Table 3), 3-aminophenol, 2,5-diaminotoluene sulfate and 2,6-diaminopyridine for instance. However, as can be seen from Figure 3, the UV spectra of these three compounds have remarkable differences, so that their identification is still possible. In the case that a diode array detector is not available, a variation of detection wavelength or the simultaneous detection at two wavelengths also leads to a clear identification.

In order to test the repeatability, 4 injections of each sample were carried out. The relative standard deviation obtained for peak areas was between 0.02 and 1.57 %, a satisfying result. The relative standard deviation for the retention time was between 0.05 and 1.30 %, also indicating a good repeatability.

According to COLIPA most hair dye formulations contain only six to eight dye intermediates.[16] The probability that some of the products in one single formulation have similar retention times is very small. However, in those cases, the UV-spectrum should allow their identification. Only in the very few cases where the spectra of the eluting substances provide no clear identification, a change in gradient composition would be necessary.

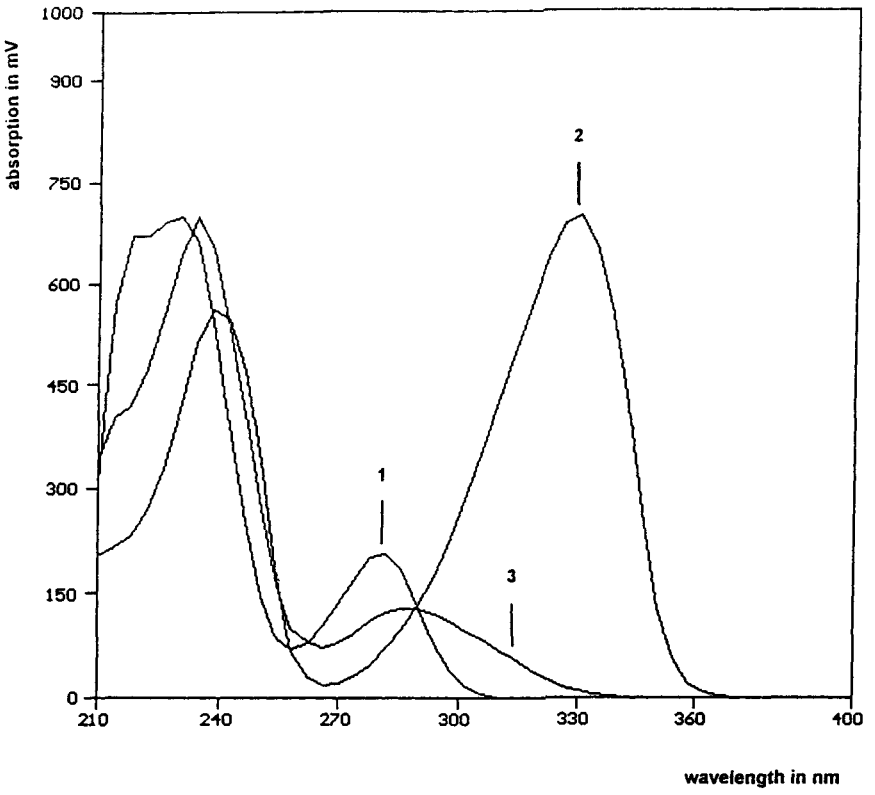

Figure 3. UV spectra of 3-aminophenol (1), 2,6-diaminopyridine (2) and 2,5-diaminotoluene sulfate (3). Spectra were obtained with the diode array detector during chromatographic separation.

Chromatography of a sample solution containing fifteen dye intermediates

An example of the separation of fifteen dye intermediates is shown in Figure 4.

Seven of the eight products previously chosen for more detailed investigation are included in this mixture of intermediates. 2,5-diaminotoluene sulfate is not included as its peak would overlap with the peak of 2,6-diaminopyridine. However, as previously explained, an identification of the two products is possible according to the two different UV spectra.

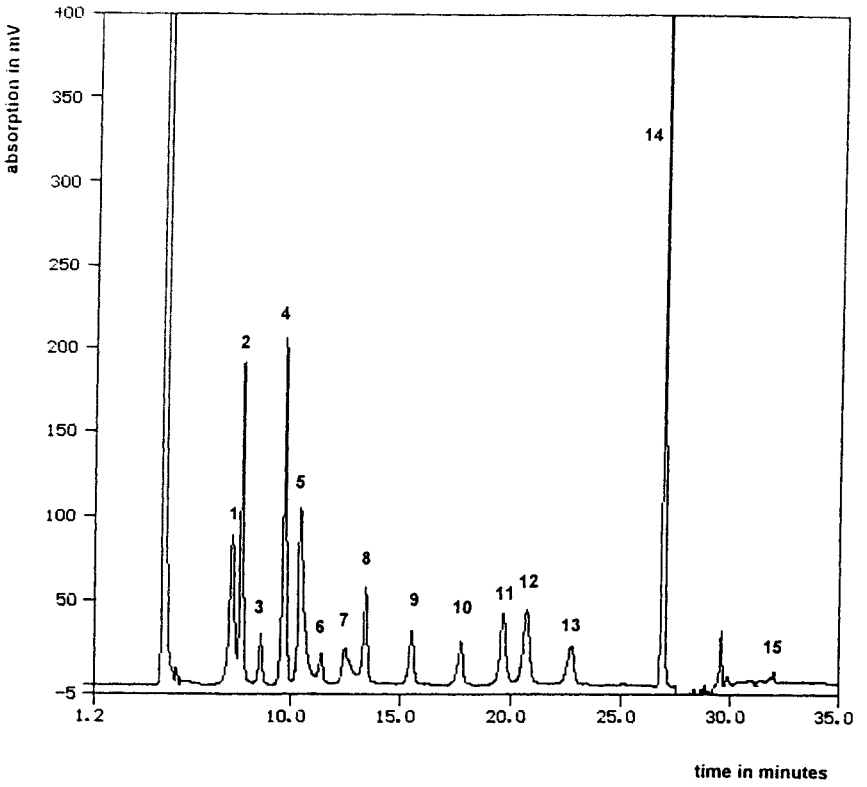

Figure 4. Chromatogram showing the separation of 15 dye forming compounds. Separation conditions are given in Table 2.

Separation characteristics are given in Table 4. From this table it can be seen, that the separation is of very good quality. The symmetry of the peaks (indicated as skewness) is satisfying for most peaks and the resolution and the capacity factors are as expected for a HPLC separation.

Calibration Curves and Limits of Detection

Calibration curves were firstly set up for single compound solutions. For each of the eight tested compounds, solutions were prepared in the range of 5 to 200 µg / mL. Data for the calibration curves are listed in Table 5. Mixtures containing several dye compounds also lead to linear calibration curves for each compound.

Table 4

Separation Properties of 15 Dye Intermediates*

Peak No.	Name of Compound	Skewness	Resolution	Capacity
1	1,4-phenylenediamine	0.85	1.26	0.72
2	4-aminophenol	0.93	2.89	0.82
3	hydroquinone	0.90	3.30	1.01
4	3-aminophenol	0.87	1.73	1.26
5	2,6-diaminopyridine	1.48	2.26	1.43
6	resorcinol	0.90	2.15	1.65
7	2-aminophenol	1.40	1.81	1.91
8	metol	0.92	5.22	2.12
9	2-nitro-1,4-phenylenediamine	0.85	4.61	2.61
10	4-nitro-1,2-phenylenediamine	0.84	3.71	3.12
11	3,4-diaminotoluene	0.94	1.82	3.57
12	2-amino-5-nitrophenol	0.89	3.14	3.82
13	4-chlororesorcinol	0.86	9.87	4.29
14	4-chloroaniline	0.92	22.74	5.27
15	hexylresorcinol	0.87	25.28	6.45

* The separation conditions are those marked in Table 2.

Figure 5 shows the case of a solution of 4-aminophenol, 2,5-diaminotoluene sulfate, resorcinol and 2-nitro-1,4-phenylenediamine. Linearity, both for single component solutions and for multi component solutions, is very good. The detection limits are calculated as the blank signal, plus three standard deviations of the blank signal, according to the calculation mode of Miller and Miller.[17] They are indicated in Table 5 as well. The values are in the range of 0.1 - 1.1 μg / mL. For resorcinol, a fairly high detection limit was obtained. This can be explained by the slope of the resorcinol calibration curve being very low, indicating a poor response for this substance at the chosen wavelength, which leads to a greater uncertainty and a higher limit of detection.

All data in Table 5 are obtained by recording the chromatograms at a wavelength of 235 nm. As the UV spectra of the recorded substances mainly have absorption maxima which are different from 235 nm, an improvement of the limit of detection can be done by measuring the peak areas at the optimum wavelength for each particular substance.

Table 5

Data for Calibration Curves and Limits of Detection for Selected Dye Intermediates

Compound	a	b	RxR	Concentration Range, µg/mL	Limit of Detection, µg/mL
3-aminophenol	1.3941	-1.8031	1.0000	12 - 120	1.02
4-aminophenol	0.8068	0.6387	0.9999	5 - 100	1.14
4-chlororesorcinol	0.3520	0.0520	1.0000	20 - 200	0.78
2,6-diaminopyridine	1.3450	0.3560	0.9997	5 - 100	0.89
2,5-diaminotoluene sulfate	0.7978	-0.0783	1.0000	12 - 120	0.42
2-nitro-1,4-phenylenediamine	2.2809	2.0465	0.9991	10 - 100	0.11
1,4-phenylenediamine	1.6033	0.4737	1.0000	20 -200	0.79
rescorcinol	0.1089	-0.0939	0.9999	20 - 200	2.88

* The separation conditions used are those marked in Table 2.
Linear regression is done: a represents the slope, b the intercept, and RxR the correlation coefficient of the regression curve $y = ax + b$.

CONCLUSION

The method described here enables the separation of a broad range of active compounds of oxidative hair dyes by reversed-phase high performance liquid chromatography. The identification of unknown compounds can be done by comparison of retention times and UV spectra of standards.

For optimum separation characteristics, samples should be dissolved in an aqueous medium containing as little methanol as possible. The minimum amount of methanol will largely depend on the solubility of the sample in pure aqueous solution. However, sample solutions containing up to 60% of methanol lead to separation characteristics which are still satisfying.

Freshly prepared sample solutions have to be immediately subjected to chromatographic separation after preparation if no antioxidant is added. In the opposite case, the tested compounds show high stability regarding storage time and storage temperature. Only UV light can cause a change in the sample composition, that can be avoided by sample storing in the dark.

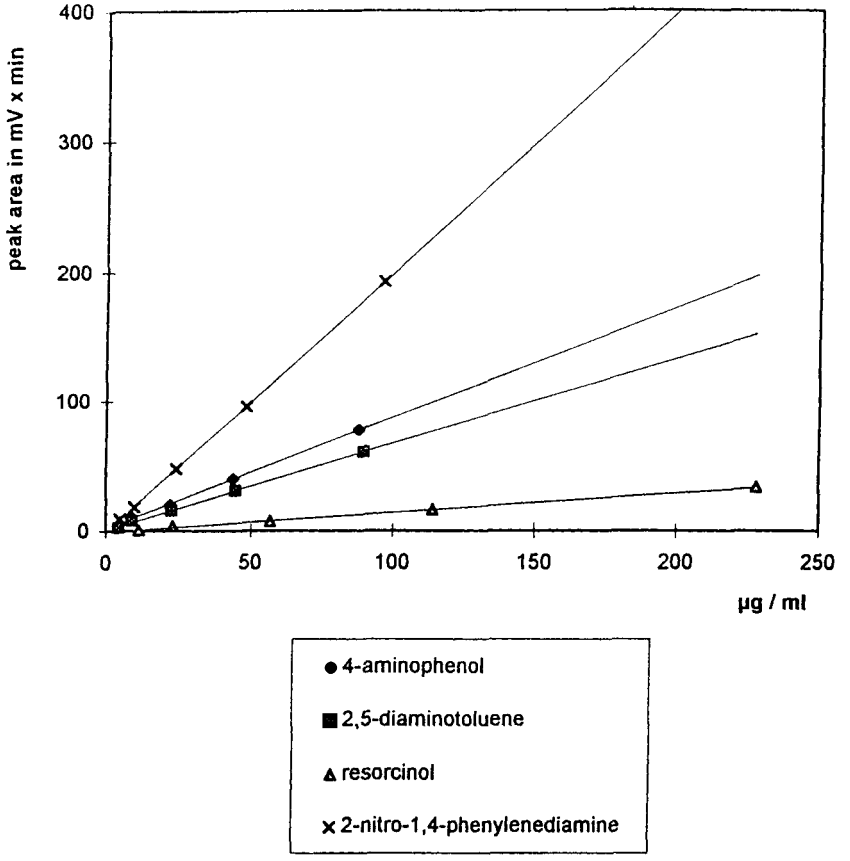

Figure 5. Calibration curves for a solution containing a mixture of 4-aminophenol, 2,5-diaminotoluene sulfate, resorcinol, and 2-nitro-1,4-phenylenediamine.

For the tested components, linear calibration curves were obtained for concentrations of up to 250 µg / mL dye forming compound. Compared to other methods published, ours enables the separation and identification of a broad range of compounds in one single run, with satisfying separation properties and with low detection limits. It has to be noted, that even while keeping the same chromatographic conditions, results can vary when using different columns. Even columns from the same brand and same lot can lead to slightly different results. For this reason a calibration is strictly necessary, from time to time, especially after replacement of columns.

To the best of our knowledge this paper is the only one in which a very significant number of hair dye intermediates are considered. There are difficulties in defining the compounds to be examined, while finding commercial standards for this kind of product can also be a problem. The work continues presently by studying the same intermediates prepared in model solutions, in order to estimate the effect of the real matrix components, used in the cosmetic industry, on their separation and determination.

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