Influence of matrix compounds on the analysis of oxidative hair dyes by HPLC

URSULA VINCENT, GUY BORDIN, and
ADELA R. RODRÍGUEZ, *European Commission, Joint Research
Centre, Institute for Reference Materials and Measurements, Retieseweg,
B-2440 Geel, Belgium.*

Synopsis

Oxidative hair dyeing products consist of a mixture of a broad spectrum of organic compounds including the hair dye's so-called active compounds and the matrix-forming compounds. Forty-seven dye intermediates commonly used in cosmetic formulations have thus been analyzed by RP-HPLC, and their chromatographic characteristics have been recorded. Since the matrix compounds could interfere with the quantitative analysis of the active compounds, eighteen matrix compounds commonly used in cosmetic formulations have been tested for their influence on dye intermediate determination. Since some of them do affect the chromatographic behavior of the dye intermediates, an isolation procedure, for separating matrix components from the dye-forming compounds, consisting of a liquid–liquid extraction by n-heptane, has been set up. In most cases, this procedure is effective for the extraction of the matrix products. Moreover, the dye intermediates are not extracted by n-heptane, and their chromatographic behavior is not altered by the extraction procedure. In summary, this study has shown that a reference method for the analysis of oxidative hair dyes should include a compulsory extraction step before submission of samples to RP-HPLC.

INTRODUCTION

Among the different methods of changing the color of human hair, oxidative hair dyeing plays an important role. Formulations consist of a wide range of organic compounds of two distinct types, i.e., the hair dye intermediates and the matrix-forming compounds. Some of the hair dyes, which are available for use in 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 substances used in hair dye formulations, with the aim of implementing the European Union Cosmetic Directive, there is need for a reliable analytical method. Initially, a high-performance liquid chromatography method was developed that enables the identification of a broad range of dye-forming compounds in standard solutions (1,2). The next step for the setup of a reliable analytical method, besides additional optimization steps, involves the investigation of possible effects of matrix components.

Due to the different and specific functions of matrix compounds in hair dye formulations, the chemical composition of matrix compounds covers a broad range, including surfactants, pH adjusters, consistency providers, antioxidants, emulsifiers, perfumes, perfume solubilizers, and preservatives. Each of these additives plays a clear role in the formulation. For instance, preservatives such as methylparaben or DEDM-hydantoin and antioxidants such as BHT, sodium sulfite, and L-ascorbic acid sodium salt have to be added to keep the product stable.

Literature on the influence of the various matrix products on the chromatographic separation of oxidative hair dyes is very rare, although it is of tremendous importance. Some authors simply do not consider their influence on the analysis (3–5), while others try to reduce matrix interference by optimizing the measurement wavelength (6).

From the point of view of the chromatographic separation of the dyes, two main situations can occur:

• a given matrix component shows a specific retentive behavior and can be detected with the UV detector.
• the matrix component forms a complex with a dye intermediate that will change the retentive behavior of the dye intermediate.

Furthermore, it must be noted that some matrix components can also interfere with the column itself, i.e., adsorb onto the stationary reversed phase, therefore changing the separation properties of the oxidative hair dyes.

Therefore, the aims of the work described in this article were to investigate possible matrix influences on the chromatographic separation of the dye-forming compounds and to set up an effective method of separating the matrix components from the dye-forming compounds. This method should not, of course, affect the chromatographic behavior of the dyes. Due to the high number of dyes and matrix compounds, a selection of products had to be made according to lists of frequently used dye intermediates and matrix products with their concentrations in formulations provided by COLIPA (Comité de Liaison Européen de l'Industrie de la Parfumerie, de Produits Cosmétiques et de Toilette; see Appendix). The forty-seven dyes and eighteen matrix components selected for these experiments are representative of four groups of dye-forming compounds, classified according to their chemical characteristics, and of five classes of additives used as matrix products in the formulations. The influence of matrix components on the dye determination has been performed on selected dyes from each group.

EXPERIMENTAL

The chromatographic procedure has been described in detail in a previous article (1).

INSTRUMENTATION

All chromatographic separations were carried out 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., Milan, Italy), an autosampler 360 with a loop of 20 ml (Kontron Instruments S.P.A.), a diode array detector 440 (Kontron Instruments S.P.A.), and a vacuum degassing system, Degasys DG 1300 (Uniflows, Japan). The

column temperature was kept constant by means of the thermostat of an electrochemical detector (Decade, Antec Leyden, Leiden, The Netherlands). Data processing was done with the Data System 450-MT2/DAD series (Kontron Instruments S.P.A.). The column was a Merck Lichrospher RP 60 Select B, 250 × 4 mm, 5-μm particle size. In some cases, a UV-Vis spectrophotometer (Lambda 7 from Perkin Elmer) was used additionally with the above equipment.

CHEMICALS

L-Ascorbic acid sodium salt (NaAsc), 3,4-diaminobenzoic acid (3,4-daba), *p*-aminophenol (4-ap), *m*-aminophenol (3-ap), *o*-aminophenol (2-ap), 2,4-diaminophenol (2,4-dap), 2,4-diaminophenol HCl (2,4-daph), 4-amino-*m*-cresol (4-a-3-mp), 2-amino-*p*-cresol (2-a-4-mp), 6-amino-*m*-cresol (2-a-5-mp), 5-amino-*o*-cresol (4-a-2-ht), 2-amino-5-nitrophenol (2-a-5-np), 2-amino-4-nitrophenol (2-a-4-np), *N,N*-diethyl-*m*-aminophenol (3-deap), *p*-phenylenediamine (1,4-pd), *m*-phenylenediamine (1,3-pd), *o*-phenylenediamine (1,2-pd), 2-nitro-*p*-phenylenediamine (2-n-1,4-pd), 4-nitro-*o*-phenylenediamine (4-n-1,2-pd), 2-chloro-*p*-phenylenediamine sulfate (2-cl-1,4-pds), *N*-phenyl-*p*-phenylenediamine (4-adp), 4,4'-diaminodiphenylamine sulfate (4,4'-dadps), resorcinol (res), 4-chlororesorcinol (chlres), 4-hexylresorcinol (hres), *p*-anisidine (1,4-ad), 2,6-diaminopyridine (2,6-dap), 2-amino-3-hydroxypyridine (2-a-3-hp), 2-methylresorcinol (2,6-dht), toluene-2,4-diamine (2,4-dat), toluene-3,4-diamine (3,4-dat), toluene-2,5-diamine sulfate (2,5-dats), 1-naphtol (1-nap), 2-naphtol (2-nap), 1,6-naphtalenediol (1,6-dhnap), 2,3-naphtalenediol (2,3-dhnap), 2,7-naphtalenediol (2,7-dhnap), phloroglucinol (phlg), pyrogallol (pg), hydroquinone (hq), pyrocatechol (pc), *p*-methylaminophenol sulfate (met), 2-methoxy-*p*-phenylenediamine sulfate (2,5-das), 3-*tert*-butyl-*p*-hydroxyanisole (3-tb-4-ha), 4-chloroaniline (4-cla), 3-methyl-1-phenyl-2-pyrazoline-5-one (3-m-1-p-2-p-5-o) were obtained from Fluka. *p*-Phenylenediamine sulfate (1,4-pds), and *m*-phenylenediamine sulfate (1,3-pds) were kindly provided by "Les Colorants Wackherr," Saint-Ouen l'Aumône, France. Sodium tetraborate decahydrate (p.a.), acetic acid 95% (suprapure), ammonia 25% (suprapure), hydrochloric acid (0.1 M), oleic acid (OA) (p.a.), n-heptane (p.a.), isopropanol (p.a.), sodium sulfite (p.a.) (SS), and polyvinylpyrrolidon (PVP) were obtained from Merck. BHT (butylated hydroxytoluene), methylparaben (MP), and lauryl sulfate (LS) were obtained from Sigma. Lauric diethanolamide (LDA), TEA-dodecylbenzenesulfonate (TDS), and Syntopon 8 D1® (ethoxylated octyl phenol-EOP) were provided by Witco S.A. Triethanolamine (TEA) was obtained from Mobi-Lab bvba, Zutendaal, Belgium. DEDM-hydantoin (DMDM), dimethicone copolyol (DC), nonoxynol-12 (NOL), and polyquaternium-11 (PQ) were kindly provided by "Keuringsdienst van Waren," Enschede, The Netherlands. n-Nonylamine 98% (NNO) was obtained from Janssen Chimica, and Oranex HT® (ORA) was provided by Spinnrad GmbH, Gelsenkirchen, Germany. Cetrimonium chloride (CC) and methanol (HPLC quality) were obtained from Fluka. Pure water (18.2 MΩ/cm quality) used for the preparation of solutions was obtained from a MilliQ Plus 185 system (Millipore, Molsheim, France).

PREPARATION OF REAGENTS

Solvents and chromatographic mobile phase

Buffer solution pH 8 (Soerensen buffer): 440 ml hydrochloric acid (0.1 N) and 2 g/l

L-ascorbic acid sodium salt (NaAsc) as an antioxidant agent were added to a 560-ml sodium teraborate solution. The solvents were mixtures of MeOH and Soerensen buffer, pH 8 in various proportions.

Mobile phase (aqueous phase B): a 0.05 M acetic acid solution was adjusted to a pH of 5.9 with a 10% ammonia solution and filtered through a 0.45-μm filter. When not in use, the eluent was stored at a temperature of 4°C to prevent microbiological growth.

Samples. The matrix products and the dye intermediates used in the experiments are given in Tables I and II, respectively. All dye intermediates and matrix component concentrations were selected according to COLIPA data.

Stock solutions of dye intermediates at a concentration of 0.1 g/100 g were prepared in mixtures of various proportions of MeOH and Soerensen buffer (containing NaAsc as an antioxidant agent), ranging from 40% to 90% Soerensen buffer. Sample solutions of dye intermediates at a concentration of 0.025 g/100 g were prepared by dilution from the stock solutions in mixtures of Soerensen buffer and MeOH ranging from 30% to 60% Soerensen buffer.

Stock solutions of matrix components were prepared in MeOH– or isopropanol–Soerensen buffer mixtures of different proportions. Sample solutions of the matrix components at various concentrations (Table I) were prepared by dilution from the stock solutions in MeOH– or isopropanol–Soerensen buffer mixtures (abbreviations are given in the Chemicals section).

Table I
Selected Matrix Compounds, Functions, Concentrations, and MeOH Proportions Used

Compound	Function	Concentration (g/100 g)	MeOH proportion (%)
Oleic acid (OA)	Surfactant, cleansing agent	5	100
Oranex HT® (ORA)	Unknown	3	100
TEA-dodecylbenzenesulfonate (TDS)	Surfactant, cleansing agent	0.5	90
Cetrimonium chloride (CC)	Surfactant, emulsifying agent	0.5	90
Lauric diethanolamide (LDA)	Surfactant, cleansing agent	2	75
BHT	Antioxidant	0.25	70
Syntopon 8 D1® (EOP)	Perfume solubilizer	6	70
Nonoxynol-12 (NOL)	Surfactant, emulsifying agent	3	70
n-Nonylamine 98% (NNO)	Unknown	25	60
Sodium lauryl sulfate (LS)	Surfactant, cleansing agent, denaturant	3	60
Polyquaternium-11 (PQ)	Antistatic agent, film former, hair fixative	2	60
Methylparaben (MP)	Preservative	0.05	60
Polyvinylpyrrolidon (PVP)	Antistatic agent, film former, hair fixative	2	60
Sodium sulfite (SS)	Antioxidant, reducing agent	2	40
Triethanolamine (TEA)	pH adjuster	1.5	40
DEDM-Hydantoin (DEDM)	Preservative	0.05	0
Dimethicone copolyol (DC)	Emollient, hair conditioning agent	2.5	*
L-ascorbic acid sodium salt (NaAsc)	Antioxidant, pH adjuster	**	**

* DC was prepared in an isopropanol–Soerensen buffer mixture with 67% isopropanol.
** The Soerensen buffer contains NaAsc 2 g/l.

Table II
Retention Times for Forty-Seven Dye Intermediates, Each of Which Was Measured as a Single
Component Solution (n = 3)

Dye	Retention time (min)	
	Mean	RSD (%)
3,4-Diaminobenzoic acid	2.75	1.54
2,4-Diaminophenol	3.95	0.17
2,4-Diaminophenol HCl	3.98	0.18
p-Phenylenediamine sulfate	5.21	0.54
Phloroglucinol	5.23	12.56
p-Phenylenediamine	5.60	9.34
Pyrogallol	5.90	3.59
2-Amino-3-hydroxypyridine	5.91	0.84
Hydroquinone	6.02	6.92
p-Aminophenol	6.13	3.46
m-Phenylenediamine sulfate	7.00	3.23
m-Phenylenediamine	7.15	6.42
2,6-Diaminopyridine	7.91	3.93
m-Aminophenol	8.04	3.43
Toluene-2,5-diamine sulfate	8.50	7.73
Resorcinol	9.23	4.67
4-Amino-m-cresol	9.70	1.82
o-Phenylenediamine	9.86	3.87
2-Methoxy-p-phenylenediamine sulfate	9.94	1.35
o-Aminophenol	10.51	5.98
Pyrocatechol	11.23	18.06
p-Methylaminophenol sulfate	11.88	2.02
2-Methylresorcinol	11.89	6.84
2-Chloro-p-phenylenediamine sulfate	12.33	2.01
Toluene-2,4-diamine	12.78	8.85
2-Nitro-p-phenylenediamine	13.14	2.20
4-Nitro-o-phenylenediamine	15.10	2.53
2-Amino-4-nitrophenol	15.50	3.01
5-Amino-o-cresol	15.66	4.11
Toluene-3,4-diamine	17.53	7.50
6-Amino-m-cresol	17.95	1.34
2-Amino-p-cresol	18.07	2.90
p-Anisidine	18.47	4.21
2-Amino-5-nitrophenol	18.66	3.14
4-Chlororesorcinol	20.44	3.70
4,4'-Diaminodiphenylamine sulfate	20.83	0.75
1,6-Naphtalenediol	25.23	2.24
2,7-Naphtalenediol	25.81	1.59
3-Methyl-1-phenyl-2-pyrazoline-5-one	26.01	6.39
4-Chloroaniline	26.82	2.50
2,3-Naphtalenediol	27.88	2.38
N-Phenyl-p-phenylenediamine	29.56	3.64
2-Naphtol	29.71	0.93
N,N-Diethyl-m-aminophenol	30.00	0.02
1-Naphtol	30.01	0.42
3-tert-butyl-4-hydroxyanisole	31.85	0.58
4-Hexylresorcinol	32.26	0.55

Sample solutions of dye intermediates at a concentration of 0.025 g/100 g in matrix media (at the different concentrations given above) were prepared by dilution from the respective stock solutions at various proportions of MeOH and Soerensen.

It has to be noted that all samples contained NaAsc as an antioxidant agent added to the Soerensen buffer to ensure the stability of the dye samples.

PROCEDURES

Reversed-phase HPLC conditions. A non-linear MeOH (A)/aqueous phase (B) gradient was used as follows: 0–25% A for 19 min, 25–80% A for 10 min, 80% A for 5 min, 80–95% A for 5 min, 95% A for 10 min, and 95–0% A for 3 min. The total flow was 1 ml/min. Between the injections, the column was equilibrated by a 25-ml mobile phase. Each analysis was repeated five times. The column temperature was kept at 48°C. The data acquisition was carried out at two or three selected wavelengths: 220 nm, 235 nm, and 290 nm, in parallel with the spectra acquisition.

Isolation of the matrix components from the final analyte solution. First, experiments using anion exchange, solid phase extraction, liquid cation exchange, and liquid–liquid extraction were carried out. The results led to the selection and the optimization of a liquid–liquid extraction procedure of the matrix components from the sample solution by n-heptane.

Two milliliters of sample solution (single-matrix product solution or mixture of dye intermediates and a matrix compound, at the concentrations given in the Experimental section) were treated with n-heptane. Depending on the matrix product, the extraction involved one to three steps. In the first step, the extraction was performed on the 2-ml sample using 20 ml n-heptane, the two phases were separated, and the resulting aqueous phase 1 was thereafter submitted to HPLC or analyzed by UV-Vis spectrophotometry. For additional extraction steps, 20 ml n-heptane were added to the resulting aqueous phase n-1, separation of the two phases was performed, and the resulting aqueous phase n was submitted to HPLC or analyzed by UV-Vis spectrophotometry.

RESULTS AND DISCUSSION

A systematic study was carried out on each of the eighteen selected matrix compounds and forty-seven selected hair dyes (Tables I and II). First, their individual retentive behavior was determined and their UV spectrum recorded, using the DAD or the UV-Vis spectrophotometer. Second, the efficiency of the liquid–liquid extraction protocol on the selected matrix compounds was checked. When a matrix compound could not be extracted from the sample solution, its influence on the retentive behavior of the dye intermediates was investigated.

CHROMATOGRAPHIC STUDY

Single solutions of dye-forming compounds. Forty-seven dye-forming compounds were subjected to chromatography and their retention times and UV spectra were recorded. Results are presented in Table II.

It comes out that 91% (43 dye intermediates) of the dye intermediates tested have their

retention time between 5 min and 30 min. Some of the tested dyes have very close retention times—4-n-1,2-pd, 2-a-4-np, and 4-a-2-ht for instance, their retention times being 15.10 min, 15.50 min, and 15.66 min, respectively. However, since their respective UV spectra show remarkable differences (8), their identification can still be carried out easily.

In order to test the repeatability, five injections of each sample were carried out. The relative standard deviation (RSD) for the retention time was between 0.2% and 1.1%, while that of the peak corrected areas (defined as the peak area divided by the retention time), recorded at 290 nm, was between 1.1% and 6.0% (9). The chromatographic behavior showed good repeatability.

The injections were repeated on three different columns in order to test the reproducibility (Table II). For thirty-six hair dye intermediates, the relative standard deviation (RSD) on the retention time was less than 5%; for nine dye intermediates, this RSD was between 6% and 10% and more than 10% for only two dyes. The reproducibility between columns was considered satisfactory.

Single solutions of matrix compounds. Eight different compounds were subjected to chromatographic separation, and their retention times and UV spectra were recorded. Results are presented in Table III. It appears that some of the tested compounds have very close retention times—BHT, OA, and ORA, for instance. However, with their respective UV spectra also showing remarkable differences (Figure 1), their identification can still be carried out easily. It must also be noted that most of the matrix compounds, with the exception of DMDM, PVP and MP, have retention times greater than 30 minutes, meaning that confusing dye intermediates with matrix products should not occur since the retention times of the hair dyes generally range from 5 to 30 minutes (1). Concerning the four dye intermediates for which retention times are out of the range of 5 to 30 min, the discrimination from the matrix compounds can also be easily made according to both retention time and UV spectrum.

In order to test the repeatability, five injections of each sample were carried out. The relative standard deviation (RSD) for the retention time was between 0.02% and 0.7%, while that of the peak corrected areas was between 0.3% and 3.7%, except for DMDM. The chromatographic behavior of the matrix compounds showed good repeatability.

Table III

Retention Times and Corrected Peak Areas for Eight Matrix Compounds, Each in a Single Solution (n = 5)

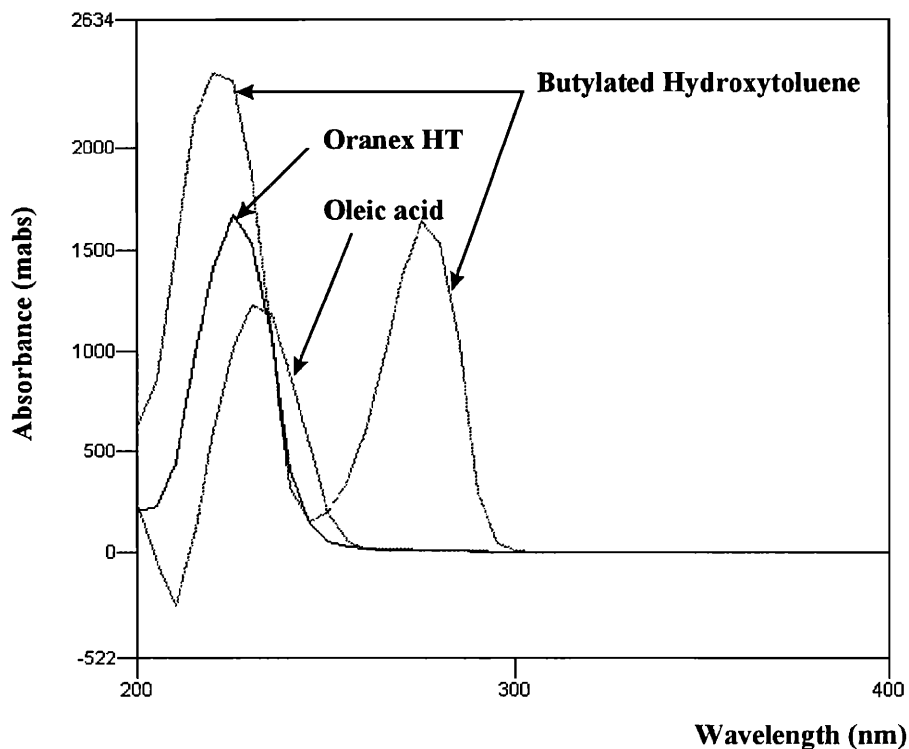| Matrix compound	Retention time (min)		Corrected peak area (AU)		λ of measurement (nm)
	Mean	RSD (%)	Mean	RSD (%)	
DMDM	6.80	0.21	4.3	12.4	220
PVP	7.10	0.24	1.1	0.2	220
MP	26.87	0.06	1.0	1.8	290
NNO	33.80	0.70	5.2	3.4	235
EOP	35.85	0.08	5.8	0.3	290
OA	37.97	0.30	20.6	2.9	235
BHT	38.11	0.02	2.5	3.7	290
ORA	38.35	0.09	12.2	0.5	235

AU: arbitrary unit.

Figure 1. UV spectra of three matrix products.

Sample solutions containing an additional matrix compound and three dye intermediates. From the forty-seven dye intermediates tested, four were chosen for further investigation based on how representative they were of their different classes of hair dyes as well as on their use in formulations.

Three sample solutions were prepared, each containing a mixture of three selected dye intermediates (1,4-pd or 4-ap, res, and 2-n-1,4-pd), the intrinsic matrix compound NaAsc as an antioxidant, and another major matrix compound, BHT, DMDM, or EOP, respectively. BHT is an important antioxidant, DMDM a commonly used preservative, and EOP a perfume solubilizer. The retention time of BHT and EOP is more than 30 min, while DMDM is eluted within about 7 min. Three control solutions, mixtures of the respective dyes without the major matrix compound, were also prepared.

All the components were at the concentrations used in formulations according to COLIPA, and five injections of each type of solution were made. For each series of measurements of control and mixture, a new column was used, as it has been shown in a preliminary study that after 50 injections, the efficiency of the column is dramatically decreased (data not reported). Table IV gives the statistical results obtained for the retention times and the corrected peak areas for each of these compounds.

Comparison of the data between mixture 1 (BHT) and control 1, mixture 2 (DMDM) and control 2, and finally mixture 3 (EOP) and control 3 leads to several observations. First of all, as shown by the results, DMDM or EOP do not interfere with the dye intermediates or with the column in a way that would change the chromatographic

Table IV
Retention Times and Corrected Peak Areas for Three Matrix Compounds and Four Dye Intermediates in
Dye-Matrix Mixtures and in Control Solutions (n = 5)

Column	Mixture	Compound	Retention time (min)		Corrected peak area (AU)	
			Mean	RSD (%)	Mean	RSD (%)
1	1	4-ap	5.59	0.85	11.5	7.2
		res	9.31	1.14	0.3	3.1
		2-n-1,4-pd	13.75	0.53	9.1	21
		NaAsc	2.18	0.38	54.7	7.8
		BHT	38.07	0.55	2.5	19.0
	Control 1	4-ap	5.57	0.40	10.6	5.9
		res	9.55	0.82	0.3	5.0
		2-n-1,4-pd	13.89	0.50	10.0	6.5
		NaAsc	2.18	0.21	56.5	8.0
2	2	1,4-pd	5.36	0.39	14.6	1.6
		res	8.32	0.34	0.5	2.9
		2-n-1,4-pd	12.91	0.55	14.4	1.1
		NaAsc	2.35	0.60	70.3	2.3
		DMDM	6.72	0.42	5.4	15.1
	Control 2	1,4-pd	5.33	0.19	15.0	2.0
		res	8.28	0.53	0.5	1.3
		2-n-1,4-pd	13.28	3.39	14.4	3.8
		NaAsc	2.35	0	80.7	1.5
3	3	4-ap	5.99	0.39	22.1	1.7
		res	9.53	0.79	0.6	1.5
		2-n-1,4-pd	14.09	0.39	20.4	1.6
		NaAsc	2.27	0.25	35.7	5.5
		EOP	36.05	0.71	6.4	0.6
	Control 3	4-ap	6.32	0.88	22.7	2.0
		res	10.26	1.99	0.6	5.9
		2-n-1,4-pd	14.84	1.42	20.3	1.6
		NaAsc	2.24	0.24	53.2	6.3

behavior (retention time, corrected peak area) of the dye intermediates. Furthermore, they do not alter the repeatability of the separation. For instance, when DMDM is added to the sample, the RSD on the retention time of the various hair dyes varies from 0.3%

to 0.5% and that of the corrected peak areas stays below the limit of 5%. For DMDM, this RSD value is of the order of what was obtained when DMDM was analyzed separately (see Table III). Nevertheless, DMDM has a retention time greater than 5 minutes and could (potentially) be confused with a dye intermediate (such as *p*-phenylenediamine, for instance). However, DMDM, which shows two chromatographic peaks at 220 nm with exactly the same UV spectrum, does not show any absorbance signal at any wavelength greater than 250 nm. Thus, a selective detection of the dye intermediates can be carried out at a wavelength greater than 250 nm.

For EOP, Tables III and IV show that EOP has a retention time much greater than 30 minutes, and thus cannot be confused with any dye intermediate. Furthermore, the peak retention times and the peak areas of the dye intermediates are not significantly changed by the matrix compound regarding either the mean values or the RSD.

Nevertheless, since EOP does not interfere with the hair dye separation in these conditions, an additional parameter, i.e., the concentration, has been checked. A broad range of concentrations of EOP were tested around the concentration used in formulations. Five RP-HPLC analyses were carried out for each concentration of EOP in a three-dye intermediate solution containing NaAsc as an antioxidant. Figure 2 shows the evolution of the mean corrected area of the peaks obtained for the three dye intermediates with increasing concentration of EOP in the sample. The graphs clearly show that the corrected areas do not vary significantly (p = 0.05) when EOP has been added to the sample solution over a concentration range of 2 g/100 g–10 g/100 g. The statistical analysis of the results (data not reported) shows good repeatability of the analysis for each selected concentration of EOP. The RSD is less than 5% for the retention time and the corrected peak area.

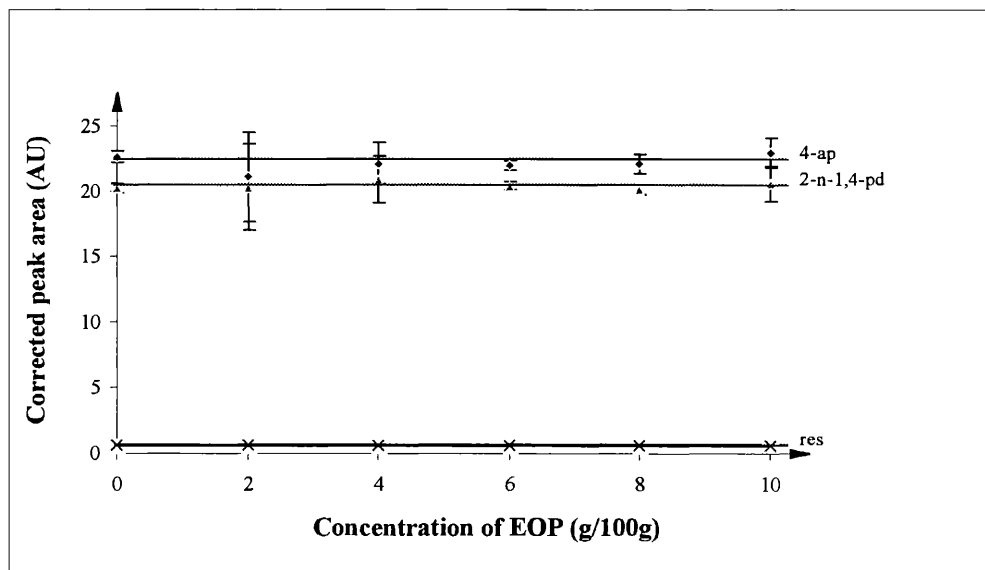Concerning BHT, the results show that the presence of this matrix compound does not

Figure 2. Influence of the concentration of EOP on the corrected peak areas of 4-ap, res, and 2-n-1,4-pd. (See text for experimental data and abbreviations.)

affect either the retention times of the dyes or their RSD, nor the mean values of the corrected peak area. Nevertheless, it is important to note that the repeatability of the measurements for one component (2-n-1,4-pd) seems to be strongly affected by the presence of BHT, with the RSD increasing from 6.4% to 21%. BHT itself has a high RSD for its peak corrected area.

Regarding these results, it appears, quite clearly, that although the matrix compound EOP does not interfere with the dye intermediates or with the column in a way that would affect the chromatographic behavior of the dye intermediates, it would be of advantage to remove BHT and DMDM from the sample solution before proceeding with the separation in order to obtain an accurate separation of the dye intermediates.

OPTIMIZATION OF THE LIQUID–LIQUID EXTRACTION METHOD

Since several matrix compounds are present in a real formulation, it is of immense interest to determine the effect of the isolation method on each of them. The optimization of the extraction protocol leads to a one-step (LDA, TDS, NNO, ORA), two-step (OA, BHT, DMDM, NaAsc), or three-step (CC, DC) extraction by n-heptane, while EOP, NOL, LS, SS, TEA, PQ, MP, and PVP are not extracted. Two different approaches were considered for testing the extraction method. BHT and EOP were submitted to extraction in a mixture containing the dye intermediates as well, whereas in a second approach, the extraction was performed on single solutions of matrix compounds.

Extraction of sample solutions containing a matrix compound and three dye intermediates. The extraction protocol has to be efficient to extract the matrix compounds, of course, but the target dyes should not be extracted. A one- to three-step extraction protocol was thus applied to sample solutions containing three selected dye intermediates (4-ap, res, and 2-n-1,4-pd), the antioxidant NaAsc, and a major matrix product (BHT or EOP). The aqueous phases obtained were submitted to HPLC, and the extraction yield was calculated according to the following equation:

$$\text{yield (\%)} = 100 - \left(\frac{\text{corrected area after extraction}}{\text{corrected area before extraction}} \times 100 \right) \tag{1}$$

Table V gives the extraction yield for each component in the solution, and the successive chromatograms obtained for the solution containing BHT before (a) and after (b) the extraction procedure are shown in Figure 3. First of all, it is clearly seen that the extraction procedure affects neither the chromatographic characteristics of the dye in-

Table V
Statistical Extraction Yield of the Components of a Dye-EOP and a Dye-BHT Mixture

Compound	One-step extraction yield (%)	Two-step extraction yield (%)
4-ap	0	0
Resorcinol	0	0
2-n-1,4-pd	0	0
NaAsc	19	50
EOP	—	0
BHT	89	100 (n.p.)

n.p.: no peak.
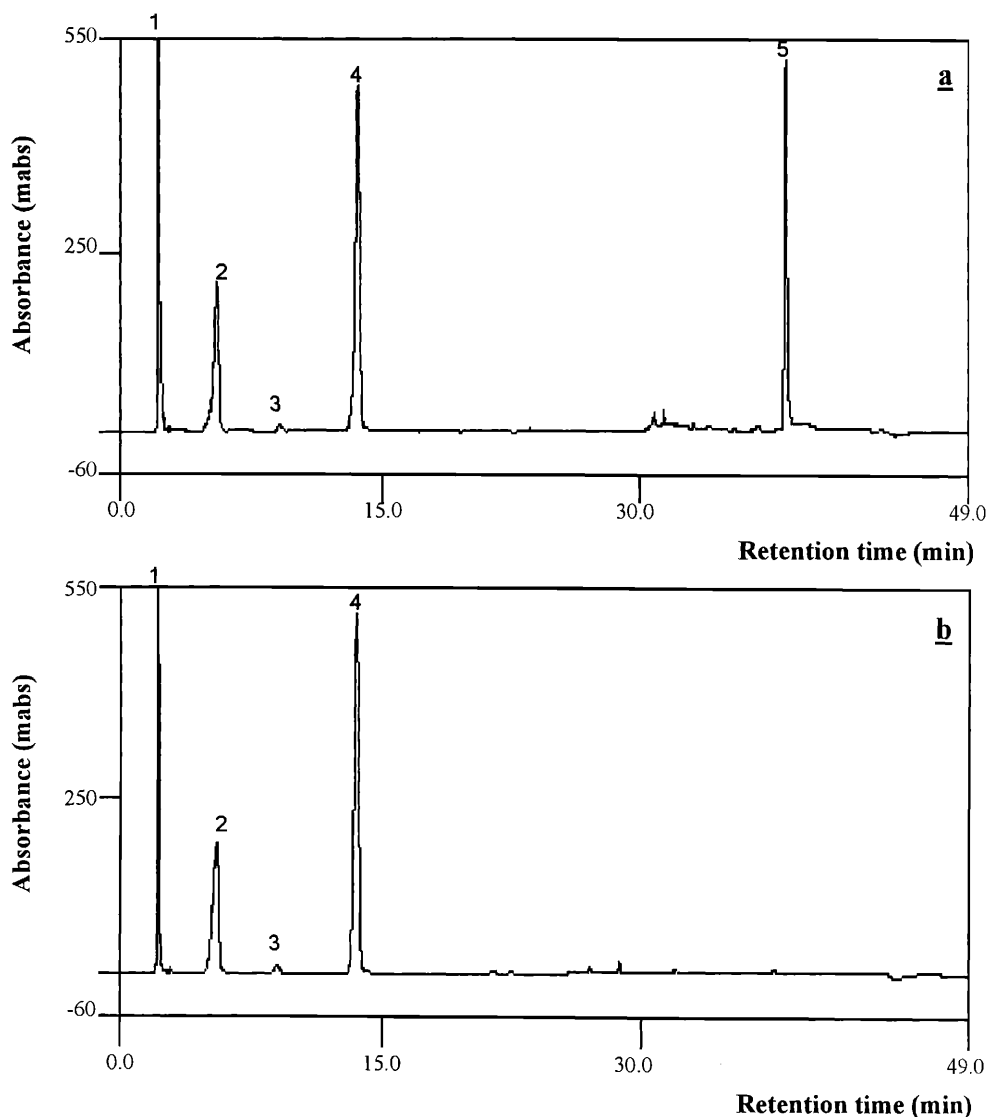
Figure 3. Successive chromatograms of a dye intermediate mixture with BHT added to the sample before and after extraction. a: Before extraction. b: After a two-step extraction. Peaks: (1) NaAsc (intrinsic matrix, antioxidant); (2) 4-ap; (3) res; (4) 2-n-1,4-pd; and (5) BHT (matrix, antioxidant). Peaks 2, 3, and 4 are unchanged between steps a and b. (See text for experimental data and abbreviations.)

termediates (retention times, corrected peak areas of peaks 2, 3, and 4) nor the repeatability of the analysis. Then, after a two-step extraction by n-heptane, 50% of the antioxidant NaAsc is extracted while 100% of BHT (peak 5) is extracted from the sample. However, it appears that EOP is not at all extracted by n-heptane. Additional experimental work has shown that EOP was 100% extractable by other tested organic solvents (trichloromethane, dichloromethane, or diethylic ether, for instance) but that they also lead to the partial extraction of the phenolic dyes (data not reported).

This extraction procedure by n-heptane is therefore very effective for the separation of

BHT from the sample and partially effective in the case of NaAsc, before the HPLC analysis. Moreover, it has to be stressed that with this extraction procedure by n-heptane, the non-extractability of the dye intermediates is ensured (9).

Extraction of single-compound matrix solutions. Due to the fact that the dye intermediates are not extracted by n-heptane, the extraction protocol was then performed on single-compound matrix solutions. The extraction protocol was applied to 16 individual solutions of matrix compounds, namely OA, NNO, ORA, DMDM, BHT, PVP, MP, LDA, TDS, DC, NOL, SS, LS, TEA, PQ, and CC (see their function in Table I). The resulting aqueous phases obtained were submitted to HPLC or analyzed by UV-Vis spectrophotometry when necessary.

The first seven compounds (OA, NNO, ORA, DMDM, BHT, PVP, and MP) show chromatographic peaks for which the chromatographic characteristics are given in Table III. For these products, the extraction yield was calculated according to equation 1. For the last nine compounds, i.e., LDA, TDS, DC, NOL, SS, LS, TEA, PQ, and CC, the final analysis could not be performed by RP-HPLC for several reasons: because LDA, NOL, and TDS are surfactants, the submission of these compounds to RP-HPLC leads to their adsorption in the system, thus provoking further contamination, and the compounds CC, LS, SS, TEA, DC and PQ do not show any chromatographic peak when submitted to RP-HPLC. As an alternative, a UV-Vis spectrophotometer was then used to test the extraction procedure on these nine compounds, by recording the spectra obtained before and after extraction. In order to determine if the UV-Vis spectrophotometer was sensitive enough for the extraction measurements, BHT, which has been shown to be extracted by n-heptane (see above), was used for comparing results obtained using both procedures.

Evaluation of the extraction yield using RP-HPLC. Figure 4 shows the successive chromatograms obtained before and after extraction of four compounds (OA, NNO, ORA, DMDM). Table VI gives the extraction yield obtained. It must be noted that the efficiency of the extraction protocol varies slightly, depending on the matrix product. OA needs a two-step extraction procedure to be fully extracted (Figure 4, 1a–c), while the peaks of NNO (Figure 4, 2a,b) and ORA (Figure 4, 3a,b) disappear after a one-step extraction procedure. Moreover, even a two-step procedure does not lead to a complete extraction of DMDM (Figure 4, 4a–c) and NaAsc. Finally, PVP and MP are not at all extracted.

The extraction protocol has therefore been shown to be efficient for isolating OA, NNO, and ORA from the sample. Concerning DMDM, the extraction yield is about 80% and MP is not extracted. Nevertheless, by analogy with EOP (Figure 2), a similar study involving the concentration as an additional parameter leads to the conclusion that DMDM and MP do not interfere with the dye intermediates or with the column in a way that would affect the chromatographic behavior of the dye intermediates (results not shown). Concerning PVP, special attention should be given to real samples containing this matrix product, as it forms a film on the column surface during the first injection, leading to a modification of the retention times of the dye intermediates. The determination of the dye intermediates has therefore to be carried out mainly according to their spectrum (results not shown).

Evaluation of the extraction yield using UV-Vis spectrophotometry. The extraction yields
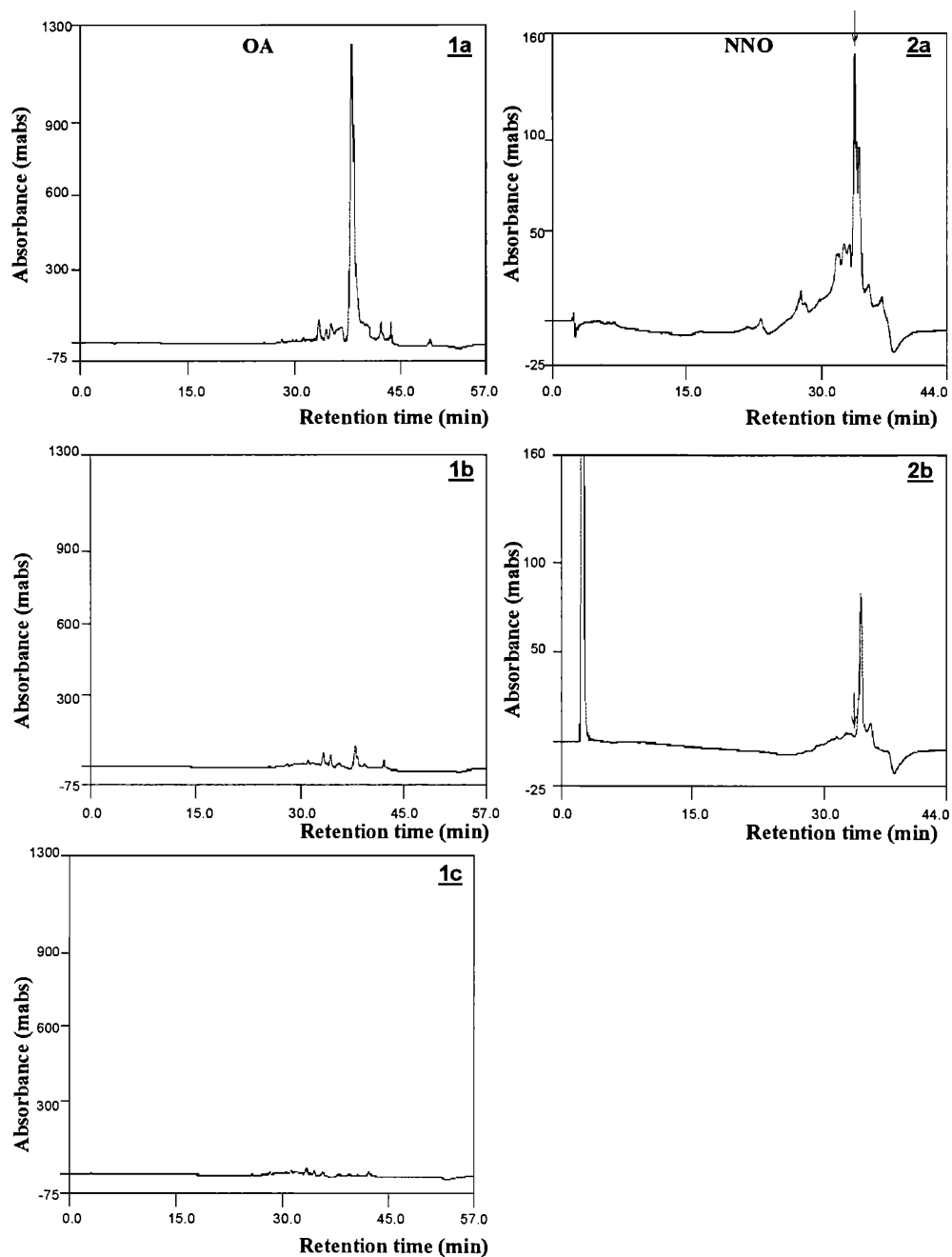
Figure 4. Successive chromatograms of four matrix product samples before and after extraction. 1: OA; 2: NNO; 3: ORA; 4: DMDM. a: Before extraction. b: After a one-step extraction. c: After a two-step extraction. (See text for experimental data and abbreviations.)

obtained are reported in Table VI. The complete extraction of BHT in a two-step extraction procedure was confirmed, and the extraction protocol has also been shown to be efficient for isolating LDA, TDS, CC, and DC from the sample. Nevertheless, once
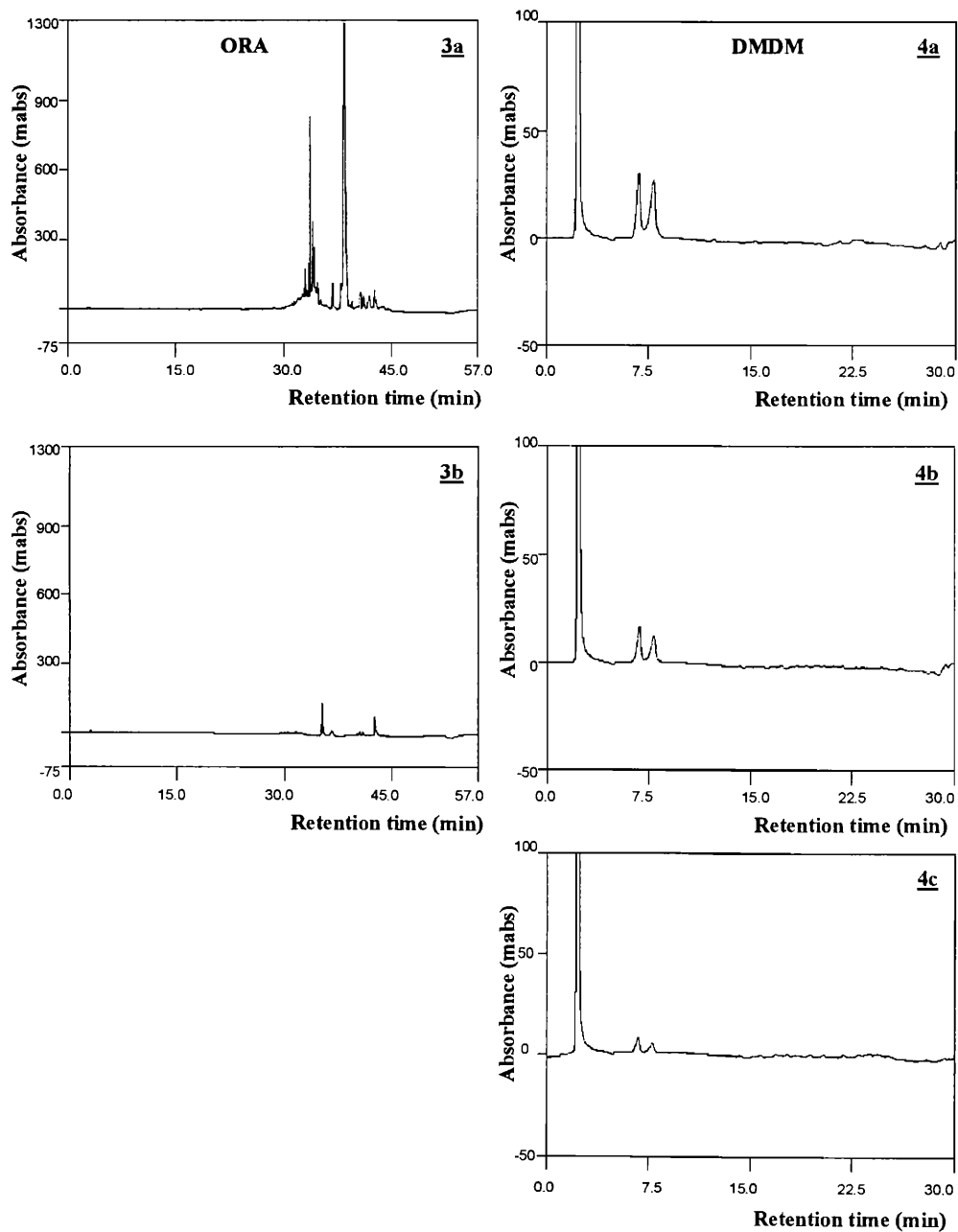
Figure 4. Continued

more, it has to be noted that the efficiency of the extraction protocol varies slightly, depending on the matrix product. LDA and TDS need a one-step extraction procedure to be fully extracted, while the complete extraction of CC and DC involves a three-step extraction procedure. Moreover, NOL, LS, SS, TEA, and PQ are not at all extracted.

Table VI
Statistical Extraction Yield of Eighteen Matrix Compounds

Evaluation using	Compound	Extraction yield (%)	Extraction step number
H P L C	OA	100 (n.p.)	2
	NNO	100 (n.p.)	1
	ORA	100 (n.p.)	1
	DMDM	80	2
	NaAsc	50	2
	EOP	0	2
	MP	0	2
	PVP	0	2
U V / V I S	BHT	100 (n.p.)	2
	LDA	100	1
	TDS	100	1
	CC	100	3
	DC	100	3
	LS	0	2
	SS	0	2
	NOL	0	2
	PQ	0	2
	TEA	0	2

n.p.: no peak.
UV/VIS: UV-Vis spectrophotometry.

CONCLUSION

The chromatographic method previously set up for the determination of hair dye intermediates has been shown to be extremely effective for the analysis of forty-seven hair dye intermediates commonly used in cosmetic formulations. Using that method, a data base of the retention time and of the spectrum has been built up for the forty-seven dyes, which allows the determination of the dyes and their discrimination. In most cases, the retention times of the hair dyes range from 5 to 30 minutes. Thus problems with confusing dye intermediates with matrix products, which generally have retention times of less than 5 minutes or greater than 30 minutes, should not be encountered. Once more, the UV spectrum constitutes a powerful criterion of discrimination when the retention time of a particular dye or a particular matrix compound is outside the expected range.

The influence of eighteen matrix products commonly used in hair dye formulations has also been investigated in relation to the determination of hair dye intermediates. Nine of the eighteen matrix compound show retentive behavior and UV-detectable peaks. Their individual retention times and UV spectra have therefore also been recorded and included in the data base with the aim of providing as complete a picture as possible of the separation of hair dyes in complex matrix media. The nine matrix compounds left over cannot be identified under these chromatographic conditions, but their UV spectra have also been recorded in order to complete the data base.

However, on the whole, the presence of some matrix compounds seems to affect the final accuracy of the quantitative measurements of some hair dyes (case of 2-n-1,4-pd), leading to the conclusion that a separation of the matrix compounds from the dye

solutions is desirable. A liquid–liquid extraction procedure by n-heptane has therefore been set up. This method was tested on all the eighteen selected matrix products and was 100% effective for eight products (LDA, TDS, CC, DC, NNO, ORA, OA, BHT) in a one- to three-step extraction procedure. DMDM and NaAsc were only partially extracted (80% and 50%, respectively), while EOP, MP, PVP, LS, SS, NOL, PQ and TEA were not extracted. Nevertheless, the non-interference of these compounds with the dye intermediates or with the column, has been proved, except in the case of PVP.

A three-step liquid–liquid extraction procedure by n-heptane, which is not toxic to the environment, is therefore suggested as a compulsory step for the separation of matrix products from the dye-containing sample before analysis by reversed-phase HPLC to avoid all problems of potential interference between the dyes and the matrix compounds or between the matrix compounds and the column in a way that would affect the chromatographic determination of the dyes. This procedure will now be applied to actual commercial formulations.

As a general conclusion, it has been shown that after applying a matrix extraction procedure using n-heptane, the RP-HPLC method, combined with diode array detection, is very promising in terms of being an efficient candidate reference method for the identification and quantitation of oxidative hair dyes.

NOTE

A spectra' database of eighteen matrix products has been recorded through the diode array detector and the UV spectrophotometer as well as a spectra' database of forty-seven dye intermediates (8). For further information about the database, email contact: vincent@irmm.jrc.be

APPENDIX

List of Some Frequently Used Matrix Products

Matrix component	Usual concentration (g/100 g)
Ammonia	6.00–9.00
Ascorbic acid	0.20
L-ascorbic acid sodium salt	0.20
BHT	0.25
Diethyleneglycolmonoether	5.00
Citric acid	0.30
Diethanolamine	2.00
Triethanolamine	0.10–1.50
TEA-dodecylbenzenesulfonate	0.50
DEDM-hydantoin	0.10

Appendix (continued)

Syntopon 8 D1®	6.00
Ethoxylated nonylphenol	3.00
Ethyl acetate	2.00
Hydroxyethyl cellulose	2.40
Isopropanol	3.00–15.00
Lauric diethanolamide	1.50–2.00
Methyl paraben	0.05
Propylene glycol	5.00–9.00
Sodium lauryl sulfate	2.95
Sulfated castor oil	4.00
Polyvinylpyrrolidon	2.00
Oleic acid	5.00
n-Nonylamine	25.00
Oranex HT®	3.00
Cetrimonium chloride	0.50

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