



EUROPEAN COMMISSION  
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL  
Directorate C - Public Health and Risk Assessment  
C7 - Risk assessment

## **SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS**

### **SCCP**

#### **Opinion on**

#### **Exposure to reactants and reaction products of oxidative hair dye formulations**

Adopted by the SCCP  
during the 6<sup>th</sup> plenary of 13 December 2005

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## 1. BACKGROUND

Commission services together with Member States agreed in April 2003 on a detailed programme of an overall strategy for the evaluation of hair dyes within the framework of the Cosmetics Directive 76/768/EEC. The strategy was published as *Information note on the use of ingredients in permanent and non-permanent hair dye formulations (dye precursors and direct dyes)* on: <http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf>

This strategy has been decided following two opinions of the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP). In its opinion of June 2001 on "The Use of Permanent Hair Dyes and Bladder Cancer Risk" SCCNFP/484/01, the SCCNFP concluded that *the potential risk for the development of bladder cancer in past users of permanent hair dyes is of concern*. The SCCNFP recommended that *the European Commission provides resources for the urgent review of the information, further epidemiological studies are performed to evaluate the possible association between bladder cancer and the use of permanent hair dyes in the EU and the European Commission takes further steps to control the use of hair dye chemicals since the potential risks of using this category of substances give cause for concern*.

In its opinion of December 2002 on "Assessment Strategies for Hair Dyes" SCCNFP/0553/02, the SCCNFP recommended an overall safety assessment strategy for hair dyes including the requirements for testing hair dye cosmetic ingredients for their potential genotoxicity and carcinogenicity.

Against this background, Commission services together with Member States agreed on a regulatory strategy to all hair dyes in use. The main element of the strategy is a tiered, modulated approach requiring industry to submit by certain deadlines files on hair dyes to be evaluated by the SCCNFP.

According to the hair dye strategy, dossiers for combinations of ingredients in permanent hair dyes have to be submitted by industry at the latest December 2007 for the evaluation in Scientific Committee on Consumer Products (SCCP). On 23 April 2004, SCCNFP forwarded its opinion on "Ring Study on Reaction Products from Typical Combinations of Hair Colouring Ingredients". It stated *that the analytical method developed for the determination of reactants and reaction products of oxidative hair dye formulations is based on sound chemistry and that is validated. However, both qualitative and quantitative information on reactants and reaction products of various hair dye formulations, under use conditions, are necessary for safety evaluation of these products*.

Meanwhile, COLIPA (European Cosmetics Toiletry and Perfumery Association) has submitted a "Technical Report Addressing Concerns regarding Exposure to Reaction Products During their Hair Dyeing Process" (January 2005) aiming at providing additional information for combinations of ingredients in hair dyes.

## 2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) share the view presented in the “Technical Report” that oxidative hair dye reaction products pose no or negligible risk to human health?*
2. *If not, the SCCP is invited to identify any additional information necessary to evaluate the overall risk to reaction products of hair dyes.*

## 3. OPINION

### 3.1. General

The report “Investigation into oxidative hair dye chemistry –Part II” is an extension of the earlier submitted study by COLIPA - Ring study on reaction products from typical combinations of hair colouring ingredients – in which a method for hair colouring *in vitro* followed by quantification of the oxidative hair dyes formed as well as unused precursors and couplers was optimised and validated. Kinetics of oxidative hair dye formation using 3 combinations of a precursor with two different couplers was reported in the earlier submitted study. The SCCNFP considered that the method employed was acceptable for the estimation of reactants and reaction products during the use of oxidative hair dyes. Furthermore, the SCCNFP expressed the need of both qualitative and quantitative information on reactants and reaction products of various hair dye formulations, under use conditions, for an appropriate safety evaluation of these products. Such data on 11 combinations of 5 precursors and 5 couplers present in oxidative hair dye formulations, as well as reaction kinetics of colour formation are reported in the present report. In addition, the results of *in vitro* percutaneous absorption studies performed using 4 reaction products (oxidative hair dyes) are also submitted for the safety evaluation.

As the chemistry and reaction kinetics of oxidative hair formation using various precursor and couplers appears to be similar, all submitted data has been considered in this evaluation, irrespective of the evaluation of some of the same data submitted earlier (SCCNFP/0808/04).

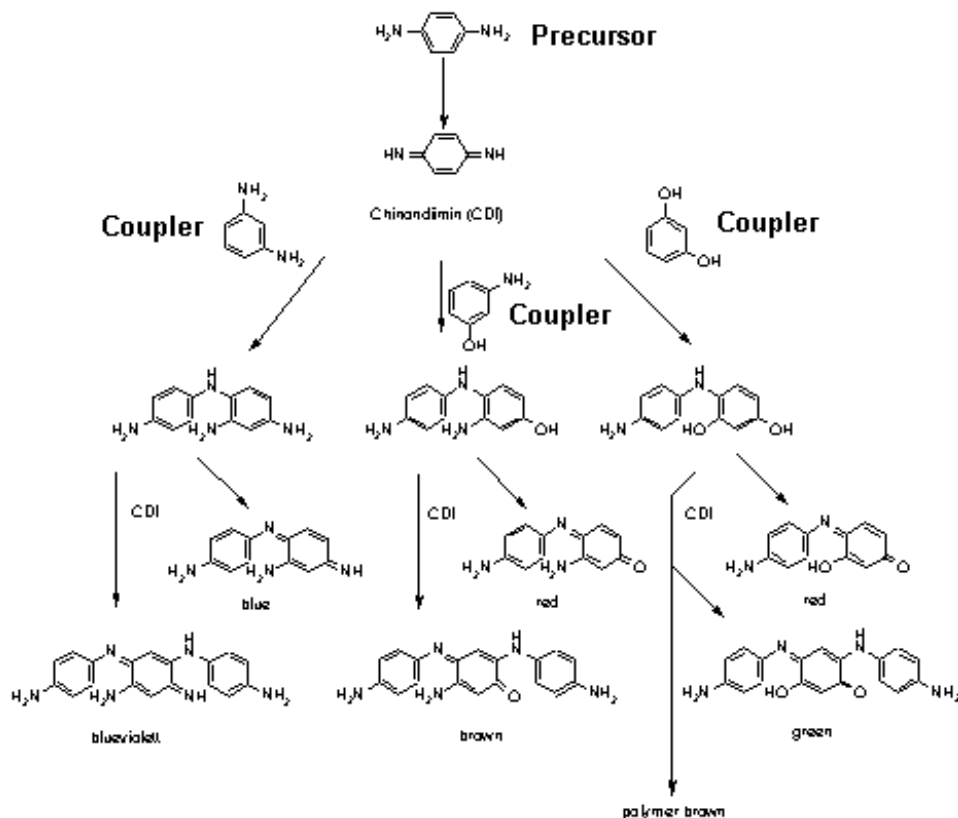
### 3.2. Chemistry of oxidative hair dye formation

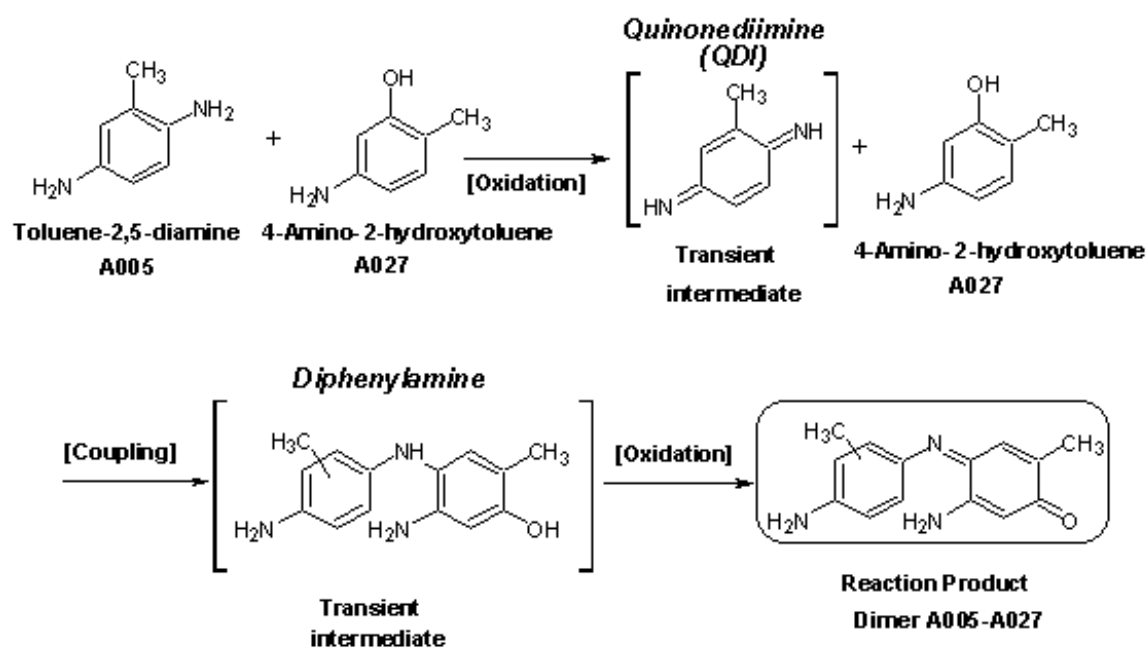
Oxidative hair dye formulations contain precursor (such as p-phenylenediamine, p-aminophenol) and coupler (such as m-aminophenol, resorcinol) molecules, which are mixed with peroxide under alkaline conditions and applied to hair. The molecules (precursor and coupler) oxidatively couple to form coloured molecules (oxidative hair dyes) as shown in Figure 1 (Ref. 1).

Couplers that have only one free position *para* to the hydroxy- or amino- activating group will couple 1:1 with a precursor, and this coupled dimeric reaction product is the final dye. An example of this is shown in Figure 2, where the precursor toluene-2,5-diamine (A5) is oxidised by alkaline hydrogen peroxide to give a transient species quinonediimine (QDI). The QDI reacts rapidly with the coupler 4-amino-2-hydroxytoluene (A27) to form a diphenylamine transient intermediate, which oxidises instantaneously to give the final red/violet dimeric reaction product

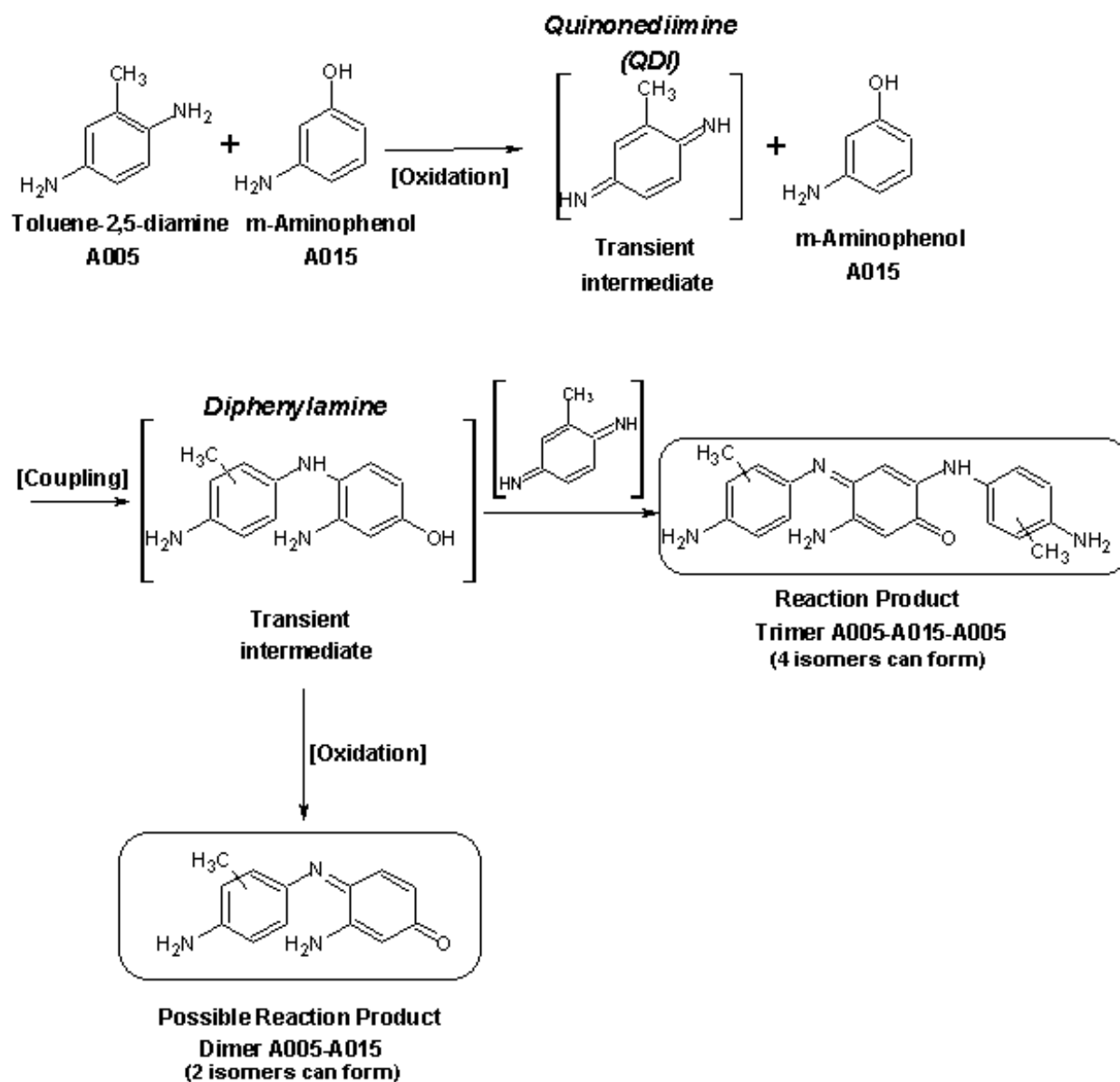
A5-A27 (Ref. 2). The reaction kinetics of the dye formation indicate that the intermediate species may not accumulate in the system (Ref. 3 -7).

**Figure 1:** Schematic presentation of oxidative hair dye formation by coupling of the precursor p-phenylenediamine with different couplers.

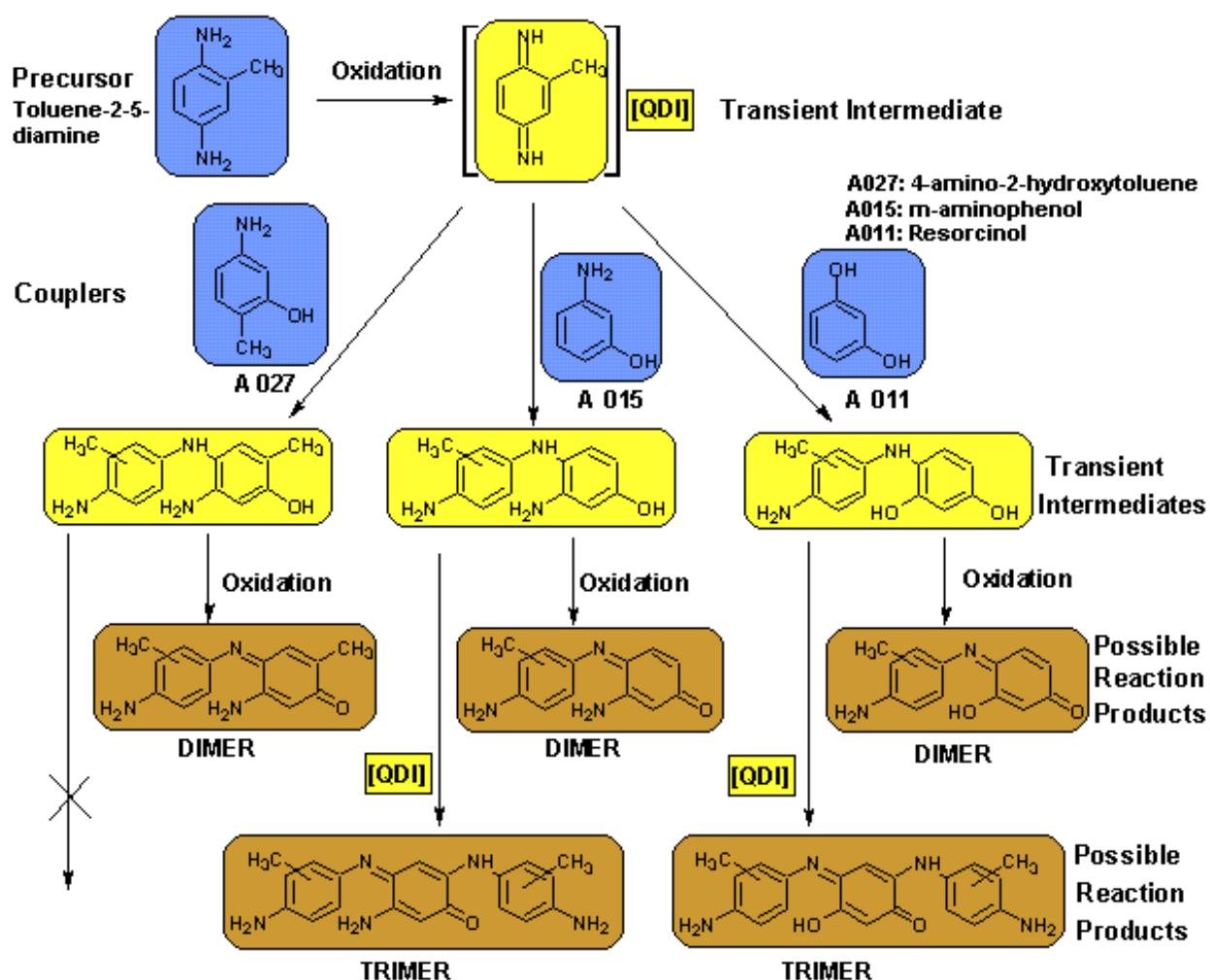


**Figure 2:** Oxidative coupling leading to a dimer species

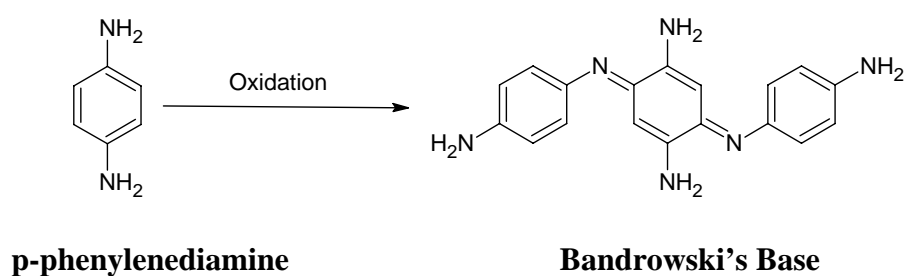
Couplers that have both positions para to the hydroxy or amino groups free can couple in a ratio of 2 precursors to 1 coupler, and the final dye will be a trimer. For example, precursor toluene-2,5-diamine (A5) is oxidised by alkaline hydrogen peroxide to give the QDI transient intermediate, which then couples with *m*-aminophenol (A15) to form a diphenylamine transient intermediate. The diphenylamine transient intermediate can rapidly oxidise to give a dimer or can further react with QDI to give a trimeric reaction product A5-A15-A5 (Figure 3).

**Figure 3:** Oxidative coupling leading to a trimer species

Marketed hair dye products almost always contain more than two dye forming ingredients. A theoretical reaction scheme for a formula containing one precursor and several couplers is shown in Figure 4. It is possible that a number of dimers and trimers may form.

**Figure 4:** Theoretical reaction scheme of the formation of oxidative hair dyes for a formula containing a precursor and several couplers

In the absence of a coupler, some precursors can self-couple. For example self coupling of p-phenylenediamine can produce Bandrowski's Base (Figure 5). As the rate of self-coupling of a precursor is a very slow reaction compared to the reaction with a coupler in the reaction mixture (Ref. 3, 8). Bandrowski's Base may not be formed in an oxidative hair dye mixture containing both p-phenylenediamine and a coupler.

**Figure 5:** Formation of Bandrowski's Base by self coupling of p-phenylenediamine



### 3.3. Methodology

#### 3.3.1. Selection of precursors and couplers and their combinations

Among a variety of precursors and couplers used to colour the hair, 11 precursor/coupler combinations chosen as a focus for this study were based on:

- tonnage used in hair colouring per year.
- class of chemical compound; precursors and couplers were selected in order to cover representative classes of chemical compounds, which are typically used in hair colouring products. For example, p-phenylenediamine (A7), p-aminophenol (A16) and pyrazole (A154) type precursors were studied.

Based on the above criteria, the following combinations were studied:

- toluene-2,5-diamine (A5) + 4-amino-2-hydroxytoluene (A27)
- p-phenylenediamine (A7) + 4-amino-2-hydroxytoluene (A27)
- p-aminophenol (A16) + 4-amino-2-hydroxytoluene (A27)
- 1-hydroxyethyl-4,5-diaminopyrazole (A154) + 4-amino-2-hydroxytoluene (A27)
- toluene-2,5-diamine (A5) + 2,4-diaminophenoxyethanol (A42)
- toluene-2,5-diamine (A5) + resorcinol (A11)
- toluene-2,5-diamine (A5) + 2-methylresorcinol (A44)
- toluene-2,5-diamine (A5) + m-aminophenol (A15)
- 1-hydroxyethyl-4,5-diaminopyrazole (A154) + m-aminophenol (A15)
- N,N-bis-hydroxyethyl-p-phenylenediamine (A50) + m-aminophenol (A15)
- toluene-2,5-diamine (A5) + 4-amino-2-hydroxytoluene (A27) + m-aminophenol (A15)

The above combinations in a cream formulation were used for the study (Table 1). Both precursors and couplers in the cream formulation were present at a concentration of 125 µmol/g (0.125 molar).

**Table 1:** Hair dye cream emulsions used in the study. Concentration of ingredients are given as % (w/w)

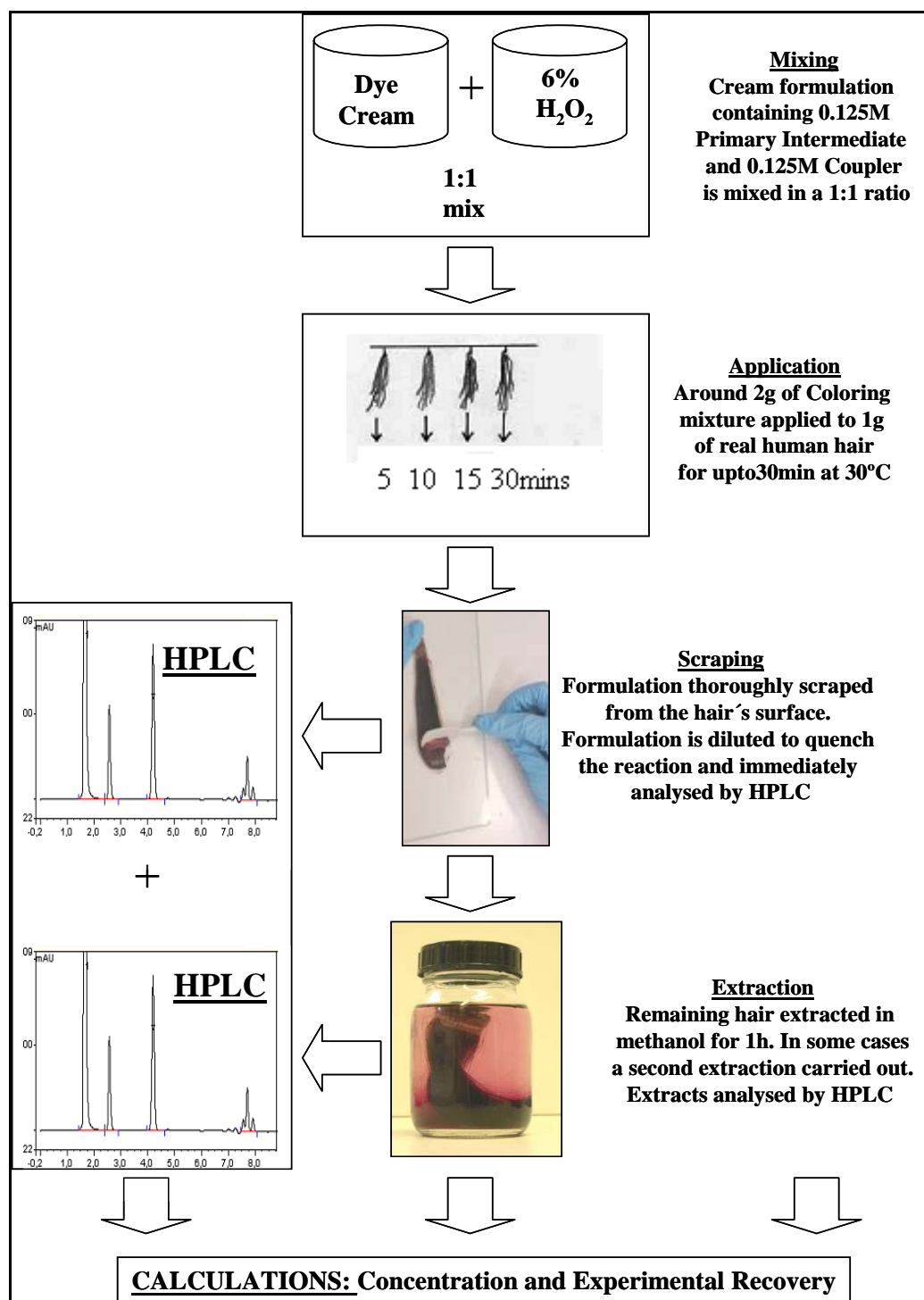
Ingredient	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
A5 (Sulphate salt)	2.75	/	/	/	2.75	2.75	2.75	/	/	2.75	5.50
A7	/	1.35	/	/	/	/	/	/	/	/	/
A16	/	/	1.36	/	/	/	/	/	/	/	/
A154	/	/	/	1.78	/	/	/	1.78	/	/	/
A50	/	/	/	/	/	/	/	/	2.45	/	/
A27	1.54	1.54	1.54	1.54	/	/	/	/	/	/	1.54
A15	/	/	/	/	/	/	1.36	1.36	1.36	/	1.36
A42	/	/	/	/	2.06	/	/	/	/	/	/
A44	/	/	/	/	/	1.55	/	/	/	/	/
A11	/	/	/	/	/	/	/	/	/	1.37	/
Cetearyl Alcohol	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Lanolin Alcohol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

## Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

<b>Ingredient</b>	<b>I</b>	<b>II</b>	<b>III</b>	<b>IV</b>	<b>V</b>	<b>VI</b>	<b>VII</b>	<b>VIII</b>	<b>IX</b>	<b>X</b>	<b>XI</b>
<b>28% Sodium Laureth Sulfate</b>	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
<b>EDTA</b>	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
<b>Sodium Sulfite</b>	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
<b>Ascorbic Acid</b>	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
<b>Ammonia (25%)</b>	4.55	4.55	4.55	4.55	4.55	4.55	4.55	4.55	4.55	4.55	4.55
<b>Water up to</b>	100	100	100	100	100	100	100	100	100	100	100

## 3.3.2. Experimental

An earlier developed protocol for the *in vitro* study, in the presence of hair, of the reaction kinetics of oxidative hair dye formation, as well as for the estimation of reactants and reaction products under use conditions was applied. The protocol, applied for the combinations A5+A27 and A7+A27, was suitable for the purpose (SCCNFP/0808/2004). The experimental methodology is presented in Figure 6.

**Figure 6:** Schematic presentation of experimental methodology

On the basis of the chemistry of oxidative hair dyes, as described before, the possible reaction products for each set of precursor and coupler were predicted and synthesised. The synthesised products were appropriately characterised and then used as reference standards for the identification and determination of these compounds in hair dye formulations.

The precursors and couplers as well as the dyes formed (reaction products) in hair dye formulations and in methanol extracts of dyed hairs were determined by high performance liquid chromatography (HPLC) with UV detection.

The experimental recoveries of the precursors/couplers were calculated by the comparison of the amounts of these present in the hair dye formulations (at time 0) with the combined amounts of precursors/couplers present in the formulations after hair dyeing, in the methanol extracts of dyed hairs and those present in the identified hair dyes. As all of the dye and the unreacted precursors/couplers from dyed hairs were not extracted in methanol, total recovery was established in 4 experiments where a cream formulation containing a radio-labelled precursor or coupler was used. In these experiments, the coloured hairs after methanol extraction were combusted to recover the remaining precursor/coupler that may be present in the form of the dye or as unreacted molecules.

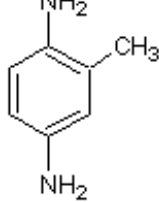
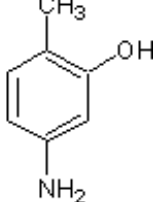
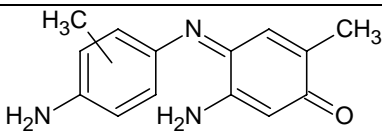
For all combinations of precursors and couplers, additional data was also generated to follow the kinetics of colour formation and the amounts of precursor/coupler present in the formulation over time.

### 3.4. Results

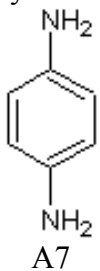
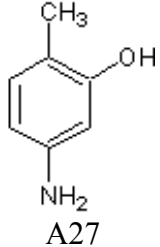
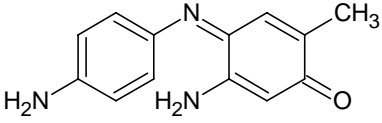
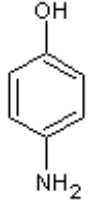
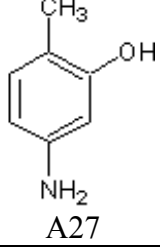
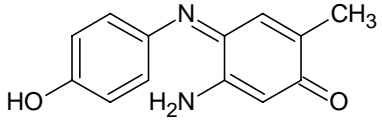
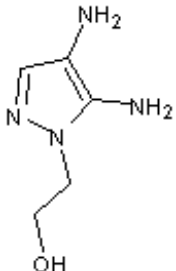
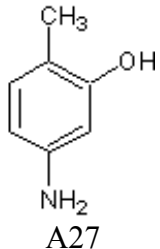
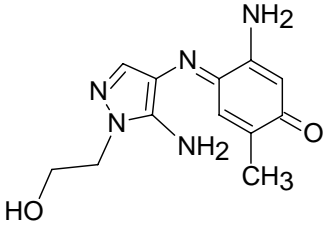
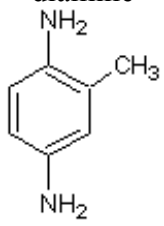
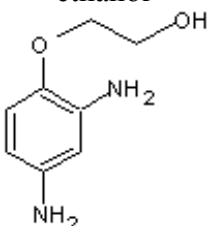
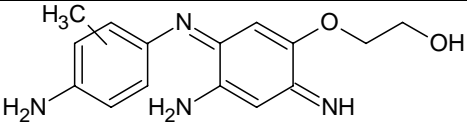
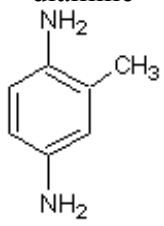
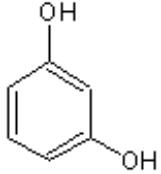
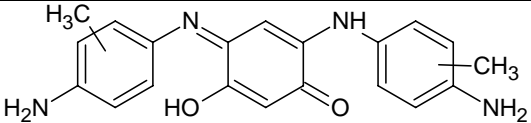
Approximately 2 g of hair dye formulations (cream formulation/6% H<sub>2</sub>O<sub>2</sub>, 1:1) were used for *in vitro* dyeing of approximately 1 g hair, as indicated in Figure 6. The identification of the reaction products, hair dyes formed by oxidative coupling of precursors and couplers in 30 min, by HPLC, revealed the presence of dimers and trimers in the formulation as described in Table 2. No other newly synthesised molecules were identified in the formulations (outside hairs) after hair dyeing or in the methanol extracts of the dyed hairs. The absence of Bandrowski's Base in the extracts of the formulation containing p-phenylenediamine was appropriately demonstrated.

The amounts of identified hair dyes formed in 30 min in various formulations (outside hairs) were determined by HPLC using respective reference compounds (synthesised in this study). The levels of hair dyes in the formulations are described in Table 3

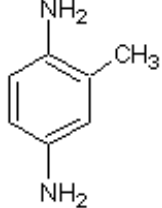
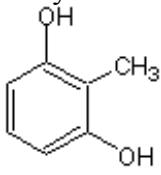
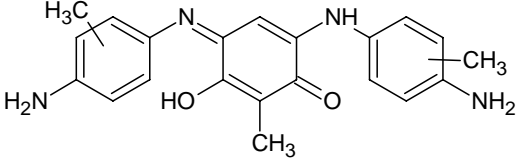
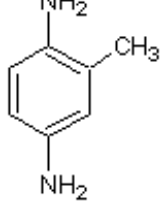
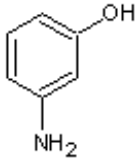
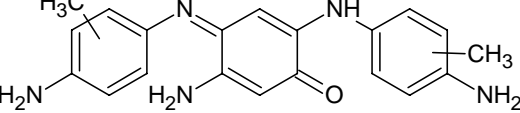
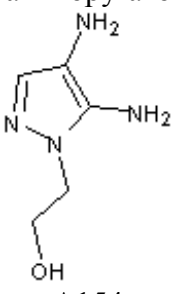
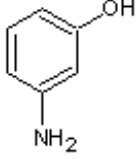
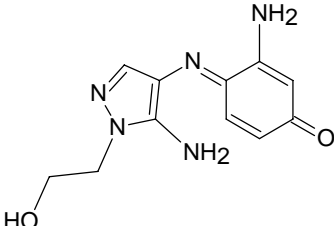
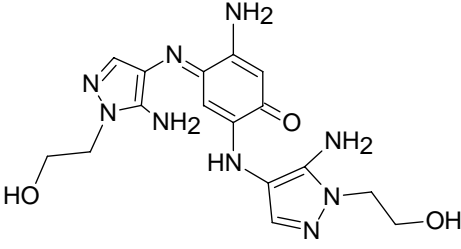
**Table 2: Hair dyes identified in the formulations containing various precursors and couplers.**

Precursor	Coupler	Reaction Product (Hair dye)
Toluene-2,5-diamine  A5	4-Amino-2-hydroxytoluene  A27	 5-Amino-4-((4-amino-2-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one, and 5-Amino-4-((4-amino-3-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one <b>Dimer A5-A27</b>

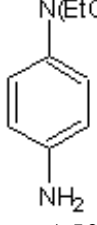
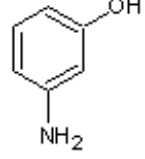
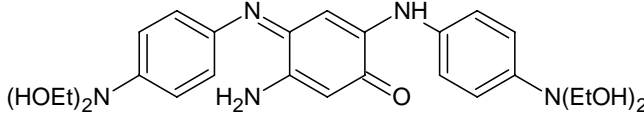
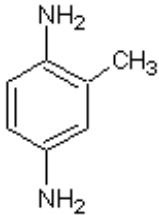
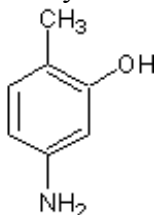
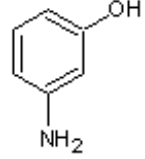
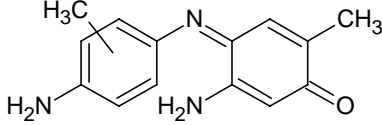
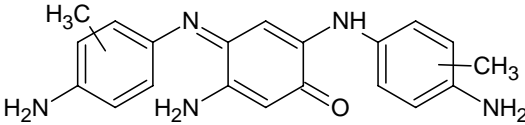
## Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

Precursor	Coupler	Reaction Product (Hair dye)
<p>p-Phenylenediamine</p>  <p>A7</p>	<p>4-Amino-2-hydroxytoluene</p>  <p>A27</p>	 <p>5-Amino-4-((4-aminophenyl)imino)-2-methyl-2,5-cyclohexadien-1-one</p> <p><b>Dimer A7-A27</b></p>
<p>p-Aminophenol</p>  <p>A16</p>	<p>4-Amino-2-hydroxytoluene</p>  <p>A27</p>	 <p>5-Amino-4-((4-hydroxyphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one</p> <p><b>Dimer A16-A27</b></p>
<p>1-Hydroxyethyl-4,5-diaminopyrazole</p>  <p>A154</p>	<p>4-Amino-2-hydroxytoluene</p>  <p>A27</p>	 <p>5-Amino-4-[5-amino-1-(2-hydroxyethyl)-1H-pyrazol-4-yl]imino]-2-methyl-2,5-cyclohexadien-1-one</p> <p><b>Dimer A154-A27</b></p>
<p>Toluene-2,5-diamine</p>  <p>A5</p>	<p>2,4-Diaminophenoxyethanol</p>  <p>A42</p>	 <p>2-(((3E)-4-Amino-3-[(methyl-4-aminophenyl)imino]-6-aminocyclohexadien-1-yl}oxy)ethanol</p> <p><b>Dimer A5-A42</b></p>
<p>Toluene-2,5-diamine</p>  <p>A5</p>	<p>Resorcinol</p>  <p>A11</p>	 <p>2-(((4-amino-3-methylphenyl)amino)-4-((4-amino-3-methylphenyl)imino)-5-hydroxy-2,5-Cyclohexadien-1-one</p> <p><b>Trimer A5-A11-A5</b></p>

## Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

Precursor	Coupler	Reaction Product (Hair dye)
<p>Toluene-2,5-diamine</p>  <p>A5</p>	<p>2-Methylresorcinol</p>  <p>A44</p>	 <p>6-[(Methyl-4-aminophenyl)amino]-4-[(methyl-4-aminophenyl)imino]-3-hydroxy-2-methylcyclohexa-2,5-dien-1-one</p> <p><b>Trimer A5-A44-A5</b></p>
<p>Toluene-2,5-diamine</p>  <p>A5</p>	<p>m-Aminophenol</p>  <p>A15</p>	 <p>5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one; 5-amino-2-(4-amino-2-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one; 5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-2-methylphenyl)imino]-2,5-cyclohexadien-1-one; and 5-amino-2-(4-amino-2-methylanilino)-4-[(4-amino-2-methylphenyl)imino]-2,5-cyclohexadien-1-one</p> <p><b>Trimer A5-A15-A5</b></p>
<p>1-Hydroxyethyl-4,5-diaminopyrazole</p>  <p>A154</p>	<p>m-Aminophenol</p>  <p>A15</p>	 <p>3-Amino-4-((5-amino-1-(2-hydroxyethyl)-1H-pyrazol-4-yl)imino)-2,5-Cyclohexadien-1-one</p> <p><b>Dimer A154-A15</b></p>  <p>(4Z)-5-amino-2-{{[5-amino-1-(2-hydroxyethyl)-1H-pyrazol-4-yl]amino}-4-{{[5-amino-1-(2-hydroxyethyl)-1H-pyrazol-4-yl]imino}}-2,5-cyclohexadien-1-one</p> <p><b>Trimer A154-A15-A154</b></p>

## Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

Precursor	Coupler	Reaction Product (Hair dye)
<p>N,N-bis-hydroxyethyl-p-phenylenediamine</p>  <p>A50</p>	<p>m-Aminophenol</p>  <p>A15</p>	 <p>5-Amino-2-[[4-[bis(2-hydroxyethyl)amino]phenyl]amino]-4-[[4-[bis(2-hydroxyethyl)amino]phenyl]imino]-2,5-cyclohexadien-1-one</p> <p><b>Trimer A50-A15-A50</b></p>
<p>Toluene-2,5-diamine</p>  <p>A5</p>	<p>4-Amino-2-hydroxytoluene</p>  <p>A27</p> <p>+</p> <p>m-Aminophenol</p>  <p>A15</p>	 <p>5-Amino-4-((4-amino-2-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one, and 5-Amino-4-((4-amino-3-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one</p> <p><b>Dimer A5-A27</b></p> <p>+</p>  <p>5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one; 5-amino-2-(4-amino-2-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one; 5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-2-methylphenyl)imino]-2,5-cyclohexadien-1-one; and 5-amino-2-(4-amino-2-methylanilino)-4-[(4-amino-2-methylphenyl)imino]-2,5-cyclohexadien-1-one</p> <p><b>Trimer A5-A15-A5</b></p>

**Table 3:** Concentrations of hair dyes in the formulations (outside hair) after hair dyeing for 30 min

Precursor and coupler combination	Level of reaction product (hair dye) in the formulation 30 min after application <sup>1</sup>	
	μmol/g	% by weight
<b>A5+A27</b>	11 (Dimer)	0.26
<b>A7+A27</b>	7 (Dimer)	0.16
<b>A16+A27</b>	6 (Dimer)	0.14
<b>A154+A27</b>	25 (Dimer)	0.65
<b>A5+A42</b>	13 (Dimer)	0.37
<b>A5+A11</b>	1 (Trimer)	0.03
<b>A5+A44</b>	0.5 (Trimer)	0.02
<b>A5+A15</b>	3 (Trimer)	0.14
<b>A154+A15</b>	13 (Dimer)	0.32

## Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

	5 (Trimer)	0.19
<b>A50+A15</b>	10 (Trimer)	0.5
<b>A5+A27+A15</b>	9 (Dimer)	0.21
	3 (Trimer)	0.10

<sup>1</sup> In case of combinations with A5, isomeric reaction products are obtained

The amounts of unreacted precursors and couplers in various formulations after 30 min hair dyeing, and in the methanol extracts of the dyed hairs, determined by HPLC, are described in table 4. The method was optimised and validated by way of a ring study using two combinations of precursor and coupler.

**Table 4:** Amounts of unreacted precursors and couplers after hair dyeing for 30 min

Precursor and coupler combination	Total $\mu$ mole applied to hair	Unreacted precursor/coupler recovered after 30 min hair dyeing			
		$\mu$ mole in the formulation	$\mu$ mole in the methanol extract	Total unreacted $\mu$ mole	(%)
<b>A5</b>	92 – 125*	9 - 28*	2 - 30*	11 - 58	(12 - 46)*
<b>A27</b>	88 – 121*	6 - 21*	7 - 32*	14 – 40	(15 - 42)*
<b>A7</b>	110 – 132*	22 - 42*	0 - 40*	33 – 69	(30 - 54)*
<b>A27</b>	107 – 143*	16 - 28*	13 - 56*	32 – 72	(30 - 58)*
<b>A16</b>	103	19	33	52	(51)
<b>A27</b>	91	11	31	42	(46)
<b>A154</b>	115	22	0	22	(19)
<b>A27</b>	115	13	11	24	(21)
<b>A5</b>	96	18	5	23	(24)
<b>A42</b>	86	17	4	21	(24)
<b>A5</b>	128	27	28	55	(43)
<b>A11</b>	126	31	71	102	(81)
<b>A5</b>	110	28	49	77	(70)
<b>A44</b>	111	27	65	92	(83)
<b>A5</b>	43	8	1	9	(21)
<b>A15</b>	43	9	15	24	(56)
<b>A154</b>	134	47	0	47	(35)
<b>A15</b>	141	30	30	60	(43)
<b>A50</b>	125	25	16	41	(33)
<b>A15</b>	131	21	64	85	(65)
<b>A5</b>	231	55	37	92	(40)
<b>A15</b>	116	30	40	70	(60)
<b>A27</b>	124	31	31	52	(42)

\* Experiments performed in 5 laboratories as a ring test.

All other results represent triplicate experiments performed in individual laboratories.

The recoveries of precursors/couplers calculated on the basis of combined amounts of these present in the formulations after hair dyeing for 30 min, in the methanol extracts and the amounts present in the respective hair dyes are described in Table 5.



**Table 5:** Experimental recoveries of precursors and couplers in *in vitro* hair dyeing experiments

Precursor and coupler combination	$\mu\text{mole Applied}$	$\mu\text{mole Recovered}$	% Recovery
A5	92 - 125*	75 - 116*	80 - 93*
A27	88 - 121*	78 - 108*	83 - 92*
A7	110 - 132*	81 - 108*	71 - 84*
A27	111 - 143*	72 - 115*	73 - 92*
A16	103	86	84
A27	91	76	84
A154	115	94	82
A27	115	95	83
A5	96	73	76
A42	86	71	82
A5	128	73	56
A11	126	111	88
A5	110	88	80
A44	111	97.5	87.5
A5	43	37	86
A15	43	38	88
A154	134	103	76
A15	141	105	75
A50	125	101	81
A15	131	119	91
A5	231	206	89
A15	116	98	84
A27	124	120	97

\*Experiments performed in 5 laboratories as a ring test.

All other results represent triplicate experiments performed in individual laboratories.

The overall recovery of A5/A44 determined in 4 experiments with the use of cream formulations containing A5 spiked with  $^{14}\text{C}$ -A5 and A44 spiked with  $^{14}\text{C}$ -A44 is described in Table 6. Adding the precursor/coupler recovered by the combustion of dyed hairs to the respective experimental recoveries described in Table 5, the overall recovery of these precursor/coupler reaches >90%. On the basis of these experiments, it is suggested in the report that the overall recoveries of all precursors/couplers used in the present investigation are around 100%.

**Table 6:** Experimental recovery of A5 and A44 determined using cream formulations with spiked  $^{14}\text{C}$ -A5 and  $^{14}\text{C}$ -A44 respectively.

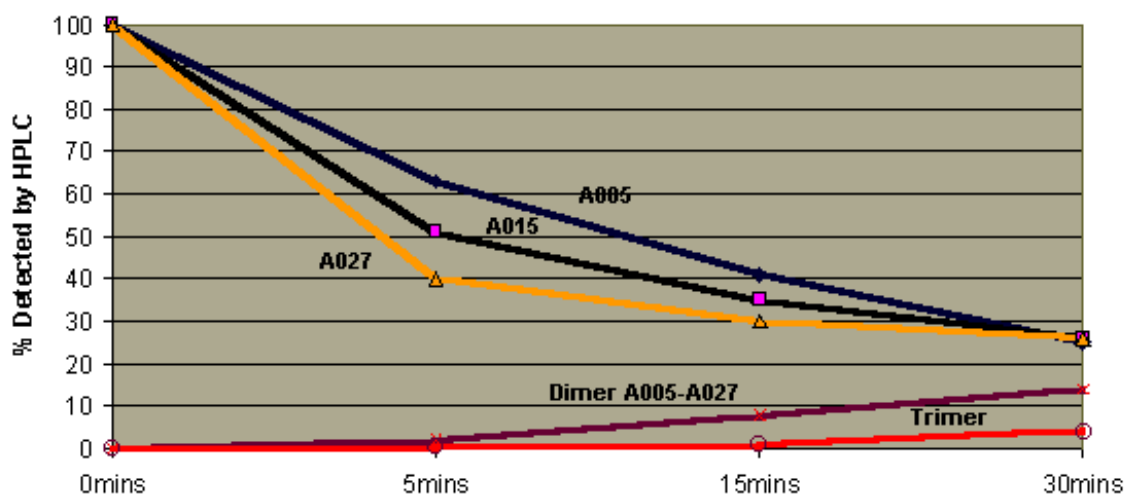
Precursor and coupler combinations	% of applied $^{14}\text{C}$ detected in			Total recovery of A5 %
	Formulation/ rinse water	Hair extract	Hair combustion	
A5 spiked with $^{14}\text{C}$ -A5 A27	51	42	11	104

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Precursor and coupler combinations	% of applied $^{14}\text{C}$ detected in			Total recovery of A5 %
	Formulation/ rinse water	Hair extract	Hair combustion	
A5 spiked with $^{14}\text{C}$ -A5 A44	18	60	26	104
A5 A44 spiked with $^{14}\text{C}$ -A44	Not reported	Not reported	17	Not reported
A5 spiked with $^{14}\text{C}$ -A5 A11	Not reported	Not reported	35	Not reported

The kinetics of colour formation by oxidative coupling of precursors and couplers were found to be similar in all combinations of precursors and couplers used in the present study. An example is shown in Figure 7.

**Figure 7:** Levels of reactants and reaction products in the formulation over time for combination A5-A15-A27.



### 3.4. Discussion

The technical report submitted is a follow up of the Ring Study Report dated October 2003 on the chemistry of oxidative hair dyes. The method described in the Ring Study for the determination of the reactants and reaction products in oxidative hair dye formulations was considered acceptable by the SCCNFP.

The objectives of the present study included:

- to understand oxidative hair dye chemistry under representative in-use conditions
- to identify the key reaction products that are formed under representative in-use conditions
- to estimate the concentrations of the key reaction products that are formed under representative in-use conditions
- to investigate the kinetics of reaction product formation

The experimental methodology for *in vitro* hair dyeing was designed to reflect consumer practices, where realistic concentrations (0.125M) of precursors and couplers in a standard cream formulation were mixed with equal volume of 6% hydrogen peroxide lotion. The mixture was incubated in the presence of human hair for 30 min at 30°C. A suitable HPLC method was used for the determination of the concentrations of reactants and reaction products in the hair dye formulations and in the methanol extracts of the dyed hairs. This methodology was used to study 11 combinations of precursors and couplers of oxidative hair dyes, chosen on the basis of chemistry type and tonnage used in hair dyeing products on the European market. For each combination, the expected reaction product(s), a dimeric and/or trimeric species, was synthesised and appropriately characterised. The synthesised reaction products were used as reference standards for the identification and quantification of hair dyes formed. The total concentrations of unreacted precursors and couplers in various experiments were 12 -84% of the applied dose (Table 4); the concentrations of the hair dyes formed were 0.02-0.65% (Table 3). However, no explanation is given for using a large excess of precursors and couplers than the required amounts, based on chemistry of oxidative hair dyes in hair dye formulations.

The hair dyes, which may be formed by oxidative coupling of various combinations of precursors and couplers, were predicted on the basis of the chemistry of oxidative hair dyes, and confirmed in this investigation. The HPLC method used for the analyses of reactants and reaction products did not reveal the presence of any additional molecules in the formulations or in methanol extracts of the dyed hairs. Furthermore, no transient intermediates or self coupling products (such as Bandrowski's Base) were detected.

The oxidative hair dye formation requires generation of quinonediimine (QDI), which is a transient intermediate with a short half-life. As the disappearance of precursor and couplers was found to be faster than the formation of hair dye molecule, an excess of these transient QDI molecules in the formulation may be expected during the whole period of hair dyeing. But absence of these molecules in the formulations (acidified with formic acid at the end hair dyeing) or in the extracts may be due to:

- QDI may not be stable at acidic pH, and/or
- QDI may be in equilibrium with the respective precursor/coupler at alkaline pH required for oxidative coupling.

Thus, exposure of the consumer with the transient QDI molecules cannot be ruled out.

The cream formulation used in the present investigation is composed of only basic ingredients but it does not reflect the marketed products, which may contain various other ingredients including some colorants. For example, declared ingredients in 3 oxidative hair dye products for non-professional use, in the European market, are described in Tables 7. Thus, it is unknown whether the reaction of hydrogen peroxide (oxidation) with some or all of the other ingredients will result in the synthesis of some new molecules to which consumer may also be exposed, besides unreacted precursor(s), coupler(s) and the hair dye(s) formed.

The experimental recovery of precursors and couplers in all investigated combinations, except that of A5 in the combination A5+A11, was demonstrated to be 71-97%. Since the dye from the dyed hairs was not extracted quantitatively in methanol, 4 experiments were performed with use of radio-labelled precursor/coupler, the methanol extracted dyed hairs were combusted, and the released radioactivity (from the hair dye and possibly trapped precursors/couplers) was

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measured. Adding this amount to the experimental recovery, the overall recoveries of precursors and couplers in each case was >90%. In case of A5+A11 combination, the experimental recovery of A5 was only 56%. However, the experiment performed with the use of radio-labelled A5 revealed that 35% radioactivity was present in the combusted hairs, thus making overall recovery of A5 to 91%. On the basis of the experiments performed with use of radio-labelled precursors/couplers, it is suggested in the report that the recovery of all precursors and couplers in various combinations was close to 100%.

The kinetics of colour formation over 30 min revealed that the amount of colour formed increases with time, while the amounts of free precursors and couplers decrease. However, the reaction products form much more slowly than the precursors and couplers disappear. The kinetics of colour formation also revealed that the exposure of the consumer to the reaction product (hair dye) is much less than the exposure to the precursor and coupler over the whole application time.

**Table 7:** Ingredients declared on 3 randomly selected oxidative hair dye products marketed in EU.

INGREDIENTS
<b>KLEURGEL/GEL COLORANT:</b> AQUA • TRIDECETH-2 CARBOXAMIDE MEA • PROPYLENE GLYCOL • HEXYLENE GLYCOL • PEG-2 OLEAMINE • POLYGLYCERYL-4 OLEYL ETHER • OLEYL ALCOHOL • ALCOHOL DENAT. • AMMONIUM HYDROXIDE • POLYGLYCERYL-2 OLEYL ETHER • OLEIC ACID • SODIUM DIETHYLAMINOPROPYL COCOASPARTAMIDE • 4-AMINO-2-HYDROXYTOLUENE • p-AMINOPHENOL • SODIUM METABISULFITE • PHENYL METHYL PYRAZOLONE • 6-HYDROXYINDOLE • AMMONIUM ACETATE • 2-METHYL-5-HYDROXYETHYLAMINOPHENOL • PENTASODIUM PENTETATE • p-PHENYLENEDIAMINE • RESORCINOL • ERYTHORBIC ACID • PARFUM. (C16497/1)
<b>ONTWIKKELCRÈME/LAIT RÉVÉLATEUR:</b> AQUA • HYDROGEN PEROXIDE • CETEARYL ALCOHOL • TRIDECETH-2 CARBOXAMIDE MEA • CETEARETH-30 • GLYCERIN • PENTASODIUM PENTETATE • SODIUM STANNATE • TETRASODIUM PYROPHOSPHATE. (C10550/1)
<b>DUBBELE HOEVEELHEID VOEDENDE NABEHANDELING/DOUBLE SOIN NUTRI-PROTECTEUR:</b> AQUA • CETEARYL ALCOHOL • GLYCERIN • BEHENTRIMONIUM CHLORIDE • AMODIMETHICONE • CETYL ESTHERS • ISOPROPYL ALCOHOL • TRIDECETH-12 • CHLORHEXIDINE DIHYDROCHLORIDE • CAMPHOR BENZALKONIUM METHOSULFATE • METHYL PARABEN • CETRIMONIUM CHLORIDE • PARFUM. (C10578/1)
<b>AROMATISCHE OLIËN CONCENTRAAT/CONCENTRÉ D'HUILE AROMATIQUE:</b> HELIANTHUS ANNUUS • PARFUM. (C 10431/1)

INGREDIENTS
<b>DEVELOPER:</b> AQUA • HYDROGEN PEROXIDE • ACRYLATES COPOLYMER • ETIDRONIC ACID • SODIUM LAURETH SULFATE • 2,6-DICARBOXYPYRIDINE • DISODIUM PYROPHOSPHATE
<b>COLOUR GEL:</b> AQUA • ISOPROPYL ALCOHOL • LAURETH-2 • COCONUT ALCOHOL • PROPYLENE GLYCOL • OLEIC ACID • ETHANOLAMINE • AMMONIUM HYDROXIDE • TOLUENE-2,5-DIAMINE • ARGININE • SODIUM LAURETH SULFATE • ASCORBIC ACID • SODIUM SULFITE • ETIDRONIC ACID • 1,3-BIS-(2,4-DIAMINOPHENOXY) PROPANE HCL • RESORCINOL • 4-CHLORORESORCINOL • HC BLUE NO. 7 • PARFUM
<b>CONDITIONER:</b> AQUA • PARAFFINUM LIQUIDUM • CETEARYL ALCOHOL • GLUCOSE • CERA ALBA • CHAMOMILLA RECUTITA • BISABOLOL • CETYL PALMITATE • CETRIMONIUM CHLORIDE • HYDROXYPROPYL METHYL CELLULOSE • LAURETH-10 • ALCOHOL DENAT. • PARFUM • PHENOXY ETHANOL • METHYL PARABEN • PROPYL PARABEN

INGREDIËNTEN/INGREDIENTS:
<b>VOEDENDE CRÈME-KLEURING / CRÈME COLORANTE NUTRITIVE:</b> AQUA • CETEARYL ALCOHOL • DECETH-3 • PROPYLENE GLYCOL • LAURETH-12 • AMMONIUM HYDROXIDE • OLETH-30 • LAURIC ACID • HEXADIMETHRINE CHLORIDE • GLYCOL DISTEARATE • ETHANOLAMINE • POLYQUATERNIUM-22 • 2-METHYL-5-HYDROXYETHYLAMINOPHENOL • SILICA DIMETHYL SILYLATE • CI 77891 • p-AMINOPHENOL • 4-AMINO-2-HYDROXYTOLUENE • ASCORBIC ACID • AMMONIUM THIOACTATE • DIMETHICONE • PENTASODIUM PENTETATE • p-PHENYLENEDIAMINE • CARBOMER • RESORCINOL • VITIS VINIFERA • PARFUM. (C20217/2) •
<b>ONTWIKKELMELK / LAIT RÉVÉLATEUR:</b> AQUA • HYDROGEN PEROXIDE • CETEARYL ALCOHOL • TRIDECETH-2 CARBOXAMIDE MEA • CETEARETH-30 • GLYCERIN • PENTASODIUM PENTETATE • SODIUM STANNATE • TETRASODIUM PYROPHOSPHATE. (C11185/1)
<b>RIJKE VERZORGENDE NABEHANDELING / SOIN-RICHE EMBELLISSEUR:</b> AQUA • CETEARYL ALCOHOL • BEHENTRIMONIUM CHLORIDE • CETYL ESTERS • TRIDECETH-12 • CHLORHEXIDINE DIHYDROCHLORIDE • AMODIMETHICONE • PERSEA GRATISSIMA • METHYL PARABEN • CITRIC ACID • CETRIMONIUM CHLORIDE • PARFUM. (C17758/1)

The dyes formed by the combinations of precursors and couplers used in the present investigation are now available for further studies, such as percutaneous absorption and mutagenicity/genotoxicity. The *in vitro* percutaneous absorption of 4 of the hair dyes synthesised in the present investigation revealed that up to 0.14 % of the applied dose may be absorbed during 30 min application (Annex 1, table 3).

A worst case scenario for the exposure from hair dyes in the dyeing process was derived from the data available. According to Table 3 the maximum content of a hair dye formed in the formulation 30 min after application was 0.65 %. In a 70 ml (=70 g) formulation this equals 455 mg hair dye formed (A154+A27 dimer). On the other hand, the maximum absorption rate of the hair dyes synthesised was 0.14 % (A16+A27, Annex 1, Table 3, Donor 1). Combination of these 2 worst case situations would yield to a maximum systemic exposure of 632 µg of a hair dye formed. It is concluded that the amount of dye that may be absorbed, and thus systemically available, may be significant in some cases. However, from the data presented in many cases systemic exposure was found to be lower. For an appropriate safety evaluation similar data for the most relevant combinations should be generated to obtain necessary information on consumer exposure.

#### 4. CONCLUSION

An analytical methodology based essentially on HPLC was developed, which allowed to follow oxidative hair dye coupling chemistry under conditions, reflecting consumer usage. The methodology applied to 11 different combinations of hair dye precursors and couplers revealed:

- Expected reaction products based on the chemistry of oxidative coupling of precursors and couplers were obtained.
- No significant additional reactions or unexpected products were detected,
- The appearance of coupling products (dimers and trimers) can be detected and quantified
- Levels of reactants (precursors and couplers) and reaction products (hair dye) can be determined in the formulation
- Extraction of dyed hair allows the quantification of reactants in the dyed hair
- The overall experimental recovery obtained from combined levels detected in the hair and in the formulation is close to 100%,.
- Self-coupling products (such as Bandrowski's Base) or transient intermediates were not detected in the hair dye formulation.
- In complex mixtures, the chemistry of the binary combination with the fastest kinetics will dominate
- The typical concentration of reaction products in the formulations after 30min varied from 0.05 % (*Combination A5+A44*) to 0.65% (*Combination A154+A27*).
- During the dyeing process, the consumer is exposed to the precursor(s), coupler(s) and expected reaction product(s).

Although transient quinonediimine intermediates, essential for the formation of oxidative coupling of precursor and coupler in hair dye formulations, were not found in the present investigation, exposure of consumers to these molecules cannot be ruled out.

The cream base used for the formulation of precursors and couplers contained only basic ingredients but that was not similar to marketed products, which may contain various other

ingredients such as colorants. Thus, the influence of these other ingredients on oxidative coupling of precursors and couplers, as well as formation of new molecules is not envisaged in this study.

The SCCP understands that over one hundred different precursors and couplers are used in oxidative hair dye formulations in the EU. Studies, similar to those presented here, with all the most relevant combinations of precursors and couplers should be performed to obtain necessary information on consumer exposure.

As the reaction products (hair dyes) of oxidative coupling of different precursor-coupler combinations (available to consumer) can be predicted, these should be synthesised. Their percutaneous absorption characteristics should be evaluated and in case of significant systemic exposure, further relevant toxicity studies are required.

The aspect of allergenicity (skin sensitisation from intermediates as well as from newly formed compounds) has not been addressed in this opinion.

## 5. MINORITY OPINION

Not applicable.

## 6. REFERENCES

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## 7. ACKNOWLEDGEMENTS

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Dr. S.C. Rastogi (Rapporteur)  
Prof. T. Sanner  
Prof. G. Speit  
Dr. J. van Engelen  
Dr. I.R. White

**Annex 1***In vitro* percutaneous absorption of oxidative hair dyes

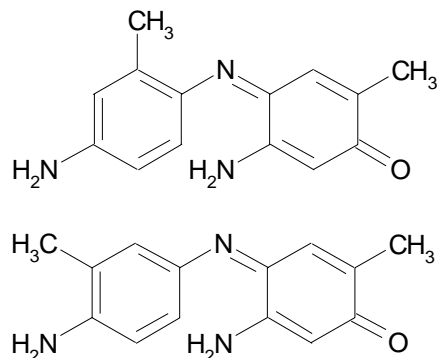
- mixture of 5-Amino-4-((4-amino-2-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one, and
- 5-Amino-4-((4-amino-3-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one (A5-A27),
- 5-Amino-4-((4-aminophenyl)imino)-2-methyl-2,5-cyclohexadien-1-one (A7-A27)
- 5-Amino-4-((4-hydroxyphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one (A16-A27)
- 5-Amino-2-[[4-[bis(2-hydroxyethyl)amino]-phenyl]amino]-4-[[4-[bis(2-hydroxyethyl)amino]-phenyl]imino]-2,5-cyclohexadien-1-one (A50-A15-A50)



## 1. Oxidative hair dyes

### 1.1. Dimer A5-A27

Chemical structure:



**Molecular formula:** C<sub>14</sub>H<sub>15</sub>N<sub>3</sub>O

**Molecular weight:** 241.30

**Chemical name:** 5-Amino-4-((4-amino-2-methylphenyl)imino)-2-methyl-2,5-cyclohexadien-1-one  
5-Amino-4-((4-amino-3-methylphenyl)imino)-3-methyl-2,5-cyclohexadien-1-one

**Name (INCI):** /  
**CAS:** not registered  
**EINECS/ELINCS:** not registered  
**Batch:** FJR0328/1  
**Appearance:** black powder  
**Melting point:** 120 – 125 °C

**Identification** by Mass spectrometry and NMR spectroscopy

**NMR content:** 86.04%  
**UV/VIS spectrum:** λ max: 282 nm and 528 nm

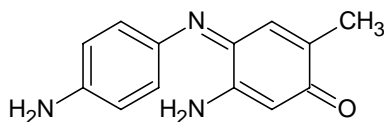
**HPLC:** Purity for two isomers:  
71.3 area%  
27.1 area%

**Solubility:** ~10 (w/w)% in DMSO  
0.11 (w/w)% in water

**Stability:** The substance on storage in dryness and darkness is considered to be stable 36 months.

**1.2 Dimer A7-A27**

**R0012325A**  
**(batch 001 D 001)**  
**A7 / A27**

General information

Empirical formula: C<sub>13</sub>H<sub>13</sub>N<sub>3</sub>O  
Molecular weight: 227.27  
Chemical name: 5-Amino-4-[(4-aminophenyl)imino]-2-methylcyclohexa-2,5-dien-1-one

Physical properties

Appearance: dark red crystals  
Log Pow: 1.11 (calculated: CLOGP v.4.2.)

Chemical identification

IR spectrophotometrie: the IR spectrum of batch 001 D 001 is in compliance with the structural formula  
UV spectrophotometrie: ε-max = 11980 at 517 nm in ethanol

Chemical characterisation

NMR spectrometryrie: the <sup>1</sup>H NMR spectrum of batch 001 D 001 is in compliance with the structural formula  
Mass spectrometrie: the ESI<sup>+</sup> and ESI<sup>-</sup> mass spectra of batch 001 D 001 is in compliance with the structural formula

Purity 99% HPLC peak area

Impurities

starting material (HPLC)	4-Amino-2-hydroxytoluene	700 µg/g
	p-phenylenediamine	500 µg/g
residual solvent (GC)	ethanol	0.2 g/100 g

Solubility (20 °C)

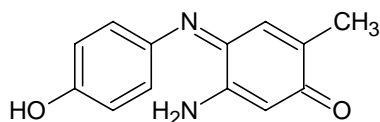
Water: < 0.5 g/100 ml  
Ethanol: # 1.5 g/100 ml  
DMSO: # 30 g/100 ml

Storage conditions

Hygroscopic product protect from humidity

**1.3 Dimer A16-A27**

**R0012228A**  
**(batch 001 L 004)**  
**A16 / A27**

General information

Empirical formula: C<sub>13</sub>H<sub>12</sub>N<sub>2</sub>O<sub>2</sub>  
Molecular weight: 228.22  
Chemical name: 5-Amino-4-[(4-hydroxyphenyl)imino]-2-methylcyclohexa-2,5-dien-1-one

Physical properties

Appearance: brown powder  
Log Pow: 1.67 (calculated: CLOGP v.4.2.)

Chemical identification

IR spectrophotometrie: the IR spectrum of batch 001 L 004 is in compliance with the structural formula  
UV spectrophotometrie: ε-max = 8760 at 469 nm in ethanol

Chemical characterisation

NMR spectrometryrie: the <sup>1</sup>H NMR spectrum of batch 001 L 004 is in compliance with the structural formula  
Mass spectrometrie: the ESI<sup>+</sup> and ESI<sup>-</sup> mass spectra of batch 001 L 004 is in compliance with the structural formula

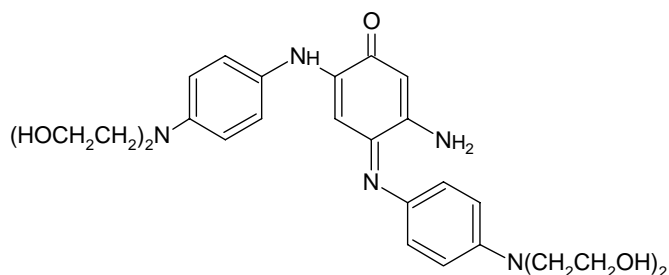
Purity 99% HPLC peak area

Impurities

starting material (HPLC)	4-Amino-2-hydroxytoluene	< 200 µg/g (not detected)
	p-aminophenol	500 µg/g
residual solvent (GC)	ethanol	0.8 g/100 g

Solubility (20 °C)

Water: < 1.5 g/100 ml  
Ethanol: # 3.5 g/100 ml  
DMSO: # 50 g/100 ml

**1.4 Trimer A50-A15-A50**

Batch Number:	PG62-104-1
Empirical Formula:	C <sub>26</sub> H <sub>33</sub> N <sub>5</sub> O <sub>5</sub>
Molecular Weight:	495.6
Chemical Name:	2,5-cyclohexadien-1-one-5-amino-2-[[4-[bis(2-hydroxyethyl)amino]-phenyl]amino]-4-[[4-[bis(2-hydroxyethyl)amino]phenyl]imino]-
Appearance:	Black Powder
Purity:	97.92%
IR Spectra (KBr):	The spectrum is in compliance with the structural formula
NMR:	<sup>1</sup> H (600Mz) is in compliance with the structural formula
Melting Point	213 –216 <sup>0</sup> C
Weight Loss:	0.33% (2hrs at 110 <sup>0</sup> C by TGA)
HPLC Peak Purity:	98.24% (Area percent, HPLC UV detection)
	Some impurities (Trace)
Solubility:	Water < 0.5%
	Ethanol 0.5%
	DMSO 1.5%

## 2 Percutaneous absorption in vitro

### 2.1 A5-A27 Dimer

Guideline:	OECD 428
Tissue:	Dermatomed fresh porcine ear skin (thickness: approximately 300 µm),
Method:	diffusion glass chambers, 6 chambers per experiment, surface area of the skin in contact with the test substance 1 cm <sup>2</sup>
Test item:	2% A5-A27 dimer (Batch no. SO-1100.029, purity 98%) in an alkaline emulsion formulation*, stability in water >7 days
Test dose:	20 µl
Receptor fluid:	Saline, 32±1°C, flow rate 1-2 ml/h
GLP:	in compliance

\*2% A5-A27 dimer, 7% cetearyl alcohol, 1% lanolin alcohol, 10% sodium laureth sulphate (28%), 4.55% ammonia (25%) and made up to 100% with water

Impedance of the skin was monitored before treatment begin and after the last sampling. All of the donor and acceptor chambers were equipped with platinum electrode connecting to a conductometer. No sudden changes in conductivity were observed, indicating the integrity of the skin.

Percutaneous absorption was studied in 2 individual experiments, 6 replicates of one concentration of the test item in each experiment. The donor chamber was filled with 1 mL of receptor fluid to determine the impedance of the skin and to collect the blank samples before the start of the experiment. After removal of the receptor fluid, 20 µL of the thoroughly mixed test item was pipetted in the each donor chamber. An additional 20 µL of the test item was pipetted in a separate beaker as the corresponding 100% reference of that chamber for the mass balance calculations. After 30 min the test item was removed by washing the skin 3 times with 1 ml of 10% shampoo solution followed by two times with 1 ml deionised water. The washings were collected and combined for the analysis. After 24 h the impedance of the skin was determined. The receptor vials were depleted and the collected eluates as well as the solution from the donor chambers were weighed and stored at -20°C until analysis.

After the incubation period, the stratum corneum was stripped using Tesa® film transparent ten times. The film pieces were collected and extracted with 3 ml 0.05 M ammonium formate buffer (pH 5.5)/acetonitrile (60:40). The test item bound to the remaining skin was extracted with 2 ml of the ammonium formate buffer/acetonitrile.

The amount of A5-A27 raw material was determined in the receptor solutions, in the combined washing solutions, in the stripping extracts and in the extracts of the remaining skin. High performance liquid chromatography employing UV detection at 286 nm was used for the determination of A5-A27

### Results

The sum of test substance found in dermis/epidermis and receptor fluid was considered as the amount of the substance penetrated the skin. The recovery and penetration rates of A5-A24 are described in the Table 1. In all cases, except experiment 2 diffusion chamber 6, the recovery of the applied A5-A24 was 78-106%. The recovery of A5-A27 in experiment 2 diffusion chamber 6

was only 56%. Disregarding the data set of experiment 2 diffusion chamber 6, the penetration rate of A5-A24 under the experimental conditions of the study was 0.025-0.157  $\mu\text{g}/\text{cm}^2$  and the % penetration of the substance was 0.009-0.059. Considering a large variation (>5 fold from minimum to maximum) in the penetration rates observed in the 12 diffusion chambers, the worst case of penetration 0.059% of the applied dose or 0.157  $\mu\text{g}/\text{cm}^2$  should be considered for safety assessment. Positive control data for the performance of method for *in vitro* percutaneous absorption of benzoic acid and testosterone were provided.

Table 1: *In vitro* percutaneous absorption and recovery of A5-A27 dimer

Diffusion chamber	Experiment 1						Experiment 2					
	1	2	3	4	5	6	1	2	3	4	5	6
% recovery	101	106	92.3	96.8	95.6	99.3	92.8	95.5	78.3	88.5	88.9	55.6
Penetration rate, $\mu\text{g}/\text{cm}^2$	0.063	0.157	0.033	0.065	0.098	0.077	0.066	0.025	0.082	0.092	0.071	0.210
% penetration	0.024	0.059	0.012	0.023	0.036	0.028	0.027	0.009	0.031	0.035	0.027	0.075

Comment: The test material Batch SO-1100 029 is not the same as used for the characterisation of A5-A27 (1.1)

Reference: Skin permeability *in vitro* absorption through porcine ear skin with A5-A27 reaction product dimer-alkaline emulsion formulation.. RCC-CCR Study No. 833001, Final report, December 22, 2004

## 2.2 Dimer A7-A27

Guideline: OECD 428  
 Tissue: Dermatomed frozen human abdominal skin from 4 females (37-46 years), thickness 200-500  $\mu\text{m}$   
 Method: Glass diffusion cells (Franz), exposed membrane area 2  $\text{cm}^2$   
 Test item: 2% A7-A27 dimer (Batch no.001D001) in a Koleston base\*  
 Test dose: 20  $\text{mg}/\text{cm}^2$ , after dilution (1:1) with 6%  $\text{H}_2\text{O}_2$ ,  
 Receptor fluid: Phosphate buffered saline containing 1% DMSO, the compatibility of the receptor with the skin was demonstrated  
 GLP: in compliance

\*Koleston is composed of 8.4% cetearyl alcohol, 3.36% sodium laureth sulphate, ammonia 1.365%, lanolin alcohol 1.19993%, disodium EDTA 0.1200%, benzoic acid 0.03600%, tocopherol 0.00007% and water 85.519%.

The experiment was performed using 8 diffusion cells mounted with intact skin membranes from 4 different subjects, duplicate experiments. The receptor chambers of the cells, containing magnetic stirrer bars, were filled with the receptor fluid and placed in a water bath at  $32 \pm 1^\circ\text{C}$ . The 1:1 v/v formulation:peroxide developer mixture was applied to the skin membranes at a nominal dose rate of 20  $\text{mg}/\text{cm}^2$ . After 30 min the formulation was washed from the skin surface and the

experimented continued up to 24 hours. The *stratum corneum* was tape stripped. The tape strips and the epidermis/dermis were extracted for the analysis of A7-A27 content.

The amount in receptor fluid samples, strip tape extracts and in the dermis/epidermis were analysed for the content of A7-A27 by a suitable high performance liquid chromatography method employing diode array and UV-visible light detection.

## Results

The sum of test substance found in dermis/epidermis and receptor fluid was considered as the amount of the substance penetrated the skin. The recovery and penetration rates of A007-A027 dimer are described in the Table 2.

Table 2: *In vitro* percutaneous absorption and recovery of dimer A7-A27

Skin donor	1	1	2	2	3	3	4	4
% recovery	98.4	95.7	95.5	95.5	92.7	91.0	92.4	91.3
Penetration rate, $\mu\text{g}/\text{cm}^2$	n.d.	n.d.	n.d.	0.012*	n.d.	n.d.	n.d.	n.d.
% penetration	n.d.	n.d.	n.d.	0.006*	n.d.	n.d.	n.d.	n.d.

\*limit of detection

The recovery of the applied A7-A27 dimer was 91-98% in all experiments. The penetration rate of the dimer under the experimental conditions of the study was below the detection limit  $0.006 \mu\text{g}/\text{cm}^2$  in all experiments except one, where penetration of the dimer was detected ( $0.006 \mu\text{g}/\text{cm}^2$ ) but that was below the limit of quantification. Positive control data for the performance of method for *in vitro* percutaneous absorption is not reported.

Reference: Étude de l'absorption percutanée in vitro du produit de couplage A7/27. L'Oréal Report No. ABS1/001. July 2004

## 2.3 Dimer A16-A27

Guideline:	OECD 428
Tissue:	Dermatomed frozen human abdominal skin from 4 females (34-58 years), thickness 200-500 $\mu\text{m}$
Method:	Glass diffusion cells (Franz), exposed membrane area $2 \text{ cm}^2$
Test item:	2% A167-A27 dimer (Batch no.001L004) in a Koleston base*
Test dose:	$20 \text{ mg}/\text{cm}^2$ , after dilution (1:1) with 6% $\text{H}_2\text{O}_2$ ,
Receptor fluid:	Phosphate buffered saline containing 1% DMSO, the compatibility of the receptor with the skin was demonstrated
GLP:	in compliance

\*Koleston is composed of 8.4% cetearyl alcohol, 3.36% sodium laureth sulphate, ammonia 1.365%, lanolin alcohol 1.19993%, disodium EDTA 0.1200%, benzoic acid 0.03600%, tocopherol 0.00007% and water 85.519%.

The experiment was performed using 8 diffusion cells mounted with intact skin membranes from 4 different subjects, 2 cells per donor. The receptor chambers of the cells, containing magnetic stirrer bars, were filled with the receptor fluid and placed in a water bath at  $32 \pm 1^\circ\text{C}$ . The 1:1 v/v

formulation: peroxide developer mixture was applied to the skin membranes at a nominal dose rate of 20 mg/cm<sup>2</sup>. After 30 min the formulation was washed from the skin surface and the experiment continued up to 24 hours. The *stratum corneum* was tape stripped. The tape strips and the epidermis/dermis were extracted for the analysis of A16-A27 content.

The amount in receptor fluid samples, strip tape extracts and in the dermis/epidermis were analysed for the content of A7-A27 by a suitable high performance liquid chromatography method employing diode array and UV-visible light detection.

## Results

The sum of test substance found in dermis/epidermis and receptor fluid was considered as the amount of the substance penetrated the skin. The recovery and penetration rates of A7-A27 dimer are described in the Table 3.

Table 3: *In vitro* percutaneous absorption and recovery of dimer A16-A27

Skin donor	1	1	2	2	3	3	4	4
% recovery	88.9	94.8	94.7	96.9	96.3	93.6	100.2	94.4
Penetration rate, µg/cm <sup>2</sup>	0.245	0.100	0.147	0.271	0.029*	0.200	0.082	0.129
% penetration	0.139	0.050	0.073	0.137	0.015*	0.105	0.044	0.070

\*limit of detection

The recovery of the applied A16-A27 dimer was 88.9-100.2% in all experiments. The penetration rate of the dimer under the experimental conditions of the study was 0.029-0.271 µg/cm<sup>2</sup> or 0.015-0.139% of the applied dose penetrated into the skin. Considering a large variation (9 fold from minimum to maximum) in the penetration rates observed in the 8 diffusion chambers, the worst case of penetration 0.139% of the applied dose or 0.271 µg/cm<sup>2</sup> should be considered for safety assessment. Positive control data for the performance of method for *in vitro* percutaneous absorption is not reported.

Reference: Étude de l'absorption percutanée *in vitro* du produit de couplage A16/27. L'Oréal Report No. ABS1/002. July 2004

## 2.4 Trimer A50-A15-A50

Guideline:	OECD 428
Tissue:	Dermatomed frozen human skin from 3 females (74, 77 and 90 years) and a 80 year male, thickness: approximately 400 µm
Method:	Glass diffusion cells, exposed membrane area 2.54 cm <sup>2</sup>
Test item:	2% A50-A15-A50 trimer (Batch no. PG62-105-1, purity 97.1%) in a koleston base (Ref. No. GTS04425, no further details available)
Test dose:	20 mg/cm <sup>2</sup> , after 1: dilution with peroxide developer (Ref. No. GTS04426, no further details available). The test dose was spiked with <sup>14</sup> C-labelled (radiolabelling in the A50 benzene ring) A50-A15-A50 trimer (Ref. No. GTS04292)
Receptor fluid:	4% polyoxyethylene 20 oleyl ether in phosphate buffered saline, the compatibility of the receptor with the skin was demonstrated



GLP: in compliance

The experiment was performed using 12 diffusion cells mounted with intact membranes from 4 different subjects. The receptor chambers of the cells, (containing magnetic stirrer bars, were filled with the receptor fluid and placed in a water bath at  $32\pm 1^\circ\text{C}$ . A pre-treatment sample (0.5 ml) was taken from each receptor chamber for analysis. An equal volume of fresh receptor fluid was added to each receptor chamber. The 1:1 v/v formulation:peroxide developer mixture was applied to the skin membranes at a nominal dose rate of  $20\text{ mg/cm}^2$ , spread over the surface using glass rods and the weight of the applied dose recorded after spreading. After 30 min the formulation was washed from the skin surface and the experiment continued up to 48 hours. Samples of the receptor fluid were taken for analysis at 0.5, 1, 2, 4, 6, 24 and 48 hours. The skin was left unoccluded during the whole experiment.

The *stratum corneum* was tape stripped and the tape strips were extracted in the liquid scintillation medium. The remaining skin after tape stripping, epidermis and dermis, was digested in liquid scintillation medium.

The amount in receptor fluid samples, strip tape extracts and in the dermis/epidermis digests were determined by liquid scintillation counting

## Results

The sum of test substance found in dermis/epidermis and receptor fluid was considered as the amount of the substance penetrated the skin. The recovery and penetration rates of A50-A15-A50 trimer are described in the Table 4. The results of two diffusion cells were not reported, as the data indicated that the skin membranes had been damaged during application of the test item.

Table 4: *In vitro* percutaneous absorption and recovery of A50-A15-A50 trimer

Diffusion cell	91	92	94	96	105	107	108	110	117	118
% recovery	106	116	111	109	111	108	105	110	109	104
Penetration rate, $\mu\text{g/cm}^2$	0.015	0.133	0.058	0.182	0.064	0.099	0.048	0.074	0.117	0.117
% penetration	0.007	0.067	0.029	0.091	0.032	0.050	0.024	0.037	0.059	0.047

The recovery of the applied A50-A15-A50 trimer was 104-117% in all experiments. The penetration rate of the trimer under the experimental conditions of the study was 0.015-0.182  $\mu\text{g/cm}^2$  and the % penetration and of the substance was 0.007-0.091. Considering a large variation (>10 fold from minimum to maximum) in the penetration rates observed in the 10 diffusion chambers, the worst case of penetration 0.091% of the applied dose or 0.182  $\mu\text{g/cm}^2$  should be considered for safety assessment. Positive control data for the performance of method for *in vitro* percutaneous absorption is not reported.

*The study authors report the mean absorption rate is  $0.044\pm 0.0235\%$  or  $0.0789\pm 0.046\text{ }\mu\text{g/cm}^2$*

Comment: The test material Batch PG62-105-1 is not the same as used for the characterisation of the trimer (1.4)

Ref.: A50/A15 coupling product: *In vitro* penetration from a formulation through human dermatomed skin. Central Toxicology Laboratory Cheshire, Report No. CTL/JV1812/Regulatory

Opinion on exposure to reactants and reaction products of oxidative hair dye formulations

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/Report. November 2004