OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

RING STUDY ON REACTION PRODUCTS FROM TYPICAL COMBINATIONS OF HAIR COLOURING INGREDIENTS

adopted by the SCCNFP on 23 April 2004
by means of the written procedure

1. Background

The oxidative hair dyes are end products of reaction between a precursor (for example p-phenylene diamine) and a coupler (for example m-aminophenol) in the presence of an oxidant such as hydrogen peroxide. A number of intermediate reaction products of short half lives are involved in the dye formation. This was described in the SCCNFP proposal for a strategy for testing hair dye cosmetic ingredients for their potential genotoxicity (SCNFP/566/02). It was also mentioned in the proposal that an adequate knowledge of the formulation of oxidative hair dye must be taken into consideration before genotoxic/mutagenic testing is performed. Furthermore, it was stressed that genotoxic/mutagenic testing of mixture of precursor(s), coupler(s) and oxidant, to which consumer is exposed, should be performed using several *in vitro* test methods.

The EU Commission has now received a study report describing the results of analysis of precursor (s), coupler(s) end products in 3 typical oxidative hair dye formulations. The analytical methodology was evaluated for an oxidative hair dye formulation by five laboratories in a ring test. The Commission requested SCCNFP to evaluate the study report for the suitability of the presented analytical methodology with respect to SCCNFP proposal on assessment strategies for hair dyes.

2. Request to SCCNFP

The SCCNFP was requested to answer following questions:

* Are the data presented in the submission sufficient for the SCCNFP to perform the risk assessment of the potential genotoxicity/mutagenicity mixtures of precursors(s), couplers and oxidant of hair dyes to which the consumer is exposed?

* If not, which kind of studies does the SCCNFP recommend to meet the requirements as laid down in the “Strategy for testing hair dye cosmetic ingredients for the potential genotoxicity/mutagenicity” (SCCNFP/0566/02) which is a part of the “Assessment strategies for hair dyes” (SCCNFP/0533/02) adopted in December 2002?

3. Study of reaction products of precursor, couplers and oxidant of hair dyes

3.1. Chemistry of hair dye formation

The chemistry of oxidative hair dye formation, taking into account the current scientific information, as well as theoretical considerations, has been elucidated in the report. 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 dye is the final product. Couplers, that have both positions *para* to hydroxy or amino groups free, can couple in a ratio of 2 molecules of precursors to 1 coupler molecule. The reaction products of the oxidative hair dye ingredients are thus a dimeric or trimeric hair dye molecules. The possibility of formation of small amounts of dye as higher oligomers and polymers (tetramer and higher) has not been ruled out. Several short-lived intermediates are generated but their measurement is not possible.

It is mentioned that Bandrowski Base, formed by self-coupling of p-phenylene diamine (PPD), does not form in commercial hair dye products as the rate of self-coupling is very slow reaction compared to reaction of PPD with coupler molecules. The rate of reaction between PPD and a standard coupler is stated to be at least 10^4-10^5 times faster than the reaction to produce Bandrowski Base.

3.2. Aim

Following scenarios were considered for the exposure with the reactants and reaction products, when consumers use oxidative hair dye formulations:

a) Some unreacted precursor and coupler remain in the formulation
b) Some unreacted precursor and coupler remain to diffuse into the hair
c) The dye (dimer/trimer) formed remains in the formulation
d) The dye (dimer/trimer) is formed in the formulation but subsequently diffuse into the hair
e) The dye (dimer/trimer) is formed in the hair

Situations a) and c) were considered of primary interest with respect to safety of consumer using an oxidative hair dye formulation. Thus, the aim of the study was to determine the concentrations of precursor(s) and coupler(s) in the formulation as well as in the hair at various time periods, after application of the hair dye formulation on the hair *in vitro.*

3.3. Method

A high performance liquid chromatography (HPLC) method was developed for the determination of the precursor (1,4-diamino-2-methylbenzene sulfate), coupler (5-amino-2-methylphenol) and the reaction product (dimer dye: DAT-PUR-quinonimine) using an oxidative hair dye formulation (hair colouring cream and 6% H_2O_2). The reaction was "quenched" (slowed), after 5 min, 10 and 15 min, by the addition of 0.1% formic acid; and the mixture was further treated for HPLC analysis as soon as possible. This method was validated, and the validated method was subjected to a ring test involving 5 laboratories. The cream formula included 2.5% (0.125 molar) 1,4-diamino-2-methylbenzene sulfate and 1.54% (0.125 molar) 5-amino-2-methylphenol.

The possible dimers and trimers of the dye to be produced by the reaction were synthesised

3.4. Investigated combinations of oxidative hair dye ingredients

Three combinations of oxidative hair dye formulas have been investigated in the study:

i) A hair cream containing 2.5% (0.125 molar) 1,4-diamino-2-methylbenzene sulfate and 1.54%, (0.125 molar) 5-amino-2-methylphenol was mixed with 6% H_2O_2 (1:1).
ii) A hair cream containing 2.5% (0.125 molar) 1,4-diamino-2-methylbenzene sulfate and 1.36 % (0.125 molar) m-aminophenol was mixed with 6% H_2O_2 (1:1).
iii) A hair cream containing 5.5% (0.25 molar) 1,4-diamino-2-methylbenzene sulfate, 1.54%, (0.125 molar) 5-amino-2-methylphenol and 36 % (0.125 molar) m-aminophenol was mixed with 6% H_2O_2 (1:1).

As indicated above, the combination i) was studied in 5 laboratories for 30 min reaction time. Both combinations ii) and iii) were studied only in two laboratories. In one laboratory, concentrations of the precursor, coupler(s) and the dye (dimer and trimer) were also determined at reaction time 5 min, 15 min and 30 min for all 3 combinations. An additional experiment was performed in which ^{14}C labelled precursor and coupler of combination i) were used to confirm the recovery of the precursor and coupler.

3.5. Results

3.5.1. Validation

The characteristics of the validated method are as follows:

Calibration curves:	Correlation coefficient > 0.999 for all 3 compounds (1,4-diamino-2-methylbenzene sulfate 0.002-0.468 mg/ml, 5-amino-2-methylphenol 0.002-0.394 mg/ml and DAT-PUR-quinonimine 0.0001-0.159 mg/ml)
Repeatability of HPLC retention time:	% RSD < 0.534 in all cases
Repeatability of Determination:	% RSD 0.095- 2.062, 0.280-1.528 and 2.926-9.451 respectively for 1,4-diamino-2-methylbenzene sulfate, 5-amino-2-methylphenol and DAT-PUR-quinonimine 0.0001-0.159 mg/ml, at all 3 concentration levels
Intermediate Precision:	For 6 mixtures, single analysis of each solution (reaction time 10 min), RSD of determination of 1,4-diamino-2-methylbenzene sulfate, 5-amino-2-methylphenol and DAT-PUR-quinonimine 0.0001-0.159 mg/ml was 8.8%, 15.8% and 11.8% at all 3 concentration levels.
Recovery:	The recovery of the 3 compounds at 3 concentration levels was 101-110% and RSD 3-9%, except in one case where recovery was 124%.

A signal-to-noise ratio of 3 in HPLC was considered as limit of detection and signal-to-noise ratio of 9 was considered as limit of quantification.

3.5.2. Ring-test: Levels of reactants and reaction products for combination i), reaction time 30 min,

The amounts of precursor and coupler used in the 5 participating laboratories varied: 92-125 µmol and 88-115 µmol respectively for precursor and coupler. The ring test of the method with cream formula i), using reaction time 30 min, revealed:

Content in the formula		
1,4-diamino-2-methylbenzene sulfate (precursor)	:	10-25% of the initial concentration
5-amino-2-methylphenol (coupler)	:	6-20% of the initial concentration
Dimer based on 1,4-diamino-2-methylbenzene sulfate:		7-14 µmol
Dimer based on 5-amino-2-methylphenol	:	8-14 µmol
Content in the hair extract		
1,4-diamino-2-methylbenzene sulfate	:	2-23% of the initial concentration
5-amino-2-methylphenol	:	7-29% of the initial concentration
Dimer based on 1,4-diamino-2-methylbenzene sulfate:		36-51 µmol
Dimer based on 5-amino-2-methylphenol	:	7-14 µmol
Recovery precursor	:	78–87%
Recovery coupler	:	82-89%.

The content of precursor in the formula at 0 min, 5 min, 15 min and 30 min, determined at one laboratory, were 100%, 50%, 40% and 22 % respectively. The content of coupler in the formula at 0 min, 5 min, 15 min and 30 min, determined in the same laboratory, were 100%, 40%, 25% and 16 % respectively. The content of dimer formed in the formula at 0 min, 5 min, 15 min and 30 min were 0 µmol, 2.5 µmol, 6 µmol and 8 µmol respectively.

3.5.3. Levels of reactants and reaction products for combination ii), reaction time 30 min

Two laboratories performed this study. Laboratory 1 used 125 µmol of precursor (1,4-diamino-2-methylbenzene sulfate) and 115 µmol of coupler m-aminophenol), while Laboratory 2 used only 43 µmol of both precursor and coupler. The results of the analysis revealed:

Content in the formula	Lab1	Lab2
Precursor:	17%	18% of the initial concentration
Coupler:	17%	21% of the initial concentration
Trimer based on precursor:	11 µmol	2 µmol
Trimer based on coupler:	5 µmol	1 µmol
Content in the hair extract		
Precursor:	20%	2% of the initial concentration
Coupler:	50%	35% of the initial concentration
Trimer based on precursor:	38 µmol	26 µmol
Trimer based on coupler:	22 µmol	13 µmol
Recovery precursor:	80%	86%
Recovery coupler:	91%	88%

The content of precursor in the formula at 0 min, 5 min, 15 min and 30 min determined in Lab1 were 100%, 53%, 28% and 17 % respectively. The content of coupler in the formula at time 0 min, 5 min, 15 min and 30 min determined in the same laboratory were 100%, 47%, 30% and

17% respectively. The content of trimer (based on coupler) formed in the formula at 0 min, 5 min, 15 min and 30 min were 0 µmol, 0.5 µmol, 1.5 µmol and 4 µmol respectively.

3.5.4. Levels of reactants and reaction products for combination iii), reaction time 30 min

Two laboratories performed this study. Laboratory 1 used 231 µmol of precursor (1,4-diamino-2-methylbenzene sulfate), 116 µmol of coupler1(m-aminophenol) and 124 µmol of coupler 2 (5-amino-2-methylphenol), while respective amounts used by the Laboratory 2 were 90 µmol, 47 µmol and 39 µmol. The results of the analysis revealed:

Content in the formula	Lab1	Lab2
Precursor:	24%	16% of the initial concentration
Coupler1:	26%	19% of the initial concentration
Coupler2:	25%	38% of the initial concentration
Dimer based on precursor:	15 µmol	2.5 µmol
Dimer based on coupler 2:	15 µmol	2.5 µmol
Trimer based on precursor:	8 µmol	2 µmol
Trimer based on coupler1:	4 µmol	1 µmol

Content in the hair extract	Lab1	Lab2
Precursor:	16%	4% of the initial concentration
Coupler1:	34%	30% of the initial concentration
Coupler2:	24%	2% of the initial concentration
Dimer based on precursor:	28 µmol	16 µmol
Dimer based on coupler 2:	28 µmol	16 µmol
Trimer based on precursor:	36 µmol	26 µmol
Trimer based on coupler 1:	18 µmol	13 µmol
Recovery precursor:	82%*	74%
Recovery coupler1:	81%*	79%
Recovery coupler2:	91%*	99%

*including 2nd extraction

The content of precursor in the formula at 0 min, 5 min, 15 min and 30 min determined in Lab 1 were 100%, 63%, 41% and 24 % respectively. The content of coupler 1 in the formula at 0 min, 5 min, 15 min and 30 min determined in the same laboratory were 100%, 51%, 35% and 26 % respectively. The content of coupler 2 in the formula at time 0 min, 5 min, 15 min and 30 min were 100%, 40%, 30% and 25 % respectively.

4. Conclusions

The analytical method developed for the determination of reactants and reaction products of oxidative hair dye formulation is based on sound chemistry. The characteristics of the validated method are in agreement with the international standards. It is not possible to perform a statistical evaluation of the ring-test due to a large variation in the amounts of precursor and coupler used by the participating laboratories, and it is also not known whether the reaction kinetics at all used concentrations will be the same. The results of the study indicate that performance of the validated method in the ring-test is rather poor, for example a large variation of precursor and coupler concentration found in the formulation after 30 min reaction (3.5.2). However, considering the complexity of chemical reactions involved in the exercise, the method may be acceptable for estimating the amounts of reactants and reaction products during the use of oxidative hair dyes. The method applied for the determination of reactants and the reaction products using two other combinations reactants (same precursor, but with another coupler and with 2 couplers) also revealed that the method is able to estimate the amounts of reactants and reaction products during the use of oxidative hair dyes.

On theoretical consideration, no attempt has been made to identify whether Bandrowski Base is formed in the oxidative hair dyes formulations.

Three main conclusions can be drawn from the present study:

- Amounts of reactants and reaction products in oxidative hair dye formulations can be estimated.
- A significant amount (approx. 20% or more of the initial concentration) of precursor(s) and coupler(s) is always present in the formulation that is not diffused into the hairs.
- The reaction products (and short-lived intermediates) are also formed in the formulation that is not diffused into the hairs.

Thus, the consumer is exposed to both reactants and reaction products, including short-lived intermediates, during the use of oxidative hair dyes.

The results of the present study also emphasise the importance of genotoxic/mutagenic testing of mixture of precursor(s), coupler(s) and oxidant, to which consumer is exposed.

5. Opinion

The SCCNFP recognises 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.