Scientific Committee on Consumer Safety

SCCS

OPINION ON

reaction products of oxidative hair dye ingredients formed during hair dyeing processes

The SCCS adopted this opinion at its 8th plenary meeting

of 21 September 2010

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members

Contact
European Commission
Health & Consumers
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

ISSN 1831-4767 ISBN 978-92-79-12750-2
doi:10.2772/26692 ND-AQ-09-022-EN-N

ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Prof. T. Platzek (chairman, rapporteur)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem (associate scientific advisor)
Prof. M.P. Vinardell
Dr. I.R. White

External expert:
Dr. M.-L. Binderup, National Food Institute, Denmark

The additional contribution from the following expert is gratefully acknowledged:
Romualdo Benigni, Experimental and Computational Carcinogenesis Unit Environment and Health Department, Istituto Superiore di Sanita, Rome, Italy

Keywords: SCCS, scientific opinion, oxidative hair dye, intermediates, reaction products, directive 76/768/ECC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on reaction products of oxidative hair dye ingredients formed during hair dyeing process, 21 September 2010

TABLE OF CONTENTS

1. BACKGROUND

Based on a mandate by the Commission the Scientific Committee evaluated the scientific paper 'Use of permanent hair dyes and bladder cancer risk' by M. Gago-Dominguez et al (Int. J. Cancer: 91, 575-579 (2001)).

The first opinion (SCCNFP/0484/01) on the use of permanent hair dyes and bladder cancer risk was adopted by the SCCNFP during its 17th Plenary Meeting on June 12 2001 with the following recommendations:

- *"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;*
- *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 view of the safety concerns expressed in relation to the use of hair dyes, the Commission agreed in April 2003 together with Member States and stakeholders on an overall strategy to regulate hair dye substances within the framework of the Cosmetics Directive. The strategy was published as an "Information note on the use of ingredients in permanent and non-permanent hair dye formulations (dye precursors and direct dyes)" on the DG ENTR website.

The main element of the strategy is a three step modulated approach that required industry to submit, by certain deadlines, safety files on hair dye substances for a risk assessment by the Scientific Committee on Consumer Safety (SCCS) that comply with modern standards of safety testing.

The hair dye strategy foresees to ban all permanent and non-permanent hair dyes for which industry has not submitted appropriate safety files and those, for which the SCCS has given a negative opinion.

The overall objective of this assessment process is to establish a positive list of hair dye substances, which are considered safe for human health and will be allowed for use by the cosmetics industry.

Epidemiological studies and developments in relation to a possible causality between use of permanent hair dyes and bladder cancer have constantly been followed by the SCCNFP and SCCP. The following opinions have been adopted as follow up to the first opinion from 2001:

- SCCNFP opinion (SCCNFP/0797/04) concerning Use of Permanent Hair Dyes and Bladder Cancer. Updated 2004 adopted 23 April 2004.
- SCCP opinion (SCCP/0930/05) on Personal Use of Hair Dyes and Cancer Risk adopted 20 September 2005.

In the framework of the hair dye strategy, the Scientific Committee also defined the requirements for the safety testing of hair dye substances. This element has been addressed in the following opinions:

- SCCNFP Proposal (SCCNFP/0553/02) Assessment Strategies for Hair Dyes adopted 17 December 2002.
- SCCNFP Proposal (SCCNFP/0566/02) for A Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential Mutagenicity/Genotoxicity adopted 4 June 2002.

- SCCP Opinion (SCCP/0971/06) on Updated Recommended Strategy for Testing Oxidative Hair Dye Substances for their Potential Mutagenicity/Genotoxicity adopted 28 March 2006.
- SCCP Opinion (SCCP/0959/05) on Review of the SCCNFP opinion on Hair Dye Strategy in the light of additional information adopted 20 June 2006.

The last step of the modulated hair dye strategy is to evaluate the reaction products and intermediates formed during the hair dyeing process.

3 opinions concerning the reaction products and intermediates of oxidative hair dyes have previously been adopted by the SCCP:

- SCCP opinion (SCCP/0941/05) on Exposure to reactants and reaction products of oxidative hair dye formulations adopted 13 December 2005.
- SCCP opinion (SCCP/1004/06) on Update of the Annex to the Opinion (SCCP/0941/05) on Exposure to reactants and reaction products of oxidative hair dye formulations adopted 20 June 2006.
- SCCP opinion (SCCP/1198/08) on Intermediates and reaction products of oxidative hair dye ingredients formed during Hair dyeing adopted 21 January 2009.

The current submission by COLIPA submitted by October 2009 complements the previous submissions on reaction products and intermediates providing additional data for evaluation by the SCCP.

2. TERMS OF REFERENCE

In the light of the current data submission and the available data base from previous submissions, the SCCS is asked to evaluate the consumer health risk by products and intermediates of oxidative hair dyes formed during the hair dyeing process.

3. OPINION

3.1 Introduction

In response to the SCCP Opinion (SCCP/0959/05) "Review of the SCCNFP opinion on Hair Dye Strategy in the light of additional information adopted 20 June 2006", industry submitted a further dossier in 2007 which was discussed by the SCCP (SCCP/1198/08). In a further comprehensive dossier in 2009 the formation of representative hair dye reaction products, intermediates and possible by-products under intended use conditions was qualitatively and quantitatively analysed. In the next step, exposure to some reaction products covering the range of physico-chemical properties was determined by *in vitro* dermal absorption studies. Systemic exposure was also studied in humans. Four reaction products were selected based on structural alerts and high exposure, and subjected to tests for genotoxicity. The results of topical carcinogenicity studies with commercial hair dye formulations were summarized. An updated review on epidemiological studies on a possible association between personal use of hair dyes and various types of cancer was also included in the submission.

3.2 Chemistry of reaction products of precursors and couplers

Forty eight oxidative hair dyes are commercially used in the EU, of which 16 are precursors and 32 are couplers (see Table 1). In several cases, salt of the listed bases are used in commercial formulations.

Table 1: Oxidative Hair Dyes for which Safety Dossier have been submitted under the EU hair dye strategy

Colipa No.	CAS No. (base)	Chemical name (base, if not indicated)	Precursor (developer, base)	Coupler	Opinion
A005	95-70-5	TOLUENE-2,5-DIAMINE	x		SCCP/1084/07
A007	106-50-3	p-PHENYLENEDIAMINE	x		SCCNFP/0129/99 SCCP/0989/06
A009	101-54-2	N-PHENYL-p-PHENYLENEDIAMINE	x		SCCP/0991/06
A011	108-46-3	RESORCINOL		x	SCCP/1117/07
A012	95-88-5	4-CHLORORESORCINOL		x	SCCS/1224/09
A015	591-27-5	m-AMINOPHENOL		x	SCCP/0978/06
A016	123-30-8	p-AMINOPHENOL	x		SCCP/0867/05
A017	90-15-3	1-NAPHTHOL		x	SCCNFP/0130/99 SCCP/1123/07
A018	83-56-7	1,5-NAPHTHALENEDIOL		x	SCCP/1060/06
A019	582-17-2	2,7-NAPHTHALENEDIOL		x	SCCP/1061/06
A022	150-75-4	p-METHYLAMINOPHENOL	x		SCCNFP/0179/99 SCCP/0963/05
A025	26021-57-8	HYDROXYBENZOMORPHOLINE		x	SCCP/0965/05
A027	2835-95-2	4-AMINO-2-HYDROXYTOLUENE		x	SCCP/1001/06

Colipa No.	CAS No. (base)	Chemical name (base, if not indicated)	Precursor (developer, base)	Coupler	Opinion
A031	55302-96-0	2-METHYL-5-HYDROXYETHYLAMINO-PHENOL		x	SCCP/0957/05
A033	533-73-3	1,2,4-TRIHYDROXYBENZENE		x	SCCP/0962/05
A039	89-25-8	PHENYL METHYL PYRAZOLONE		x	SCCP/1033/06
A042	70643-19-5	2,4-DIAMINOPHENOXYETHANOL		x	SCCP/0979/06
A043	61693-42-3	3-AMINO-2,4-DICHLORPHENOL		x	SCCP/1074/07 SCCP/1205/08
A044	608-25-3	2-METHYLRESORCINOL		x	SCCP/1002/06
A050	54381-16-7	N,N-BIS(2-HYDROXYETHYL)-p-PHENYLENEDIAMINE	x		SCCP/0983/06
A053	1004-74-6	2,4,5,6-TETRAMINOPYRIMIDINE	x		SCCNFP/0695/03 SCCP/1118/07
A074	2835-99-6	4-AMINO-m-CRESOL	x		SCCP/0898/05
A075	2835-98-5	6-AMINO-m-CRESOL	x		evaluation pending
A079	81892-72-0	1,3-BIS-(2,4-DIAMINOPHENOXY)-PROPANE		x	SCCP/1098/07
A080	93841-25-9	HYDROXYETHYL-p-PHENYLENE DIAMINE	x		SCCP/0666/03 SCCP/1124/07
A084	83763-47-7	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE		x	SCCP/0958/05 SCCP/1172/08 SCCS/1250/09
A094	80419-48-3	5-AMINO-6-CHLORO-o-CRESOL		x	SCCNFP/0732/03 SCCS/1225/09
A098	94158-14-2	HYDROXYETHYL-3,4-METHYLENEDIOXYANILINE		x	SCCP/0951/05
A099	84540-47-6	2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE		x	SCCP/1034/06
A101	56216-28-5	2,6-DIMETHOXY-3,5-PYRIDINEDIAMINE		x	SCCP/0908/05
A111	3131-52-0	DIHYDROXYINDOLE		x	SCCNFP/0657/03 SCCP/0952/05
A117	110102-85-7	5-AMINO-4-CHLORO-o-CRESOL		x	SCCNFP/0659/03 SCCP/1120/07
A121	128729-30-6	HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-p-PHENYLENEDIAMINE)	X		SCCNFP/0340/00 SCCP/1051/06
A128	2380-86-1	6-HYDROXYINDOLE		x	SCCNFP/0667/03 SCCP/0947/05
A129	91-56-5	ISATIN		x	SCCP/0876/05
A130	90817-34-8	3-AMINO-2-METHYLAMINO-6-METHOXYPYRIDINE		x	SCCNFP/0643/03 SCCP/1121/07

Colipa No.	CAS No. (base)	Chemical name (base, if not indicated)	Precursor (developer, base)	Coupler	Opinion
A132	16867-03-1	2-AMINO-3-HYDROXYPYRIDINE		x	SCCP/1126/07
A136	141-86-6	2,6-DIAMINOPYRIDINE		x	evaluation pending
A138	149330-25-6	2,6-DIHYDROXYETHYLAMINO-TOLUENE		x	SCCNFP/0697/03
A143	1603-02-7	2,5,6-TRIAMINO-4-PYRIMIDINOL SULFATE	x		SCCNFP/0710/03 SCCP/1122/07
A147	29539-03-5	DIHYDROXYINDOLINE HYDROBROMIDE		x	SCCNFP/0669/03
A153	5697-02-9	1-ACETOXY-2-METHYLNAPHTHALENE		x	SCCP/1163/08
A154	155601-30-2	1-HYDROXYETHYL-4,5-DIAMINOPYRAZOLE SULFATE	x		SCCP/0990/06
A155	63969-46-0	2,2'-METHYLENEBIS-4-AMINOPHENOL	x		SCCP/1142/07
A156	7469-77-4	2-METHYL-1-NAPHTHOL		x	SCCP/1163/08
A157	223398-02-5	4-FORMYL-1-METHYLQUINOLINIUM-p-TOLUENESULFONATE		x	SCCP/0923/05
A158	149861-22-3	2-AMINO-5-ETHYLPHENOL	X		evaluation pending
A159	857035-95-1	2,3-DIAMINODIHYDROPYRAZOLO PYRAZOLE DIMETHOSULFONATE	x		evaluation pending

The chemistry of oxidative hair dye formation has been described in an earlier opinion of the scientific committee (SCCP/0941/05). An example of the formation of a coloured reaction product molecule by a combination of a precursor and coupler in an oxidative hair dye is shown in Figure 1.

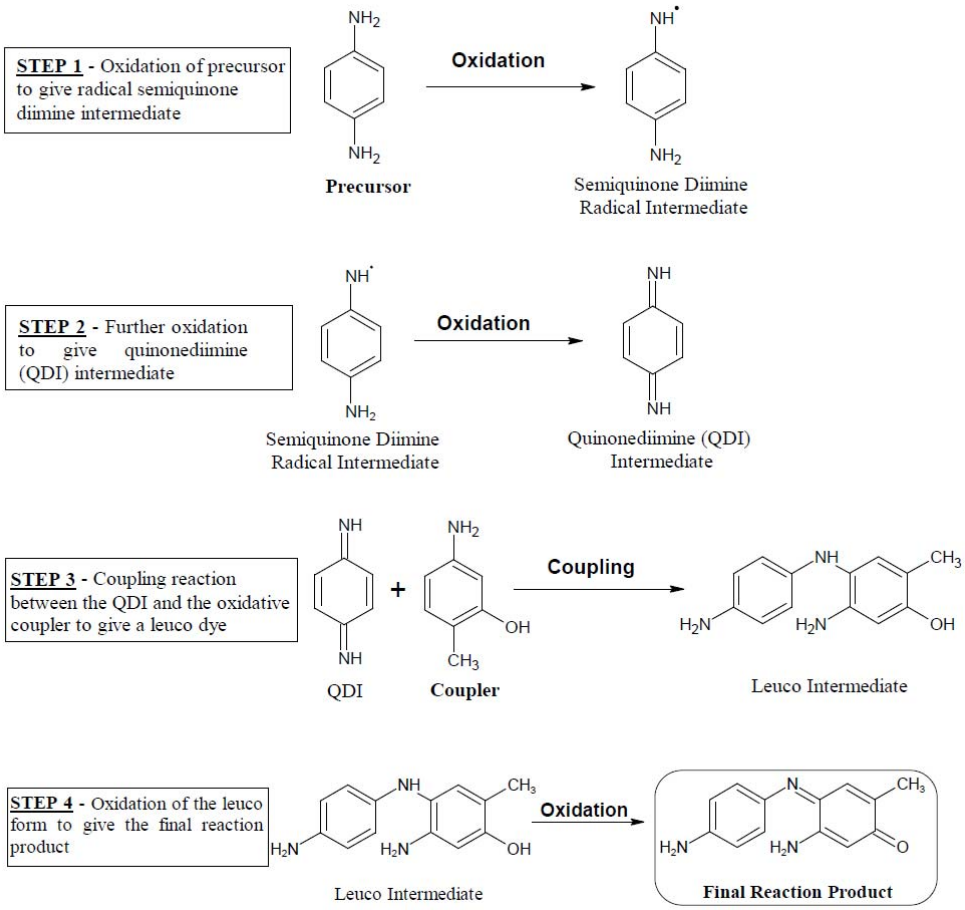


Figure1: Proposed mechanism of oxidative hair dye formation

The cosmetic industry performed several studies in the past years to improve the knowledge concerning reaction products of precursors and couplers of oxidative hair dyes to which consumers are exposed. These studies include:

- Development of a suitable methodology for the identification and determination of both intermediates and reaction products,
- Investigation of intermediates and reaction products formed by the combinations of several precursors and couplers of oxidative hair dyes in relevant matrices in the absence or presence of hair, under conditions simulating the hair dyeing process,
- Synthesis of several relevant reaction products and studies on their physico-chemical properties,
- Exposure assessment of some reaction products based on dermal absorption studies performed *in vitro.*

These studies demonstrated that the combinations of precursors and couplers mainly produce dimers and trimers as reaction products and that no self coupling products such as Bandrowski's base are formed in the presence of a coupler. Furthermore, no additional reaction products, other than those predicted, were found. A study performed with three precursors (p-toluenediamine, p-aminophenol, and 1-hydroxyethyl-4,5-diaminopyrazole) and three couplers (2-methylresorcinol, 4-amino-2-hydroxytoluene and 2,4-diaminophenoxyethanol) in a hair dye formulation revealed that the reaction products of oxidative coupling were in agreement with the theoretical predictions based on the reaction kinetics, and that the fastest coupling reactions dominate the chemistry in the formulation.

A number of direct dyes used in commercial oxidative hair colouring products are nitro-substituted aminobenzene derivatives (nitro direct dyes). According to the applicant, the nitro group strongly deactivates the molecule so that they remain inert under conditions of hair colouring, although some of these dyes have a similar structure to oxidative hair dyes. This was demonstrated in two studies. In the first study, HC Yellow 2 and 2-Amino-6-chloro-4-nitrophenol in commercial hair dye formulations were not found to affect the kinetics of reaction products formation. No additional reaction products could be detected. However, this may have been due to the low sensitivity of the analytical method. In another study, the stability of 4 dyes (B099, 2-amino-6-chloro-4-nitrophenol; B051, 4-amino-3-nitrophenol; B098, HC Violet 2; B028, sodium picramate) in an oxidative environment (dyes dissolved in water/ammonia/ isopropanol, mixed (1:1) with 6% hydrogen peroxide) was evaluated by their UV/Vis spectra recorded at time 0-30 min. As no change in the absorption of the dyes in the region 200-800 nm was observed, it was concluded that these dyes were stable in the oxidative environment.
Although this is a plausible conclusion under the experimental condition used, it cannot be excluded that some of these dyes might couple with precursors in oxidative hair dye formulations. Both 2-amino-6-chloro-4-nitrophenol and sodium picramate (2-amino-4,6-dinitrophenol sodium salt), which have a free position *para* to the amino group in the benzene ring, could theoretically couple with the precursors in oxidative hair dye formulations. Sodium picramate (B028) is under safety evaluation by SCCS and no information on its stability under oxidative conditions has been submitted.

A review of the literature shows that for all kinetic studies carried out with oxidative precursors plus couplers and hydrogen peroxide as the oxidant the relative rates of each step are as shown in Figure 2 (taken from Corbett 1969):

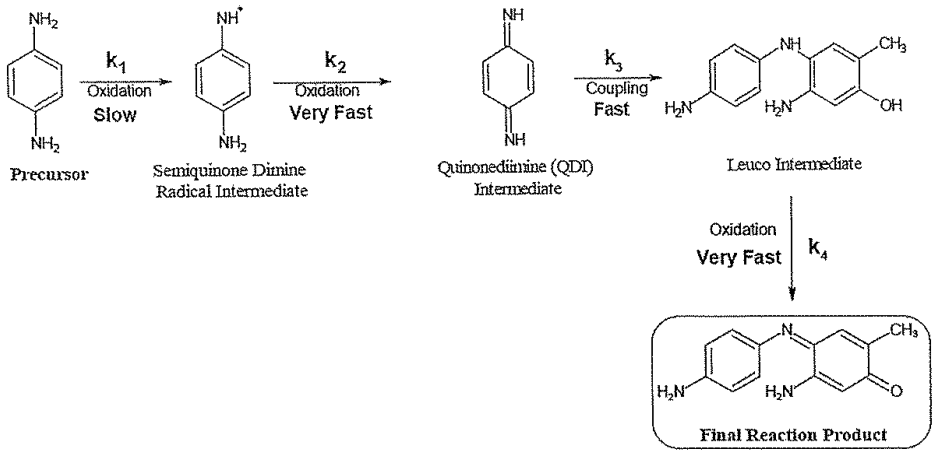


Figure 2: Relative reaction rates of various steps of oxidative hair dye formation

The rate of each step depends on the reaction conditions and especially the strength of the oxidant used. In the case of hair colouring products, the reaction proceeds in the presence of alkaline peroxide. In a series of studies on various oxidative coupling reactions under these conditions, Corbett (1972) has experimentally calculated the relative rates of each step and shown that:

$$k2 \text{ and } k4 > k3 >> k1$$

Corbett (1972) estimated that the rate of oxidation of p-phenylenediamine (PPD) to produce the quinonediimime intermediate (QDI) proceeds at a rate of approximately 0.002 min^{-1}. When this is compared to the rate with which QDI subsequently reacts with a coupler (Table 2), it is possible to calculate the steady state concentration of QDI. The theoretical

maximum concentration of PPD-QDI in an oxidative hair dye formulation was calculated to be $23x10^{-9}$ molar or 2 ppb. The commercial hair colouring products contain antioxidants, which slow down the formation of QDI and further reduce the theoretical maximum concentration of QDI to below 2 ppb. Thus, <2 ppb PPD-QDI may be present in oxidative hair dye formulations during the hair dyeing process, but the suspected build up of QDI in the hair dye formulation (SCCP/0941/05) is theoretically not possible. A recent study, where the mass balance of the precursor and reaction product was 100% at all times during the reaction, provided additional experimental evidence that there was no build up of QDI. In addition, no QDI was detected in the oxidative hair dye formulations by a method employing a combination of analytical techniques (cyclic voltametry, electron spin resonance spectroscopy and UV/Vis spectroscopy). Finally, no leuco intermediates (Figure 2) of oxidative hair dyes were identified in the formulations under conditions simulating hair dyeing.

Table 2: Rate of formation of p-phenylenediamine (PPD)-QDI and rate of its coupling with some couplers (from Corbett, 1969)

Estimated Rate of QDI formation from alkaline peroxide = $0.002min^{-1}$
Estimated Rate of QDI reaction with PPD = 28.7 L $mole^{-1}$ min^{-1}
Theoretical Max Concentration of QDI (no coupler) = 35×10^{-6} molar
Comparative Rate of Coupling with QDI

Coupler	Rate of Coupling with QDI	Relative Rate of Coupling with QDI
PPD	~ 30	1
Resorcinol	150,000	5000
1-Naphthol	74,000	2500
m-Aminophenol	55,000	1800

* Theoretical Max Concentration QDI (in presence of Coupler) = 23×10^{-9} molar or 2 ppb
* Theoretical Max Concentration QDI (in presence of Antioxidant) < 23×10^{-9} molar or 2 ppb

The chemical class of precursor and coupler determines the chemistry and the resulting reaction product structure. Tables 3 and 4 summarise the combinations that have been studied. According to the applicant, these include:

- Examples of major precursor classes
- Examples of major coupler classes
- Highest tonnage precursors and couplers
- Most frequent combinations
- Combinations leading to dimeric reaction products
- Combinations leading to trimeric reaction products

The precursor and couplers studied can be grouped as follows:

Precursors

1. p-Phenylenediamines which include p-phenylenediamine (A007), p-toluenediamine (A005) and N,N-bis-hydroxyethyl-p-phenylenediamine (A050)
2. p-Aminophenols which include p-aminophenol (A016) and 4-amino-m-cresol (A074)
3. Heterocyclic diamines which include 1-hydroxyethyl-4,5-diaminopyrazole (A154) and 2,4,5,6-tetraaminopyrimidine (A053)

Couplers

1. Resorcinols which include resorcinol (A011) and 2-methylresorcinol (A044)

2. Blocked m-aminophenols leading to dimeric reaction products which include 4-amino-2-hydroxytoluene (A027) and unblocked m-aminophenols leading to trimeric reaction products which include m-aminophenol (A015)
3. m-Phenylenediamine derivatives which include 2,4-diaminophenoxyethanol (A042)
4. Pyridines which include 2-amino-3-hydroxypyridine (A132) and 2,6-Dihydroxy-3,4-dimethylpyridine (A099)
5. Naphthols which include 1-naphthol (A017), 2,7-dihydroxynaphthalene (A019) and 1-acetoxy-2-methylnaphthalene (A153)

The combinations of the seven precursors and ten couplers studied so far are described in Tables 3 and 4. Precursors and couplers with a variety of substituents such as hydroxy, amino, imino, carbonyl, hydroxyethyl, hydroxyethoxy and alkyl groups were included. The qualitative and quantitative information on the reaction products (dimers and trimers) formed under simulated hair dyeing conditions using glass surface instead of scalp is listed in Table 3. Details of the HPLC analytical method used to quantitatively follow the various oxidative combinations and the resulting formation of the reaction products have been provided in previous opinions of the scientific committee (SCCNFP/0808/04, SCCP/0941/05). When precursors and couplers (both at approximately 62.5 µmol/g) in an oxidative hair dye formulation react for 30 min in the presence of hair (ratio of hair dye formulation to hair 2:1, w/w), the reaction products formed were distributed in two compartments:

- within the hair and
- in the hair dye formulation surrounding hair including the hair surface.

The skin (scalp) may be exposed to the free reaction products in the hair dye formulation, remaining on the surface and outside hair. The concentrations of the specific reaction products in the hair dye formulation were 0.02 – 0.65% (Table 3); and the total concentrations of unreacted precursors and couplers in various experiments were 12 - 84% of the applied dose (SCCP/0941/05). The SCCS notes that the impact of the scalp as an additional compartment for the formation and distribution of precursors, couplers and reaction products *in vivo* is not modelled in this experimental set-up.

Table 3: Concentrations of reaction products formed from various combinations of precursors and couplers of oxidative hair dyes outside hairs in experiments simulating hair dyeing.

Oxidative combination	Reaction Product	Concentration (% w/w) in the formulation [a]
p-toluenediamine (A005) + 4-amino-2-hydroxytoluene (A027)	Dimer A005-A027	0.26
p-toluenediamine (A005) + m-aminophenol (A015)	Trimer A005-A015-A005	0.14
p-toluenediamine (A005) + resorcinol (A011)	Trimer A005-A011-A005	0.03
p-toluenediamine (A005) + 2-methylresorcinol (A044)	Trimer A005-A044-A005	0.02
p-toluenediamine (A005) + 2,4-diaminophenoxyethanol (A042)	Dimer A005-A042	0.37
p-phenylenediamine (A007) + 4-amino-2-hydroxytoluene (A027)	Dimer A007-A027	0.16
N,N-dihydroxyethyl-p-phenylenediamine (A050) + m-aminophenol (A015)	Trimer A050-A015-A050	0.50
p-aminophenol (A016) + 4-amino-2-hydroxytoluene (A027)	Dimer A016-A027	0.14
1-hydroxyethyl-4,5-diaminopyrazole (A154) + 4-amino-2-hydroxytoluene(A027)	Dimer A154-A027	0.65
1-hydroxyethyl-4,5-diaminopyrazole (A154) +m-aminophenol (A015)	Dimer A154-A015 Trimer A154-A015-A154	0.32 0.19

Oxidative combination	Reaction Product	Concentration (% w/w) in the formulation [a]
Precursor p-toluenediamine (A005) Couplers 4-amino-2-hydroxytoluene (A027)+ m-aminophenol (A015)	Dimer A005-A027 Trimer A005-A015-A005	0.21 0.10
Precursors: p-toluenediamine (A005) + p-aminophenol (A016) + 1-hydroxyethyl-4,5-diaminopyrazole (A154) Couplers 2-methylresorcinol (A044) + 4-amino-2-hydroxy-toluene (A027) + 2,4-diaminophenoxyethanol (A042)	Dimer A005-A027 Dimer A005-A042 Dimer A016-A027 Dimer A016-A042 Dimer A154-A027 Dimer A154-A042	[b] [b] [b] [b] [b] [b]

[a] in the hair dye formulation on the surface and outside hairs
[b] only qualitative analysis was performed.

The concentrations described in the table above represent the levels of reaction products obtained after 30 min.

Figure 3 shows the kinetic plot for the appearance of Dimer A005-A027 from the oxidative combination of p-toluenediamine (A005) and 4-amino-2-hydroxytoluene. (A027). In this example, the formation of the reaction product is approximately linear over time. A second example based on the formation of Trimer A050-A015-A050 from the oxidative combination of N,N-bis(2-hydroxyethyl)-p-phenylenediamine (A050) and m-aminophenol (A015) also showed a linear rate of formation over the 30 min application time (Figure 4).

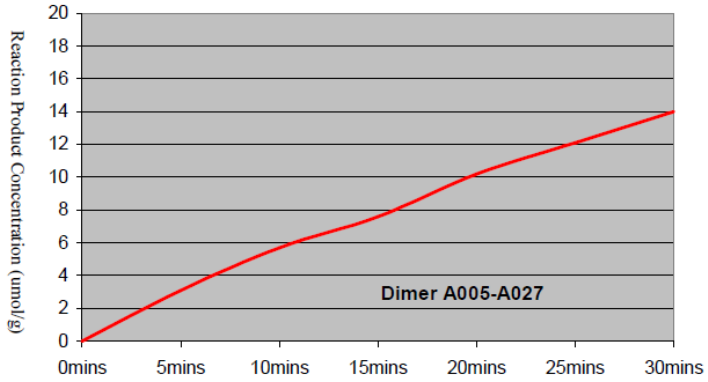


Figure 3: Formation of Dimer A005-A027 in hair dye formulation over time:– Area under curve = external exposure.

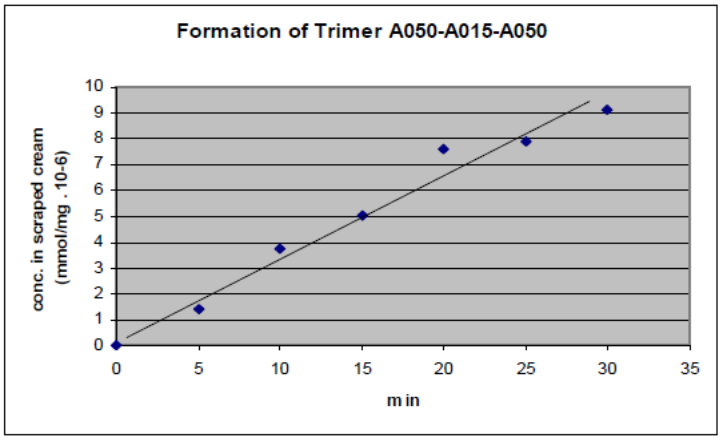


Figure 4: Formation of Trimer A050-A015-A050 over time.

Similar kinetic plots were also reported for some other relatively fast coupling reactions.
For relatively fast forming reaction products, the applicant estimated that the average reaction product concentration over the whole application time would be around 50% of the maximum concentration. This time averaged concentration may represent the maximum external exposure of the consumer to the reaction product and can also be calculated as the area under the curve in Figures 3 and 4.

Slower forming reaction products (e.g. Trimer A050-A011-A050) may not show a linear rate of formation and so in these cases, the maximum concentration after 30 min application time was considered as the average concentration to avoid underestimating potential external exposure.

The estimated maximum external (topical) exposure from reaction products, formed by various combinations of precursors and couplers of oxidative hair dyes, is reported in Table 4.

Table 4: Estimated maximum external (topical) exposure from reaction products, formed from various combinations of precursors and couplers of oxidative hair dyes outside hairs in experiments simulating hair dyeing.

Oxidative combination	Reaction Product	Estimated maximum external (topical) exposure % (w/w)
p-toluenediamine (A005) + 4-amino-2-hydroxytoluene (A027)	Dimer A005-A027	0.12
p-toluenediamine (A005) + m-aminophenol (A015)	Trimer A005-A015-A005	0.07
p-toluenediamine (A005) + resorcinol (A011)	Trimer A005-A011-A005	0.03
p-toluenediamine (A005) + 2-methylresorcinol (A044)	Trimer A005-A044-A005	0.02
p-toluenediamine (A005) + 2,4-diaminophenoxyethanol (A042)	Dimer A005-A042	0.29
p-toluenediamine (A005) + 2amino-3-hydroxy pyridine (A132)	Trimer A005-A132-A005	0.16
p-phenylenediamine (A007) + m-aminophenol (A015)	Dimer A007-A015	0.02
	Trimer A007-A015-A007	0.03
p-phenylenediamine (A007) + 4-amino-2-hydroxytoluene (A027)	Dimer A007-A027	0.08
p-phenylenediamine (A007) + resorcinol (A011)	Trimer A007-A011-A007	0.03
p-phenylenediamine (A007) + 1-naphthol (A017)	Trimer A007-A017-A007	0.14
N,N-dihydroxyethyl-p-phenylenediamine (A050) + m-aminophenol (A015)	Trimer A050-A015-A050	0.26
N,N-dihydroxyethyl-p-phenylenediamine (A050) + resorcinol (A011)	Trimer A050-A011-A050	0.02
p-aminophenol (A016) + 2,4-diaminophenoxyethanol (A042)	Dimer A016-A042	0.14
p-aminophenol (A016) + 4-amino-2-hydroxytoluene (A027)	Dimer A016-A027	0.07
p-aminophenol (A016) + m-aminophenol (A015)	Dimer A016-A015	0.05
	Trimer A016-A015-A016	0.06
p-aminophenol (A016) + 2-amino-3-hydroxypyridine (A132)	Trimer A016-A132-A016	0.14
4-amino-m-cresol (A074) + 4-amino-2-hydroxytoluene (A027)	Dimer A074-A027	0.08
1-hydroxyethyl-4,5-diaminopyrazole (A154) + 4-amino-2-hydroxytoluene(A027)	Dimer A154-A027	0.33
1-hydroxyethyl-4,5-diaminopyrazole (A154) + m-aminophenol (A15)	Dimer A154-A015	0.16
	Trimer A154-A015-A154	0.10
1-hydroxyethyl-4,5-diaminopyrazole (A154) +1-naphthol (A017)	Dimer A154-A017	0.16
2,4,5,6-tetraaminopyrimidine (A053) + 2,6,dihydroxy3,4-dimethylpyridine (A099)	Dimer A053-A099	0.08

Oxidative combination	Reaction Product	Estimated maximum external (topical) exposure % (w/w)
4-amino-m-cresol (A074) + 1-acetoxy-2-methylnaphthalene (A153)	Dimer A074-A153	0.20
1-hydroxyethyl-4,5-diaminopyrazole (A154) + 2,4-diaminophenoxyethanol (A042)	Dimer A154-A042	0.33
1-hydroxyethyl-4,5-diaminopyrazole (A154) + resorcinol (A011)	Trimer A154-A011-A154	0.06
2,4,5,6-tetraaminopyrimidine (A053) + 2-methylresorcinol (A044)	Dimer A053-A044 Trimer A53-A44-A53	0.02 0.04
2,4,5,6-tetraaminopyrimidine (A053) + 2,7-dihydroxynaphthalene (A019)	Dimer A053-A019	0.03

Summary and discussion of chemistry of reaction products of precursors and couplers

The methodology described earlier (SCCP/0941/05) on the investigation of reaction products of precursors and couplers of oxidative hair dyes under conditions simulating the hair dyeing process, was applied for the qualitative and quantitative analysis of 30 reaction products formed by 26 different combinations of seven precursors and ten couplers. In addition, one experiment was performed where only a qualitative analysis was done on the products formed by the reaction of more than one precursor and/or coupler in an oxidative hair dye formulation. The precursors and couplers used represented the highest tonnage precursors and couplers with a variety of substituents, such as hydroxy, amino, imino, carbonyl, hydroxyethyl, hydroxyethoxy and alkyl groups. The reaction products formed were of both dimeric and trimeric structures of a large range of molecular weight and water solubility. It was demonstrated that the other components of commercial hair dye formulations, including two direct dyes, did not affect the kinetics of reaction products formation. No build up of intermediates was observed. It was also revealed that the reaction products of oxidative coupling were in agreement with the theoretical predictions based on the reaction kinetics and that the fastest coupling reactions dominate the chemistry in hair dye formulations.

The presence of direct dyes in oxidative hair dye formulations (HC Yellow 2 and 2-amino-6-chloro-4-nitrophenol) has been shown not to affect the kinetics of reaction products formation. No additional reaction products, other than those predicted were found. Furthermore, it was demonstrated that 4 direct dyes (B099, 2-amino-6-chloro-4-nitrophenol; B051, 4-amino-3-nitrophenol; B098, HC Violet 2; B028, sodium picramate) were stable in alkaline oxidative environment. Sodium picramate (B028) is still under safety evaluation as a hair dye by SCCS. The absence of reaction product of 2-amino-6-chloro-4-nitrophenol in the above mentioned study may be due to low sensitivity of the analytical method. Thus, stability of all direct dye in oxidative hair dye formulations needs to be checked by more sensitive methods.

According to the applicant, the concentrations of precursors and couplers used in the studies were the typical concentrations in the marketed products (approximately 62.5 μ mol/g in an oxidative hair dye formulation after mixing with hydrogen peroxide). It should, however, be noted that the worst case scenario of the concentration of some active ingredients, for example p-phenylenediamine (A007) and toluene-2,5-diamine (A005) in hair dye formulations after mixing with hydrogen peroxide, can be >185 μmol/g (A007) and 327 μmol/g (A005) according to the maximum allowed concentrations of these ingredients (2 % (A007) and 4 % (A005)). To estimate relevant exposure concentrations of reaction products, the selected concentrations of the active ingredients, precursors and couplers, in the reaction mixture should represent the worst case scenario.

The consumer is exposed both to unreacted precursors and couplers of oxidative hair dyes and the respective reaction products. The concentrations of the specific reaction products in the hair dye formulation after 30 min under conditions of simulating hair dyeing were 0.02 – 0.65% (Table 3); and the total concentrations of unreacted precursors and couplers in various experiments were 12 - 84% of the applied dose (SCCP/0941/05).

The applicant estimated the total external (or topical) exposure by reaction products from the time averaged concentration which is also represented by the area under the kinetic plot graph. The maximum exposure concentration from the fast-forming reaction products (e.g. dimers) is considered as the area under the curve for 30 min exposure. For the slow-forming reaction products (e.g. trimers) the maximum concentration obtained after 30 min application of hair dyeing formulation was considered as maximum exposure concentration. The estimated exposure concentrations of reaction products are described in Table 4.

All studies submitted were performed using a 30 min application period. However, the SCCS is also aware that application of certain hair dye products is recommended for >30 min to achieve the expected hair colour.

The exposure assessment, (based on *in vitro* hair dyeing simulation using a glass surface) has not evaluated the impact of an additional compartment, the scalp, also available for the distribution of hair dye formulation (*in situ* dermal absorption of the reaction products formed) during hair dyeing by the consumer.

Although studies on the reaction products of precursors and couplers with several substituents have been performed, these do not include precursor/couplers with chloro substituents. The reaction products of these may have significant different physico-chemical and toxicological properties compared to those studied so far. These oxidative hair dyes with chloro-substituents are 4-chlororesorcinol (A012; CAS No. 95-88-5), 3-amino-2,4-dichlorphenol (A043; CAS No. 61693-42-3), 5-amino-6-chloro-o-cresol (A094; CAS No. 84540-50-1) and 5-amino-4-chloro-o-cresol HCl (A117; CAS No. 110102-85-7). All of the chloro substituted oxidative dyes are couplers. In the case of A012 and A117, the chloro group is a leaving group during the coupling process hence the final reaction product does not contain a chloro group and have the same structure as the equivalent reaction products from A011 (resorcinol) and A027 (4-amino-2-hydroxytoluene).
In the case of the low tonnage couplers A094 and A043, the final reaction products will contain a chloro substituent. The substitution of a particular reaction product with a chloro group would slightly increase both molecular size and hydrophobicity. Indeed the calculation of the molecular size and log D (at pH 10) of two similar reaction products (Dimer A007-A027; molecular weight 229; Log D -0.03) and Dimer A007-A094; molecular weight 263; Log D +0.76) illustrate this tendency. However, the substitution of a reaction product with a chloro group is not expected to have a dramatic effect on penetration. The reaction products already studied cover the full range of molecular sizes and hydrophobicities and the chloro-substituted reaction products would not fall outside of this range. Reaction products studied for dermal penetration cover a range of molecular weights of 229 to 490 and Log D values at pH 10 of +1.8 to -3.5. On the other hand, genotoxicity of chloro substituted reaction products may differ.

3.3 Exposure assessment by *in vitro* dermal absorption studies

In previous submissions, industry presented *in vitro* dermal penetration data for 9 reaction products obtained using 1% of reaction products in typical hair dye formulations. However, studies on the kinetics of oxidative hair dye chemical reactions, performed on selected combinations of oxidative hair dye ingredients, showed that a concentration of 1% was too high when compared to the actual reaction products concentrations achieved under conditions relevant to hair dye use, which were in the range of 0.016 - 0.33%.

For further dermal penetration studies, fourteen reaction products were selected in order to be representative of commercial oxidative hair colours and covering the range of physico-chemical properties.

- molecular weights (Fig. 5)
    - small dimers (eg A007-A027 with MW 230)
    - large trimers (eg A050-A011-A050 with MW 490)

- hydrophobicity (Fig. 6)
    - most hydrophobic (eg A074-A027; calculated Log D at pH 10 of +1.8)
    - least hydrophobic (eg A050-A011-A050; calculated Log D at pH 10 of -3.5)

- compounds with a wide range of the most common structural moieties and substituents.

- examples of oxidative dyes from all of the major chemical classes

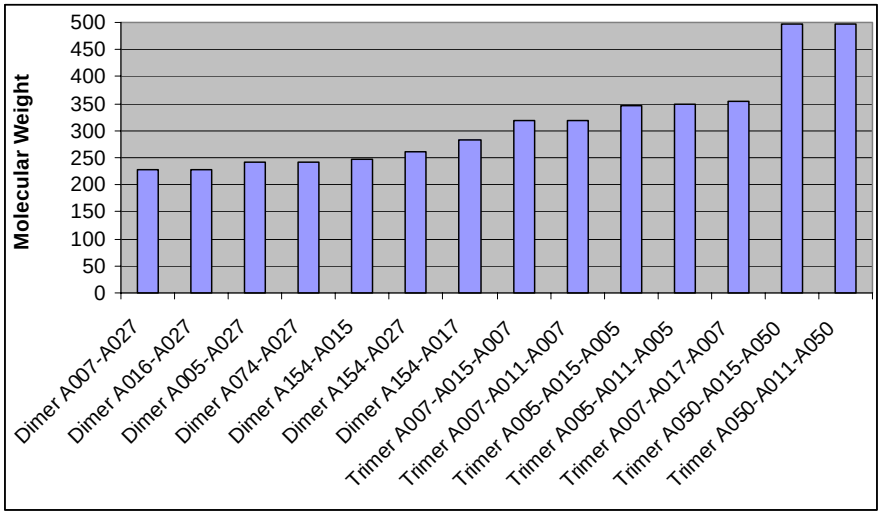


Figure 5: Range of reaction product molecular weights covered by studies

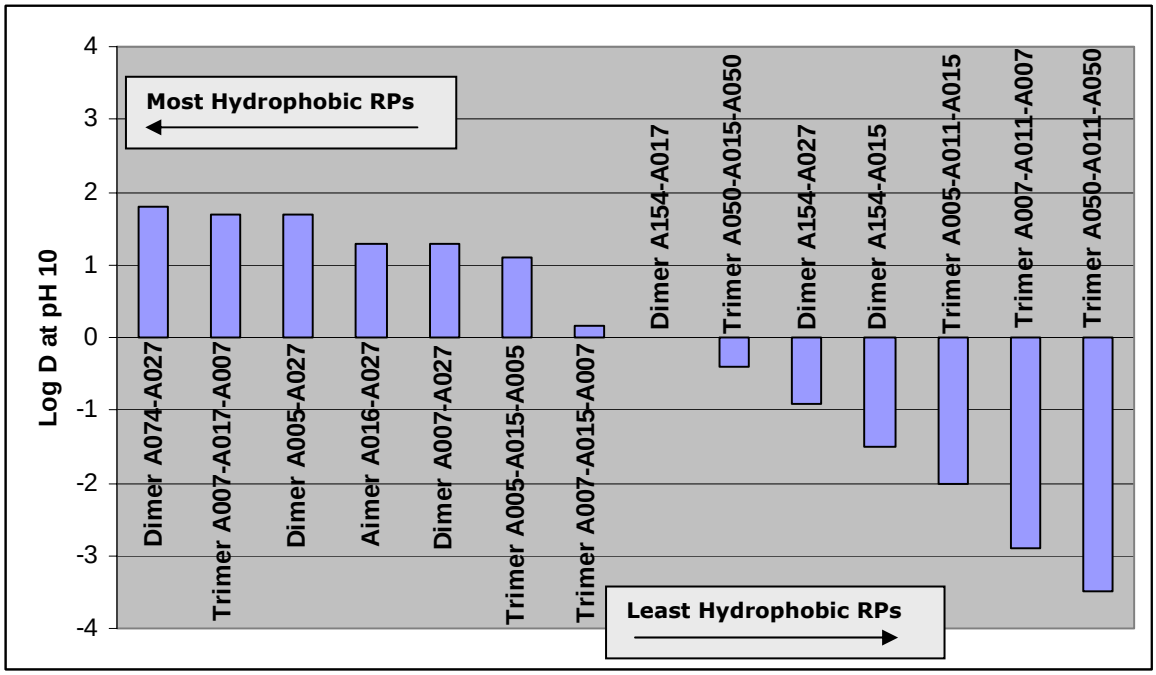


Figure 6: Range of hydrophobicities (based on Log D at pH 10) covered by studies

Although studies had also been foreseen with A005-A042, A005-A044-A005 and A053-A099, they could not be conducted due to difficulties in synthesizing samples of the reaction products and/or difficulties in formulating a test sample with the reaction product due to extremely low solubility.

In vitro 72 hr dose-response dermal penetration studies

Selection of test concentrations

As described in chapter 3.2, studies on the kinetics of oxidative coupling over time were conducted with a range of representative hair dye precursor/coupler combinations in order to evaluate the rate of formation of reaction products over a 30 minute exposure period. These studies showed that the concentration of most reaction products in the formulation increased linearly over time.

The reaction product concentration measured at the end of the colour development period represents the maximum external concentration to which the consumer is exposed. For relatively fast forming reaction products showing a linear increase in reaction product concentration over time, the average reaction product concentration for the entire application time period is approximately 50% of the maximum concentration. Slower forming reaction products (e.g. A050-A011-A050) may not show a linear rate of formation and in these cases, the maximum reaction product concentration (measured at the end of the kinetics study) was considered to be the relevant concentration for evaluation of percutaneous penetration.

For most reaction products three concentrations were tested, including the relevant consumer exposure concentration and the concentration of 1%. For A074-A027 only one additional (higher) concentration and for A005-A027 only the concentration relevant to consumer exposure was evaluated.

These concentrations were selected in order to allow the estimation of consumer exposure under realistic conditions and to determine whether there is a linear relationship between

the reaction product concentration in the hair colorant formulation and its dermal penetration rate.

Table 6 summarizes the reaction product concentrations that were used in the *in vitro* dermal penetration studies.

Experimental procedure

In most cases the reaction products were added to typical hair dye base formulations which were then mixed with a developer formulation containing hydrogen peroxide or, for those reaction products unstable in the presence of peroxide, a placebo formulation not containing peroxide. In two studies (A005-A027 and A074-A027) the formulation containing the reaction products was not mixed with a developer formulation.

Frozen dermatomed human skin samples (thickness 300-500 µm) were used for most studies, except for studies on A005-A027 and A074-A027, where fresh dermatomed pig skin was used (ear skin, 300-500 µm in thickness).

The formulations were applied to the skin samples, pre-mounted in diffusion cells, at a nominal dose of 20 mg/cm². Following 30 minutes of contact, the skin surface was washed off with a mild detergent. Samples of receptor fluid were taken at regular time intervals for 72 hr.

At the end of each experiment, the surface of the skin was washed, the *stratum corneum* was tape stripped and the epidermis was separated from the dermis by heat. In two studies (A005-A027 and A074-A027) tape stripping was not performed prior to heat separation of the epidermis and dermis.

The amounts of reaction product in the receptor fluid, dermis, epidermis, *stratum corneum* (tape strips) and skin washes were determined by liquid scintillation counting (LSC), HPLC/MS (A154-A017) or HPLC with UV/VIS detection (A005-A027 and A074-A027).

Table 5: Limits of detection for analytical methods used to determine exposure to reaction products

Analytical Method	Limit of detection [pg/cm²]	Number of reaction products analysed
LSC	2.1-4.7	11
HPLC/MS	92-177	1
HPLC (UV/Vis)	12 000-14 000	2

When the reaction product concentration found in the receptor fluid was below the limits of detection at the later sampling times, the amount of reaction product found in the epidermis after a 72 hr experimental period was considered not to be systemically bioavailable. In such cases, only the amounts in the receptor fluid and dermis were considered to be bioavailable.

When detectable amounts of reaction product continued to penetrate into the receptor fluid beyond 24 to 48 hr, it was assumed that the skin may serve as a reservoir for continued penetration of the reaction product. In such cases, the total amount found in the epidermis (without the *stratum corneum*), dermis, and receptor fluid was considered to be bioavailable.

Results from dermal penetration studies

Table 6 summarizes the results from dermal penetration studies conducted on 14 different hair dye reaction products. Reported are

- the estimated dermal bioavailability (expressed as ng/cm^2);
- the adjusted human systemic exposure per day (µg/day), assuming one hair colouring event every 28 days (this number is relevant for risk assessment of genotoxic carcinogens).

The majority of the reaction product was recovered from the rinsing solutions after 30 min exposure (86 – 105%). For most reaction products there was no evidence that the amounts found in skin compartments represent a skin reservoir. For these, the amounts of reaction product found in the receptor fluid and in the dermis were included in the calculation of systemic bioavailability. For 5 reaction products (A154-A017, A007-A017-A007, A074-A027, A005-A015-A005, and A016-A027), the possibility of a skin reservoir could not be excluded. Accordingly, the amounts of reaction product found in the receptor fluid and dermis, as well as the amount in the epidermis, were included in the calculation of systemic bioavailability.

The amounts of reaction product considered to be bioavailable were in the range of 3.27 to 717.79 ng/cm^2 (mean +1SD). Assuming that oxidative hair dyes are used once every 28 days on a scalp with a surface area of 580 cm^2 (SCCP, 2006), the accordingly adjusted daily exposure ranged from 0.07 to 14.86 µg/day (mean+1SD).

Summary and discussion of *in vitro* dermal absorption studies

In vitro skin penetration studies were investigated for 14 oxidative hair dye reaction products.

Exposure time: 30 min

Despite the SCCP recommendation (SCCP1198/08) that a 45 min exposure period should be used for the reaction product studies, the 30 min skin exposure time could be accepted, as a 50% increase in exposure time can be expected to result in exposures in the same range as those reported in the Table 6. Furthermore, the SCCS used the mean + SD figure according to its recent opinion on dermal absorption (SCCS/1358/10) to cover variabilities in the dermal absorption studies.

Time-averaged concentration based on kinetics studies

Time-averaged concentrations obtained from kinetics experiments were used for the dermal penetration studies. For relatively fast forming reaction products with linear kinetics it is reasonable to assume that the average reaction product concentration over the whole application time would be around 50% of the final concentration achieved. For slower forming reaction products, the final concentrations measured at the end of the kinetics study were used for the dermal penetration studies since kinetics may not be linear in these cases.

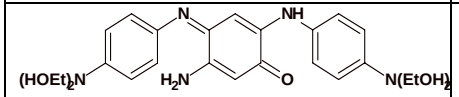
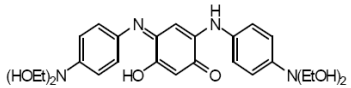
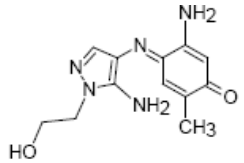
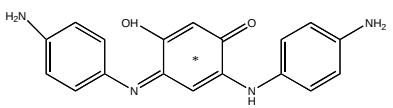
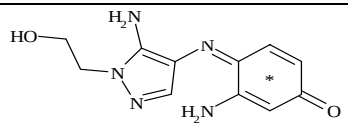
Use of peroxide in formulation

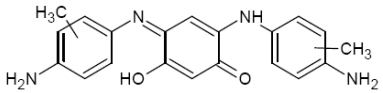
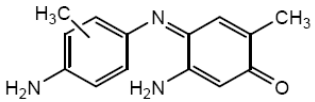
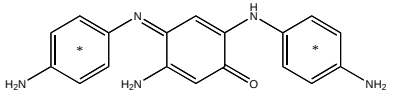
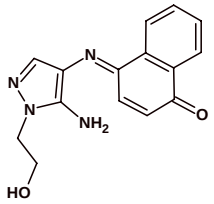
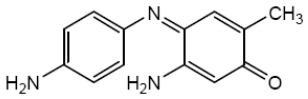
Peroxide is used in oxidative hair dye formulations to initiate the oxidation of the precursor molecule ultimately leading to the formation of the final reaction product. For the reaction product A005-A011-A005, it has been demonstrated that the presence of peroxide does not influence the penetration behaviour of the reaction product. In the present submission, some studies were carried out with peroxide, whilst others were not. In cases where peroxide was present, it was ensured that the criteria for 3 hour stability of the reaction product in the dosing formulation were met (i.e., >96% stability over 30 minutes).

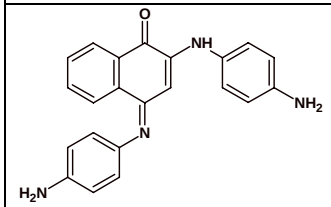
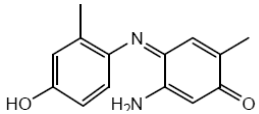
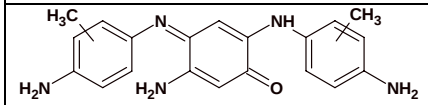
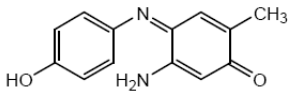
The amounts of reaction product considered to be bioavailable were in the range of 3.27 to 717.79 ng/cm^2 (mean +1SD). This corresponds to 1.9 to 416 µg absorbed dose (i.e. dose potentially bioavailable) per hair dye application (i.e. 0.03 to 6.9 µg/kg bw). Assuming that

oxidative hair dyes are used once every 28 days on a scalp with a surface area of 580 cm^2 (SCCP, 2006), the accordingly adjusted daily exposure ranged from 0.07 to 14.86 µg/day (mean+1SD) corresponding to 1.13 to 248 ng/kg bw/d.

Table 6: Exposure assessment by in vitro dermal absorption studies

Reaction Product	Reaction product concentration in the final formulation to which consumers are exposed based on kinetic studies (%)	Reaction product concentrations tested in 72 h dermal penetration study (%)	Limit of Detection in receptor fluids (pg/cm²)	Estimated Bioavailability Mean +1 SD (ng/cm²)	Exposure per day (µg/day) (adjusted bioavailable dose x 580 cm² / 28 days)
 Trimer A050-A015-A050	0.26	1.0; 0.5; **0.25**	2.8	3.27	0.07
 Trimer A050-A011-A050	0.016	1.0; 0.1; **0.05**	2.6	4.30	0.09
 Dimer A154-A027	0.33	1.0; **0.35**; 0.1	92.6	12.53	0.26
 Trimer A007-A011-A007	0.03	1.0; 0.1; **0.03**	4.1	7.54	0.16
 Dimer A154-A015	0.16	1.0; 0.4; **0.16**	170	10.54	0.22

Reaction Product	Reaction product concentration in the final formulation to which consumers are exposed based on kinetic studies (%)	Reaction product concentrations tested in 72 h dermal penetration study (%)	Limit of Detection in receptor fluids (pg/cm²)	Estimated Bioavailability Mean +1 SD (ng/cm²)	Exposure per day (µg/day) (adjusted bioavailable dose x 580 cm² / 28 days)
 Trimer A005-A011-A005	0.03	1.0; 0.3; **0.1**	3.8	16.21	0.34
 Dimer A005-A027	0.12	**0.1**	9200 HPLC (UV/Vis)	17.78	0.37
 Trimer A007-A015-A007	0.03	1.0; 0.3; **0.1**	4.7	11.61	0.24
 Dimer A154-A017	0.16	1.0; 0.3; **0.15**	177* (HPLC/MS)	7.79	0.16
 Dimer A007-A027	0.08	1.0; 0.3; **0.1**	2.9	44.30	0.92

Reaction Product	Reaction product concentration in the final formulation to which consumers are exposed based on kinetic studies (%)	Reaction product concentrations tested in 72 h dermal penetration study (%)	Limit of Detection in receptor fluids (pg/cm²)	Estimated Bioavailability Mean +1 SD (ng/cm²)	Exposure per day (µg/day) (adjusted bioavailable dose x 580 cm² / 28 days)
 Trimer A007-A017-A007	0.14	0.9; **0.15**; 0.05	2.1	95.25	1.97
 Dimer A074-A027	0.08	0.5; **0.1**	8000 HPLC(UV/Vis)	263.7	5.46
 Trimer A005-A015-A005	0.07	1.0; 0.3; **0.1**	2.0	461.89	9.57
 Dimer A016-A027	0.07	1.0; 0.3; **0.1**	2.2	717.79	14.87

*Value corresponds to the limit of quantification. The limit of detection was not determined.
Bold Blue: relevant concentration tested in skin penetration studies for estimation of systemic bioavailability.

3.4 Exposure assessment determined by studies in humans

3.4.1 Human systemic exposure to a [^{14}C]-para-phenylenediamine-containing oxidative hair dye

Introduction

A mass balance study was conducted in sixteen human volunteers to quantify the systemic exposure of human consumers to oxidative hair dye ingredients, hair dye reaction products and their respective metabolites, following a single use of a PPD-based hair colouring product. Subjects received a dark-shade, [^{14}C]-PPD-labelled oxidative hair dye, containing PPD (A007), meta-aminophenol (A015) and resorcinol (A011). These particular hair dye ingredients are amongst the highest tonnage oxidative hair dyes in the EU market.
This investigation aimed to quantify, using a sensitive LC-MS/MS method, the presence of PPD and its acetylated metabolites as well as reaction products and their possible acetylated metabolites in human plasma and urine. The following overview will focus on the presence of A007-A011-A007 and A007-A015-A007.

Materials and methods

The hair of sixteen human volunteers was dyed with a commercial, dark-shade oxidative hair dye containing on-head concentrations of 1% [^{14}C]-para-phenylenediamine (PPD, A007), 0.5% resorcinol (A011) and 0.5% meta-aminophenol (A015), under conditions used in hairdressing salons. The dyeing process was performed by experienced, professional hairdressers who adhered to labelling instructions, but otherwise followed their individual professional practices. The development time of the oxidative hair dye was 30 min. The mean application rate was 0.65 ± 0.11 g [^{14}C]-PPD-equivalents per study subject. After dyeing, the hair was rinsed, washed, dried and clipped. All hair, washing water and study materials were collected and the radioactivity determined for the mass balance.

Blood samples were taken prior to hair dyeing (base-line control) and at 2, 4, 6, 10, 24 and 48 hr after hair dyeing. Urine was collected prior to hair dyeing (base-line control) and for the periods 0-12, 12-24, 24-48 hr post-hair dye application. Presence of [^{14}C] was analysed in all study materials, plasma and urine samples by scintillation counting. In addition, plasma and urine samples were analysed by LC-MS/MS techniques for the anticipated A007-A011-A007 (Figure 7) and A07-A015-A007 (Figure 8).

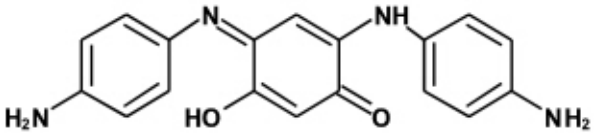


Figure 7: Chemical structure of the oxidative hair dye A007-A011-A007 resulting from the reaction of PPD (A007) with the coupler resorcinol (A011)

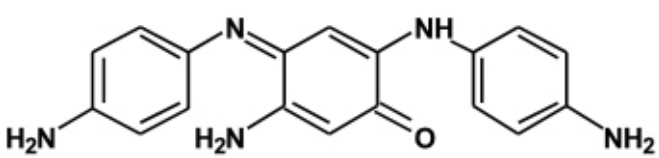


Figure 8: Chemical structure of the oxidative hair dye A007-A015-A007 resulting from the reaction of PPD (A007) with the coupler m-aminophenol (A015).

Human skin and liver may metabolise arylamines to their respective N-acetylated metabolites (Nohynek et al, 2005; Stanley et al, 2005); therefore urine and plasma were analysed by LC-MS/MS for the presence of mono- and di-acetylated derivatives of A007-A015-A007 and A007-A011-A007. The lower limits of quantification (LOQ) of the analytical methods are summarised in Table 7.

Table 7: Lower limits of quantification (LOQ) in human urine and plasma of hair dye reaction products and their potential metabolites

Substance	Urine (ng/mL)	Plasma (ng/mL)
Reaction product A007-A015-A007	1.28	0.1
Mono-N-acetyl-A007-A015-A007	1.28	0.16
Di-N,N'-acetyl-A007-A015-A007	0.8	0.32
Reaction product A007-A011-A007	0.4	0.1
Mono-N-acetyl-A007-A011-A007	0.5	0.16
Di-N,N'-acetyl-A007-A011-A007	4.0	0.8

Results

Mass balance
On the basis of [^{14}C] application and recovery, the overall mass balance for the study was 96.21 ± 1.57%.

Plasma
The C_{max} was 97.4 ± 61.5 ng/ml and was reached after 2 h. Plasma residues mainly consisted of N,N'-diacetylated PPD with C_{max} values at 2.5 hr after hair dyeing ranging from 24 to 388 ng/mL. PPD, N-mono-acetylated PPD, A007-A015-A007, A007-A011-A007 and their respective mono- or diacetylated metabolites were absent in most plasma samples at their respective limits of quantification. Two (subjects #5, 24 hr sample and #12, 2 hr sample) out of 96 samples, contained nominal amounts of A007-A015-A007 slightly above the limit of quantification (LOQ_{plasma}) (0.102 and 0.175 ng/mL, respectively). Three (subjects #5, 48 hr sample; #12, 2 hr; #14, 4 hr) out of 96 samples showed traces of mono-acetyl-A007-A015-A007 slightly above the LOQ_{plasma} (ranging from 0.164 to 0.258 ng/mL), while a single sample (Subject #8, 24 hr sample) contained diacetyl-A007-A015-A007 slightly above the LOQ_{plasma} (0.323 ng/mL). However, given their magnitude (close to the LOQ_{plasma}) and rare occurrence, these isolated values suggest low human systemic exposure to these reaction products.

Urine
The mean total amount of PPD equivalents excreted in the urine over 48 h was 5.77 ± 3.23 mg corresponding to 69.2 ± 34.8 µg PPD equivalents per kg bw. Urine samples mainly contained N,N'-diacetyl-PPD with maximal concentrations detected in the 0-12 hr urine samples. Maximal concentrations ranged from 620 to 4500 ng/mL. PPD and N-monoacetyl-PPD were present in some samples at far lower concentrations when compared with those of N,N'-diacetyl-PPD, with maximal concentrations in the 0-12 hr urine amounting to 29.6 and 6.9 ng/mL, respectively. Taking into account the urine volumes excreted, the mean total amount excreted over 48 hours amounted to 8.8 µg, 4.4 µg and 3067 µg for PPD, N-monoacetyl- PPD and N,N'-diacetyl-PPD, respectively. On a molar basis the sum of PPD and its metabolites excreted is about 16 µmol.

Hair dye reaction products A007-A015-A007 and A007-A011-A007 were not detectable in most urine samples. Exceptions were 2/48 samples (subjects #11 and #13, 0-12 hr urine samples) which contained A007-A015-A007 slightly above the LOQ_{urine} of 1.28 ng/mL (1.4 and 1.3 ng/mL, respectively) and 4/48 samples (subjects #7, #11, #12 and #13, 0-12 hr urine samples) which showed concentrations of A007-A011-A007 in the range of 0.5 to 2.0 ng/mL (LOQ_{urine} of 0.4 ng/mL).

With the exception of a single sample (subject #13, 0-12 hr urine) that showed traces of N-monoacetylated-A007-A015-A007 at the LOQ_{urine} (1.3 ng/mL), no mono- or diacetylated reaction products were detectable in the urine.

Ref.: Meuling, de Bie 2009

3.4.2 Additional information

Exposure to 2 commercial hair dye products (brown-red colour shade and black-brown colour shade) was investigated in 2 females in Germany (Schettgen *et al.*, 2010). Urinary samples were collected for a time period of 48 h after personal application of the hair dye creams which were labeled to contain toluene-2,5-diamine. The concentrations of the indicated ingredient were unknown. The urine samples were heated with 37% hydrochloric acid for 1 h at 80 °C in order to hydrolyse the amine conjugates and then analysed for aromatic diamines as well as for o-toluidine and 4-aminobiphenyl using a GC-MS method. The cumulative excreted amounts of toluene-2,5-diamine were 700 µg and 1500 µg, respectively.

Ref.: Schettgen *et al.*, 2010

3.4.3 Summary and discussion of studies in humans

A reliable way to determine systemic bioavailability of substances topically applied to humans is to understand their excretion (Wester & Maibach, 1996). Given that topically absorbed [^{14}C]-PPD-based hair dyes are excreted in the urine and not the faeces (Hueber-Becker *et al.*, 2004), urinary excretion may be used to demonstrate and quantify human systemic exposure to starting materials and reaction products of oxidative hair dyes.

The LC-MS/MS-based analytical methods used in the present study for detection of metabolites and potential hair dye reaction products in human plasma and urine were sensitive (LOQ_{plasma} 0.1 - 0.8 ng/mL; LOQ_{urine} 0.4 - 4.0 ng/mL, see Table 7), ensuring that even trace exposures can be detected.

After hair dyeing with an oxidative hair dye containing PPD, results show that the principal metabolite in the plasma after topical human exposure is N,N'-diacetyl-PPD. This metabolite has been shown to be non-genotoxic (Garrigue *et al.*, 2006).

A few plasma samples (5/96 samples from 4/16 volunteers) and urine (4/48 samples from 4/16 volunteers) contained trace levels of the two expected reaction products and/or their acetylated metabolites at or slightly above the respective LOQ_{plasma}. The maximum amounts of urinary excretion of RP A007-A015-A007 and/or its acetylated metabolites are summarised in Table 8. The maximum urinary excretion of RP A007-A011-A007 and/or its acetylated metabolites is summarised in the Table 9.

Table 8: Human exposure in two subjects (presence of urinary residues after a single hair dyeing event) to the hair dye reaction product A007-A015-A007 and its metabolites

Subject No.	Collection interval (hr)	Urine volume (mL)	A007-A015-A007 (ng/mL)	Ac-A007-A015-A007 (ng/mL) †	Total RP excreted (ng) ††	Mean time-averaged exposure (ng/day) †††
#11	0-12	976	1.41	-	1374	49.1
#13	0-12	714	1.34	1.29	1881	67.2

† N-acetylated hair dye reaction product
†† Cumulative excretions for reaction product + acetylated reaction product metabolite
††† use frequency of oxidative hair dyes of once every 4 weeks (28 days)

Table 9: Human exposure in four subjects (presence of urinary residues after a single hair dyeing event) to the hair dye reaction product A007-A011-A007

Subject No.	Collection interval (hr)	Urine volume (mL)	A007-A011-A007 (ng/mL)	Total reaction product excreted (ng)	Mean time-averaged exposure (ng/day) †
#7	0-12	1152	0.48	553	19.7
#11	0-12	976	1.07	1046	37.4
#12	0-12	1698	0.63	1065	38.0
#13	0-12	714	2.05	1462	52.2

† use frequency of oxidative hair dyes of once every 4 weeks (28 days)

Given that a potential health concern related to the use of oxidative hair dyes is an increased cancer risk due to systemic absorption of hair dye reaction products, the risk assessment should be based on the total human systemic exposure to these substances. Since oxidative hair dyes are not used on a daily basis but at 6- to 8-week intervals, risk assessment should therefore use a recommended time-averaged human daily exposure to potentially carcinogenic chemicals (Murdoch *et al.*, 1992; COC, 2003; IPCS, 1992; Renwick, 2004). With the conservative assumption that oxidative hair dyes are used once every 28 days (ie at 4-week intervals), the daily mean time-averaged exposure may be estimated by dividing the exposure from a single hair colouring event by a factor of 28.

The human exposure to hair dye reaction products, including that to A007-A011-A007 and A007-A015-A007, has previously been estimated on the basis of *in vitro* skin penetration studies (see Chapter 3). In Table 10, the results obtained in these *in vitro* studies are compared with the exposure levels determined in the present clinical study.

Table 10: Human systemic exposure to reaction product A007-A015-A007 and A007-A011-A007 and their metabolites relative to estimated exposure on the basis of *in vitro* skin penetration studies

Exposure Model	Reaction Product Exposure			
	A007-A015-A007 (ng/event)	A007-A011-A007 (ng/event)	A007-A015-A007 (ng/day) †††	A007-A011-A007 (ng/day) †††
In vitro Skin penetration study Mean absorbed dose	6734 (11.61 x 580)	4373 (7.54 x 580)	240	156
In vivo Maximum excretion found in the human study†	1881	1462	67	52
Mean exposure values estimated for the human study taking into account samples without detectable levels††	826	417	30	15

† Results from Subject #13 who had the highest levels of reaction product A007-A015-A007 plus mono-acetylated metabolite and the highest level of reaction product A007-A011-A007 in the 0-12 hr urine sample
†† For the volunteers with urine concentrations below the LOQ_{urine}, 0-12 hr samples were assumed to contain reaction product A007-A011-A007 or A007-A015-A007 at 50% LOQ_{urine} according to Beal (2001). For the few subjects with urine levels above the LOQ_{urine} in 0-12 hr urine samples, the measured values were used.
††† use frequency of oxidative hair dyes of once every 4 weeks (28 days)

The measured exposure levels from the subject with the highest excretion of reaction products (subject #13) were about 2 times lower than the mean exposure levels estimated by the *in vitro* studies (see Table 10). When taking into account that the RP A007-A015-A007 and A007-A011-A007 could be present in 0-12 hr urine samples (the time period when reaction products appeared in the urine of some subjects) of subjects where values were below the LOQ_{urine} (1.28 and 0.4 ng/mL, respectively), and assuming

that these samples contained RP levels at 50% LOQ_{urine} as recommended by Beal (2001), estimated mean systemic exposures for A007-A015-A007 and A007-A011-A007 would be 826 and 417 ng per hair dyeing event, respectively, or 30 and 15 ng/day, respectively, when adjusting for the 28-day interval between exposures (equals 0.50 and 0.25 ng/kg bw/d). This suggests that human exposure to hair dye reaction products can be predicted to be 8 to 10 fold lower than in the respective *in vitro* skin penetration studies (see Table 10).

When comparing human systemic exposure to reaction products (about 1 µg per event) with that of N,N'-diacetyl-PPD, the principal metabolite of PPD (about 2 mg per event), exposure to reaction products is 3 orders of magnitude lower based on 0-12 h urine samples. This confirms the predictions from *in vitro* skin penetration studies which also indicate that dermal penetration of reaction products is substantially lower than that of the corresponding precursors/couplers (see Chapter 5). It is also noteworthy that, despite sensitive analytical methods, only four urine samples contained measurable levels at or slightly above the LOQ_{urine}.

In conclusion, the results of this study show that, following a single hair dyeing event with a dark-shade, oxidative hair dye, human exposure to the reaction products A007-A011-A007 or A007-A015-A007 is low. The results demonstrate that *in vitro* skin penetration studies with reaction products can overestimate human systemic exposure. Given that *in vitro* skin penetration studies suggested that the RP A007-A011-A007 and A007-A015-A007 had greater potential to penetrate into or through human skin, leading to higher systemic exposure estimates than the results from this human study have demonstrated. This may hold true also for human exposure estimated for other hair dye reaction products on the basis of *in vitro* skin penetration studies.

The results of the above study (Meuling, de Bie 2009) are supported by a recent paper (Schettgen et al, 2010) who showed the cumulative excretion of 700 to 1500 µg toluene-2,5-diamine corresponding to 5.7 and 12.3 µmol respectively after exposure to two different commercial hair dye products. The values are similar to those obtained for PPD containing hair dyes (16 µmol, sum of PPD and its metabolites, see 3.4.1.).

Interestingly it was shown that during the hydrolysis step 0.5 – 1 ‰ of the 2,5-toluenediamine can be desaminated to form o-toluidine. The concentration of o-toluidine reached a maximum of 3.4 µg/l. This was far above the values found in the general population (95^{th} percentile: 0.23 µg/l) and in non-smokers (95^{th} percentile: 0.19 µg/l) as reported by Kuetting *et al.* in 2009. The excretion of o-toluidine by non-smokers may in part be explained by exposure to toluene-2,5-diamine containing hair dyes.

3.5 Genotoxicity of reaction products

3.5.1 Systemic exposure of hair dye reaction products compared to their precursors and couplers

The goal of this comparison by industry was to select the reaction products with the highest absolute and relative human exposures for genotoxicity testing. In this approach the mean values of the skin penetration data of the reaction products were used.

As shown in chapter 3.2. human external (topical) exposure associated with the use of oxidative hair dyes is limited to precursors/couplers and their reaction products, with topical exposure concentrations significantly higher for precursors/couplers than for their corresponding reaction products. Human systemic exposure to some representative reaction products was evaluated in a series of *in vitro* skin penetration studies using concentrations based on the results obtained in kinetic studies of the hair dye chemical reactions.

Similarly, human systemic exposure to hair dye precursors/couplers was investigated by conducting *in vitro* skin penetration studies. These studies have been included in the corresponding hair dye ingredient safety dossiers that were submitted during the last decade in the framework of the European Hair Dye Strategy and were assessed by the SCCNFP, SCCP, and SCCS.

Using these two sets of data, systemic exposure to the examined hair dye reaction products was compared with systemic exposure to their corresponding precursors/couplers.

Human systemic exposure to hair dye reaction products

Systemic exposure data obtained from *in vitro* skin penetration studies on hair dye reaction products at concentrations comparable to the human exposure situation are summarized in Table 11. Detailed study conditions used as well as interpretation of data are described in Chapter 3.3.

According to the applicant, human systemic exposure to the hair dye reaction products investigated ranged from 1.3 to 253 µg per hair colouring event i.e. 0.05 to 9.04 µg/day, when taking the mean value of the percutaneous absorption study and by adjusting to the use frequency of oxidative hair dyes of once every 4 weeks (division by 28).

Table 11: Systemic exposure dose to hair dye reaction products

Reaction Product	External exposure concentration (%)[1]	Concentration tested in skin penetration study (%)	Mean Systemic Exposure Dose (µg/hair colouring event)[2]	Exposure per day (adjusted bioavailable dose µg/day)[3]
A050-A015-A050	0.26	0.25	1.3	*0.05*
A050-A011-A050	0.016	0.05	1.6	*0.06*
A154-A027	0.33	0.35	1.6	*0.06*
A007-A011-A007	0.03	0.03	2.6	*0.09*
A154-A015	0.16	0.16	2.7	*0.10*
A005-A011-A005	0.03	0.1	3.9	*0.14*
A005-A027	0.12	0.1	9.4	*0.34*
A007-A015-A007	0.02	0.1	4.5	*0.16*
A154-A017	0.16	0.15	16.2	*0.58*
A007-A027	0.08	0.1	16.2	*0.58*
A007-A017-A007	0.14	0.15	37.5	*1.34*
A074-A027	0.08	0.1	101.8	*3.64*
A005-A015-A005	0.07	0.1	177.0	*6.32*
A016-A027	*0.07*	*0.1*	*253.1*	*9.04*

[1] derived from kinetic studies of hair dye chemical reactions (see chapter 3.2.)
[2] skin penetration rate (µg/cm^2) * 580 cm^2 (scalp surface area)
[3] considering use frequency of oxidative hair dyes of once per 28 days

Human systemic exposure to hair dye precursors and couplers

Human systemic exposure doses for precursors/couplers were estimated from the respective *in vitro* skin penetration studies conducted under oxidative conditions and in the presence of other precursors/couplers when available (Table 12). No *in vivo* data has been used for calculation of human systemic exposure to precursors/couplers even when such data was used for their risk assessment, in order to compare systemic exposure doses obtained under similar experimental models.
Human systemic exposure to the precursors/couplers for which skin absorption data is available for the corresponding reaction products ranged from 63 to 18130 µg per hair colouring event i.e. 2.2 to 647.5 µg/day, assuming a use frequency of oxidative hair dyes of once every 4 weeks.

Table 12: Systemic exposure dose to hair dye precursors and couplers

Precursor/ Coupler	Concentration tested in skin penetration study (free base, %)	Mean Systemic Exposure Dose (µg per hair colouring event) 1	Exposure per day (adjusted bioavailable dose. µg/day)[2]	*Study Reference*
A005	2.5	18130	647.5	*L'Oreal P39 (1997)*
A007	2.0	6131	219.0	*Hueber-Becker (2004)*
A011	1.25	603	21.5	*Inveresk 774075 (1995)*
A015	1.2	2535	90.5	*L'Oreal 16011 (1999)*
A017	2	2076	74.1	*CTL JV1809 (2005)*
A027	1.5	1601	57.2	*Cosmital KP152 (2005)*
A050	2.5	63	2.2	*CTL JV1798 (2005)*
A074	1.5	498	17.8	*Cosmital KP176 (2008)*
A154	2	2204	78.7	*Cosmital KP089 (2004)*

[1] skin penetration rate (µg/cm²) 580 cm² (scalp surface area)
[2] considering use frequency of oxidative hair dyes of once per 28 days

Comparison of the human systemic exposure to hair dye reaction products with exposure to corresponding hair dyes

On the basis of the results of *in vitro* percutaneous penetration studies, ranges of exposure to hair dye reaction products and precursors/couplers are 0.05 - 9.04 µg/day and 2.2 - 647.5 µg/day, respectively. The precursors/couplers covered in this analysis include the ingredients most widely used in the market place (based on tonnage).
In order to directly compare systemic exposure doses of RP with those of their corresponding hair dye precursors/couplers, the ratios between systemic exposure doses (SED) of precursors/couplers (Table 12) and SED of corresponding reaction product (Table 11) were calculated and are reported in Table 13. The mean exposure ratio was 706, with a median value of 170 and individual ratios ranging from 5 to 4649.

These systemic exposure ratios demonstrate that human systemic exposure to hair dye reaction products frequently is much lower than human systemic exposure to precursors/couplers.

Table 13: Comparison of Systemic Exposure Dose (SED) of Hair Dye Reaction Products (RP) with SED of corresponding precursors/couplers

Reaction Product (A-B or A-B-A)	SED RP (µg/day)[1]	SED A (µg/day)	SED B (µg/day)	Ratio SED A / SED RP	Ratio SED B / SED RP
A050-A015-A050	0.05	2.2	90.5	48	*1950*
A050-A011-A050	0.06	2.2	21.5	39	*377*
A154-A027	0.06	78.7	57.2	1378	*1001*
A007-A011-A007	0.09	219.0	21.5	2358	*232*
A154-A015	0.10	78.7	90.5	816	*939*
A005-A011-A005	0.14	647.5	21.5	4649	*155*
A005-A027	0.34	647.5	57.2	1920	*170*
A007-A015-A007	0.16	219.0	90.5	1362	*563*
A154-A017	0.58	78.7	74.1	136	*128*
A007-A027	0.58	219.0	57.2	378	*99*
A007-A017-A007[2]	1.34	219.0	74.1	163	*55*
A074-A027[2]	3.64	17.8	57.2	5	*16*
A005-A015-A005[2]	6.32	647.5	90.5	102	*14*
A016-A027[2]	*9.04*	- [3]	*57.2*	- [3]	*6*

[1] Skin penetration rate (µg/cm^2) * 580 cm^2 (scalp surface area) / 28 days (use frequency)
[2] Reaction Products selected for genetic toxicity testing
[3] No adequate *in vitro* skin penetration data available on A016

The four reaction products with the highest absolute and relative human exposures (A016-A027, A074-A027, A007-A017-A007, A005-A015-A005) were selected for testing in genotoxicity studies.

3.5.2. Genotoxicity studies with Dimer A016-A027

Genotoxicity studies in vitro

Bacterial Reverse Mutation Test

Guideline: OECD 471 (1997)
Species/strain: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102
Replicates: triplicates in 2 individual experiments
Test substance: reaction product A16-A27
Solvent: DMSO
Batch: R0012228A 001L005
Purity: > 99 %
Concentrations: Experiment 1: 0.32, 1.6, 8, 40, 200 and 1000 µg/plate without and with S9-mix
Experiment 1a: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate with S9-mix for TA98 only
Experiment 2: 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate without and with S9-mix
Treatment: Experiment 1: direct plate incorporation with 72 h incubation without and with S9-mix
Experiment 2: direct plate incorporation with 72 h incubation without S9 mix

pre-incubation method with 60 minutes pre-incubation and 72 h incubation with S9-mix.

GLP: In compliance
Date: December 2008 - May 2009

A016-A027, dissolved in DMSO, was tested in bacterial tester strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537, in two independent experiments both in the presence and in the absence of a metabolic activation system. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Experiment 1 and experiment 2 in the absence of S9-mix were performed as a plate incorporation assay; experiment 2 in the presence of S9-mix was performed as a pre-incubation assay.
Concentrations for experiment 1 were selected on the results from a toxicity range-finder experiment. Toxicity was evaluated up to the prescribed maximum concentration of 5000 µg/plate on the basis of a thinning of the bacterial background lawn and a reduction in the number of revertant colonies. Evidence of toxicity was observed at 1000 µg/plate and above in the absence and presence of S9-mix. These data were considered to be acceptable for mutation assessment and are presented as the TA100 mutagenicity data for experiment 1. Experiment 2 treatments of all tester strains were performed in the absence and presence of S9-mix with the maximum test concentration of 5000 µg/plate.
Appropriate reference mutagens were used as positive controls and showed distinct increases in induced revertant colonies. Concurrent vehicle controls were performed as negative controls, and their mean numbers of revertant colonies all fell within acceptable ranges

Results
Following treatment of strain TA98 with A016-A027 in the presence of S9-mix, statistically significant increases in revertant numbers were observed (at the 1% level using Dunnett's test) in each experiment. These increases were relatively small in magnitude, but were reproducible on each treatment occasion and in each case demonstrated evidence of a concentration-relationship. These increases were therefore considered to be indicative of a mutagenic activity of A016-A027 in strain TA98 following metabolic activation in this assay system.
None of the other strain treatments produced a biologically relevant increase in the number of revertant colonies sufficient to be considered as indicative of any mutagenic activity of A016-A027.

Conclusion
Under the experimental conditions used A016-A027 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: Williams, 2009

In vitro gene mutation assay (_hprt_ locus)

Guideline:	OECD 476 (1997)	
Cells:	mouse lymphoma L5178Y cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	reaction product A16-A27	
Solvent:	DMSO	
Batch:	R0012228A 001L005	
Purity:	> 99%	
Concentrations:	experiment 1:	15, 30, 60, 75, 90, 105, 120 and 135 µg/ml without S9-mix 15, 30, 60, 120, 180 and 210 µg/ml with S9-mix
	experiment 2:	20, 40, 60, 75, 90, 100, 110 and 120 µg/ml without S9-mix

	20, 40, 80, 100, 120, 140, 160, 180, 210 and 250 µg/ml with S9-mix
Treatment	3 h both without and with S9 mix; expression period 7 days and a selection period of 12 days.
GLP:	In compliance
Date:	December 2008 - July 2009

A016-A027 dissolved in DMSO was assayed for mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. The assay was performed in two independent experiments using duplicate cultures each. Liver S9 fraction from Arachlor 1254-induced rats was used as exogenous metabolic activation system. In all experiments cells were treated for 3 h in the absence and presence of metabolic activation system, followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Test concentrations were based on the results of a cytotoxicity range finder experiment measuring relative survival, relative to the concurrent vehicle control cell cultures. In the presence of S9-mix, the highest concentration to provide >10% relative survival (RS) was 300 µg/ml, which gave 16% RS. In the absence of S9-mix, RS could not be calculated because no colonies were observed on the survival plates for the vehicle control. Day 0 cell count data in the absence of S9-mix showed some evidence of toxicity at 75 µg/ml and above and plate count data for treated cultures indicated that extreme toxicity (<10% survival) was observed at 150 µg/ml.
In experiment 1 concentrations ranging from 15 to 200 µg/ml were tested in the absence of S9-mix and from 15 to 400 µg/ml in the presence of S9-mix. In experiment 2, concentrations ranging from 20 to 160 µg/ml in the absence of S9-mix and from 20 to 250 µg/ml in the presence of S9-mix, were tested. The known mutagens 4-nitroquinoline 1 oxide (NQO) and benzo[a]pyrene (B[a]P) were used as positive controls without S9-mix and with S9-mix, respectively. Concurrent vehicle controls were performed as negative controls, and their mutant frequencies fell within acceptable ranges.

Results
In experiment 1 the highest concentrations analysed for viability and 6-TG resistance (giving ≥10% relative survival) were 120 µg/ml in the absence of S9-mix and 210 µg/ml in the presence of S9-mix, which yielded 13% and 10% relative survival to concurrent control values, respectively. In experiment 2, the highest concentrations analysed for viability and 6-TG resistance were 120 µg/ml in the absence of S9-mix and 250 µg/ml in the presence of S9-mix, which yielded 19% and 25% relative survival, respectively.
When tested up to toxic concentrations both in experiments 1 and 2 a biologically relevant increase in mutant frequency compared to concurrent controls was not observed neither in the absence nor in the presence of S9-mix. A statistically significant increase in mutant frequency was observed at one intermediate concentration (120 µg/ml) in the presence of S9-mix in experiment 1 but this small, isolated increase was not reproduced between experiments at similar or higher concentrations producing similar levels of cytotoxicity. In both experiments, all mutant frequency values in the absence and presence of S9 fell within the historical negative control ranges, based on the last 20 experiments performed in this laboratory. These isolated observations were therefore considered not to be biologically relevant.

Conclusion
Under the experimental conditions used, A016-A027 did not induce mutations at the *hprt* locus of L5178Y mouse lymphoma cells and consequently is not genotoxic (mutagenic) in this gene mutation test.

Ref.: Lloyd, 2009

In vitro micronucleus test in human lymphocytes

Guideline:	draft OECD 487
Cells:	human lymphocytes from 2 healthy, non-smoking female volunteers
Replicates:	duplicate cultures in a single experiment
Test substance:	reaction product A16-A27
Solvent:	DMSO
Batch:	R0012228A 001L005
Purity:	> 99 %
Concentrations:	3h treatment time: 80, 110, 140 and 160 µg/ml without S9-mix 40, 60 and 110 µg/ml with S9-mix 24 h treatment: 7.5, 10, 12.5, and 15 µg/ml without S9-mix
Treatment	48 h PHA followed by 3 + 21 h treatment with and without S9-mix 48 h PHA followed by 21 h treatment without S9-mix
GLP:	In compliance
Date:	December 2008 - June 2009

A016-A027 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Blood from two healthy, non-smoking female volunteers was used for each experiment in this study. The mitogen phytohaemagglutinin (PHA) was included in the culture medium in order to stimulate the lymphocytes to divide, and blood cultures were incubated at 37° C for 48 h and rocked continuously.
The concentration selection for the micronucleus test was based on the cytotoxicity data from a range-finder experiment measuring replication index (RI). The top dose for micronucleus analysis was to be the one at which at least approximately 50% (typically 45-55%) reduction in RI occurred or the highest dose tested. Cells were treated either for 3 h with 21 h recovery both in the absence and presence of S9-mix at concentration ranging from 20 – 200 µg/ml or for 24 h in the absence of S9-mix only at concentration ranging from 2.5 – 30 µg/ml. Cytochalasin B was added directly to all 24 h cultures at the time of treatment and for the 3 h cultures after removal of the test chemical during the recovery period of 21 h.
Concurrent negative (vehicle) controls were employed. Mitomycin C (MMC) and vinblastine (VIN) were employed in the absence of rat liver S9-mix as clastogenic and aneugenic positive control chemicals, respectively. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S9-mix.

Results
In the absence of S9-mix treatment with A016-A027 for 3 h resulted in a statistically significant ($p \leq 0.01$) increase in the percentage binuclear cells with micronuclei compared to those observed in concurrent vehicle controls for all concentrations analysed. However, these increases were not concentration related and not exceeding the historical vehicle control range. As such, these increases were considered to be of no biological significance.
In the presence of S9-mix treatment with A016-A027 for 3 h resulted in a statistically significant increase at the highest dose only (110.0 µg/ml). Again a dose response relationship was not observed and the positive value did not exceed the historical control value. As such it was considered that this isolated increase was spurious and of no biological significance. After treatment with A016-A027 for 24 h frequencies of micronucleated binucleate (MNBN) cells were found, which were similar to and not significantly higher than those observed in concurrent vehicle controls.

Conclusion
Under the experimental conditions used A016-A027 did not induce an increase in cells with micronuclei and, consequently is not genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.: Whitwell, 2009

Genotoxicity studies *in vivo*

***In vivo* unscheduled DNA synthesis (UDS) test**

Guideline:	OECD 486
Species/strain:	male Wistar rats
Group size:	4 rats per dose
Test substance:	R0012228A
Batch no:	R0012228A 001L005
Purity:	99 %
Dose level:	0, 1000 and 2000 mg/kg bw
Route:	oral gavage
Vehicle:	30% DMSO/70% PEG 400
Sacrifice times:	4 h and 16 h after dosing
GLP:	in compliance
Date:	January 2009 - August 2009

The test item A016-A027 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. The test item was formulated in 30% DMSO / 70% PEG 400. Test concentrations were based on the results of a pre-experiment on acute toxicity. Rats were treated orally and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, and 24 h after start of treatment. The highest dose level of 2000 mg/kg bw (the maximum dose level recommended by guidelines) was estimated to be suitable. In the main experiment mice were exposed orally to 0, 1000 and 2000 mg/kg bw. The volume administered orally was 10 ml/kg. The rats of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2, 4 (4 h sacrifice only), and 16 h (16 h sacrifice only).
Hepatocytes for UDS analysis were collected approximately 4 h and 16 h after administration of A016-A027. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat from at least 3 treated rats.
Appropriate reference mutagens N,N'-dimethylhydrazine dihydrochloride (80 mg/kg bw) and 2-acetylaminofluorene (100 mg/kg bw) administered orally were used as positive controls, whilst negative control animals received the vehicle.

Results
In the pre-experiment on acute toxicity with exposure up to 2000 mg/kg bw, reduction of spontaneous activity, ruffled fur and orange urine was found up to 24 h after administration. In the main experiment identical toxic sign were found at all doses tested.
The coloured urine and the clinical symptoms confirmed bioavailability of A016-A027 after oral administration. The viability of the hepatocytes was not substantially affected by the *in vivo* treatment with the test item.
Neither a biological increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 4 h and the 16 h treatment time.
The positive control substances revealed distinct increases in the number of nuclear and net grain counts, thus showing the validity of the test system used.

Conclusion

Under the experimental conditions reported A016-A027 did not induce DNA-damage leading to unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: Reichenbach, 2009

Bone marrow micronucleus test in rat

Guideline:	OECD 474
Species/strain:	Wistar rat
Group size:	6 rats/sex/group
Test substance:	R0012228A
Batch no:	R0012228A 001L005
Purity:	> 99 %
Dose level:	0, 500, 1000 and 2000 mg/kg bw
Route:	orally
Vehicle:	30% DMSO/&0% PEG 400
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance
Date:	January 2009 - August 2009

The test material A016-A027, formulated in an appropriate vehicle (30% DMSO + 70% PEG 400), was investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on the results of a pre-experiment on acute toxicity. Rats were treated orally and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, 24, 30 and 48 h after start of treatment. The highest dose level of 2000 mg/kg bw (the maximum dose level recommended by guidelines) was estimated to be suitable. In the main experiment mice were exposed orally to 0, 500, 1000 and 2000 mg/kg bw. The volume administered orally was 10 ml/kg. The rats of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2-4, 6, 24 and 48 h after treatment. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE).
As a positive control, 20 mg/kg bw cyclophosphamide was administered orally, whilst negative control animals received the vehicle.

Results
Treatment with cyclophosphamide produced a substantial increase in erythrocytes with micronuclei, thus showing the validity of the test system used.
In the a pre-experiment on acute toxicity with exposure up to 2000 mg/kg bw, reduction of spontaneous activity, ruffled fur and orange urine was found up to 24 h after administration. In the main experiment identical toxic signs were found at all doses tested.
After treatment with the test item the ratio PCE/NCE was not substantially decreased as compared to the mean ratio of the vehicle control, thus indicating that A016-A027 did not exert any cytotoxic effects in the bone marrow. However, the orange colored urine of all treated animals and the clinical signs observed after treatment indicated the systemic distribution of A016-A027 and thus its bioavailability.
Biologically relevant or statistically significant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment and neither for males nor for females.

Conclusion
Under the experimental conditions used A016-A027 did not induce a biologically relevant increase in the number of PCEs with micronuclei of treated rats and, consequently, A016-A027 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref.: Honarvar, 2009a

3.5.3. Genotoxicity studies with Dimer A074-A027

Genotoxicity studies *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102
Replicates:	triplicates in 2 individual experiments
Test substance:	A74-A27 reaction product dimer
Solvent:	DMSO
Batch nr.:	SO-1100.027
Purity:	96 %
Concentrations:	Experiment I: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
	Experiment II: 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	Experiment I: direct plate incorporation with 72 h incubation without and with S9-mix
	Experiment II: pre-incubation method with 60 minutes pre-incubation and 48 h incubation with S9-mix.
GLP:	In compliance
Date:	September 2006 – January 2007

Dimer A074-A027, dissolved in DMSO, was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) tester strains TA98, TA100, TA102, TA1535 and TA1537. The assay was performed in two independent experiments both in the presence and in the absence of a metabolizing system. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Each concentration and the controls were tested in triplicate. Experiment I was performed as plate incorporation assay, experiment II as pre-incubation assay. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as experiment I.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies. Concurrent untreated and solvent controls were performed as negative controls.

Results
Toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation at concentrations of 2500 – 5000 µg/plate depending on the tester strain. Occasionally precipitation was observed at 5000 µg/plate.
In both experiments A074-A027 treatment did not result in a biologically relevant increase in revertant colonies in any of the five tester strains neither in the absence nor in the presence of S9-mix.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies showing the validity of the test system used.

Conclusion
Under the experimental conditions used A074-A027 was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: Sokolowski, 2007

In vitro* gene mutation assay (*hprt* locus*)

Guideline:	OECD 476 (1997)	
Cells:	mouse lymphoma L5178Y cells	
Replicates:	duplicate cultures in 3 independent experiments	
Test substance:	A74-A27	
Solvent:	DMSO	
Batch:	SOL20332-1	
Purity:	99.4 %	
Concentrations:	experiment I:	4.4; 8.8; 17.5; 35.0; and 52.5 µg/ml with and without S9-mix
	experiment II:	1.3; 2.5; 5.0; 10.0; and 15.0 µg/ml without S9-mix 0.5; 1.0; 2.0; 3.0; and 4.0 µg/ml with S9-mix
	experiment III:	0.625; 1.25; 2.5; 5.0; 10.0; and 15.0 µg/ml with S9-mix
Treatment	experiment I:	4 h treatment with and without S9-mix, expression period 5 days and a selection period of 10-15 days.
	experiment II:	24 h treatment µg/ml without S9-mix, expression period 5 days and a selection period of 10-15 days. 4 h treatment with S9-mix, expression period 5 days and a selection period of 10-15 days.
	experiment III:	4 h treatment with S9-mix, expression period 5 days and a selection period of 10-15 days.
GLP:	In compliance	
Date:	March 2009 - September 2009	

A74-A27, dissolved in DMSO, was assayed for mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. The assay was performed in three independent experiments using duplicate cultures each. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. The cells were exposed to the test item for 4 hours in the first experiment with and without metabolic activation and in the second experiment for 4 hours with and for 24 hours without metabolic activation. Since the maximum concentration of the second experiment with metabolic activation was not analysed due to technical error, this experimental part was repeated. Experiment III was performed with a treatment time of 4 hours with metabolic activation. Treatment was followed by an expression period of 5 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures
Concurrent negative and solvent controls were employed. The known mutagens ethylmethane sulfonate (EMS) and 7,12-dimethylbenz(a)anthracene (DMBA) were used as positive controls without S9-mix and with S9-mix, respectively.

Results
No precipitation of the test item was observed in experiments I, II, and III. The recommended toxic range of approximately 10-20 % survival compared to the concurrent negative controls was only occasionally reached.
When tested up to toxic concentrations both in experiments I, II and III a biologically relevant increase in mutant frequency compared to concurrent controls was not observed neither in the absence nor in the presence of S9-mix. The induction factor of three times the corresponding solvent control was exceeded at 35.0 µg/ml in the first experiment without metabolic activation (culture I), and at 17.5 µg/ml with metabolic activation (culture II). In the second experiment the induction factor was exceeded at 2.0 µg/ml in the presence of metabolic activation in culture I. However, these increases were not reproduced in the parallel cultures and were therefore considered as biologically

irrelevant. Furthermore, the above described isolated increases showed no dose-dependency and could not be reproduced in experiment III where no relevant increases in mutant frequency were observed.

Conclusion
Under the experimental conditions used, A74-A27 did not induce mutations at the *hprt* locus of L5178Y mouse lymphoma cells and consequently is not genotoxic (mutagenic) in the gene mutation test.

Ref.: Wollny, 2009a

In vitro micronucleus test in V79 cells

Guideline:	draft OECD 487
Cells:	Chinese hamster V79 cells
Replicates:	duplicate cultures in a single experiment
Test substance:	A74-A27 reaction product dimer
Solvent:	DMSO
Batch:	SO-1100.027
Purity:	96 %
Concentrations:	15.2, 30.5 and 60.9 µg/ml without S9-mix 3.8, 7.6, 15.2, 30.5 and 60.9 µg/ml with S9-mix
Treatment	4 h treatment with and without S9-mix; harvest time 24 hours after the beginning of treatment
GLP:	In compliance
Date:	October 2006 – February 2007

A74-A27, dissolved in DMSO, was investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. The study was conducted in the absence of cytochalasin B.
The dose selection of the micronucleus test was based on the toxicity data and the occurrence of test item precipitation. The pre-experiment for toxicity was performed following the current OECD Guideline no. 473 for chromosomal aberration studies. With respect to the solubility A74-A27 the concentration of 1950 µg/ml (approx. 8 mM) was applied as top concentration for treatment of the cultures. The treatment period in the main test was 4 h without and with S9-mix. Harvest time was 24 hours after the beginning of culture.
Concurrent negative and solvent controls were employed. Colcemid was used as positive control without metabolic activation and cyclophosphamide was used in presence of the metabolizing system.

Results
Precipitation of the test item in culture medium was observed at 243.8 µg/ml and above in the absence of S9-mix and at 121.9 µg/ml and above in the presence of S9-mix. No relevant increase in the osmolarity or change in pH value was observed.
Both in the absence and in the presence of S9-mix, biologically relevant, dose dependent and statistically significant increases in the percentage of V79 cells with micronuclei were observed.

Conclusion
Under the experimental conditions used, A074-A027 did induce an increase in V79 cells with micronuclei in vitro both in the presence and absence of metabolic activation and, consequently is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.: Höpker, 2007

Genotoxicity studies *in vivo*

Bone marrow micronucleus test in mouse

Guideline:	OECD 474
Species/strain:	NMRI mouse
Group size:	5 mice/sex/group
Test substance:	A74-A27
Batch no:	SOL20332-1
Purity:	99.4 %
Dose level:	0, 500, 1000 and 2000 mg/kg bw
Route:	orally
Vehicle:	30% DMSO/70% PEG 400
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance
Date:	January 2009 - April 2009

The test material A74-A27, formulated in an appropriate vehicle (30% DMSO + 70% PEG 400), was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the results of a pre-experiment on acute toxicity. Mice were treated orally and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, 24, 30 and 48 h after start of treatment. The highest dose level of 2000 mg/kg bw (the maximum dose level recommended by guidelines) was estimated to be suitable. In the main experiment mice were exposed orally to 0, 500, 1000 and 2000 mg/kg bw. The volume administered orally was 10 ml/kg. The mice of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2-4, 6, 24 and 48 h after treatment. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE).
As a positive control, 40 mg/kg bw cyclophosphamid was administered orally, whilst negative control animals received the vehicle (30% DMSO + 70% PEG 400) per os.

Results
Treatment with cyclophosphamide produced a substantial increase in erythrocytes with micronuclei, thus showing the validity of the test system used.
In the a pre-experiment on acute toxicity with exposure up to 2000 mg/kg bw, reduction of spontaneous activity, ruffled fur and orange urine was found up to 30 h after administration in the 2000 mg/kg bw group; in the 1000 mg/kg bw group only orange coloured urine was observed. In the main experiment reduction of spontaneous activity, ruffled fur and orange urine were found in the 2000 mg/kg bw group and orange coloured urine only in the other treatment groups.
After treatment with the test item the ratio PCE/NCE was not substantially decreased as compared to the mean ratio of the vehicle control, thus indicating that A74-A27 did not exert any cytotoxic effects in the bone marrow. However, the orange coloured urine of all treated animals and the clinical signs observed after treatment indicated the systemic distribution of A74-A27 and thus its bioavailability.
Biologically relevant or statistically significant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment and neither for males nor for females.

Conclusion
Under the experimental conditions used A74-A27 did not induce a biologically relevant increase in the number of PCEs with micronuclei of treated mice and, consequently, A74-A27 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: Honarvar, 2009b

3.5.4. Genotoxicity studies with Trimer A005-A015-A005

Genotoxicity studies *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535a and TA1537
Replicates:	triplicates in a single experiment
Test substance:	A005-A015-A005
Solvent:	DMSO
Batch:	PG59-120-1
Purity:	> 97.1 % (area% at 254 nm)
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with 72 h incubation without and with S9-mix
GLP:	In compliance
Date:	January 2009 - October 2009

Trimer A005-A015-A005, dissolved in DMSO, was tested in bacterial tester strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537 in a single experiment both in the presence and in the absence of a metabolic activation system. Each concentration and the controls were tested in triplicate. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
A pre-experiment for toxicity was performed with all strains both without and with S9-mix to select the concentrations for the main experiment. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. The pre-experiment was performed as a plate incorporation assay. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as the main experiment. Since a positive response was obtained a second experiment was not performed.
Appropriate reference mutagens were used as positive controls and showed distinct increases in induced revertant colonies. Concurrent vehicle controls were performed as negative controls, and their mean numbers of revertant colonies all fell within acceptable ranges.

Results
The plates incubated with A005-A015-A005 showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used. No toxic effects, evident as a reduction in the number of revertants, occurred in any strain up to the highest concentration except TA102 with S9-mix where a reduction in the number of revertants was observed at 1000 µg/plate and above.
A dose dependent increase in revertant colony numbers was observed following treatment with A005-A015-A005 in strain TA98 in the presence of S9-mix. A biologically relevant increase in revertant colonies was not observed in strain TA98 in the absence of S9-mix nor in any of the other strains used neither in the absence nor in the presence of S9-mix.

Conclusion
Under the experimental conditions used A005-A015-A005 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: Sokolowski, 2009a

In vitro gene mutation assay (*hprt* locus)

Guideline:	OECD 476 (1997)
Cells:	Chinese hamster V79 cells
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	A005-A015-A005
Solvent:	DMSO
Batch:	PG59-132-2
Purity:	97.1 % (HPLC, area at 254 nm)
Concentrations:	experiment I: 0.75, 1.5, 3, 6 and 12 µg/ml without S9-mix 1.5, 3, 6, 12 and 24 µg/ml with S9-mix experiment II: 3, 6, 9, 12 and 15 µg/ml without S9-mix 9, 12, 15, 18 and 21 µg/ml with S9-mix
Treatment	4 h treatment with and without S9-mix, expression period 7 days and a selection period of 8 days.
GLP:	In compliance
Date:	January 2009 - October 2009

A005-A015-A005, dissolved in DMSO, was assayed for mutations at the *hprt* locus of Chinese hamster V79 cells both in the absence and presence of metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. The study was performed in two independent experiments, using duplicate cultures and identical experimental procedures.
Test concentrations were based on the results of a pre-test on toxicity. In this pre-test the colony forming ability of approximately 500 single cells after treatment with A005-A015-A005 was observed and compared with controls. Toxicity of A005-A015-A005 was indicated by a reduction of the cloning efficiency. In the pre-test and the main experiment, the cells were exposed to A005-A015-A005 for 4 hours with and without metabolic activation. Treatment was followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as relative cloning efficiency of the treated cultures relative to the relative cloning efficiency of the solvent control cultures.
Concurrent negative and solvent controls were employed. The known mutagens ethylmethane sulfonate (EMS) and 7,12-dimethylbenz(a)anthracene (DMBA) were used as positive controls without and with S9-mix, respectively.

Results
The recommended toxic range of approximately 10-20 % survival compared to the concurrent negative controls was not reached. The highest applied concentration was limited by the solubility properties of A005-A015-A005 in DMSO.
In both experiments a biologically relevant increase in mutant colony numbers was not observed up to the maximum concentrations tested which were limited by toxicity. EMS (0.15 mg/ml) and DMBA (1.1 µg/ml) were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion
Under the experimental conditions used, A005-A015-A005 did not induce mutations at the hprt locus of V79 cells and consequently is not genotoxic (mutagenic) in the gene mutation test.

Ref.: Wollny, 2009b

In vitro micronucleus test in human lymphocytes

Guideline:	draft OECD 487
Cells:	human peripheral blood lymphocytes from a healthy, non-smoking 25 year old male volunteers

Replicates:	duplicate cultures in a single experiment	
Test substance:	A005-A015-A005 (WR802376)	
Solvent:	DMSO	
Batch:	PG59-120-1	
Purity:	97.7 %	
Concentrations:	4 h treatment	without S9-mix: 5, 10 and 35 µg/ml
		with S9-mix: 1, 7.5 and 20 µg/ml
	24 h treatment	without S9-mix: 1, 15 and 25 µg/ml
Treatment	48 h PHA followed by 4 + 20 h treatment with and without S9-mix	
	48 h PHA followed by 24 h treatment without S9-mix	
	harvest time 24 hours after the beginning of treatment	
GLP:	In compliance	
Date:	December 2008 - July 2009	

A005-A015-A005, dissolved in DMSO, has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in human erythrocytes. Blood from a healthy, 25 year old non-smoking male volunteer was used in this study. The mitogen phytohaemagglutinin (PHA) was included in the culture medium for 48 h in order to stimulate the lymphocytes to divide. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
A solubility test was conducted to determine the maximum soluble concentration or workable suspension using water. Since a workable stock concentration of 500 mg/ml was achieved in DMSO, DMSO was selected as the solvent. The dose selection for the micronucleus test was based on cell proliferation and cytotoxicity evaluated as an effect of A005-A015-A005 on the cytokinesis blocked proliferation index or replication index tested up to the maximum prescribed dose of 10 mM (= 3470 µg/ml).
The treatment period in the main test was either 4 h without and with S9-mix or 24 h continuously without S9-mix. Harvest time was 24 h after the beginning of treatment. Cytochalasin B (final concentration 6 µg/ml) was added during the recovery period in the cultures treated for 4 h and present continuously during treatment in the cultures treated for 24 h.
Concurrent negative and solvent controls were employed. Mitomycine C, vinblastin and cyclophosphamide were used as positive controls.

Results
The doses for the micronucleus assay were chosen in a preliminary cytotoxicity assay. In this assay substantial toxicity (≥ 55±5% cytotoxicity relative to the solvent control) was observed at dose levels ≥ 104.1 µg/ml in the non-activated 4-hour exposure group, at dose levels ≥ 1.041 µg/ml in the S9-activated 4 h exposure group, and at dose levels ≥ 34.7 µg/ml in the non-activated 24 h exposure group.
The results for the positive and negative controls indicate that all criteria for a valid assay were met.
Under the 3 treatment conditions both the results found for the cytokinesis blocked proliferation index as the % cytotoxicity indicated to exposure of the human peripheral blood lymphocytes.
In all 3 treatment conditions, both in the absence and in the presence of S9-mix, biologically relevant, dose dependent and statistically significant increases in the percentage of human peripheral blood lymphocytes with micronuclei were not observed.

Conclusion
Under the experimental conditions used, A005-A015-A005 did not induce an increase in human peripheral blood lymphocytes with micronuclei *in vitro* both in the presence and absence of metabolic activation and, consequently A005-A015-A005 is not genotoxic (clastogenic and/or aneugenic) in human peripheral blood lymphocytes.

Ref.: Gudi, 2009a

Genotoxicity studies *in vivo*

***In vivo* unscheduled DNA synthesis (UDS) test**

Guideline:	OECD 486
Species/strain:	male Wistar HsdCpb:WU rats
Group size:	4 rats per dose
Test substance:	A005-A015-A005
Batch no:	PG59-120-1
Purity:	97.1 % (HPLC, area at 254 nm)
Dose level:	0, 375 and 750 mg/kg bw
Route:	oral gavage
Vehicle:	30% DMSO/70% PEG 400
Sacrifice times:	4 h and 16 h after dosing
GLP:	in compliance
Date:	December 2008 - October 2009

The test item A005-A015-A005 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. The test item was formulated in 30% DMSO / 70% PEG 400. Test concentrations were based on the results of a pre-experiment on acute toxicity. Rats were treated orally up to 1000 mg/kg bw and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, and 24 h after start of treatment. In the main experiment mice were exposed orally to 0, 375 and 750 mg/kg bw. The volume administered orally was 10 ml/kg. The rats of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2, 4 (4 h sacrifice only), and 16 h (16 h sacrifice only).
Hepatocytes for UDS analysis were collected approximately 4 h and 16 h after administration of A005-A015-A005. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat from at least 3 treated rats.
Appropriate reference mutagens N,N'-dimethylhydrazine dihydrochloride (80 mg/kg) and 2-acetylaminofluorene (100 mg/kg) administered orally were used as positive controls, whilst negative control animals received the vehicle.

Results
The viability of the hepatocytes was not substantially affected by the treatment with A005-A015-A005 at any of the treatment periods or dose groups. The inter-individual variations obtained for the yield and the viability of the isolated hepatocytes were in the range of the historical laboratory control.
In the pre-experiment on acute toxicity with exposure up to 1000 mg/kg bw A005-A015-A005, reduction of spontaneous activity and ruffled fur was seen at every dose tested up to 24 h after exposure. Additionally, at the highest dose tested 2 rats died and occasionally apathy was observed. Consequently, the maximum tolerated dose level of 750 mg/kg bw was chosen to be suitable as top dose for the main experiment. In the main experiment ruffled fur and brown coloured urine was reported after A005-A015-A005 treatment. The brown coloured urine of rats treated with A005-A015-A005 confirms its bioavailability and systemic distribution.
Neither a biological increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 4 h and the 16 h treatment time.

The positive control substances (DMH, 80 mg/kg bw and 2-AAF, 100 mg/kg bw) revealed distinct increases in the number of nuclear and net grain counts, thus showing the validity of the test system used.

Conclusion
Under the experimental conditions reported A005-A015-A005 did not induce DNA-damage leading to unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: Honarvar, 2009c

3.5.5. Genotoxicity studies with Trimer A007-A017-A007

Genotoxicity Studies *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102
Replicates:	triplicates in a single experiment
Test substance:	A007-A017-A007
Solvent:	DMSO
Batch nr.:	GF814775
Purity:	98 % (HPLC, area at 254 nm)
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with 72 h incubation without and with S9-mix
GLP:	In compliance
Date:	January 2009 - October 2009

A007-A017-A007, dissolved in DMSO, was tested in bacterial tester strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537, in a single experiment both in the presence and in the absence of a metabolic activation system. Each concentration and the controls were tested in triplicate. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
A pre-experiment for toxicity was performed with all strains both without and with S9-mix to select the concentrations for the main experiment. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. The pre-experiment was performed as a plate incorporation assay. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as the main experiment. Since a positive response was obtained in this experiment a second experiment was not performed.
Appropriate reference mutagens were used as positive controls and showed distinct increases in induced revertant colonies. Concurrent vehicle controls were performed as negative controls, and their mean numbers of revertant colonies all fell within acceptable ranges.

Results
The plates incubated with A007-A017-A007 showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used. Minor toxic effects, evident as a reduction in the number of revertants, occurred in the absence of metabolic activation in strain TA1535 at 2500 and 5000 µg/plate and in strain TA1537 at 5000 µg/plate.
A dose dependent increase in revertant colony numbers was observed following treatment with A007-A017-A007 in strains TA98 and TA1537 in the presence of S9-mix. A biologically relevant increase in revertant colonies was not observed in strain TA98 and

TA1537 in the absence of S9-mix nor in any of the other strains used neither in the absence nor in the presence of S9-mix.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Conclusion
Under the experimental conditions used A007-A017-A007 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: Sokolowski, 2009b

In vitro gene mutation assay (_hprt_ locus)

Guideline:	OECD 476 (1997)
Cells:	Chinese hamster V79 cells
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	A007-A017-A007 (WR A021232)
Solvent:	DMSO
Batch nr.:	GF814775
Purity:	98 % (HPLC, area at 254 nm)
Concentrations:	experiment I: 0.125, 0.25, 0.5, 1, 2 and 2.5 µg/ml without S9-mix 1.25, 2.5, 5, 10, 15 and 20 µg/ml with S9-mix experiment II: 1, 1.5, 1.75, 2, 2.3 and 2.5 µg/ml without S9-mix 5, 10, 12.5, 15, 17.5 and 20 µg/ml with S9-mix
Treatment	4 h treatment with and without S9-mix, expression period 7 days and a selection period of 8 days.
GLP:	In compliance
Date:	December 2008 - October 2009

A007-A017-A007, dissolved in DMSO, was assayed for mutations at the *hprt* locus of Chinese hamster V79 cells both in the absence and presence of metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. The study was performed in two independent experiments, using duplicate cultures and identical experimental procedures.
Test concentrations were based on the results of a pre-test on toxicity. Toxicity of A007-A017-A007 is indicated by a reduction of the cloning efficiency. In the pre-test and the main experiment, the cells were exposed to A007-A017-A007 for 4 h with and without metabolic activation. Treatment was followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as relative cloning efficiency of the treated cultures relative to the relative cloning efficiency of the solvent control cultures.
Concurrent negative and solvent controls were employed. The known mutagens ethylmethane sulfonate (EMS) and 7,12-dimethylbenz(a)anthracene (DMBA) were used as positive controls without and with S9-mix, respectively.

Results
In most cases the recommended toxic range of approximately 10-20 % survival compared to the concurrent negative controls was reached.
In both experiments a biologically relevant increase in mutant colony numbers was not observed up to the maximum concentrations tested which both without and with S9-mix. The mutant frequency generally remained well within the historical range of solvent controls, except at the maximum analyzable concentration of the first culture of the first experiment with metabolic activation. This isolated increase was not considered a relevant effect since it was neither reproduced in the parallel culture of the same experiment nor in the second experiment with metabolic activation, at comparable levels of cytotoxicity.
EMS (0.15 mg/ml) and DMBA (1.1 µg/ml) were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion
Under the experimental conditions used, A007-A017-A007 did not induce mutations at the *hprt* locus of V79 cells and consequently is not genotoxic (mutagenic) in the gene mutation test.

Ref.: Wollny, 2009c

In vitro micronucleus test in human lymphocytes

Guideline:	draft OECD 487	
Cells:	human peripheral blood lymphocytes from a healthy, non-smoking 25 year old male volunteers	
Replicates:	duplicate cultures in a single experiment	
Test substance:	A007-A017-A007 (GTS25169)	
Solvent:	DMSO	
Batch nr.:	GF814775	
Purity:	97.4 % (HPLC, area at 254 nm)	
Concentrations:	4 h treatment	without S9-mix: 1.25, 2.5 and 5 µg/ml with S9-mix: 2.5, 5 and 10 µg/ml
	24 h treatment	without S9-mix: 1.25, 2.5 and 5 µg/ml
Treatment	48 h PHA followed by 4 + 20 h treatment with and without S9-mix 48 h PHA followed by 24 h treatment without S9-mix harvest time 24 h after the beginning of treatment	
GLP:	In compliance	
Date:	December 2008 - July 2009	

A007-A017-A007, dissolved in DMSO, was investigated in the absence and presence of metabolic activation for the induction of micronuclei in human erythrocytes. Blood from a healthy, 25 year old non-smoking male volunteer was used in this study. The mitogen phytohaemagglutinin (PHA) was included in the culture medium for 48 h in order to stimulate the lymphocytes to divide. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
A solubility test was conducted to determine the maximum soluble concentration or workable suspension using water. Since a workable stock concentration of 500 mg/ml was achieved in DMSO, DMSO was selected as the solvent. The dose selection for the micronucleus test was based on cell proliferation and cytotoxicity evaluated as an effect of A007-A017-A007 on the cytokinesis blocked proliferation index or replication index tested up to the maximum prescribed dose of 10 mM (= 3540 µg/ml).
The treatment period in the main test was either 4 h without and with S9-mix or 24 h continuously without S9-mix. Harvest time was 24 h after the beginning of treatment. Cytochalasin B (final concentration 6 µg/ml) was added during the recovery period in the cultures treated for 4 h and present continuously during treatment in the cultures treated for 24 h. Concurrent negative and solvent controls were employed. Mitomycine C, vinblastin and cyclophosphamide were used as positive controls.

Results
The doses for the micronucleus assay were chosen in a preliminary cytotoxicity assay. In this assay substantial toxicity (≥ 55±5% cytotoxicity relative to the solvent control) was observed at dose levels ≥ 10.62 µg/ml in the non-activated 4 and 24 hour exposure groups and at dose levels ≥ 35.4 µg/ml in the S9-activated 4 h exposure group.
The results for the positive and negative controls indicate that all criteria for a valid assay were met. Under the 3 treatment conditions both the results found for the cytokinesis blocked proliferation index as the % cytotoxicity indicated to exposure of the human peripheral blood lymphocytes.
In all 3 treatment conditions, both in the absence and in the presence of S9-mix, biologically relevant, dose dependent and statistically significant increases in the percentage of human peripheral blood lymphocytes with micronuclei were not observed.

Conclusion
Under the experimental conditions used, A007-A017-A007 did not induce an increase in human peripheral blood lymphocytes with micronuclei in vitro both in the presence and absence of metabolic activation. and, consequently A007-A017-A007 is not genotoxic (clastogenic and/or aneugenic) in human peripheral blood lymphocytes.

Ref.: Gudi, 2009b

Genotoxicity studies *in vivo*

***In vivo* unscheduled DNA synthesis (UDS) test**

Guideline: OECD 486
Species/strain: male Wistar HsdCpb:WU rats
Group size: 4 rats per dose
Test substance: A007-A017-A007 (WR A021232)
Batch: GF814775
Purity: 98 % (HPLC, area at 254 nm)
Dose level: 0, 500 and 1000 mg/kg bw
Route: oral gavage
Vehicle: 30% DMSO/70% PEG 400
Sacrifice times: 4 h and 16 h after dosing
GLP: in compliance
Date: February 2009 – January 2010

The test item A007-A017-A007 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. A007-A017-A007 was formulated in 30% DMSO / 70% PEG 400. Test concentrations were based on the results of a pre-experiment on acute toxicity. Rats were treated orally up to 2000 mg/kg bw and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, and 24 h after start of treatment. In the main experiment mice were exposed orally to 0, 500 and 1000 mg/kg bw. The volume administered orally was 10 ml/kg. The rats of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2, 4 (4 h sacrifice only), and 16 h (16 h sacrifice only).
Hepatocytes for UDS analysis were collected approximately 4 h and 16 h after administration of A007-A017-A007. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat from at least 3 treated rats.
Appropriate reference mutagens N,N´-dimethylhydrazine dihydrochloride (80 mg/kg) and 2-acetylaminofluorene (100 mg/kg) administered orally were used as positive controls, whilst negative control animals received the vehicle.

Results
The viability of the hepatocytes was not substantially affected by the treatment with A007-A017-A007 at any of the treatment periods or dose groups. The inter-individual variations obtained for the yield and the viability of the isolated hepatocytes were in the range of the historical laboratory control.
In the pre-experiment on acute toxicity with exposure up to 2000 mg/kg bw A007-A017-A007, reduction of spontaneous activity, ruffled fur and orange to dark red stained urine was seen at every dose tested up to 24 h after exposure. Additionally, at the highest doses tested 3 rats in the 1500 and 3 rats in the 2000 mg/kg bw dose group died. Occasionally apathy, abdominal position and eyelid closure was observed in these groups.

Consequently, the maximum tolerated dose level of 1000 mg/kg bw was chosen to be suitable as top dose for the main experiment. In the main experiment reduction of spontaneous activity, ruffled fur and coloured urine was reported after A007-A017-A007 exposure. One rat died in the 1000 mg/kg bw dose group 16 h after treatment. The coloured urine of rats treated with A007-A017-A007 confirms its bioavailability and systemic distribution.
Neither a biological increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 4 h and the 16 h treatment time.
The positive control substances (DMH, 80 mg/kg bw and 2-AAF, 100 mg/kg bw) revealed distinct increases in the number of nuclear and net grain counts, thus showing the validity of the test system used.

Conclusion
Under the experimental conditions reported A007-A017-A007 did not induce DNA-damage leading to unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: Honarvar, 2009d

3.5.6. Summary and Discussion on genotoxicity testing of reaction products

Four representative reaction products have been selected for genotoxicity studies on the basis of exposure and structural alerts (A016-A027, A074-A027, A005-A015-A005, A007-A017-A007). These four compounds were identified having the highest absolute and relative human exposure and considered to address all structural alerts (aromatic amine, secondary amine, benzoquinone imine moiety). Testing included both dimers (A16-A027, A074-A027) and trimers (A007-A017-A007 and A005-A015-A005).

The studies were performed according to Good Laboratory Practice (GLP), following the respective OECD guidelines and covering the relevant endpoints of genotoxicity test batteries, i.e. gene mutations, clastogenicity and aneugenic potential as described in the study reports. An overview of the test results is given in Table 14.

Table 14: Overview of genotoxicity test results of the four reaction products tested

Reaction Product	*In vitro* Tests			*In vivo* Tests	
	Ames	hprt	MN	MN	UDS
A016-A027	+	-	-	-	-
A074-A027	-	-	+	-	Not performed
A005-A015-A005	+	-	-	Not performed	-
A007-A017-A007	+	-	-	Not performed	-

Ames: Bacterial Reverse Mutation Test
hprt: mammalian cell gene mutation assay (hprt locus)
MN: Micronucleus test
UDS: In vivo unscheduled DNA synthesis

A016-A027
A016-A027 did induce gene mutations in bacteria. In a gene mutation test in mammalian cells (*hprt* locus) treatment with A016-A027 did not result in an increased mutant frequency.
In the *in vitro* micronucleus test increases in the percentages of cells with micronuclei were occasionally observed. However, these increases were always within the historical control values. The unscheduled DNA synthesis test to confirm the results found in the gene mutation assay in bacteria was negative as was the *in vivo* micronucleus.
Consequently, A016-A027 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

A074-A027
A74-A27 did not induce gene mutations both in bacteria and mammalian cells (*hprt* locus). In the *in vitro* micronucleus test treatment with A74-A27 resulted in an increase in the percentage of V79 cells with micronuclei. The *in vivo* micronucleus was negative. Consequently, A74-A27 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

A005-A015-A005
A005-A015-A005 was positive in a gene mutation test in bacteria inducing mutations in *Salmonella typhimurium* strain TA98. In a gene mutation test in mammalian cells (*hprt* locus) treatment with A005-A015-A005 did not result in an increased mutant frequency.
A005-A015-A005 exposure did not induce an increase in human peripheral blood lymphocytes with micronuclei in *in vitro.* The unscheduled DNA synthesis test to confirm the positive result found in the gene mutation assay in bacteria was negative. Consequently, A005-A015-A005 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

A007-A017-A007
A007-A017-A007 was positive in a gene mutation test in bacteria inducing mutations in *Salmonella typhimurium* strains TA98 and TA1537. In a gene mutation test in mammalian cells (*hprt* locus) treatment with A007-A017-A007 did not result in an increased mutant frequency. A007-A017-A007 exposure did not induce an increase in human peripheral blood lymphocytes with micronuclei *in vitro.* The unscheduled DNA synthesis test to confirm the positive result found in the gene mutation assay in bacteria was negative.
Consequently, A007-A017-A007 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Discussion on the outcome of genotoxicity tests
Three of the four tested reaction products were positive in some *Salmonella* strains of the Ames test. None of these positive findings could be confirmed in the *hprt* locus gene mutation assay performed with mammalian cells, or in the *in vivo* UDS test performed in rats. In these UDS assays the systemic bioavailability of the dyes was confirmed by the observed systemic toxic effects and/or the occurrence of discoloured urine.

One reaction product induced genotoxic effects in the micronucleus test *in vitro* (performed with V79 cells). However, this finding was not confirmed in an *in vivo* bone marrow assay performed in rats which studied the same endpoint. The systemic bioavailability of the dye was again confirmed in this study by the observed systemic toxic effects and the occurrence of discoloured urine.

Table 15: Overview of genotoxicity test results performed for the four reaction products tested as compared with the respective precursors/couplers

Reaction Product	*In vitro* Tests			*In vivo* Tests	
	Ames	**tk/hprt**	**MN/CA**	**MN**	**UDS**
A016** p-aminophenol	incon-clusive	incon-clusive	+	incon-clusive	-
A027 4-amino-2-hydroxytoluene	-	+	+	-	-
A074 4-Amino-3-methylphenol	-	-	Not performed	-	-
A005 Toluene-2,5-diamine	+	-	+	-	-
A015 *m*-Aminophenol	+	-	+	-	Not performed

A007 p-Phenylenediamine	+	-	+	-	-
A017 1-Naphthol	-	+	+	-	-
A016-A027	+	-	-	-	-
A074-A027	-	-	+	-	Not performed
A005-A015-A005	+	-	-	Not performed	-
A007-A017-A007	+	-	-	Not performed	-

Ames: Bacterial Reverse Mutation Test
tk: mammalian cell gene mutation assay (*tk* locus)
hprt: mammalian cell gene mutation assay (*hprt* locus)
CA: chromosome aberration test
MN: Micronucleus test
UDS: In vivo unscheduled DNA synthesis
** Only acceptable tests from a large number of tests were incorporated. p-Aminophenol is genotoxic *in vitro* and *in vivo* inducing clastogenic effects under standard test conditions at high doses in the presence of toxic effects. The biological significance of these effects and their relevance for exposed humans was discussed. There is evidence that p-aminophenol is metabolized in the skin to acetaminophen which is clastogenic *in vitro* and *in vivo* in standard genotoxicity tests.

Table 15 is a compilation of the results of the reaction products and their respective precursors/couplers. A common result for both precursors/couplers and the reaction product is the positive outcome in one or more *in vitro* tests which was not confirmed *in vivo*. In the case of the trimer A005-A015-A005 the reaction product induced gene mutations in bacteria as did the precursor and coupler A005 and A015. On the other hand, the dimer A016-A027 induced gene mutations *in vitro* while A016 and A027 were found to be clastogenic. Similarly, the trimer A007-A017-A007 is not clastogenic while A007 and A017 are clastogenic. Therefore, it can be deduced that it is not possible to predict the specific outcome of the tests of the reaction product on the basis of the results of the respective precursors/couplers.

3.6 (Q)SAR (quantitative) structure-activity relationship

(Q)SAR is a qualitative relationship that relates a (sub)structure of a molecule to the presence or absence of a property or activity of interest. Results obtained from valid qualitative or quantitative structure-activity relationship models ((Q)SARs) may indicate the presence or absence of a certain dangerous property.

To be able to estimate the value of (Q)SAR data one has to know what the specific (Q)SAR model exactly predicts and what the results mean. Therefore, the (Q)SAR model must have (i) a defined endpoint of regulatory importance, (ii) an unambiguous algorithm giving the regulator insight in the mathematical and statistical procedure and full detail of the training set, (iii) a defined domain of applicability with for the regulator a clear definition of the descriptor and structure spaces and (iv) a mechanistic interpretation. Together with (v) measures of goodness-of-fit, robustness and predictivity, these items form the 'Setubal' principles, developed at a workshop in Setubal (Portugal) which were later adopted by the OECD (CEFIC, 2002; OECD, 2003, 2004). In fact these principles, rephrased into OECD principles, describe the criteria to which a correctly performed (Q)SAR evaluation has to meet. Many (commercial available) (Q)SAR models still have problems to settle to these principles.

For ecotoxicological endpoints, several QSARs are recommended in the Technical Guidance Documents TGD (EC, 2003). For human health effects, non-testing methods have rarely been used, and where they have been used, it is generally in the form of grouping rather than QSAR. (ECHA 2008).

In practice, the defined endpoint and insight in the applicability domain is of the highest importance. The applicability domain describes the borders of the (Q)SAR model in which a substance has to fall to get a reliably prediction. The applicability domain is determined by a specific endpoint (e.g. Ames test) and entry of data of chemicals which were tested with this test (learning set). For chemicals outside the domain the prediction of the model is of unknown reliability. In other words (Q)SAR predictions are uninterpretable if the applicability domain of the model used is not known. Yet this may be acceptable as long as so much information of the model (applicability domain) is available that the regulator is able to explain the results and knows on the basis of which data the result is positive or negative.

The applicant used the commercial software program DEREK which is a knowledge-based expert system. The set of 30 reaction products compiled in Table 4 was evaluated for structural alerts using DEREK to screen for potential toxicological hazards for these compounds. A DEREK screen for structural alerts in the corresponding precursors/couplers was also conducted to allow for a comparison across these sets of compounds. The key systemic toxicity endpoints associated with structural alerts identified by the DEREK screen were genotoxicity and carcinogenicity. The other systemic toxicity endpoints associated with structural alerts in reaction products were methaemoglobinaemia (the alert was for simple aniline compounds and compounds likely to be metabolised or hydrolysed to form a simple aniline are intended to be identified by this alert) and hepatotoxicity (the alert was for acetaminophen or an analogue). These alerts were present in both precursors/couplers and reaction products. Furthermore, the benzoquinone imine moiety is present across the spectrum of structures formed from all the major classes of oxidative dyes.

Non-genotoxicity endpoints

For the aromatic amine (aniline-like compounds), both the precursors of the p-aminophenol class (which are structures similar to acetaminophen) as well as the reaction products formed from these precursors contain this alert. Considering the very low exposure to reaction products and the toxicological basis for these alerts, the use of oxidative hair dyes was judged to not pose a relevant health risk for either methaemoglobinaemia or hepatoxicity.
The benzoquinone imine moiety can react via redox mechanism and via Michael addition. An example is given by Diclofenac-induced hepatotoxicity which is the consequence of bioactivation to a benzoquinone imine (Poon *et al.*, 2001). But this is also related to high doses and insofar not relevant for hair dye reaction products.

Genotoxicity

Several individual precursors and couplers used in oxidative hair colouring contain structural alerts for genotoxicity (aromatic amines, secondary amines). The DEREK analysis of reaction products identified the alerts for carcinogenicity involving genotoxic mechanisms that had been already identified in the precursors/couplers. About half of the reaction products as well as some of the precursors/couplers contain secondary amine functional groups. The secondary amine is flagged as a carcinogenicity alert by DEREK. Secondary amines may also give rise to nitrosamine formation, i.e. under acidic conditions after oral exposure in the stomach (this exposure pathway is not relevant for cosmetics).

During the process of updating the safety dossiers for oxidative hair dye ingredients for the purpose of the European hair dye strategy, the substances have been tested for genotoxicity and some are positive in *in vitro* genotoxicity assays. However, most of them - amongst them all precursors/couplers used in the EU - have been demonstrated as being non-genotoxic *in vivo.* These studies have been assessed by SCCNFP, SCCP and SCCS.

The DEREK search for structural alerts identifies additional genotoxicity alerts in some precursors (e.g., alerts for *in vitro* chromosome damage with p-phenylenediamine and for phenols substituted in the *para* position with a nitrogen or oxygen, as in p-aminophenol-type precursors). The proposed mechanistic basis for these alerts involves oxidation to electrophilic quinone-type intermediates.

Comment and conclusion
Expert systems based on structural alerts generate many false positives but they cannot identify non-active compounds (ECETOC, 2003). For the *in vitro* mutagenicity endpoint (Q)SAR may be considered a promising tool. However, the use of QSAR in the regard of reaction products was of limited value since the arylamine structure which is a structural element of many hair dye precursors and reaction products, is identified as an alert. For the assessment of arylamine-containing complex molecules it is desirable to develop SAR for *in vivo* genotoxicity in the future.

3.7 Carcinogenicity

The International Agency for Research on Cancer (IARC) evaluated occupational exposures of hairdressers and barbers and personal user of hair colorants in addition to some hair dyes and cosmetic colorants in 1992 (IARC, 1993). It was concluded that there is limited evidence that occupation as a hairdresser or barber entails exposures that are carcinogenic and that there is inadequate evidence that personal use of hair colorants entails exposures that are carcinogenic. The conclusion with regard to occupational exposures was primarily based on an increased risk of bladder cancer. Eight individual hair dyes were evaluated. One of them showed sufficient evidence for carcinogenicity in animal experiments (HC Blue No. 1) while limited evidence for carcinogenicity was found for four of the hair dyes evaluated (2-amino-4-nitrophenol, 2-amino-5-nitrophenol, 2-nitro-para-phenylenediamine, D&C Red No. 9). None of these hair dyes are at present used in hair dyes in EU and with the exception of HC Blue No. 1 these substances are banned[1].

IARC repeated its evaluation in 2008 (IARC, in press). It was noted that many new epidemiological studies on cancer had been published since the last IARC assessment. IARC reconfirmed its conclusions from the evaluation in 1992 (Baan *et al.* 2008).

3.7.1 Animal studies

The Industry has discussed 5 experiments with topical application of permanent hair dye formulations after mixing with hydrogen peroxide.

Studies with rats

Study 1
Kinkeln and Holzmann (1973) studied one oxidative hair dye formulation (3.0% p-toluenediamine + 0.75% 2,4-diaminoanisole + 0.75% resorcinol) and a 4% solution of p-toluenediamine. Each group consisted of 50 male and female Sprague-Dawley rats. The rats received twice weekly applications of mixtures of the above formulations for 24 months. A placebo formulation at similar pH and with similar hydrogen peroxide content served as control substance. The treated area was approximately 9 cm² of shaved dorsal skin. After mixing with an equal volume of a 6% solution of hydrogen peroxide, 0.5 g of the dye formulations were left on the skin for 0.5 hours, after which the treated skin sites were rinsed. No evidence for adverse effects, toxicity (either systemic or at the site of application) or carcinogenic activity of the dyes was observed in the study.

[1] Directive 76/768/EEC, annex II: 2-amino-4-nitrophenol, entry 383; 2-amino-5-nitrophenol entry 384; 2-nitro-*para*-phenylenediamine, entry 1319; D&C Red No. 9, entry 1305

Comment
2,4-diaminoanisole is classified according to CLP as carc. cat 1B, mut. cat 2.

Study 2
Burnett and Goldenthal (1988) have carried out a multi-generation reproduction and carcinogenicity study with Sprague-Dawley rats exposed topically to six different commercial oxidative hair colouring formulations. The hair dyes were applied twice weekly throughout the growth, mating, gestation and lactation phases of F_0 parents to the weaning of F1a and F2b litters. Sixty of each sex, male and female, weanlings selected from the F1a litters was used for a lifetime (24 months) carcinogenicity study. The hair dye formulations were applied twice weekly. The precursors and couplers were mixed with an equal volume of a 6% solution of hydrogen peroxide and 0.5 ml of the mixture was applied to shaved skin on a surface of approximately 5 cm² and left without rinsing. F2b litters were treated under the same conditions and used for the evaluation of reproductive parameters. After 24 months of treatment, the animals were killed and a complete list of organs and tissues was subjected to histopathology. Variations in the incidence of mammary tumours and pituitary adenomas, both common tumours in Sprague Dawley rats, were observed but these were concluded to be spontaneous and unrelated to treatment. Overall, the study showed no evidence for reproductive toxicity, systemic toxicity or carcinogenic activity (either systemic or at the site of application) of any hair dye.

Comment
Two of the hair dye formulations contained 2,4-diaminoanisole (2.0% and 4.0%) which is classified according to CLP as carc. cat 1B, mut. cat 2.

Studies with mice

Study 1
Groups, 28 each of male and female Swiss Webster mice, received a weekly topical treatment of two different hair dye mixtures for two years (Giles and Chung, 1976). These were mixed with an equal volume of 6% hydrogen peroxide and applied (50 µl) to the clipped intrascapular skin. Control groups were treated with water-isopropanol mixed with 6% peroxide. Positive control groups received 9,10-dimethylbenzanthracene (DMBA) at 0.005% and 0.02% in acetone. The study found no evidence for a carcinogenic effect (either systemic or at the site of application) of the hair dye formulations tested, whereas DMBA-treated groups showed a high incidence of skin tumours, demonstrating the sensitivity of the test system to known skin carcinogens.

Comment
Both hair dye formulations contained 0.2% and 0.6% 2,4-toluenediamine (4-Methyl-m-phenylenediamine) which is classified according to CLP as carc. cat 1B, mut. cat 2.

Study 2
Burnett *et al*. (1980) studied nine different commercial oxidative hair dye formulations. The hair dyes were applied to groups 50 each of male and female Swiss Webster mice. Treatment was once weekly for at least 20 months and hair dye formulation (50 µl) was applied to approximately 1 cm² of clipped skin, after mixing with an equal volume of a 6% solution of hydrogen peroxide. No rinsing off was performed. Negative control groups (three groups each of 50 male and females) were shaved but not treated. The study found no signs of toxicity (either systemic or at the site of application) or carcinogenicity of any investigated hair dye.

Comment

The experiment involved 12 treatment groups (9 permanent hair dye formulations and 4 non-oxidative hair dye formulations and 3 control groups. Some of the formulations contained 2,4-diaminoanisole which is classified according to CLP as carc. cat 1B, mut. cat 2.

Study 3
Jacobs *et al.et al.* (1984) investigated the toxicity and carcinogenicity of two commercial oxidative and twelve non-oxidative hair dye formulations. Groups, 60 each of male and female Swiss mice, were topically treated once per week for 20 months, sacrificed and evaluated. Final hair dye formulation (50 µl) was applied to a clipped skin area of approximately 1 cm^2 without rinsing. Animals of two control groups were shaved only and received no treatment. Although there was an increased incidence of malignant lymphoma in three treated groups when compared to control group 2, the differences were not significant when compared with control group 1. In addition, the incidence remained within historical control values for animals of this strain, sex and age. Overall, the study found no evidence for systemic toxicity or carcinogenicity (either systemic or at the site of application) of any investigated hair dye formulation.

Comment
Some of the formulations contained Disperse Blue 1 which is classified according to CLP as carc. cat 1B.

General comment on animal studies with topical application of oxidative hair dye formulations

Commercial oxidative hair dye formulations were investigated in five studies via the topical route. A positive control was used only in one study with mice where it was shown that the carcinogen DMBA induced a significant increase in skin tumours at the site of contact (Giles and Chung, 1976). Although all the other studies included known carcinogens (2,4-diaminoanisole, Disperse Blue 1, 2,4-toluenediamine (4-Methyl-m-phenylenediamine) classified according to CLP as carc cat 1B, the applicant stated that none of the experiments showed evidence for systemic toxicity or carcinogenicity (either systemic or at the site of application). It should also be noted that many of the colorants actually in use as hair dye ingredients were present in concentrations much lower that the maximum permitted concentration.

The applicant claimed that in the studies the quantity of hair dye applied (mg hair dye/kg body weight) was comparable to the quantity to which humans may be exposed during a hair colouring event. In this respect it should be noted that in carcinogenicity studies in general, the animals are exposed to much higher concentrations that normally occur in the human situation. The reason is the low sensitivity of animal studies as the group size is only of the order of 50 animals implying that the tumour frequency has to be high in order to be detected. In order to correct for the low sensitivity the highest dose tested should in general show some toxicity although it should not exceed the "highest tolerable dose".

As pointed out above the observation that no toxicity and no carcinogenicity was observed with recognized genotoxic carcinogens classified both as carcinogen and mutagen in EU clearly demonstrate that the sensitivity of the animal experiments was too low to detect any potential carcinogenicity of oxidative hair dye formulations. No conclusion can be drawn from the studies with regard to carcinogenicity of oxidative hair dye formulations in humans.

3.7.2 Human studies

Studies concerning personal use of hair dye published after 1992 will be discussed in this chapter.

Bladder cancer

Cohort Studies

A large prospective cancer mortality study (Cancer Prevention Study II; CPS II) conducted by the American Cancer Society has been published (Thun *et al.*, 1994; Altekruse *et al.*, 1999). This study collected data specific to personal use of permanent hair dyes in over 570,000 US women and found no association between permanent hair dye use and mortality from bladder cancer. Henley and Thun (2001) further extended the follow-up time for evaluation for this cohort. At this analysis there were a total of 336 bladder cancer deaths and the results continued to indicate no increased risk of bladder cancer associated with ever use of permanent hair dye. In a recent study Mendelsohn and coworkers (2009) followed 70366 Chinese women for an average of 7 years. No increased risk of bladder cancer was reported among hair dye users. The number of cases was, however small (19 unexposed/13 exposed).

Case-Control Studies

One case–control study from the Los Angeles area (Gago-Dominguez *et al.*, 2001) suggested a possible association between personal use of permanent hair dyes among women and the risk of bladder cancer (odds ratio (OR) = 1.8(1.01-3.3)[2], which was stronger for slow acetylators (OR= 2.9 (1.2-7.5)) (Gago-Dominguez *et al.,* 2003). The finding (Gago-Dominguez *et al.*, 2001) is supported by Kelsey *et al*., 2005 who showed a significant association of TP53 inactivation (i.e., mutation and nuclear accumulation) in bladder tumours of hair dye users since aromatic amines contained in hair dyes would be expected to produce transition mutations.

The association between permanent hair dye use and bladder cancer is partly supported by a study from New Hampshire (Andrew *et al.,* 2004). Among users of permanent hair dyes the risk of bladder cancer showed a non-significant increase among women in general (relative risk (RR) =1.5 (0.8-2.7)) and a significant increase among those that had started to use permanent hair dyes before the age of 37 (RR= 2.3 (1.1-4.6)) or started to use permanent hair dyes more than 31 years ago (RR=2.6 (1.1-6.3)). On the other hand, a study from Texas (Lin *et al.*, 2006) showed no significant increase in bladder cancer among users of permanent hair dyes either in the whole study or in any of the subgroups. Additionally, Kogevinas *et al.* (2006) did not find any increased risk of bladder cancer among permanent hair dye users in a hospital based case-control study from Spain. Serretta *et a*l. (2006) did not find any effect of hair dye use on superficial transitional cell carcinoma of the bladder in Italy. No differentiation was, however made between permanent hair dyes and other types of hair dyes.

Three meta-analyses have been published evaluating personal use of hair dyes and bladder cancer (Huncharek and Kupelnick, 2005; Takkouche *et al.*, 2005; Kelsh *et al.*, 2008). The summary relative risk estimates reported were 1.01, 1.01, and 0.97, respectively. Huncharek and Kupelnick (2005) performed in addition sensitivity analyses examining the influence of hair dye type, colour, and study design. The sensitivity analyses yielded statistically significant RRs ranging from 1.22 (1.11-1.51) to 1.50 (1.30-1.98), indicating that personal use of hair dye products increases bladder cancer risk by

[2] The numbers in brackets after the risk estimate represent the 95% confidence interval

22% to 50% vs. non-use. Kelsh *et al.* (2008) conducted an examination of bladder cancer risk in relation to permanent hair dye use, use of dark colour dye, duration of hair dye use and lifetime applications. No statistically significant positive associations were observed across any of these exposure metrics. Takkouche *et al.* (2009) performed a meta-analysis of the risk of bladder cancer among hairdressers and related workers and reported a RR of 1.30 (1.20-1.42).

Haematopoietic cancers

Lymphoma

Lymphoma is a broad term that includes a number of different cancers involving lymphocytic cells and the lymphatic system. There are two main groups of lymphomas - Hodgkin's disease and non-Hodgkin's lymphoma (NHL). All Hodgkin's disease lymphomas and most NHL are B-cell lymphomas.

Hodgkin's Disease

Cohort Studies
The American Cancer Society (CPS II) study collected data specific to personal use of permanent hair dyes and found no increased risk of death from Hodgkin's disease in over 570,000 US women included in the study (Thun *et al.*, 1994). Another prospective cohort study on incidence of Hodgkin's disease was the Harvard Nurses' Health Study (Grodstein *et al.*, 1994). This study included over 99,000 nurses and found no increased risk for Hodgkin's disease in relation to personal use of permanent hair dye.

Case-Control Studies
Several case–control studies of personal use of hair dyes have also been conducted, and these have not found any statistically significant association between Hodgkin's disease and either permanent or semi-permanent hair dye use (Miligi *et al.*, 1999; Tavani *et al.*, 2005; Benevente *et al.*, 2005; de Sanjose *et al.*, 2006). Only one study reported a statistically significant increased risk of Hodgkin's disease among female users of permanent hair dyes (Zahm *et al.*, 1992).

Takkouche *et al.* (2005) performed a meta-analysis of the risk of Hodgkin's disease in relation to personal use of hair dyes and reported a RR of 0.88 (0.54-1.42). The meta-analysis involved four studies (Grodstein *et al.*, 1994; Thun *et al.*, 1994; Zahm *et al.*, 1992; Tavani *et al.*, 2005). Takkouche *et al.* (2009) performed a meta-analysis of the risk of Hodgkin's disease among hairdressers and related workers and reported a RR of 1.22 (0.96-1.55).

Non-Hodgkin's Lymphoma (NHL)

NHL is a collective term applied to a group of many (depending on classification, more than forty different) lymphomas, all of which are clinically and biologically distinct subtypes. The classification or nomenclature given to these various NHL's has changed over the years. The current official classification system is known as the REAL/WHO system and is based on cell type, appearance, morphology, genetic features and immunologic phenotype.

There have been a large number of epidemiologic studies on personal use and occupational exposure to hair dye and NHL. Interpretation of the results is complicated by the changing nomenclature for the various NHL's over the years, gender differences in results in the various studies, combining data for all NHL subtypes rather than obtaining data and calculating risk for each specific subtype of NHL.

Cohort Studies
The American Cancer Society (CPS II) studied the association between mortality from NHL and permanent hair dye use (Thun *et al.*, 1994; Altekruse *et al.*, 1999). Overall, there were no associations between ever use of permanent hair dye or duration of use and NHL mortality. Likewise, no increase in NHL was found in the Nurses' Health Study (Grodstein *et al.*, 1994) and in the study on Chinese women (Mendelsohn *et al.*, 2009).

Case-Control Studies
Since 1992, 12 papers described results from case-control studies of NHL and personal use of hair dye (Zahm *et al.*, 1992; Linos *et al.*, 1994; Holly *et al.*, 1998; Miligi *et al.*, 1999; Schroeder *et al.*, 2002; Zhang *et al.*, 2004; Chiu *et al.*, 2004; Tavani *et al.*, 2005; Benavente *et al.*, 2005; de Sanjose *et al.*, 2006; Chiu *et al.*, 2007; Morton *et al.*, 2007 (The results from Benevente *et al.*, 2005 has later been included in the paper of de Sanjose *et al.*, 2006)).

Two recent comprehensive review articles (Takkouche 2005, Zhang 2008) have been published on personal use of hair dyes and various cancers, including NHL. One study article listed above (Chiu *et al.*, 2007) has not been considered in any of the review articles. This study analyzed tumour tissue samples obtained from a previous case-control study for the presence or absence of the chromosomal translocation 5(14;18). Hair dye use was not associated with either t(14;18)-positive or t(14;18)-negative subtype.

Takkouche *et al.* (2005) performed a meta-analysis for NHL based on 12 studies (Stavraky *et al.*, 1981; Cantor *et al.*, 1988; Zahm *et al.*, 1992; Linos *et al.*, 1994; Holly *et al.*, 1998; Miligi *et al.*, 1999; Schroeder *et al.*, 2002; Zhang *et al.*, 2004; Chiu *et al.*, 2004; Tavani *et al.*, 2005; Grodstein *et al.*, 1994; and Altekruse *et al.*, 1999) and showed that there was a statistically significant increased risk of NHL associated with ever users of hair dye (RR=1.23 (1.07-1.42)). The results for permanent dye use only (RR=1.13 (0.99-1.29)), based on 6 studies) and for intensive exposure (defined as more than 200 lifetime exposures to hair dye, RR=1.07 (0.90-1.28), based on 6 studies) did not show any statistically significant association. Takkouche *et al.* (2005) point out the fact that the restriction of the analysis to intensive exposure to hair dyes and to exclusive use of permanent hair dye did not strengthen the risk further, and they comment that this is "consistent with the absence of a causal effect".

The review of Zhang *et al.* (2008) included 4,461 NHL cases and 5,799 controls from the International Lymphoma Epidemiology Consortium (InterLymp). Three studies were from the USA, the Connecticut Women's NHL Study (CT) (Zhang *et al.*, 2004), the National Cancer Institute (NCI)/Surveillance, Epidemiology) and End Results (SEER)' Multi-Center Case-Control Study (NCI/SEER), (Morton *et al.*, 2007) and the Epidemiology of NHL Study from the University of California at San Francisco (UCSF) (Holly *et al.*, 1998). The three U.S. studies collectively represent a total of six sites from the SEER program (Connecticut, San Francisco-Oakland, Iowa, Detroit, Seattle-Puget Sound, and Los Angeles). The case-control study from Europe (the EpiLymph International Case-Control Study of Lymphomas) (de Sanjose *et al.*, 2006) represents geographic sites from six countries (Germany, Italy, France, Ireland, Czech Republic, and Spain). Each study collected detailed information on hair dye use (including duration of use, total number of applications, year of use, and type and colour of hair-dye used) and included histologically confirmed incident NHL cases.

Among women, 75% of the cases and 70% of the controls reported ever having used hair dyes. An increased risk of NHL was observed among women who started using hair dyes before 1980 compared with non-users (OR=1.3 (1.1-1.4)). Stratification by NHL subtype, hair dye use was associated with an increased risk of follicular lymphoma (FL) and chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) but not other NHL

subtypes. The increased risk of FL (OR=1.4 (1.1-1.9)) and CLL/SLL (OR=1.5 (1.1-2.0)) associated with hair dye use was mainly observed among women who started use before 1980 with a significant trend in risk with duration of use (P=<0.01, 0.02 respectively). For women who began using the products in 1980 or after, an increased risk of FL was limited to users of dark-coloured hair dyes (OR=1.5 (1.1-2.0)) with ORs of 1.5 (1.1-2.1) for permanent dark-coloured hair dyes and 1.7 (1.1-2.4) for non-permanent dark-coloured hair dyes. Results from the analyses showed that risk of CLL/SLL was increased mainly for European women who used hair dyes, not US women (de Sanjose *et al.*, 2006, OR= 1.6 (1.1-2.5), Morton *et al.*, 2007 OR=1.1 (0.6-2.24), Holly *et al.*, 1998 OR=0.8 (0.4-1.4), Zhang *et al.*, 2004 OR=1.3 (0.7-2.4)) whereas the association with follicular lymphoma was seen in both geographic regions. Although the duration and frequency of hair-dye use in the control group were similar between European women and US women, duration of use among European women with CLL/SLL was greater than that for US women (p values ranged from 0.006 to 0.06 for various hair-dye products). Other factors, such as differences in hair-dye formulations, classification of rare NHL subtypes, or chance variations, also may have contributed to the observed difference in risk pattern between the European and US populations. Among men, approximately 10% of cases and 10% controls had ever used hair dyes. Risk of NHL was not associated with hair dye use before or after 1980 among men.

It is concluded that the results indicate that personal hair dye use may play a role in the risk of FL and CLL/SLL in women who started use before 1980 and that an increase in risk of FL in women starting use in 1980 or after cannot be excluded.

Takkouche *et al.* (2009) performed a meta-analysis of the risk of NHL among hairdressers and related workers and reported a RR of 1.11 (1.00-1.19).

Leukaemia

In the SCCP Opinion (SCCP/093/05) it was concluded that "some studies indicate excess risk for acute leukaemia and chronic lymphoid leukaemia (CLL) for users of hair dye". As CLL is already covered in the section on NHL, no discussion on CCL is included below.

Cohort studies
Grodstein *et al.* (1994) using the Nurses Health Study (women aged 30-55 years in 1976, followed until 1990), reported no increase in the risk leukaemia after use of permanent hair dyes.

Altekruse *et al.* (1999) reported from the American Cancer Society cohort study (CPSII) some indication for a possible increased risk of death from all leukaemia among women using permanent hair dyes (RR=1.1 (0.9-1.3)). The risk increased with duration of use: 1 – 9 years RR=0.9 (0.7-1.2), 10 – 19 years RR=1.2 (0.9-1.5), 20+ years RR=1.3 (1.0-1.7), p-value for trend 0.04.

Mendelsohn and coworkers (2009) found no increased risk of leukaemia among hair dye users in China. The number of cases was, however, small (20 unexposed/9 exposed).

Case control studies
Several studies have been published in relation to pre-leukeamic conditions and hair dye use.

Essential thrombocythemia (ET) is a myeloproliferative disease caused by clonal proliferation of the myeloid stem cell. Terminal transformation to acute leukaemia is known to occur as a possible sequela to the disease. Odds ratio (OR) estimates suggest an association between ET and hair dye use. Thus, while OR for ever use of hair dye was 1.5 (0.7-3.2), the OR using dark colour was 2.1 (0.9-3.8) and for those using dark hair dye for periods longer than 10 years OR = 5.3 (1.4-19.91) (Mele *et al.*, 1996).

Elevated risks for the dysmyelopoietic syndrome were found in two studies from Japan with relative risks among women of 2.50 (0.97-6.41) (Ido *et al.*, 1996) and 2.88 (1.38-6.01) (Nagata *et al.*, 1999), respectively. In the study by Nagata and co-workers the the relative risk was 1.99 (1.17-3.38) for both sexes together. The risk increased with duration of use (10+ years RR = 4.10 (1.64-10.23) and total frequency of use (70+ uses RR = 3.08 (1.22-7.75)). No increase was found in a small group of men (OR=1.23 (0.53-2.88)). It is uncertain whether or not there is an overlap between the cases from the two Japanese studies. No answer to a request to the author was received.

Mele *et al.* (1994, 1995) using patients from three hospitals in Italy found no statistically significant increased risks for acute promyelocytic leukaemia, acute myeloid leukaemia, acute lymphocytic leukaemia or chronic myeloid leukaemia.

Miligi *et al.* (1999) found no increased risk of leukaemia among hair dye users in general. The risk was, however, increased among women using dark permanent products (2.0 (1.1-3.8)).

Rauscher *et al.* (2004) performed a population-based case-control study of acute leukaemia in 1986–1989 in USA and Canada. There was a modest positive association for ever use of hair dyes (OR= 1.3 (1.0-1.8)). The increase was stronger among those only using permanent dye (OR=1.6 (1.1-2.4)). However, the use of dark shade permanent hair dyes was not associated with higher risk (OR=1.6 (0.78-3.2) than the use of light shade permanent hair dyes (OR=1.8 (1.1-3.1)). The risk increased for long duration (15+ years) of use (OR=1.9 (1.1-3.6)). The greatest odds ratio was for 15 or more years of using hair dyes and six or more times per year (OR=2.4 (1.0-5.8)). First use before 1970 represented a higher risk (OR=1.7 (1.0-3.0)) than first used after 1979 (OR=1.2 (0.51-2.9)). When stratified by leukaemia subtype, ever use of permanent hair dyes was associated with an OR of 1.6 (1.1- 2.5) for myelocytic leukaemia, and the trends in risk with duration and frequency were similar to the trends observed for all leukaemia subtypes combined. For lymphoblastic leukemia, the OR for ever use of permanent dyes was 2.0 (0.9- 4.6). There was a suggestion of a dose response for both duration and frequency, with the OR reaching
4.6 (1.5-14) for 15 or more years of use and the OR reaching 3.8 (1.2-12) for six or more applications per year. The authors conclude that long duration of permanent dye use may have a larger impact on the risk of adult acute leukaemia and other haematopoietic cancers than prior epidemiological data suggest.

Takkouche *et al.* (2009) performed a meta-analysis of the risk of leukaemia among hairdressers and related workers and reported a RR of 1.11 (1.03-1.19).

Multiple myeloma

Cohort studies
The American Cancer Society (CPS II) studied the association between mortality from multiple myeloma and permanent hair dye use (Thun *et al.*, 1994; Altekruse *et al*., 1999). Overall, there were no associations between ever use of permanent hair dye or duration of use and multiple myoloma mortality. Likewise, no increase in NHL was found in the Nurses' Health Study (Grodstein *et al.*, 1994) and in the study on Chinese women (Mendelsohn *et al.*, 2009).

Case control studies
Koutros *et al.* (2009) studied the association between personal hair dye use and risk of multiple myeloma among women in USA. The study involved 175 cases of multiple myeloma and 679 controls. No association between ever reporting hair colouring product use and myeloma risk among all users: OR=0.8 (0.5-1.1), semi-permanent users: OR=0.7 (0.4-1.2), permanent users: OR=0.8 (0.5-1.1), or dark permanent users:

OR=0.8 (0.5-1.3). There were no significant associations among women who used hair dyes before 30 years of age, who started use before 1980, who had ≥240 lifetime applications, or for dark permanent users with 28 or more years of use.

Takkouche *et al.* (2005) performed a meta-analysis of the risk of multiple myeloma in relation to personal use of hair dyes and reported a RR of 1.14 (0.86-1.52). The meta-analysis involved six studies (Grodstein *et al.*, 1994; Thun *et al*., 1994; Zahm *et al.*, 1992; Brown *et al.*, 1992; Herrinton *et al.*, 1994; Tavani *et al.*, 2005). Takkouche *et al.* (2009) performed a meta-analysis of the risk of multiple myeloma among hairdressers and related workers and reported a RR of 1.62 (1.22-2.14).

Other cancer sites

Takkouche *et al.* (2005) performed a meta-analysis of the risk of cancer at several sites. Only sites where more than three studies are available and are described below. For other sites only one or two studies have been found and it is difficult to draw any conclusion.

Skin cancer.
No increase was found in old studies of malignant melanoma (Holman and Armstrong, 1983, 1985; Osterlind *et al.*, 1988). In a recent study Feizy and Toosi (2009) reported an increased risk of basal cell carcinoma (BCC) among women patients that used hair dyes more frequently than controls (OR=3.7 (1.5-9.5).

Breast cancer.
No increase was found in the case of female breast cancer (RR=1.06 (0.96-1.18)) based on 14 studies

3.7.3 Summary and discussion

Animal studies
The applicant provided 5 experiments (2 studies with rats and 3 studies with mice) with topical application of permanent hair dye formulations after mixing with hydrogen peroxide. No toxicity or carcinogenicity was observed in any of the experiments even though recognized genotoxic carcinogens classified both as carcinogen and mutagen in the EU were present in all 5 experiments. This clearly demonstrates that the sensitivity of the animal experiments have been too low to detect any potential carcinogenicity of oxidative hair dye formulations. No conclusion can be drawn from the studies with regard to carcinogenicity of oxidative hair dye formulations in humans.

Human studies
Although the epidemiological evidence that personal use of hair dyes entails exposures that are carcinogenic is inadequate, some epidemiological studies on personal use of hair dyes may raise concern.

Some concern for bladder cancer has been raised on the basis of American studies. However, no increased risk has been found in a European study.

Some concern has been raised in relation to haematological malignancies. An increased risk of non-Hodgkin's lymphoma (NHL) was observed among women who started using hair dyes before 1980 compared with non-users. Upon stratification by NHL subtype, hair dye use was associated with an increased risk of follicular lymphoma (FL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) but not other NHL subtypes. The risk of CLL/SLL was increased mainly for European women who used hair dyes, and not in US women. The results indicate that personal hair dye use may play a role in the risk of FL and CLL/SLL in women who started use before 1980 and that an increase in risk of FL in women starting use in 1980 or after cannot be excluded. Risk of NHL was not associated with hair dye use among men.

In a population-based case-control study of acute leukaemia in USA and Canada a modest positive association for ever use of hair dyes was observed. The increase was stronger among those only using permanent dye and the risk increased with number of years of hair dye use and number of use per year. First use before 1970 represented a higher risk than first use after 1979.

In the period from 1980, a number of colorants have been banned in EU as well as in individual European countries on the basis of reported carcinogenic effects in animal studies. In USA, a ban of specific colorants in cosmetic preparations is seldom used, while US FDA requires that the label should contain information on possible carcinogenic effects. Another difference between the use of hair colorants in USA and EU is that it is more common in USA that hairdressers mix colorants themselves. Under such conditions, the control of the dyes used and their purity is limited.

3.8 Risk Assessment and Discussion

During the last decade, considerable progress has been made in assessing the safety of hair dyes. Concerns on bladder cancer raised by the scientific community and the public were taken up by the SCCNFP in 2001. The European Commission addressed concern by legislative measures. The Cosmetics Industry has performed a considerable number of chemical and toxicological studies for precursors and couplers as well as for reaction products and possible intermediates. All hair dye substances for which a safety dossier conforming to current standards was not provided have been banned.

A comprehensive dossier on reaction products was sent to the European Commission in 2009 and was reviewed by the SCCS. Studies on exposure and on genotoxicity were supplied as well as an updated review on epidemiology.

This risk assessment of reaction products is focused on genotoxicity. General toxicity is not considered a concern for the consumer due to the low exposure (0.03 to 6.9 µg/kg bw) which takes place only once per month. As no data has been made available for this endpoint, sensitization is not addressed in this opinion.

Chemistry

A representative set of oxidative dye combinations was studied under conditions mimicking consumer usage and using a quantitative HPLC-based analytical method in order to identify and quantify the formation of reaction products in hair colouring formulations. Reactions of seven precursors and ten couplers were investigated. The study of 26 oxidative dye combinations covered the major chemical classes of precursors and couplers. The reaction products formed were of both dimeric and trimeric structures and of a large range of molecular weight and water solubility. It was demonstrated that other components of commercial hair dye formulations, including two direct dyes, did not affect the kinetics of reaction products formation. No build up of intermediates was observed. It was shown that the reaction products of oxidative coupling were in agreement with the theoretical predictions based on the reaction kinetics and that the fastest coupling reactions dominate the chemistry in hair dye formulations. No additional reaction products were detected. Furthermore, studies were also carried out to show that exposure to intermediate species and self-coupling products (e.g. Bandrowski's Base) is negligible.

The maximum concentrations of specific reaction products in the hair dye formulation after 30 min to which the consumer may be exposed were 0.02 – 0.65% (w/w). Time-averaged concentrations were obtained from kinetics experiments. For relatively fast forming reaction products with linear kinetics it was assumed that the average reaction product concentration over the whole application time would be around 50% of the final concentration achieved resulting in exposure concentrations between 0.015% and

0.33%. For slower forming reaction products, the maximum reaction product concentration concentrations measured at the end of the kinetics study were used for the dermal penetration studies since kinetics may not be linear in these cases.

Exposure assessment

In vitro studies

The methodology of exposure assessment used for hair dye reaction, i.e. percutaneous absorption *in vitro,* is considered adequate. *In vitro* skin penetration was investigated for 14 oxidative hair dye reaction products. A 30 min skin exposure time was used for dermal absorption experiments, despite the SCCP recommendation that a 45 min exposure period should be used for the reaction product studies (SCCP/1198/08). However, a 50% increase in exposure time can be expected to result in exposures in the same range as those reported. Furthermore, SCCS used the mean + SD figure in this assessment according to its recent opinion on dermal absorption (SCCS/1358/10) to cover study variabilities. Time-averaged concentrations obtained from the kinetics experiments described above were used for the dermal penetration studies. For slower forming reaction products, the final concentrations measured at the end of the kinetics study were used for the dermal penetration studies since kinetics may not be linear in these cases. The percutaneous absorption rates in the *in vitro* skin penetration studies of the 14 representative reaction products evaluated ranged from 3.27 to 717.79 ng/cm^2 (mean +1SD). This corresponds to 1.9 to 416 µg absorbed dose (i.e. dose potentially bioavailable) per hair dye application (i.e. 0.03 to 6.9 µg/kg bw).

In vivo Studies

A study with human volunteers was performed to quantify the systemic exposure of human consumers to oxidative hair dye ingredients, hair dye reaction products and their respective metabolites, following a single use of a PPD-based hair colouring product. Subjects received a dark-shade, [^{14}C]-PPD-labelled oxidative hair dye, containing 1% PPD (A007), 0.5% m-aminophenol (A015) and 0.5% resorcinol (A011) (on-head concentrations). Presence of [^{14}C] was analysed in all study materials, plasma and urine samples by liquid scintillation counting. In addition, plasma and urine samples were analysed by LC-MS/MS techniques for presence of PPD and its acetylated metabolites as well as the anticipated reaction products A007-A011-A007 and A007-A015-A007 and their possible acetylated metabolites. 4/48 plasma samples from 4/16 volunteers contained trace levels of the two expected reaction products and/or their acetylated metabolites at, or slightly above, the respective LOQ_{plasma}.

Exposure was estimated based on urinary excretion. The limits of quantification in the urine (LOQ_{urine}) for the reaction products A007-A011-A007 and A007-A015-A007 were 0.4 to 1.28 ng/ml, respectively.The maximum excretion values found were 1881 ng/event (A007-A015-A007) and 1462 ng/event (A007-A011-A007).
Assuming that all samples contained at least levels at 50% LOQ_{urine} the estimated mean systemic exposures for A007-A015-A007 and A007-A011-A007 would be 826 and 417 ng per hair dyeing event, respectively. In comparison, for PPD, N-monoacetyl-PPD and N,N'-diacetyl-PPD the mean total amount excreted over 48 hours amounted to 8.8 µg, 4.4 µg and 3067 µg, respectively. On a molar basis the sum of PPD and its metabolites excreted is about 16 µmol.

These results are further supported by a recent study where exposure to 2 commercial hair dye products containing toluene-2,5-diamine was investigated using a GC-MS method. The cumulative excreted amounts of toluene-2,5-diamine (sum of amine and conjugate) were 700 µg and 1500 µg, corresponding to 5.7 and 12.3 µmol, respectively.

Comparison of in vitro and in vivo study results on exposure
When comparing the *in vitro* and *in vivo* study results it appears that exposure is overestimated by *in vitro* studies: when taking the maximum *in vivo* exposure, the factor is 3 to 4, for the mean *in vivo* exposure figure factors of 8 to 10 were calculated.

Comparison of exposure to reaction products and precursors/couplers

In order to directly compare systemic exposure doses of reaction products with those of their corresponding hair dye precursors and couplers, the ratios between systemic exposure doses were calculated on the basis of the results of *in vitro* percutaneous penetration studies. The mean exposure ratio was 706, with a median value of 170 and individual ratios ranging from 5 to 4649. These systemic exposure ratios demonstrate that human systemic exposure to hair dye reaction products is frequently much lower than the exposure to their corresponding hair dye precursors and couplers.

When comparing human systemic exposure to reaction products *in vivo* (about 1 µg per event) with that of N,N'-diacetyl-PPD, the principal metabolite of PPD (about 2 mg per event), exposure to reaction products is 3 orders of magnitude lower based on 0-12 h urine samples. This confirms the above *in vitro* results.

It is concluded that when a consumer uses a commercial hair colouring product, exposure is primarily to unreacted precursors and couplers. In addition, exposure to reaction products was observed while no exposure to intermediates was noted.

When discussing a possible carcinogenic risk of reaction products it should be understood that for genotoxic carcinogens the mean daily exposure has to be taken into account. The relevant exposure of reaction products in this regard is estimated to be 1 to 248 ng/kg bw/d based on the *in vitro* percutaneous absorption studies. This is in the majority of investigated substances above the threshold of toxicological concern for genotoxic carcinogens that was discussed recently in EU scientific committees (2.5 ng/kg bw/d). (http://ec.europa.eu/health/archive/ph_risk/committees/documents/sc_o_001.pdf)

However, when using the very limited in vivo data (of only 2 reaction products, A007-A015-A007 and A007-A011-A007) the estimated mean systemic exposure figures would be 826 and 417 ng per hair dyeing event, respectively, or 30 and 15 ng/day (corresponding to 0.50 and 0.25 ng/kg bw/d), respectively, when adjusting for the 28-day interval between exposures.

Genotoxicity

The reaction products with the highest absolute and relative human exposures, based on the mean values of the skin penetration data, were selected for genotoxicity testing. The precursors/couplers from which the reaction products were formed, have been tested for genotoxicity in studies assessed by SCCNFP, SCCP and SCCS. Although some precursors/couplers are positive in *in vitro* genotoxicity assays they all have been demonstrated as being non-genotoxic on the basis of *in vivo* test.

The genotoxicity test results of the reaction products and the results of their respective precursors/couplers were compared. A common result for both precursors/couplers and the reaction product is the positive outcome in one or more *in vitro* tests which was not confirmed *in vivo*. This can be expected due to the similarity of structural alerts. Yet, sometimes the same and sometimes different genotoxic endpoints were positive for precursors/couplers and their reaction products. In the case of the trimer A005-A015-A005 the reaction product induced gene mutations in bacteria as did the precursor and coupler A005 and A015. On the other hand, the dimer A016-A027 induced gene mutations *in vitro* while A016 and A027 were found to be clastogenic. Similarly, the

trimer A007-A017-A007 is not clastogenic while A007 and A017 are clastogenic. Therefore, it can be deduced that it is not possible to predict the specific outcome of the tests of the reaction product on the basis of the results of the respective precursors/couplers. A final conclusion on the possible genotoxic hazard of any compound can be drawn only on the basis of testing. Since testing of all possible hair dye reaction products may not feasible, some degree of uncertainty remains.

In the case of exposure studies, it has been agreed to cover all relevant classes of hair dye reaction products with regard to commercial classes and physico-chemical properties. Consequently, 14 reaction products were investigated in dermal absorption studies. In contrast, for genotoxicity testing only 4 compounds have been selected and tested. However, also with regard to potential genotoxicity, physico-chemical properties may play an important role. It is thus recommended to enlarge analogously the database on genotoxicity of reaction products by including further compounds of additional structural and physico-chemical properties. Additionally, the tonnage on the market and the magnitude of exposure should be used as a selection criterion. It is understood that in vitro testing of further reaction products might generate further positive test results which according to the present knowledge cannot be overruled without in vivo studies which are no longer permitted.(Q)SAR (quantitative) structure-activity relationship
The applicant used the commercial software program DEREK which is a knowledge-based expert system to evaluate the set of 26 reaction products (Table 4).

The structural alerts for methaemoglobinaemia and hepatotoxicity were the aromatic amine and benzoquinone imine moiety. Based on (Q)SAR analyses and considering the very low exposure to reaction products the use of oxidative hair dyes was judged to not pose a relevant health risk for either methaemoglobinaemia or hepatoxicity.

The structural alerts for genotoxicity (aromatic amines, secondary amines) were found in reaction products and in several precursors/couplers. The secondary amine is also flagged as a carcinogenicity alert by DEREK. Additional genotoxicity alerts were identified in some precursors (e.g. the *para* position for phenols substituted with a nitrogen or oxygen).

For the *in vitro* mutagenicity endpoint (Q)SAR may be considered a promising tool. The use of (Q)SAR in the regard of reaction products was, however, of limited value since the arylamine structure which is a structural element of many hair dye precursors and reaction products, is identified as an alert. Moreover, expert systems based on structural alerts generate many false positives but they cannot identify non-active compounds (ECETOC, 2003). For the assessment of arylamine-containing complex molecules it is desirable to use or to develop SAR for *in vivo* genotoxicity in the future.

A critical fault of commercial expert systems is the lack of insight in the applicability domain. The applicability domain is determined for a specific endpoint (e.g. Ames test) and entry of data of chemicals which were tested with this test (learning set). Predictions are un-interpretable if the applicability domain of the model used is not known. If SAR for *in vivo* genotoxicity will be used or developed, it is a requirement that the SAR satisfies the OECD principles and above all that the applicability domain is known.

Carcinogenicity

Animal studies

Five experiments (two studies with rats and three studies with mice) with topical application of permanent hair dye formulations after mixing with hydrogen peroxide were performed. No toxicity or carcinogenicity was observed in any of the experiments even though recognized genotoxic carcinogens classified both as carcinogen and mutagen in EU were present in all five experiments. This clearly demonstrates that the sensitivity of

the animal experiments has been too low to detect any potential carcinogenicity of oxidative hair dye formulations. No conclusion can be drawn from the studies with regard to carcinogenicity of oxidative hair dye formulations in humans.

Human studies
The epidemiological assessment has been concentrated on bladder cancer and haematological malignancies since no evidence was found linking personal use of hair dyes to a cancer risk at other sites.
It is concluded that there is an indication of extra risk of bladder cancer for women in USA using permanent hair dyes frequently and for a long time. No increased risk of bladder cancer was found in a European study.
Although the published data on haematological malignancies are conflicting, especially when all types of hair dyes are considered, it is concluded that some studies indicate excess risks for chronic lymphoid leukaemia/small lymphocytic lymphoma and acute leukaemia for users of hair dyes.
The risk of developing a malignant disease in relation to personal use of (permanent) hair dye is primarily related to users starting to dye their hair before 1980 although an increase in risk in women starting use after 1980 cannot be excluded. Next to the use of only permanent hair dyes, the risk increases with number of years of hair dye use and number of uses per year.

4. CONCLUSION

In the light of the current data submission and the available data base from previous submissions, the SCCS was asked to evaluate the consumer health risk by products and intermediates of oxidative hair dyes formed during hair dyeing processes.

- The use of oxidative hair dye formulations results in consumer exposure to precursors and couplers as well as to their reaction products. Exposure to reaction products is considerably lower compared to that from precursors and coupler. No exposure to intermediates was noted.

- The percutaneous absorption rates in the *in vitro* skin penetration studies of the 14 representative reaction products evaluated ranged from 3.27 to 717.79 ng/cm^2 (mean +1SD). This corresponds to 1.9 to 416 µg absorbed dose (i.e. dose potentially bioavailable) per hair dye application (i.e. 0.03 to 6.9 µg/kg bw).

- In the risk assessment of reaction products general toxicity is not considered a concern due to the low and intermittent exposure (on average once per month).

- As no data has been made available for this endpoint, sensitization risk is not addressed in this opinion.

- For genotoxicity, a common result for both precursors/couplers and the reaction product is the positive outcome in one or more *in vitro* tests which was not confirmed *in vivo*. It can be deduced that it is not possible to predict the specific outcome of the tests of the reaction product on the basis of the results of the respective precursors/couplers. A final conclusion on the possible genotoxic hazard can be drawn only on the basis of testing.

- The use of (Q)SAR in the regard of reaction products was of limited value since the arylamine structure which is a structural element of many hair dye precursors and reaction products, is identified as an alert.

For the assessment of arylamine-containing complex molecules it is desirable to use or to develop in the future SAR for *in vivo* genotoxicity which satisfies the OECD principles and has a known applicability domain.

- With regard to carcinogenicity of oxidative hair dye formulations in humans, no clear-cut conclusion can be drawn from the studies.
  A definite answer to the question of a causal relationship between personal hair dye use and cancer cannot be expected by epidemiology alone. But from the evaluation of the available studies it can be deduced that for current users of hair dyes marketed in the EU no clear indications for an excess of cancer risk have been demonstrated. This judgement is in line with the evaluation of IARC in 2008: The Working Group considered the epidemiological evidence inadequate, and concluded that personal use of hair colorants is "not classifiable as to its carcinogenicity in humans" (Group 3) (Baan *et al*. 2008).

- It is common practice that oxidative hair dye formulations contain more than one precursor and coupler. Thus, the use of oxidative hair dye may result in exposure of several reaction products simultaneously. This combined exposure of several reaction products has not been considered in this opinion.

Based on the data yet available, the SCCS raises no major concern regarding genotoxicity and carcinogenicity of hair dyes and their reaction products currently used in the EU. However, at present the data base on genotoxicity on reaction products underpinning this conclusion is small and therefore some degree of uncertainty remains. Enlargement of the data base with data on additional reaction products would strengthen the above conclusions drawn from the limited data currently available and further reduce the level of uncertainty. At present, confirmation of safety regarding genotoxicity and carcinogenicity could only be achieved by the use of *in vivo* studies, which, however, are no longer permitted according to EU legislation. In the future, modern methodologies (e.g. skin models, -omics, SAR) may allow the assessment of safety without animal experimentation.

5. MINORITY OPINION

Not applicable

6. REFERENCES

References to 3.2.

Corbett JF, Benzoquinone Imines. Part 1. p-Phenylenediamine-Ferricyanide and p-Aminophenol-Ferricyanide Redox Systems. J. Chem. Soc. (B) 1969: 207-212

Corbett JF, Benzoquinone Imines. Part IX. Mechanism and Kinetics of the Reaction of p-Benzoquinone Di-imines with m-Aminophenols. J. Chem. Soc. Perkin II 1972: 539-548

SCCNFP/0808/04 Ring study on reaction products from typical combinations of hair colouring ingredients. Adopted by the SCCNFP on 23 April 2004

SCCP/0941/05 SCCP opinion on Exposure to reactants and reaction products of oxidative hair dye formulations adopted 13 December 2005.

SCCP/1004/06 SCCP opinion on Update of the Annex to the Opinion (SCCP/0941/05) on Exposure to reactants and reaction products of oxidative hair dye formulations adopted 20 June 2006.

SCCP/1198/08 SCCP opinion on Intermediates and reaction products of oxidative hair dye ingredients formed during Hair dyeing adopted 21 January 2009.

References to 3.3.

CRL study No. 782458 (2007). The *In Vitro* Percutaneous Absorption of Radiolabelled A50/A15/A50 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

CRL study No. 782683 (2007). The *In Vitro* Percutaneous Absorption of Radiolabelled A50/A11/A50 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

CRL study No. 783891 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A154/A027 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

CRL study No. 780707 (2007). The *In Vitro* Percutaneous Absorption of Radiolabelled A005/A011/A005 Product at 3 Concentrations in a Hair Dye Through Human Skin

Harlan study No. 1084900 (2009). Skin Permeability *in vitro* Absorption Through Porcine Ear Skin with A5-A27 Reaction Product Dimer (0.1 %)

CRL study No. 783598 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A007/A11/A007Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

DTL study No. JV2035 (2009). A154/A015 Coupling Product *in vitro* Penetration from a Formulation Through Dermatomed Human Skin

CRL study No. 783582 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A007/A015/A007 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

DTL study No. JV2014 (2009). A154/A017 Coupling Product *in vitro* Penetration from a Formulation Through Dermatomed Human Skin

CRL study No. 783776 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A007/A027 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

CRL study No. 783561 (2008). The *In Vitro* Percutaneous Absorption of Radiolabelled A007/A017/A007 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

Harlan Study No. 1139002 (2009). *In Vitro* percutaneous Absorption Through Frozen Porcine Ear Skin With A74-A27 Reaction Product Dimer (0.1 and 0.5%)

CRL study No. 783577 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A005/A015/A005 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

CRL study No. 782641 (2009). The *In Vitro* Percutaneous Absorption of Radiolabelled A16/A27 Coupling Product at Three Concentrations in a Hair Dye Through Human Skin

The SCCP's Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation, 6th Revision. Adopted by the SCCP during the 10th plenary meeting of 19 December 2006

SCCS/1358/10 Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients.

References to 3.4.

Beal SI. Ways to fit a PK model with some data below the quantification limit. J. Pharmaco. Pharmacodyn. 28: 481-504, 2001.

COC (2003). Committee on Carcinogenicity of Chemicals, Consumer Products and the Environment, UK. Scientific approach on a strategy for the risk assessment of chemical carcinogens. Available at: http://www.advisorybodies.doh.gov.uk/

Delaney EJ. An impact analysis of the application of the threshold of toxicological concern concept on pharmaceuticals. (Reg. Toxicol. Pharmacol. 49: 107-124, 2007.

Garrigue JL, Ballantyne M, Kumaravel T, Lloyd M, Nohynek GJ, Kirkland D, Toutain H. In vitro genotoxicity of para-phenylenediamine and its N-monoacetyl or N,N'diacetyl metabolites. Mut. Res. 608: 58-71, 2006.

Hueber-Becker F, Nohynek GJ, Meuling WJA, Benech-Kieffer F, Toutain H. Human systemic exposure to a [^{14}C]-para-phenylenediamine-containing oxidative hair dye

and correlation with in vitro percutaneous absorption in human or pig skin. Food Chem. Toxicol. 42: 1227-1236, 2004.

IPCS. International Program on Chemical safety. Assessing Human Health Risks of Chemicals: Derivation of Guidance Values for Human Health-Based Exposure Limits. Environmental Health Criteria No. 170. Avalaible at: http://www.inchem.org/documents/ehs/ehc/ehc170.htm

Kuetting B, Goen T, Schwegler U, Fromme H, Uter W, Angerer J, Drexler H. Monoarylamines in the general population- A cross-sectional population-based study including 1004 Bavarian subjects. Int J Hyg Environ Health 212: 298-309, 2009.

Meuling WJA, de Bie ATHJ. Consumer exposure to an actual, dark-shade oxidative hair dye. A [^{14}C]-PPD labelled mass balance study. TNO Report V7852| 2009

Munro IC, Renwick AG, Danielewska-Nikiel B. The threshold of toxicological concern (TTC) in risk assessment. Toxicol. Lett. 180: 151-158, 2008.

Murdoch DJ, Krewski D, Wargo, J. Cancer risk assessment with intermittent exposure. Risk Analysis 12: 569-577, 1992.

Nohynek GJ, Duche D, Garrigues A, Meunier PA, Toutain H, Leclaire J. Under the skin: biotransformation of para-aminophenol and para-phenylenediamine in reconstructed human epidermis and human hepatocytes. Toxicol. Lett. 158: 196-212, 2005.

Renwick AG. Risk characterisation of chemicals present in food. Toxicol. Lett. 149: 163-176, 2004.

Schettgen T, Heinrich K, Kraus T, Gube M. Determination of 2,5-toluylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Archives of Toxicology DOI 10.1007/s00204-010-0563-3, 2010.

Stanley LA, Skare JA, Doyle E, Powrie R, D'Angelo D, Elcombe CR. Lack of evidence for metabolism of p-phenylenediamine by human hepatic cytochrome P450 enzymes. Toxicology 210: 147-157, 2005.

Wester RC, Maibach HI. Percutanous absorption: short-term exposure, lag time, multiple exposures, model variations and absorption from clothing. In Dermatotoxicology, (1996) 5th Ed. Eds. Marzulli FN and Maibach HI. 38-48. Taylor and Francis, Washington DC, US.

References to 3.5

COSMITAL Study No. KP 089 (2004). Cutaneous absorption of WR 18247 through pig skin in vitro.

COSMITAL Study No. KP 152. (2005) Cutaneous Absorption of 1.5 % 4-Amino-2-hydroxytoluene (=WR23032) in a typical hair dye formulation with hydrogen peroxide and reaction partner (WR23005) through pig skin *in vitro*.

COSMITAL Study No. KP 176. (2008) Cutaneous Absorption of 1.5 % 4-Amino-m-crersol (WR23533) in a typical hair dye formulation with hydrogen peroxide and reaction partner 4-Amino-2-hydroxytoluene (A027; WR23032) through pig skin *in vitro*.

CTL Study No. JV1798 (2005). N,N-Bis(2-Hydroxyethyl)-p-Phenylenediamine: *In Vitro* Penetration from a Formulation through Human Skin,

CTL Study No. JV1809 (2005). 1-Naphthol: *In Vitro* Penetration From A Formulation Through Human Skin

Hueber-Becker F, Nohynek GJ, Meuling WJ, Benech-Kieffer F, Toutain H (2004). Human systemic exposure to a [^{14}C]-para-phenylenediamine-containing oxidative hair dye and correlation with in vitro percutaneous absorption in human or pig skin. Food Chem Toxicol, 42(8):1 227-36

Inveresk Study No. 774075, Report No. 24566 (2005). The *In Vitro* Percutaneous Absorption of Radiolabelled Resorcinol Through Human Skin.

L'Oréal Study No. 16 011 (1999). *In Vitro* Percutaneous Absorption of 3-Aminophenol.

L'Oreal Study No. P39 (1997). In vitro transcutaneous absorption of Toluene 2.5-diamine sulfate.

R. Gudi, 2009a: In Vitro Human Lymphocyte Micronucleus Assay with A005-A015-A005, Study Number AC22XX.348.BTL, Bioreliance, Rockville/USA, April 2009

R Gudi, 2009: In Vitro Human Lymphocyte Micronucleus Assay with A007-A017-A007, Study Number AC22XW.348.BTL, Bioreliance, Rockville/USA, April 2009

V. Höpker, 2007: In vitro Micronucleus Assay in Chinese Hamster V79 cells with Dimer A74-A27, RCC-CCR Study Number 1047702, Roßdorf/Germany, February, 2007
N. Honarvar, 2009a: Micronucleus Assay in Bone Marrow Cells of the Rat with R0012228A (reaction product A016-A027), Harlan-CCR Study Number 1237401.
N. Honarvar, 2009b: Micronucleus Assay in Bone Marrow Cells of the Mouse with A74-A27, Harlan-CCR Study Number 1233100, Roßdorf/Germany, April 2009
N. Honarvar, 2009c: In vivo Unscheduled DNA Synthesis in Rat Hepatocytes with 5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one (A005-A015-A005), Harlan-CCR Study Number 1230304, Roßdorf/Germany, August 2009
N. Honarvar, 2009d: In vivo Unscheduled DNA Synthesis in Rat Hepatocytes with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone (A007-A017-A007), Harlan-CCR Study Number 1243800, Roßdorf/Germany, August 2009
M. Lloyd, 2009. Mutation at the *hprt* locus of mouse lymphoma L5178Y cells (MLA) using the Microtitre fluctuation technique with R0012228A (reaction product A16/A27), Covance Study Number 8200616.
M. Reichenbach, 2009. In vivo Unscheduled DNA Synthesis in Rat Hepatocytes with R0012228A (reaction product A016/A027), Harlan-CCR Study Number 1237402.
A. Sokolowski, 2007; Salmonella typhimurium reverse mutation assay with Dimer A74-A27, RCC-CCR Study Number 1047701, Roßdorf/Germany, January 2007
A. Sokolowski, 2009; Salmonella typhimurium Reverse Mutation Assay with 5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one (A005/A015/A005), RCC-CCR Study Number 1230301, Roßdorf/Germany, August 2009
A. Sokolowski, 2009b; Salmonella typhimurium Reverse Mutation Assay with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone (A007/A017/A007), Harlan RCC Study Number 1230401, Roßdorf/Germany, August 2009
J. Whitwell, 2009. Induction of micronuclei in cultured human peripheral blood lymphocytes with R0012228A (reaction product A16/A27), Covance Study Number 8200598.
L. Williams, 2009. Reverse mutation in five histidine-requiring strains of Salmonella typhimurium with R0012228A (reaction product A16/A27), Covance Study Number 8200617.
H.-E. Wollny, 2009a: Cell Mutation Assay at the Hypoxanthine – Guanine – Phosphoribosyl Transferase Locus (HPRT) in Mouse Lymphoma L5178Y Cells with A74-A27, Harlan-CCR Study Number 1243500, Roßdorf/Germany, Draft August 2009
H.-E. Wollny, 2009b: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 5-amino-2-(4-amino-3-methylanilino)-4-[(4-amino-3-methylphenyl)imino]-2,5-cyclohexadien-1-one (A005-A015-A005), Harlan-CCR Study Number 1230302, Roßdorf/Germany, August 2009
H.-E. Wollny, 2009c: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone (A007/A017/A007), Harlan-CCR Study Number 1230402, Roßdorf/Germany, August 2009

References to 3.6.

CEFIC (2002) Report from the workshop on regulatory acceptance of QSARs for human health and environmental endpoints, pp 58. Setubal, Portugal, March 4-6. Report available from rbu@cefic.be.
EC (2003). Technical guidance documents on risk assessment in support of Commission Directive 93/67/EEC on risk assessment for new notified substances, Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances, and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. EUR 20418 EN. European Commission, Joint Research Centre, Ispra, Italy.

ECETOC (2003). (Q)SARs: Evaluation of the commercially available software for human health and environmental endpoints with respect to chemical management applications. Technical Report No. 89, Brussels, Belgium.

ECHA (2008). Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals Guidance for the implementation of REACH. European Chemicals Agency, 2008

Poon GK, Chen Q, Teffera Y, Ngui JS, Griffin PR, Braun MP, Doss GA, Freeden C, Steams RA, Evans DC, Baillie TA, Tang W. Bioactivation of Diclofenac Via Benzoquinone Imine Intermediates-Identification of Urinary Mercapturic Acid Derivatives in Rats and Humans. Drug Metab. Disposition 29: 1608-1613, 2001.

OECD (2003) Organisation for Economic Co-operation and Development ad-hoc expert group on (Q)SARS: Summary conclusion of the 1st meeting and draft work plan. OECD Headquarters, Paris, France.

OECD (2004) The report from the expert group on (quantitative) structure-activity relationships [(Q)SARs] on the principles for the validation of (Q)SARs. 2nd meeting of the ad hoc expert group on QSARs. OECD series on Testing and Assessment, number 49. OECD Headquarters, Paris, France.

References to 3.7.

Altekruse SF, Henley SJ, Thun MJ. Deaths from hematopoietic and other cancers in relation to permanent hair dye use in a large prospective study (United States). Cancer Causes and Control 10: 617-625, 1999.

Andrew AS, Schned AR, Heaney JA, Karagas MR. Bladder cancer risk and personal hair dye use. Int. J. Cancer 109: 571-586, 2004.

Baan R, Straif K, Grosse Y, Secrean B, El Ghissassi F, Bouvard V, Benbrahin-Tallaa L, Cogliano V (2008). Special Report: Policy - Carcinogenicity of some aromatic amines, organic dyes, and related exposures. Lancet Oncology. 9: 322-323.

Benavente Y, Garcia N, Domingo-Domenech E, Alvaro T, Font R, Zhang Y, de Sanjose S. Regular use of hair dyes and risk of lymphoma in Spain. Int. J. Epidemiol. 34: 1118-1122, 2005.

Brown LM, Everett GD, Burmeister LF, Blair A. Hair dye use and multiple myeloma in white men. Am J Public Health 82: 1673-1674, 1992.

Burnett CM, Goldenthal EI. Multigeneration reproduction and carcinogenicity study in Sprague-Dawley rats exposed topically to oxidative hair colouring formulations containing p-phenylenediamine and other aromatic amines. Food Chem. Toxicol. 26: 467-474, 1988.

Burnett CM, Jacobs MM, Seppala A, Shubik P. Evaluation of the toxicity and carcinogenicity of hair dyes. J. Toxicol. Environ. Health (1980) 6: 247-257, 1980.

Cantor KP, Blair A, Everett G, VanLier S, Burmeister L, Dick FR, Gibson RW, and Schuman L. Hair dye use and risk of leukaemia and lymphoma. Am. J. Pub. Health 78:570-571, 1988.

Chiu BC-H, Weisenburger DD, Zahm SH, Cantor KP, Gapstur SM, Holmes F, Burmeister LF, Blair A. Agricultural pesticide use, familial cancer, and risk of non-Hodgkin lymphoma. Cancer Epidemiol Biomarkers Prev. 13: 525-31, 2004.

Chiu BC-H, Dave BJ, Blair A, Gapstur SM, Chmiel JS, Fought AJ, Zahm SH, Weisenburger DD. Cigarette smoking, familial hematopoietic cancer, hair dye use, and risk of 5(14:18)-defined subtypes of non-Hodgkin's lymphoma. Am. J. Epidemiol. 165: 652-659, 2007.

de Sanjose S, Benavente Y, Nieters A, Foretova L, Maynadie M, Cocco PL, Staines A, Vornanen M, Boffetta P, Becker N, Avaro T, Brennan P. Association between personal use of hair dyes and lymphoid neoplasms in Europe. Am. J. Epidemiol. 164: 47-55, 2006.

Feizy V, Toosi S. Relation of basal cell carcinoma to hair dye use. J Am Acad Derm. 61: 532, 2009.

Gago-Dominguez M, Castelao JE, Yuan J-M, Yu MC, Ross RK. Use of permanent hair dyes and bladder-cancer risk. Int. J. Cancer 91: 575-579, 2001.

Gago-Dominguez M, Bell DA, Watson MA, Yuan J-M, Castelao JE, Hein DW, Chan KK, Coetzee GA, Ross RK, Yu MC. Permanent hair dyes and bladder cancer: risk modification by cytochrome P4501A2 and N-acetyltransferases 1 and 2. Carcinogenesis 24: 483-489, 2003.

Giles AL, Chung CW. Dermal carcinogenicity study by mouse skin painting with 2,4-toluenediamine alone or representative hair dye formulations. J. Toxicol. Environ. Health 1: 433-440, 1976.

Grodstein, F Hennekens CH, Colditz GA, Hunter DJ, Stampfer MJ. A prospective study of permanent hair dye use and hematopoietic cancer. J. Natl. Cancer Inst. 86: 1466-1470, 1994.

Herrinton LJ, Weiss NS, Koepsell TD, Daling JR, Taylor JW, Lyon JL. Exposure to hair-coloring products and the risk of multiple myeloma. Am J Public Health. 84: 1142-1144, 1994.

Henley SJ, Thun MJ. Use of permanent hair dyes and bladder-cancer risk. Int. J. Cancer 94: 903-904, 2001.

Holly EA, Lele C, Bracci PM. Hair-colour products and risk for non-Hodkgin's lymphoma: a population-based study in the San Francisco Bay Area. Am. J. Public Health 88: 1767- 1773, 1998.

Holman CDJ, Armstrong BK. Hutchinson's melanotic freckle melanoma associated with nonpermanent hair dyes. Br J Cancer 48: 599-601, 1983.

Holman CD, Armstrong BK. Hutchinson's melanotic freckle melanoma and the use of nonpermanent hair dyes. Br J Cancer 52: 135, 1983.

Hunchareck M, Kupelnick B. Personal use of hair dyes and the risk of bladder cancer: results of a meta-analysis. Public Health Reports 120: 31-38, 2005.

Ido M, Nagata C, Kawakami N, Shimizu H, Yoshida Y, Nomura Y, Mizoguchi H. A casecontrol study of myelodysplastic syndromes among Japanese men and women. Leuk. Res. 20: 727-731, 1996.

International Agency for Research on Cancer. Occupational Exposures of Hairdressers and Barbers and Personal Use of Hair Colourants; Some Hair Dyes, Cosmetic Colourants, Industrial Dyestuffs and Aromatic Amines. World Health Organization, Lyon. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 57, 1993.

International Agency for Research on Cancer. Some Industrial and Cosmetic Dyes, and Related Exposures. World Health Organization, Lyon. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 99. In press.

Jacobs MM, Burnett CM, Penicnak AJ, Herrera JA, Morris WE, Shubik P, Apaja M, Granroth G. Evaluation of the toxicity and carcinogenicity of hair dyes in Swiss mice. Drug Chem. Toxicol. 7: 573-586, 1984.

Kelsey KT, Hirao T, Hirao S, Devi-Ashok T, Nelson HH, Andrew A, Colt J, Baris D, Morris JS, Schned A, Karagas M. TP53 alterations and patterns of carcinogen exposure in a U.S. population-based study of bladder cancer. Int. J. Cancer 117: 370–375, 2005.

Kelsh MA, Alexander DD, Kalmes RM, and Buffler PA. Personal use of hair dyes and risk of bladder cancer: a meta-analysis of epidemiologic data. Cancer Causes Control 19:549-558, 2008.

Kinkel HJ and Holzmann S. Study of long-term percutaneous toxicity and carcinogenicity of hair dyes in rats. Food Cosm. Toxicol. 11: 641-648, 1973.

Kogevinas M, Fernandez F, Garcia-Closas M, Tardon A, Garcia-Closas R, Serra C, Carrato A, Castanao-Vinyas G, Yeager M, Chanock SJ, Lloreta J, Rothman N, Real FX, Dosemici M, Malats N, Silverman D. Hair dye use is not associated with risk for bladder cancer: Evidence from a case-control study in Spain. Eur. J. Cancer 42: 1448-1454, 2006.

Koutros S, Baris D, Bell E, Zheng T, Zhang Y, Holford TR, Leaderer BP, Landgren O, and Zahm SH. Use of hair coloring products and risk of multiple myeloma among U.S. women. Occup. Environ. Med. 66: 68-70, 2009.

Lin J, Dinney CP, Grossman HB, Wu X. Personal permanent hair dye use is not associated with bladder cancer risk: Evidence from a case-control study. Cancer Epidemiol. Biomarkers Prev. 15: 1746-1749, 2006.

Linos A, Kiamouris C, Foukenelis T, Kourousis C, Hatzis C, Stefanoudaki K. A case-control study of non-Hodgkin's lymphoma. J. Epidemiol. 139: S46, 1994.

Mele A, Szklo M, Visani G, Stazi MA, Castelli G, Pasquini P, Mandelli F. Hair dye use and other risk factors for leukaemia and preleukaemia: a casecontrol study. Am J Epidemiol. 139: 609-619, 1994.

Mele A, Stazi MA, Pulsoni A, Visani G, Monarca B, Castelli G, Rocchi L, Avvisati G, and Mandeli F. Epidemiology of acute promyelocytic leukemia. Haematologica 80: 405-408, 1995.

Mele A, Visani G, Pulsoni A, Monarca B, Castelli G, Stazi MA, Gentile G, and Mandelli F. Risk factors for essential thrombocythemia: A case-control study. Cancer 77: 2157-2161, 1996.

Mendelsohn, JB, Li Q-Z, Ji B-T, Shu X-O, Yang G, Li H-L, Lee KM, Yu K, Rothman N, Gao Y-T, Zheng Q and Chow W-H. Personal use of hair dye and cancer risk in a prospective cohort of Chinese women. Cancer Sci. 100:1088-1091, 2009.

Miligi L, Costantini AS, Crosignani P, Fontana A, Masala, G, Nanni O. Ramazzotti V, Rodella S, Stagnaro E, Tumino R, Vigano C, Vindigni C, Vineis P. Occupational, environmental, and life-style factors associated with the risk of hematolymphopoietic malignancies in women. Am. J. Ind. Med. 36: 60-69, 1999.

Morton LM, Bernstein L, Wang SS, Hein DW, Rothman N, Colt JS, Davis S, Cerhan JR, Severson RK, Welch R, Hartge P, Zahm SH. Hair dye use, genetic variation in Nacetyltransferase 1 (NAT1) and 2 (NAT2), and risk of non-Hodgkin lymphoma. Carcinogenesis 28: 1759-1764, 2007.

Nagata C, Shimizu H, Hirashima K, Kakishita E, Fujimura K, Niho Y, Karasawa M, Oguma S, Yoshida Y, Mizoguchi H. Hair dye use and occupational exposure to organic solvents as risk factors for myelodysplastic syndrome Leuk. Res. 23: 57-62, 1999.

Osterlind A, Tucker MA, Stone BJ, Jensen OM. The Danish case-control study of cutaneous malignant melanoma, IV: no associationwith nutritional factors, alcohol, smoking or hair dyes. Int J Cancer. 1988; 42: 825-828, 1988.

Rauscher GH, Shore D, Sandler DP. Hair dye use and risk of adult acute leukaemia. Am. J. Epidemiol. 160: 19-25, 2004.

Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CK, Schuman LM, Tolbert PE, Rothman N, Cantor KP and Blair A. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin's lymphoma (United States). Cancer Causes Control 13:159-168, 2002.

Serretta V, Morgia G, Altieri V, Pavone-Macaluso M, Scuto F, Allegro R, Di Lallo A, Cindolo L, Melloni D. Preliminary report of a multicentric study on environmental risk factors in Ta-T1 transitional cell carcinoma of the bladder. Urol Int. 77: 152-158, 2006.

Stavraky KM, Clarke EA, and Donner A. A case-control study of hair-dye use and cancers of various sites. Br. J. Cancer 43:236-239, 1981.

Takkouche B, Etminan M, Montes-Martinez A. Personal use of hair dyes and risk of cancer. JAMA 293: 2516-2525, 2005.

Takkouche B, Regueira-Méndez C, Montes-Martínez A. Risk of cancer among hairdressers and related workers: a meta-analysis Inter J Epidemiology 38: 1512-1531, 2009.

Tavani A, Negri E, Franceschi S, Talamini R, Serraino D, La Vecchia C. Hair dye use and risk of lymphoid neoplasms and soft tissue sarcomas. Int. J. Cancer 113: 629-631, 2005.

Thun MJ, Altekruse SF, Namboodiri MM, Calle EE, Myers DG, Heath CW. Hair dye use and risk of fatal cancers in U.S. women. J. Natl. Cancer Inst. 86: 210-215, 1994.

Zahm SH, Weisenburger DD, Babbitt PA, Saal RC, Vaught JB, Blair A. Use of hair colouring products and the risk of lymphoma, multiple myeloma, and chronic lymphocytic leukaemia. Am. J. Public Health 82: 990-997, 1992.

Zhang Y, Holford TR, Leaderer B, Boyle P, Zahm SH, Flynn S, Tallini G, Owens PH, and Zheng T. Hair-colouring product use and risk of non-Hodgkin's lymphoma: a population-based case-control study in Connecticut. Am. J. Epidemiol. 59:148-154, 2004.

Zhang Y, De Sanjose S, Bracci PM, Morton LM, Wang R, Brennan P, Hartge P, Boffetta P, Becker N, Maynadie M, Foretova L, Cocco P, Staines A, Holford T, Holly EA, Nieters A, Benavente Y, Bernstein L, Zahm SH, Zheng T. Personal Use of Hair Dye and the

Risk of Certain Subtypes of Non-Hodgkin Lymphoma. Am J Epidemiol 167: 1321-1331, 2008.

References to 3.8.

SCCP/1198/08 SCCP opinion on Intermediates and reaction products of oxidative hair dye ingredients formed during Hair dyeing adopted 21 January 2009.

SCCS/1358/10 Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients

Baan R, Straif K, Grosse Y, Secrean B, El Ghissassi F, Bouvard V, Benbrahin-Tallaa L, Cogliano V (2008). Special Report: Policy - Carcinogenicity of some aromatic amines, organic dyes, and related exposures. Lancet Oncology. 9: 322-323.