EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

UPDATED RECOMMENDED STRATEGY FOR TESTING OXIDATIVE HAIR DYE SUBSTANCES FOR THEIR POTENTIAL MUTAGENICITY/GENOTOXICITY

(SCCP'S NOTES OF GUIDANCE)

Adopted by the SCCP
during the 7th plenary of 28 March 2006

TABLE OF CONTENTS

1. INTRODUCTION

In the 3rd plenary SCCP meeting of 15 March 2005, a temporary working group on mutagenicity/genotoxicity was established to update the existing opinion on "Recommended strategy for testing hair dyes for their potential genotoxicity/mutagenicity/carcinogenicity" [SCCNFP/0720/03]. The resulting final opinion will subsequently be taken up in the updated Notes of Guidance of the Committee, 2006.

As stated in several previous opinions, hair dyeing formulations belong to 3 categories, i.e. for temporary, semi-permanent and permanent colouring of hair.

- The formulations for temporary dyeing of hair generally comprise of water soluble acid dyes and water soluble pigments, which are deposited on the surface of the hair. These colours are removable by a single effective shampooing.
- The formulations for semi-permanent dyeing of hair contain, for the most part, simple derivatives of nitroanilines, nitrophenylenediamines and nitroaminophenols. These molecules penetrate into the cuticle and partially into the cortex of the hair. As a result, the colouring effects can resist 5-10 shampooings.
- The third category, namely oxidative or permanent hair dye formulations contain the so-called oxidative or permanent hair dye substances. The latter are the subject of the current opinion. All other ingredients in the permanent hair dye formulation, not being precursors or couplers, follow the general SCCP guidance for genotoxicity / mutagenicity testing of cosmetic ingredients [SCCNFP/0755/03]. The oxidative hair dye formulations are marketed as two component kits. One component contains the dye precursors (such as p-phenylenediamine, 2,5- diaminotoluene, N,N-bis(2-hydroxymethyl)-p-phenylenediamine, p-aminophenol etc.) and couplers (such as resorcinol, chlororesorcinol, methyl resorcinol, alpha-naphthol, m-aminophenol, etc.) in an alkaline soap or syndet base. The other component is a stabilised solution of hydrogen peroxide. The two components are mixed immediately prior to use. The precursors and peroxide diffuse into the hair shaft, where colour formation takes place after a cascade of chemical reactions. The dye precursors are oxidised by hydrogen peroxide to p-benzoquinone imines/diimines, which are reactive intermediates in the colour formation. The couplers, which are relatively stable to hydrogen peroxide, undergo rapid reaction with the intermediates resulting in dinuclear, trinuclear or polynuclear colour molecules. These molecules are usually too large to escape from the hair structure. The hydrogen peroxide present in the oxidative hair dye formulations also serves as bleaching agent for the natural pigment of the hair.

  The colour formation (shades) is dependent on precursors present in the dyeing solution, its pH and the time of contact with the hair [Zviak 1986; Corbett 1984; Corbett 1991]. The shade is further determined by addition of other colorant molecules, being semi-permanent or temporary. Oxidative hair dyes are resistant to fading by shampooing and re-colouring of hair is only required every 4 to 6 weeks, due to hair growth. The time of contact of the dyeing solution with the hair scalp may vary from 15-45 minutes. Oxidative hair dye formulations represent the major segment of the hair colouring market.

In particular the assessment of the genotoxic/mutagenic/carcinogenic potential of oxidative hair dye formulations is rather complex, because dye precursors, intermediates and final reaction products are involved at the same time. Therefore, it is the opinion of the SCCP that the hazard of the oxidative hair dye molecules should be evaluated not only by testing the individual colour

ingredients but also the combination of those ingredients that form together the reaction products as occurring during the application of permanent hair dye formulations on hair and scalp.

2. RECOMMENDED MUTAGENICITY / GENOTOXICITY TESTING

2.1 Preamble

1. Mutations are defined as heritable changes of the amount or the structure of the genetic material of a cell or an organism. These alterations may involve:

   a. genes (intragenic changes, gene loss or changes stretching beyond a gene)
   b. chromosome structure (intra- and inter-chromosomal changes)
   c. chromosome number (aneuploidy and polyploidy).

2. Genotoxicity is used as a generic term, which includes the induction of mutations (gene mutations, structural chromosome mutations, genome mutations) as well as so-called indicator effects which are related to the induction of mutations (e. g. DNA damage, DNA repair, recombination). Genotoxicity tests comprise mutagenicity tests and indicator tests (e.g. Unscheduled DNA Synthesis or UDS-test, Sister Chromatid Exchange or SCE-test, Comet-assay).

3. During the risk assessment process of chemicals it is necessary to pay attention to both mutagenicity and indicator tests. Whereas basic testing almost always relies on mutagenicity tests, indicator tests are usually used in follow-up testing when further information is needed (e.g. for clarification of the significance of positive findings). In addition to the characterization of the intrinsic genotoxic potential of chemicals, the extent of human exposure may drive the need for additional testing.

   Originally, mutagenicity/genotoxicity testing aimed at detection of germ cell mutagens. In current regulatory practice emphasis is put on screening for possible carcinogenic substances ("genotoxic carcinogens").

2.2 SCCNFP/0720/03 testing strategy

In 2003, the SCCNFP recommended for oxidative hair dyes 6 *in vitro* tests in order to obtain information on (1) gene mutations, (2) structural chromosome aberrations (clastogenicity), (3) numerical chromosome aberrations (aneugenicity), (4) DNA damages and (5) carcinogenicity:

1.	bacterial reverse mutation test:	EC B.13/14	OECD 471
2.	*in vitro* mammalian chromosome aberration test:	EC B.10	OECD 473
3.	*in vitro* mammalian cell gene mutation test:	EC B.17	OECD 476
4.	UDS in mammalian cells *in vitro*:	EC B.18	OECD 482
5.	*in vitro* micronucleus test:		OECD 487 (draft)
6.	*in vitro* SHE (Syrian Hamster Embryo) cell transformation assay:		OECD 495

In the event of positive results, *in vivo* tests were to be considered, but only after thorough review of the *in vitro* results and of all the available data on toxicokinetics and toxicodynamics of the oxidative hair dye under consideration [SCCNFP/0720/03].

2.3 Further progress

Since its publication, the SCCNFP opinion [SCCNFP/0720/03] has been commented extensively [BfR 2004] and the majority of industry's remarks were bundled in a paper issued by Kirkland et al. [Kirkland et al. 2005].
In order to examine the progress in current knowledge and the different arguments put forward, a number of SCCP members discussed all newly available information with invited experts in the field.

2.4 Discussion

2.4.1 Basic mutagenicity/genotoxicity testing

The regulations and/or guidance on biocides [98/8/EC], food additives [SCF 2001a], food contact materials [SCF 2001b] and cosmetic ingredients in general [SCCNFP/0755/03], all mention a basic set of mutagenicity/genotoxicity studies containing 3 *in vitro* tests. These are:

1.	bacterial reverse mutation test:	EC B.13/14	OECD 471
2.	*in vitro* mammalian cell gene mutation test:	EC B.17	OECD 476
3a.	*in vitro* micronucleus test:		OECD 487 (draft)
3b.	*in vitro* mammalian chromosome aberration test:	EC B.10	OECD 473

Hair dyes are the only category of substances for which a standard testing battery of 6 *in vitro* tests has been recommended [SCCNFP/0720/03].

From the extensively reviewed scientific literature, it appears [Kirkland et al. 2005] that the majority of mutagens, including clastogens, can be detected either by the *in vitro* induction of micronuclei, as being positive in the bacterial reverse mutation test, or as inducing mutations in the *in vitro* mammalian cell gene mutation test. Therefore, in order to detect clastogens, the previously proposed chromosomal aberration test [SCCNFP/0720/03] can be omitted.

Therefore, the strategy of using a basic 3-test battery (namely 1. and 2, plus 3a. or 3b.) is considered by the SCCP as being sufficiently safe to detect mutagens.

Nevertheless, taking into consideration the concerns expressed in previous opinions with respect to the potential carcinogenicity of oxidative hair dyes [SCCNFP/0484/01, SCCNFP/0495/01, SCCNFP/0797/04, SCCP/0930/05], additional provisions with respect to dermal absorption and metabolic activation have to be taken into account.

(i) <u>Dermal absorption:</u>
Dermal absorption should be addressed as a first step in safety evaluation. Performing state of the art *in vitro* dermal absorption studies is of key importance.
For the formed products further testing may, in certain cases, not be necessary (see further on).

(ii) Metabolic activation:

The available information on the biotransformation of the compound under investigation should be provided.

Metabolic activation has to be considered in all cases and it is essential to include an exogenous source of metabolic activation in the study protocol of an *in vitro* genotoxicity test in accordance with the EC B / OECD guidelines.

Species differences do exist and enzyme competence is of primary importance. When liver S9 is used in tests with bacterial or mammalian cells, one must be aware that some key phase I and phase II enzymes could be absent or not sufficiently active because of missing co-factors. Primary hepatocytes tend to perform better than cell lines but their performance is depending on several factors including species, gender, freshness of the cell preparation, cell culture conditions and other factors.

In case N-acetylation of the exocyclic aminogroup is involved in activation, respectively detoxification (e.g. in aromatic amines, azo-dyes, ...), functional expression of N-acetyltransferase activity (NAT1 and NAT2), which is known to be tissue- and species-specific, is necessary [Hein 2002]. Even so the expression of sulfotransferase activity (SULT1A1 and SULT1A2) may be important (e.g. in amino-, amido- and nitro-arenes) [Glatt 2000, Glatt and Meinl 2004, Muckel et al. 2002].

Although still under scientific development, tests using genetically engineered Salmonella typhimurium strains or Chinese hamster cell lines expressing human SULT and NAT isoenzymes, could be performed [Glatt and Meinl 2005]. Fundamental questions, however, with respect to cellular uptake, robustness of test, etc., are not yet completely solved. Justification should be given for the chosen methodology.

2.4.2 Assessment of mutagenicity/genotoxicity on a case-by-case basis

The 3-test battery, including considerations on dermal absorption and metabolic activation, forms the basis of the mutagenicity/genotoxicity risk assessment strategy for oxidative hair dye formulations. QSAR (Quantitative Structure-Activity Relationship) data can provide useful additional information and may also be a tool for priority setting [Franke et al. 2001, Benigni and Passerini 2002, Benigni 2005].

In the case of a positive *in vitro* result or whenever doubt exists, additional testing may be necessary on a case-by-case basis allowing the necessary flexibility.
The potential to induce mutagenic / genotoxic effects has to be excluded for (i) the precursors and couplers, (ii) the formed products and (iii) the intermediates formed.

(i) Precursors and couplers

For these substances, the basic set of 3 *in vitro* tests with special attention for dermal absorption and metabolic activation, is recommended.

When a positive outcome is found in one (or more) of these *in vitro* assays, further *in vivo* testing is necessary.

This is in particular the case when, according to the dermal absorption results, systemic exposure cannot be excluded.

(ii) Formed products

When, on the basis of the *in vitro* dermal absorption results of the final products formed, systemic exposure to one (or more) of these molecules occurs, the standard 3-test battery for ingredients should be carried out. Additional testing may be necessary. When no or only minimal dermal absorption occurs (estimated towards criteria set e.g. on the basis of the Threshold of Toxicological Concern or TTC-concept [Kroes et al. 2005]), no further testing is necessary for the final products formed.

(iii) Intermediates

If concern exists that reactive intermediates could be formed, additional testing data should be made available or relevant scientific justification for the unsuitability of additional testing should be provided in order to create more confidence to support the substance under consideration.

Actually, no validated *in vitro* tests are available to cover this aspect. Consequently, only *in vivo* testing is feasible at this moment, e.g. the application of the oxidative hair dye mixture on the skin of experimental animals and subsequent performance of a comet assay or a micronucleus test. The SCCP encourages further developments in this field.

Before performing an *in vivo* test, it must be known whether relevant exposure of the target organ(s) occurs in that particular *in vivo* test. Especially, when bone marrow is used as the target organ, relevant systemic availability has to be demonstrated. Relevant data on toxicokinetics have to be provided and intraperitoneal application should be considered.

3. CONCLUSION

The recommended base set of *in vitro* mutagenicity/genotoxicity assays for oxidative hair dye substances, consists of 3 tests (1 + 2 and 3a or 3b):

1.	a bacterial reverse mutation test:	EC B.13/14	OECD 471
2.	an *in vitro* mammalian cell gene mutation test[1]:	EC B.17	OECD 476
3a.	an *in vitro* micronucleus test:		OECD 487 (draft)
3b.	an *in vitro* mammalian chromosome aberration test:	EC B.10	OECD 473

The following tests are considered to provide additional useful information / confirmation, but are not considered forming part of the basic requirements:

an UDS in mammalian cells *in vitro*:	EC B.18	OECD 482
an *in vitro* SHE cell transformation assay:		OECD 495

Should the *in vitro* testing battery show positive results and/or should QSAR results, physicochemical data or any other indication suggest potential mutagenicity or genotoxicity, additional testing may be required.

[1] Preferentially, the mouse lymphoma thymidine kinase assay, because it detects gene mutations and chromosomal effects.

The choice of the additional *in vitro* / *in vivo* tests will be performed on a case-by-case basis, taking into account the following considerations:

1) Sound *in vitro* dermal absorption studies generate indispensable data on the potential systemic availability of starting material and reaction products.

2) Metabolic activation should be considered in the decision making process.

3) Although not considered as first choice, it could be necessary to perform *in vivo* mutagenicity/genotoxicity assays in which the oxidative hair dye mixture is applied on the skin of experimental animals (e.g. nude mice) followed by a micronucleus test and/or a comet assay on the skin. Further development, standardization and validation of these methods are needed, but their use is encouraged.

4. REFERENCES

98/8/EC - Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. *Official Journal L123, 24/04/1998 p.1.*

Benigni R., Passerini L. Carcinogenicity of the aromatic amines: from structure activity relationships to mechanisms of action and risk assessment. Mutation Research 511, 191-206 (2002).

Benigni R. Structure-activity relationship studies of chemical mutagens and carcinogens: mechanistic investigations and prediction approaches. Chemical Reviews, 105, 1767-1800 (2005).

BfR - (Bundesinstitut für Risikobewertung). Comment on the "Proposal for Recommended Mutagenicity / Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC" of the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers (SCCNFP), 09 Dec 2003 [SCCNFP/0755/03]. Issued 12/02/2004.

Corbett J.F. Chemistry of hair colorant processes – science as an aid to formulation and development. Journal of the Society of Cosmetic Chemists 35, 297-310 (1984).

Corbett J.F. Hair coloring processes. Cosmetics & Toiletries 106, 53-57 (1991).

EC B.10 - Mutagenicity - *in vitro* mammalian chromosome aberration test. Commission Directive 2000/32/EC of 19 May 2000 adapting to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal L 136, 08/06/2000 p.35.*

EC B.13/14 - Mutagenicity - reverse mutation test bacteria. Commission Directive 2000/32/EC of 19 May 2000 adapting to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal L 136, 08/06/2000 p.57.*

EC B.17 - Mutagenicity - *in vitro* mammalian cell gene mutation test. Commission Directive 2000/32/EC of 19 May 2000 adapting to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal L 136, 08/06/2000 p.65.*

EC B.18 - DNA damage and repair - unscheduled DNA synthesis - mammalian cells *in vitro.* Commission Directive 88/302/EEC of 18 November 1987 adapting to technical progress for the ninth time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal L 133, 30/05/1988 p.64.*

Franke R., Gruska A., Giuliani A., Benigni R. Prediction of rodent carcinogenicity of aromatic amines: a quantitative structure-activity relationships model. Carcinogenesis 22(9), 1561-1571 (2001).

Glatt H. Sulfotransferases in the bioactivation of xenobiotics. Chemico-Biological Interactions 129 (1-2), 141-170 (2000).

Glatt H., Meinl W. Pharmacogenetics of soluble sulfotransferases (SULTs). Naunyn Schmiedebergs Arch Pharmacol. 369(1), 55-68 (2004).

Glatt H., Meinl W. Sulfotransferases and acetyltransferases in mutagenicity testing: technical aspects. Methods Enzymol.400, 230-49 (2005).

Hein D.W. Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. Mutation Research 506-507, 65-77 (2002).

Kirkland D.J., Henderson L., Marzin D., Müller L., Parry J.M., Speit G., Tweats D.J., Willams G.M. Testing strategies in mutagenicity and genetic toxicology : An appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutation Research 588, 88-105 (2005).

Kroes R., Kleiner J., Renwick A. The threshold of toxicological concern concept in risk assessment. Toxicological Sciences 86, 226-230 (2005).

Muckel E., Frandsen H., Glatt H.R. Heterologous expression of human N-acetyltransferases 1 and 2 and sulfotransferase 1A1 in Salmonella typhimurium for mutagenicity testing of heterocyclic amines. Food Chem Toxicol. 2002 Aug;40(8):1063-8

OECD 471 - OECD Guideline for testing of chemicals - Guideline 471 : Bacterial Reverse Mutation Test. Organization for Economic Cooperation and Development, Paris, adopted 26 May 1983, last updated 21 July 1997.

OECD 473 - OECD Guideline for testing of chemicals - Guideline 473 : *In vitro* Mammalian Chromosomal Aberration Test. Organization for Economic Cooperation and Development, Paris, adopted 26 May 1983, last updated 21 July 1997.

OECD 476 - OECD Guideline for testing of chemicals - Guideline 476 : *In vitro* Mammalian Cell Gene Mutation Test. Organization for Economic Cooperation and Development, Paris, adopted 4 April 1984, last updated 21 July 1997.

OECD 482 - OECD Guideline for testing of chemicals - Guideline 482 : Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro.* Organization for Economic Cooperation and Development, Paris, adopted 23 October 1986.

OECD 487 - OECD Guideline for testing of chemicals - Draft Guideline 487 : *In vitro* Micronucleus Test. Organization for Economic Cooperation and Development, Paris, draft approved 14 June 2004.

OECD 495 - OECD Guideline for testing of chemicals - Draft Guideline 495 : *In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay. Organization for Economic Cooperation and Development, Paris, draft approved

SCCNFP/0484/01, Final : Opinion on the use of permanent hair dyes and bladder cancer risk, *adopted by the SCCNFP during the 17th plenary meeting of 12 June 2001*.

SCCNFP/0495/01, Final : Opinion concerning the safety review of the use of certain azo-dyes in cosmetic products, *adopted by the SCCNFP during the 19th plenary meeting of 27 February 2002*.

SCCNFP/0720/03, Final : Updated recommended strategy for testing hair dyes for their potential genotoxicity/mutagenicity/carcinogenicity, *adopted by the SCCNFP during the 24th plenary meeting of 24-25 June 2003*.

SCCNFP/0755/03, Final : Recommended Mutagenicity/Genotoxicity tests for the safety testing of cosmetic ingredients to be included in the annexes to Council Directive 76/768/EEC (SCCNFP Notes of Guidance), *adopted by the SCCNFP on 23 April 2004 by means of the written procedure.*

SCCNFP/0797/04, Final : Opinion concerning use of permanent hair dyes and bladder cancer, *adopted by the SCCNFP on 23 April 2004 by means of the written procedure*.

SCCP/0930/05 : Opinion on personal use of hair dyes and cancer risk, *adopted by the SCCP during the 5th plenary meeting of 20 September 2005*.

SCF (Scientific Committee on Food). Guidance on Submission on Food Additive Evaluations by the Scientific Committee on Food : SCF/CS/ADD/GEN/26/Final, 12th July 2001a.

SCF (Scientific Committee on Food). Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact material prior to its authorisation. SCF/CS/PLEN/GEN/100 Final, 19 December 2001b.

Zviak C. The Science of Hair Care. Marcel Dekker Inc., New York and Basel, pp.273-286 (1986).

5. ACKNOWLEDGEMENTS

Members of the working group are acknowledged for their valuable contribution to this opinion. The members of the working group are:

Prof. G. Degen	Prof. J. Revuz	
Dr. B. Jazwiec-Kanyion	Prof. V. Rogiers	(chairperson and rapporteur)
Prof. V. Kapoulas	Prof. T. Sanner	
Prof. J.-P. Marty	Prof. G. Speit	
Prof. T. Platzek	Dr. J. van Engelen	
Dr. S.C. Rastogi	Dr. I.R. White	

Invited experts

Prof. A. Carere
Prof. H.-R. Glatt
Dr. Herbold
Prof. R. Sram