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

OPINION

CONCERNING

"REPORT FOR ESTABLISHING THE TIMETABLE FOR PHASING OUT ANIMAL TESTING FOR THE PURPOSE OF THE COSMETICS DIRECTIVE" ISSUED BY ECVAM (30/04/2004)

adopted by the SCCNFP on 1 July 2004 by means of the written procedure
INTRODUCTION

As stated in the overview and executive summary, the aim of the report issued by ECVAM and titled "Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive" of 30/04/2004 is to provide an objective overview of the current status of alternative methods/strategies and the prospects for their validation and regulatory acceptance so that they can be used for replacing animal tests in the safety assessment of cosmetic products as required by the EU cosmetics Directive.

In order to achieve this, the currently used animal tests have been sub-divided in 11 areas and each of those areas has been investigated by a group of experts in the field. The groups were asked to include in their individual section:

- a table summarizing the inventory of the most valuable alternative methods currently known to be available,
- the identification of the gaps left by the in vitro methods compared to the animal test,
- recommendations for achieving full replacement of animal tests,
- the time estimated as necessary to achieve full replacement of animal tests.

With regard to this complex aim of the document, the SCCNFP would like to make the following comments:

1) Alternative methods are not restricted to replacement tests, while the 7th Amendment of the cosmetic products clearly demands non-animal tests (see Art.4a: "Member States shall prohibit : ... the performance on their territory of animal testing ...".

It should be made clear in the first lines of the report and emphasized in the executive summary that the definition of an alternative method is based on the 3R principle of Russell and Burch [Russell et al. 1959] and not on replacement alone. Currently it is not obvious that the alternative methods/strategies will fit into the provisions of the 7th amendment.

The SCCNFP wants to clearly give the message to the Commission that total abolishment of animal tests within 10 years is not feasible from an objective scientific point of view. Even the alternative strategies discussed in the document, which are estimated to take more than 10 years for further development, still include an animal test in the final tier. In its "Opinion on the BUAV-ECEAE* report on "The way forward - action to end animal toxicity testing", adopted in January 2004, the CSTEE† also discusses several aspects of the development of alternative methods and finally comes to the conclusion that, for the foreseeable future, the use of live animals in toxicity testing is essential in order to perform reliable risk assessments [CSTEE 2004].

2) The terms in vitro, in vivo, ex vivo, in silico, replacement test should be clearly defined and used correctly and consistently throughout the document, especially keeping in mind the provisions of the 7th amendment of the cosmetic directive [2003/15/EC]. An in vitro method is not necessarily a non-animal test. The starting point of many cell cultures, tissue cultures, auxiliary mixes (such as the S9 mix), as well as some components of the culture medium, are animal-derived, which means that there is no total replacement. The terms are not always correctly used and this is very likely to lead to misinterpretation and wrong conclusions.

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* British Union for the Abolition of Vivisection - European Coalition to End Animal Experiments
† Scientific Committee on Toxicity, Ecotoxicity and the Environment
3) The chapters of the document are unacceptably different in quality and layout. The document reflects the way it has been composed, namely by different people with different interests. The SCCNFP acknowledges that the involvement of all the stakeholders is the strength of the document, but not enough time has been devoted to consistency, coherence and quality of the final document.

It is the opinion of the SCCNFP that each chapter should first provide a description of the known mechanisms of the typical effects to be observed in vivo, a description of the current in vivo tests and their endpoints in the different areas of use. This should be followed by a list of the available alternatives, with their individual:

- short descriptions, scientific relevance and purpose
- method developer(s)
- known users
- status of validation and/or standardization
- fields of application and limitations
- recommended use in the options of animal replacement
- on-going developments
- efforts needed to complete validation
- key references

Since only one or two chapter attain this quality level, the actual document should be revised seriously by a small team of competent people.

4) Not all the sections in the document answer the relevant questions. The identification of the gaps left by the in vitro methods compared with the animal test, is often neglected. However, this is the starting point of the whole future development of alternatives. In many cases (especially for systemic toxicity), all the gaps are not identified, due to insufficient knowledge of the underlying mechanisms of human and animal toxicity. These shortcomings should also be highlighted for each chapter. In these cases, before the development of an alternative method can even be considered, a basic research effort has to be involved.

5) It is to be regretted that the previous inventory made by ECVAM [Worth et al. 2002], which gave a comprehensive overview of the available alternative methods until 2002, has not been used as a solid basis for a detailed update. Although the timetables proposed at that time were too optimistic, the inventory as such was correct and could have been used as a starting document. Only the inclusion of missing tests or further developments would have been required, leaving more time available to specifically concentrate on picking out the most promising methods with their individual timetables, as well as on the prioritising of tests.

6) It is not clear what the exact sources are to be for the developmental work to be done. It is not mentioned whether ECVAM, industry, the academic world, tests from the USA, EU projects, ... are to be involved. It is not clear who is going to do what, in particular when gaps are not known or poorly identified. When even no proposals for method development exist, responsibilities for developing and financing strategies for research in these areas should be defined. The Commission should establish, in analogy with the validation responsibility of ECVAM, a responsible unit or committee for the overall research strategy to fulfill the requirements of the 7th Amendment [2003/15/EC] to the Cosmetics Directive [76/768/EEC].

7) The chapter authors were asked to give an indication of the time necessary for full validation, assuming that optimal conditions are met. It is strongly advised to give an indication
on those so-called "optimal conditions". What is the order of magnitude of the financial funding and the staff requirements? There is no use in giving an indication of time line if the "optimal conditions" are not feasible.

8) There has been a final stakeholders meeting and it seems that the final document, composed by the chapters produced by the individual working groups, has been adapted in terms of timeframes involved. It is recommended to include the stakeholders' comments as an annex to the final document, and not to change deadlines agreed upon within the individual working groups.

9) In chapters 2 and 4, there is reference to the previous version of the Notes of Guidance. Especially when discussing human volunteer studies, it must be emphasized that the most recent version of the Notes (SCCNFP/0690/03) is different in content and layout and does no longer contain the detailed information on these specific opinions. Consequently, reference should be made to the individual opinions on the subject.

10) Finally, the SCCNFP would like to point out that the following fields are missing throughout the document:

- absorption through mucosa
- respiratory sensitisation
- in the repeated dose toxicity and developmental toxicity sections, two of the "3R"s (refinement and reduction) have been neglected.
- upcoming alternative technologies, such as the use of stem cells, genomics/proteomics/... and tissues and cells derived from transgenic rats and in particular transgenic mice, have also been overlooked
- some areas of reproductive/developmental toxicity have not been covered by the proposed tests in section 11 including (i) reproductive behaviour, (ii) parturition, (iii) postnatal functional development and (iv) gonadotropin hormones

The SCCNFP would like to mention that in Chapter 2, under point 2.2, the following should be added in order to complete the definition of risk characterisation:

"For non-threshold carcinogenic effects, risk characterisation is performed by determination of the lifetime risk after chronic exposure during lifetime. The risk is determined by linear extrapolation downwards from a dose descriptor to the exposure levels accounted in the human situation. The dose descriptor for non-threshold carcinogenic effects is obtained from long-term animal carcinogenicity tests."

The SCCNFP has concentrated in this opinion on Chapter 3 of the ECVAM document - titled "Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive" of 30/04/2004 - since it contains the inventory of existing alternative methods. The type of proposed alternative strategy is discussed comparing each with the expectations / recommended outcome of the in vivo experiments.

In the Annex, some minor remarks are given which are not significant enough to be included in the body text of this opinion.
DISCUSSION PER CHAPTER

1. ACUTE TOXICITY

The term "acute toxicity" is used to describe the adverse effects on health that may result from a single exposure to a substance via the oral, dermal or inhalation route [ECB 2003].

The goal of an acute toxicity test is to provide a reliable basis for acute hazard classification and/or a valid estimate of appropriate starting doses for non-lethal in vivo studies. In the classical acute toxicity tests, as designed in the late twenties, the lethal endpoint was related to the calculation of an LD$_{50}$ or LC$_{50}$ value, while the non-lethal endpoints served to identify specific target-organ and target-system toxicities [Worth et al. 2002]. Therefore the statement in the report that those studies "basically serve to classify ..." is not correct. The non-lethal clinical and organ toxicity endpoints have been neglected by the authors of this chapter.

The section on acute toxicity describes the existing in vivo acute oral and inhalation test methods in detail. In contrast to the detailed description of the in vivo methods, the in vitro methods are only briefly described. There is no clear, scientific identification of the data gaps compared with the in vivo tests. The only identified gaps are 1) the lack of knowledge on the effects occurring at dose levels close to the lethal dose and 2) the quality of the classification depending on the slope of the dose-response curve. It is not clear why the table in Worth and Balls [2002] is not taken into consideration, since this table is clearer and more complete than the data presented.

It is the SCCNFP's opinion that the following aspects must also be covered by alternative methods in this field:

1. Representation of the complex ADME$^\dagger$ process
2. Modelling of toxicity of all mammalian organs and their interactions including body and organ weight
3. Modelling of the various outcomes of toxicity within one organ
4. Modelling adequately toxification, detoxification, induction, saturation and repair

Assessing human toxicity by screening chemicals to rank chemical toxicity in silico with QSAR$^\S$ and QSTR$^\S$ has been hardly considered. However, this type of predictive programme, based on chemical structure and analysis of large current toxicological data sets, can become an initial tool, which provides useful starting information. Once appropriately developed, it should be a quick and easy screening method for oral, dermal and inhalation acute toxicity tests (determination of LD$_{50}$/LC$_{50}$).

It becomes clear that there is a lack of validated alternative methods for complete replacement of animal use in the field of acute toxicity testing. Much faith is resting on a new EU-funded project, the A-cute-tox programme. This project must still be started up and while it aspires to be a strategy for prediction of human acute oral systemic toxicity and total animal replacement for the acute toxicity tests for regulatory purposes, the validation of such a programme will be lengthy. The 10 years minimum timeframe seems to be rather optimistic and unrealistic.

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$^*$ Median Lethal Dose 50%
$^\dagger$ Median Lethal Concentration 50%
$^\ddagger$ Absorption, Distribution, Metabolism, Excretion
$^\S$ Quantitative Structure-Activity Relationship
$^\S$ Quantitative Structure-Toxicity Relationship
2. **SKIN IRRITATION/CORROSION**

**Skin irritation**
Dermal irritation is defined as the production of reversible damage of the skin following the application of a test substance for up to 4 hours [OECD 404, 2002]. Besides the numerous adaptations performed with the objective of refining the tests and reducing the number of animals, there are some reconstructed human skin models, skin explants and organ cultures and QSAR approaches, of which some are good candidates for validation within the years to come. Also several testing strategies, combining *in vitro* methods, *in vivo* methods and testing on human volunteers, have been worked out in order to assess the skin irritation potential of substances.

Even for the alternatives to the animal tests for assessing irritative potential, the following shortcomings can be noted:

- only acute (single dose) effects are able of being detected;
- models have not been developed for assessing adverse effects following repeated exposure (as is the case in real life for cosmetics) and for determining the reversibility of these effects. Although wrongly indicated in the executive summary, reversibility data are indispensable for the purpose of classification and labelling;
- methods measuring the inflammatory response as well as the time course of an irritative response are only at research level;
- there is a clear need for more mechanistically based endpoints that are more predictive of skin irritation than are simple cytotoxicity determinations. There is not much information on the possible mechanisms of skin irritation in the document.

The realistic estimation of the authors is that there would be an international regulatory position for partial replacement for skin irritation by 2007/2008. The full-integrated approach (incorporating issues such as reversibility, dose-response assessments, ...) is a long-term project, which will take in total at least 10 years.

Meanwhile, the newly published ATP 29* [2004/73/EC] should be mentioned, since it contains new reduction and refinement provisions in the *in vivo* skin irritation test.

**Skin corrosion**
Skin corrosion consists of the potential of a substance to cause irreversible damage to skin, namely, visible necrosis through the epidermis and into the dermis, following the application of a test substance for up to four hours. Three alternative methods have been taken up in Annex V to the dangerous substances directive [67/548/EEC and its adaptations to technical progress], which means that animal tests should not be performed for this endpoint.

As far as the report is concerned, it must be stated that this chapter gives a clear view of the actual situation, with detailed and useful information on the individual alternative methods.

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* 29th Adaptation to Technical Progress of Dir. 67/548/EEC on the classification and labelling of dangerous substances.
However, an important aspect, which has not been discussed, is the problem of the possible formation of a corrosive metabolite out of a non-corrosive mother compound. This cannot be covered by the available replacement *in vitro* corrosivity tests.

### 3. **Eye irritation**

*In vivo* eye irritation tests have been developed to assess the potential of a test substance to cause chemosis, discharge and/or redness to the conjunctiva, swelling of the iris and/or opacity to the cornea, after a single application. The range of criteria for injury and inflammation covered by the Draize rabbit eye test is unlikely to being replaced by one single *in vitro* test.

This chapter on eye irritation displays the highest level of quality and completeness of the whole report. It describes the *in vivo* mechanisms of eye irritation, the Draize rabbit eye test with its purpose for the different areas in which it is used, and then focuses on the available alternatives, with their individual:

- short descriptions, scientific relevance and purpose
- method developer(s)
- known users
- status of validation and/or standardization
- fields of application and limitations
- recommended use in the options of animal replacement
- on-going developments
- efforts needed to complete validation
- key references

As stated before, it is the SCCNFP's opinion that this way of structuring the (sub)chapters should be respected throughout the whole document.

The described methods cover isolated organs, organotypic methods - CAM* methods, human corneal epithelial methods, cell based cytotoxicity methods, cell function based assays, SAR†'s for eye irritation and some other alternative methods. The "efforts needed to complete validation" are not specified for every method and the latest B5. method in Annex V of Dir. 67/548/EEC [2004/73/EC] is not yet incorporated in this current document, but otherwise it is extremely interesting, well-structured and complete.

The SCCNFP endorses the timetables presented by the authors, meaning that, if the recommendations are followed, a validated method may be expected in +6 years.

In the meantime, the new strategy published in Annex V to Dir. 67/548/EEC may be practised for the reduction and refinement approach of animal testing. Thus, the *in vivo* rabbit eye test needs only to be performed as a last step, i.e. when the assessment on all other tiers has produced a negative result, to assess mildly to moderately irritant compounds.

Finally, COLIPA‡ should declare the estimated time line of its own research program, so that the method obtained through this project could be validated as soon as possible. In this regard, coordination with current initiatives elsewhere is recommended.

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* Chorio-Allantoic Membrane  
† Structure-Activity Relationship  
‡ European Cosmetic Toiletry and Perfumery Association
The authors estimate that the achievement of regulatory acceptance will certainly take more than 6 years (at least to 2010).

4. **SKIN SENSITISATION**

Key steps to be considered when discussing sensitising properties are the protein binding, metabolism, and cellular/immunological events leading to sensitisation.

The document starts by summing up the animal tests, including the Local Lymph Node Assay (LLNA), the Magnusson Kligmann Maximisation test, the Buehler test. This list is followed by the human volunteer studies, after which *in vitro* and QSAR methods are mentioned.

The document states that the QSAR methods have been developed further than the *in vitro* methods. However, it is not clear how they have to be validated and how testing guidelines could be developed for QSAR methods. It is the opinion of the SCCNFP that in order to render QSARs a useful *screening tool* for regulatory safety testing, a common and transparent database is required, accompanied by a well-elaborated decision tree and unambiguous rules.

With regard to the *in vitro* methods, a great deal of focused research remains necessary to identify the most relevant parameters and to standardise testing protocols. Every *in vitro* test will only be able to cover certain steps of the multi-stage mechanism of skin sensitisation. Moreover, advances in basic immunological research continue to increase the number of potential test parameters, e.g. the upregulation or downregulation of the expression of cell membrane proteins and cell-cell signalling molecules, such as interleukins, and changes in the antigen uptake process.

The SCCNFP would like to make the following comments on this chapter:

1) respiratory sensitisation is missing; it is not only important for cosmetics (e.g. shaving foams and/or other hygienic products containing proteases), but also for chemicals in general.
2) when discussing the human volunteer studies, it must be emphasized that the most recent version of the Notes of Guidance of the SCCNFP (SCCNFP/0690/03) does not contain the detailed information any more, but that reference should be made to the individual SCCNFP opinions on the subject;  
3) the LLNA has been taken up in Annex V to Dir. 67/548/EEC [2004/73/EC];
4) the document mentions an interesting *in vivo / in vitro* comparison, but only a reference to the BfR website is stated here. The outcome of that comparison is crucial information and should be added in this chapter;

The authors' forecast for alternative methods for skin sensitisation is 12-14 years for regulatory acceptance within the EU (2016-2018). The SCCNFP estimates that it will take more than 15 years.

5. **SKIN ABSORPTION / PENETRATION**

According to the OECD Draft Guideline 428, *in vitro* methods for skin absorption measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to
measure diffusion only, or fresh, metabolically active skin, to simultaneously measure diffusion and skin metabolism [OECD 428].

The presented chapter is nothing more than an incomplete note. The text is not structured as requested. The references should be updated and restricted to the relevant ones.

Instead of stating the existing in vivo methods, their in vitro counterparts and their applicability and limitations, a number of references are given (referring too much to the same method).

The in vitro method has been adopted by the OECD together with the in vivo method, with the clear position that sometimes in vivo confirmation will be necessary. Therefore the SCCNFP is not convinced that this method has been designed to being considered as a standard stand-alone method. The "Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients" issued by the SCCNFP in 2003 [SCCNFP/0750/03], already tackle a number of specific provisions, but experience has taught that the method needs further refinement in order to harmonise the validity of the results obtained in the performance of a given skin absorption/penetration study in the cosmetic field.

Specialised training for laboratories is needed to improve specific skills that are a prerequisite for general acceptance of the results obtained by this type of in vitro test. A first step forward could be the characterisation of the vehicle applied on the skin. 2-3 prototype formulations could be used to obtain a realistic skin absorption profile for the active ingredient tested, according to its physico-chemical characteristics (in particular, its Log P<sub>o/w</sub>) and its in use conditions.

An aspect that is neglected is the absorption through mucosa. Nevertheless, there has been a case in the past where a low molecular weight quaternary ammonium compound appeared to be relatively harmless, but displayed severe lethal neurotoxic effects when administered through the classical Draize rabbit eye irritation test and thus absorbed through the mucosa of the eye [Moss et al. 1991].

It is the SCCNFP's opinion that the subchapter on skin absorption/penetration should be part of the overall chapter on toxicokinetics.

The timetable is missing. It should also include an indication of the time required for adaptation of the existing OECD method into a protocol that can be used for cosmetic products. This could be done through an updated opinion of the SCCNFP in cooperation with ECVAM and the OECD. It is estimated to take more than 3 years.

6. **SUBACUTE AND SUBCHRONIC TOXICITY**

Repeated dose toxicity is a consequence of the persistent or progressively deteriorating dysfunction of cells, organs or multiple organ systems, resulting from long-term exposure to a chemical [Worth et al. 2002].

Although in the introduction to this report, the right questions are asked, the available in vitro methods mentioned are not complete when compared to the list stated in section 11 of the Worth and Balls publication [Worth et al. 2002]. In this publication, the section is named "target organ and target system toxicity", which appears to be a more appropriate title.
The SCCNFP agrees with the conclusion that “inter species differences limit the usefulness of animal studies for predicting long-term target organ and target system effects in humans”. However, over the last decades, toxicologists gained a huge body of experience that these animal studies are a reliable basis for ensuring the safety of man coming into contact with chemical substances.

The subchronic toxicity study in rodents is considered the foundation of the safety assessment of cosmetic ingredients. Firstly, it gives an indication of target organ toxicity following the complex in vivo process of absorption, distribution, metabolism and excretion (ADME, i.e. toxicokinetics). Secondly, this study gives a quantitative figure, the NOAEL*, which is used as quantitative descriptor of the toxic risk in general. Together with the second key element, i.e. systemic exposure, the safety can be described as MoS† in a quantitative way.

Therefore, the following aspects must be covered by alternative methods in this field:

1. Representation of the complex ADME process
2. Modelling of toxicity of all mammalian organs and their interactions including body and organ weight
3. Modelling of the various outcomes of toxicity within one organ
4. Mimicking hormone-controlled effects
5. Modelling adequately toxification, detoxification, induction, saturation and repair
6. Modelling long-term effects

Even the various in vitro alternative tests meant to “cover five of the most common targets for toxicity” are far from being validated. The SCCNFP sincerely doubts, however, whether these methods studying five individual types of target organ toxicity will ever be sufficient to replace one sub-chronic study, the more so because additional target organs and their interactions will also have to be considered. In addition, all toxic effects are dose-dependent, and there is to date no accepted model available to transfer in vitro toxicity concentration data into in vivo target concentrations for the various endpoints that have to be considered.

From the experience gained it can be expected that evaluations made solely on the available in vitro systems in this field regularly would result in false-positive and false-negative assessments of substances. It is foreseeable that the toxic potential will be overlooked in those numerous situations where no adequate in vitro models exist. Furthermore, it is likely that in other cases the toxic potential found in vitro is of no relevance for the in vivo situation. It is realized that subchronic studies are not the gold standard either, but at this moment they are the best instruments available.

The SCCNFP agrees with the conclusion that such alternative tests may be used to study special toxicological issues (e.g. mechanisms of toxicity), but is of the opinion that they are unfit for predictive purposes concerning toxic effects in general. In addition, many animals may be required to provide the organ systems for some of these alternative tests.

Finally, the SCCNFP would like to make the following additional comments on this chapter:

1) In the overview of the methods, the origin of the cells would be useful information.

* No Observed Adverse Effect Level
† Margin of Safety
2) The deadline of "> 10 years" is unrealistic, since it is not foreseeable. Instead, one should consider here considerable improvement of the "other" 2R's, namely refinement and reduction.
3) The tiered approach of Worth and Balls [Worth et al. 2002] could be mentioned here, besides the achievements made between that publication and today.

7. **GENOTOXICITY / MUTAGENICITY**

Mutagenicity refers to the induction of permanent transmissible changes in the structure of the genetic material of cells or organisms. These changes (mutations) may involve a single gene or a block of genes. Genotoxicity is a broader term that refers to the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome, such as the spindle apparatus and topoisomerase enzymes [Worth et al. 2002].

*In vivo* genotoxicity tests are performed to determine whether the potential for genotoxicity detected *in vitro* is realised *in vivo*.

The document appears complete and well-structured.

The SCCNFP would like to point out that several opinions have been issued with regard to mutagenicity/genotoxicity testing strategies in the cosmetic field [SCCNFP/0690/03, SCCNFP/0720/03, SCCNFP/0755/03] and therefore, it is strongly advised to incorporate those in the current document.

A total replacement in the field of genotoxicity/mutagenicity is not considered to be feasible within the next 12 years (> 2016) and will depend, besides the development of *in vitro* tests on skin models, on the progress in the fields of toxicokinetics and toxicogenomics.

8. **UV-INDUCED EFFECTS**

This section is specifically designed for cosmetic products, since some of the product types may, by their usage pattern, involve exposure to UV-light (UV-filters in sunscreens, UV-filters to ensure light stability of cosmetics, ...).

The majority of the methods developed to assess UV-induced effects are adaptations of the classical methods. Section 8 gives a clear overview of the available methods and their current status. Since they are adaptations of the classical methods without UV-radiation, their validation will depend on the results achieved in those other areas. The available methods can be summarised as:

**For acute phototoxicity (deadline no problem, test available):**

1) 3T3 NRU PT* test: Annex V, B41.
2) Red Blood Cell Phototoxicity test
3) Human 3-D skin model *in vitro* phototoxicity test

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* Neutral Red Uptake Phototoxicity Test
For photochemical genotoxicity (deadlines between 0 and 6 years):

1) Reverse gene/point mutation (B.13/14): photo-Ames test
2) Forward gene/point mutation: photo-hypoxanthine-guanine phosphoribosyl transferase assay and photo-mouse lymphoma assay: L5178YTK+/- Mouse Lymphoma assay and the HPRT* in Chinese hamster cell lines
3) Photo-clastogenicity (B.10): Photo chromosome aberration test

For photo-sensitisation (deadline 8-15 years for in vitro tests):

1) GPMT† employing additional UV-vis irradiation.
2) In future, photo-LLNA‡ and photo-MEST§

In the specific area of photomutagenicity/photogenotoxicity, the validation processes are reported to being faced with several problems, such as the fact that there is no comparison possible between in vivo and in vitro data.

9. TOXICOKINETICS AND BIOTRANSFORMATION

As defined in the presented chapter, toxicokinetic studies are designed to obtain species-, dose- and route-dependent data on the concentration-time course of parent compound and its metabolites, e.g. in blood, urine, faeces and exhaled air. From these data, kinetic parameters can be derived by appropriate techniques.

The major problem in developing them is caused by the complexity of the living organism and by the fact that, for new molecules, no information at all is available on the possible effects and target organs.

In silico methodology becomes a useful screening tool, that, however, must be further developed and validated.

In this chapter a tiered approach of alternative methods for toxicokinetic studies in animals has been described. The tiered approach is comprised of three steps: Tier 1. Likelihood of systemic exposure from cosmetic ingredients, Tier 2. Determination of the distribution of the compound and its biotransformation, and Tier 3. Determination of the potential of a compound to produce adverse effects.

Some OECD guidelines (adopted or under development) as well as EU adopted test methods employing in vitro/ex-vivo methodology are available to partly fulfil the requirement of replacing toxicokinetics study in vivo. The principles and the strategies for the development of new methods and their validation have been described. However, in vitro methods for several endpoints are not available. The authors prefer to develop in silico methods as alternatives. According to the authors, the estimated time for the validation of most of the required methods is 5 years. The development and validation of certain methods will require longer period: 1) OECD guideline 417 for excretion (>10 years), and for pulmonary absorption (estimated time 7-8 years). It is also envisaged by the authors that development and validation of integrated human volunteer-based approaches will also take more than 10 years.

* Hypoxanthine-guanine PhosphoRibosyl Transferase
† Guinea Pig Maximisation Test
‡ Local Lymph Node Assay
§ Mouse Ear Swelling Test
It should be noted that some important barrier functions, i.e. blood brain barrier and blood testes barrier functions were considered, but no strategy for developing \textit{in vitro} methods has been described.

The authors propose an overall testing strategy based on toxicokinetics. In this respect, it must be emphasized that all other areas in the testing of \textit{in vitro} systemic toxicity rely on the results of these \textit{in vitro} toxicokinetic studies. E.g. if a compound will never reach the kidneys, no \textit{in vitro} nephrotoxicity studies will be required.

This new approach will have the following consequences:

- the \textit{in vitro} toxicokinetics area will need the highest priority in basic research and the development of new alternative methods;
- \textit{in vitro} alternatives will have to be developed for the three exposure routes: inhalation, dermal exposure and oral exposure. The dermal exposure route is currently being covered by the \textit{in vitro} dermal absorption test, which could be fully validated for use in the cosmetic area in the years to come. For the oral route, some promising tests have been developed, but they are not yet in the same stage as the dermal absorption test. They also only include one single cell type. \textit{In vitro} alternatives for the inhalation route are unfortunately still in a preliminary basic research phase;
- once the \textit{in vitro} absorption methods are fully operative, they will provide a figure of exposure that should be the basis for further testing. In certain cases, no testing would be necessary at all. The determination of such limit values, possibly per specific type of chemicals, could also be considered as a field that needs special attention.

The SCCNFP supports the tiered approach described for toxicokinetics. However, although \textit{in silico} methods can be placed in the forefront as screening methods providing complementary information, the development of \textit{in vitro} methods still is strongly recommended, as various regulatory mechanisms active in \textit{in vitro} test systems, may not yet be considered in \textit{in silico} methods.

Before this strategy, detailed data on both, the toxicokinetics as well as metabolism of cosmetic ingredient used to be of less importance, with the exception of cases having high dermal absorption. As of today, however, they will be at the forefront of risk assessment and exposure will be the decisive part of further testing.

The deadlines, as stated by the authors, appear to be extremely short and very unrealistic. It is strongly advised to include a much larger chapter on the mechanisms that still have to be elucidated \textit{in vivo} and on the many data gaps that still exist when comparing the outcome of the existing \textit{in vitro} methods with the results obtained from \textit{in vivo} tests. With regard to Tier 1, dermal absorption \textit{in vitro} could be available by 2006. An \textit{in vitro} alternative for the oral route can optimistically be foreseen by 2008-2010, while for the inhalation route, more than 10 years should be envisaged.

Since developments of Tiers 2 and 3 come after development of Tier 1, the time involved is difficult to predict.
10. Carcinogenicity

Substances are defined as carcinogenic if they induce tumours (benign or malignant), increase their incidence or malignancy, or shorten the time of tumour occurrence when they are inhaled, ingested, dermally applied or injected [ECB 2003].

According to the authors, the process of carcinogenesis is now recognised as resulting from the transition of normal cells into cancer cells via a sequence of stages and complex biological interactions.

The authors give an overview of the few existing in vitro tests in the field of carcinogenicity. They conclude that the modelling of such complex adverse effects cannot be accomplished at present, or within 10 years, by the use of non-animal tests. A deadline for full replacement cannot be established.

In the "Inventory of the alternative methods currently available", a cross reference should be made to the chapter on genotoxicity/mutagenicity. Indeed, genotoxicity tests, cell transformation and gap junction intercellular communication tests are important in the identification of potential carcinogens.

With regard to reduction and refinement, several transgenic models are described. The mention of newborn mice is missing. It should also be explicitly stated in the report conclusions that these tests cannot be used for quantitative risk assessment since they do not predict the endpoint target organ.

11. Reproductive Toxicity

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle, including the impairment of reproductive function, the induction of adverse effects in the embryo, such as growth retardation, malformation and death.

The authors conclude that, due to the complexity of the mammalian reproductive cycle, it is not possible to model the whole cycle in one in vitro system. More specifically, the following limitations are cited in the text:

- Many of the well-developed tests rely on tissues derived from intact mammals.
- The proposed tests can only cover single aspects of developmental toxicity.
- New tests have to be developed since the currently available tests are not sufficient.
- Test guidelines assessing the reproductive and developmental hazard of chemicals test strategies have yet to be developed. Consequently, it is not possible to foresee whether all aspects of developmental and reproductive toxicology have been covered by the tests proposed.
- In addition, some areas of reproductive/developmental toxicity that are not covered by the proposed tests have been identified and include (i) reproductive behaviour, (ii) parturition, (iii) postnatal functional development and (iv) gonadotropin hormones.

The SCCNFP would like to emphasize that the overview provided in section 11 is an actual EU research project of ECVAM (starting 2004). It will only show within 5 years whether the work strategy may lead to solutions or not. It is clear that it is currently not foreseeable if and when tests on reproductive and developmental toxicity can be replaced. Full replacement is certainly not foreseen in the next 10 years.
In Table 1 of the ECVAM report, the first rows are prone to misinterpretation since *in vitro* embryotoxicity tests do not replace the whole field of developmental toxicity testing.

12. EXECUTIVE SUMMARY

As a major remark, the SCCNFP would like to state that there is considerable inconsistency between the executive summary and Chapter 3 of the presented ECVAM document (especially with regard to carcinogenicity).

- Classification of dangerous substances is not only based on hazard identification, but also on dose-response assessment. Therefore, the SCCNFP cannot agree with the way of linking hazard assessment with classification and labelling, while dose-response assessment is only considered being of value for risk assessment. This is a hidden way of claiming that full replacement is available, while, in fact, we can only consider it as partial replacement. The most striking example is point 2 of the executive summary (skin irritation/corrosion): it is impossible to have full replacement for classification and labelling without having any indication of the reversibility, which is taken into account in the classification system as described in Annex VI of Dir. 67/548/EEC [2001/59/EC].

- With regard to point 5 (skin absorption/penetration), the SCCNFP does not agree that the TG428 can be considered as a full alternative to the *in vivo* test in all cases.

- Point 6 (subacute and subchronic toxicity), last sentence: it must be stressed that this methodology needs much more than 10 years plus much more emphasis should be put on the fact that the methods involved are still at R&D level. It is a key element.

- As stated before, the SCCNFP does not agree with the deadlines specified for toxicokinetics, which are much too optimistic, viewing the large R&D effort that still has to be invested in that area. The shortcomings of the *in vitro* compared to the *in vivo* tests are largely underestimated.

- The table at the end of the executive summary needs some serious adaptation. First of all, the column "time necessary to achieve ESAC validation" gives deadlines for only partial aspects of the full replacement. Since this is the first figure encountered in the table, it gives the wrong impression that animal testing will soon be abolished. In addition, key information consists of estimates of the "optimal conditions" mentioned throughout all the sections presented.

Unanswered questions include:
"How many staff do we need?"
"How much funding is necessary?"
"Who determines and coordinates the strategy?"
"Who are the researchers and developers involved (industry, academia, …)?"
"What will be the role of the different stakeholders, incl. ECVAM?"
GENERAL CONCLUSION

1) The existing *in vivo* animal toxicity tests have shown to identify the effect(s) of concern and the relationship between the dose, or level of exposure to a substance, and the incidence and severity of the observed effect(s).

The results of these tests serve to provide the Competent Authorities of the Member States with a useful tool for classification and/or further risk assessment. This is a very important starting point, which should not be neglected.

2) *In vitro* methods replacing animal tests must provide the same level of knowledge. However, many of the currently described *in vitro* methods and proposed here for replacement of *in vivo* methods, historically only served research purposes, and no regulatory purposes. It is necessary that tests must be developed and validated for the purpose of safeguarding human health and safety. *In vitro* methods usually provide specific, but very restricted information, which will have to be combined with complementary knowledge and results from other *in vitro/* usually *in vivo* tests. They only form a small part of the jigsaw puzzle that should give the whole picture obtained through one *in vivo* experiment. The fact that they might not have been designed to form part of this puzzle in the first place, often leads to some practical problems.

3) The SCCNFP would like to state that the presented document gives a good overview of the existing alternative methods to animal testing. However, the document lacks coherence in the quality and level of detail displayed in the different sections. Another important issue to be mentioned is the incomplete identification of the gaps when moving from *in vivo* to *in vitro* methods in the different fields of toxicity discussed. Knowledge on these gaps will certainly play a crucial role in the further development of (new) alternative methods. Regulators should be involved in this gap identification process.

4) The timetable summarising all tests in the executive summary is unacceptable and unrealistic. Indeed, the deadlines for full replacement are, on several occasions, very optimistic and unrealistic, especially since it is proposed that the authors may count on "optimal conditions", without placing any limitation on funding and staff. It must not be forgotten that this document must enable the European Commission to establish realistic timetables for the abolition of animal testing for cosmetic ingredients. Experience from the past has taught that validation of alternative methods is a slow process. Limited funding in the field is available. However, the financial constraints are not the only ones. Scientifically, some problems have to be recognized and solved. Top scientists are not always involved in alternative research and the field seems to lack new ideas and developments. Young people should be stimulated more effectively. The hope that industry would have come up with a number of "in-house" alternative tests and would have shared these with the rest of the scientific community, has not been fulfilled.

5) Crucial information that is missing in most of the document (except for the eye irritation section), is the applicability of alternative methods, e.g. in the cell or tissue cultures that use culture media with a specific composition and specific physico-chemical properties, not all test compounds can be easily added to these complex systems. Therefore, tests should be developed to be used as a standard test for regulatory purposes, as is the case for the currently accepted *in vivo* methods.

Moreover, in the cosmetic field, more and more developments are made in the field of nanotechnology, which means that the form/size of the compounds to be added to the test
system, will also need special attention. At this moment, this aspect is even easily neglected in the field of *in vivo* toxicology.

**SPECIFIC CONCLUSIONS**

As far as acute toxicity is concerned, there is a clear lack of validated alternative methods for complete replacement of animal use. Much faith is resting on the new EU-funded A-cute-tox programme, which however still awaits a lengthy validation period. The 10 years minimum timeframe is estimated to be rather optimistic and unrealistic.

Skin and eye irritation and skin corrosion clearly are the most developed fields in alternative methods. Their advantage is that only one specific target organ needs to be studied and only local toxicity is involved. The expected effect is well-defined. The result is a yes/no answer to the question "Is the substance irritating ?" or "Is the substance corrosive ?". This is much easier than assessing systemic toxicity, where the scientists do not know which organs will be targeted and through which mechanisms the effect(s) will occur. The results of systemic toxicity tests are no simple yes/no answers to a couple of well-defined questions.

In the case of skin sensitisation, chapter 4 clearly shows that a large effort in the field of basic immunological research will be necessary before the alternative methods can be developed. The timetable, as presented by the authors, brings us to 2016-2018 for alternative methods for skin sensitisation.

For skin absorption/penetration, a subchapter that should form part of the chapter on toxicokinetics, the forecast for the availability of an alternative method suitable for use in the cosmetic field, is set on 2 years.

With regard to the subacute and subchronic toxicity testing, the SCCNFP is convinced that the currently available *in vitro* tests meant to cover five of the most common targets for toxicity, will, once validated, not suffice to obtain the same level of knowledge obtained through a classical subchronic animal study. From the starting position that consumer safety shall not be impaired, replacement of the animal subchronic toxicity study in a foreseeable time is considered unrealistic. Therefore, no deadline can be determined for alternative replacement strategies in this field.

The mutagenic/genotoxic area has the huge advantage of long-term experience with ready-to-use *in vitro* tests. These methods have already been taken up in Annex V to Dir. 67/548/EEC many years ago. Nevertheless, even in this experienced *in vitro* area, there still are some general crucial limitations of the tests. These have been identified as being due to the absence of toxicokinetic characteristics and/or the use of cell lines not relevant to predict genotoxicity at target organs. The current situation is that no single *in vitro* test can fully replace an existing *in vivo* animal test. A total replacement in the field of genotoxicity/mutagenicity is not considered feasible within the next 12 years (> 2016). It will depend, besides the development of *in vitro* tests on skin models, on the progress in toxicokinetics and toxicogenomics.

Since the majority of the methods developed to assess UV-induced effects are adaptations of the classical methods, their validation will strongly depend on the results achieved in those other areas. The only exception is the 3T3 NRU phototoxicity test, which has been taken up in Annex V to Dir. 67/548/EEC.
In the past, toxicokinetic and biotransformation data used to be auxiliary when compounds had been shown to exert adverse effects during repeated dose toxicity tests. Therefore, they were not automatically taken up in every classical animal testing strategy. Moreover, biotransformation data were often used to prove that an adverse effect observed in a laboratory animal, would never occur in a human being.

In moving from *in vivo* to *in vitro* toxicity testing strategies, however, toxicokinetics are now becoming the ultimate starting point to identify the possible organs/tissues of concern, the level of possible exposure, etc. Therefore, this area needs to be given absolute priority and a maximum of resources.

Carcinogenicity tests cannot be replaced by alternative methods yet. Reduction/refinement alternatives are important to identify substances that may need to be further studied in long-term carcinogenicity tests. Results from such alternative methods may either increase or decrease concern of carcinogenic potential.

Finally, for reproduction toxicity tests, new approaches have been proposed, but, due to the complexity of the mammalian reproductive cycle, it is not possible to model the whole cycle into one *in vitro* system. A full replacement strategy is therefore not foreseen in the next 10 years. Even more, some areas of reproductive/developmental toxicity that are not covered by the proposed tests have been identified and include (i) reproductive behaviour, (ii) parturition, (iii) postnatal functional development and (iv) gonadotropin hormones.

The deadlines, stated in the report, were apparently not approved by all experts in the working groups. The SCCNFP is of the opinion that they are much too optimistic and not realistic.
<table>
<thead>
<tr>
<th>Human health effect</th>
<th>FULL replacement test(s) / strategy available June 2004</th>
<th>Foreseeable time involved (expected year of potential results)</th>
<th>PARTIAL replacement test(s) / strategy available June 2004</th>
<th>Foreseeable time involved for partial replacement (expected year of potential results)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acute toxicity</td>
<td>None</td>
<td>&gt; 2014</td>
<td>QSAR models and <em>in vitro</em> tests under investigation</td>
<td>- Not foreseeable</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Awaiting more information through the FP6 A-cute-Tox programme</td>
</tr>
<tr>
<td>2. Skin irritation/corrosion</td>
<td>Skin irritation : None</td>
<td>&gt; 2014</td>
<td>3 <em>in vitro</em> tests under validation</td>
<td>2007-2008</td>
</tr>
<tr>
<td></td>
<td>Corrosion : 3 <em>in vitro</em> tests EU method B.40*</td>
<td>&lt; 2004†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Eye irritation</td>
<td>14 <em>in vitro</em> tests under validation</td>
<td>&gt; 2010</td>
<td>individual <em>in vitro</em> tests useful in screenings / reduction strategies</td>
<td>2006-2010</td>
</tr>
</tbody>
</table>

* Taken up in Annex V to the Dangerous Substance Directive
† With the exception of the detection of non-corrosive mother compounds metabolised into corrosive molecules
<table>
<thead>
<tr>
<th>Human health effect</th>
<th>FULL replacement test(s) / strategy available June 2004</th>
<th>Foreseeable time involved (expected year of potential results)</th>
<th>PARTIAL replacement test(s) / strategy available June 2004</th>
<th>Foreseeable time involved for partial replacement (expected year of potential results)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Skin sensitisation</td>
<td>None</td>
<td>&gt; 2019</td>
<td>QSAR models and <em>in vitro</em> methods</td>
<td>2016-2018</td>
</tr>
<tr>
<td>5. Skin absorption / penetration</td>
<td>OECD 428* adapted for cosmetics</td>
<td>&gt; 2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Subacute and subchronic toxicity</td>
<td>None</td>
<td>Not foreseeable &gt;&gt; 2014</td>
<td>various <em>in vitro</em> methods meant to cover five of the most common targets for toxicity</td>
<td>Not foreseeable</td>
</tr>
<tr>
<td>7. Genotoxicity / mutagenicity</td>
<td>None</td>
<td>&gt; 2016</td>
<td><em>in vitro</em> mutagenicity / genotoxicity tests taken up in Annex V to Dir. 67/548/EEC</td>
<td>&lt; 2004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human health effect</th>
<th>FULL replacement test(s) / strategy available June 2004</th>
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<th>PARTIAL replacement test(s) / strategy available June 2004</th>
<th>Foreseeable time involved for partial replacement (expected year of potential results)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9. Toxicokinetics and biotransformation</td>
<td>None</td>
<td>Not foreseeable</td>
<td>percutaneous absorption pulmonary absorption <em>in vitro</em> oral absorption, distribution, excretion, metabolism</td>
<td>see section 5 2011-2012 &gt;2014</td>
</tr>
</tbody>
</table>
| 10. Carcinogenicity                     | None                                                    | Not foreseeable                                               | None R&D level                                           | - Not foreseeable  
- Awaiting results through the FP6 ReProTect programme                            |
| 11. Reproductive toxicity               | None                                                    | Not foreseeable                                               | MM, EST, WEC (partial embryotoxicity *in vitro*) All other areas of developmental toxicity : R&D level | < 2004  
Not foreseeable                                                                       |

* Endorsed by ESAC (ECVAM Scientific Advisory Committee)
**TABLE 2 : ALTERNATIVE METHODS NOT LISTED IN ANNEX V(67/548/EEC)**

<table>
<thead>
<tr>
<th>Alternative method(s)</th>
<th>Reference(s)</th>
<th>Applicability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic stem cell test for embryotoxicity</td>
<td>Anon. 2002</td>
<td>In the context of complete developmental toxicity testing, the embryotoxicity data can be derived from the results from these tests</td>
</tr>
<tr>
<td>Micromass embryotoxicity assay</td>
<td>Genschow et al. 2002</td>
<td></td>
</tr>
<tr>
<td>Whole rat embryo embryotoxicity assay</td>
<td>Brown et al. 1995</td>
<td></td>
</tr>
<tr>
<td>CORROSITEX continuous time monitor assay</td>
<td>15th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, December 2000</td>
<td>Additional corrosivity test, although only valid for testing specific classes of chemicals, such as organic bases and inorganic acids.</td>
</tr>
<tr>
<td>Skin absorption: In Vitro Method, OECD TG 428*</td>
<td>OECD 428</td>
<td>Replacement method for determining percutaneous / dermal absorption. Still needs some adaptation for use in the cosmetic field</td>
</tr>
</tbody>
</table>

**FINAL OPINION OF THE SCCNFP**

The SCCNFP was requested to identify 1) validated alternative methods that do not use animals, reduce the number of animals used or reduce the suffering caused; 2) those cases where full replacement alternatives are not yet available (as provided by Article 7(2) and (3) of Directive 86/609/EEC) to offer consumers a level of protection equivalent to that of the conventional methods which they are intended to replace, and are not listed in Annex V of Directive 67/548/EEC.

These methods are listed in Table 2.

In table 1 a timeframe is provided for full and partial replacement of animal testing.

---

† Equivalent EU method B.xx foreseen in Annex V to the Dangerous Substances Directive (67/548/EEC) through its 30th Adaptation to Technical Progress
REFERENCES


Anon. 
Statement on the Scientific Validity of the Embryonic Stem Cell Test (EST) - an In Vitro Test for Embryotoxicity. 
17th meeting of ECVAM Scientific Advisory Committee, 16-17 October 2001, EC JRC, IHC, ECVAM, Ispra, Italy. 
Alternatives To Laboratory Animals 30, 265-268 (2002).

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Screening chemicals for reproductive toxicity: the current alternatives. The report and recommendations of an ECVAM/ETS workshop (ECVAM Workshop 12) 

CSTEE (Scientific Committee on Toxicity, Ecotoxicity and the Environment) 
Opinion of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) on the BUAV-ECEAE report on "The way forward - action to end animal toxicity testing" 

ECB (European Chemicals Bureau) 

**Moss J.N., Hsu A., Lange B. and Scribner H. E.**
The need for animal testing - Report of a case (short communication)

**OECD 404** - OECD Guideline for testing of chemicals - Guideline 404: Acute Dermal Irritation/Corrosion


**OECD 428** - OECD Guideline for testing of chemicals - Draft Guideline 428: Skin absorption: In vitro method

**Russell B**, Russell WMS, Burch RL.
The principles of Humane Experimental Technique.


**SCCNFP/720/03, 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.


**Worth A.P. and Balls M.**
Alternatives To Laboratory Animals 30 Supplement 1, 1-125 (2002).

**Zijlstra J.A., Schmid B.**
Validation of three in vitro/teratogenicity test systems using identical coded compounds. III the post-implantation rat embryo culture.
Teratology 44, 31A (1991)
ANNEX:

SOME MINOR REMARKS IN SPECIFIC SECTIONS OF CHAPTER 3:

Section 1: acute toxicity

- OECD numbers are referred to, while the European B-numbers of Annex V to Dir. 67/548/EEC are lacking; these should be added;
- the in vivo dermal toxicity test is not mentioned and should be added;
- reference nr.13 (Guideline 433 of the OECD) is incomplete;
- in many descriptions, the exact identification of the cells is lacking (human, animal origin??);

Section 2: skin irritation/corrosion

ECB references of the definitions of the in vivo toxicity terms could be stated besides the OECD definitions.

Section 3: skin sensitisation

On p.2: 1999/45/E\textit{G} should be 1999/45/E\textit{C};

Section 11: reproductive toxicity

The OECD numbers are mentioned; the B-numbers of Annex V to Dir. 67/548/EEC are lacking and should be added.

SOME MINOR REMARKS WITH REGARD TO THE EXECUTIVE SUMMARY:

Last sentence of point 3 (eye irritation):
"as a compromise" should be deleted, and
"more than 6 years" should be replaced by "a period in excess of 6 years"

Under point 4 (skin sensitisation): 9th line: "may not achieve ESAC endorsement": replace by "are very unlikely to achieve ESAC endorsement"

The usage of the wording "tiers" and "stages" is confusing throughout the executive summary. In our opinion, the term "tiered approach" should be employed for strategies, which move from non-animal (in silico, existing data), over ex vivo and in vitro to in vivo approaches. E.g. in Section 7: genotoxicity/mutagenicity, replace "stage" by "tier". The term "stage" can then be used instead of "tier" in the case of the toxicokinetics approach, where the three "stages" defined don't go from non-animal to in vivo, but cover different aspects of the toxicokinetics. Within each "stage", an individual tiered approach is possible.