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

MEMORANDUM

CONCERNING

THE ACTUAL STATUS OF ALTERNATIVE METHODS TO THE USE OF ANIMALS IN THE SAFETY TESTING OF COSMETIC INGREDIENTS

adopted by the SCCNFP during the 20th plenary meeting
of 4 June 2002

1. Introduction

One of the major mandates of the SCCNFP, defined by the Commission (DG XXIV/1890/98, 20 May 1998), is to act as a resource of scientific expertise to the European Commission with regard to the development of alternative methods. As such the SCCNFP advises the European Commission on the status of alternative methods to animal testing of cosmetics on an on-going basis and particularly, in accordance with Art. 4,1(i) of Council Directive 76/768/EEC, amended by Council Directive 93/85/EEC.

In particular, the Commission has requested the SCCNFP to assess the possibility to replace safety data obtained on the basis of animal tests with data obtained using alternative methods and to indicate those end-points for which no alternative methods are yet available (doc. n° 16831 of 11 August 1998).
The SCCNFP therefore closely follows the scientific developments of alternative methods by academia, industry and public institutions and this in a broader context in order to identify the alternative methods that are applicable to the safety evaluation of cosmetic ingredients and finished products. Also scientific discussion meetings are organised with ECVAM and COLIPA scientists to evaluate the results of pre-validation and validation studies and their applicability to the cosmetics sector.

For the moment, the number of validated alternative methods, fitting into the 3Rs concept of Russell and Burch (Reduction, Replacement, Refinement) (1) and available for the practical application in regulatory testing and risk assessment of cosmetic ingredients is limited.
According to the "Notes of Guidance for Testing of Cosmetic Ingredients for their Safety Evaluation" (SCCNFP/0321/00 Final), the specific toxicity studies necessary for the safety evaluation of cosmetic ingredients include acute toxicity, percutaneous absorption, skin irritation, eye irritation, skin sensitisation and photosensitisation, subchronic toxicity, mutagenicity/genotoxicity, phototoxicity/photoirritation, photomutagenicity/ photogenotoxicity, human data, toxicokinetics and metabolism data, long-term toxicity and carcinogenicity.

Only for some of these areas, appropriate alternatives currently exist and these are present in different stages of development :

- formally validated tests accepted by SCCNFP;
- tests equivalent to formally validated tests and accepted by SCCNFP;
- tests under validation, not yet completed or not successful (not accepted by SCCNFP).

In a number of specific toxicological fields of key importance for the safety evaluation of cosmetic ingredients appropriately validated alternative tests are lacking. These include in particular subchronic toxicity, long-term toxicity, carcinogenicity and toxicokinetics. For the field of photomutagenicity/photogenotoxicity the *in vitro* methodology is quite well developed.

The existing techniques will not be discussed here but in another part of the SCCNFP Notes of Guidance in which the new testing strategy, in particular for testing of hair dye cosmetic ingredients (SCCNFP/0566/02, 17 April 2002), is being presented.

2. Formally validated 3R methods accepted by the SCCNFP

Formally validated methods are those alternatives that have followed the validation process, as set up by ECVAM and the independent ECVAM Scientific Advisory Committee (ESAC) , including test development, prevalidation (informal interlaboratory study), validation (formal interlaboratory study with coded substances), independent assessment and progression toward regulatory acceptance (2).
These methods are based on a so-called prediction model, an algorithm for converting the results obtained into a statement about the *in vivo* toxicity under study (3).
Currently 5 formally validated 3R methods exist that have been also accepted by the SCCNFP: 4 corrosivity tests and 1 phototoxicity test.

2.1. Skin Corrosivity

- The rat skin transcutaneous electrical resistance (TER) assay, using excised rat skin as a test system and its electrical resistance as an endpoint, has been endorsed by ESAC (31 March 1998; http://www.iivs.org/news/ratskin.html) (4)
The method is taken up in Annex V method B.40 of the Dangerous Substances Directive (Directive 67/548/EEC), thereby making its use for chemicals mandatory.
The Draft New OECD Guideline 430 (March 2002) on *in Vitro* Skin Corrosion: TER is still under consideration by the OECD Member States.

- EPISKIN™ and EpiDerm™, two commercialised human skin models consisting of reconstructed human epidermal equivalent using cell viability (MTT-test) as an endpoint, have been endorsed by ESAC (31 March 1998 and 14-15 March 2000, respectively); http://www.iivs.org/news/ratskin.html) (4) (5)
These methods are taken up in Annex V of the Dangerous Substances Directive 67/548/EEC and are mandatory for skin corrosion testing of chemicals in the EU.
The Draft New OECD Guideline 431 (March 2002) on *In Vitro* Skin Corrosion: Human Skin Model Test still is under consideration by the OECD Member States.

- Corrositex™ is a commercial system of reconstituted collagen matrix taking colour or physical change in indicator as an endpoint. The model was prevalidated and validated by ECVAM-funded studies and had an unacceptably high underprediction rate. Consequently, it was endorsed by ECVAM only for skin corrosion testing of acids, bases and derivatives.
It has not been taken up in the Dangerous Substances Directive. The Revised Draft Updated OECD Guideline 404 (June 2001) on Acute Dermal Irritation/Corrosion is still under consideration by the OECD Member States.

2.2. Phototoxicity

The 3T3 neutral red uptake (3T3 NRU) test for phototoxic potential uses 3T3 fibroblasts and UV-A irradiation.
It has been endorsed by ESAC (1-2 October 1997; http://www.iivs.org/news/3t3.html) and a statement on its use for the particular purpose of testing UV-filters has been issued (7).

The 3T3 NRU test is taken up in Annex V, method B41 of the Dangerous Substances Directive 67/548/EEC making it mandatory for chemical testing of phototoxic potential.
The Draft New OECD Guideline 432 (March 2002), namely Draft Proposal for a New Guideline: 432 *In Vitro* 3T3 NRU Phototoxicity test is still under consideration by the OECD Member States.

3. 3R methods equivalent to formally validated tests and accepted by the SCCNFP

These 3R methods have been accepted by ESAC as being equivalent to formally validated tests. They include a test for skin sensitisation, the murine local lymph node assay (LLNA), and an *in vitro* percutaneous absorption test.

3.1. Skin Sensitisation : LLNA

The murine local lymph node assay is a refinement test, thus still an *in vivo* test on mice, providing reduction of the number of animals used and refinement in the methodology in comparison with the traditional guinea pig-based methods (guinea pig maximisation test and the Buehler test). It is more rapid, quantitative and objective.
In principle, the assay evaluates the extent to which a chemical contact allergen stimulates the proliferation of lymphocytes in lymph nodes draining the site of chemical application. A chemical is regarded as a skin sensitiser if it induces a stimulation of ≥ 3 fold that found in vehicle treated controls.
The LLNA has been formally validated in the USA and has been endorsed as scientifically valid by ESAC (14-15 March 2000;
http://iccvam.niehs.nik.gov/methods/llnadocs/llna_val.htm) (8)
It forms the basis of the Draft Revised New OECD Guideline 429 on Skin Sensitisation: Local Lymph Node Assay (June 2001).

3.2. Percutaneous Absorption

The *in vitro* methodology for percutaneous absorption testing is based on the use of Franz-cells. It measures the diffusion of substances across excised human or pig skin, which may be of full or partial thickness. In the case of non-viable skin only diffusion can be measured. When fresh skin is used both, diffusion and skin metabolism, can be assessed. This methodology has not been formally validated, but the cosmetic and the pesticides industry have provided the necessary in use data to create confidence in the methodology. There is now the Draft OECD Test Guideline 428 on Skin Absorption: *in Vitro* Method (9) and a Draft OECD Guidance Document for the Conduct of Skin Absorption Studies (10). Both, are still under consideration by the OECD Member States.
The SCCNP has accepted the *in vitro* methodology to evaluate percutaneous absorption of cosmetic ingredients (20 January 1999) and has defined an additional set of basic criteria for cosmetic ingredients (23 June 1999) (SCCNFP/0167/99 Final).

4. 3R methods under validation (not yet completed or not successful)

A number of alternative methods exist that either have not yet been taken completely through the formal validation process or were not successful in this respect.
To the former category belong embryotoxicity tests, and acute lethal toxicity tests; to the latter eye and skin irritation testing.

4.1 Embryotoxicity tests

Since the field of developmental toxicity is very complex, it is expected that the various stages cannot be mimiced using one alternative method.
Embryotoxicity has been studied separately and so far three embryotoxicity tests have been formally prevalidated and validated. In addition, a prediction model has been developed to classify the chemicals into non, weak/moderate, strong embryotoxic substances.
The existing alternative tests consist of the whole embryo culture (WEC), the micromass (MM) test and the embryotoxic stem cell test (EST).
The last two tests were considered scientifically valid by ESAC (16-17 October 2001) for distinguishing into the 3 just mentioned categories of embryotoxicity whereas the WEC test was considered scientifically valid for identifying strong embryotoxic chemicals. The ESAC statements will now be published and the areas of application defined (ESAC meeting 3 June 2002).
The 3 alternative embryotoxicity tests have not yet discussed within the SCCNFP.

4.2. Acute lethal toxicity

Reduction and refinement alternatives of the LD_{50} method have been accepted at the OECD and EU level. These are :

- Acute Oral Toxicity – Fixed Dose Method, Updated OECD Guideline 420 (20 December 2001) and B.1bis in Annex V to Directive 67/458/EEC;
- Acute Oral Toxicity – Acute Toxic Class Method, Updated OECD Guideline 423 (20 December 2001) and B.1tris in Annex V to Directive 67/458/EEC;
- Acute Oral Toxicity – Up- and Down Procedure, Updated OECD Guideline 425 (20 December 2001) not yet an equivalent in Annex V to Directive 67/458/EEC.
- Currently, a formal validated replacement alternative for acute lethal toxicity does not yet exist. A validation study of a basal cytotoxicity test will be soon initiated by ICCVAM and ECVAM principally based on the mass of data generated before by FRAME, MEIC and the Halle and Gores Registry of Cytotoxicity (11-26).

4.3. Eye irritation tests

A list of alternative methods has been compiled by ECVAM (table 1) (27) providing a good overview (28-38). These methods are in different stages of development but it has been shown by all the validation exercises run until now that it is not possible to formally establish the scientific validity of a single or more replacement tests, applicable across the full range of eye irritation potency (39). It is generally considered that a battery of alternative tests is required for the

assessment of eye irritation since there are multiple mechanisms of eye irritation. These tests should model then the different mechanisms and provide complementary results.
Generally spoken the BCOP-test (bovine cornea opacity-permeability test) seems good for neutral organics and the RBC (red blood cell) and NRU (neutral red uptake) tests for surfactants. For alcohols and esters no good methodologies are yet available (39).
According to the strategy proposed by the OECD, new chemicals can be classified as irritating to the eye on the basis of a tiered testing strategy including structure-activity relationships, physicochemical tests and *in vitro* tests. Animal testing is then only necessary as a last confirmation step of negative results (6). Consequently, this strategy critically depends on the availability of one or more scientifically validated *in vitro* tests for inclusion in this strategy. However, currently these are not available.

4.4. Irritation tests

Also alternative tests for skin irritation (Table 2) belong to the category of tests for which a lot of prevalidation efforts have been done (40). However, these were not leading to a successful outcome of starting a formal validation study.

5. Lacking domains of alternative methods

For biokinetic endpoints, besides the *in vitr*o percutaneous absorption test mentioned before, not much is yet in a sufficient state to be taken up in validation. For biotransformation a selection of methodology and prevalidation of computer-based approaches and *in vitro* culture tests remain to be done.
For the important and large area of target organ toxicity and systemic toxicity a lot of development work is necessary. For neurotoxicity some relevant data exist and a tiered approach on the sequential assessment of basal cytotoxicity and neurospecific endpoints is recommended by ECVAM (27). However, it must be noted that until today no single *in vitro* method for neurotoxicity has been formally validated.
Also in several other areas not many tests are available that can currently be used in regulatory testing. Strategies are being proposed by ECVAM for nephrotoxicity testing and a study is running on the identification of possible *in vitro* endpoints. For the other organs no specific methods are yet available (27). As already mentioned, for repeat-dose toxicity testing, no generally accepted alternative methods are available for replacing chronic testing in animals, although this is an important issue in the safety evaluation of cosmetics consuming a large number of animals.
An ECVAM workshop on novel, advanced *in vitro* methods for long-term testing was held in 1999 and the report was recently published (41). Several models exist for long-term testing in liver, kidney and central nervous system, but no validation is yet been done.
For genotoxicity and carcinogenicity, *in vitro* mutagenicity tests are quite well developed as far a genototoxic compounds are concerned. Tests for detecting non-genotoxic carcinogens is another issue. As long as these do not exist, *in vivo* rodent studies will remain necessary.
For reproductive toxicity, 3 embryotoxicity methods have been endorsed by ESAC (see before), but these are only a small part of the tests needed in reproductive toxicity testing, which usually is performed *in vivo* and consumes much animals.

6. References

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Table1: *in vitro* methods for eye irritation

Method	Test system	Endpoint	Reference
Bovine corneal opacity and permeability (BCOP) test	Excised cornea from the bovine eye	Opacity and permeability of the cornea	28
Hen's egg test-chorioallantoic membrane (HET-CAM)	Hen's egg	Damage to chicken chorioallantoic membrane	29
Chorioallantoic membrane-trypan blue staining (CAM-TBS)	Hen's egg	Damage to chicken chorioallantoic membrane	30
Isolated rabbit eye (IRE) test	Isolated rabbit eye	Corneal swelling, corneal opacity and fluorescein retention	31
Isolated chicken eye (ICE) test	Isolated chicken eye	Corneal swelling, corneal opacity and fluorescein retention	32
Fluorescein leakage (FL) test	Madin-Darby Canine Kidney (MDCK) cells	Damage caused to the tight junctions in MDCK monolayers	33
Neutral red uptake (NRU) test	3T3-L1 cells	Cell viability	34
Neutral red release (NRR) test	Rabbit corneal fibroblasts or mouse embryonic fibroblasts or normal human epidermal keratinocytes	Damage to the cell membrane	35
Red blood cell (RBC) haemolysis test	RBCs from calf blood samples	Damage to cytoplasmic membrane (haemolysis) in combination with damage of liberated cellular proteins (denaturation)	36
Agarose Diffusion Method	L929 mouse fibroblast cells	Cell death	37
EpiOcular™	Reconstituted human corneal epithelium	Cell viability, release of inflammatory mediators; permeability (MTT, IL-1, IL-1α, PGE2, LDH, and sodium fluorescein permeability)	38

From ECVAM report April 2002

Table 2 : *in vitro* methods for skin irritation

Method	Test system	Endpoint	Applicability	Formal Status
EPISKIN™human skin model (commercial system)	reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	general; a few materials may interfere with MTT reduction	Protocol modification and prevalidation (validation study under discussion)
Epi<<<Derm™human skin model (commercial system)	reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	general; a few materials may interfere with MTT reduction	Protocol modification and prevalidation (validation study under discussion)
Pig ear test	pig ear	Trans-epidermal water loss (TEWL)	general	Further development necessary
Mouse skin integrity function test (SIFT)	excised mouse skin	TEWL and electrical resistance	general; a few materials may interfere with either TEWL or ER determination	Protocol modification and prevalidation (validation study under discussion)

From ECVAM report April 2002