The use of non-animal alternatives in the safety evaluations of cosmetics ingredients by the Scientific Committee on Consumer Safety (SCCS)

M.P. Vinardell

Dept. Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona (Spain)

Corresponding author: M.P. Vinardell

Dept. Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona (Spain), email: mpvinardellmh@ub.edu

Tel: +34934024505; Fax +34934035901

Abstract

In Europe, the safety evaluation of cosmetics is based on the safety evaluation of each individual ingredient. Article 3 of the Cosmetics Regulation specifies that a cosmetic product made available on the market is to be safe for human health when used normally or under reasonably foreseeable conditions. For substances that cause some concern with respect to human health (e.g. colorants, preservatives, UV-filters), safety is evaluated at the Commission level by a scientific committee, presently called the Scientific Committee on Consumer Safety (SCCS).

According to the Cosmetics Regulations, in the EU, the marketing of cosmetics products and their ingredients that have been tested on animals for most of their human health effects, including acute toxicity, is prohibited. Nevertheless, any study dating from before this prohibition took effect is accepted for the safety assessment of cosmetics ingredients. The in vitro methods reported in the dossiers summited to the SCCS are here evaluated from the published reports issued by the scientific committee of the Directorate General of Health and Consumers (DG SANCO); responsible for the safety of cosmetics ingredients. The number of studies submitted to the SCCS that do not involve animals is still low and in general the safety of cosmetics ingredients is based on in vivo studies performed before the prohibition.

Highlights

- SCCS safety evaluations of cosmetics ingredients are based on in vivo studies from before the animal ban.
- Dermal absorption is the most common study done in vitro, although animals are also used.
- Few in vitro studies of toxicokinetics were included in the dossiers.
- Studies on human volunteers were also included for skin and eye irritation, dermal absorption and toxicokinetics.

Key words

Animal alternatives, cosmetics ingredients, safety evaluation, animal ban, in vitro, in vivo

1. Introduction

The safety evaluation of cosmetics in Europe is based on the evaluation of each individual ingredient. Article 3 of the European Cosmetics Regulations specifies that a cosmetic product made available on the market is to be safe for human health when used normally or under reasonably foreseeable conditions. Cosmetics products have rarely been associated with serious health hazards; however, this does not mean that the use of cosmetics per se is safe. Particular attention needs to be paid to long-term safety aspects, since cosmetics products may be used extensively over a large part of the human lifespan and sensitive groups of the population such as children, old people, pregnant women, etc. may be affected. Therefore, safety-in-use for cosmetics products has been established in Europe by controlling the ingredients via their chemical structures, toxicity profiles, and patterns of exposure.

The safety of those substances that cause some concern with respect to human health (e.g. colorants, preservatives,UV-filters, etc.) is evaluated at the Commission level by a scientific committee, presently called the Scientific Committee on Consumer Safety (SCCS). The substances are detailed in the Annexes of Regulation (EC) No. 1223/2009, which replaced the previous Directive from 11 July 2013 onwards (European Commission, 2009).

The SCCS was established in 2008 to substitute the former Scientific Committee of Consumer Products (SCCP). Before 1997, the recommendations proposed by the Scientific Committee on Cosmetology at the Commission’s request were included in EC Reports. Between 1997 and 2004, all Scientific Committee opinions were published on the Internet and can be accessed through the Committee's website. All SCCS opinions can easily be located through the substance category of the ingredient involved and the adoption date.

One of the responsibilities of the SCCS is to recommend guidelines for the cosmetics and raw materials industries to develop adequate studies for the safety evaluation of

cosmetics. The SCCS evaluates the dossiers submitted by industry through the Directorate General of Health and Consumers (DG SANCO). The cosmetics ingredients evaluated by the SCCS correspond to those in the Annexes of the Regulations and to substances forbidden in Annex II, restricted substances in Annex II, and colorants, preservatives and UV-filters in Annexes IV, V and VI respectively.

Determination of the toxic potential of a cosmetics product is based on a series of toxicity studies and forms part of the hazard identification. Alternative methods, replacing animal testing, have been mandatory in Europe to evaluate cosmetics ingredients since March 2013, according to a Commission Decision. However, at present, the majority of toxicological tests still involve the use of animals, as is also the case for other chemical substances. Traditionally, toxicological data that are relevant to human health have been obtained by studying the toxicological profiles on animals of the substances under consideration, using the same exposure route as that in humans (topical, oral or inhalation).

When a dossier containing information on a cosmetics product is submitted to the SCCS for evaluation, the manufacturer should provide the Commission with information on: acute toxicity (if available); irritation and corrosivity to skin and eye; skin sensitisation; dermal / percutaneous absorption; repeat dose toxicity; mutagenicity / genotoxicity; carcinogenicity; reproductive toxicity; toxicokinetics; photo-induced toxicity; and human data (SCCS/1501/12).

One consideration before toxicological studies are accepted for evaluation is whether the studies have been carried out according to guidelines and following Good Laboratory Practice (GLP). In some cases, this information is not present and the SCCS asks for further information before making an opinion.

According to the Cosmetics Regulation (European Commission, 2009), it is prohibited in the EU to market cosmetics products and their ingredients if they have been tested on animals for most human health effects, including acute toxicity. This imposes on the

cosmetics industry the need for alternative approaches to the safety testing of the ingredients of consumer products. After a meeting of experts organised by the European Centre for the Validation of Alternative Methods (ECVAM), the alternative methods that existed at the time and had been applied to cosmetics were reviewed (Adler et al., 2011, Hartung et al., 2011).

The 7th amendment to the EU Cosmetics Directive prohibits the launching of animal-tested cosmetics on the European market after 2013. The European Commission invited stakeholders (industry, non-governmental organisations, EU member states and the Commission's SCCS) to identify scientific experts in five areas of toxicological: toxicokinetics, repeat dose toxicity, carcinogenicity, skin sensitisation, and reproductive toxicity. The experts selected were asked to analyse the status of and prospects for alternative methods, and to provide a scientific estimate of the time necessary to achieve full replacement of animal testing. In short, the experts confirmed that it would take at least another 7-9 years for the complete replacement of the current in vivo animal tests used for the skin sensitisation safety assessment of cosmetics ingredients for skin sensitisation. However, the experts were also of the opinion that alternative methods may provide hazard information, i.e., to differentiate between sensitisers and non-sensitisers, before 2017. This would, however, not provide complete information on what safe exposure is, because the relative potency of a sensitiser would still not be known. For toxicokinetics, the timeframe was 5-7 years to develop the models still lacking to predict lung absorption and renal/biliary excretion; and even longer to integrate the methods to fully replace animal toxicokinetic models. For the systemic toxicological endpoints of repeat dose toxicity, carcinogenicity and reproductive toxicity, the time necessary for full replacement could not even be estimated (Adler et al., 2011).

CAAT-Europe assembled experts from Europe, America and Asia to design a scientific roadmap for future risk assessment approaches, considering that the animal use for cosmetics testing for the European market has been banned. The key recommendations proposed

focused on improving existing methods, the combination of hazard testing and toxicokinetics predictions and the developing of integrated test strategies among others. Important points are the data quality, and the scientific background of a test method. Information from each test system should be mapped along adverse outcome pathways (Leist et al. 2014).

2. Methodology

The study material consisted of SCCS opinions issued between April 2008 and March 2013 concerning cosmetics ingredients. No confidential data were used, as all the information came from opinions downloaded from the Committee's website. There are different types of opinions and in some cases there are addenda to previous opinions. In this study, only full opinions were considered: addenda or specific opinions for a particular item, such as microbial resistance, were not taken into account.

Each opinion was analysed with respect to each of the different sections, taking note of whether the procedure used was based on the use of animals or non-animal models. The percentage of non-animal models was compared to that of animal models and the use of human data was also noted.

A total of 103 dossiers were analysed: 75 corresponded to hair dyes and 28 to other ingredients in cosmetics including UV filters, fragrances and preservatives, among others.

3. Results and Discussion

SCCS opinions are currently organised into hair dyes, cosmetics ingredients and nanomaterials; but over the period evaluated in the present study, the opinions were organised into fragrances, hair dyes, preservatives, UV-filters and other substances. In this paper, for comparative purposes, we distinguish between hair dyes and other ingredients, but we have also grouped the two categories together. The number of

SCCS opinions depends on the type of cosmetics; hair dyes were the most numerous with 75 substances evaluated.

Studies performed on animals could be included only if they were performed before the ban on animal use in March 2009, except for repeat dose studies which were permitted until March 2013. After that date, new studies were required not to use animals.

3.1. Acute toxicity

Studies of acute toxicity are not always necessary for the dossiers summited to the SCCS, but they are usually included in those supplied by industrial sources and in all cases the studies were performed on laboratory animals. The oral route was the most common, but the dermal route was also used occasionallyand in a few cases information about the inhalation route was also supplied. All the accepted methods for determining acute oral toxicity are based on in vivo experiments that estimate the LD50 value (i.e., the single dose of a substance that can be expected to cause death in 50% of the animals in an experimental group). Considering the prohibition on the use of animals for cosmetics ingredients and building on the results of a previous international validation study, a follow-up study was organised by the ECVAM to assess whether the 3T3 Neutral Red Uptake cytotoxicity assay could identify substances not requiring classification as acute oral toxicants under the EU regulations. The assay exhibited high sensitivity (92%–96%) but relatively low specificity (40%–44%). It could thus prove to be a valuable part of an integrated testing strategy: a read-across argument or weight-of-evidence (WoE) approach to identifying non-toxic chemicals (LD50 > 2000 mg/kg) (Prieto et al., 2013). In the dossiers supplied by industry sources for SCCS evaluation over the period 2009-2013, no assays to predict acute toxicity were performed in vitro.

3.2. Eye irritation

Eye irritation is one of the classic studies performed on animals, usually rabbits, as reported many years ago (Draize et al., 1944). The method has been highly controversial and much effort has gone into developing alternative methods (Vinardell and Mitjans, 2008). However, the validated in vitro methods focus on distinguishing corrosive and more irritant chemicals from non-irritants, and they do not make categorisation possible, in contrast to the in vivo method. In the dossiers submitted to the SCCS, nearly all the studies were performed on albino rabbits; only a few used in vitro methods. The majority of the in vivo studies performed on rabbits followed the OECD guidelines, which were adopted in 1981 and updated successively in 1987, 2002 and then recently in 2012 (OECD, 2012). However, some studies adhered to no specific guidelines and were not even performed under GLP conditions; some used guinea pigs as the animal model.

Among the in vitro methods reported in the dossiers related to different ingredients, we found the isolated chicken eye (ICE) and the bovine corneal opacity and permeability (BCOP) tests; two validated methods that appear in the OECD guidelines (OECD, 2013a,b). These are in vitro tests used to identify chemicals (individual substances or mixtures) as either: 1) causing "serious eye damage" (category 1 of the Globally Harmonised System for the Classification and Labelling of Chemicals (GHS)); or 2) not requiring classification for eye irritation or serious eye damage according to the GHS. Other methods that are used include the Het-Cam: a method that has not been validated but which is very widely used by the cosmetics industry due to its low cost; and neutral red uptake in cell cultures (Spielmann et al., 1996). When comparing the results for hair dyes with those for other ingredients, we observed that in the former case there were no studies on human volunteers whereas in the latter case human studies represented 9% of the total. When we considered all the ingredients together, the percentage of human studies was just 3% (Figure 1). The use of human volunteers

in studies of eye irritation is not considered ethical by the SCCS, as indicated in many opinions.

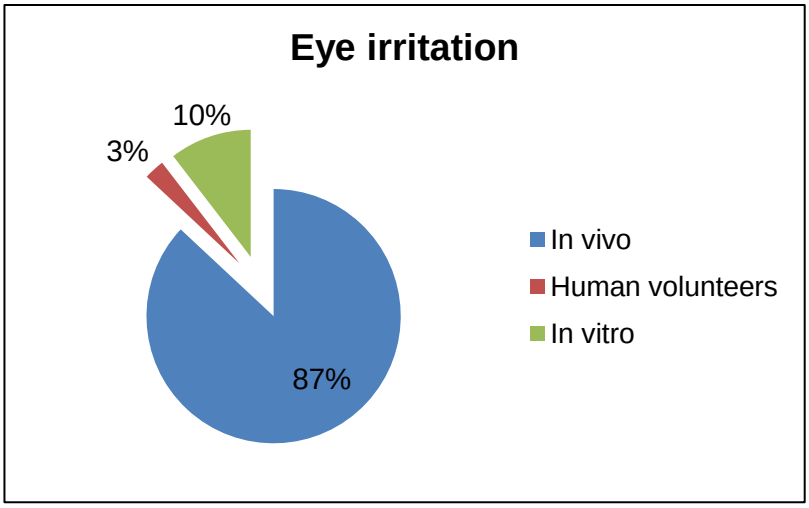

Figure 1. Percentage of eye irritation studies performed in vivo, in vitro and on human volunteers.

The need for alternative approaches to replace the in vivo Draize rabbit eye test for the evaluation of the eye irritation of cosmetics has been recognised by the cosmetics industry for many years. There has been extensive research into the development of different assays, some of which have been formally validated; but no single in vitro assay has been validated as a full replacement for the Draize rabbit eye test. Although not formally validated, several other in vitro models have been used for over a decade by the cosmetics industry as valuable tools in a WoE approach to the safety assessment of ingredients and finished products. Cosmetic Europa, formerly COLIPA, organised a scientific meeting in 2008 to review the use of alternative approaches and to set up a decision-tree approach for their integration into tiered testing strategies for the hazard and safety assessment of cosmetics ingredients and their use in products (McNamee et al., 2009). The conclusion was that confidence in the evaluation of eye irritation potential is increased through the use of combinations of assays to obtain a classification of the irritancy potential (from non-irritant to severe). A combination was proposed of both recognised accepted and non-validated assays, together with all

other available information, in a tiered approach based on a WoE evaluation of eye irritation. General acceptance of such an approach is necessary for animal studies to be replaced by it.

3.3. Skin irritation

In the case of skin irritation, the accepted method was adopted in 1981 and updated in 2002 (OECD, 2002). The method is based on the use of rabbit, in a way similar to that used in the Draize eye test, and this was the most commonly used method in these evaluations. However, other species such as guinea pig or mouse were used to a lesser extent for the evaluation of hair dyes. In the case of other substances, the use of human volunteers was observed. The use of in vitro methods has been very limited: to TER (rat skin transcutaneous electrical resistance test) and to the use of reconstructed epidermis models. The percentage of the different methods used to assay all the ingredients is shown in Figure 2. The use of in vitro methods was even less common than the use of human volunteers.

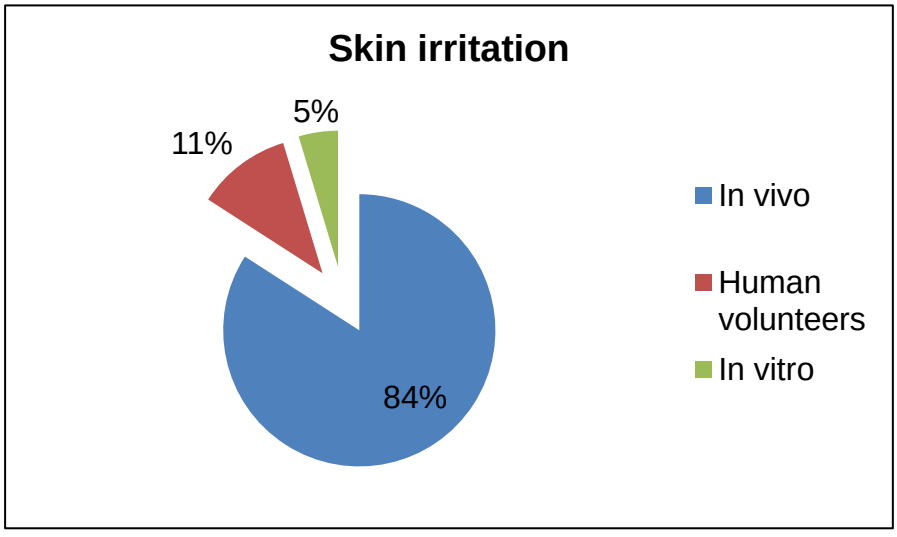

Figure 2. Percentage of skin irritation studies performed in vivo, in vitro and on human volunteers.

A number of in vitro skin irritation tests have been officially validated and are accepted in the OECD guidelines such as OECD439 (OECD, 2013c). The methods are based on reconstructed human epidermis. Taking the EpiSkin^{TM} method as an example, the SCCS expressed concerns over potential interference with colour formation from reducing substances, hair dyes and colourants (SCCP/1145/07). After studying additional data supplied by an industry source, the SCCS expressed the opinion that the modified EpiSkin™ method did not sufficiently show that the 3-(4,5)-dimethyl-2-thiazolyl-2,5-dimethyl-2Htetrazolium bromide (MTT) test could be used as a suitable endpoint to test colour ingredients/hair dyes for their potential skin irritation. A different endpoint, not involving optical density quantification, should be sought [SCCS/1392/10].

For skin corrosion testing, at present 5 validated in vitro alternatives have been included in the Regulations: the TER (OECD, 2013d) and tests on reconstructed human epidermis (EpiSkin™, EpiDerm™, SkinEthic™ and EST-1000 (epidermal skin test-1000) (OECD, 2013e).

Similarly to the case of eye irritation, Cosmetic Europe (formerly COLIPA) has devised a decision tree. One of the conclusions of the COLIPA workshop and Project Team Safety Assessment 2009/2013, was that the good correlation between in vitro and in vivo skin irritation assays, together with the substantial in-house experience with the former, allows for confidence in the outcomes of these assays, such that in-house safety assessments of new products can be made without the use of animal testing. A decision tree for hazard assessment and classification, using a WoE approach throughout, involves stepwise evaluation of: firstly, physicochemical characteristics, (Q)SAR and existing data, to identify and rule out corrosive chemicals from further testing; secondly, in vitro corrosivity; and finally, in vitro irritation, to distinguish between irritants and non-irritants. Once a chemical has been classified as corrosive, irritant or non-irritant, its safety assessment can then be evaluated using a second decision tree approach. Corrosive chemicals should be tested in an in vitro corrosivity test at the use

concentration and, if shown to be non-corrosive, tested for irritation using an RHE in vitro irritation model. Chemicals classed as irritants can be retested at the usage concentration, since they may not be irritants at lower concentrations or when used in the final formulation. Human confirmatory testing of the formulation is only carried out on a case-by-case basis. In conclusion, the evaluation of the skin irritation potential of new chemicals to be used in cosmetics can be confidently accomplished using only alternative methods (Macfarlane et al., 2009).

3.4. Skin sensitisation

For skin sensitisation, the studies were mostly performed in vivo (81%) and a small percentage on humans using the patch test method (Figure 3).

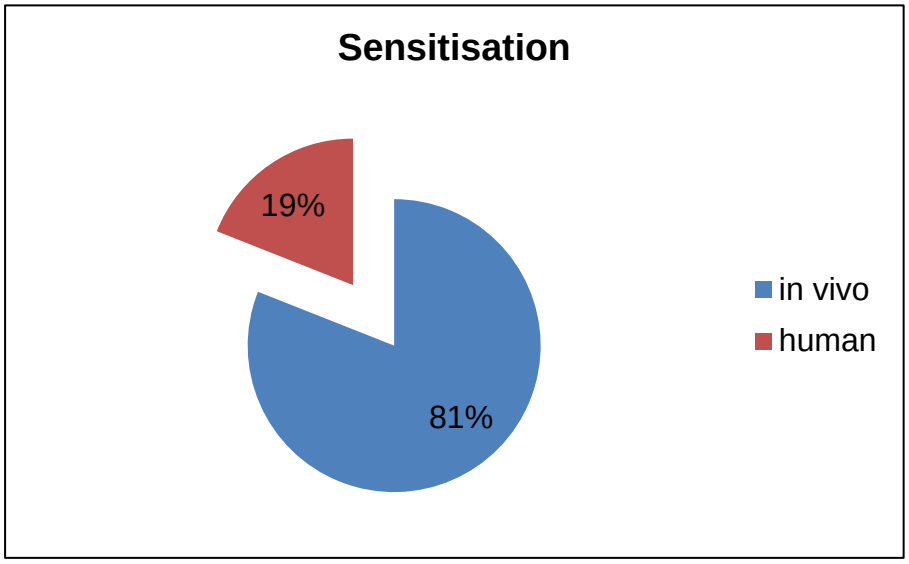

Figure 3. Percentage of sensitisation studies performed in vivo and on human volunteers.

Officially accepted animal testing methods for assessing skin sensitisation potential include: the mouse Local Lymph Node Assay (LLNA) and its non-radioactive modifications (LLNA-DA and the LLNA-BrdU Elisa) (OECD, 2010); the Guinea Pig Maximisation Test (GPMT) by Magnusson & Kligman; and the Buehler occluded patch

test in the guinea pig (OECD, 1992). The mouse and guinea pig methods differ with respect to the endpoints used: whereas the mouse LLNA measures the responses provoked during the induction of sensitisation, the two guinea pig tests measure challenge-induced elicitation reactions in previously sensitised animals. The Buehler method is less sensitive than the GPMT and scientific justification should be given if the Buehler test is used [SCCS/1501/12]. The mouse LLNA was used more than the methods based on guinea pigs (Figure 4).

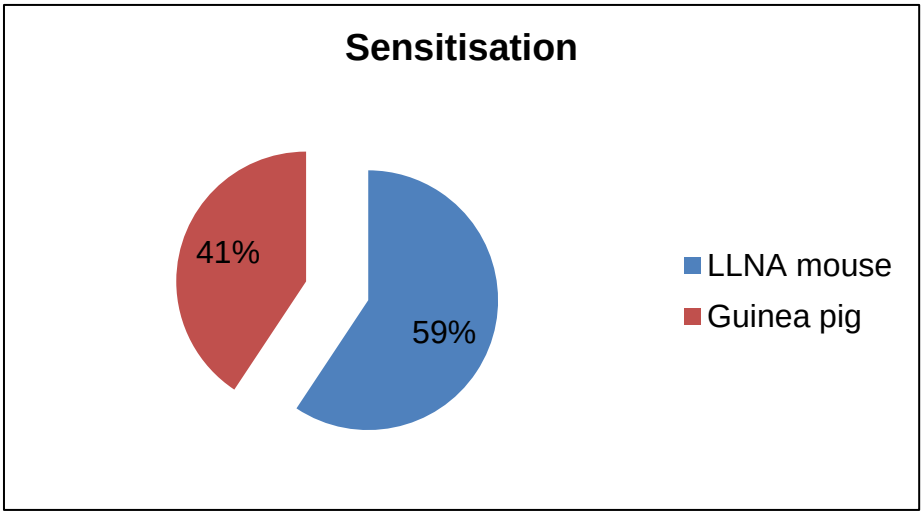

Figure 4. Percentage of sensitisation studies performed on mice and guinea pigs.

The LLNA is considered a reduction and refinement method compared to the traditional guinea pig tests since it provides advantages in terms of animal welfare, but it cannot more be used for evaluation of ingredients in cosmetics.

The most commonly used in vivo method was the LLNA. The basic principle underlying the mouse LLNA is that sensitisers induce a primary proliferation of lymphocytes in the auricular lymph nodes that drain the chemical application site. This proliferation is proportional to the dose applied and provides a measure of sensitisation.

As opposed to the skin or eye irritation studies, animal sensitisation studies were permitted until March 2013 under European legislation, because they correspond to repeat dose toxicity. Of the studies presented, none were in vitro; nevertheless, there are two validated methods that are currently in the final phase of OECD approval.

356 Those two methods are the Direct Peptide Reactivity Assay (DPRA) (Gerberick et al.,
357 2004, 2007) and KeratinoSens™ (Natsch et al., 2014; Delaine et al., 2011). The DPRA
358 addresses the process of haptenation, i.e., the covalent binding of low-molecular-
359 weight substances (haptens) to skin proteins, which is considered to be the molecular
360 initiating event of skin sensitisation. KeratinoSens™ addresses the activation of the
361 antioxidant/electrophile response element (ARE)-dependent pathway in keratinocytes;
362 a biological mechanism covered by the second key event of skin sensitisation. Both
363 test methods provide mechanistic information considered relevant for the assessment
364 of the skin sensitisation potential of chemicals.
365 The human studies were performed by old methods (Marzulli and Maibach, 1986;
366 Kligman, 1966; Kligman and Epstein, 1975) based on the maximisation response in
367 volunteers. The human repeat insult patch test (HRIPT) consists of 2 phases, or
368 sometimes 3. Phase I is the induction phase, where the product is applied to the skin 9
369 times over the course of 3 weeks. This is followed by a two-week rest period, after
370 which the skin is exposed to the product again in phase II: the elicitation phase. A
371 response in phase II is usually allergic in nature and phase III is used to verify and
372 better define the reaction. The different methods available have different application
373 phases, but the resulting predictions of allergy and irritation response are the scientific
374 goals. Use of the HRIPT is considered unethical by the SCCS.
375
376 3.5. Dermal absorption
377 Dermal absorption is a well-established in vitro method that is described in the OECD
378 guidelines and there is a special SCCS memorandum that describes the procedure
379 (SCCS/1358/10). Despite the existence of an in vitro protocol, some studies were
380 performed on animals and human volunteers (Figure 5).
381 The in vivo studies were performed on rats, but in some cases rabbits were also used.
382 The in vitro method can use skin from humans or pigs, according to the SCCS
383 recommendations. Human skin is the better choice but is not always readily available.

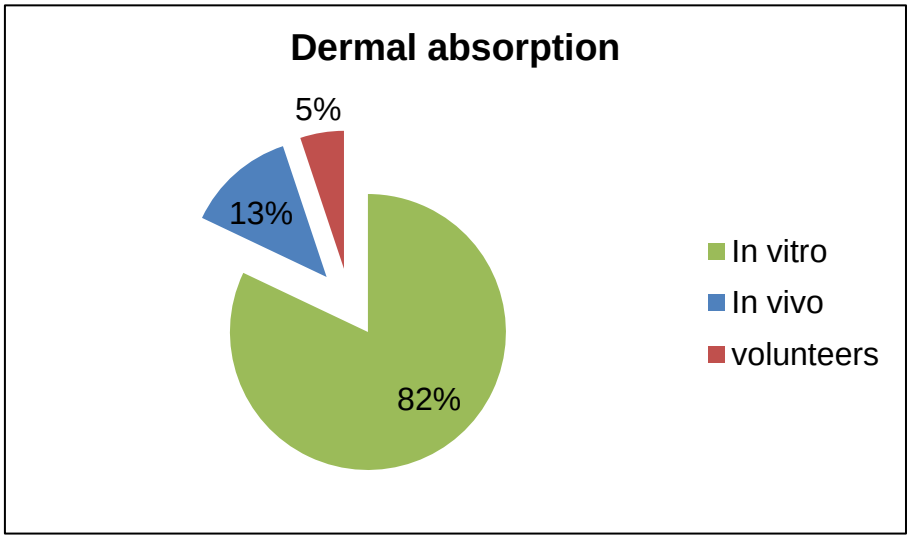

Figure 5. Percentage of dermal absorption studies performed in vitro, in vivo and on human volunteers.

Alternatively, pig skin may be used as it shares essential permeation characteristics with human skin. However, 12 studies (11.65%) used rat skin, despite high levels of absorption having been demonstrated for this skin; it is some 2 to 10 times more permeable than human skin due to differences in the thickness of the epidermis (Ross et al., 2000).

Another option is to use cultured or reconstructed human skin models; but such systems are not yet recommended for in vitro testing, on the basis of an insufficient barrier function (Bouwstra et al., 2008). Some studies propose the use of a fully differentiated human skin trilayer that could have multiple applications such as in vitro drug absorption tests and regenerative therapies (Monfort et al., 2013); but such engineered skin has not yet been validated.

3.6. Genotoxicity

In the assessment of genotoxicity there are many in vitro methods that provide information on three major genetic endpoints: mutagenicity at a gene level,

411 chromosome breakage and/or rearrangements (clastogenicity), and numerical
412 chromosome aberrations (aneugenicity) (Pfuhler et al. 2010).
413 Due to the diverse nature of the mechanisms involved in genotoxicity, it is known that
414 no single test can detect all genotoxic effects. In this sense, the SCCS recommended
415 recently the combination of two assays the Bacterial reverse Mutation Test (OECD,
416 1997) as a test covering gene mutations and In vitro Micronucleus Test (OECD 2014)
417 as a test for both structural (clastogenicity) and numerical (aneugenicity) chromosome
418 aberrations. The combination of these two assays would cover the three genotoxicity
419 endpoints described above, as the bacterial test detects gene mutations and the in vitro
420 micronucleus assay detects both structural and numerical chromosome aberrations.
421 Except for special cases for which the Ames test is not suitable, the SCCS
422 recommends the combination of the two assays for the base level testing of cosmetic
423 substances (SCCS/1532/14).
424 These two assays have been used for evaluating genotoxicity in all the dossiers
425 evaluated by the SCCS, together with other in vitro and in vivo methods, the last
426 performed before the ban for animals use.
427
428 3.7. Carcinogenicity
429 Studies of carcinogenicity were not included in all the dossiers. Of the dossiers
430 evaluated, only 37 included studies of carcinogenesis; mostly in vivo, with only 22%
431 performed in vitro (Figure 6).
432 The in vivo studies were performed on mice, rats and hamsters; and by different
433 routes: oral, dermal and inhalation (Mallye et al., 2001). In most cases the method did
434 not follow any guidelines, despite the corresponding OECD Guideline being adopted in
435 1981 and recently revised (OECD, 2009).
436 The in vitro studies correspond to the in vitro cell transformation assay (CTA) in
437 BALB/c3T3 (Mascolo et al., 2010; Matthew et al., 1993) and the Syrian hamster
438 embryo cell (SHE) assay (Jones et al., 1988).

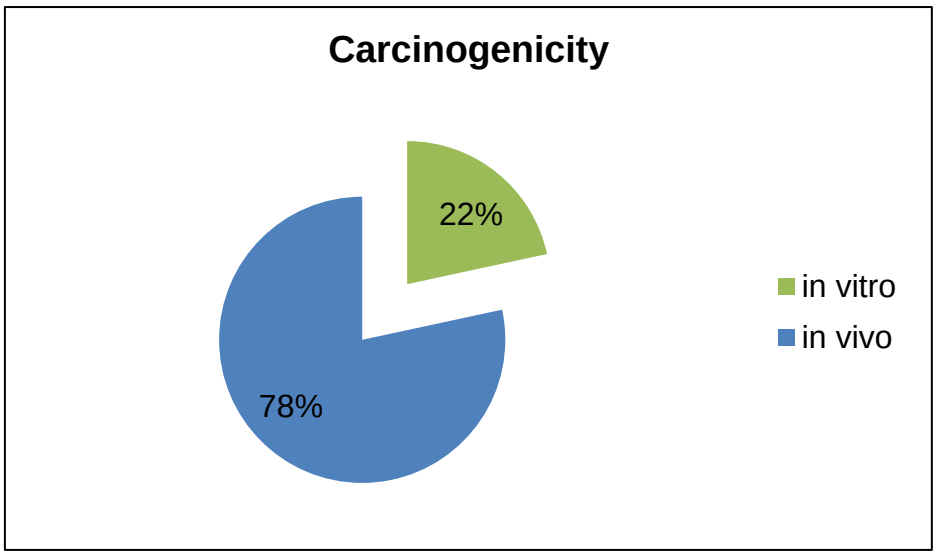

Figure 6 . Percentage of carcinogenicity studies performed in vivo and in vitro.

The BALB/c 3T3 model represents one of the best-known CTAs and is regarded as a useful tool to screen single chemicals or complex mixtures for carcinogenicity. Of the in vitro testing methods, CTAs appear to be one of the most suitable tools to predict the carcinogenic properties of chemicals (Lilienblum et al., 2008). Matthews et al. (1993) published a comprehensive review comparing the results obtained for 147 compounds in the BALB/c3T3 transformation test with those from animal bioassays; a good correlation was shown with good sensitivity but poor specificity.

SHE cell transformation has been used almost since it was first reported as an in vitro test to determine potential carcinogenicity of chemical/physical agents. Many groups worldwide have used this assay to study the carcinogenic capacity of a wide variety of chemical/physical agents and several inter-laboratory studies have been conducted to evaluate the assay (Isfort, 1996).

These methods are not yet accepted, but there are some validation studies (Corvi et al., 2012; Pant et al., 2012). Drafts of the guideline protocols are available online: http://www.oecd.org/env/ehs/testing/Draft%2017%20October%202012.pdf.

3.8. Toxicokinetic studies

The Toxicokinetic studies included different procedures and were usually performed in vivo on different animals or humans (Fig 7).

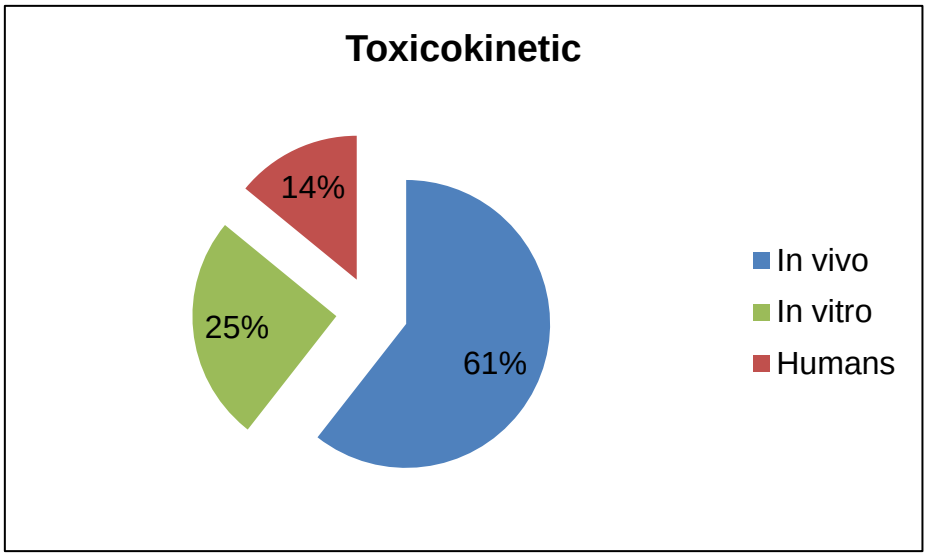

Figure 7. Percentage of toxicokinetic studies performed in vivo, in vitro or on human volunteers.

In vitro methods to study these phenomena should be based on different aspects of the process (absorption, metabolism, etc.).

The process of absorption has been studied in the TC-7 cell line, which is a clone of CaCo-2 cells, usually used in in vitro studies of oral absorption (Gres et al., 1998). In total, 10 hair dyes were studied. A study sponsored by the ECVAM evaluated the reproducibility (between-laboratory and within-laboratory variability) and the predictive capacity of two in vitro cellular systems—the Caco-2/ATCC parental cell line and the Caco-2/TC7 clone—at estimating the oral fraction absorbed (Fa) in humans (Prieto et al., 2010). The study concluded that good estimations of human Fa for five well-absorbed compounds was demonstrated; while moderately and poorly absorbed compounds were overestimated.

In the studies presented to assess the toxicokinetic effects of cosmetics ingredients, there were studies of metabolism in hepatocytes obtained from humans, rats or mice. These isolated cells (Klieber et al., 2010) or 3D models (Godoy et al., 2013) have been used in many studies to demonstrate effects on metabolism in vitro. Some studies of metabolism have been performed on keratinocytes or reconstructed epidermis. The use of reconstructed epidermis has been demonstrated to be a good strategy for studying metabolism in vitro (Hewitt et al., 2013; Götz et al., 2012a,b).

3.9. Phototoxicity

Phototoxicity studies were carried out on products that are especially exposed to solar radiation, such as UV filters, but also on some other products, such as some hair dyes, preservatives, etc. In all, only 35 of the products were studied for phototoxicity. One third of the studies were in vitro and nearly half were in vivo: the rest were on human volunteers (Figure 8).

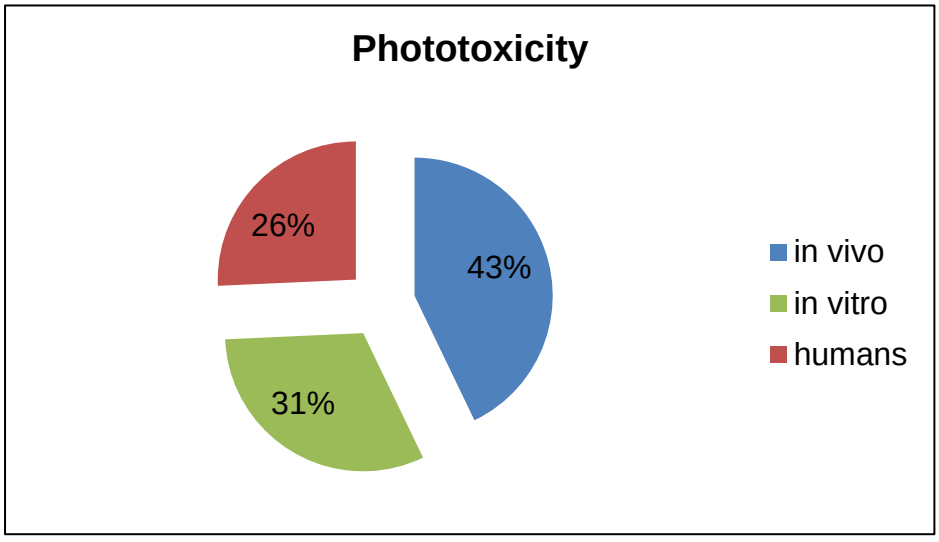

Figure 8. Percentage of phototoxicity studies performed in vivo, in vitro and on human volunteers.

Some studies of phototoxicity are related to the photomutagenicity response or photoallergy, rather than phototoxicity. All the hair dyes studied used in vivo studies in guinea pigs. The studies on human volunteers corresponded to UV-filters, and some preservatives, fragrances and other substances. In total, 9 substances were assessed in humans.

Only five studies corresponded to the validated and accepted method of 3T3-NRU phototoxicity (ECVAM, 1998; Spielmann et al., 1998; Gaspar, 2013; Ceridono et al., 2013). It is surprising that so few studies were performed using this method, considering it was the first validated in vitro method to be accepted by the OECD (OECD, 2004).

A recent study has established a non-animal photosafety assessment approach for cosmetics using in vitro photochemical and photobiochemical screening systems The photochemical properties were assessed in by UV/VIS spectral analysis, reactive oxygen species (ROS) assay and 3T3 neutral red uptake phototoxicity testing (3T3 NRU PT). These in vitro screening systems individually provide false predictions; however, a systematic tiered approach using these assays was proposed to provide photosafety assessment without any false-negatives (Onoue et al. 2013).

Conclusions

The toxicological studies of new cosmetics ingredients should at present be in vitro. However, safety evaluation can be based on in vivo studies performed before the European ban on the use of animals came into effect. The evaluations of different cosmetics ingredients performed by the SCCS are mostly based on in vivo studies from before the ban. At the moment, the total number of in vitro studies is small compared to that of studies on laboratory animals. We believe the near future will see an increase in the use of in vitro methods. There are some validated and accepted methods, but there are not methods for all the studies required; there are no validated and accepted methods for repeat dose toxicity, toxicokinetics and others.

550 **References**

551 Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., et al.,
552 2011. Alternative (non-animal) methods for cosmetics testing: current status and future
553 prospects-2010. Arch. Toxicol. 85, 367-485. DOI: 10.1007/s00204-011-0693-2

554 Bouwstra, J.A., Groenink, H.W., Kempenaar, J.A., Romeijn, S.G., Ponec, M., 2008.
555 Water distribution and natural moisturizer factor content in human skin equivalents are
556 regulated by environmental relative humidity. J. Invest. Dermatol. 128, 378-388.

557 Corvi, R., Aardema, M.J., Gribaldo, L., Hayashi, M., Hoffmann, S., Schechtman, L.,
558 Vanparys, P., 2012. ECVAM prevalidation study on in vitro cell transformation assays:
559 general outline and conclusions of the study. Mut. Res. 744,12-19.

560 Ceridono, M., Tellner, P., Bauer, D., Barroso, J., Alépée, N., Corvi, R., et al., 2012. The
561 3T3 neutral red uptake phototoxicity test: practical experience and implications for
562 phototoxicity testing--the report of an ECVAM-EFPIA workshop. Regul. Toxicol.
563 Pharmacol. 63, 480-488. DOI: 10.1016/j.yrtph.2012.06.001.

564 Delaine, T., Niklasson, I.B., Emter, R., Luthman, K., Karlberg, A.T., Natsch, A., 2011.
565 Structure--activity relationship between the in vivo skin sensitizing potency of
566 analogues of phenyl glycidyl ether and the induction of Nrf2-dependent luciferase
567 activity in the KeratinoSens in vitro assay. Chem. Res. Toxicol. 15, 1312-1318. DOI:
568 10.1021/tx200196s

569 Draize, J.H., Woodward, G., Calvery, H.O., 1944. Method for the study of irritation and
570 toxicity of substances applied topically to the skin and mucous membranes. *J.*
571 *Pharmacol. Exp. Ther.* **82,** 377–390.

572 ECVAM, 1998. ECVAM Statement on the scientific validity of the 3T3 NRU PT test (an
573 in vitro test for phototoxic potential) ATLA, 26 , 7–8.

574 EU, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the
575 Council of 30 November 2009 on cosmetic products. Oficial Journal of the European
576 Union. L 342,59-209.

577 Gaspar,L.R., Tharmann, J., Campos, P.M., Liebsch, M., 2013. Skin phototoxicity of
578 cosmetic formulations containing photounstable and photostable UV-filters and vitamin
579 A palmitate. Toxicol. in Vitro, 27, 418–425. DOI: 10.1016/j.tiv.2012.08.006

580 Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin,
581 J.P., 2004. Development of a peptide reactivity assay for screening contact allergens.
582 Toxicol. Sci. 81, 332-343.

583 Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G, Lepoittevin,
584 J.P., 2007. Quantification of chemical peptide reactivity for screening contact allergens:
585 A classification tree model approach. Toxicol. Sci. 97, 417-427.

586 Godoy, P., Hewitt, N.J., Albrecht, U., Andersen, M.E., Ansari, N., Bhattacharya, S., et
587 al., 2013. Recent advances in 2D and 3D in vitro systems using primary hepatocytes,
588 alternative hepatocyte sources and non-parenchymal liver cells and their use in
589 investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch. Toxicol. 87,
590 1315-1530. DOI: 10.1007/s00204-013-1078-5

591 Götz, C., Pfeiffer, R., Tigges, J., Blatz, V., Jäckh, C., Freytag, E.M., Fabian, E.,
592 Landsiedel, R., Merk, H.F., Krutmann, J., Edwards, R.J., Pease, C., Goebel, C., Hewitt,
593 N., Fritsche, E., 2012a. Xenobiotic metabolism capacities of human skin in comparison
594 with a 3D epidermis model and keratinocyte-based cell culture as in vitro alternatives
595 for chemical testing: activating enzymes (Phase I).Exp. Dermatol. 21, 358-363. DOI:
596 10.1111/j.1600-0625

597

598 Götz, C., Pfeiffer, R., Tigges, J., Ruwiedel, K., Hübenthal, U., Merk, H.F., Krutmann, J.,
599 Edwards, R.J., Abel, J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012b.
600 Xenobiotic metabolism capacities of human skin in comparison with a 3D-epidermis
601 model and keratinocyte-based cell culture as in vitro alternatives for chemical testing:
602 phase II enzymes. Exp. Dermatol. 21, 364-369. DOI: 10.1111/j.1600-0625

603 Grès, M.C., Julian, B., Bourrié, M., Meunier, V., Roques, C., Berger, M., Boulenc, X.,
604 Berger, Y., Fabre, G., 1998. Correlation between oral drug absorption in humans, and
605 apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line:
606 comparison with the parental Caco-2 cell line. Pharm. Res. 15, 726-733.

607 Hartung, T., Blaauboer, B.J., Bosgra, S., Carney, E., Coenen, J., Conolly, R.B., Corsini,
608 E., Green, S., Faustman, E.M., Gaspari, A., Hayashi, M., Wallace, H. A., Hengstler,
609 J.G., Knudsen, L.E., Knudsen, T.B., McKim, J.M., Pfaller, W., Roggen, E.L. 2011. An
610 expert consortium review of the EC-commissioned report "alternative (Non-Animal)
611 methods for cosmetics testing: current status and future prospects - 2010".ALTEX. 28,
612 183-209.

613 Hewitt, N.J., Edwards, R.J., Fritsche, E., Goebel, C., Aeby, P., Scheel, J., Reisinger,
614 K., Ouédraogo, G. et al., 2013. Use of human in vitro skin models for accurate and
615 ethical risk assessment: metabolic considerations.Toxicol. Sci. 133,, 209-217. DOI:
616 10.1093/toxsci/kft080

617 Isfort, R.J., Kerckaert, G.A., LeBoeuf, R.A., 1996. Comparison of the standard and
618 reduced pH Syrian Hamster Embryo (SHE) cell in vitro transformation assays in
619 predicting the carcinogenic potential of chemicals. Mut. Res. 356,11-63.

620 Jones, C.A., Huberman, E., Callaham, M.F., Tu, A., Halloween, W., Pallota, S., Sivak,
621 A., Lubet, R.A., Avery, M.D., Kouri, R.E., Spalding, J., Tennant, R.W., 1988. An
622 Interlaboratory Evaluation of the Syrian Hamster Embryo Cell Transformation Assay
623 Using Eighteen Coded Chemicals. Toxicol.In Vitro, 2, 103-116.

624 Klieber, S., Torreilles, F., Guillou, F., Fabre, G., 2010. The use of human hepatocytes
625 to investigate drug metabolism and CYP enzyme induction. Methods Mol. Biol. 640,
626 295-308. DOI: 10.1007/978-1-60761-688-7_16.

627 Kligman, A.M., 1966. The identification of contact allergens by human assay. III. The

628 maximization test. A procedure for screening and rating contact sensitizers. J. Invest.
629 Dermatol. 47, 393-409.

630 Kligman, A.M., Epstein, W., 1975. Updating the maximization test for identifying contact
631 allergens. Contact Dermatitis 1: 231-239.

632 Leist, M., Hasiwa, N., Rovida, C., Daneshian, M., Basketter, D., Kimber, I., Clewell, H.,
633 Gocht, T., Goldberg, A., Busquet, F., Rossi, A.M., Schwarz, M., Stephens, M.,
634 Taalman, R., Knudsen, T.B., McKim, J., Harris, G., Pamies, D., Hartung, T. 2014.
635 Consensus report on the future of animal-free systemic toxicity testing. ALTEX. 31,
636 341-56.

637 Lilienblum, W., W. Dekant, W., Foth, H., Gebel, T., Hengstler, J.G. , Kahl, R., Kramer,
638 P.J. , Schweinfurth, H., Wollin K.-M., 2008. Alternative methods to safety studies in
639 experimental animals: role in the risk assessment of chemicals under the new
640 European chemicals legislation (REACH). Arch. Toxicol. 82, 211–236. DOI:
641 10.1007/s00204-008-0279-9

642 Macfarlane, M., Jones, P., Goebel, C., Dufour, E., Rowland, J., Araki, D., Costabel-
643 Farkas, M., Hewitt, N.J., Hibatallah, J., Kirst, A., McNamee, P. Schellauf, F., Scheel, J.,
644 2009. A tiered approach to the use of alternatives to animal testing for the safety
645 assessment of cosmetics: Skin irritation. Reg.Toxicol.Pharmacol. 54,188–196.

646 Malley, L.A., Kennedy, G.L., Elliott, G.S., Slone, T.W., Mellert, W., Deckardt, K., Kuttler,
647 K.,Hildebrand, B., Banton, M.I., Parod, R.J., Griffiths, J.C., 2001. Chronic toxicity and

oncogenicity of N-methylpyrrolidone (NMP) in rats and mice by dietary administration. Drug Chem. Toxicol. 24, 315-338.

Marzulli, F.N., Maibach, H.I., 1980. Contact allergy: predictive testing of fragrance ingredients in humans by Draize and Maximization methods. J. Environ. Pathol. Toxicol. 3, 235-245.

Mascolo, M.G., Perdichizzi, S., Rotondo, F., Morandi, E., Guerrini, A., Silingardi, P., Vaccari, M., Grilli, S., Colacci, A., 2010. BALB/c 3T3 cell transformation assay for the prediction of carcinogenic potential of chemicals and environmental mixtures. Toxicol. In Vitro. 24, 1292-1300. DOI: 10.1016/j.tiv.2010.03.003

Matthews, E.J., Spalding, J.W., Tennant, R.W., 1993. Transformation of BALB/c-3T3 Cells: B. Transformation Responses of 168 Chemicals Compared with Mutagenicity in Salmonella and Carcinogenicity in Rodent Bioassays. Environ. Health Persp. 101, 347-482.

McNamee, P., Hibatallah, J., Costabel-Farkas, M., Goebel, C., Araki, D., Dufour, E., Hewitt, N.J., Jones, P., Kirst, A., Le Varlet, B., Macfarlane, M., Marrec-Fairley, M., Rowland, J., Schellauf, F., Scheel, J., 2009. A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: Eye irritation. Reg. Toxicol. Pharmacol. 54, 197–209.

Monfort, A., Soriano-Navarro, M., García-Verdugo, J.M., Izeta, A., 2013. Production of human tissue-engineered skin trilayer on a plasma-based hypodermis. J. Tissue Eng. Regen. Med. 7, 479-490.

Natsch, A., Emter, R., Gfeller, H., Haupt, T., Ellis, G., 2014. Predicting skin sensitizer potency based on in vitro data from KeratinoSens and kinetic peptide binding: Global vs. domain-based assessment. Toxicol. Sci. DOI: 10.1093/toxsci/kfu229

672 OECD, 1992.Test No. 406: Skin Sensitisation, OECD Guidelines for the Testing of
673 Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264070660-en

674 OECD (1997),Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the
675 Testing of Chemicals, Section 4, OECD Publishing.
676 DOI: 10.1787/9789264071247-en

677 OECD, 2002,Test No. 404: Acute Dermal Irritation/Corrosion, OECD Guidelines for the
678 Testing of Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264070622-en

679 OECD, 2004.Test No. 432: In Vitro 3T3 NRU Phototoxicity Test, OECD Guidelines for
680 the Testing of Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264071162-
681 en

682 OECD, 2009. Test No. 451: Carcinogenicity Studies, OECD Guidelines for the Testing
683 of Chemicals, Section 4, OECD Publishing.DOI: 10.1787/9789264071186-en

684 OECD, 2010.Test No. 429: Skin Sensitisation: Local Lymph Node Assay, OECD
685 Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. DOI:
686 10.1787/9789264071100-en

687 OECD, 2012.,Test No. 405: Acute Eye Irritation/Corrosion, OECD Guidelines for the
688 Testing of Chemicals, Section 4, OECD Publishing.
689 DOI: 10.1787/9789264185333-en

690 OECD, 2013a. Test No. 438: Isolated Chicken Eye Test Method for Identifying i)
691 Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring
692 Classification for Eye Irritation or Serious Eye Damage, OECD Guidelines for the
693 Testing of Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264203860-en

694 OECD, 2013b.Test No. 437: Bovine Corneal Opacity and Permeability Test Method for
695 Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring

696 Classification for Eye Irritation or Serious Eye Damage, OECD Guidelines for the
697 Testing of Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264203846-en.

698 OECD, 2013.Test No. 439: In Vitro Skin Irritation - Reconstructed Human Epidermis
699 Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD
700 Publishing. DOI: 10.1787/9789264203884-en

701 OECD, 2013d.Test No. 430: In Vitro Skin Corrosion: Transcutaneous Electrical
702 Resistance Test Method (TER), OECD Guidelines for the Testing of Chemicals,
703 Section 4, OECD Publishing. DOI: 10.1787/9789264203808-en

704 OECD, 2013e.Test No. 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis
705 (RHE) Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD
706 Publishing.DOI: 10.1787/9789264203822-en.

707 OECD (2014),*Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD
708 Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.
709 DOI: 10.1787/9789264224438-en

710 Onoue, S., Suzuki, G., Kato, M., Hirota, M., Nishida, H., Kitagaki, M., Kouzuki, H.,
711 Yamada, S., 2013. Non-animal photosafety assessment approaches for cosmetics
712 based on the photochemical and photobiochemical properties. Toxicol. in Vitro 27,
713 2316–2324.

714 Pant, K., Bruce, S.W., Sly, J.E., Kunkelmann, T., Kunz-Bohnenberger, S., Poth, A.,
715 Engelhardt, G., Schulz, M., Schwind, K.R., 2012. Prevalidation study of the Syrian
716 hamster embryo (SHE) cell transformation assay at pH 6.7 for assessment of
717 carcinogenic potential of chemicals. Mut. Res. 744, 54-63. DOI:
718 10.1016/j.mrgentox.2011.12.005

719 Pfuhler, S., Kirst, A., Aardema, M., Banduhn, N., Goebel, C., Araki, D., Costabel-
720 Farkas, M., et al., 1998. The International EU/COLIPA In Vitro Phototoxicity Validation

721 Study: Results of Phase II (Blind Trial). Part 1: The 3T3 NRU Phototoxicity
722 Test.Toxicol. in Vitro, 12, 305–327.

723 Prieto, P., Hoffmann, S., Tirelli, V., Tancredi, F., González, I., Bermejo, M., De Angelis,
724 I., 2010. An exploratory study of two Caco-2 cell models for oral absorption: a report on
725 their within-laboratory and between-laboratory variability, and their predictive capacity.
726 Altern Lab Anim. 38, 367-86.

727 Prieto, P., Cole, T., Curren, R., Gibson, R.M., Liebsch, N., Raabe, H., Tuomainen,
728 A.M., Whelan, M., Kinsner-Ovaskainen, A., 2013. Assessment of the predictive
729 capacity of the 3T3 Neutral Red Uptake cytotoxicity test method to identify substances
730 not classified for acute oral toxicity (LD50 > 2000 mg/kg): Results of an ECVAM
731 validation study. Reg. Toxicol. Pharmacol. 65, 344–365.

732 Ross, J.H., Dong, M.H., Krieger M.I., 2000. Conservatism in pesticide exposure
733 assessment. Reg. Toxicol. Pharmacol. 31, 53-58.

734 SCCP/1145/07 Memorandum on the in vitro test EPISKIN™ for skin irritation testing,
735 adopted by the SCCP during the 14th plenary meeting on 18 December 2007.

736 SCCS/1358/10 SCCS (Scientific Committee on Consumer Safety), basic criteria for
737 the in vitro assessment of dermal absorption of cosmetic ingredients, 22 June 2010.

738 SCCS/1392/10SCCS (Scientific Committee on Consumer Safety), memorandum
739 (addendum) on the in vitro test EPISKIN™ for skin irritation testing, 14 December
740 2010.

741 SCCS/1501/12: The SCCS's Notes of Guidance for the testing of cosmetic ingredients
742 and their safety evaluation, 8th revision, adopted by the SCCS during the 17th plenary
743 meeting of 11 December 2012.

744 SCCS/1532/14, SCCS (Scientific Committee on Consumer Safety), Addendum to the
745 SCCS's Notes of Guidance (NoG) for the Testing of Cosmetic Ingredients and their
746 Safety Evaluation 8th Revision (SCCS/1501/12), 9 April 2014, SCCS/1532/14, revision
747 of 22 October 2014.

748 Spielmann, H., Liebsch, M., Kalweit, S., Moldenhauer, F., Wirnsberger, T., Holzhutter,
749 H-G., Schneider, B., Glaser, S., Gerner, I., Pape, W.J.W., Kreiling, R., Krauser, K.,
750 Miltenburger, H.G., Steiling, W., Luepke, N.P., Muller, N., Kreuzer, H., Murmann, P.,
751 Spengler, J., Bertram-Neis, E., Siegemund, B., Wiebel, F.J., 1996. Results of a
752 validation study in Germany on two in vitro alternatives to the Draize eye irritation test,
753 the HET-CAM test and the 3T3 NRU cytotoxicity test. ALTA 24, 741–858.

754 Spielmann, H., Balls, M., Dupuis, J., Pape, W.J., Pechovitch, G., de Silva, O.,
755 Holzhütter, H.-G. , Clothier, R., Desolle, P., Gerberick, Fi, Liebsch, M.,, Lovell, W.W.,
756 Maurer, T., Pfannenbecker, U., Potthastl, J.M., Csato, M., Sladowsk, D., Steiling, W.,
757 Brantom, P. 1998. The International EU/COLIPA In Vitro Phototoxicity Validation Study:
758 Results of Phase II (Blind Trial). Part 1: The 3T3 NRU Phototoxicity Test.Toxicol. in
759 Vitro, 12, 305–327.

760 Vinardell, M.P., Mitjans, M., 2008. Alternative methods for eye and skin irritation tests:
761 an overview. J. Pharm. Sci. 97, 46-59.

762 **Legends to figures**

763 Figure 1. Percentage of eye irritation studies performed in vivo, in vitro and on human
764 volunteers.

765 Figure 2. Percentage of skin irritation studies performed in vivo, in vitro and on human
766 volunteers.

767 Figure 3. Percentage of sensitisation studies performed in vivo and on human
768 volunteers.

769 Figure 4. Percentage of sensitisation studies performed on mice and guinea pigs.

770 Figure 5. Percentage of dermal absorption studies performed in vitro, in vivo and on
771 human volunteers.

772 Figure 6 . Percentage of carcinogenicity studies performed in vivo and in vitro.

773 Figure 7. Percentage of toxicokinetic studies performed in vivo, in vitro or on human
774 volunteers.

775 Figure 8. Percentage of phototoxicity studies performed in vivo, in vitro and on human
776 volunteers.

777

778

779

780

781

782

783

784