Alternatives to Animal Testing: A Review of Trends and Perspectives

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ABSTRACT: *Alternative test methods have the potential to reduce animal testing; however, the extent to which in vitro methods can be replaced is questionable. This article summarizes validated alternatives to test the safety of cosmetic ingredients. It also illustrates how great a challenge it is to devise a proper alternative method.*

For years, progress has been made in developing alternatives to animal testing as society has internationally sought methods to that end. Regulatory mandates have played a key role as well; the 7th Amendment to the Cosmetic Directive (2003/15/EC) was adopted by European Union (EU) institutions in 2003 and imposes strict deadlines to abolish in vivo animal studies for cosmetic ingredient testing. The other crucial regulation pressing for alternative methods is REACH, which was adopted by the European Council and the European Parliament in December 2006. It is worth mentioning that the ban on the testing of chemicals on animals refers strictly to compliance with requirements of the Cosmetic Directive.

To facilitate the development of non-animal tests for the EU, the European Center for Validation of Alternative Methods (ECVAM) was established in 1991. Scientific advice for the validation process is subsequently provided by experts from all member states in ECVAM's Scientific Advisory Committee (ESAC). All available methods are considered during the validation process, whereby their reliability and relevance are established. The general rules to validate an alternative method have been agreed upon at an international level and this complex process consists of several stages.[1] Alternative test methods have the potential to reduce animal testing; however, it is questionable as to what extent in vitro methods can be replaced since some testing requires the involvement of a whole organism. This article summarizes validated alternatives to test the safety of cosmetic ingredients. It also illustrates how great a challenge it is to devise a proper alternative method.

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ECVAM

ECVAM was established to facilitate the development of non-animal tests; this was in response to Directive 86/609/EEC in the EU,[2] in which Article 23 states that the European Commission should encourage research for the development and validation of alternative methods. ECVAM became a unit of a Joint Research Centre of the EU Commission in Italy, and at an international level, ECVAM strictly cooperates with other organizations such as the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which has been established in United States, as well as the Organization for Economic Cooperation and Development (OECD).

Safety Assessment of Cosmetics in the EU

The assessment of cosmetic product safety in the EU is regulated by provisions of Directive 76/768/EEC.[3] In 1993, the 6th Amendment was added, indicating that the testing of ingredients or combinations of ingredients on animals should be banned as of Jan. 1, 1998; this date would be postponed where alternative methods of testing had not been scientifically validated.[4]

The 7th Amendment to Directive 2003/15/EC then established challenging timelines for phasing out animal testing.[5] It should be noted that REACH also favors validated, appropriate alternative methods to conventional animal testing; Article 25 (1) of the regulation states: "In order to avoid unnecessary animal testing, testing on vertebrate animals for the purpose of this Regulation shall be undertaken only as a last resort."

Validating Alternative Methods

The process of validating an alternative method aims to establish its relevance and reliability for a particular purpose.[6] The crucial elements of this process are the scientific basis of the system and its predictive possibilities.[7] The prediction model for the validation process can be defined as an algorithm established to convert data collected from in vitro experiments into predictions of a chemical substance's influence on an organism.

As noted, the validation of the particular method is complex and consists

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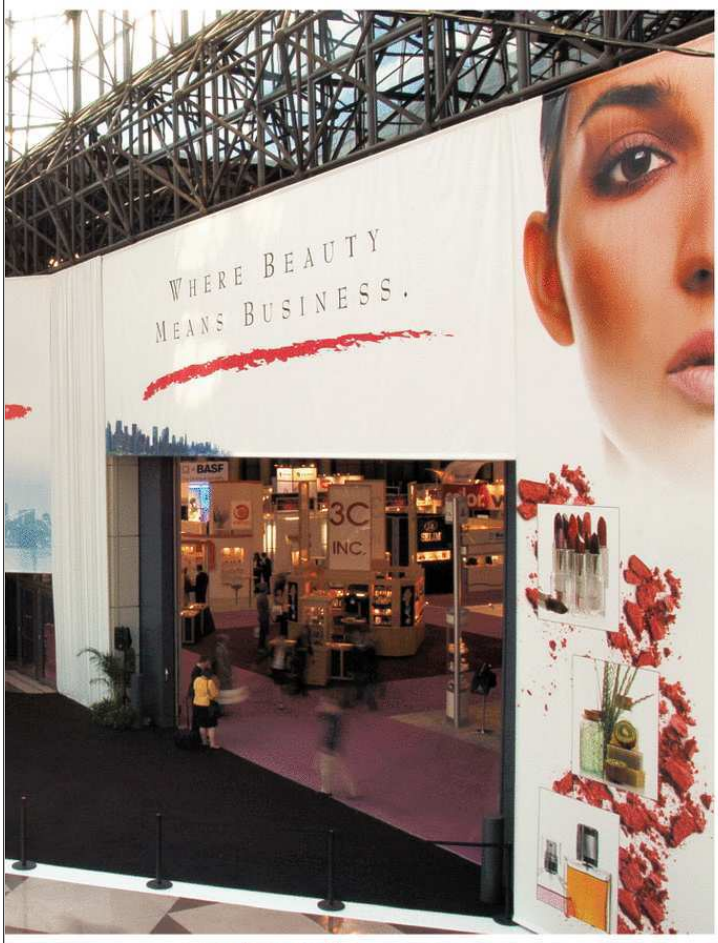

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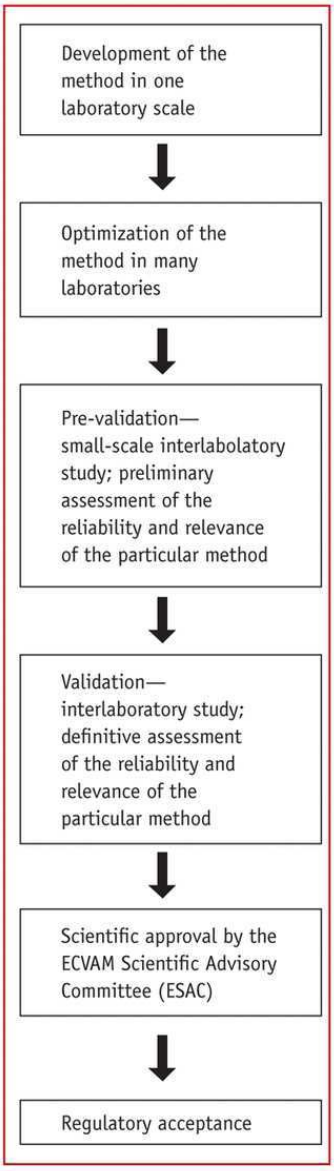

Figure 1. Schematic of the validation process

of several steps and levels (see **Figure 1**). First, the method is studied and refined in one laboratory; depending on the method, this stage can take months to years. The study then is expanded to many laboratories that perform the same study on a larger scale.[8] The next step connects to the pre-validation process, which makes an initial assessment of the reliability and relevance of the method; a more finalized assessment is reached during a formal validation stage. During the pre-validation phase, researchers determine whether the method has the potential to successfully pass a validation study. This level of optimization ensures that resources are not wasted on methods with little chance for success.

The next stage is pre-validation, during which pre-existing information obtained from in vivo studies is useful in that it enables comparisons if a validated method is relevant. The main disadvantage of older in vivo methods, however, is that they often do not fulfill modern criteria concerning documentation, which can prolong the validation process.[9] Upon meeting all requirements, the alternative method is accepted by ESAC and moves to the final step: regulatory acceptance of the validated alternative method, and ECVAM plays a crucial role in this step as well.[10] With so many steps involved to validate and accept a new alternative method, it can take up to six years to complete the process.

Toxicological Assessment of Cosmetics

According to the Cosmetic Directive, to evaluate the safety of a cosmetic product, manufacturers should consider the general toxicological profiles of the ingredients, their chemical structure, and the level of exposure consumers will have to them. The first step in safety evaluation is to examine analyses made of the particular ingredients in the cosmetic formulation, followed by assessing their risk by reviewing recent literature and studies related to the toxicological aspects of the ingredients. In some cases, a reassessment of the ingredient safety profile may be required.

Seeking Alternative Methods

In some industries, toxicological studies for alternative methods have already been conducted and the methods have successfully been validated. In others, the methods are still under discussion. For more complicated fields, safety assessment via in silico methods may be useful to manage complex scientific issues. Following are some of the methods for which validated alternatives exist, as well as an overview of works in progress (see **Table 1**).

Acute toxicity: To develop in vitro tests that can completely replace acute toxicity testing, the A-Cute-Tox integrated project under the EU 6th Framework Program was developed. Within the framework of this project, approximately 60 chemicals have been studied, taking into consideration absorption, metabolism, distribution and toxicity data. Additional studies of 50 compounds will take place in 2009. The project involves 35 research groups culled from universities and the industry. The aim of these studies is to develop an in vitro testing strategy to replace the fixed dose and acute toxic class methods, and the up-and-down procedure.

While these methods are described in the revision of "SCCP Notes of Guidance" as validated refinement and reduction methods, they are not total replacement methods, either.[11] Ultimately, the most crucial objective of the A-Cute-Tox project is to elaborate a replacement method for oral acute toxicity testing, which can be expected early in 2011. In addition, methods for inhalation and dermal toxicity are important to the cosmetics industry as well.

Skin irritation/corrosion: The skin irritation study undertaken by ECVAM was completed with success in May 2006, at which time the Summary Report of the ECVAM Skin Irritation Validation Study (SIVS) was published.

Table 1. Existing alternative methods and proposed methods

Validated alternatives	Non-validated alternatives
Replacement methods:	• eye irritation
• skin irritation/corrosion	• repeated-dose toxicity
• percutaneous penetration	• toxicokinetics
• mutagenicity and genotoxicity	• reproductive toxicity
• phototoxicity	• carcinogenicity
Refinement/reduction methods:	
• skin sensitization	• acute toxicity

The aim of SIVS was to study three in vitro test systems—EpiSkin[a], EpiDerm[b] and the skin integrity function test—to replace the Draize skin irritation test.[12] In April 2007, ESAC fully accepted the EpiSkin model and issued the following statement: "The EpiSkin method is considered to be a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by 3-(4,5)-dimethyl-2-thiazolyl-2,5-dimethyl-2H-tetrazolium bromide (MTT) reduction, and for being used as a replacement for the Draize skin irritation test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and non-skin irritating substances."[13]

At the same time, however, the Scientific Committee on Consumer Products (SCCP) emphasized the need for additional studies to support the EpiSkin method for the safety assessment of cosmetic ingredients present in the Annexes of Directive 76/768/EEC. In regard to skin corrosion, three alternative methods have been validated: the rat skin transcutaneous electrical resistance (TER) test, EpiSkin and EpiDerm. The EpiDerm model can be used to identify skin irritants due to its high specificity; however, ESAC suggested further studies to improve its level of sensitivity.[14] The TER test was granted regulatory approval as a replacement for the in vivo skin corrosivity test. It allows for the identification of corrosive chemical substances and mixtures, taking into account that the corrosivity potential of a substance may be predicted from its effects on the transcutaneous electrical resistance of rat skin and from its effects on skin penetration of sulforhodamine B dye. The method can be also used to identify hazardous materials or classify a material's corrosive potential in order to fulfill the regulatory requirements set forth by REACH.

Eye irritation: The close cooperation between ECVAM, ICCVAM and COLIPA is apparent in work toward finding alternatives for eye irritation tests. While no validated alternative method yet exists, substantial progress has been made. The validation program on alternative eye irritation testing was managed by ECVAM but many in vitro tests will be necessary to assess the complex mechanisms involved in eye irritation in vivo.[15, 16] Methods such as Bovine Cornea Opacity Permeability (BCOP) and Isolated Chicken Eye (ICE) have been elaborated but are not yet validated. The BCOP is a biologically complex ex vivo model for evaluating the potential ocular irritancy/toxicity of a substance, which is measured by a substance's ability to induce corneal opacity and corneal permeability to fluorescein. The ICE method assesses damage caused by the test substance by determination of corneal swelling, opacity and fluorescein retention. These methods are appropriate to replace animal methods studying severe irritants and are not useful for the estimation of mild and nonirritants.

Skin sensitization: Currently there are no validated alternate methods to replace in vivo studies for skin sensitization. Due to its complexity, researchers have concluded that more than one in vitro test should be employed to cover

[a] *EpiSkin is a registered trademark of SkinEthic.*
[b] *EpiDerm is a registered trademark of MatTek.*

the complicated mechanisms that occur during skin sensitization.[17] Moreover, broader studies are being conducted to improve the predictive performance of existing methods such as Local Lymph Node Assay. Aimed at the elaboration of alternative methods for skin sensitization, the integrated project Sens-it-iv, supported by a grant from the European Commission, has been initiated.[18] This project is a cooperation between university scientists, industry, small and medium enterprises, and associations.

Percutaneous penetration: Replacement tests for in vitro dermal absorption methodologies have been evaluated. These tests determine the amount of a given chemical that penetrates the skin, and whether the chemical has the potential to absorb into the circulatory system. The permeation process of chemical compounds through the skin is complex and depends on a number of different factors such as lipophilicity, concentration, molecular weight, duration of exposure and thickness of the epidermis. The in vitro methods that have been developed are based mainly on measuring chemical diffusion across excised pig or human skin, employing flow through or static diffusion cells.[19] Additionally, the reconstituted human epidermis model (RHE) has been introduced and is currently in the evaluation process.[20]

Upon meeting all requirements, the alternative method is accepted by ESAC and moves to the final step: regulatory acceptance.

Photo-induced toxicity: The design of tests for predicting acute phototoxicity is another example of cooperation between ECVAM and COLIPA. The primary replacement method used widely in the cosmetics industry to predict the phototoxic potential of chemicals is the 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT). The 3T3 NRU PT test can be used to determine the phototoxic activity of a substance induced by the combination of the substance and light. The method is based on comparison of the substance's cytotoxic activity when tested after the exposure, as well as in the absence of exposure to a non-cytotoxic dose of light. [21]

Repeated dose toxicity: One of the most difficult animal tests to replace is the repeated dose toxicity test, and there no validated alternative yet exists. The Predictomics project, which has been initiated within the framework of the FP6 Research Program—a European Union program employing 14 partners from nine European countries—aims to develop a novel platform for anticipating liver and kidney toxicity.[22]

Another project that could prove useful for the cosmetics industry is Predict-IV, within the 7th Framework Program, which designs strategies to improve the assessment of chemical safety involving non-animal test systems, mechanistic toxicology, cell biology as well as in silico modeling.

Mutagenicity and genotoxicity: Alternative in vitro methods for assessing mutagenicity/genotoxicity exist but many of them yield a high rate of false positive results that require confirmation using animal tests;[23] thus, they must be improved. Among the methods recommended by the SCCP are: the In Vitro Mammalian Cell Gene Mutation Test, the Bacterial Reverse Mutation Test, and the In vitro Micronucleus Test, which was recently validated. Another direction of research in this area is aimed at optimizing in vitro micronucleus and Comet assays in primary skin cells and human skin models; here again, cooperation between COLIPA and ECVAM is apparent. In many cases new methods should be developed to properly evaluate the mutagenic potential of a substance.

Carcinogenicity: The primary alternative method available for in vitro carcinogenicity testing is the cell transformation assay (CTA), which is an in vitro system for detection of the carcinogenic potential of substances; this method is now pre-validated by ECVAM. Unfortunately, no validated alternatives exist for genotoxic and non-genotoxic carcinogens. To develop tests to predict the carcinogenicity of chemical substances, the FP6 Integrated Project Carcinogenomics has been formed by the European Union.

Toxicokinetics: An important aspect to the predictability of alternative methods is toxicokinetics. At present, there are no validated alternatives that can encompass processes related to absorption, distribution, metabolism and excretion. The complexity of living systems requires integrated testing strategies. The absorption barrier model for the examination of compounds from the gastrointestinal tract is being studied by ECVAM. Another important factor that plays a key role in toxic effects is the metabolism of the substance but most in vitro studies lack metabolizing data. Studies in this field are in process.

Reproductive toxicity: The development of methods to test for reproductive toxicity occurs within the framework of the integrated ReProTect project initiated by ECVAM; however, validated alternative methods to cover the broad field of reproductive toxicity are lacking. Existing tests to study embryotoxicity such as the Whole Embryo Culture test (WEC), the MicroMass test (MM) and the Embryotoxic Stem Cell Test (EST) need further investigation and are within the framework of ECVAM studies.

Conclusions

During recent years, progress in the validation of alternative test methods has been made. New dimensions in the development of alternative in vitro and in silico methods, or the two combined, are obtainable via the implementation of three large integrated projects: ReProTect, Sens-it-iv and A-Cute-Tox. The availability of alternative methods has become a key issue for the cosmetics industry especially since the testing and marketing ban was implemented in European cosmetic legislation. The testing ban on ingredients will take place step-by-step, as soon as methods are validated; however, the deadline for all human

health effects was March 11, 2009, with the exception of repeated-dose toxicity, reproductive toxicity, and toxicokinetics, with an expected deadline of March 11, 2013. This means that testing cosmetic ingredients on animals is forbidden except for in these three fields of toxicity.

For more complicated fields, safety assessment via in silico methods may be useful.

Presently, fully validated replacement methods to animal testing exist for skin corrosion and irritation testing, phototoxicity, percutaneous penetration and genotoxicity/mutagenicity. For acute toxicity and skin sensitization, only refinement and reduction alternatives have been developed. The design of alternative methods to replace animal testing in other fields of toxicological studies remains a challenge for scientists and the industry.

There are still tests that, considering the complexity of living organisms, are difficult to replace by a model system. Taking into account these conditions, there is a need for common efforts between university researchers and those within the industry to elaborate and validate new alternative methods.

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