JRC Scientific and Technical Reports

ECVAM Technical Report on the Status of Alternative Methods for Cosmetics Testing (2008-2009)

A report prepared in the framework of Directive 2003/15/EC (7th Amendment to the Cosmetics Directive)

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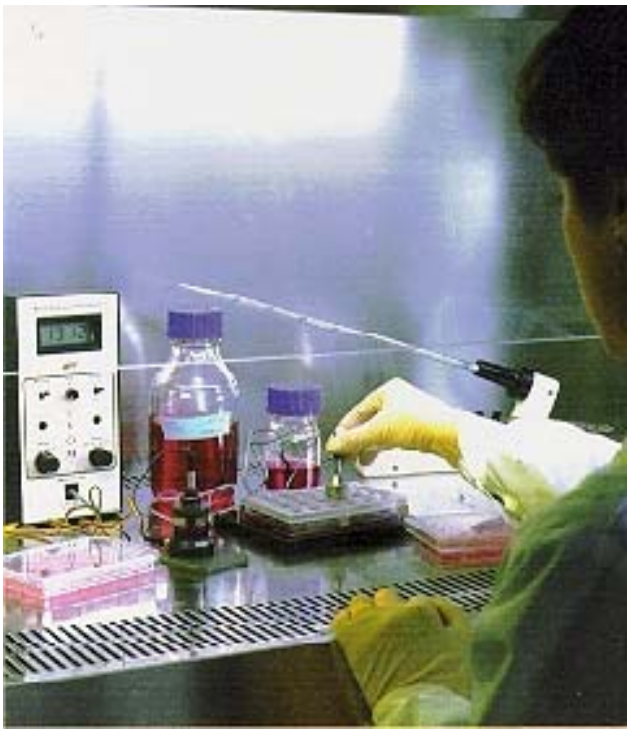

EUR 24413 EN - 2010

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JRC58991

EUR 24413 EN
ISBN 978-92-79-16021-9
ISSN 1018-5593
DOI 10.2788/99101

Luxembourg: Publications Office of the European Union

Printed in Italy

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EXECUTIVE SUMMARY

The ECVAM technical report presents the progress made in the development and validation of alternative methods for the human health effects relevant to the Cosmetics Directive. It provides an update on the activities described by ECVAM in 2005[1], 2006 and 2007[2]

The report intends to present the latest scientific and technical developments in the field during 2008-2009. As required by Directive 2003/15/EC, the seventh amendment to Directive 76/768/EEC, developments in refinement and reduction methods are also described (EU, 2003).

The status in 2009 on the development, validation and regulatory acceptance of alternative methods in the different human health-related effects of concern to the Cosmetics Directive is as follows:

For acute local effects, such as acute phototoxicity and skin corrosion, validation studies have led to the regulatory acceptance of the methods. In the field of skin irritation, the EC had the lead at OECD level for a new draft OECD test guideline on *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method. In parallel, a new EU Test Method B.46 based on the ECVAM-validated reconstructed human epidermis assays (EpiSkin™, EpiDerm™ and SkinEthic™RHE) was included in the EU Test Method Regulation COM 440/2008.
For skin penetration, OECD adopted in 2004 a test guideline based on diffusion of chemicals into and across skin to a fluid reservoir.

In the area of genotoxicity, the micronucleus test *in vitro* has been successfully validated in 2006 and the draft OECD Test Guideline should soon be adopted, now that new data on the different measures of cytotoxicity to be used, while performing the micronucleus test, have been generated by an EU working group. Several *in vitro* genotoxicity tests are in use since decades. However, these tests are prone to an unacceptable rate of false positive results, leading to unnecessary follow up animal experiments. Following the recommendation of an ECVAM workshop aiming at improving the predictivity of the *in vitro* testing battery, ECVAM has been involved: 1) in the publication of a list of chemicals to be used in the evaluation of the performance of new or modified tests and in the analysis thereof; and 2) in the analysis of published data for top concentration and upper limit of cytotoxicity considerations in mammalian cell genotoxicity testing, which seems to be a reason for false positives. Furthermore a prevalidation of the micronucleus test and the comet assay applied to reconstructed human skin models is ongoing under the coordination of COLIPA.

In the area of eye irritation, from twelve methods considered as most advanced, ten methods underwent validation. In particular, two organotypic assays (the BCOP and the ICE assays), out of the four (BCOP, ICE, HET-CAM and IRE) which underwent validation, were endorsed by ESAC in 2007 as scientifically valid to identify severe eye irritants, and OECD TGs on these two assays were adopted in 2009.
Furthermore, from the four cytotoxicity- /cell function- based assays (NRR, FL, CM, RBC) evaluated in an ECVAM retrospective validation study, the CM was recommended by ESAC to be used in top-down (discriminating severe eye irritants from all other classes) and bottom-up (discriminating non-

[1]ECVAM (2005) Cosmetics technical report. ECVAM contribution. Available at: http://ec.europa.eu/enterprise/cosmetics/doc/antest_ecvam_2005v2.pdf (accessed on 29.11.2007).

Eskes and Zuang (2005) Alternative (Non-animal) Methods for Cosmetics Testing: Current Status and Future Prospects**.** *ATLA* **33** Suppl. 1, pp.228.

[2] ECVAM technical report 2006-2007.Available at: http://ecvam.jrc.ec.europa.eu/ under "Publications", "ECVAM and EU Policies", accessed on 25.05.2009.

irritants from all other classes) approaches for defined applicability domains, and the FL was recommended to be used in a top-down approach. Finally, from the two other assays (LVET and Ocular Irritection®) which underwent external validation and submitted to ECVAM for evaluation, the ESAC recommended that existing LVET data pertaining to the applicability domain of detergents and cleaning products are recommended as scientifically useful within a weight of evidence approach, as also foreseen in the CLP regulation, but that LVET is not recommended for the prospective generation of new data. ESAC also recommended LVET as reference standard against which to develop *in vitro* assays for detergents and cleaning products.
The Ocular Irritection® test method, on the other hand, further to the evaluation of the submission, ECVAM requested to provide additional information in a revised submission.
A prospective validation study on two Reconstructed human Tissue models (EpiOcular™ and SkinEthic™ HCE) which were submitted to ECVAM by COLIPA, was initiated in December 2008 and is currently on-going.

Finally, testing strategies which combine the validated assays are currently under development and evaluation, with the aim to completely replace the animal test for eye irritation.

For acute oral toxicity, previous findings from Halle's Registry of Cytotoxicity (Halle, 2003) and the completed NICEATM/ECVAM validation study (http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm) showed that the prediction from *in vitro* cytotoxicity data of low systemic toxicity is much better than the prediction of high systemic toxicity. Since 87% of new chemicals (New Chemicals Database) are non-toxic substances (Bulgheroni *et al.* 2009), the chance to correctly classify a new non-toxic substance with this *in vitro* assay is very high. Indeed, the preliminary results of the validation study to identify non-toxic substances (rat oral LD_{50} > 2000 mg/kg b.w.) show a high negative predictive value for the 3T3/NRU cytotoxicity assay. The results of the prevalidation of the testing strategy carried out within the ACuteTox project will become available only in June 2010.

The endpoints described above are those falling under the 2009 deadline with regard to the animal testing ban and marketing ban on cosmetics tested on animals. Those which fall under the 2013 deadline of the marketing ban are repeated-dose toxicity (including skin sensitisation and carcinogenicity), toxicokinetics and reproductive toxicology.

Under the new circumstances of the Cosmetics Directive, where only alternative methods can be used, toxicokinetics and metabolism *in vitro* and *in silico* test systems are of crucial importance to discard artifactual findings secondary to the *in vitro* environment. A collaborative effort between industry, academia, the European Commission and the three validation bodies (ECVAM, ICCVAM and JACVAM) was therefore set up to validate an *in vitro* metabolic competent test system as an important building block in integrated testing strategies for the complex endpoints.

For the endpoint on carcinogenicity, three variants of the cell transformation assay *in vitro* were validated according to modules 1 to 4 of the ECVAM modular approach and will shortly be submitted for ESAC peer review. In the field of skin sensitisation, three promising *in vitro* methods [the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U939 Skin Sensitisation Test (MUSST)] were sufficiently optimised by industry and were accepted in 2009 by ECVAM for entering prevalidation.
In the area of reproductive toxicology, some promising methods, which were developed under Reprotect, an EU-funded collaborative research project in which ECVAM was also involved, will be submitted to ECVAM for (pre)validation and some *in vitro* methods for the identification of endocrine disruptors are currently under validation. A reduction in the number of animals used for reproductive toxicity testing is envisaged with the ongoing work that advocates a modular approach to the extended

one-generation reproduction study (Moore *et al*., 2009). For the complex endpoints, the lack of enough robust and relevant methods to fully replace the animal tests remains a challenge**.**

Table 1 represents the current international state-of-play in the validation and acceptance of alternative methods for the human health effects of concern to the Cosmetics Directive.

Table 2 represents the test (pre-)submissions received at ECVAM during 2008-beginning of 2010.

Table 1. International Validation and Acceptance of Alternative Methods 1998-2010[3]

Test Method per human health effect	**Current status**	**Regulatory acceptance**			
		US	**Japan**	**OECD**	**EU**
Eye irritation					
Bovine Corneal Opacity and Permeability (BCOP) Test Method	ICCVAM peer review and report (2007); ESAC statement (2007)	US acceptance in 2008		OECD TG 437 (2009)	EU Test Method B.47
Isolated Chicken Eye (ICE) Test Method	ICCVAM peer review and report (2007); ESAC statement (2007)	US acceptance in 2008		OECD TG 438 (2009)	EU Test Method B.48
EpiOcular assay	ECVAM-COLIPA validation on going (2008-2011)				
SkinEthic assay	ECVAM-COLIPA validation on going (2008-2011)				
Fluorescein Leakage assay	ECVAM validation completed. ESAC statement 2009			*SPSF submitted to OECD (2010) Draft TG in preparation*	
Cytosensor Microphysiometer assay	ECVAM validation completed. ESAC statement 2009	Recommendations to Federal Agencies: in progress		*SPSF submitted to OECD (2010) Draft TG in preparation*	
Neutral Red Release assay	ECVAM validation completed in 2009 (negative outcome)				
Red Blood Cell test	ECVAM validation completed in 2009 (negative outcome)				
Low Volume Eye Test	ECVAM validation	Transmittal of ICCVAM			

[3] Updated April 2010

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
	completed in 2007. ESAC statement 2009. ICCVAM international peer review (2009)	reccomendations to U.S. Federal agencies: in progress. Acceptance in US expected late 2010			
Cytotoxicity test: Short Time Exposure (STE) test	JaCVAM-sponsored validation study planning.				
Cytotoxicity test: SIRC cells	JaCVAM peer review ongoing				
Cytotoxicity test: three-dimensional dermal model (MATREX)	JaCVAM peer review ongoing				
Use of anesthetics, analgesics, and humane endpoints in the Draize eye test; Recommen-dations for routine use	NICEATM-ICCVAM. ICCVAM international peer review (2009)	Transmittal of ICCVAM recommendation s to U.S. Federal agencies: in progress. Acceptance in U.S. expected late 2010		*SPSF submitted to OECD (2010)*	
In vitro methods for identification of moderate and mild irritants and substances not labeled as irritants: Recommend-dations for further optimization and studies BCOP ICE IRE HET-CAM	NICEATM-ICCVAM. ICCVAM international peer review (2009)	Transmittal of ICCVAM recommendation s to U.S. Federal agencies: in progress. Acceptance in U.S. expected late 2010			
In vitro approach for categorization of anti-microbial cleaning products: recommend-dations for further studies	NICEATM-ICCVAM ICCVAM international peer review (2009)	Transmittal of ICCVAM recommendation s to U.S. Federal agencies: in progress. Acceptance in U.S.expected late 2010			
Skin Corrosion					
CORROSITEX Skin Corrosivity Test	ICCVAM peer review and report (1999); ESAC statement (2000)	US acceptance in 2000		OECD TG 435 (2006)	
EpiSkin Skin Corrosivity Test	ECVAM validation; ESAC			OECD TG 431 (2004)	EU Test Method B.40

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
	statement (1998); ICCVAM review and report (2002)				Bis
EpiDerm Skin Corrosivity Test	ECVAM validation; ESAC statement (1998); ICCVAM review and report (2002)			OECD TG 431 (2004)	EU Test Method B.40 Bis
SkinEthic Skin Corrosivity Test	ECVAM validation/ESAC statement (2006)			OECD TG 431 (2004)	EU Test Method B.40 Bis
Rat Skin TER Corrosivity Test	ECVAM validation; ESAC statement (1998); ICCVAM review and report (2002)			OECD TG 430 (2004)	EU Test Method B.40
EST-1000 (CellSystems)	ESAC statement (2009)			OECD TG 431 (2004)	EU Test Method B.40 Bis
Skin irritation					
EpiSkin Skin Irritation Test	ECVAM validation; ESAC statement (2007)		Accepted by Japanese Regulatory Acceptance Board	OECD TG 439 approved by OECD WNT, March 2010; Adoption by OECD JM foreseen Sept. 2010	EU Test Method B.46
EpiDerm Skin Irritation Test	ECVAM validation; ESAC statement (2008)			OECD TG 439 approved by OECD WNT, March 2010; Adoption by OECD JM foreseen Sept. 2010	EU Test Method B.46
SkinEthic RHE	ECVAM validation; ESAC statement (2008)			OECD TG 439 approved by OECD WNT, March 2010; Adoption by OECD JM foreseen Sept. 2010	EU Test Method B.46
LabCyte EPI-MODEL24 *in vitro* test method	JaCVAM-sponsored validation study			OECD Peer review	

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
	completed 2009			finalised (May 2010)	
Investigation of *in vitro* dermal irritation assays to evaluate false negative corrosives from in vitro corrosivity tests EpiDerm EPISKIN SkinEthic RHE	NICEATM-ICCVAM. Study in progress				
Skin absorption/ penetration					
In vitro diffusion method for measuring skin absorption				OECD TG 428 (2004)	EU Test Method B.45
Skin sensitisation					
Local Lymph Node Assay for skin sensitisation	ICCVAM peer review and report (1999); ESAC statement (1999)	US acceptance in 1999		OECD TG 429 (2002)	EU Test Method B.42
Reduced Local Lymph Node Assay for skin sensitisation	ESAC statement (2007); ICCVAM peer review and report (2008)	US acceptance in 2010		Under discussion within the revision of TG 429	
MUSST	ECVAM validation started in 2009				
h-CLAT	ECVAM validation started in 2009				
Direct Peptide Reactivity Assay	ECVAM validation started in 2009				
Updated Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)	NICEATM-ICCVAM. ICCVAM International peer review (2008)	U.S. acceptance (2010)		OECD TG 429 (updated) approved by OECD WNT, March 2010; Adoption by OECD JM foreseen Sept. 2010	
Nonradioactive LLNA protocol (LLNA: BrdU-Flow Cytometry)	NICEATM-ICCVAM. ICCVAM international				

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
	peer review, 2009 Data audit and interlaboratory study planned				
Nonradioactive LLNA protocol (LLNA: BrdU-ELISA)	JaCVAM (validation study); NICEATM-ICCVAM (international peer review 2009)	Transmittal of ICCVAM recommendations to U.S. Federal agencies: in progress	Acceptance by Japanese Regulatory Acceptance Board pending	OECD TG 442B approved by OECD WNT March 2010. Adoption by OECD JM foreseen Sept. 2010	
Nonradioactive LLNA protocol, LLNA:DA	JaCVAM (validation study); NICEATM-ICCVAM (international peer review 2009)	Transmittal of ICCVAM recommendations to U.S. Federal agencies: in progress	Accepted by Japanese Regulatory Acceptance Board (2008)	OECD TG 442A approved by OECD WNT March 2010. Adoption by OECD JM foreseen Sept. 2010	
Harmonized performance standards for LLNA	NICEATM-ICCVAM; ECVAM; JaCVAM Endorsed by ESAC and ICCVAM	U.S. acceptance in 2010		Draft OECD update to TG 429 approved by OECD WNT March 2010; Adoption by OECD JM foreseen Sept. 2010	
Acute phototoxicity					
3T3 NRU Phototoxicity Test (photo-irritation)	ESAC Statement (1998)			OECD TG 432 (2004)	EU Test Method B.41
3T3 NRU Phototoxicity Test: Application to UV Filter Chemicals	ESAC Statement (1998)			OECD TG 432 (2004)	EU Test Method B.41
Tiered testing strategy to predict phototoxicity (3T3 NRU PT and reconstructed human epidermis models)	ECVAM feasibility study completed				
Produced reactive oxygen species	JaCVAM-sponsored				

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
(ROS) and photostability study	validation study planning				
Test method battery to predict phototoxicity (yeast growth inhibition phototoxicity assay and red blood cell photohemolysis assay)	JaCVAM Japanese Regulatory Acceptance Board recommended additional work be performed				
Acute Toxicity					
Up and Down Procedure (UDP)	ICCVAM peer review and report (2001); ESAC statement (2007)	Yes, agency acceptance		OECD TG 425 (2001)	
Fixed Dose Procedure (FDP)	ICCVAM (2001); ESAC statement (2007)			OECD TG 420 (2001)	EU Test Method B.1 Bis
Acute Toxic Class Method (ATC)	ICCVAM (2001); ESAC statement (2007)			OECD TG 423 (2001)	EU Test Method B.1 Tris
In vitro cytotoxicity test (3T3 Neutral Red Uptake) to estimate starting doses for oral acute systemic toxicity	NICEATM-ECVAM Validation Study; ICCVAM peer review and report (2008)	Yes, agency endorsement		Guidance document adopted in 2010	
In vitro cytotoxicity test (NHK Neutral Red Uptake) to estimate starting doses for oral acute systemic toxicity	NICEATM-ECVAM Validation Study; ICCVAM peer review and report (2008)	Yes, agency endorsement		Guidance document adopted in 2010	
In vitro cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD_{50} > 2000 mg/kg b.w	ECVAM follow-up validation study completed (2009) (BRD in preparation)				
Granulocyte macrophage-Colony Forming Unit for Predicting Acute Neutropenia in Humans	ECVAM validation ESAC statement (2006)				
Up-and-Down Procedure (acute dermal toxicity)	NICEATM-ICCVAM. Collecting acute dermal toxicity data for use in computer simulations for future validation				
Acute inhalation toxicity	OECD TG 403 (Revised version			OECD TG 403 (2009)	

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
	adopted, 2009)				
Inhalation toxicity - acute toxic class method	Adopted as OECD Test Guideline (TG) 436 (2009)			OECD TG 436 (2009)	
Genotoxicity/ mutagenicity					
In vitro micronucleus test	ECVAM validation; ESAC statement (2006)			OECD TG 487 approved by OECD WNT by written procedure (2009) Adoption by OECD JM foreseen Sept. 2010	Mentioned in Annex VIII of Reg. 1907/2006
Comet assays in vitro and in vivo (2 test methods)	JaCVAM validation study in progress on *in vivo* and *in vitro* methods; coordination with ICCVAM and ECVAM				
Genotoxicity assays (COMET in vitro and micronucleus test in vitro) in 3D skin models (2 tests)	COLIPA/ECVAM Validation study ongoing				
Improvement of the in vitro test battery for genotoxicity	ECVAM Analysis of cytotoxicity top dose in in vitro genotoxicity tests on going				
Carcinogenicity					
Cell Transformation Assay pH 6.7	ECVAM prevalidation completed. ESAC peer review to initiate in 2010				
Cell Transformation Assay pH 7	ECVAM prevalidation completed. ESAC peer review to initiate in 2010				
Cell Transformation Assay Balb/c 3T3 cell	ECVAM prevalidation completed. ESAC peer review to initiate in 2010				
BHAS cell transformation	JaCVAM validation study				

Test Method per human health effect	Current status	Regulatory acceptance			
		US	**Japan**	**OECD**	**EU**
assay	in progress with ECVAM participation				
Toxicokinetics/ metabolism					
In vitro hepatic biotransformation enzyme induction [Hepa RG (in-house); Hepa RG (commercial) and cryopreserved human hepatocytes] (3 test methods)	Validation study on-going (ECVAM led with ICCVAM and JaCVAM involvement)				
Reproductive toxicology					
In Vitro Embryotoxicity Tests employing micromass culture, rat embryo cultures, and embryonic stem cells	ECVAM validation completed. ESAC statement in 2001				
LUMI CELL® estrogen receptor transcriptional activation assay: agonist and antagonist protocols	International validation study in progress (NICEATM, JaCVAM, ECVAM)				
MELN ® estrogen receptor transcriptional activation assay: agonist and antagonist protocols	Validation study will start in 2010 (ECVAM)				
Extended-F1 generation test	Validation under the OECD umbrella (with ECVAM participation) completed (2009)				
CertiChem MCF-7 cell proliferation assay for the detection of human estrogen receptor agonists and antagonists	NICEATM-ICCVAM. International validation study in progress. Independent peer review planned March 2011				
Stably transfected human estrogen receptor-αtranscriptional activation assay for detection of estrogenic	Validation under the OECD umbrella (JaCVAM coordinated) completed			OECD TG 455 (2009)	

Test Method per human health effect	Current status	Regulatory acceptance			
		US	Japan	OECD	EU
agonist-activity of chemicals					
Stably transfected human estrogen receptor-αtranscriptional activation assay for detection of anti-estrogenic activity of chemicals	International validation study in progress (JaCVAM coordinated)				

Table 2. Test (pre-)submissions received at ECVAM from 2008 to first quarter of 2010

Test Method per human health effect	Year of submission to ECVAM	Status of test method
Rat recombinant androgen receptor binding assay for the detection of compounds with (anti)androgenic potential	2010	Full submission awaited
Transactivation assay for the detection of compounds with (anti) androgenic potential using PALM cells	2010	Full submission awaited
Test system for the evaluation of organophospates (OPs)- induced neurotoxicity following inhibition of critical esterases, acetylcholinesterase (AChE) and neuropathy target esterase (NTE) measured by i*n vitro* approach	2009	Full submission awaited
ERalpha CALUX® assay for the prediction of the *in vivo* estrogenic activity of chemicals	2009	Full submission awaited
VitroDerm RhE model for skin irritation testing	2009	Incomplete pre-submission
EST-1000 RhE model for skin irritation testing	2009	Full submission awaited
GreenScreen for genotoxicity testing	2009	Full submission awaited
Biobide- Automated screening to detect cardiotoxicity in Zebrafish	2009	Full submission awaited
Direct Peptide Reactivity Assay for skin sensitisation testing	2009	Under validation
Myeloid U939 Skin Sensitisation Test (MUSST)	2009	Under validation
Chemical reactivity measurement using the Glutathione (GSH and GSSG) peptide reactivity HPLC-MS assay	2009	Test did not progress to validation

Test Method per human health effect	Year of submission to ECVAM	Status of test method
Chemical reactivity measurement using the cysteine or lysine peptide binding HPLC assay	2009	Under validation
MELN® transactivation assay for the detection of compounds with (anti) estrogenic potential	2009	Full submission awaited
Ocular Irritection® assay for eye irritation testing	2009	Under external validation
In vitro hepatic biotransformation enzyme induction - HepaRG (commercial) for toxicokinetics/metabolism testing	2008	Under validation
In vitro hepatic biotransformation enzyme induction - HepaRG (in-house) for toxicokinetics/metabolism testing	2008	Under validation
In vitro hepatic biotransformation enzyme induction - cryopreserved human hepatocytes for toxicokinetics/metabolism testing	2008	Under validation
In vitro diffusion method for measuring skin absorption	2008	Full submission awaited
Valitox test for acute toxicity	2008	Full submission awaited
CPA-I test for respiratory sensitisation testing	2008	Test did not meet ECVAM's priorities
EpiOcular™ model for eye irritation testing	2008	Under validation
SkinEthic™Human Corneal Epithelium (HCE) for eye irritation testing	2008	Under validation
Assay for the assessment of DNA modifying agents for genotoxicity testing	2008	Test did not meet ECVAM's priorities
Test for the identification of endocrine disrupting chemicals for reproductive toxicity testing	2008	Further clarifications on test pre-submission awaited
The Chorioallantoic Membrane Vascular Assay (CAMVA) for eye irritation testing	2008	Full submission awaited
human Cell Line Activation Test (h-CLAT) for skin sensitisation testing	2008	Under validation
VITOSENS- Skin sensitisation testing	2008	Under external validation
The Porcine Corneal Opacity Reversibility Assay (PorCORA) for eye irritation testing	2008	Submission awaited
SkinEthic RHE for skin irritation testing	2008	Validated and regulatory accepted
Modified EpiDerm for skin irritation testing	2008	Validated and regulatory accepted

INTRODUCTION

The animal testing ban of cosmetic ingredients came into force on 11 March 2009, for all human health effects. The marketing ban of cosmetic ingredients tested on animals became effective at the same date for all human health effects with the exception of repeated-dose toxicity (including skin sensitisation and carcinogenicity), reproductive toxicity and toxicokinetics. For these specific health effects, a deadline of 10 years after entry into force of the Directive is foreseen, i.e., 11 March 2013, irrespective of the availability of alternative non-animal tests, but which could be postponed through Codecision procedure in case of technical problems in meeting this deadline. Noteworthy, in Europe, according to Directive 2003/35/EC, cosmetic finished products cannot be tested on animals anymore since 2003 (EU, 2003).

Article 9 of Directive 2003/15/EC amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products for the seventh time stipulates that "every year the Commission shall present a report to the European Parliament and the Council on progress made in the development, validation and legal acceptance of alternative methods……". The ECVAM technical report is prepared to support the Commission Annual report by providing information on the current status of alternative methods.

During 2008-2009, ECVAM progressed its activities related to validation, development and optimisation of alternative methods. Furthermore, during the last two years ECVAM became also very proactive in the regulatory acceptance of alternative methods.

The present report is divided into two parts. The first part covers the human health effects which fall within the 2009 deadline according to the marketing ban imposed by the Cosmetics Directive and the subsequent reports on establishing a timetable for phasing out animal testing (EC, 2004a). The second part describes those which fall within the 2013 deadline. It should be kept in mind that according to the 7th Amendment of the Cosmetics Directive, the animal testing ban will be effective in 2009 for all the human health endpoints, irrespective of the availability of alternative methods.

1. HUMAN HEALTH EFFECTS FALLING UNDER THE 2009 DEADLINE

1.1 ACUTE TOXICITY

In the area of acute toxicity, one of the activities during 2008-2009 was the continuation of the EU Integrated Project ACuteTox.

The work carried out in this project consisted in the generation of *in vitro* data using different cell systems and *in silico* models (http://www.acutetox.org/). By the end of 2008, 57 chemicals were tested with a number of functional tests covering absorption, distribution, excretion, metabolism and specific organ toxicity, such as haemato-, neuro-, nephro- and hepatotoxicity. The data generated were stored in a novel database developed (Acutoxbase) within the project. This database contains a full set of data regarding the 97 ACuteTox reference chemicals, including molecular structure, physicochemical properties and summary descriptions on use, toxicity and *in vivo* biokinetics. Moreover, the database contains data sets from acute oral toxicity studies *in vivo*, from human blood poisoning reports, from *in vitro* experiments and different SOPs for *in vitro* assays (Kinsner-Ovaskainen *et al*., 2009).

During the five years of the project, a very large number of *in vitro* assays were used to test a set of 57 chemicals. The generated data were used to assess the within-laboratory variability, the preliminary predictive capacity, and in some cases also the between-laboratory variability of each *in vitro* assay.

The outcome of this phase of the project is a large toolbox of *in vitro* methods, some of them evaluated to the level of prevalidation.

Moreover, based on a statistical analysis of this large dataset, a list of *in vitro* and *in silico* methods was selected in the course of 2009, and three proposals for *in vitro* tiered testing strategies were defined. The choice of assays was done according to their reproducibility and reliability, and most importantly, according to their potential to classify chemicals into the different acute oral toxicity categories (EU and GHS).

- Neutral Red Uptake in 3T3 mouse fibroblasts (general cytotoxicity).
- Cytokine release (IL-1, TNF-α, IL-6) in human whole blood (immunotoxicity).
- Cytomic panel for Cytotoxicity Screening (Ca^{2+} uptake, mitochondrial and plasma membrane potential) in A.704 (human kidney adenocarcinoma), HepG2 (human hepatoma), SH-SY5Y (human neuroblastoma) cells.
- Cytomic panel Oxidative stress (intracellular peroxidative activity, intracellular levels of superoxide anion, oxidized DNA base 8-oxoguanine) in HepG2, SH-SY5Y and A.704 cells.
- Inhibition of colony forming unit efficiency in human cord blood-derived cells stimulated with granulocyte/monocyte-colony stimulating factor (CBC/CFU-GM).
- A multiparametric high content analysis in rat brain aggregates, comprising (i) mRNA expression for the genes heat-schock protein 32 (HSP32), glial fibrillary acidic protein (GFAP), neurofilaments (NF-H), and myelin basic protein (MBP); (ii) the rate of global RNA synthesis (i.e., uridine incorporation into total RNA) (neurotoxicity).
- MTT assay in rat hepatocytes (metabolism).
- Kinetic parameters: volume of distribution, protein binding, clearance (*in vitro* metabolic stability assay), and oral absorption (using *in vitro* Caco-2 cells and neuronal networks – human intestinal absorption [HIA]) for the estimation of the oral dose from the effective concentration observed *in vitro.*
- The estimation of compound passage through the blood-brain barrier using neuronal networks (LogBB) (for neurotoxicity assays).

Further work will focus mainly on the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of the combination that gives the best prediction. During this phase, the methods identified as promising building blocks for the testing strategy, will be challenged with a new set of coded 32 compounds.

The proposed combinations will be challenged retrospectively with the new data generated during this exercise, in order to identify the best performing combination (2010).

As a follow-up study to the international validation study on the prediction of acute toxicity by cytotoxicity assays and taking into consideration the high prevalence of non-toxic substances in the New Chemicals Database (87% with LD_{50} > 2000 mg/kg), ECVAM has commissioned in 2008, a validation study in which 57 industrial chemicals (of which 43% are cosmetic ingredients) were tested to assess the predictive capacity of the validated 3T3/ NRU cytotoxicity assay to discriminate between toxic/hazardous (LD_{50} < 2000 mg/kg) and non-toxic (LD_{50} > 2000 mg/kg) substances. The validated protocol of the test was performed manually at Health & Safety Laboratory (UK) and was also established on the robotic testing facility at the Institute for Health and Consumer Protection, and this automated version was evaluated using the same set of chemicals. A third laboratory in the US assessed an abbreviated version of the validated 3T3/NRU protocol, which is less costly and more industry-friendly. The testing was finalised in June 2009 and data analysis is ongoing. The preliminary results show relatively high negative predictive values (90%). However, it is important to note that the rate of false positives is also high (65%).

Following the initiatives undertaken by the pharmaceutical industry to waive the acute oral toxicity testing before going to clinical studies by using information from other *in vivo* studies, ECVAM performed in 2008 an investigation to identify non-toxic compounds (LD50 > 2000 mg/kg) using information from 28-days repeated dose toxicity studies. A Non Observed Adverse Effect Level threshold was set that allowed the correct identification of 63% of non-toxic compounds, while only less than 1% of harmful compounds were misclassified as non-toxic. The proposed approach could have an immediate impact for the testing of cosmetic ingredients as it could filter more than 50% of substances (Bulgheroni *et al*. 2009).

In addition, during 2009, ECVAM has performed a retrospective data analysis of multi-route acute studies in order to evaluate the concordance among regulatory classifications for acute, dermal and/or inhalation toxicity. 1569 chemicals retrieved from the New Chemicals Database were included in this anlaysis. The results showed that the concordance among oral and dermal route for non classified substances is 100% and only for one substance the dermal classification was more severe than for the oral route (manuscript in preparation). Similar findings have been recently published by Creton *et al.* (2010) with a small chemical data set, confirming that acute dermal studies do not add value beyond oral data for hazard classification of chemicals.

1.2 SKIN IRRITATION/CORROSION

The ECVAM skin irritation validation study (SIVS) ended successfully in May 2006 (Spielmann *et al*., 2007; Eskes *et al*., 2007). Further to peer-review of the study by the ECVAM Scientific Advisory Committee (ESAC), in April 2007, ESAC issued a statement on the scientific validity of the EpiSkin™ assay as being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by 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 irritant and no-label (non-skin irritant) test substances (ESAC, 2007a).

The EpiDerm™ test method was considered to reliably identify skin irritants due to its high specificity, but negative results might require further testing [e.g. according to the tiered strategy, as described in the OECD TG 404, (OECD, 2004a)]. The ESAC recommended that improvement of the EpiDerm™ protocol should be made to increase the level of sensitivity. Thus, the protocol of the EpiDerm™ assay was optimised by the test developers and the assay underwent an external validation study in view of upgrading the test to a full replacement test. The study was submitted to ECVAM in April 2008 in view of ESAC peer review. In November 2008, upon completion of the peer review process, ESAC issued a statement on the scientific validity of the modified EpiDerm™ test method as a full replacement test (ESAC, 2008a).

Finally, the similar SkinEthic™ RHE test method for skin irritation testing underwent an external catch-up validation study, was independent peer-reviewed and an ESAC statement on its scientific validity as a full replacement test was issued in November 2008 (ESAC, 2008a).

An EU Test Method on Reconstructed human Epidermis (RhE) test methods for skin irritation testing was drafted and submitted to the EU National Coordinators of Testing Methods in December 2007. Test Method B.46 *In Vitro* Skin Irritation: Reconstructed Human Epidermis Model Test, including the three validated RhE test methods (EpiSkin™, EpiDerm™ and SkinEthic™ RHE) was officially introduced into the EU Test Method Regulation (COM regulation 440/2008/EC) in July 2009 (EC, 2009). The consequent adoption of three validated *in vitro* test methods as stand-alone tests for replacing the skin irritation animal test will be of utmost importance to meet the challenges of the 7th Amendment to the Cosmetics Directive. The Commission also submitted a test guideline to the OECD which has been included in the OECD Test Guidelines Programme. Work in this forum is progressing

well: two OECD expert meetings for discussing the draft OECD TG were already held and the draft TG will be submitted to the WNT for adoption in March 2010.

Recently, ESAC issued a statement (ESAC, 2009a) on the scientific validity of the EpiSkin™, the EpiDerm™ and the SkinEthic™ RHE test methods as being reliable and relevant stand-alone tests for predicting rabbit skin irritation under the EU CLP regulation adopting the GHS classification system (EC, 2008). In the EU, the UN GHS system as applicable to all authorities is directly transposed and implemented through the Regulation on the Classification, Labelling and Packaging of Substances and Mixtures (CLP 498 Regulation EC 1272/2008) which was adopted in December 2008, came into force on 20 January 2009 and will, after a transitional period, replace the previous EU legislations for the classification of 500 substances and mixtures (i.e. preparations). In agreement with the UN GHS system, the CLP system continues to use one irritant category. However, according to the new rules for skin irritation classification and labelling (C&L), the cut-off
503 score to distinguish between no-category and category 2 substances was shifted to 2.3 (UN GHS or CLP) from a value of 2.0 (previous EU system). Consequently substances with an *in vivo* score between 2.0 and 2.3 that were considered irritant under the previous EU system are now considered non-irritants under UN GHS, which does not use the optional Category 3 (Griesinger *et al.*, 2010).

A presubmission of another RhE test method for skin irritation testing [Epidermal Skin Test (EST)-1000] was received at ECVAM in September 2009 and a further submission of an RhE test method (StratiCELL EPI/001) is expected soon.

This raises the question on the level of priority that should be given to the validation of additional test methods which are similar to the already validated and accepted ones.

In the field of skin corrosion, an additional similar test method (EST-1000) to the validated ones (EpiSkin™, EpiDerm™ and SkinEthic™ RHE skin corrosion tests) was endorsed as being able to distinguish between corrosive and non-corrosive chemicals within the context of OECD Test guideline 431 in 2009 (ESAC, 2009b).

To advance the development of new biomarkers which could be used in reconstructed human epidermis test methods, ECVAM assessed two promising new predictive technologies in the framework of its in-house research: toxicogenomics as well as toxico-metabonomics. The outcome of the toxicogenomics approach was published in 2007 (Borlon *et al*., 2007).

1.3 EYE IRRITATION

Substantial progress has also been made in the field of eye irritation as described below. ECVAM is working in close collaboration with ICCVAM, COLIPA and the industry in general, in order to streamline and harmonise global validation efforts and avoid duplication of work.

1.3.1 Testing Strategies

Promising testing strategies for eye irritation were identified during an ECVAM Expert Meeting involving more than 30 participants from industries, CROs, regulators, academia and animal welfare organisations in 2005 (Scott *et al*., 2010). The expert meeting was convened to allow developers/users to nominate methods to be considered as a basis for an overall testing strategy. Assays were evaluated and categorised on the basis of their applicability domains (e.g., categories of severity of irritation, modes of action, chemical class, physicochemical compatibility). The analyses were based on the data developed from current practice and published studies, expert opinion, the ability to predict depth of

injury (within the applicable range of severity), modes of action that could be addressed and compatibility with different physico-chemical forms. The difficulty in predicting the middle category of irritancy (e.g. R36, GHS Categories 2A and 2B) was recognized. The testing scheme proposes using a Bottom-Up (begin with using test methods that can accurately identify non-irritants) or Top-Down (begin with using test methods that can accurately identify severe irritants) progression of *in vitro* tests, based on expected irritancy of substances as primarily identified by their physico-chemical properties. Irrespective of the starting point, the approach would identify non-irritants and severe irritants, leaving all others to the (mild/moderate) irritant GHS 2/R36 categories.

Fifteen assays were nominated which can be divided in four major groups: cytotoxicity- and cell function- based assays, reconstructed human tissue models, organotypic assays and other assays.

The performances and applicability domains of individual alternative methods which are considered sufficiently promising to populate the proposed testing strategies were, or are currently being, determined through validation studies (Zuang *et al*., in press).

1.3.2 Evaluation of in vitro assays

Two organotypic assays, the Bovine Corneal Opacity and Permeability (BCOP) assay, and the Isolated Chicken Eye (ICE) test were endorsed by ESAC in April 2007 for the identification of ocular corrosives and severe irritants (ESAC, 2007b). Such statement was based on the results and conclusions from the ICCVAM retrospective validation study carried out in collaboration with ECVAM from 2003-2006. OECD Test Guidelines on the two test methods were adopted in 2009. For the two other organotypic assays evaluated, the Hen's Egg Test on the Chorio-allantoic Membrane (HET-CAM) assay and the Isolated Rabbit Eye (IRE) test, ESAC requested that further work was performed before a statement on their scientific validity to identify ocular corrosives and severe irritants could be made. With regard to the evaluation of the four organotypic assays for identifying mild or non irritants, a retrospective analysis of the collected data was carried out by ICCVAM. In May 2009, the ICCVAM Ocular Peer Review Panel recommended BCOP for the identification of non-classified materials. None of the test methods was recommended for full replacement, because none of the methods is able to identify the mild/moderate ranges of ocular irritancy.
At ECVAM, follow-up work on further improvements of the prediction models and analyses for prediction of all ranges of irritancy, using data mining techniques, is currently ongoing.

The retrospective validation of four cytototoxicy- and cell function- based assays, i.e., the Neutral Red Release (NRR), the Red Blood Cell (RBC), the Fluorescein Leakage (FL) and the Cytosensor Microphysiometer (CM) test methods took place between May 2006 and October 2008. The study was based on the retrospective collection of existing data compiled according to the ECVAM modular approach to validation and weight-of-evidence principles (Hartung *et al*., 2004; Balls *et al*., 2006). Based on the final results, recommendations were made by the Validation Management Group on the validity of the NRR (INVITTOX Protocol 54 and PREDISAFE™) and FL (INVITTOX Protocol 120) to initiate a bottom-up approach, discriminating chemicals not classified as irritants (GHS and EU non-classified) from all irritant classes, and the CM (INVITTOX Protocol 102 modified) and FL (INVITTOX Protocol 71) to initiate a top-down approach, discriminating severe irritants (GHS Cat 1, EU R41) from all other classes, for defined applicability domains.

In July 2009, ESAC endorsed the CM (INVITTOX Protocol 102 modified) and the FL (INVITTOX Protocol 71) as scientifically valid for use as an initial step within a top-down approach to identify ocular corrosives and severe irritants (GHS Cat 1, EU R41, EPA Cat I) from all other classes, for water-soluble chemicals (substances and mixtures). Furthermore, the CM (INVITTOX Protocol 102 modified) was considered to have been scientifically validated for use as an initial step within a bottom-up approach to identify chemicals not classified as irritants (GHS no category, EU no category,

EPA Cat IV) from all irritant classes, only for water-soluble surfactants, and water-soluble surfactant-containing mixtures (ESAC, 2009c).

On the basis of a thorough evaluation of the data compiled in the course of the ECVAM validation study, the ESAC concluded that the CM test method does not correctly identify moderate and mild ocular irritants (EU: R36; GHS: Cat 2A/B; EPA: Cat II/III). Therefore, the test method can only be employed to make decisions on two of the categories of the eye irritation classification schemes. Consequently, ESAC did not recommend this test method as a full replacement method. It should be noted in this context that the top-down and bottom-up approaches foresee the theoretical possibility of a *default* mild/moderate categorization (e.g. EU R36 or GHS Cat 2) of all those chemicals neither identified as ocular corrosives and severe irritants nor as "not classified" in the first two tiers of the strategy. However, since the false negative rate of the CM was high (9-55%) when initiating a top-down approach and the false positive rate was high (50-69%) when initiating a bottom-up approach, the possibility to use the method for default categorization was excluded. The test method can thus not be considered a full replacement method on its own using the top-down and bottom-up approaches.

For the FL (INVITTOX Protocol 71), additional testing and further refinement, in particular with respect to variability and definition of the applicability domain, by expanding the dataset of tested chemicals and direct comparison with *in vivo* data was recommended. With regard to the NRR (INVITTOX Protocol 54 and PREDISAFE™), RBC (INVITTOX Protocols 37 and 99) and FL (INVITTOX Protocols 82, 86 and 120), ESAC considered that the available evidence was insufficient to support a recommendation that they are ready for consideration for regulatory use.

Similarly, the available evidence for Fluorescein Leakage INVITTOX Protocol 71 did not support a recommendation for its use to initiate a bottom-up approach for regulatory use.

Two Reconstructed human Tissue (RhT) models, the SkinEthic™ Human Corneal Epithelium (HCE) and the EpiOcular™ OCL-200 model were positively reviewed by the ECVAM Eye Irritation Task Force who recommended protocol improvements prior to enter a formal validation study. The two assays have then undergone protocol optimisation and assessment in a multi-laboratory trial coordinated by COLIPA. Subsequently, the results were submitted to ECVAM in 2008 and were assessed in view of planning a prospective validation study. In December 2008, the ECVAM-COLIPA validation study on the two RhT models was initiated. The validation study foresees the testing of 104 coded chemicals in three runs and in three laboratories. Six Validation Management Group meetings took already place since December 2008, where strategic decisions were taken including the definition of a study design and the type and number of chemicals to be tested, in view of starting the experimental phase of the study at the earliest possible date (Freeman *et al*., in press).

The Ocular Irritection® assay, an *in vitro* method that mimics the biochemical phenomena of corneal proteins denaturation and disruption caused by irritant substances acting on the cornea, was submitted to ECVAM in January 2009. Further to the evaluation of the submission, ECVAM requested in May 2009 to provide additional information in a revised submission. In order to fill the remaining data gaps identified in the submission, an external validation study on the Ocular Irritection® assay will be initiated beginning of 2010, with a view to submit the study to ECVAM for peer review by ESAC.

1.3.3 Evaluation of a refinement method

The Low Volume Eye Test (LVET) was first submitted to ECVAM in 2003. Further to ECVAM's request for additional information and the recommendations from the ECVAM Task Force on Eye Irritation, a final dossier based on retrospective evidence compiled according to the Modular Approach was submitted to ECVAM by the test submitter in February 2007. The dossier was peer-reviewed by ESAC, who finally came to a consensus in July 2009. ESAC recommended the use of existing LVET

data for the use domain of household detergent and cleaning products and their main ingredients (e.g. surfactants) for making eye irritation classification and labelling decisions in a weight of evidence approach, and as reference data for the validation of *in vitro* test methods. However, ESAC recommended not generating new LVET data (ESAC, 2009d).

1.3.4 Mechanistically-based assays

Beside the development of testing strategies, ECVAM is also closely following the development of mechanistically relevant assays which cover other aspects of ocular toxicity that occur *in vivo* in order to address the currently existing mechanistic gaps (e.g. reversibility of effects due to tissue remodelling and modelling of the inflammatory responses or identification of mild irritant effects).

1.4 GENOTOXICITY AND MUTAGENICITY

The successful validation of the *in vitro* micronucleus test led to EU regulatory acceptance and to the rapid integration of the test into the REACH legislation. An OECD *ad hoc* expert meeting for the finalisation of the draft test guideline (TG 487) was held in Atlanta in October 2007. In 2008, the OECD WNT provisionally approved the draft Test Guideline, pending the outcome of the cytotoxicity measure [Relative Population Doubling (RPD) or Relative Increase in Cell Counts (RICC)] performance assessment, since the US had objected that the proposed methods for cytotoxicity measurements in draft TG 487, had not been adequately substantiated. To resolve this issue, a European Working Group coordinated by the UK agreed to develop data demonstrating the performance of these cytotoxicity measurement approaches. The US agreed to accept these methods if, after reviewing the data, it concurs that the developed data do substantiate their use. The experimental work was finalised and the results were presented at the International Workshop on Genotoxicity Testing (IWGT) which was held in Basel in August 2009. Test Guideline 487 has been approved by written procedure by the OECD Working Group of National Coordinators on 28 November 2009 and will therefore soon be adopted, which will lead to its widespread international application. According to the standard procedure, the micronucleus test *in vitro* will be introduced into the EU Test Method Regulation.

Since the regulatory accepted *in vitro* assays produce a high rate of false positives, which need to be confirmed by animal testing, ECVAM organised a workshop in April 2006 to address this issue (Kirkland *et al*., 2007). The workshop focused on the possibilities to improve the current battery of *in vitro* tests in order to have a lower false positive rate resulting from the *in vitro* tests. This is a crucial step in the development of a strategy not using *in vivo* tests by 2009. The recommendations developed during the workshop are currently followed up by different groups and organisations, including COLIPA, ECVAM and NC3Rs. One of the recommendations was to identify chemicals that could be used in the evaluation of the performance of new or modified tests. Therefore, an expert panel was convened by ECVAM and a list of chemicals was published (Kirkland *et al.,* 2008). These chemicals are now used in a project led by COLIPA and carried out by Covance (UK), which has the objective to improve current *in vitro* mammalian cell genotoxicity assays. The project addresses whether cell types, clones or isolates that are less susceptible to "false positive" results than others, can be identified, whilst still allowing the detection of all important *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens. The preliminary data that have been presented at the IWGT in Basel indicate that p53 compromised rodent cell lines over-estimate genotoxic potential in the micronucleus test. Therefore, IWGT suggests using p53 competent cells in *in vitro* micronucleus or chromosome aberration tests.

Another recommendation of the ECVAM workshop was to evaluate, if the current requirements for testing (top concentration and highest level of toxicity) could be modified so as to reduce the number of "false positive" results and as such avoid unnecessary additional animal testing, whilst still allowing the detection of all important *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens. To address

this issue ECVAM has commissioned an analysis of published data for top concentration and upper limit of cytotoxicity considerations in mammalian cell genotoxicity testing. The objective of the analysis was to determine whether concentrations as 10mM are needed to detect *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens using *in vitro* mammalian cell tests or whether a lower level can be justified. The analysis suggests that about 5% of chemicals would be missed by the testing battery, if the top concentration was reduced to 1mM. However, a preliminary analysis shows that, among these chemicals, some are misleading positives and other are picked up when newly tested, using current testing guidelines (manuscripts are in preparation). These data have been presented at the IWGT, where a consensus was reached that there are reasons to reduce the 10mM top dose. This topic is also under discussion in the revision of the ICH guidance for genotoxicity testing and data interpretation for pharmaceuticals intended for human use.

Another recommendation that had been made by the nominated expert sub-working group in 2005 was the introduction of an additional *in vitro* step using skin models (Maurici *et al*., 2005). Taking on this recommendation, the COLIPA Task Force Genotoxicity and ECVAM have started a study on the optimisation of the *in vitro* micronucleus, and the Comet assays in reconstructed human epidermis models. The optimisation and the transferability phases are now completed, and the within- and between-laboratory reproducibilities are currently under evaluation.

ECVAM is also currently evaluating possibilities for achieving reduction in animal use. With an aim to evaluate reduction opportunities, ECVAM has conducted a survey to collect data on reduction opportunities in the Micronucleus Test *in vivo* and the Chromosomal Aberration Test *in vivo* from industry and CROs. The goal of this activity was to investigate current practice with regard to animal use (number of animals, single or both sex, use of negative and positive controls, etc). Based on the results of the ECVAM survey, it became clear that, although some opportunities for reduction already exist, they are not being implemented by everybody. The outcome of this survey served as the basis for the ECVAM workshop on "Reduction in Regulatory Genotoxicity Testing: Identification and Implementation Opportunities" which was held in June 2008. The objectives of the Workshop were to evaluate: 1) how to reduce the number of animals in standard genotoxicity testing, 2) whether the number of animals can be reduced by applying smarter testing strategies, and 3) to brainstorm how these reduction possibilities can be promoted and implemented. Reduction opportunities, such as omission of positive and/or negative control animals, evaluation of one gender only, analysis of two time points in the same animal, integration of a genotoxicity assay in the 28-days repeated dose toxicity assay, combination of genotoxicity assays in the same animals were assessed and discussed (Pfuhler *et al*., in press).

International validation studies on the comet assay *in vitro* and *in vivo* are currently ongoing under the coordination of JaCVAM.

1.5 UV-INDUCED EFFECTS

ACUTE PHOTOTOXICITY

The validated 3T3-NRU *in vitro* phototoxicity test (3T3-NRU-PT) gained regulatory acceptance in all EU Member States in 2000 (EC, 2000) and in the OECD Member States in 2004 (OECD, 2004b). The test is now widely used in the chemical and cosmetic industries.

Subsequently, the usefulness of reconstructed human skin model assays as a supplementary assay to the 3T3-NRU PTwas demonstrated in several studies (Jones *et al.*, 2003; Lelièvre *et al*., 2007; Liebsch *et al*., 1995; Liebsch *et al.*, 1997; Liebsch *et al*., 2005; Portes *et al.,* 2002; Jírová *et al.,* 2005).

These studies showed that a true phototoxic potential of a chemical which is correctly predicted in the 3T3-NRU-PT may be modified and modulated when applied topically to the skin at low concentrations (e.g. in formulations) and additional information on phototoxicity linked to barrier function, tissue distribution and bioavailability of the chemical in the skin may be needed.

The reconstructed human skin model assays represent a useful tool for confirmation of non-phototoxic and phototoxic samples identified by the 3T3 NRU PT. However, no test method was submitted to ECVAM yet.

Further investigations are needed in the extrapolation of *in vitro* results to the human situation. In a limited study focused on a specific group of chemicals, it was shown that in certain cases, the human situation may be underpredicted (Kejlová *et al*., 2007) and a safety factor should be considered for extrapolation.

Recently, the Safety Ad Hoc Group (SAHG) of the European Federation of Pharmaceutical Industries and Associations (EFPIA) commissioned a survey of member companies to understand the triggers for photosafety testing and how *in vitro* hazard characterization translated to *in vivo* risk. Data from 10 EFPIA member companies relative to 361 compounds (97% of which had data for the 3T3-NRU-PT) indicated that the *in vitro* photosafety assays are over predicting animal photosafety *in vivo* and human photosafety risk in the clinic. These findings raise concerns regarding the use of in vitro photosafety assays for assessment of chemical photosafety of pharmaceuticals for regulatory purposes, and further underline the usefulness of reconstructed human skin models as supplementary assays to the 3T3-NRU-PT (Lynch & Wilcox, 2010).

Recent research studies report the characterization of alternative cell lines and models for phototoxicity assessment. A study performed on L-929 cells showed that this cell line presented a phototoxic response similar to that of 3T3 cells, suggesting that the L-929 cell line could be a valuable alternative to 3T3 cells in the *in vitro* screening for phototoxicity assessment (Ray *et al.*, 2008). In addition, a comparison study of solar radiation-induced cellular damage between *ex vivo* porcine skin organ culture and *in vitro* reconstructed human epidermis suggested that both cultures are good surrogates to human skin tissues and are relevant tools for phototoxicity studies (Bacqueville & Mavon, 2009).

Additionally, a research paper reported the use of E. Coli as an alternative and low cost *in vitro* test system for the screening of the phototoxic potential of chemicals and cosmetic products (Verma *et al.*, 2008).

Finally, an *in vitro* high-throughput cell-free assay, which monitors the generation of reactive oxygen species (ROS) from test compounds by combining the use of a multiwell plate and a quartz reaction container was developed and assessed in-house in the laboratory that developed the test, and suggested to be an effective tool as a first screen to predict the phototoxic risk of chemicals (Onoue *et al.,* 2008).

New developments on acute phototoxicity were presented at the 7th World Congress on Alternatives & Animal Use in the Life Sciences, held in Rome on 30 August to 3 September, 2009.
In particular, Kandárová reviewed and discussed existing findings on the potential and usefulness of 3D reconstructed human skin equivalents to estimate phototoxic damage (Kandárová, 2009).
In addition, the results of an *in vitro* phototoxicity test performed with the reconstructed human skin model KeraSkin™ showed high predictivity of phototoxic potential for nine chemicals, suggesting that KeraSkin™ could be a valuable alternative *in vitro* test method for phototoxicity (Ryu *et al.,* 2009).

A comparative analysis of the phototoxic predictivity of three reconstructed human skin equivalents, Epiderm™, KeraSkin™ and Melaskin™ was performed with 8 chemicals, showing the same results

and good correspondence with *in vivo* data for the three models, suggesting that they could efficiently discriminate between phototoxic and non-phototoxic chemicals (Sohn *et al.,* 2009).

Finally, an *in vitro* strategy for phototoxicity testing was presented based on complementary endpoints: (I) supercoiled circular DNA for assessment of photoreactivity, (II) yeast Saccharomyces cerevisiae for evaluation of photocytotoxicity and photomutagenicity, (III) use of normal cultured human skin cells where photogenotoxicity can be detected using the comet assay (IV) and reconstructed skin. Such a strategy should allow the evaluation of compounds with very different physicochemical properties (Marrot *et al.,* 2009).

However, all these test systems are still in the research and development phase and for most of them further work is needed to optimise them before they can be considered for validation by ECVAM.

1.6 SKIN ABSORPTION/PENETRATION

In vitro tests for the determination whether a chemical penetrates through skin were accepted at OECD level in 2004 (OECD, 2004c) and at EU level in 2008. *In vitro* methods for skin absorption measure the diffusion of chemicals across excised human or pig skin in flow through or static diffusion cells. An OECD guidance document for the conduct of skin absorption studies describes the circumstances in which the use of the *in vitro* method would be appropriate (OECD, 2004d).

In 2007, preliminary results on an *in vitro* test based on reconstituted human epidermis (RhE) models were submitted to ECVAM for evaluation. The advantages of this test method are the adequate availability of RhE models, the shorter duration of the experiments and possibly also the investigation of skin metabolism in skin tissue of human origin (Schäfer-Korting *et al*., 2006). Another important advantage is the much lower variability in results due to standardized production of the RHE models when compared to the results obtained with excised skin, as was demonstrated in the European FP5 EDETOX project (http://www.ncl.ac.uk/edetox/abouttheedetoxproject.html, van der Sandt *et al*., 2004).
Furthermore, by introducing skin models also for skin absorption, beside skin corrosion and skin irritation, would allow comparing results for specific chemicals across endpoints more easily.

In 2008, the performance of RhE models for the determination of skin absorption was further evaluated by testing nine substances, covering a wider range of physico-chemical properties, in ten laboratories under strictly controlled conditions (Schäfer-Korting *et al.*, 2008). The results showed that the permeation properties of RHE exceeded that of human epidermis and pig skin, yet the ranking of substance permeation through RHE models reflected the permeation through human epidermis. RHE models showed lower variability of data and sufficient correlation between all preparations examined. Thus, RHE models seem appropriate alternatives to human and pig skin for *in vitro* assessment of permeation and penetration of substances. An expanded substance panel and an increase in the number of experiments are needed to progress the assay through validation.

Recent studies report the development of a modified growth method for cultured skin substitutes. These skin substitutes, developed under anchored conditions, were shown to present better barrier properties than those grown by standard methods, thus being more suitable as an *in vitro* skin permeability model (Barai *et al.*, 2008).

In addition, recent studies report the development of a predictive model for estimating the cumulative mass of a chemical absorbed into and across the skin from cosmetic/dermatological formulations, which could provide a tool for assessment of *in vitro* permeation (Gregoire *et al.*, 2009).

Here again, all these test systems are still in the research and development phase and for most of them further work is needed to optimise them before they can be considered for validation by ECVAM.

2. HUMAN HEALTH EFFECTS FALLING UNDER THE 2013 DEADLINE

2.1 SKIN SENSITISATION

The assessment of the skin sensitisation potential of chemicals still relies on the use of animals due to the lack of validated partial replacement or full replacement methods. It is increasingly aknowledged that the complexity of the mechanisms underlying this human health effect will require the combination of *in silico*, *in chemico* and *in vitro* approaches to ensure the safe use of products.

At research level, ECVAM is strongly involved in the EU FP6 Integrated Project "Sens-it-iv" (2005-2010) (http://www.sens-it-iv.eu) which aims to contribute to the replacement of animal experimentation by *in vitro* assays for identifying skin and respiratory sensitisers in relation with the use of safe ingredients by the chemical, cosmetic and pharmaceutical industries. In October 2008, the Annual General Assembly of the project constituted an important milestone for the project, since it marked the passage from the science to the technology module in which more emphasis will be given to test methods development rather than research. A number of promising *in vitro* methods and biomarkers are currently under investigation for their potential to discriminate a) between sensitisers and irritants b) between respiratory and skin sensitisers, with the ultimate goal to combine them into a testing strategy able to deliver information on potency. ECVAM is providing support in order to bring these methods up to a level of development/standardisation which comply with its criteria for entering prevalidation.

With regard to validation activities, three partial replacement tests, namely the Direct Peptide Reactivity Assay (DPRA), 2) the human Cell Line Activation Test (h-CLAT) and 3) the Myeloid U939 Skin Sensitisation Test (MUSST), have been formally submitted to ECVAM. These tests, developed by Colipa associated Industries, underwent extensive optimisation through ring trials within Colipa. Further to the evaluation by ECVAM, the tests entered phase III of a prevalidation study in which the laboratory reproducibility and the preliminary predictive capacity will be assessed with a set of coded chemicals. It is envisaged that these tests, once formally assessed for their reliability, may play a role as building blocks in a future testing strategy for the full replacement of current animal tests.

Consideration is also given to the availability of validated non-radioactive modifications of the LLNA to improve its regulatory use that would imply further reduction in the number of animals used for the assessment of the skin sensitization potential of chemicals. To be able to properly assess such variants of the standard LLNA, and on the basis of the recommendation of an ECVAM workshop on: "An Evaluation of Performance Standards and Non-radioactive Endpoints for the Local Lymph Node Assay" (Basketter *et al.,* 2008) ECVAM has been working in close collaboration with ICCVAM on the development of a harmonized set of Performance Standards (PS) for the LLNA. The ECVAM harmonised PS for the LLNA were endorsed as scientifically valid by ESAC during its 29th meeting (ESAC, 2008b). The inclusion of the harmonised PS in the proposed revision of the OECD TG 429 is currently on-going.

In addition, the reduced Local-Lymph Node Assay which uses fewer animals than the tradional LLNA is also being included in the revised OECD TG 429. The reduced LLNA was validated by ECVAM and was endorsed as scientifically valid by the ESAC during its 26th meeting (ESAC 2007c).

In the field of respiratory sensitisation, an expert meeting was convened at ECVAM in April 2008 (Roggen *et al*., 2008) to consider what progress has been made in the light of the recommenations

derived from the 2006 Workshop and the published report (Kimber *et al*., 2007) and to explore how advances in the development of methods for the identification of skin sensitising chemicals could be exploited for the evaluation of chemical respiratory allergens. The workshop participants concluded that beside some progress in the QSAR field, little progress has been made since the previous workshop. This is mainly attributable to the lack of a generally accepted animal model, the complexity of the lung and the yet poorly understood underlying biological mechanisms. Therefore, research is still needed to fill these gaps. Among the current activities in the area of skin sensitisation which could be exploited also in the field of respiratory sensitisation it was suggested that the Peptide Binding-Assay, DC-based assay and the LLNA may provide useful tools to identify respiratory sensitisation hazard. However, there remains the need for additional work to determine whether these methods can reliably discriminate between skin and respiratory sensitisers.

2.2 SUBACUTE AND SUBCHRONIC TOXICITY

ECVAM was involved in the European STREP project PREDICTOMICS (http://www.predictomics.com/) that aimed at developing a novel platform for anticipating liver and kidney chronic toxicity elicited by drugs and xenobiotics. The final goal was to identify specific early mechanistic markers of toxin induced cell alterations by integrating genomic, proteomic and cytomic analysis. The project ended in December 2007 and the main achievements were the following (http://www.predictomics.com/):

1. Revised version of gene expression fingerprint profile for steatosis induced in cell cultures.
2. Development of an assay based on HepG2 cells to detect steatosic potential of drugs using a multiparametric flow cytometry approach.
3. Development of a high-content assay based on bioimaging to detect steatosic potential of drugs by using a multiparametric image analysis approach.
4. A prediction model for steatosis based on transcriptomic profiles and cytomic analysis, in the course for being prevalidated.
5. Development of several new fluorescent bile-acid probes to investigate interference in bile-acid transport by drugs.
6. Development of flow cytometric kinetic assays suitable to follow on real time the uptake and efflux of bile-acid fluorescent derivatives and the interference of xenobiotics on these processes. A prediction model for cholestasis based on cytomic analysis has been developed, in the course for being prevalidated (Rohacova *et al*., 2008; Rohacova *et al*., 2009).
7. The impact of microvascular endothelial cells on proximal tubular endothelial cells has been further delineated, thus providing a very well characterised renal co-culture model.
8. Microarray analysis has been carried out for twelve nephrotoxins in Human Kidney (HK)-2 cells.
9. Mechanistic information on several nephrotoxins has been provided. A number of pathways indicative of toxicity or protection against damage have been identified e.g. the p53 pathway (senescence and cancer) and the Nrf2 (Nuclear factor-erythroid 2-related factor 2) pathway (oxidative stress). Additionally pathways involved in CsA (cyclosporine A) induced epithelial mesenchymal transition (EMT) have been uncovered (Jennings *et al*., 2009).
10. Standard operating procedures for HK-2 cell culture, treatment and microarray analysis have been produced under the supervision of ECVAM.
11. A list of markers with potential value in detecting renal injury has been generated.
12. A preliminary prediction model for nephrotoxicity based on transcriptomic profiles has been developed.

In summary, two *in vitro* models were developed to study steatosis and cholestasis (main drug-induced liver diseases) using model cholestatic and steatotic hepatotoxins (Gomez-Lechon *et al*., 2007). Models were also challenged with five coded drugs. A human proximal tubule model (HK-2 cells) has

been established for the purposes of conducting gene expression microarrays with the Affymetrix HGU-133 plus 2 platform. Using 12 nephrotoxic compounds, a number of marker genes have been identified which are potential markers of early response to toxicity.

At OECD level, the revision of OECD test guideline on chronic *in vivo* toxicity studies (TG 452) took place during 2008 and is still under discussion at the OECD WNT.

In the field of immunotoxicity, a study to prevalidate a set of *in vitro* assays was finalised in April 2008. Human Peripheral Blood Mononuclear Cells (PBMC), murine, or rat splenocytes were used. For each of the three species, basal cytotoxicity was assessed before commencing the tests using the LDH-release endpoint. Human PBMC were stimulated with anti-CD3/anti-CD28, murine splenocytes with anti-CD3/anti-CD28, concanavalin A (Con A), lipopolysaccharide (LPS), and anti-CD40/IL-4, and rat splenocytes with Con A and LPS. Cell proliferation and production of IFN-γ and TNF-α were taken as endpoints. Seven compounds, for which good human and/or animal data are available, were selected: six with well-known effects on the immune system (benzo(a)pyrene (BaP), cyclophosphamide (CY), methotrexate, rapamycin (RAP), dexamethasone (DEX), and urethane) and one chemical classified as non-immunotoxic (D-mannitol). Each species/stimulant/endpoint combination was evaluated by using criteria of fold-stimulation and threshold levels for proliferation and cytokine production. For 10 of the 21 combinations tested, these criteria were met, and they were evaluated for their ability to identify immunosuppressive activity.
The percentage of correct predictions of immunosuppressive activity was established by using criteria of suppressed response. This prediction was made using the results of all 10 species/stimulant/endpoint combinations. Five out of 6 positive compounds could be identified as immunosuppressive (correct prediction >30%) and the non-immunotoxic compound came out as negative. These results suggest that *in vitro* assays are able to detect immunosuppression, holding promise for further evaluating these assays using a wide range of chemicals.

Further to two ECVAM workshops on chronic toxicity and physiology-based pharmacokinetic modeling (PBPK), a FP7 collaborative project Predict-IV started in May 2008. This project addresses strategies to improve the assessment of drug safety in the early stage of development and late discovery phase, by an intelligent combination of non animal-based test systems, cell biology, mechanistic toxicology and in-silico modelling, in a rapid and cost effective manner. The aim is to identify general pathways resulting in toxicity that are independent of cell/tissue type. Metabonomics, high content imaging and other endpoints capturing deregulation of essential cellular processes will be combined to develop a testing strategy with predictive capacity (http://www.predict-iv.toxi.uni-wuerzburg.de/).
The three main target organs under investigation are the liver, the kidney and the Central Nervous System. The activities are relevant to other fields beside drug development, such as the prediction of repeated dose toxicity of cosmetic ingredients.

During 2008, a COLIPA-DG RTD Joint Research Inititiative has been established and has resulted into a new FP7 call in 2009, focusing on repeated dose toxicity. The ultimate long-term goal is the replacement of repeated dose toxicity testing in human safety assessment.

2.3 TOXICOKINETICS AND METABOLISM

Toxicokinetics and metabolism are of importance for most of the other toxicological endpoints and considered as a crucial aspect for the predictivity of alternative tests. The complexity of physiological regulations and impact networks within a living animal will require tiered approaches and integrated testing strategies to answer all questions related to systemic toxicities. The details for an approach specifically relevant for cosmetics are outlined in Coecke *et al.* (2005). Details are given below on how the different information sources can be gathered to carry out both, quantitative and qualitative

evaluations of toxicity, based on a tiered approach including toxicokinetic and metabolism information, and as follow-up to the recommendations made in 2005 (Coecke *et al*., 2005).

2.3.1 Barrier models

ECVAM was involved in the studies of absorption barrier models. In particular, an ECVAM prevalidation study on *in vitro* models for the prediction of gastro-intestinal absorption was finalised in 2008 and a publication of the outcome of the study is in preparation (Prieto *et al.,* submitted). Ten test chemicals for which *in vivo* oral absorption data are available were tested in two laboratories. Atenolol, cimetidine and propranolol were included as reference compounds for low, medium and high intestinal absorption, respectively. Transport experiments were independently carried out in the two laboratories. Median CVs of 10.5% and 15.5% were found for each laboratory. Concerning between-laboratory reproducibility, comparable response levels were found for the three references compounds and for paracetamol while, for the other chemicals, lower reproducibility was obtained, in particular for those actively transported. No significant differences in Papp values were reported between the two cell lines investigated. Due to the limited number of chemicals tested, a model with a real predictive value in terms of *in vivo* absorption is not realistic; nevertheless a preliminary prediction model has been established using two mathematical models available from the literature. Good *in vitro-in vivo* correlation was obtained for well absorbed compounds, while moderate and low absorbed compounds were rather overestimated. Both Caco-2 models were more reliable to identify compounds that use passive diffusion than active transport to cross the gastro-intestinal barrier (since Caco-2 cells under- or overexpress only some of the transporters present in the intestinal mucosa), and for skin barrier (alternative tests for percutaneous absorption were adopted as OECD Test Guidelines in 2004; see 1.6 above). The data generated by such models are crucial input into more global kinetic models used to predict the fate of chemicals *in vivo*. The feasibility study to evaluate the performance of five selected *in vitro* blood-brain barrier (BBB) models for predicting the uptake into the brain was finalised in 2008. Overall, the results of the ECVAM study point out that the new *in vitro* BBB model (4d/24w), suitable to automation (Culot *et al*., 2008), constitute an opportunity to considerably increase the rate at which BBB permeability data can be generated. In addition, the combination of this *in vitro* BBB model (4d/24w) with SHSY5Y neuroblastoma cells as target cells, represents a convenient approach to explore the importance of the BBB in neurotoxicity assessment. (Hallier-Vanuxeem *et al*., 2009). In collaboration with Sovicell GmbH, ECVAM is evaluating a hybrid system that combines an *in vitro/in silico* model. Results are being generated with the same set of 16 compounds and will be compared with the permeability data obtained in the ECVAM BBB study with the 5 different *in vitro* models.

2.3.2 Biotransformation

Metabolism (biotransformation) plays in many cases a key role in the origin of toxic effects and is also critical when evaluating inter and intra-species differences. Toxic effects can also be increased through processes which impact the metabolic capacity of systems. Therefore, it is also important to have alerts when some compounds have critical effects on the overall metabolic capacities of cellular system by inducing it (Coecke *et al*. 1999), inhibiting it, or by direct metabolism-mediated cytotoxic effects (Coecke *et al.* 2006; Jacobs *et al.*, 2008).

Most of the *in vitro* systems are lacking metabolising capacities and this has been recognised as being a bottle-neck in *in vitro* test development. A strategic approach to have an indication of the likelihood that metabolism-mediated effects play a role is by trying to combine *in vitro* systems with a metabolic competent source (i.e. a system that models hepatic biotransformation. Alternatively, in a tiered approach (Coecke *et. al*, 2005), an indication of metabolism-mediated toxicity can alert and lead to specific decision points when assessing systemic effects.

Exposure to drugs, occupational and industrial chemicals, or environmental pollutants, can lead to either the induction or the inhibition of biotransformation. Due to susceptibility of Cytochrome P450

(CYP) to being induced, i.e. increases in enzymatically active microsomal protein content after exposure to specific chemicals, these enzymatic systems have been shown to be involved in various side effects, such as profound endogenous hormonal disturbances, increased liver weight, drug-drug interactions and exacerbated toxic effects. Therefore, routine evaluation of the inducing potential of a given chemical on these pathways is essential for human safety assessment (Coecke *et al*., 1999, Coecke *et al.* 2006). Expert teams on metabolism, toxicokinetics and induction were established to further the developments in the field of toxicokinetics and metabolism with an aim to validate the necessary test systems.

The prevalidation on the applied use of freshly isolated human hepatocyte cultures, assessed by looking at the predictive capacity for liver enzyme induction involving three independent laboratories, was completed. The corresponding report is being finalised and submitted.
On 4-5 September 2008, ECVAM organised a stakeholder meeting in the field of Toxicokinetics and Metabolism. During this stakeholder meeting, all participants agreed that a validation study that would provide a standard for human hepatic metabolism and toxicity would be very beneficial. In order to approach this question, initially the potential for cytochrome P450 (CYP) induction at doses which are of clinical relevance applied to the *in vitro* systems will be assessed. The CYP enzymes are known to be the predominant enzymes involved in metabolism and toxicity as compared to other biotransformation enzymes. Therefore, the assessment of the presence of different CYP isoforms is an essential part of metabolic competence assessment. Induction has a complex underlying mechanism (gene activation followed by *de novo* protein synthesis) and for this reason, it is a good indicator for high quality metabolic competent systems that can be used long-term. The results of this study are envisaged to be the starting point for a novel *in vitro* platform for assessing metabolism and toxicity.

Two human hepatic metabolic competent test systems are under evaluation: the HepaRG test system and cryopreserved human hepatocytes. For both test systems a test method has been established for the determination of CYP activities in HepaRG cells cultured in 96-well plates and in cryopreserved human hepatocytes cultured in 48-well plates. The endpoint “induction of CYPs” is measured after 48 hours in the HepaRG test system, and after 72 hours in the human cryopreserved hepatocytes, following treatment with test compounds and two reference compounds (β-naphthoflavone and rifampicin), using a cocktail of prototypical substrates for different CYP isoforms incubated directly with the two test systems. Phenacetin, midazolam, bupropion and diclofenac will be used as probe substrates present in the cocktail to evaluate the induction of CYP1A2, CYP3A4/5, CYP2B6 and CYP2C9, respectively. ECVAM is practically participating with its own laboratoratories in this study.

A comparative study of rat and human *in vitro* metabolising systems (microsomes versus homogenates) on a set of chemical compounds is completed. The goals of the project were to characterise, with human and rat liver homogenate or microsomal preparations, the metabolic stability (i.e. disappearance of the parent substance from incubations with human liver preparations and appropriate cofactors for metabolism) and the appearance of metabolic products and their tentative identification. Using this approach important background information was gained for characterising the studied substances in order to predict, in the end, their behaviour in the *in vivo* situation. Equally important is that the data obtained illustrate similarities, but also quantitative and qualitative differences between homogenate and microsomes, as well as between human and rat. The basic approach suits equally well for the characterization of metabolic capability of any tissues or cell cultures from various animals. A follow-up study to assess the same 55 compounds in the above described human metabolic competent test system is planned. The results of this study have served in the process of drafting a new test guideline for the 2-generation reproductive toxicity study by introducing in the guideline different paragraphs related to toxicokinetics and metabolism including a specific mentioning of the need to assess *in vitro* metabolic processes to assess species differences

2.3.3 Physiologically-based pharmacokinetic modelling (PBPK)

An ECVAM workshop report on *Physiologically-based Kinetic Modelling (PBK): Meeting the 3Rs Agenda* (Bouvier d'Yvoire *et al.*, 2007) describes the strategy proposed by the participants to:

- to better define the potential role of PBK modelling, as a set of techniques capable of contributing to the reduction, refinement and replacement of the use of laboratory animals in the risk assessment process of potentially toxic chemicals;
- to discuss the need for technical improvement in PBK modelling and its applications; and
- to identify the need to increase understanding and, potentially, acceptance by the regulatory authorities, of the capabilities and limitations of PBK modelling techniques in toxicological risk assessment.

It furthermore also intended to provide the non-expert reader with an overview of the field in relation to the Three Rs, with some key references.

As a follow-up to the tiered strategy proposed by the Toxicokinetic and Metabolism expert panel (Coecke *et al.*, 2005) focussing on Cosmetics, the practical use of PBK models has been discussed at a recent meeting on Toxicokinetics and Metabolism held on 4-5 March 2009. One of the very interesting PBPK Model Equation Generators called MEGen developed at the Health and Safety Laboratory is currently being reviewed by ECVAM (Loizou *et al*., 2008; Loizou, 2009). Necessary steps need to be undertaken to adapt MEGen to the needs related to the toxicological requirements of the Cosmetic Directive.

ECVAM commissioned to RIVM the development of a pilot database with kinetic parameters of compounds used as reference substances in various *in vitro* toxicity testing (pre)validation programmes. The toxicokinetic properties of compounds can form valuable information in human risk assessment. *In vivo* as well as *in vitro*, biological targets are exposed to concentrations of the compounds or their metabolites. Concentrations and their time course, mostly determined in blood or plasma, provide the most direct link between the observed or predicted *in vivo* effects and the effects observed *in vitro*. Accurate quantitative knowledge of the *in vivo* concentration-time relationship is therefore a prerequisite for the correct interpretation of *in vitro* toxicity testing results. Classical compartmental modelling parameters were chosen to describe the *in vivo* kinetic properties as they fulfil the needs for prediction of *in vivo* concentration time profiles under linear conditions. Protein binding parameters were added to facilitate calculation such as unbound substance concentrations.
Beside an input module (storage template) for the database, a retrieval template was developed to be put on the ECVAM website to facilitate further use of kinetic data. The database was filled with human and rat kinetic parameters (mainly based on intravenous and oral administration) for 100 substances following assessment of their reliability. Kinetic data
were collected on classical compartmental modelling parameters, which describe the absorption, distribution and elimination (metabolism and excretion) phase. Typical classicalcompartmental modelling parameters are systemic bioavailability, absorption rate constant, volume of distribution and elimination rate constant. This pilot database contains kinetic parameters for internal exposure estimation, which may facilitate quantitative *in vitro- in vivo* extrapolation and will be essential data in PBK efforts.

2.4 CARCINOGENICITY

The cell transformation assay (CTA) was identified, beside the gap junction communication assay (GJIC), as the only available *in vitro* carcinogenicity assay. In 2007, the OECD published a Detailed Review Paper (DRP) on the CTAs (OECD, 2007). The DRP concluded that, based on the available data, the performances of the SHE and the Balb/c 3T3 CTAs were considered adequate for recommending that they are included into official OECD Test Guidelines. Using the draft DRP as a

basis, and following the recommendations of a previous workshop (Combes *et al.,* 1999), and an expert meeting on cell transformation held at ECVAM in 2004, it became clear that a formal validation of the assays, in particular focusing on the reproducibility and the development of a standardised protocol, were needed. Consequently, ECVAM coordinated and sponsored a prevalidation study of CTA in both SHE (pH 6.7) and Balb/c 3T3 cells. Due to promising preliminary results, the prevalidation of a third variant of the assay (SHE pH 7.0) was initiated in January 2008. The aim of this prevalidation study was to assess the reproducibility of these three standardised protocols. In order to evaluate whether the tests would meet the criteria requested by the ECVAM principles on test validity, the modular approach of validation was followed (Hartung *et al*., 2004). In this study four modules were fully assessed: 1) test definition, 2) within-laboratory reproducibility, 3) transferability and, 4) between-laboratory reproducibility. In addition, the data generated add to the understanding of the predictive capacity of the CTA, which was previously addressed by the OECD DRP evaluation (OECD, 2007). Each *in vitro* test was conducted according to the same agreed protocol in three different laboratories. The evaluation of the results on the reproducibility of the three assay variants was completed and the prevalidation reports were prepared with the intention to be submitted to an ESAC peer review panel for a final assessment of the scientific validity of the cell transformation assays in accordance to modules 1-4. The outcome of the study will be published in a special issue of Mutation Research (in preparation).

A new variant of the CTA based on a derived Balb/c 3T3 clone, Bhas 42, is under validation by JaCVAM and ECVAM is involved in the Validation Management Team.

Besides the CTA, the use of toxicogenomics-based tests is being explored in collaboration with external partners. Furthermore, the FP6 Integrated Project CARCINOGENOMICS was initiated and aims at developing *in vitro* methods predictive for carcinogenicity in major target organs, i.e. liver, lung and kidney. In the frame of the CARCINOGENOMICS project, several initiatives were taken to involve regulators in the discussions on the conditions that newly developed methods have to meet for regulatory acceptance in the area of carcinogenicity testing. Among these, a workshop on Genomics in Cancer Risk Assessment was organised last August 27-28th in Venice, as a satellite symposium of the 7th World Congress on Alternatives and Animal Use held in Rome. The state-of-the-art in chemical cancer risk assessment was explored and the possible contribution of genomics and systems toxicology with respect to tackling the current flaws and uncertainties was discussed. Most importantly, the workshop was successful in bringing together leading experts from academia, industry and regulatory authorities for this and in identifying the road lying ahead: by taking the systems toxicology approach, generating mechanistic information on toxic responses from bioassays consisting of human cells, and linking this with growing insights in molecular processes underlying human pathophysiologies.

2.5 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

2.5.1 Sixth EU framework programme integrated project "REPROTECT"

Due to the high impact of reproductive toxicity studies on the number of animals used for the detection of reproductive and developmental toxicants, ECVAM drafted and initiated an integrated project called "ReProTect" with the aim to stimulate further development and optimization of *in vitro* models for toxicological safety testing in this area (http://www.reprotect.eu/). Several tests mentioned in the Cosmetics Report (Bremer *et al*., 2005) have been included in order to make them available for entering the validation process. ECVAM is providing scientific/technical support to the project coordinator as well as relevant information on the development of testing strategies. Moreover, ECVAM sponsored the statistical analysis of the data produced in order to evaluate the within- and between-laboratory variabilities of the developed/optimised assays and will host the project database after the project's effective end. Importantly, the project delivered a test battery of 14 successfully

developed *in vitro* tests, which have been challenged with 10 compounds under blind conditions. The predictive outcome was quite impressive and a manuscript has been accepted for publication.

Several tests will be presented in a special issue of the journal *Reproductive Toxicology* that will be ready in 2010.

ECVAM received submission of test methods which assess the capacity of test chemicals to influence hormonal pathways.

Research Area "Fertility":

- **Computer-assisted sperm analysis as a building block in a testing strategy to replace one/two generation studies (OECD TGs 415 and 416) (W.P. I.1).** This test was optimised and successfully transferred to a second laboratory. More than 40 chemicals were analysed for their effects on sperm motility. An independent statistical evaluation demonstrated the reliability and transferability of the test between two laboratories. A formal validation study will be considered. However, a first expert review noticed that the IC_{50} values were extremely high. A further evaluation to consider the relevance of the test in testing strategies is necessary.

- **Cell lines employed in studies on steroidogenesis.** A genetically engineered BLT1 Leydig tumor cell line was developed within ReProTect. Several chemicals were tested in one laboratory using the new genetically engineered cell line. SOPs using BLT1-L17 cells as a tool for a Leydig cell toxicity test are available. The statistical evaluation is currently taking place and if the results are promising, a decision on the formal validation of the test will be taken.

- **Sertoli cell lines.** The establishment of SOPs for the Sertoli cell line SerW3, as well as for the primary cells was finalised and 10 test chemicals were evaluated in two independent laboratories. The data are now analysed by an independent statistician in order to decide on further validation. However, due to the internal reorganization of the responsible ReProTect partner that has participated in the study, follow-up studies could not be foreseen.

- **The follicle culture bioassay (FBA), Mouse/Bovine oocytes as building blocks in a testing strategy to replace one/two generation studies (OECD TGs 415 and 416)** Granulosa/Thecal cells (W.P. I.5) and Folliculogenesis (W.P. I.6).
  The test development for oocytes bovine maturation (IVM)/fertilization (IVF) and pre-implantation embryo (IVP) was concluded by producing the SOP and by testing 16 chemicals. The evaluation of the transferability of the IVM test was planned. The transferability and the evaluation of the within-laboratory variability will be published in the special issue of *Reproductive Toxicology*.
  Twenty-six compounds were tested under the developed mouse oocytes assay's (MEA) SOP and the statistical analysis of the data generated is ongoing. SOPs are now available for the detection of adverse effects on steroidogenesis. For the follicle culture bioassay (FBA), the raw data generated after testing more than 20 well-known chemicals were transferred to the appointed statistician. The final report and the submission of a scientific manuscript are planned. The development of the Granulosa test is concluded. Unfortunately, the statistical analysis revealed a high within-laboratory variability of the test, most probably due to the instability of the genetically engineered cells developed.

Research Area "Implantation":

- **Endometrium/implantation and oviduct studies (W.P. II.1)**

The main objective of the WP is to establish cell culture models and explants specific for composing the endometrium. Two targets were considered: the human endometrial endothelial and stromal cells. Moreover, it has been planned to detect estrogen receptor, apoptosis markers of oxidative stress and cell death, drug metabolising cytochrome P450 and drug transporters on the target cells.
Preliminary SOPs for human endometrial stromal/endothelial cells are prepared but a delay in the work planned has to be expected due to the inconsistency of the scientific results. The development of a test based on the use of the human endometrial explants and cell lines is ongoing. The SOPs were established and the definition of the most predictive endpoints was carried out. Moreover, the testing phase has already started with few selected compounds and the data generated were sent to the statistician.

- **Placental toxicity**
  The identification of important cell types in the process of placentation is ongoing. Furthermore, the placenta is also considered a valuable organ for studies of fetal exposure by the placenta perfusion. Two laboratories are currently involved in the transferability study of placental perfusion and some chemicals were already tested. In order to study the transport of cells derived from placenta, the establishment of the BeWo cell system is under evaluation. The SOPs for culturing and treatment of throphoblast-like cell lines and chorionic villous explants were delivered. The testing phase is already initiated with some selected compounds and it is planned to test additional substances in order to evaluate the within-laboratory variability of the assays.
- The development of an *in vitro* system based on the use of pericyte cells is ongoing and the SOP was delivered. Few compounds have been already tested in order tocontinue with the test optimisation.

Research Area "Prenatal development":

- **Early embryonic development: The embryonic stem cell test (EST), the use of human embryonic stem cells (hESC) for embryotoxicity studies and the proteome signature for toxicology studies**
  The differentiation of murine embryonic into neural cells was characterised by analysis of the neuron-specific marker. Differentiation protocols for dose-response profiles are available and are being challenged with selected reference compounds. Furthermore, murine embryonic stem cells were successfully differentiated into chondrocytes, which are another target tissue for teratogenic compounds. However, when the validated EST was challenged with various embryotoxicants, the test demonstrated a high rate of underprediction. The applicability domain of the test requires careful definition. The inclusion in the project of the validated EST was performed in order to extend the existing chemicals database by testing another 13 well-defined substances and consequently, better define the applicability domain for the assay. Due to the misclassification of the tested compounds by the EST, it was decided to organise a joint workshop between ECVAM and ReProTect in order to bring together European scientists from industry and academia actively working with the EST, and to discuss the present status of the various derivates of the validated embryonic stem cell test. The aim was to identify how much flexibility could be introduced for *in vitro* tests based on embryonic stem cells. The workshop also aimed to critically review the mode of action in order to better describe the applicability domain of the EST (Marx-Stoelting *et al.*, 2009).

  In order to identify toxicological signatures of developmental toxicants at proteome level, lysates of murine embryonic stem cells differentiated into cardiomyocytes (treated with valproic acid, diphenylhydantoin and lithium chloride) were analysed for changes in their proteome. Protein biomarker signatures from mESC (mouse embryonic stem cells)

differentiated into cardiomyocytes, and hESC differentiated into neurons, are now available, and the results will be published soon.

- **Late prenatal development**
  The objective of this work is the introduction of a metabolic incubation system into the validated WEC (Whole Embryo Culture). The inclusion of a metabolic competent system into WEC was concluded by testing few chemicals under the developed SOP.

Research Area "Cross-Cutting technologies":

- **Quantitative Structure/Activity Relationships (QSARs)**
  The development of QSARs focused on the blood testis barrier, thus complementing and supporting the building blocks: Leydig cells, Sertoli cells and spermatogenesis. In addition, the development of QSAR models for placental transfer and steroid receptor binding/transactivation was planned. The planned work for the QSARs activities in the project was concluded. QSARs model for metabolic activation, endocrine disruptor and placental barrier are now available within the project.

- **Biotransformation**
  In the ReProTect project, it was planned to study the possibility to define a metabolic competent system that can work in conjunction with *in vitro* tests developed/optimised in ReProTect. The tasks related to this topic focused on the development of a system based on the use of hepatocytes able to work in conjunction with the validated embryonic stem cell test. The SOP is now ready, but only two compounds were tested. In order to evaluate the robustness of the developed protocol, more experiments should be performed.

- **Estrogen/Androgen receptor binding assays, which could be used as building blocks to replace the uterotrophic test and the Hershberger test (OECD draft TG) and one/two generation studies (OECD TGs 415 and 416)**
  ECVAM has followed the recommendations of the ICCVAM/NICEATM report on the status of *in vitro* methods for detecting endocrine disrupting functions of chemicals (ICCVAM-NICEATM, 2002). Four transcriptional tests (PALM, MELN, ER-CALUX®, AR-CALUX®) and two receptor binding assays based on recombinant proteins were selected by the ECVAM Task Force on Endocrine Disruption. The optimisation of the tests mentioned above, was finalised and the tests are currently analysed by an independent biostatistician. The data analysis will decide if a formal validation study should be initiated. However, the MELN, as well as the ER-CALUX system, are both already mentioned in the OECD performance-based guideline for the testing of chemicals: "Stably transfected Transcriptional Activation (TA) assay for the detection of estrogenic (agonist and antagonist) activity of chemicals", which is delivered to the WNT as background document. ReProTect has successfully delivered the MELN test which is able to detect estrogenic and anti-estrogenic compounds. The test has been submitted to ECVAM for validation.
  Due to the good performance of the Pan Vera receptor binding test, optimised within the ReProTect project, a formal validation exercise under the lead of the US EPA, has started. A Standard Project Submission Form on Human Recombinant Estrogen Receptor Alpha Binding Assays was submitted to the OECD in September 2007. ECVAM has funded a participating European laboratory and is currently working out the data transfer to the US EPA.

  ECVAM is participating with its own laboratories in the LUMICELL validation study. LUMICELL is able to detect (anti)-estrogenic compounds. The validation study is led by ICCVAM, and JACVAM is also participating. The experimental phase is now finalised, the results are being analysed and a peer review is in preparation

Another *in vitro* test assessing estrogenic activity, the stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals (STTA), has been adopted by OECD Council on 7 September 2009 and is now available as OECD TG 455. ECVAM has been in the peer review panel of the validation study.

2.5.2 Other activities

Due to a high number of animals needed for assessing reproductive toxicity only for the implementation of REACH, ECVAM also held a workshop in September 2006, on possible refinement methods for reproductive toxicity, which could significantly reduce the number of animals used. The most promising approach identified at the workshop was the extended one-generation study proposed by ILSI-HESI in order to evaluate agrochemicals (Cooper *et al.* 2006). The workshop analysed the study design of the extended F1 generation study for its suitability to assess also industrial chemicals. It was felt that with the introduction of a trigger concept this reduction/refinement method will significantly contribute to the reduction of animals for reproductive toxicity safety assessments, since the added value of the second generation seems to be very limited. Nevertheless the discussion is still ongoing. In addition, the relevance of developmental toxicity in a second species was questioned and would require further investments into retrospective validation exercises.

ECVAM organised a workshop on cardiotoxicity in order to discuss possibilities to reduce the number of animals for cardiotoxicity assessment, but also to increase the predictive capacity of existing methods. The workshop report was published in September 2009 (Stummann *et al*., 2009).The experts came to the conclusion that alternative methods assessing cardiomyopathy, i.e. contractility toxicity, ischaemia toxicity, secondary cardiotoxicity and valve toxicity are at a nascent stage. Alternative tests in the field of drug-induced arrhythmia are relatively advanced, but are mainly performed in addition to *in vivo* studies with the aim to improve drug safety and are focusing strongly on hERG blockage or QT prolongations. Prediction of proarrhythmic potential in humans could probably be improved by including alternative assays (*in silico* or *in vitro*) covering additional mechanisms of the arrhythmic toxicities.

3. NON-TESTING METHODS

Non-testing methods include qualitative and quantitative structure-activity relationship models (SARs and QSARs; collectively referred to as (Q)SARs), as well as less formalised chemical grouping methods that form the basis of chemical category and read-across approaches. All of these methods are based on the premise that the properties (including toxicological activities) of a chemical can be directly predicted from its molecular structure or inferred from the properties of similar compounds whose activities are known. The concepts and applications of non-testing methods are explained elsewhere (Worth, 2010). Background information on JRC activities on non-testing methods (computational toxicology) can be found on the JRC-IHCP website (http://ecb.jrc.ec.europa.eu/qsar).

Non-testing methods have been found useful to help to make predictions of human health endpoints, including the endpoints covered by the requirements of the Cosmetics Directive. Under certain circumstances, the information provided by a non-testing method can be considered sufficient to avoid the need for animal testing; in other cases, the information can supplement other available data in weight-of-evidence assessments of chemical hazard and risk.

During 2008-2009, JRC activities on non-testing methods have focussed on: a) the development of technical guidance documents, and publication of state-of-the-art reviews; b) the development,

assessment and dissemination of software tools; c) developing and promoting Integrated Testing Strategies (ITS).

Since 2009, ECVAM's innovation pillar also includes these activities of the JRC and will in particular focus on integrating its in vitro, non-testing and omics activities with modelling and statistical approaches for ITS development and, eventually, their validation.

3.1 DEVELOPMENT OF GUIDANCE ON THE USE OF NON-TESTING METHODS

Guidance on the regulatory use of (Q)SARs and grouping approaches (use of chemical categories and read-across), developed mainly for the purposes of REACH, is given in ECHA (2008). The drafting of this guidance was led by the JRC, and eventually adopted by the competent authorities, the Commission and ECHA. As background to this guidance, a series of case studies has also been published (Worth & Patlewicz, 2007). More specific guidance on the use of non-testing methods in ecotoxicity assessment (setting of environmental quality standards) has also been published (Crane *et al*, 2008).

According to the REACH guidance (ECHA, 2008), it is possible to use data from (Q)SAR models instead of experimental data if each of four main conditions is fulfilled: i) the model used is shown to be scientifically valid; ii) the model used is applicable to the chemical of interest; iii) the prediction (result) is relevant for the regulatory purpose; and iv) appropriate documentation on the method and result is given.

To help the registrants of chemicals provide appropriate documentation on (Q)SARs, several QSAR Reporting Formats have been developed by the JRC in consultation with experts nominated by Member State authorities and the OECD. This includes a format to document the characteristics and validation on the model, the QSAR Model Reporting Format (QMRF), and a format to document the generation and interpretation of the prediction, the QSAR Prediction Reporting Format (QPRF). To evaluate the reliability of an individual prediction, important aspects of QPRF are transparency in the documentation of the training set, the chemical space it represents, and the location of chemical of interest within that space. Guidance on how to demonstrate the validity of a (Q)SAR model is provided in the ECHA (2008). Further guidance on how to document models in the form of QMRFs and their predictions in the form of QPRFs is given on the JRC website (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools).

The two main approaches for grouping chemicals are the "analogue approach" and the "category approach". The "analogue approach" is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent. As the number of chemicals being grouped increases, the potential for developing hypotheses and making generalisations about the trends also increases. The "category approach" is used when the grouping is based on a more extensive range of analogues and more endpoints, such that trends across properties can be detected. The term read-across is used to describe a method for filling data gaps in either the analogue or category approaches.

Grouping approaches have been applied to industrial chemicals, but in comparison, these approaches have been less widely used in the regulatory assessment of cosmetic ingredients. It is therefore concluded that read-across could be used more extensively to support the hazard assessment of cosmetic ingredients.

In addition to guidance documentation, the JRC has also written reviews on the state-of-the-art of existing models for various endpoints, including the following human health effects which are relevant to the assessment of cosmetics: a) skin and eye irritation/corrosion (Gallegos Saliner *et al.*, 2008); b) skin sensitisation (Alenius *et al.*, 2008; Patlewicz & Worth, 2008; Patlewicz *et al.*, 2008a, 2008b;

Roberts & Patlewicz, 2009); c) mutagenicity and carcinogenicity (Benigni *et al.*, 2008a, 2008b); d) mammalian toxicity (Tsakovska *et al.*, 2008); and e) reproductive toxicity (Cronin & Worth, 2008).

3.2 IMPLEMENTATION OF NON-TESTING METHODS VIA PUBLICLY ACCESSIBLE SOFTWARE

To promote the acceptance of non-testing methods, the JRC has been developing and making freely available user-friendly software that provides a means of applying grouping approaches as well as (Q)SARs for various endpoints. An overview of these software tools is given in Pavan & Worth (2008). These software tools are accessible via the JRC website (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools).

The Toxtree software implements (Q)SAR models and decision tree approaches for hazard estimation and mode-of-action evaluation. The current version (July 2009) implements: a) the Cramer rules for applying the Threshold of Toxicological Concern (TTC) concept (Patlewicz *et al.*, 2008c) and an extended Cramer scheme; b) the Verhaar rules for aquatic modes of action; and c) the BfR rules for predicting the presence and absence of skin irritation and corrosion potential (Gallegos Saliner *et al.*, 2007); d) the BfR rules for predicting the presence and absence of eye irritation and corrosion potential (Tsakovska *et al.*, 2007); e) the Benigni-Bossa rulebase for predicting mutagenicity and carcinogenicity (Benigni *et al.*, 2008a); and f) the ToxMic rulebase, which makes predictions of mutagenicity and carcinogenicity based on *in vivo* micronucleus assay data.

To provide a tool for comparing and grouping chemicals, the Toxmatch software has been developed (Gallegos Saliner *et al.*, 2008a; Patlewicz *et al.*, 2008d). Toxmatch applies methods of chemical similarity analysis to support the formation of chemical categories as well as the application of read-across. The use of Toxmatch to apply read-across in the assessment of developmental toxicity has been demonstrated by Enoch *et al.* (2009).

The Decision Analysis by Ranking Techniques (DART) tool encodes a number of partial order and total order methods for the ranking of chemicals (Pavan & Todeschini, 2008a, 2008b, 2008c). These methods can be used to group and sort chemicals according to their relative levels of concern, or to group chemicals according to different toxicological profiles. The ranking can be based on molecular descriptors and/or experimental (including *in vitro*) properties. The methods can be used not only to prioritise chemicals for further testing, but also to group chemicals (Pavan & Worth, 2008a).

To provide a reliable resource of QMRFs, the JRC is also developing a QSAR Model Database (QMDB) which is an inventory of robust summaries of (Q)SARs that can be searched, for example, by endpoint or by chemical (sub)structure. This database is accessible at: http://qsardb.jrc.it/qmrf/

3.3 DEVELOPMENT AND PROMOTION OF INTEGRATED TESTING STRATEGIES

The concepts of integrated testing are explained in a number of papers (e.g. van Leeuwen *et al.*, 2007). The JRC has carried out detailed reviews of the state-of-the-art in Integrated Testing Strategies (ITS) for various endpoints, including skin and eye irritation/corrosion (Gallegos Saliner & Worth, 2007) and skin sensitisation (Patlewicz & Worth, 2008).

In the REACH guidance on chemical safety assessment (ECHA, 2008), ITS have been developed for all REACH endpoints. Since these strategies are generally described at a general level, considerable research is still needed to develop endpoint-specific strategies in more detail, and to assess their impacts, including their benefits (e.g. reduced animal testing) and potential costs (e.g. consequence of incorrect predictions). Some of this work is being carried out within the framework of the OSIRIS Integrated Project (http://www.osiris-reach.eu), in which the JRC-IHCP is a partner.

As an illustration of research needed to further develop endpoint-specific ITS, the JRC has developed ITS for skin irritation, as a means of exploring the applicability of novel concepts in the design and evaluation of ITS. The work is further described in Hoffmann *et al.* (2008).

The use of non-testing methods within ITS implies the need for computational tools and a structured workflow to facilitate their application. A generic workflow for the implementation of non-testing methods has been developed (Bassan & Worth, 2008).

A major outstanding issue is the practical question of how to validate ITS. Some preliminary ideas have been developed by ECVAM in collaboration with the EPAA (Kinsner-Ovaskainen *et al*, 2009a), but this is a question which will be further considered and discussed. A second ECVAM-EPAA workshop on ITS took place on 12-13 October 2009, with the aims to: 1) discuss to which extent the existing validation principles are applicable to the validation of testing strategies (based on selected case studies); and 2) develop a draft approach for validation of ITS and apply it to the selected case studies. A paper is currently in preparation.

Based on the discussion held, the participants agreed that there is an obvious difference between the ITS seen as information gathering process, and testing strategies consisting of alternative methods, combined in a predefined way and designed as a replacement of an existing animal test. It was generally agreed that only the latter should be formally validated. Also, distinction has to be made between the validation of single methods that are used as building blocks of a testing strategy and the validation of a testing strategy as a whole.

3.3.1 Validation of building blocks

When assessing the single building block of a testing strategy the following need to be considered:

a. The test needs to be reliable and, at least, biologically relevant.
b. The reliable building block will be integrated via a testing strategy.
c. In some cases, the assessment of predictive capacity of the single building block may not be necessary, as long as the predictive capacity of the whole testing strategy is demonstrated.

The evaluation of an individual test method to qualify it as a building block would involve at least the first 4 modules of validation: Test definition; within-laboratory reproducibility; transferability; between-laboratory reproducibility.

In addition:

d. The biological relevance of the parameter of interest would need to be established.
e. The chemical selection fits the biological relevance.
f. The ability to measure the parameter of interest (not the human health effect) would have to be assessed.

Test method developers should be encouraged to develop and submit to ECVAM not only tests designed as a full replacement of an animal method, but also tests which could be used as part of a testing strategy (building blocks).

3.3.2 Validation of ITS as a whole

With regard to the validation of a testing strategy it was concluded that:

An Integrated Testing Strategy seen as information gathering process (understood also as a weight-of-evidence approach) cannot be validated.
A testing strategy consisting of test methods that are combined in a predefined way and designed for replacement of an animal test used for regulatory purposes can be validated, but a detailed list of principles to be applied in the construction and assessment of testing strategies should be developed.

In order to successfully validate a testing strategy:

a. Industry needs to share information on how a given testing strategy is foreseen to be used in safety assessment,

b. ECVAM needs to share with industry and regulators the plans for validation of the testing strategy.

c. Regulators should be involved in these discussions.

4. INTERNATIONAL COLLABORATIONS

ECVAM continues to work closely with its main international partners, such as ICCVAM (USA) and JaCVAM (Japan). Relationships with Health Canada were strengthened in the framework of the International Cooperation on Alternative Test Methods (ICATM).
In April 2009 representatives from the validation bodies signed a Memorandum of Cooperation promoting enhanced international cooperation and coordination on the scientific validation of non- and reduced-animal toxicity testing methods.
ECVAM liaisons are regularly participating in the different ICCVAM working groups and ICCVAM liaisons are attending the validation management group meetings of ECVAM-coordinated validation studies. The more recent validation studies are usually led by one of the validation bodies, and set up in a way to share, and not duplicate, the work amongst all participating validation bodies.

ECVAM is also closely working with the OECD by either taking the lead for the drafting of new or revised OECD Test Guidelines, by participating in OECD expert groups or by coordinating OECD validation studies. ECVAM staff is also regularly sent on secondment to the OECD.
Finally, ECVAM representatives are actively supporting the various platforms of the European Partnership on Alternative Approaches to Animal Testing (EPAA).

5. CONCLUSIONS

The political and regulatory environment considerably changed over the last seven years in relation to the development, validation and legal acceptance of alternative methods. Beside being an ethical desire, the need for alternative methods has also become a legal necessity, as stipulated in the 7th Amendment of the Cosmetics Directive and REACH.

Most of the endpoints falling under the 2009 deadline are covered with validated alternative methods. Those human health effects which are not yet completely covered in the sense of a complete replacement of the animal test(s) are acute toxicity, eye irritation and genotoxicity.

Within the integrated project A-Cute-Tox, several promising in vitro methods were evaluated and combined in *in-vitro* testing strategies in view to completely replace *in vivo* testing for acute toxicity testing of chemicals. The strategies are currently under evaluation and the final results will become available in June 2010. The initial results of the ECVAM validation study on the NRU cytotoxicity test to identify non-toxic substances, which are most relevant for use in cosmetics, showed that the test method has a high negative predictive value of 90%, but also a high rate of false positives (65%).

For genotoxicity and mutagenicity, *in vitro* methods are in legislation. However, in the current test strategy for genotoxicity, positive results obtained from these methods need confirmation by animal testing. The negative results on the other hand, are used to classify the substance as not genotoxic. In this field, the ECVAM activities are based on the improvement of the test strategy in order to reduce the number of false positives resulting from *in vitro* tests and thus to avoid subsequent animal testing. If animal experiments can no longer be carried out from 2009 onwards to confirm the positive *in vitro* results, an economical problem emerges for industry, as too many active cosmetic ingredients will be discarded without need. Several approaches, which are currently followed, look promising for making solutions available within 2010-2011.

In the field of eye irritation, the evaluation of the individual tests by retrospective and prospective validation studies will allow determining the most suitable strategies that utilise the strengths of specific *in vitro* assays for the classification of test chemicals according to their irritation potential. The bottom-up or top-down approaches described above identify chemicals not classified as irritant as well as ocular corrosives/severe eye irritants, respectively. A default mild/moderate irritant classification (GHS Cat. 2 or EU R36) may be acceptable for the remaining chemicals, if the overall accuracy of a test method is still relatively high at each tier of the approaches (i.e. generating not too many false postives in the first tier of a bottom-up approach and not too many false negatives in the first tier of a top-down approach), or if most of the chemicals that are not classified as irritant or are ocular corrosives/severe eye irritants are correctly identified using a combination of multiple tests in each tier. Otherwise the development and validation of *in vitro* methods capable of detecting mild/moderate irritants (GHS Cat. 2 or EU R36) will be necessary, for inclusion as a last tier in both the top-down and bottom-up approaches.

For the complex endpoints, several research initiatives were launched over the last 5 years to make new approaches available for validation.
These projects included the development and optimisation of alternative methods in the fields of reproductive toxicology (Re-Pro-Tect; 2004-2009), acute toxicity (A-cute-Tox; 2004-2009), skin and respiratory sensitisation (Sens-it-iv; 2005-2010), carcinogenicity (Carcinogenomics; 2006-2011) and chronic toxicity (Predict-i.v.; 2008-2013) and more recently the COLIPA-DG RTD Joint Research Inititiative, which resulted into a new FP7 call in 2009, focusing on repeated dose toxicity.

Those research projects which will soon come to an end will deliver some promising *in vitro* methods which may enter the ECVAM validation process in the near future and new approaches to combine the different tests in the most optimal way are needed for these endpoints. Although a considerable reduction in the number of animals used has already been achieved by using alternative methods, full replacement of the animal test(s) by 2013 remains of a problem, despite the huge amount of work and efforts which are currently invested in many different aspects of research, development, optimisation and validation of alternative methods and testing strategies.
A more positive attitude towards the acceptance of stringently-validated alternative methods at the regulatory level would also be most beneficial.

Finally, the substitution of animal safety tests with highly modern animal-free technology which ensures the same level of protection of the population, should remain a common international goal, driven by strong ethical considerations.

ACRONYMS

A.704	Human, kidney, adenocarcinoma
AR-CALUX®	Human osteoblastic osteosarcoma U2 OS cell line transfected with the human androgen receptor
BALB/c 3T3	Mouse Embryonic Fibroblasts
BBB	Blood-Brain Barrier
BCOP	Bovine Corneal Opacity and Permeability
Caco-2	Human colonic adenocarcinoma cell line
CFU-GM	Colony Forming Unit-Granulocyte Macrophage
CM	Cytosensor Microphysiometer
COLIPA	European Cosmetic, Toiletry and Perfumery Association
CRO	Contract Research Organisation
CsA	Cyclosporine A
CTA	Cell Transformation Assay
CYP	Cytochrome P450
DART	Decision Analysis by Ranking Techniques
DNA	Deoxyribonucleic Acid
DPRA	Direct Peptide Reactivity Assay
EC	European Commission
ECB	European Chemical Bureau
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EMT	Epithelial Mesenchymal Transition
EPA	Environmental Protection Agency
EPAA	European Partnership on Alternative Approaches to animal testing
ER-CALUX®	Human breast cancer cell line T47D cells, expressing endogenous estrogen receptors
ESAC	ECVAM Scientific Advisory Committee
EST	Embryonic Stem cell Test
EU	European Union
FL	Fluorescein Leakage
GFAP	Glial fibrillar acidic protein
GHS	Globally Harmonised Classification System
GJIC	Gap Junction Intercellular Communication

HCE	Human Corneal Epithelium
h-CLAT	human Cell Line Activation Test
HepaRG	Human hepatoma cells
HepG2 cell/protein	Human liver cell line (hepatocellular cacinoma)
hESC	Human Embryonic Stem Cells
HET-CAM	Hen's Egg Test on the Chorioallantoic Membrane
HIA	Human intestinal absorption
HK-2	Human Kidney-2 cell line
HSP32	Heat shock protein 32
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	Isolated Chicken Eye
IFN-γ	Interferon- Gamma
IL-1	Interleukin-1
IL-6	Interleukin-6
IRE	Isolated Rabbit Eye
ITS	Integrated Testing Strategies
JACVAM	Japanese Centre for the Validation of Alternative Methods
JRC	Joint Research Centre
L929	Mouse fibroblast cell line
LD_{50}	Lethal Dose killing 50% of the animals
LDH	Lactate dehydrogenase
LLNA	Local Lymph Node Assay
LVET	Low Volume Eye Test
MELN	MCF-7 human breast cancer cells (ERα+) transfected with the estrogen responsive gene ERE-ß Glob-Luc-SVNeo
MBP	Myelin basic protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUSST	Myeloid U939 Skin Sensitisation Test
NC3Rs	National Centre for the 3Rs (replacement, refinement, reduction)
NF-H	Neurofilament- high
NHEK	Normal Human Epidermal Keratinocytes
NICEATM	The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
Nrf2	Nuclear factor-erythroid 2
NRR	Neutral Red Release

NRU	Neutral Red Uptake
NRU PT	Neutral Red Uptake Phototoxicity Test
OECD	Organisation for Economic Cooperation and Development
PALM	Human prostate adenocarcinoma cell line Androgen Luciferase MMTV
PBMC	human Peripheral Blood Mononuclear Cells
PLS	Partial Least Square analysis
PS	Performance Standards
QMDB	QSAR Model Database
QMRF	QSAR Model Reporting Format
QPRF	QSAR Prediction Reporting Format
(Q)SAR	(Quantitative) Structure-Activity Relationship
RBC	Red Blood Cell
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
RhE	Reconstructed Human Epidermis
RhT	Reconstructed Human Tissue
ROS	Reactive Oxygen Species
SerW3	Sertoli cell line
SHE	Syrian Hamster Embryonic cell line
SHSY5Y	Human neuroblastoma cells
SMEs	Small and Medium-sized Enterprises
SOP	Standard Operating Procedure
STREP	Specific Targeted Research Project
TNF-α	Tumor Necrosis Factor alpha
TG	Test Guideline
TTC	Treshold of Toxicological Concern
WEC	Whole Embryo Culture

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European Commission

EUR 24413 EN– Joint Research Centre – Institute for Health and Consumer Protection
Title: ECVAM Technical Report on the Status of Alternative Methods for Cosmetics Testing (2008-2009). A report prepared in the framework of Directive 2003/15/EC (7th Amendment to the Cosmetics Directive)
Author(s): Valérie Zuang, João Barroso, Susanne Bremer, Silvia Casati, Mara Ceridono, Sandra Coecke, Raffaella Corvi, Chantra Eskes, Agnieszka Kinsner, Cristian Pellizzer, Pilar Prieto, Andrew Worth and Joachim Kreysa
Luxembourg: Publications Office of the European Union
2010–52 pp. – 21 x 29,5 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-16021-9
DOI 10.2788/99101

Abstract
The ECVAM technical report presents the progress made in the development and validation of alternative methods for the human health effects relevant to the Cosmetics Directive. It provides an update on the activities described by ECVAM in 2005, 2006 and 2007.

The report intends to present the latest scientific and technical developments in the field during 2008-2009. As required by Directive 2003/15/EC, the seventh amendment to Directive 76/768/EEC, developments in refinement and reduction methods are also described (EU, 2003).

Most successes in the development of alternative methods are in acute local toxicity and short-term testing, such as e.g. skin and eye irritation/corrosion, phototoxicity and skin penetration The test methods consuming a high number of animals, however, are in long-term testing and systemic toxicity, such as e.g. reproductive toxicity and repeated dose toxicity. In these complex fields, several research initiatives are ongoing. However full replacement approaches are still lacking.

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EUROPEAN COMMISSION

LB-NA-24413-EN-C

Publications Office

ISBN 978-92-79-16021-9