4. MATERIALS AND METHODS

4.1. MATERIAL

4.1.1. CELL LINES AND RECONSTRUCTED HUMAN SKIN MODELS

4.1.1.1. Mouse fibroblast cell line Balb/c 3T3

A permanent mouse fibroblast cell line Balb/c 3T3, clone 31, from the American Type Culture Collection (ATCC) was used in the study. Basic characteristics and culture conditions are given in Table 4. Because the cells were used in the phototoxicity studies, it was necessary to evaluate their sensitivity to UV light regularly according to the quality control procedure described in paragraph 4.2.4.1.

Since the cell sensitivity to UV light may increase with the number of passages, cells of the passage number less than 90 were used in experiments. The cell line meets assay acceptance criteria if viability of irradiated cells (UVA dose of 5J/cm^2), is at least 80 % viability of non-irradiated controls. At 9 J/cm^2 (UVA) the reduction of cell viability should not exceed 50% (OECD TG 432).

Table 4. Basic characteristics of the cell line and culture conditions.

Cell type	
ATCC Balb/c 3T3, Clone 31	Cell line used in the EU/COLIPA validation study and ECVAM "Special Study on UV filters“ (Spielmann *et al*., 1998a; Spielmann *et al*., 1998b)
Culture conditions:	Sterile (incubator and sterile flow laminar hood required), 37°C; 7,5 % CO_2; 95 % humidity
Culture Medium for routine culture:	Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine, supplied with: 10 % NBCS (New Born Calf Serum) 4 mM Glutamine 100 IU Penicillin 100 µg/mL Streptomycin
Culture Medium for freezing	Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine, supplemented with: 20 % NBCS (New Born Calf Serum) 7 - 10 % DMSO (Dimethyl Sulfoxide)
Trypsinisation:	Trypsine/EDTA solution (0.05 %) Phosphate buffered saline (PBS) without Ca^{++} and Mg^{++}

Complete culture media were kept at 4° C and stored for no longer than two weeks.

4.1.1.2. Reconstructed human epidermal model EpiDerm

The reconstructed human skin model EpiDerm (EPI-200) is produced by MatTek Corporation (Ashland, MA, USA) and consist of normal human epidermal keratinocytes cultured to form a multi-layer, highly differentiated model of the human epidermis *in vitro* (Canon *et al*., 1994). The model consists of organised basal, spinous, granular and cornified layers analogous to those found *in vivo*.

The EpiDerm model is available in several sizes and modifications. The tissues are cultured 10 days on specially prepared cell culture inserts and provided as kits containing 24 skin models. The inserts are during the shipment embedded in an agarose gel containing nutrient solution and the whole system is cooled by 2-3 ice packs at temperature approximately 4-6 °C. The manufacturer also provides DMEM-based culture medium and MTT assay components (MTT concentrate, MTT diluent, PBS, isopropanol, 24 well plates and MTT protocol).

Table 5. Basic characteristics of the reconstructed human epidermal model EpiDerm.

Specification		**Remark**
Insert type	plastic insert with chemically modified, collagen-coated membrane	
Tissue surface: EPI-200	0.63 cm^2	used in skin irritation, skin corrosion, phototoxicity, barrier studies and histology
EPI-606	4 cm^2	used for confocal laser microscopy, EPI-606 is larger alternative of EPI-200.
Biological origin of cells	foreskin or abdomen	single donor (neonatal or adult)
Typical histology	9-12 cell layers (Boelsma *et al*., 2000)	

The manufacturer controls each epidermal batch. Tissues as well as culture media are tested for viral, bacterial, fungal and mycoplasma contamination. The quality acceptance criteria are met, if the optical density (OD) of formazan produced in the MTT assay (measured at 570 nm) is higher than 1.0. In addition, tissue barrier is evaluated in the time course assay by determination of the ET50 value (time required to reduce tissue viability to 50% after application of cytotoxic chemical). The assay is performed with Triton X-100 (1%) and the ET50 value must fall inside of the historically established range.

4.1.1.3. Reconstructed human epidermal model EPISKIN

The EPISKIN^{SM} (Standard Model) is produced by EPISKIN-SNC (Lyon, France). Each epidermal unit is an organotypic culture made of adult human keratinocytes which become stratified epidermis after a 13-days culture period (Tinois *et al*., 1991; Cotovio *et al*. 2005).

The epidermis model is prepared by seeding adult human keratinocytes onto a collagen (type I) matrix filmed with type IV collagen (as a dermis surrogate), previously fixed in a polycarbonate insert (Tionis *et al*., 1991). These inserts are specifically designed to keep the epidermis in a fixed position in the culture wells. This allows for gas exchanges and easy access to culture media during experiments. Epithelial differentiation is achieved by an air-emersion step that leads to a fully differentiated three-dimensional epidermis with a stratum corneum (Tinois *et al*, 1991). The inserts (containing the EPISKIN model) are embedded in an agarose gel containing nutrient solution and shipped in multi-well plates at room temperate. Assay medium is supplied with the model in sufficient amount and is cooled with gel refrigerant during the shipment.

Table 6. Basic characteristics of the reconstructed human epidermal model EPISKIN

Specification		**Remark**
Insert type	culture insert is manufactured from resistant plastic material. Collagen I sheet serves as a supportive membrane	tailor made for EPISKIN
Tissue surface:	1.07 cm^2	used in barrier studies, histology studies and for confocal laser microscopy
Biological origin of cells	abdomen of adult donors	several donors
Typical histology	8-10 cell layers (Boelsma *et al*., 2000)	

Quality control of tissue batches performed by EPISKIN-SNC includes histology analysis (H-E staining) and evaluation of the differentiation stage of the model, evaluation of tissue viability (using MTT assay) and IC50 assay (determination of the concentration, which causes the tissue viability decrease to 50 %) performed with sodium dodecyl sulphate. For each batch the manufacturer provides a conformity certificate. The model is manufactured according to ISO 9001 standards (Cotovio *et al*., 2005).

4.1.1.4. Reconstructed human epidermal model SkinEthic

The SkinEthic reconstituted human epidermal model is produced by SkinEthic Laboratories (Nice, France) and consists of normal human epidermal keratinocytes cultured to form a multilayer, differentiated model of the human epidermis *in vitro.*

The model is produced in several sizes in a serum-free, chemically defined medium (Rosdy and Clauss, 1990; Rosdy *et al*., 1994). The culture inserts containing the skin models are embedded in an agarose gel containing nutrient solution and shipped in multi-well plates at room temperature. Assay medium is supplied with the model in a sufficient amount and is cooled with gel refrigerant (below 10 °C) during the shipment. All epidermal models used in later described studies were fully differentiated epidermal cultures grown on the air-liquid interface for 17 days.

Table 7. Basic characteristics of the reconstructed human epidermal model SkinEthic

Specification		**Remark**
Insert type	plastic insert with polycarbonate membrane	
Tissues surface	0.63 cm^2 *	Model used in skin irritation and skin corrosion studies
	0.5 cm^2	Model used in barrier studies and histology
	4 cm^2	used for confocal laser microscopy
Biological origin of cells	foreskin or abdomen	Single donor (neonatal or adult)
Typical histology	5-7 cell layers (Boelsma *et al*., 2000)	

** Production of model size 0,63 cm^2 was terminated in the middle of 2005*

The manufacturer controls each production batch. Information on histology (hematoxylin-eosin staining) and viability of tissues (determined in MTT-test) is provided as standard quality control. The tissue batch meets acceptance criteria, if the optical density (OD) of formazan produced in the MTT assay is higher than 0.8 (measured at 570 nm) and histology sections show a minimum of 4 healthy viable cell-layers under the stratum corneum.

Tissues as well as culture media are tested for viral, bacterial, fungal and mycoplasma contamination. Recently, determination of the ET50 value after exposure to Triton X-100 (1%) became part of the quality control.

4.1.1.5. Reconstructed human epidermal model EST - 1000

The EST-1000 reconstituted human epidermal model was introduced on the market in 2004 by Cell Systems, GmbH, St. Katharinen, Germany. The model consist of normal, human epidermal keratinocytes cultured to form a multi-layer, highly differentiated model of the human epidermis *in vitro* (Hoffmann *et al*., 2005).

The EST-1000 model consists of organised basal, spinous, granular and cornified layers analogous to those found *in vivo*. The tissues (surface 0,63 cm^2) are cultured in transwell culture inserts and shipped as kits. Culture medium and multiwell plates are provided with the model. The epidermal models used in the Skin corrosion study were fully differentiated three-dimensional reconstituted human epidermal cultures grown on the air-liquid interface.

Table 8. Basic characteristics of the reconstructed human epidermal model EST-1000.

Specification		**Remark**
Insert type	polycarbonate, polycarbonate membrane	
Tissue surface:	0.63 cm^2	Used in the skin corrosion study
Biological origin of cells	foreskin or abdomen	(neonatal or adult)
Typical histology	Not yet sufficiently published	

The inserts containing the EST-1000 model are embedded in an agarose gel containing nutrient solution and shipped to customers in multi-well plates. While tissues are shipped at room temperature, assay medium is cooled with gel refrigerant during the shipment.

The manufacturer controls quality of each epidermal batch. Information on histology (hematoxylin-eosin staining) and viability of tissues (MTT-test) is provided for each batch as standard quality control. Tissues as well as culture media are tested for viral, bacterial, fungal and mycoplasma contamination.

4.1.2. TEST CHEMICALS

4.1.2.1. Skin corrosion studies

Skin corrosion study using the reconstructed human skin model SkinEthic

As described in the paragraph 3.2.1, the skin corrosion study using the reconstructed human skin model SkinEthic was divided into the 2 phases.

The main objective of phase I was to evaluate performance of the SkinEthic model (correct prediction, inter-tissue variability) as well as the applicability of the assay to a wide range of chemical classes before considering any further testing. Selected chemicals (twenty non-coded chemicals from the ECVAM skin corrosion validation studies (Fentem *et* al., 1998, Liebsch *et* al., 2000) had to challenge the test protocol and thus allow assessment of tissue quality with regard to barrier function. For the specification of these chemicals see Table 9.

Table 9. Chemicals tested in Phase I

No.	Chemical name	CAS No:	In vivo Class	Solid, liquid (S/L)	Supplier	Chemical class	Interaction with MTT (+ / -)
1	SDS (20 % aq)	151-21-3	NC	L	Aldrich	Surfactants/soaps	-
2	Boron trifluoride dihydrate	13319-75-0	C	L	Aldrich	Inorganic acids	+
3	Benzylacetone	2550-26-7	NC	L	Aldrich	Neutral organics	-
4	H_2SO_4 (10 % wt.)	7664-93-9	C*	L	Aldrich	Inorganic acids	-
5	HCl (14,4 % wt.)	7647-01-0	C	L	Aldrich	Inorganic acids	-
6	n-Heptylamine	111-68-2	C	L	Aldrich	Organic bases	+
7	2,4-Xylidine	95-68-1	NC	L	Aldrich	Organic bases	-
8	Tetrachloroethylene	127-18-4	NC	L	Aldrich	Neutral organics	-
9	Octanoic acid (caprylic acid)	124-07-02	C	L	Aldrich	Organic acids	-
10	55/45 Octanoic/decanoic acid	68937-75-7	C	L	Unichema	Organic acids	-
11	4-Amino-1,2,4-triazole	584-13-4	NC	S	Aldrich	Organic bases	-
12	2-tert-Buthylphenol	88-18-6	C	L	Aldrich	Phenols	+
13	1,2- Diaminopropane	78-90-0	C	L	Aldrich	Organic bases	+
14	Phenethyl bromide	103-63-9	NC	L	Aldrich	Electrophiles	+
15	4-(Methylthio)-benzaldehyde	3446-89-7	NC	L	Aldrich	Electrophiles	+
16	Potassium hydroxide 10% aq.	1310-58-3	C	L	Aldrich	Inorganic bases	-
17	Isostearic acid	30399-84-9	NC	L	Unichema	Organic acids	-
18	Acrylic acid	79-10-7	C	L	Acros	Organic acid	-
19	1,6-dibromohexane	97-53-0	NC	L	Aldrich	Brominated	+
20	8N KOH	1310-58-3	C	L	Aldrich	Inorganic bases	+

C - corrosive; NC - non corrosive; S - solid; L - liquid
C - The classifications given in OECD Test Guideline 431 and in paper published by Fentem et al. (2001) are not in agreement with the classification according to 67/548/EEC Annex I*

The aim of Phase II was to evaluate the test performance of the SkinEthic skin corrosivity test with regard to its predictivity and interlaboratory reproducibility. For that purpose we followed recommendations of the OECD TG 431 which requires correct prediction of the 12 reference substances listed in Table 10.

Table 10. OECD reference chemicals - Phase II

No	Chemical name	CAS No:	In vivo Class (C/NC)	Remarks on data supporting classification / general comments
1.	1,2-Diaminopropane	78-90-0	C*	Interaction with MTT was observed.
2.	Acrylic Acid	79-10-7	C*	Published data with EpiDerm or EPISKIN model missing (corrosive on EpiDerm after 3 min at ZEBET - unpublished experiment).
3.	2-tert-Buthylphenol	88-18-6	C	Borderline C/NC chemical, as judged from the proximity of the chemical to the classification boundary (Barratt *et al*., 1998). Interaction with MTT.
4.	Potassium hydroxide (10% aq)	1310-58-3	C	Corrosive, but supporting data do not enable unequivocal classification as either R34 (II/III) or R 35 (I); more probable to be R 34 (II/III) (Barratt *et al*., 1998).
5.	Octanoic acid (caprylic acid)	124-07-02	C	Borderline C/NC chemical, as judged from the proximity of the chemical to the classification boundary (Barratt *et al*., 1998).
6.	Sulfuric acid (10% wt.)	7664-93-9	C	According to the classification mentioned in OECD Test Guideline 431, the chemical is classified as corrosive. According to Annex I of the Directive 67/548/EEC in range of concentration 5 -15 % the chemical is classified as irritant.
7.	4-Amino-1,2,4-triazole	584-13-4	NC	Non irritant.
8.	Eugenol	97-53-0	NC	Borderline NC/C chemical, as judged from the proximity of the chemical to the classification boundary (SAR analysis) (Barratt *et al*., 1998). Interaction with MTT.
9.	Phenethyl bromide	103-63-9	NC	Interaction with MTT.
10.	Tetrachloroethylene	127-18-4	NC	Classified as C in one of three EPISKIN laboratories in the validation study (Fentem *et al*., 1998). Very high scores for irritation *in vivo* in rabbits (ECETOC, 1995).
11.	Isostearic acid	30399-84-9	NC	Non irritant.
12.	4-(Methylthio)-benzaldehyde	3446-89-7	NC	Interaction with MTT.

C - severely corrosive; C - corrosive; NC - Non corrosive*

Although the OECD TG 431 does not require testing under blind conditions, decision was taken to test the samples coded to increase the scientific reliability of the study. All test chemicals were purchased by ZEBET at the BfR. Coding and distribution was performed by the BfR Biostatistical Unit.

One chemical (sulphuric acid 10%) was excluded from coding. It was decided, that this chemical needs to be tested, in addition to the one hour exposure, also with four hour exposure, in order to evaluate, if the result from Phase I (false negative) could be improved by a change of the prediction model. This extended exposure time would be consequently applied only for low concentrated solutions of inorganic acids.

Skin corrosion study using the reconstructed human skin model EST-1000

Since the main objective of the study was to screen performance of the EST-1000 model in the skin corrosion test and to prove the transferability of the skin corrosion protocol, the reference chemicals endorsed by the OECD TG 431 were used for testing (Table 10). Moreover, this approach allowed a direct comparison of the EST-1000 performance with the SkinEthic, EpiDerm and EPISKIN reconstructed human epidermis models.

4.1.2.2. Skin irritation studies

Skin irritation study using the reconstructed human skin model EpiDerm

As described in paragraph 3.3.2.1 the first step in the study was to adapt the new EPISKIN skin irritation protocol (Portes *et al*., 2002; Cotovio *et al*., 2005) to the EpiDerm model. For the adaptation of the protocol to EpiDerm model and its optimisation, 20 non-coded chemicals from phase III of the ECVAM prevalidation study were used (see Table 11, chemicals marked with asterisk). The same set of chemicals was used by Portes *et al*. and Cotovio *et al*. when developing and optimising the protocol with EPISKIN model. Thus, testing of this reference set enabled direct comparison between the protocol performance on both models.

In the second phase of the study that aimed for assessment of the predictive power of the new method, additional test substances had to be included (Table 11, chemicals without asterisk). To ensure reliable reference *in vivo* rabbit data, all chemicals were selected from the ECETOC database No. 66 (ECETOC, 1995). The classification of tested chemicals was performed by an expert of the BfR (Berlin, Germany), applying EU and GHS classification rules (Table 12). The final classifications are given in Table 13.

Table 11. Test chemicals - specification.

No.	Chemical name	CAS-No.	Phys. state	Purity (%)	Supplier	Chemical group according to ECETOC database
1*	2,4-Xylidine	95-68-1	liquid	98.1	Bayer AG.	amines
2*	2-Methyl-4-phenyl-2-butanol	103-05-9	liquid	100	Givaudan-Roure	alcohols
3*	3,3-Dithiodipropionic acid	1119-62-6	solid	99	Aldrich	Sulphur-containing compound
4*	3-Chloronitrobenzene	121-73-3	solid	99.6	Bayer AG.	halogen. aromatics
5*	4,4-Methylene-bis(2,6-ditertbutyl)phenol	118-82-1	solid	98	Aldich	phenolic derivatives
6*	4-Amino -1,2,4-triazole	584-13-4	solid	96.7	Aldrich	miscellaneous
7*	cis-Cyclooctene	931-87-3	liquid	95	Fluka	hydrocarbons
8*	Soap from 20/80 coconut oil/tallow	-	pellets	-	Unichema	soaps/surfactants
9	1,6 - Dibromohexane	629-03-8	liquid	98	ACROS Organics	brominated derivatives
10	2-ethoxy ethyl methacrylate	2370-63-0	liquid	99	Aldrich	acrylates/methacrylates
11	Benzyl acetate	140-11-4	liquid	>99	ACROS Organics	esters
12	Benzyl benzoate	120-51-4	liquid	>99	ACROS Organics	esters
13	Benzyl salicylate	118-58-1	liquid	99	LANCASTER	esters
14	Benzylalcohol	100-51-6	liquid	99	ACROS Organics	alcohols
15	Dipropylene glycol	25265-71-8	liquid	99	ACROS Organics	alcohols
16	Erucamide	112-84-5	solid	90	ACROS Organics	amides
17*	Hydroxycitronellal	107-75-5	liquid	98.7	Givaudan-Roure	aldehydes
18	Isopropanol		liquid	99.8	Merck	alcohols
19	Isopropyl myristate	110-27-0	liquid	98	Sigma	esters
20	Isopropyl palmitate	142-91-6	liquid	99.8	CAELO	esters
21	Lauric acid	143-07-7	solid	>99	ACROS Organics	fatty acids
22	Methylstearate	112-61-8	solid	99	INC Biomedicals	esters
23	n-Butyl propionate	590-01-2	liquid	>99	ACROS Organics	esters
24	Sodium bicarbonate	144-55-8	solid	>99.5	ACROS Organics	alkalis
25	Sodium bisulphite	7631-90-5	solid	>97	Aldrich	inorganics
26*	1,1,1-Trichloroethane	71-55-6	liquid	>99.95	Aldrich	chlorinated solvents
27*	10-Undecenoic acid	112-38-9	liquid	98.8	IFF	acids
28*	1-Brompentane	110-53-2	liquid	99	Aldrich	brominated derivatives
29*	dl-Citronellol	106-22-9	liquid	98.7	Givaudan-Roure	alcohols
30*	d-Limonene	5989-27-5	liquid	98.8	Givaudan-Roure	miscellaneous
31*	Heptanal	111-71-7	liquid	95	IFF	aldehydes
32*	Lilestralis/lilial	80-54-6	liquid	97.8	Givaudan-Roure	aldehydes
33*	Methylpalmitate	112-39-0	liquid	99	Aldrich	esters
34*	Potassium hydroxide (5% aq.)	1310-58-3	liquid	r.g	Mallindkrot	alkalis
35	Tetrachloroethylene	127-18-4	liquid	99	ACROS Organics	chlorinated solvents
36*	SDS (50% aq.)	151-21-3	susp.	r.g	Sigma	soaps/surfactants
37	1-Bromohexane	111-25-1	liquid	>99	ACROS Organics	brominated derivatives
38	alpha-Terpineol	98-55-5	liquid	99	ACROS Organics	alcohols
39	Cinnamaldehyde	104-55-2	liquid	>98	ICN Biomedicals	aldehydes
40	Eugenol	97-53-0	liquid	>99	ICN Biomedicals	phenolic derivatives
41	Linalol	78-70-6	liquid	97	LANCASTER	alcohols
42	Linalyl acetate	115-95-7	liquid	>95	Merck	esters
43	Methyl laurate	111-82-0	liquid	99	LANCASTER	esters
44	SDS (20% aq.)	151-21-3	liquid	r.g.	Sigma	soaps/surfactants
45	Tallow propylene polyamine	68911-79-5	pellets	>95	Elf atochem	amines

**chemicals tested in Phase I*

Table 12. Test chemicals - Individual erythema and oedema scores computed for each animal and *in vivo* experiment listed in ECETOC database no. 66. (ECETOC, 1995).

No.	Chemical	Individual scores for erytheme						Individual scores for oedeme					
		Animal No.						Animal No.					
		1	2	3	4	5	6	1	2	3	4	5	6
1	2,4-Xylidine	1	1	1				0.7	0.7	0			
2	2-Methyl-4-phenyl-2-butanol	1.7	1.7	1	1			1	0.3	0.3	0.7		
3	3,3-Dithiodipropionic acid	0	0	0	0			0	0	0	0		
4	3-Chloronitrobenzene	0	0	0				0	0	0			
5	4,4-Methylene-bis(2.6-ditertbutyl)phenol	0	0	0				0	0	0			
6	4-Amino -1.2.4-triazole	0	0	0	0	0	0	0	0	0	0	0	0
7	cis-Cyclooctene	2	1	2	1.7	1	1.3	0.7	0	0.7	0.7	0	0.3
8	Soap from 20/80 coconut oil/tallow	1	1	1.3				1	1	1			
9	1,6 - Dibromohexane	0.7	1	1				0	0	0			
10	2-ethoxy ethyl methacrylate	1	1.7	2				0	0	0			
11	Benzyl acetate - I	1.7	0	1.3				1	0	0.7			
	Benzyl acetate - II	1.7	0	0.7	1			0	0	0	0		
12	Benzyl benzoate - I	0	0	0				0	0	0			
	Benzyl benzoate - II	0	1.7	1	2			0	0.3	0.3	1		
13	Benzyl salicylate - I	0.3	0.7	0				0	0	0			
	Benzyl salicylate - II	1	0.3	1	0			0.3	0	0	0.3		
14	Benzylalcohol - I	1.7	1	1.3				0.3	0	0.3			
	Benzylalcohol - II	1	1.7	1	2			0	0.7	0.3	0.7		
15	Dipropylene glycol - I	0	0	1				0	0	0			
	Dipropylene glycol - II	0	0	0	0			0	0	0	0		
16	Erucamide	0	0	0				0	0	0			
17	Hydroxycitronellal - I	1	1	0.7				0.3	0.3	0			
	Hydroxycitronellal - II	1.7	0	0.7	1			0.3	0	0	0		
18	Isopropanol	1.7	0.3	0.3				0	0	0			
19	Isopropyl myristate	1	1	1				0.7	0	0			
20	Isopropyl palmitate	1	1.7	1				0	0.7	0			
21	Lauric acid	1	0	0.3				0	0	0			
22	Methylstearate	1	2.3	1				0	2	0			
23	n-Butyl propionate	1.7	0.7	1	1			0	0	0	0		
24	Sodium bicarbonate	0.3	0	0				0	0	0			
25	Sodium bisulphite	1	1	1				0	0	0			
26	1,1,1-Trichloroethane	4	4	3				2	1.3	1.3			
27	10-Undecenoic acid	2	2	2	2			1	1.3	1	0.3		
28	1-Brompentane	3.7	1.7	2.7				2.7	0	2.7			
29	dl-Citronellol - I	2	2	2				2	2.7	2			
	dl-Citronellol - II	2	2	2	2			2.7	2.7	1.3	1.3		
	dl-Citronellol – II	2	2	2	2			2	2	1.3	1.3		
30	d-Limonene - I	2	2	2				1.3	2	1.3			
	d-Limonene - I	2	2	1.7	2			1.3	0.7	1.3	2		
31	Heptanal	4	4	2.7	2.3			2	2.3	3	2		
32	Lilestralis/lilial - I	1.7	2	2.3				2	2.7	3			
	Lilestralis/lilial - II	2	1.7	2	2			1.7	1.7	2.3	1		
33	Methylpalmitate	1.5	3	3				1	2.5	2.5			
34	Potassium hydroxide (5% aq.)	3	3	3.7				2	2	2			
35	Tetrachloroethylene	4	4	4				2	1.7	1.3			
36	SDS (50% aq.)	4	4	3.7				2	2	2.3			
37	1-Bromohexane	2.7	2	2.7				0	0.7	2			
38	Alpha-Terpineol - I	1.7	2	1.3				2	2.3	3			
	Alpha-Terpineol - II	2	2.7	2	2			3	3	2.7	1.7		
	Alpha-Terpineol - III	2	2	1.7	2			2.7	2	0.7	3		
39	Cinnamaldehyde	2	2	2	2.3			2	2	1.3	2.3		
40	Eugenol	2	2	2	2			1.7	1.3	1	1		
41	Linalol - I	2	2	1.7				2	1.3	1			
	Linalol - II	2	2	2	2			1.7	1.7	1	1.3		
	Linalol - III	1	2	2	1.7			0	1	0.7	0		
42	Linalyl acetate - I	1.7	2	2				1.7	2	2			
	Linalyl acetate - II	2	2	2	1.7			2	2	2	1.7		
43	Methyl laurate	2	2	2				2	2	2			
44	SDS (20% aq.)	3.3	4	4				2.3	3	3.7			
45	Tallow propylene polyamine - I	2	0.7	2				1.3	0	2			
	Tallow propylene polyamine - II	1.3	3.7	0				3.7	3.7	0			

Table 13. Test chemicals – classification *in vivo*.

No.	Chemical	CAS-No.	EU Class			GHS Class		
			EXP 1	EXP 2	EXP 3	EXP 1	EXP 2	EXP 3
1	2,4-Xylidine	95-68-1	NI			NI		
2	2-Methyl-4-phenyl-2-butanol	103-05-9	NI			NI		
3	3,3-Dithiodipropionic acid	1119-62-6	NI			NI		
4	3-Chloronitrobenzene	121-73-3	NI			NI		
5	4,4-Methylene-bis(2.6-ditertbutyl)phenol	118-82-1	NI			NI		
6	4-Amino -1.2.4-triazole	584-13-4	NI			NI		
7	cis-Cyclooctene	931-87-3	NI			SLI		
8	Soap from 20/80 coconut oil/tallow	no data	NI			NI		
9	1,6 - Dibromohexane	629-03-8	NI			NI		
10	2-ethoxy ethyl methacrylate	2370-63-0	NI			NI		
11	Benzyl acetate	140-11-4	NI	NI		NI	NI	
12	Benzyl benzoate	120-51-4	NI	NI		NI	NI	
13	Benzyl salicylate	118-58-1	NI	NI		NI	NI	
14	Benzylalcohol	100-51-6	NI	NI		NI	NI	
15	Dipropylene glycol	25265-71-8	NI	NI		NI	NI	
16	Erucamide	112-84-5	NI			NI		
17	Hydroxycitronellal	107-75-5	NI	NI		NI	NI	
18	Isopropanol	67-63-0	NI			NI		
19	Isopropyl myristate	110-27-0	NI			NI		
20	Isopropyl palmitate	142-91-6	NI			NI		
21	Lauric acid	143-07-7	NI			NI		
22	Methylstearate	112-61-8	NI			NI		
23	n-Butyl propionate	590-01-2	NI			NI		
24	Sodium bicarbonate	144-55-8	NI			NI		
25	Sodium bisulphite	7631-90-5	NI			NI		
26	1,1,1-Trichloroethane	71-55-6	R 38			I		
27	10-Undecenoic acid	112-38-9	R 38			SLI		
28	1-Brompentane	110-53-2	R 38			I		
29	dl-Citronellol	106-22-9	R 38	R 38	R 38	SLI	SLI	SLI
30	d-Limonene	5989-27-5	R 38	NI		SLI	SLI	
31	Heptanal	111-71-7	R 38			I		
32	Lilestralis/lilial	80-54-6	R 38	NI		I	SLI	
33	Methylpalmitate	112-39-0	R 38*			I*		
34	Potassium hydroxide (5% aq.)	1310-58-3	R38*			I*		
35	Tetrachloroethylene	127-18-4	R 38			I		
36	SDS (50% aq.)	151-21-3	R38*			I*		
37	1-Bromohexane	111-25-1	R 38			I		
38	alpha-Terpineol	98-55-5	R 38	R 38	R 38	I	I	SLI
39	Cinnamaldehyde	104-55-2	R 38			SLI		
40	Eugenol	97-53-0	R 38			SLI		
41	Linalol	78-70-6	R 38	R 38	NI	SLI	SLI	SLI
42	Linalyl acetate	115-95-7	R 38	R 38		SLI	SLI	
43	Methyl laurate	111-82-0	R 38			SLI		
44	SDS (20% aq.)	151-21-3	R38*			I*		
45	Tallow propylene polyamine	68911-79-5	R 38	R 38		SLI	I	

I / (R38) = Irritant; NI = Non-Irritant; SLI = Slight irritant; Exp- experiment

** **p**ossibly corrosive*

Skin irritation study using the reconstructed human skin model SkinEthic

As with the EpiDerm model, the performance of the SkinEthic model with the "common skin irritation protocol", was evaluated using the twenty non-coded chemicals from phase III of the ECVAM pre-validation study (Fentem *et al*., 2001) (Table 11, chemicals marked with an asterisk). This set of chemicals was extensively tested on EpiDerm and EPISKIN models with several protocol modifications and prediction models (Fentem *et al*., 2001, Portes *et al.*, 2002; Kandárová *et al*., 2004; Cotovio *et al*., 2005). In the second phase of the study (evaluation of inter-laboratory transferability) six chemicals were selected, coded and tested between two laboratories - ZEBET and Experimental Toxicology - Schering AG, Berlin, Germany (see Table 14).

In addition, analysis of IL-1α release into the culture medium was performed in order to investigate if the IL-1α can contribute to the prediction of the irritation potential of chemicals.

Table 14. Characterisation of chemicals used in Phase II.

No.	Chemical	CAS No.	Purity (%)	Chemical Type	Solid/ Liquid	Supplier	In vivo classification			
							EU Class		GHS Class	
							Exp1	Exp2	Exp1	Exp2
1	Lilestralis	80-54-6	97.8	Aldehyde	Liquid	Givaduan-Roure	R38	NI	I	SLI
2	Hydroxycitronellal	107-75-5	98.7	Aldehyde	Liquid	Givaduan-Roure	NI	NI	NI	NI
3	Isopropyl myristate	110-27-0	98	Ester	Liquid	Sigma	NI	-	NI	-
4	Sodium bicarbonate	144-55-8	>99.5	Alcalis	Solid	ACROS Organics	NI	-	NI	-
5	Lauric acid	143-07-7	>99	Fatty acid	Solid	ACROS Organics	NI	-	NI	-
6	1-Bromohexane	111-25-1	>99	Brominated deviate	Liquid	ACROS Organics	R38	-	I	-

I = Irritant; NI = Non-Irritant; SLI = Slight irritant, Exp - experiment

4.1.2.3. Phototoxicity studies

In phototoxicity studies, mainly cosmetic UV filters (sunscreens) and plant extracts were evaluated. Sunscreens efficiently absorb and/or scatter solar ultraviolet radiation and therefore may undergo photo-fragmentation, photo-isomerisation and can form reactive intermediates and undesirable products of photo-degradation. These substances may not necessarily act as acute photo-toxins, if the penetration into the deeper (viable) epidermal layers does not happen, and they can still be safely used in low concentrations.

The validated *in vitro* method (3T3 NRU PT) may, however, predict these substances as potential phototoxins although when tested on human skin, no response would be observed. Because of the presence of the functional stratum corneum and barrier

similar to those found in human skin, the reconstructed human skin model is considered as a suitable tool for screening of such substances before any confirmatory test on human volunteers is performed.

For the evaluation of this hypothesis, several substances with potency of being light activated were evaluated in 3T3 NRU PT (Table 15). When the result was positive, additional phototoxicity test on human skin model EpiDerm was performed.

Table 15. Specification of test chemicals used in Phototoxicity studies.

Name	INCI name	Class	CAS-No.	Supplier
Eusolex 9020	Butyl methoxy-dibenzoylmethane	UV filter	70356-09-1	Merck
Eusolex 232	Phenylbenzimidazole Sulfonic Acid	UV filter	27503-81-7	Merck
Eusolex 4300	Benzophenone-3	UV filter	131-57-2	Merck
Eusolex 6300	4-Methylbenzylidene Camphor	UV filter	36861-47-9	Merck
Benzophenone-4	2-hydroxy-4-methoxynemzophenone -5 sulphonic acid	UV filter	4065-45-6	ACROS Organics
Titanium Dioxide	Titanium Dioxide (TiO_2)	UV filter	13463-67-7	Sigma
Litsea cubeba	Litsea cubeba	Plant oil	68855-99-2	Biomedica
Bergamot oil	Bergamot oil	Plant oil	89957-91-5	Sigma
	Bergamot oil	Plant oil	89957-91-5	Schupp
	Bergamot oil	Plant oil	89957-91-5	Aroma
	Bergamot oil	Plant oil	89957-91-5	Biomedica

4.1.3. REAGENCIES AND SOLUTIONS

Tissue morphology

Formaldehyde 4.3% (ready to use)	Kugel Medizintech. Vertriebs, Germany
Xylene 100%	Sigma-Aldrich, Germany
Ethanol p.a. (70%, 80%, 90%, 96%)	Sigma-Aldrich, Germany
TissueTek™ OCR embedding medium	Science Services, Germany
Hematoxylin solution	Reagena, Finland
Eosine solution	Reagena, Kuopio, Finland

Lipid analysis

Methanol (HPLC grade)	Labscan, Ireland
Ethanol (HPLC grade)	Labscan, Ireland
Chlorform (HPLC grade)	Labscan, Ireland
Diethyl Ether (HPLC grade)	Labscan, Ireland
Hexyl acetate (HPLC grade)	Labscan, Ireland
Aceton (HPLC grade)	Labscan, Ireland
Hexane (HPLC grade)	Labscan, Ireland
Ether (HPLC grade)	Labscan, Ireland
Dichlormethane (HPLC grade)	Labscan, Ireland
Milpore H_2O	--
$CuSO_4$. 5H2O	Sigma-Aldrich, Germany
$CuCH_3(COO)_2$	Sigma-Aldrich, Germany
H_3PO_4	Sigma-Aldrich, Germany

STANDARDS for HPTLC:

Ceramide 1 (CER1)	Cosmoferm B.V, the Netherlands
Ceramide 2 (CER2)	Cosmoferm B.V, the Netherlands
Ceramide 3 (CER3)	Cosmoferm B.V, the Netherlands
Ceramide 4 (CER4)	Cosmoferm B.V, the Netherlands
Ceramide 6 (CER6)	Cosmoferm B.V, the Netherlands
Cholesterol (CHOL)	Sigma-Aldrich, Germany
Free Fatty Acides (FFA)	Sigma-Aldrich, Germany
Triglycerides (TG)	Sigma-Aldrich, Germany
Cholesterol sulphate (CHOL SO_4)	Sigma-Aldrich, Germany
Cholesterol ester (CE)	Sigma-Aldrich, Germany
Cerebrosides (CEREB)	Sigma-Aldrich, Germany

Barrier studies and TEER measurements

Triton X-100, 1%	Sigma-Aldrich, Germany
PBS (ready to use)	PAN Biotech, Germany
MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (1%)	Sigma-Aldrich, Germany
Isopropanol	Sigma-Aldrich, Germany

Confocal laser scanning microscopy

Sodium fluorescein in EtOH (0,5 %)	Merck - Schuchardt, Germany
Sodium fluorescein in H_2O (0,5 %)	Merck - Schuchardt, Germany

Skin corrosion studies

PBS (ready to use)	PAN Biotech, Germany
MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (1%)	Sigma-Aldrich, Germany
Isopropanol	Sigma-Aldrich, Germany
8N KOH (positive control)	Sigma-Aldrich, Germany

Skin irritation studies

PBS (ready to use)	PAN Biotech, Germany
MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (1%)	Sigma-Aldrich, Germany
Isopropanol	Sigma-Aldrich, Germany
5% SDS (positive control)	Sigma-Aldrich, Germany
Quantikine, IL-1 α immunoassay	R&D Systems, Germany

Phototoxicity studies

PBS with and without Ca^{2+} and Mg^{2+}	PAN Biotech, Germany
Acetic acid	Sigma-Aldrich, Germany
Ethanol	Sigma-Aldrich, Germany
Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride)	Sigma-Aldrich, Germany
MTT 1%	Sigma-Aldrich, Germany
Deionized water	PAN Biotech, Germany
Sesame oil	Bufa, The Netherlands
Isopropanol	Sigma-Aldrich, Germany

4.1.4. DEVICES

Histology and immunohistology

Parrafin embedding station; Type EG 1120 (60°C)	Leica, The Netherlands
Cold plate; Type EG 1130; (-8°C)	Leica, The Netherlands
Microtom; Type RM 2135	Leica, The Netherlands
Water Bath; Type HI 1210 (37°C)	Leica, The Netherlands
Hot plate; Type HI 1120 (40°C)	Leica, The Netherlands
Microscope (Axioplan 2, Imaging)	Zeiss, Germany
Digital camera (AxioCAM)	Zeiss, Germany
Pipettes and tips	NeoLab, The Netherlands
Embedding cassettes	Kugel Medizintech. Vertriebs, Germany

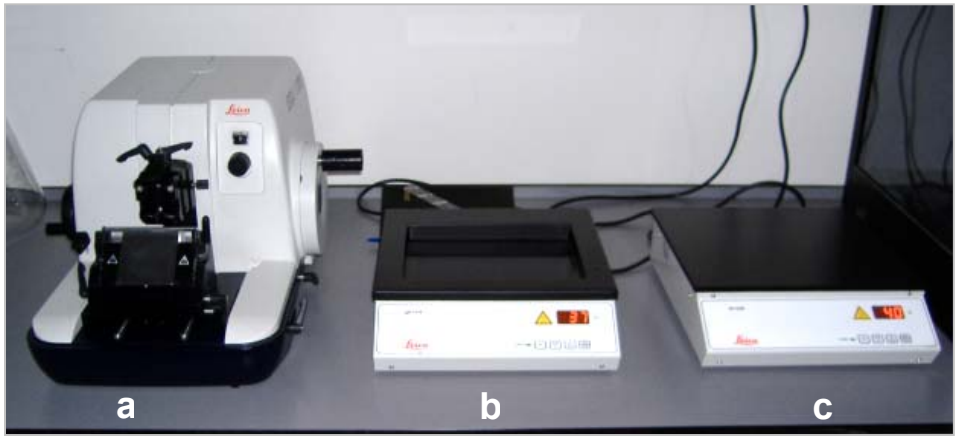

Figure 13. Equipment for cutting of paraffin embedded tissues:
a) microtom, b) water bath, c) hot plate.

Lipid analysis

Linomat, Type IV (For HPTLC and FTIR analysis)	CAMAG, Germany
Microbalance, Type Termobalance TG 50	Mettler, Germany
Metal pans for the lipid content determination	Mettler, Germany
Evaporator, supplied with N_2 stream	Marius Instrumenten, Germany
Regulated heating plate, Type Termoplate S (25 -200°C)	Desaga, Germany
Incubator (60°C)	Desaga, Germany
Scanner GS710	Bio-Rad, The Netherlands
Software for lipid analysis - *Quantity One*	Bio-Rad, The Netherlands
Pipettes and tips	NeoLab, The Netherlands

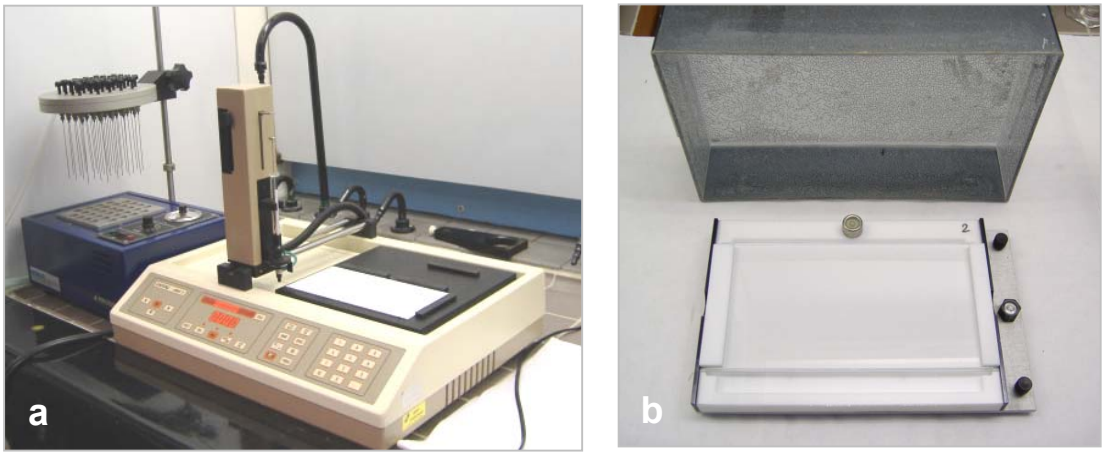

Figure 14. Equipment for lipid analysis using HP TLC method:
a) Linomat and sample evaporator supplied with nitrogen gas,
b) equipment for the TLC plate development.

TEER assay and barrier evaluation

Volt/ohm-meter Millicell-ERS for measurement of: - transepithelial voltage and - resistance of cells grown on microporous membranes. Specification: - alternating current 8AC-square -wave current (±20 μA or ±2 μA at 12.5 Hz) - resistance range 0-20 kOhm, - membrane voltage range ±199,9 mV - silver/silver chloride (Ag/AgCl) electrode	Millipore, USA
Incubator (5% CO_2 ; 37°C ; 95 % humidity)	Heraeus, Germany
Water bath (37°C)	GFL GmbH, Germany
Spectrophotometer (filter 570nm)	Dynatech, Germany
Digital Thermometer (0-70°C)	NeoLab ,Germany

Figure 15. Volt/ohm-meter Millicell-ERS.

Confocal laser scanning microscopy

Dermatological Confocal laser scanning microscope, Stratum	Optiscan, Australia
Pipettes and tips	NeoLab, Germany

Skin corrosion and irritation studies

Laminar flow hood	Heraeus, Germany
Incubator (5% CO_2 ; 37°C ; 95 % humidity)	Heraeus, Germany
Water Bath (37°C)	GFL GmbH, Germany
Refrigerator (4°C, -20°C)	AEG, Germany
Spectrophotometer (filter 570nm)	Dynatech, Germany
Pipettes and tips	NeoLab, Germany
Digital Thermometer	NeoLab, Germany
Plate shaker	IKA Labortechnik, Germany

Phototoxicity studies

Solar simulator (UV/VIS), Type SOL 500, Filter H1	Dr. Hoenle, Germany
Dosimeter, Type 37	Dr. Hoenle, Germany
Laminar flow hood	Heraeus, Germany
Incubator (5% CO_2 ; 37°C ; 95 % humidity)	Heraeus, Germany
Water Bath (37°C)	GFL GmbH, Germany
Refrigerator (4°C, -20°C)	AEG, Germany,
Spectrophotometer (filter 570 nm)	Dynatech, Germany
Pipettes and tips	NeoLab, Germany
Plate shaker	IKA Labortechnik, Germany

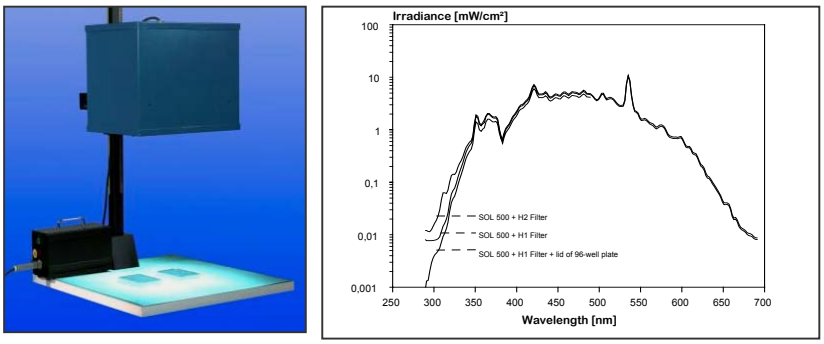

Figure 16. UV/VIS light simulator SOL 500 and its irradiance spectrum.

4.2. METHODS

4.2.1. CHARACTERISATION OF RECONSTRUCTED HUMAN SKIN MODELS

4.2.1.1. Tissue morphology

Samples of the reconstructed human epidermal models were excised from the inserts, washed with PBS to allow removal of pH indicators and rests of culture medium and cut into four pieces (width approximately 0.2 cm). Two parallel samples were embedded in paraffin and the other two were embedded in TissueTek™ OCR compound (snap-frozen) for further analysis.

EMBEDDING IN PARAFFIN:

Tissue samples were placed into embedding cassettes and fixed in buffered 4% paraformaldehyde overnight. Thereafter, the cassettes were transferred into 70% ethanol and stored at 4°C until use. On a day of experiment, tissues were gradually de-hydrated (80% EtOH, 45 min; 90% EtOH, 45 min; 96% EtOH, 45 min, 100% EtOH, 45 min; Xylene 100%, 45 min) and embedded in paraffin.

HEMATOXYLIN EOSINE STAINING:

Vertical sections (5 µm) were cut using a standard Microtom (Leica), depariffinized, rehydrated and stained with Hematoxylin-Eosin (H&E) solution using following steps:

1. Paraffin slides were placed in slide holder and exposed to following solvents:
   3 x 3 min 100% Xylene
   3 x 3 min 100% Ethanol
   1 x 3 min 95% Ethanol
   1 x 3 min 80% Ethanol
   1 x 5 min deionised water
2. Hematoxylin staining:
   1 x 3 min Hematoxylin (1%)
   rinse once in deionised water
   1 x 5 min Tap water
3. Eosine staining:
   1 x 30 sec Eosine (1%)
   1 x 3 min rinse in deionised water and dry the slides at room temperature.
4. A drop of liquid gelatine was pipetted on a slide and a glass coverslip was placed on the area to protect the sample.

4.2.1.2. Lipid analysis

Lipid extraction from stratum corneum

The lipids were extracted from the samples of skin models using different mixtures of chloroform and methanol. Reconstructed epidermis models were mechanically separated from the dermal substrate with forceps, dried in the exicator and stored under nitrogen at -20°C. The harvested material was extracted according to Ponec *et al*. (2000b), see Figure 17. The extracts were stored at -20°C until use.

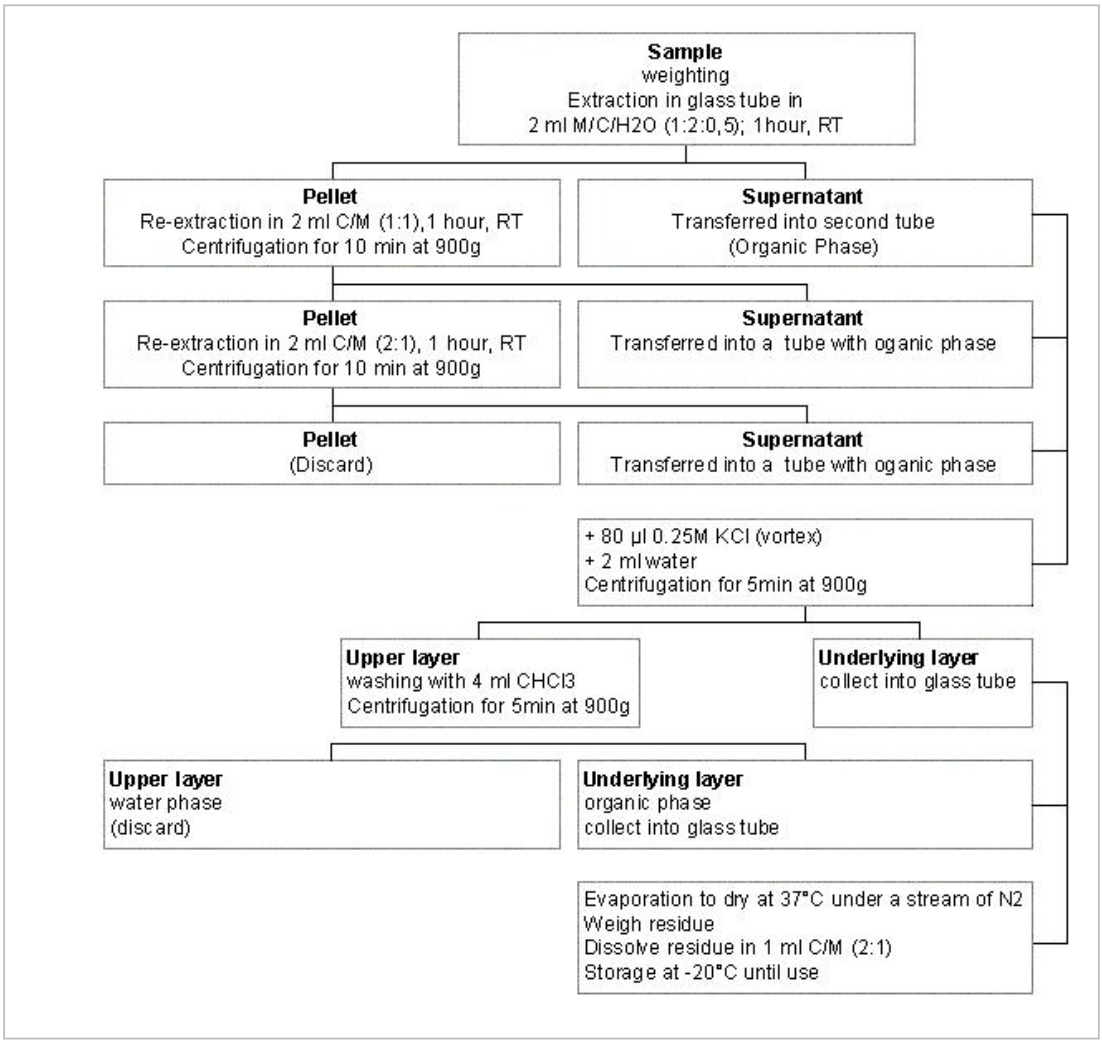

Figure 17. Extraction procedure according to Ponec *et al*. (2000b).

Determination of the lipid concentration

For further steps in the lipid analysis procedure, the lipid concentration in a sample (new or long-stored) must be determined. Metal pans (Mettler, Germany) were washed with chloroform/methanol solution (2:1) and dried on a hot plate. The weight of empty pans was determined using microbalance (Termobalance TG 50, Mettler, Germany). 80µl of the lipid extract (in chloroform/methanol) was applied into each pan using a microsyringe (3 pans were used for one test sample). The pans were kept on a hot plate (60°C) until the solvent completely evaporated. Weights of the pans with lipid residues were determined using microbalance and lipid content per sample was calculated.

High performance thin layer chromatography

For the separation of lipids from the complex mixture, high performance thin layer chromatography (HPTLC) was used. The separation was achieved by different affinity of the substances to a mobile phase.

Initially, HPTLC silica gel plates were rinsed with 2 rinsing solutions (ethyl acetate /methanol; 40/60 and chloroform/diethylether/ethyl acetate 30/50/20). After each rinse step, the plate was dried at 60°C to allow the evaporation of the solvents. Before using in the experiment, the plate was 10 min activated at 120 °C, cooled down to room temperature and only thereafter used in the experiment. Alternatively, the plate was kept in an exicator until next day.

Samples and standards were applied with a microsyringe on TLC plates using a spraying device Linomat (CAMAG, Germany). On both sides of the plate, 15, 10, 8, 5, and 2 mg of standard lipid mix were applied. Test sample was applied in amounts of 30, 15 and 5 mg on silikagel plate. This test design allowed to test three samples on one TLC plate. The TLC plates were developed with 6 different solvents according to the separation protocol given in Table 16.

Table 16. Development system for lipid separation.

Step	Distance (mm)	Solvent	Approximate developing time
1	30	H / C / A = 8 / 90 / 2	2 min (+ 10 min drying at 60°C)
2	10	C / A / M = 76 / 8 / 16	20 s (+ 10 min drying at 60°C)
3	60	H / C / HA / EA / A / M = 6 / 80 / 1 / 10 / 4	12 min (+ 10 min drying at 60°C)
4	15	C / A / M = 76 / 4 / 20	45 min (+ 10 min drying at 60°C)
5	70	H / C / HA / EA / M = 8 / 80 / 1 / 6 / 6	19 min (+ 10 min drying at 60°C)
6	90	H / DE / EA = 78 / 18 / 4	21 min (+ 15 min drying at 120°C)

H – hexane, C – chloroform, A – acetone, M – methanol, HA - hexyl acetate, EA - ethyl acetate, DE – diethyl ether

Following the last drying step (15 min at 120°C), the TLC plates were stained by means of pasteur pipette with a mixture of $CuSO_4$. $5H_2O$ / $CuCH_3(COO)_2$ (ratio 2:6). The TLC plates were thereafter placed on a hot plate and heated approximately 10 min at 80°C until first lipid spots occurred. At this temperature, purple-rose bands of cholesterol and sterol deviates (lanosterol of cholesterolsulphate) can be detected. The plates were scanned and thereafter heated to 120°C. At this temperature all saturated compounds were visible, while unsaturated appear upon 160°C. The plates were again scanned and finally heated at 170°C to allow for a detection of unsaturated rests of lipids presented in sample. The result obtained at 170°C was taken for an analysis using the Quantity One - software provided by Bio-Rad (The Netherlands).

4.2.1.3. Evaluation of the barrier function

For the evaluation of the barrier function of reconstructed human skin models, two endpoints were selected and evaluated concurrently:

1) decrease of trans-epidermal electrical resistance (TEER),
2) determination of the ET 50 value after exposure to Triton X-100 (1% w/v).

Measurement of electrical resistance is frequently used for the evaluation of tightness of the cell monolayers. In the present study, the resistance of the cell layers of the reconstructed human skin models was monitored by means of Millicell-ERS, connected to a pair of chopstick electrodes (for technical details see paragraph 4.1.4 Devices). The experimental design is given in Figure 18.

The reconstructed human epidermal models were stored and pre-incubated according to manufacturers recommendations. On the day of experiment, skin samples were aseptically transferred into 6-well plates pre-filled with 5 ml of pre-warmed PBS (37°C) for measurement of resistance values. Plates were kept in a water bath to maintain the constant temperature of 37°C. Into each insert, 0.5 ml of pre-warmed PBS (37°C) was pipetted and exactly after 5 min resistance values (in kΩ) were measured.

Thereafter, the PBS was aspirated, and the tissue surface dried with cotton tip. Two tissues per time-point were treated with a constant dose (160 µl/cm^2 ± 1µL) of Triton 1% (for the specific volume doses can be found in Table 17). Two tissues serving as negative controls were dosed with sterile deionized water and were exposed to Triton for 4 hours. All cultures were incubated at 37 ± 1ºC in a humidified atmosphere (5 ± 1% CO_2 in air) for the appropriate length of exposure time.

Table 17. Specification of volume applied per different model size.

Model	Tissue surface (cm^2)	Volume of Triton applied (µl)	Exposure time (h)
EpiDerm	0.63	100	2, 4, 6, 8, 10
EPISKIN	1.1	175	2, 4, 6, 8, 10
SkinEthic	0.5	80	2, 4, 6, 8, 10

Following exposure, tissues were rinsed once with pre-warmed (37°C) PBS. The rests of PBS were gently removed with a cotton tip. Thereafter, inserts were transferred into the 6-well plates pre-filled with 5 ml of pre-warmed PBS (37°C) for measurement of TEER in the same manner as described above. Finally, inserts were placed into 24 well plates pre-filled with MTT medium (1.0 mg/ml in DMEM; 300 µl/well) and incubated for 3 hours at 37 ± 1ºC in a humidified atmosphere of 5±1% CO_2.

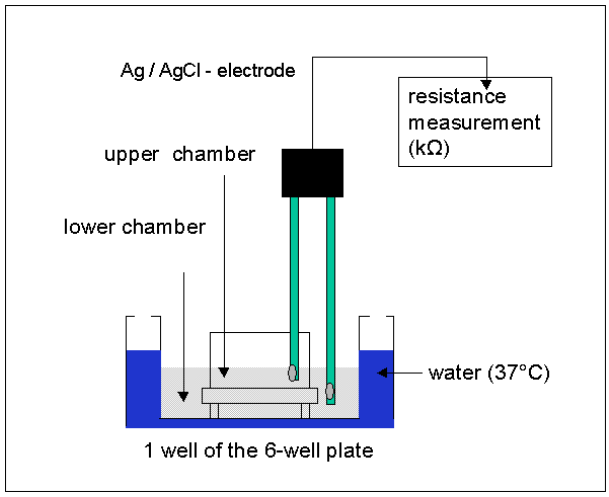

Figure 18. Schematic design of the apparatus for determination of resistance.

At the end of the MTT assay, the tissues were removed from the MTT medium, rinsed with PBS, blotted on absorbent material, and immediately transferred to a 24-well plate containing isopropanol (2 ml per well) to extract the formazan product (reduced MTT). The plates were shaken for 2 hours at room temperature to improve the extraction conditions. Thereafter, three aliquots of 200 µl aliquots of the isopropanol extract were transferred to a 96-well plate, and the optical density was measured at 570 nm using a microtitre plate reader.

Relative tissue viability was calculated as a percentage of the viability of the mean of negative control, the dose response curves were constructed and ET 50 values calculated. The results of the TEER assay were expressed as:

1.) a total decrease of the TEER values (in $k\Omega.cm^2$) at measured time-points,

2.) a relative decrease related to control tissues (in %) at measured time-points.

4.2.2. SKIN CORROSION STUDIES

The *in vitro* skin corrosion test is based on the knowledge that necrotic damage of viable skin cells (skin corrosion) shows a high correlation with skin cell cytotoxicity, which occurs rapidly after brief exposure of the skin barrier (*stratum corneum*) to a corrosive chemical (Liebsch *et al*., 2000). The correlation between histopathological findings and immediate cytotoxicity (measured as reduction of MTT conversion) has been demonstrated by Perkins *et al*. (1996).

The *in vitro* skin corrosion assays consist of a topical exposure (3 min and 1 h) of the test material to the reconstructed human epidermal tissues followed by immediate determination of the cytotoxic effect. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity, measured by production of formazan from MTT. Viability of tissues treated with a test chemical is compared with that of untreated controls and the percent of viability is calculated. This value is used for the classification according to prediction model.

All skin corrosion studies described in this work are based on the EpiDerm skin corrosion protocol developed and validated by ZEBET at the BfR (Liebsch *et al*., 2000) and are taking into account requirements of the OECD TG 431.

4.2.2.1. Skin corrosion study using SkinEthic model

Special technical steps in the protocol

Evaluation of the MTT conversion caused by chemicals bound into the tissue

Initially, the ability of a test substance to directly reduce MTT (yellow stain which is used for determination of viability of cells/tissues (Mossman, 1983)) was assessed. The test material was added into a 1.0 mg/ml MTT solution in Dulbecco's modified eagle medium (DMEM). For liquids 50 µl, and for solids 25 mg, of the test materials were added to the MTT solution (1ml) and the mixtures were incubated in the dark at 37 °C for 60 min. If the MTT solution turned to blue/purple, it was assumed the test chemical had reduced the MTT. However, only those test materials that remain bound to the tissue after rinsing present a problem, resulting in a false MTT reduction. To evaluate if residual test material was binding to the tissue, a functional check using freeze-killed control tissues was performed (for details see Liebsch *et al*., 2000). Freeze-killed control tissues were prepared by a process of two times freezing (-20°) and thawing of viable tissues of the batch used in the concurrent skin corrosion experiment.

Two tissues per time-point were exposed to 50 µl (liquids) or 25 mg (solids) of each MTT reducing test chemical. When the chemical remained bounded to the tissue, blue formazan crystals were eventually formed. Consequently, the optical density of the extracted formazan was measured and the mean value was subtracted from the mean value obtained with viable tissues treated with the same chemical.

Application technique for solids

Solids were ground with a mortar and pestle, and the powder was applied on the tissue surface with a 25 mg calibrated spoon (Aesculap), to give a constant bulk volume of about 25 ± 3 mg. After application to the tissue surface, 25 µl sterile H_2O was added to wet the test material. If necessary, the applied material was spread to match the size of the tissue. The “spoon application technique” used in this study was already successfully used in the ECVAM skin corrosion study (Liebsch *et al*., 2000) with the EpiDerm model. In addition, in the routine (*in vivo*) toxicological praxis is the application of solids performed similarly.

Application technique for liquids

During adaptation of the EpiDerm protocol to the SkinEthic model, it was observed that spreading of hydrophilic liquids is difficult due to high surface tension. In the first experiments, this technical problem resulted in variability and under-predictions. Therefore, it was decided to use the “mesh application technique” for the spreading of all liquids in a similar manner as described for the EpiDerm model (Kandárová *et al*., 2004). Here the chemical is directly applied on the tissue and a nylon mesh (8 mm diameter) is placed on the top. This technique helps to keep the chemical evenly distributed on the surface during the entire exposure.

Experimental procedure

SkinEthic reconstructed human models (0.63 cm^2) were shipped on Mondays and arrived at the laboratories usually on Tuesdays. The skin corrosion test was performed on Tuesdays or Wednesdays. Upon receipt, tissues were kept at room temperature (18-22°C) on the shipping agarose until use. On the day of testing, tissues were removed from transport agarose and conditioned by a 3 h pre-incubation in 1 ml assay medium (37 °C, 5% CO_2, saturated humidity) in 6-well plates, to allow release of metabolites, cytokines and cellular debris accumulated during the shipment. After replacement of the medium, 50 µl (liquids and semisolids) or 25 mg (solids) of the neat test chemical were applied topically on 3 replicate tissues per chemical. Three tissues were dosed with 50 µl of H_2O (ultra-pure or distilled), serving as negative control, and 3 tissues were dosed with 50 µl 8N potassium

hydroxide, serving as positive control. Tissue dosing was performed consecutively at 1 min intervals, which was the time needed to rinse of chemicals.

After exposure, the tissues were carefully rinsed in a gentle stream of PBS from a washing bottle, by filling and emptying the culture insert twenty times. Washed and blotted inserts were collected in a 24-well “holding plate” pre-filled with 0,3 ml of maintenance medium. Thereafter, the inserts were transferred into a new 24-well plate containing 0,3 ml freshly prepared MTT medium (1 mg MTT/ml assay medium) and incubated for 3 h at 37 °C, 5% CO_2 and saturated humidity.

After 3 h, all tissues were rinsed with PBS, gently blotted and transferred to a new 24-well plate. Two ml of formazan solvent (isopropanol, analytical grade) were added for completely immersing the inserts. Extraction was either performed at room temperature for 2 h on a plate shaker, or, alternatively, overnight without shaking, carefully sealed to prevent evaporation of the solvent. Following extraction, three 200 µl aliquots of each extract were transferred to a 96-well plate and the optical density determined in a plate spectrophotometer at 570 nm (OD_{570}). The relative tissue viability of each tissue was calculated as a percentage of the viability of the mean of the negative controls.

The acceptance criteria for the test were following: (1) optical density (OD_{570}) of formazan extracted from negative controls had to be more than 0.8; (2) coefficient of variation (CV) of identically treated tissues had to be less than 30%, with exception of cases with OD below 0.3; (3) the positive control had to be classified as “corrosive” in the experimental run. The mean of three identically treated inserts was used for the classification of the chemical according to the PM. All procedures were documented in “method documentation sheets”, for GLP-compliant documentation.

Prediction model

The corrosivity potential of the test material was predicted from the mean tissue viabilities obtained after 3 min and 1 h treatments, and compared to the negative control tissues concurrently treated with H_20. A chemical was classified as “corrosive” if the relative tissue viability was decreased below 50% after 3 min of exposure to a test material. In addition, test materials classified “non-corrosive” after 3 min (viability > 50%) were classified “corrosive” if the relative tissue viability was decreased below 15 % after exposure for 1 h.

4.2.2.2. Skin corrosion study using EST-1000 model

The skin corrosion assay with EST-1000 model was performed according to the standard operation (SOP) procedure developed for SkinEthic epidermis. This method became later a base for EST-1000 skin corrosion SOP. In general, the two standard operation procedures differ only by pre-incubation techniques: SkinEthic tissues can be kept on the shipping agar at room temperature overnight, while for the EST-1000 model overnight pre-incubation is recommended by the manufacturer.

4.2.3. SKIN IRRITATION STUDIES

Similarly as the *in vitro* skin corrosion test, the skin irritation test is based on the knowledge that damage of viable skin cells shows a correlation with skin cell cytotoxicity, which occurs after exposure of the skin barrier to an irritating chemical. However, *in vitro* skin corrosion and *in vitro* skin irritation assays differ significantly by their test design.

The *in vitro* skin irritation assay consists of a topical exposure (15 min) of the test material to the reconstructed human epidermal tissues followed by 42 hours of postincubation. In comparison to *in vitro* skin corrosion test, design of the *in vitro* skin irritation protocol enables long-term development of the cell damage or tissue recovery after the exposure to test chemical. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity, measured by production of formazan from MTT at the end of the treatment. Viability of tissues treated with a test chemical is compared with that of untreated controls. The relative tissue viability is used for the final classification according to prediction model.

The EpiDerm skin irritation protocol (Kandárová *et al*., 2004) was developed on a base of EPISKIN protocols (Portes *et al*., 2002, Cotovio *et al*., 2003 personal communication; Cotovio *et al*., 2005). Both EpiDerm and EPISKIN assays are currently evaluated in an ECVAM skin irritation validation study. The EpiDerm standard operation procedure protocol was later adapted to the SkinEthic reconstructed human epidermis (Kandarova *et al*., 2006b). In the latter protocol, release of interleukins into the culture medium was additionally assessed to investigate whether the second endpoint could improve the prediction of irritants.

4.2.3.1. Skin irritation study using EpiDerm model

Special technical steps in the protocol

Application technique for liquids

In the EPISKIN protocol (Portes *et al*., 2002; Cotovio *et al*., 2005), 10 µl or 10 mg of test substance were applied onto the EPISKIN surface (0.38 cm^2). Since the surface of EpiDerm tissues is larger (0.63 cm^2), it was important to find the optimum dose for providing the best agreement with results obtained with EPISKIN. The effects of applying 10, 15, 20 and 25 µl of liquid or 10, 15, 20 and 25 mg of solid test substances were compared, and the best results were obtained with the highest doses (25 µl or 25 mg). To improve spreading of liquids, a nylon mesh (diameter 8 mm) was placed on the surface of the tissue after the test substance was applied. This technique helped to keep the chemical evenly distributed for the entire exposure period. However, possible interactions between the nylon mesh and the test substance had to be considered for each test sample. In case of reaction between the test material and the nylon, the mesh shouldn't be used.

Application technique for solids

Solids were ground with a mortar and pestle, and the powder was applied with a “sharp spoon” (Aesculap) to give a constant bulk volume of about 25 ± 2 mg, rather than applying an amount of constant weight. 25 µl sterile water was added to moisten the test material. If necessary, the applied material was spread to match the size of the tissue. This application technique was also used for the EpiDerm model in the ECVAM skin corrosion validation study (Liebsch *et al*., 2000).

Application technique for waxy materials and highly viscous liquids

Waxy materials (like soaps and tallow) were applied on a stainless-steel disc (8mm diameter) and placed on the surface of the tissue. Highly viscous chemicals were applied with a positive displacement pipette. The test substance was distributed on the surface by using a bulb-headed probe. To improve spreading, a nylon mesh was placed on the tissue surface, provided that there was no indication of a reaction between the mesh and the test material.

Rinsing technique

A refined rinsing technique was used to reduce false-positive predictions for chemicals that cannot be washed off easily and may therefore affect cell viability during the 42-hour post-incubation period. This technique consists of three steps that permit better removal of applied substances at the end of the exposure period. Each tissue was rinsed

10 times in a soft stream of PBS from a washing bottle by filling and emptying the culture insert. Subsequently, the tissue was completely submerged and shaken three times in 150 ml PBS, and finally, again rinsed once with a stream of PBS from a washing bottle.

Experimental procedure

EpiDerm kits were shipped from the USA on Mondays, and usually arrived in Berlin on Tuesday afternoon. The best reproducibility and results were obtained when the tissues were used on the day of arrival or following overnight storage at 4–6°C on the agarose used for their transport. Initially, the ability of a test substance to directly reduce MTT was assessed by adding the test material to 1ml of MTT solution (1.0 mg/ml) in DMEM. Liquid (25 µl) and solid (25 mg) test materials were added to the MTT solution and the mixtures were incubated for 60 minutes at 37°C in 5% CO_2 in air, with saturated humidity. If the MTT solution turned to blue/purple, it was assumed that the test chemical had reduced the MTT (Mossmann, 1983). However, only those test materials that remained bound to the tissue after rinsing presented a problem, by giving a false reduction of MTT. To evaluate whether residual test material binds to the tissue, a functional check using freeze-killed control tissues was performed (Liebsch *et al*., 2000). None of the MTT-reducing chemicals evaluated was significantly present in the tissues after the 42-hour post-incubation period, so no corrections had to be performed.

On the day of experiment, tissues were aseptically removed from the transport agarose and conditioned by a 1-hour incubation in 0.9 ml assay medium (at 37°C in 5% CO_2 , 95% air, saturated humidity) in 6-well plates, in order to release transport stress-related compounds and any debris accumulated during shipment. The tissues were then transferred to 0.9ml fresh assay medium and exposed topically to the test chemicals. Liquids (25 ± 1 µl) were applied with a micropipette, and a nylon mesh (8-mm diameter) was placed over the surface of the tissue. Solids were applied with a 25 ± 2 mg calibrated spoon and wetted with 25 µl sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Waxy test materials were first applied to a stainless-steel disc and than placed on the tissue. Each test chemical was applied to three tissues. In addition, three tissues served as negative controls and were dosed with 25 µl sterile water, and three tissues served as positive controls and were exposed to 5% sodium lauryl sulphate (SLS). To prevent chemical contamination across the wells of the 6-well plate, volatile substances were tested on separate plates. Dosing was performed consecutively at 60-second intervals (the time needed for the rinsing procedure).

After 15 minutes of exposure, each tissue was carefully rinsed with Ca^{2+} and Mg^{2+}-free PBS, by using the rinsing technique described above. Blotted inserts were than transferred to new 6-well plates containing 0.9 ml fresh maintenance medium. The surface of each tissue was dried with a sterile cotton tip. Tissues were post-incubated for 42 hours

(at 37°C, in 5% CO_2 in air and with saturated humidity) to allow development of cell damage, which was subsequently assessed in the MTT assay.

When the 42 hours post-incubation was completed, blotted tissues were transferred to 24-well plates containing 0.3 ml freshly prepared MTT medium (1 mg MTT/ml) and incubated for 3 hours (at 37°C, in 5% CO_2 in air and with saturated humidity). The tissues were then rinsed twice with PBS and transferred to new 24-well plates. 2 ml isopropanol (analytical grade) was added to each well to completely immerse the inserts. The plates were sealed with parafilm and formazan extraction was performed at room temperature for 2 hours on a plate shaker. Thereafter, two 200 µl aliquots of isopropanol extract per tissue were transferred to a 96-well plate, and optical density was measured at 570 nm, using isopropanol as a blank. The relative viability was calculated as the percentage of the mean viability of negative controls. The mean of the three values from identically treated tissues was used to classify the chemical according to the PM.

Prediction model

The PM applied in the study is based on a comparison of the cell viability of treated tissues with that of negative controls (treated with water) by using the MTT assay. If the tissue viability of three exposed tissues is reduced to less than 50% of control values, the chemical is classified as an “irritant” (Portes *et al*., 2002).

4.2.3.2. Skin irritation study using SkinEthic model

Experimental procedure

SkinEthic epidermal tissues were shipped from the producer in Nice on Mondays and arrived in Berlin on Tuesday morning. Upon receipt tissues were aseptically removed from the transport agarose, transferred into the maintenance medium (1ml) and conditioned for 2 hours (37°C, 5% CO_2, humidified atmosphere). Conditioning allowed release of the transport stress related compounds and debris accumulated during shipment. Tissues were then transferred to the fresh growth medium (1 ml) and conditioned in the incubator overnight.

Next day, tissues were topically exposed to the test chemicals. Liquids (20 ± 1 µl) were applied with a micropipette and a nylon mesh (diameter = 8 mm) was placed on the surface of the tissue. Solids were applied with a 20 mg ± 2 mg calibrated spoon and wetted with 20 µl sterile water. If necessary, the mixture was gently spread on the surface of the epidermis with a microspatula. Due to high hydrophobic surface of the SkinEthic tissues the nylon mesh was used as a spreading support for all liquid chemicals and in addition for all solids fully solubilized after wetting with 20 µl of water. Waxy test materials were first

applied to a stainless-steel disc and than placed on the tissue. Each test chemical was applied to 3 tissues. In addition, three tissues serving as negative controls were dosed with 20 µl sterile water and three tissues serving as positive controls were exposed to 5% (aq.) sodium dodecyl sulphate (SDS). To prevent chemical contamination across the wells of the 6 well plate, volatile substances were tested on separate plates.

Dosing was performed consecutively at 60 second intervals. After 15 minutes of exposure each tissue was carefully rinsed with Ca^{2+} and Mg^{2+}-free PBS. The washing procedure used for EpiDerm (10 times washing of tissues with the stream of PBS from the washing bottle followed by shaking of the tissues three times in 150 ml of PBS plus an additional washing of the tissue from washing bottle) was in some cases not sufficient for SkinEthic epidermal model. Some solid chemicals adhered to the surface of the tissue even after extensive washing procedure. Therefore, mechanical removal of rests of solid substances (e.g. 3-chlornitrobenzene) was sometimes necessary. Blotted inserts were than transferred to new 6 well plates containing 1 ml of fresh growth medium. The efficacy of washing procedure was controlled using a binocular microscope.

Tissues were post-incubated for the next 24 hours (5% CO_2, 37°C, saturated humidity). Afterwards the 24 h old growth medium was changed (in Phase II collected for IL-1 alpha analysis) and tissues were postincubated in new growth medium for next 18-20 hours. When the 42 +/- 2 hours postincubation was completed, blotted tissues were transferred to 24-well plates containing 0.3 ml freshly prepared MTT medium (1mg MTT / ml) and incubated for 3 hours at 5% CO_2, 37°C and saturated humidity. Tissues were then rinsed twice with PBS and transferred to new 24 well plates. Two ml isopropanol (analytical grade) were added to each well, completely immersing the inserts. Plates were sealed with parafilm and formazan extraction was performed at room temperature for 2 hours on a plate shaker. Two aliquots (200 µl) per tissue of isopropanol extract were transferred to a 96-well plate. Optical density was measured at 570 nm using isopropanol as a blank. The relative viability was calculated as percentage of the mean viability of negative controls. The mean of the three values from identically treated tissues was used to classify the chemical according to the prediction model.

Prediction model

The PM applied in the study is based on a comparison between the cell viability of treated tissues with that of negative controls (treated with water) by using the MTT assay. If the tissue viability of three exposed tissues is reduced to less than 50% of control values, the chemical is classified as an “irritant” (Portes *et al*., 2002; Kandárová *et al*., 2004; Cotovio *et al*., 2005).

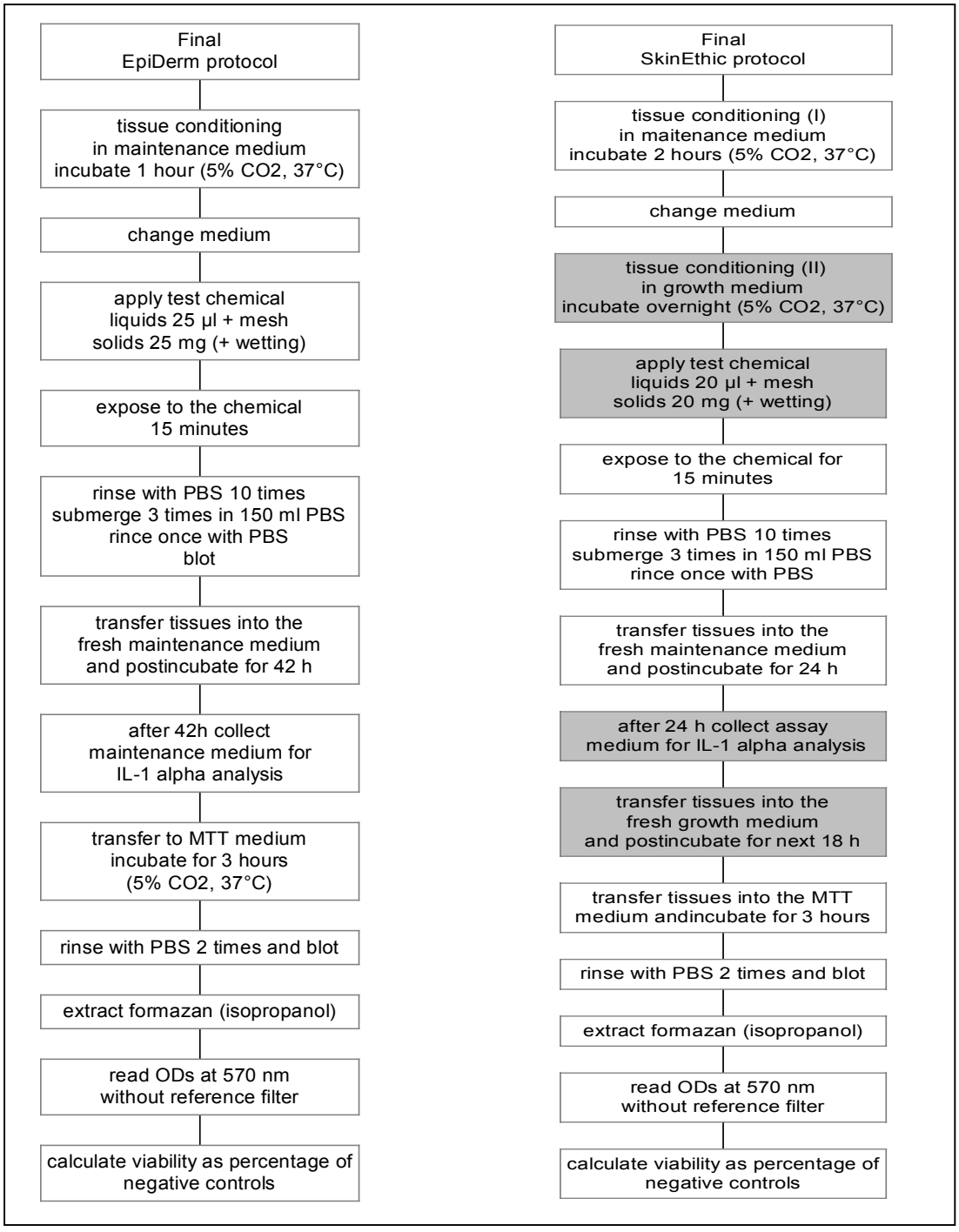

Figure 19. Technical differences between EpiDerm and SkinEthic skin irritation protocol. The main differences between the two protocols are marked with grey colour.

4.2.4. PHOTOTOXICITY STUDIES

4.2.4.1. In vitro 3T3 NRU phototoxicity test

The *in vitro* 3T3 NRU phototoxicity test is based on a comparison between the cytotoxicity of a chemical when tested in the presence and in the absence of non-cytotoxic dose of simulated solar light.

In this test, cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation. 3T3 NRU phototoxicity test is an officially validated method for the assessment of the phototoxicity of chemicals (OECD, 2004b)

Preparation of cultures

A permanent mouse fibroblast cell line, Balb/c 3T3, clone 31, from the American Type Culture Collection (ATCC), was used in the study. Sensitivity to UV and visible light was controlled regularly according to the quality control procedure described later in the text. Because the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest passage number were used.

Balb/c 3T3 cells used for the phototoxicity test were seeded in culture medium at seeding density is 1 x 10^4 cells per well. For each test chemical, cells were seeded identically in two separate 96-well plates, which were then taken concurrently through the entire test procedure under identical culture conditions except for the time period where one of the plates was irradiated (+Irr) and the other one was kept in the dark (-Irr).

Cells from frozen stock cultures were seeded in culture medium at an appropriate density and subcultured at least once before they were used in the *in vitro* 3T3 NRU phototoxicity test

Media and culture conditions

For Balb/c 3T3 cells Dulbecco's Modified Eagle's (DMEM) Medium supplemented with 10% new-born calf serum (NBCS), 4 mM glutamine, penicillin (100 IU), and streptomycin (100 μg/mL), and humidified incubation at 37 °C, 7,5% CO_2 is recommended. It is particularly important that cell culture conditions assure a cell cycle time within the normal historical range of the cell line used.

Preparation of test substances

Preparation of the test substance concentrations was performed immediately prior to use. All chemical handling and the initial treatment of cells were performed under light conditions that avoid photo-activation or degradation of the test substance prior to irradiation.

The ranges of concentrations of each chemical tested in the presence (+Irr) and in the absence (-Irr) of light was adequately determined in dose range-finding experiments. The highest concentration of the test substance should not alter the physiological test conditions (e.g. osmotic and pH stress should be avoided). For relatively insoluble substances that are not toxic at concentrations up to the saturation point the highest achievable concentration was tested. The maximum concentration of a test substance did not exceed 1000 μg/mL. A geometric dilution series of 8 test substance concentrations with a constant dilution factor was used.

Test chemicals were dissolved in physiologically balanced buffer solution PBS with Ca^{2+} and Mg^{2+} (free from protein components, light absorbing components e.g., pH-indicator colours and vitamins to avoid interference during irradiation). Test chemicals of limited solubility in PBS were pre-dissolved in recommended solvents - dimethylsulphoxide (DMSO) and Ethanol (ETOH) - and consequently diluted with PBS. The final concentration of DMSO or EtOH did not exceeded 1%. The solvent was present at a constant volume in all cultures, i.e. in the negative (solvent) controls as well as in all concentrations of the test chemical.

Irradiation Conditions

In all phototoxicity studies performed the same source of light was used (mercury-metal halide arc lamp from Dr. Hönle (Typ SOL- 500 from Dr. Hönle, Planegg, Germany). The intensity of light (irradiance) was controlled before each phototoxicity test using a calibrated UV-meter (Dr. Hönle, Type 37). Irradiance was adjusted to 1.7 mW/cm^2 which is the energy needed within the time period of 50 min to achieve a dose of 5 J/cm^2 (as measured in the UVA range). This dose is non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite chemicals to elicit phototoxic reactions.

Assay quality controls

Radiation sensitivity of the cells, establishing of historical data:

Cells were controlled regularly for sensitivity to the light source by assessing their viability following exposure to increasing doses of irradiation. Several doses of irradiation, including levels substantially greater than those used for the 3T3 NRU phototoxicity test were used in this assessment. Cells were seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test and irradiated the next day. Cell viability was determined one day later using Neutral Red uptake assay.

The test met the quality criteria if the irradiated negative/solvent controls showed a viability of more than 80% when compared with non-irradiated negative/solvent control and if the highest non-cytotoxic dose (5 J/cm^2 [UVA]) classified the reference chemicals correctly.

Viability of solvent controls:

The absolute optical density ($OD_{540\ NRU}$) of the Neutral Red extracted from the solvent controls indicates whether the 1x10^4 cells seeded per well have grown with a normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of the untreated controls is $\geq$ 0.3.

Positive control:

A known phototoxic chemical was tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) is recommended by the OECD TG 432 as a suitable positive control. Following test acceptance criteria were defined for CPZ:

CPZ irradiated (+Irr): IC_{50} = 0.1 to 2.0 μg/ml,

CPZ non-irradiated (-Irr): IC_{50} = 7.0 to 90.0 μg/mL,

The Photo Irritation Factor (PIF) should be > 6.

Experimental procedure

100 μL culture medium was dispensed into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, 100 μL of a cell suspension of 1xl0^5 cells/mL in culture medium (= 1x10^4 cells/well) was dispensed. Two plates were prepared for each series of individual test substance concentrations. Cells were incubated for 24 h until they formed a half-confluent monolayer. This incubation period allowed for cell recovery, adherence, and exponential growth.

After incubation, culture medium was decanted from the cells and wells were gently washed with 150 µL of the buffered solution used for incubation. 100 µL of the buffer containing the appropriate concentration of test chemical or solvent (solvent control) was added into each well. 8 different concentrations of the test chemical was used in each experiment (see Figure 20). The plates with test solutions were incubated in the dark (37°C, 7.5% CO_2, 95 % humidity) for 60 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank	blank	blank	Blank	blank	blank	blank	blank	blank
B	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
C	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
D	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
E	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
F	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
G	blank	**NC**	C1	C2	C3	C4	C5	C6	C7	C8	**NC**	blank
H	blank	blank	blank	blank	blank	blank	Blank	blank	blank	blank	blank	blank

Figure 20. Experimental design of the 96- well plate in the 3T3 NRU-PT test.
NC- negative/solvent control, (C1 - C8) - range of 8 concentrations

From the two plates prepared for each series of test substance concentrations and the controls, one plate was selected for the determination of cytotoxicity (-Irr), and one for the determination of photo-cytotoxicity (+Irr). To perform the +Irr exposure, the cells were irradiated at room temperature for 50 minutes through the lid of the 96-well plate. Non-irradiated plate (-Irr) was kept at room temperature in a dark box for 50 min.

Afterwards, test solutions were decanted and plates carefully washed twice with 150 µL of the buffered solution used for incubation, but not containing the test material. Finally, the buffer was replaced with culture medium and plates were incubated overnight (18-22 h; 37°C; 7.5% CO_2; 95 % humidified atmosphere).

The next day, cells were examined for growth, morphology, and integrity of the monolayer using a phase contrast microscope. Changes in cell morphology and effects were recorded. Afterwards, cells were washed with 250 µL of the pre-warmed buffer and buffer was removed by gentle tapping. 200 µL of a 50 µg/mL Neutral Red (NR) in medium without serum was added into each well and plates were incubated for 3 h (37°C; 7.5% CO_2; 95 % humidified atmosphere).

At the end of the incubation period, NR medium was removed, and cells washed with 250 µL of the buffer. Buffer excess was removed by blotting or centrifugation. Exactly 250 µL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 2 part

acetic acid) was added into each well. Microtiter plates were gently shaken on a plate shaker for 20 min until NR has been extracted from the cells and a homogeneous solution was formed. The optical density of the NR extract was measured at 540 nm, using blanks as a reference. Data were saved in electronic file format for subsequent analysis using Phototox software, version 2.0.

Data analysis

To enable evaluation of the data, a Photo Irritation Factor (PIF) or Mean Photo Effect (MPE) are calculated. In the study the "Phototox software, version 2.0." (Holzhütter, 1989; Peters and Holzhütter, 2003) was used for all calculations. The software calculates automatically the basic measures of phototoxicity: PIF (Photo-Irritation Factor) and MPE (Mean Photo Effect).

Briefly, for the calculation of the PIF and MPE the set of discrete dose-response values has to be approximated by an appropriate continuous dose-response curve. Fitting of the curve to the data is commonly performed by a non-linear regression method.

A **Photo Irritation Factor** (PIF) is calculated using the following formula:

$$PIF = \frac{IC_{50}\,(\text{-Irr})}{IC_{50}(\text{+Irr})}$$

If an IC_{50} in the presence or absence of light cannot be calculated, a PIF cannot be determined for the test material.

A **Mean Photo Effect** (MPE) is based on comparison of the complete concentration response curves. It is defined as the weighted average across a representative set of photo effect values

$$MPE = \frac{\sum_{i=1}^{n} w_i \; PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

The photo effect (PE_{ci}) at any concentration (C) is defined as the product of the response effect (RE_c) and the dose effect (DE_c), i.e. $PE_c = RE_c \times DE_c$. The response effect (RE_c) is the difference between the responses observed in the absence and presence of light, i.e. $RE_c = R_c\,(\text{-Irr}) - R_c\,(\text{+Irr})$.

The dose-effect is given by

$$DE_c = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

where C* represents the equivalence concentration, i.e. the concentration at which the +Irr response equals - Irr response at concentration C. If C* cannot be determined since the response values of the +Irr curve are systematically higher or lower than R_C(-Irr) the dose effect is set to 1. The weighting factors w_i are given by the highest response value, i.e. w_i = MAX {Ri (+Irr), Ri (-Irr)}. The concentration grid C_i is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the +Irr experiment the residual part of the +Irr curve is set to the response value “0”. Depending on whether the MPE value is larger than a properly chosen cut-off value (MPE_c= 0.15) or not, the chemical is classified as phototoxic (OECD, 2004).

Prediction model

Based on the OECD TG 432, a test substance with:

a PIF < 2 or an MPE < 0.1 predicts:	“No phototoxicity”
a PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts:	“Probable phototoxicity” and
a PIF > 5 or an MPE > 0.15 predicts:	“Phototoxicity”.

If phototoxic effects are observed only at the highest test concentration (especially for water soluble test chemicals), additional considerations may be necessary for the assessment of hazard. These may include data on skin absorption, and accumulation of the chemical in the skin and / or data from other tests, e.g. testing of the chemical in *in vitro* animal or human skin, or skin models.

4.2.4.2. In vitro phototoxicity test using EpiDerm model

The test consists of topical exposure of the EpiDerm model to the test material followed by an irradiation experiment (+UVA) and a dark control experiment (-UVA). After irradiation and rinsing, the tissues are incubated for 21 hrs followed for the determination of the cytotoxic effect in MTT-assay. The cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity, measured by the production of formazan from MTT.

Tissue viability obtained with each of the five concentrations of the test chemical is compared with that of untreated controls and the % degree of viability inhibition is calculated. For prediction of phototoxic potential the concentration responses obtained in the presence and in the absence of UV irradiation are compared.

Test sample preparation and test concentrations

According to solubility (see Table 18), chemicals were applied either as solutions in water, or as solutions or suspensions in sesame oil. Poorly water soluble test materials (category 5-7) were tested dissolved or suspended in sesame oil. Water soluble test materials (category 2-4) were tested dissolved in water.

Table 18. Range of solubility (according to ZEBET Standard Operating Procedure for 3T3 NRT PT).

DESCRIPTIVE TERM	RANGE OF SOLUBILITY	% (w/v)	CATEGORY
very soluble	> 1000 mg/ml	>100.00	1
freely soluble	> 100 mg/ml - 1000 mg/ml	>10.00	2
soluble	> 30 mg/ml - 100 mg/ml	>3.00	3
sparingly soluble	> 10 mg/ml - 30 mg/ml	>1.00	4
slightly soluble	> 1 mg/ml - 10 mg/ml	>0.10	5
very slightly soluble	> 0.1 mg/ml - 1 mg/ml	>0.01	6
practically insoluble	0.1 mg/ml and lower	<0.01	7

Concentration series

Five concentrations of the test material were tested in each experiment. The highest concentration of a test material should show cytotoxicity in non-irradiated tissues. Since many test chemicals are likely to absorb UV, they can act as "UV-filter". Therefore, the highest applied concentration of a test substance should not exceed 20%.

Application of test sample

To be close to the human *in vivo* test and to achieve a constant application area, a patch technique was initially developed using paper disks (d = 8 mm, normally used in Finn chambers for human patch tests). However, experiments performed at Procter & Gamble, Beiersdorf AG and ZEBET revealed that the patch technique is only an improvement when oil is used as vehicle, since the pads soaked with aqueous solutions dried during over-night exposure.

Later the patch was replaced by nylon mesh. The mesh does not interact with the majority of test chemicals and helps to keep the chemicals spread evenly on the tissue surface during the whole exposure period without any influence. 50 µL (solutions in water) or 20 µL (solutions in oil) were topically applied to the EpiDerm™ tissues, the mesh was

placed on the surface and the test chemical was gently spread using the bulb headed pasteur pipette.

Assay quality controls

UVA Sensitivity of the Epi-200 Tissues

The sensitivity of the tissues to UV light must be within the established acceptance range. The historical $ID_{50\ UVA}$ (dose of UVA light reducing the tissue viability to 50 %) for EpiDerm tissues falls in the range of ~12-18 J/cm^2. The test is meeting criteria if up to 6 J/cm^2 (UVA) reduction of viability do not exceed 20%.

Negative Control

The absolute OD of the negative control tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after shipping procedure and under specific conditions of the assay. Tissue assay is meeting the acceptance criterion if the mean optical density (OD_{570}) of the two negative control tissues is $\geq$ 0.8.

Positive Control: Chlorpromazine (CPZ)

It is not necessary to include a positive control into each phototoxicity test as this reduces the number of concentrations of the test chemical. However, an experiment with five concentrations of Chlorpromazine (dissolved in H_20) ranging from 0.001% up to 0.1% should be repeated on a regular basis.

Test is meeting acceptance criteria if a dose dependent reduction of cell viability (only in the UVA-irradiated tissues) is observed between range of CPZ concentrations 0.00316% and 0.0316%.

Maximum inter-tissue viability difference of tissue couples

According to the historical data base of ZEBET the mean difference between untreated tissue duplicates is 7 - 9%. A difference of more than 30% (i.e. exceeding the 99% confidence interval) between two tissues treated identically should be regarded as a rejection criterion, and re-testing of the chemical is recommended if the resulting viability is near to the classification cut-off.

Experimental Procedure

Epi-200 kits were shipped from Boston on Monday; and usually arrived at ZEBET on Tuesday afternoon. Upon receipt of the EpiDerm tissues, the sealed 24 well plates, assay medium, PBS, MTT diluent, and MTT extracting solution were placed into the

refrigerator (4°C). The vial containing the MTT concentrate was placed in the freezer (-20 °C).

On a testing day, tissues were aseptically transferred into 6-well plates pre-filled with 0.9 ml of assay medium and pre-incubated (37°C, 5% CO_2, saturated humidity) one hour to allow release of interleukins and products of the metabolism accumulated in the tissues. During the pre-incubation period, for each test chemical, series of five concentrations were prepared.

At the end of the pre-incubation period skin models were transferred into new 6-well plates (pre-filled with new 0.9 ml assay medium) and dosed with a test chemical. For one test chemical, twelve epidermal tissues were used in the (-UVA) cytotoxicity part and 12 tissues in the (+UVA) phototoxicity part of the test. Both parts of the test were dosed identically (see Figure 21). Once all tissues have been dosed, plates were covered with lids and incubated over night (18-24 h; 37°C, 5% CO_2, saturated humidity).

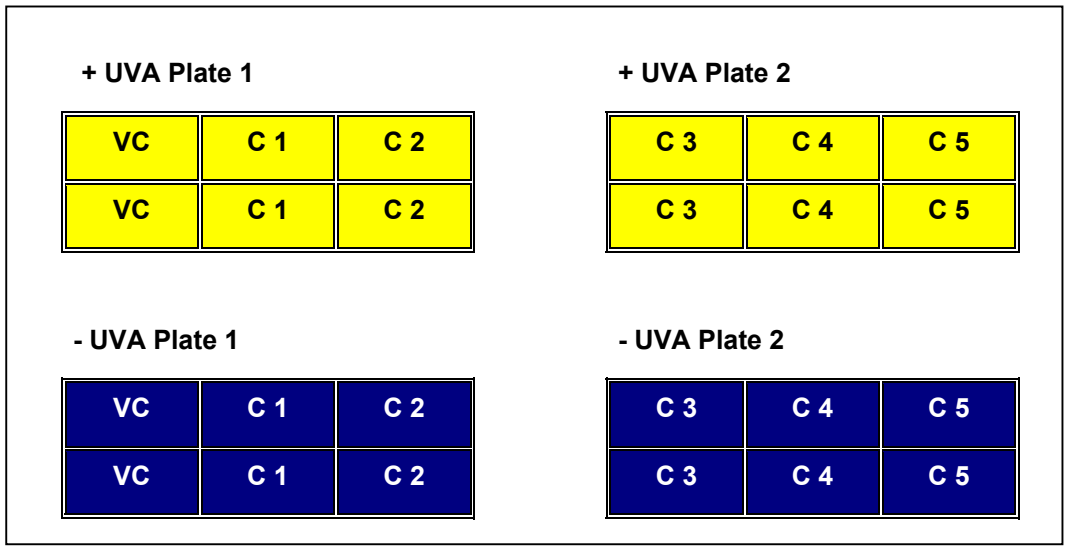

Figure 21. Plate design of the H3D phototoxicity test.
VC – vehicle control; C1 – the lowest concentration, C5 – the highest concentration

Next day the application pads/meshes were removed from the surface of the EpiDerm model. Two plates (=12 tissues) were irradiated for 60 min with 1.7 mW/cm^2 (= 6 J/cm^2) at room temperature. Ventilation with a fan prevented condensation under the lid. The other 2 plates (-UVA) were kept in the dark at room temperature in black boxes, until the irradiation period was completed.

After UVA irradiation was completed, each insert was rinsed with sterile PBS from washing bottle. Washed and blotted tissues were transferred into the new 6-well plates containing 0.9 ml of the fresh assay medium. All plates (+UVA) and (UVA) were incubated over night (18-24 hrs; 37°C, 5% CO_2, saturated humidity).

When the post-incubation period was completed, blotted tissues were transferred to 24-well plates containing 0.3 ml freshly prepared MTT medium (1 mg MTT/ml) and

incubated for 3 h (at 37°C, in 5% CO_2 in air and with saturated humidity). The tissues were then rinsed twice with PBS and transferred to new 24-well plates. 2 ml of isopropanol (analytical grade) was added to each well to completely immerse the inserts. The plates were sealed with parafilm and formazan extraction was performed at room temperature for 2 hours on a plate shaker.

Thereafter, three 200 µl aliquots of isopropanol extract per tissue were transferred to a 96-well plate, and optical density was measured at 570 nm, with isopropanol as a blank. For each concentration of a test chemical, the mean OD of the tissue couple treated with a tested concentration was determined and expressed as relative percentage viability of the untreated vehicle controls. Identical calculations were performed for the (+UVA) part of the test and the (-UVA) part of the test.

Prediction model

A chemical was predicted to have a phototoxic potential if one or more test concentrations of the (+UVA) part of the experiment reveal a decrease in viability exceeding 30% when compared with identical concentrations of the (-UVA) part of the experiment. Prediction of phototoxicity is supported if, in addition, the (+UVA) induced reduction in tissue viability shows a dose response relationship.

4.2.5. STATISTICAL METHODS

The special statistical analyses in studies described below were performed using the statistical software package SPSS®, version 11.5 (SPSS, Chicago, IL, USA).

4.2.5.1. Skin Corrosion

In the first phase (intra-laboratory assessment of the test performance) no special statistical methods were necessary. However, in phase II (inter-laboratory trial) the need for advanced statistical evaluation arose, since in addition to determination of sensitivity, specificity and accuracy, the concordance between laboratories and sources of variability had to be determined.

The General Linear Model (GLM) multivariate procedure was used for analysis of intra- and inter-laboratory variances. The GLM Multivariate procedure is based on the general linear model, which provides a unifying framework for well-known approaches such as multiple regression and analysis of variance, and which also enriches the variety of models that may be routinely fitted to data (Everitt and Dunn, 2001; Landau and Everitt, 2003).

The GLM multivariate procedure operates with the items “factor” and “variable”. All “factors” are usually determined by the assay design and are fixed. In the skin corrosion study, the factors are “chemicals”, “laboratories” and “runs”. Inter-laboratory variability is represented by the factor “laboratories”, and the factor “runs” is an indicator of intra-laboratory variability. It is assumed that factors have linear relationships to the dependent variables (here, 3min and 1-h exposure times) and each factor can have a different effect on the value of the dependent variables. The contribution of all three factors to the assay variability was calculated and expressed by significance values.

Effects with an error probability value smaller than 0.05 were assumed to be significant, and values below 0.01 were regarded as highly significant. The relative mean square values were calculated for both time-points as well, since this is a more straightforward way to assess the percentile contribution of each factor to the variability of the assay (Everitt and Dunn, 2001; Landau and Everitt, 2003).

4.2.5.2. Skin Irritation

The prediction model applied in the skin irritation studies is based on a comparison of the cell viability of treated tissues with that of negative controls (treated with water) by using the MTT assay. If in this specific test design (exposure to the test chemical for 15 minutes and 42 hours post-incubation), the cell viability of three exposed tissues is reduced to less than 50% of control values, the chemical is classified as “irritant”(Portes *et al*., 2002).

The classification of each test chemical according to the defined PM provides information on whether the classification of the chemical is classified falsely or correctly. However, it does not provide any information on the likelihood of assigning the test chemical to one of the two classification classes (Non-irritant or Irritant). To obtain this infotmation, the probability for correct classification was estimated for each of the tested chemicals according to the Bayes’ Rule.

Figure 22 explains this procedure. A chemical is classified in the group for which the probability is the greatest. The sum of the probabilities of the two classes is 100%, since each chemical must belong to one of the two classes. For each class, it is possible to determine the point that represents the mean of all viabilities. These points are called group centroids. The distance from this point can be computed for each case, and a chemical is assigned to the specific class to which it is nearest. By using these distances for the classification, probability values of correct classifications were derived.

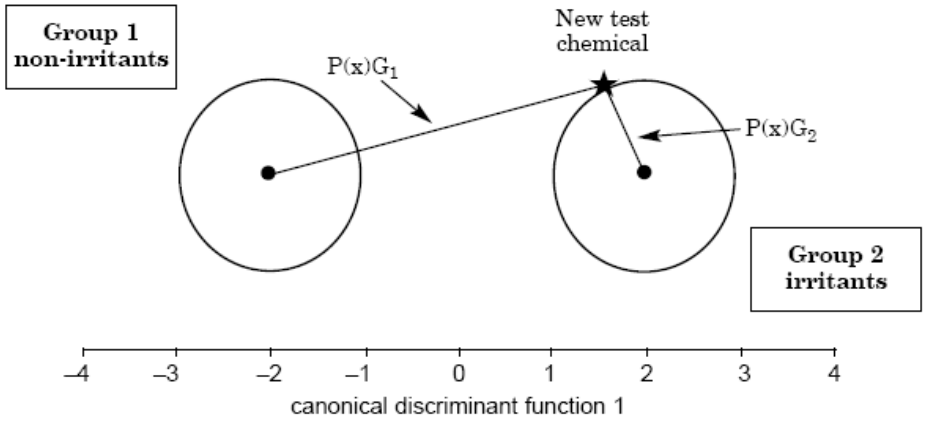

Figure 22. Classification graph with one function contributing to group separation

The figure is an illustration of the use of the canonical discriminant function, adapted from Genschow *et al*. (2004). Each circle indicates a group centroid. In this example, the left centroid indicates the mean value for the non-irritating chemicals (G1), and the right centroid shows the mean for irritating chemicals (G2). The canonical discriminant function is a linear combination of the discriminating variables. In this function, it is assumed that the means were as different as possible. The new test chemical is expected to belong to the group centroid to which it is closest. The new test chemical (Marked with an asterisk) is most likely to belong to the irritating chemicals (G2). Assigning a new test chemical to the group with the largest probability is equivalent to assigning it to the group with the smallest generalised distance.