

Development of a multiparametric *in vitro* model of skin sensitization

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ABSTRACT: Most animal experiments on cosmetics safety are prohibited and since March 2013, this obligation includes sensitization tests. However, until now there has been no validated alternative *in vitro* method. In this work, 400 compounds used in the cosmetic industry were selected to cover the greatest diversity of structures, biological activities and sensitizing potential. These molecules were submitted to a series of tests aimed at reproducing essential steps in sensitization and to distinguish between sensitization and irritations, i.e., transcutaneous permeation (factor A), haptentation (factor B), sensitization cytokines production (factor C) and acute toxicity (factor D). The transcutaneous diffusion was measured on human skin explants using Franz cells. Haptentation was tested in solution on human serum albumin. Sensitization cytokine production was investigated by measurement of interleukin-18 release by keratinocytes. Acute toxicity was determined using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide⁷⁵ cell viability test. As only sufficiently stable, soluble and detectable compounds are usable, 33, 72, 68 and 68 molecules were finally tested on factors A, B, C and D, respectively, and 32 were completely screened by the four factors. The individual correlation of the four factors with the reference *in vivo* tests was limited but the combination of these factors led to a correlation between *in vivo* and *in vitro* assays of 81.2% and the safety of the test (risk of false negative) reached 96.8%. The techniques employed are simple and inexpensive and this model of four tests appears as a promising technique to evaluate *in vitro* the skin sensitization potential of unknown molecules. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: skin sensitization; *in vitro* models; cosmetic compounds; skin permeation; xenobiotic haptentation; interleukin 18; MTT Test

Introduction

A list of 26 allergens, whose presence in cosmetics must be mentioned on packaging, has been established by the European Union (EU) (Directive 2003/15/EC). The European guidelines for cosmetic compounds 1223/2009 (Regulation EC No. 1223/2009) also requires that the sensitization potential of any new substance or formulation be documented as a combination of active molecule (s) and passage enhancer(s) can reveal a new sensitization activity. For the cosmetic industry, the situation was complicated by the decision since March 2009 to ban all *in vivo* assays. Different *in vitro* models have been validated to evaluate skin corrosion or irritation (Deshmukh *et al.*, 2012; Fentem and Botham 2002; Kidd *et al.*, 2007). In contrast, for other parameters, such as the sensitization potential, there is no completely validated *in vitro* model (Basketter *et al.*, 2013; Mehling *et al.*, 2012).

Skin sensitization is a complex process that starts by the transdermal permeation of the xenobiotic. Most of the molecules able to cross the skin barrier are too small to have an intrinsic sensitization activity and this activity frequently depends of their potential to establish stable covalent links with endogenous proteins (Divkovic *et al.*, 2005). Bound to the carrier molecule, the hapten forms a complex whose size is sufficient to trigger an immune response. In skin, the immune complex is then detected by keratinocytes that react by the production of cytokines and leads to the activation of a specific subset of dendritic immune cells also designated as Langerhans cells. As antigen-presenting cells, Langerhans cells migrate towards lymphocytes and activate the immune response. A subsequent

exposure to the same xenobiotic leads to a rapid activation of the specific lymphocyte(s) clone(s) and to the induction of an inflammatory sensitization reaction (Rustemeyer *et al.*, 2006). *In vivo*, sensitization is evaluated using three main models described in detail in International Organization for Standardization (ISO), Organization for Economic Co-operation and Development (OECD) or American Society for Testing and Materials (ASTM) guidelines: the guinea pig maximization test (GPMT) (ISO 10993-10, 2010), the mouse local lymph node assay (LLNA) (OECD Guidelines, test no. 429, 2010) and the human patch test (HPT) (ASTM D6355 – 07, 2012) but controversial results are frequent between these three tests whose principles and sensitivity are very different (Basketter and Scholes 1992; Kimber *et al.*, 2001). The complexity and

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multiparametric character of sensitization also makes it very difficult to reproduce *in vitro*. Different approaches have been proposed. Simplified models, such as permeation in human skin explants (Davies *et al.*, 2011), haptenation reaction (Mutschler *et al.*, 2009) or cell culture based models (Corsini *et al.*, 2009) taking into consideration only one parameter of the sensitization process, can hardly reproduce the complexity of *in vivo* mechanisms. Integrated testing strategies (ITS) combining multiple *in vitro*, *in silico* and even *in chemico* approaches (Hoffmann *et al.*, 2008; Jaworska *et al.*, 2011, 2013; Wanner *et al.*, 2010) have been developed to approach the complexity of human physiology and generally provide reliable *in vivo* extrapolations from *in vitro* data (Hartung *et al.*, 2013). However, the cost and technical requirements of highly sophisticated multicellular human skin models (Aeby *et al.*, 2010), represents a major limitation for industrial use. According to EU guidelines, the obligation to switch to *in vitro* sensitization tests has been mandatory for cosmetic products since 2013 (Regulation EC no. 1223/2009). It is now urgent to propose strategies to evaluate *in vitro* skin sensitization using multiparametric approaches and techniques keeping the cost of such tests within reasonable limits (Basketter *et al.*, 2007; ICCVAM, 2013; Maxwell *et al.*, 2011; Natsch *et al.*, 2009).

In the present study, we developed an *in vitro* sensitization test based on four key parameters defined to allow the differentiation between sensitization and acute irritation. This model was tested over a series of molecules selected for their use in the cosmetic industry, covering the greatest diversity of molecular structures and including agents of all degrees of sensitizing potentials. The results have been compared to those from the literature obtained using GPMT, LLNA and/or HPT *in vivo* reference tests.

Material and Methods

Chemicals

All sensitizers and non-sensitizers tested, caffeine, human serum albumin (HSA), urea, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and salts for Ringer's buffer were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Acetonitrile, isopropanol and orthophosphoric acid were from Carlo Erba (Val de Reuil, France). Cell culture medium (RPMI) and supplements (glutamine, antibiotics/antimycotic solution and fetal calf serum) were provided by Lonza (Levallois-Perret, France).

Definition of the Data Bank of Cosmetic Sensitizers and Non-sensitizers

This first part of the project was aimed at defining a series of 400 different small molecules used in the cosmetic industry. The molecules were selected based on six criteria:

- Diversity of structure, biological activity and mechanisms of action
- Variety of sensitization potential
- Quality of *in vivo* sensitization activity data
- Availability as pure compounds
- Potential detection by classical analytical methods: high-performance liquid chromatography (HPLC) or capillary gas chromatography (CPG)
- Compatibility with a vehicle allowing their administration on living cells.

In a first step, we focused on the ICCVAM list (ICCVAM test methods, 2006), the International Cosmetic Ingredient Dictionary

and Handbook (2010), including 13 000 molecules and the list of the 26 allergens recognized by the European Union (Directive 2003/15/EC).

In a second step, we took advantage of the data base "CosIng" created by the EU, including data on all cosmetic ingredients produced since the "Cosmetics Directive" 76/768/EEC (Commission Decision 2006/257/EC) and information from the Scientific Committee for Consumer Products (SCCP Fragrances, 2013). These two lists cover 22 000 ingredients regularly associated with reliable *in vivo* toxicological data. All mixtures and complex solutions were systematically excluded from the list and only pure compounds of defined chemical structure and CAS number were kept. To form a homogeneous list, we only focused on molecules that have been submitted to GPMT, LLNA and/or HPT. However, contradictory results were frequently encountered. For that reason, the molecules were classified based on four different criteria by rank of priority, i.e., inclusion in the EU list of allergens or R43 classification according to the European Substance Information System leading to a direct identification as sensitizer, reports from specific studies, reports from LLNA studies and reports from GPMT studies. As the advantage/risk approach cannot be applied to cosmetic compounds, we decided to classify the molecules in two categories, i.e., sensitizers and non-sensitizers. Finally, the sensitizer category covered molecules of very different activities ranging from low to extreme.

General Design of the *In Vitro* Model

A detailed analysis of the skin sensitization process led to an identification of four key steps that can be reproduced by specific *in vitro* tests assuming that only a combination of these tests can generate a complete model of sensitization (Fig. 1). The first of these parameters is the kinetics and intensity of the transcutaneous flux of the xenobiotic (permeation). Indeed, a potential sensitizer should remain inactive if it does not penetrate into the skin in a sufficient amount over a specific time. This factor (noted A) can be evaluated by measuring the flow through human skin explants using Franz flow cells. The drawback to this approach is that an analytical technique must be developed for each cosmetic molecule studied. Afterwards we focused on molecules for which the development and validation of an analytical procedure by HPLC coupled with ultraviolet (UV) detection and in certain instances, gas chromatography coupled with mass spectrometry (CPG/MS) made reaching a sufficient level of sensitivity possible. As mentioned elsewhere, small cosmetic molecules are rarely capable of triggering an immune response by themselves and need to bind with endogenous compounds, generally proteins, to elicit the sensitization process. It was then decided to evaluate this haptenation step (factor B) by using an *in vitro* partition assay between bound and free molecules in the presence of a representative protein. As an analytical procedure was developed for each tested molecule, it was then possible to assay the free form of the molecule after separation of the bound fraction. The complex formed with the hapten is capable of activating both keratinocytes and/or dendritic cells. The response of keratinocytes can be easily evaluated using either cultured cell lines or reconstructed skin patches. Dendritic cell culture is much more delicate and expensive, and as our target was to develop a test simple enough to be employed in screening studies, we decided to exclude this parameter from our model. There is another key parameter where we needed to select an adequate molecular marker to distinguish between sensitization and irritation reactions. We

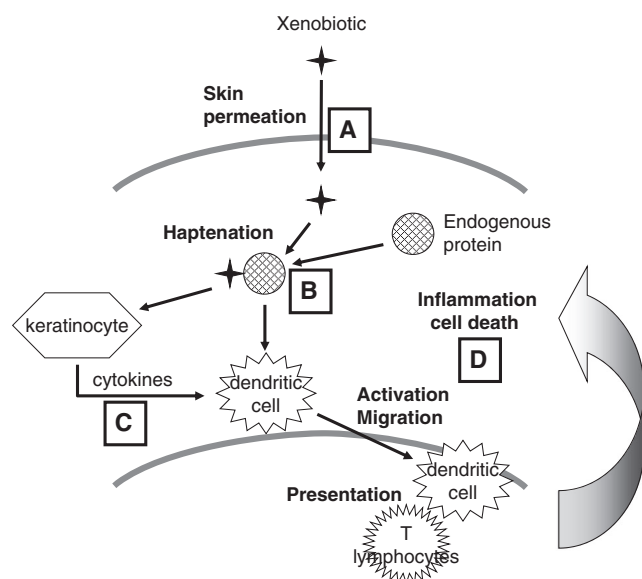


Figure 1. General pathway of sensitization induction showing the four key steps selected to design the present *in vitro* model. (A) Skin permeation. (B) Haptentation. (C) Production of sensitization specific cytokine (interleukin 18). (D) Acute toxicity.

decided to use interleukin 18 (IL18) as a marker of sensitization (factor C). To define standard values of IL18, tested compounds must be employed at sublethal concentrations. The cytotoxic activity of the molecules was then selected as a complementary parameter (factor D) assuming that an irritation reaction should be associated to a high and immediate cytotoxicity whereas a potential sensitization reaction should be associated to a low immediate cytotoxicity.

Measure of Skin Permeation (Factor A)

Skin permeation was used as an indirect evaluation of the real amount the xenobiotic capable to accumulate into the skin for a length of time sufficient to induce the sensitization reaction. This parameter was measured using Franz diffusion cells (Fig. 2). Human skin resulting from abdominoplasties was obtained by agreement with a French BioBank (Biobanque de Picardie, CHU Amiens, Salouel, France). After removing the subcutaneous fat, the full-thickness abdominal skin of women was mounted horizontally on the Franz cells with the dermis facing down, between the donor and receptor compartments. The exposed skin surface area was 2.0 cm², and the receptor compartment volume was 6 ml. The donor medium consisted of 0.1 ml of xenobiotic-containing Ringer's buffer (pH 6.8, containing 6.72 g l⁻¹ NaCl, 2.1 g l⁻¹ NaHCO₃, 0.243 g l⁻¹ MgCl₂ 6H₂O, 0.176 g l⁻¹ CaCl₂ 2H₂O, 0.416 g l⁻¹ KH₂PO₄, 0.054 g l⁻¹ K₂HPO₄ in deionized water) and the receptor medium was only Ringer buffer (pH 6.8). The Franz diffusion cells were connected to a circulating water bath, which yielded a tissue temperature of 32 °C comparable to the physiological temperature of the skin surface. The receptor side content was mixed using a magnetic stirring device to ensure appropriate homogenization of the released xenobiotic throughout the experiment. Just before administration of the tested molecule on the membrane surface, a 1000 µl sample was collected from the receptor chamber. One day (24 h) after beginning the experiment, a 1 ml aliquot of the receptor medium was collected and the amount of xenobiotic was determined using

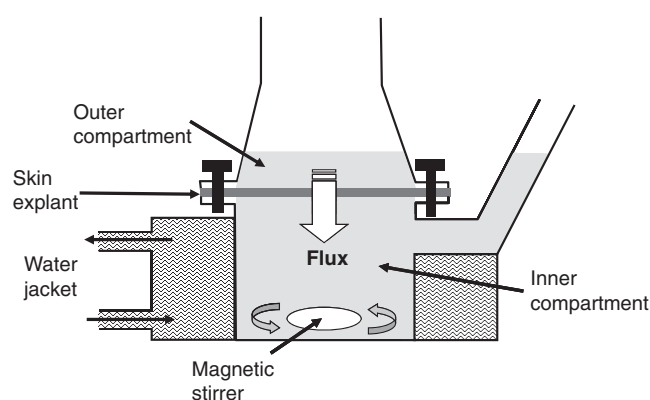


Figure 2. Schematic representation of Franz diffusion cells used to measure the skin permeation of xenobiotics.

the chromatographic facilities. Samples were frozen and stored at - 20 °C until further analysis. Data analyses of cumulative amounts of xenobiotic permeation studies (minimum three measures) were used to calculate the transdermal drug flow.

The skin flow can be experimentally determined from the following equation:

$$J = (dQ/dt)/A$$

where J is the flow (µg cm⁻² h⁻¹), A is the diffusion area of skin tissue (cm²) through which drug permeation occurs, and dQ/dt is the amount of drug passing through the skin over time.

To compare the flows of numerous molecules rapidly and efficiently, we chose to use a single endpoint flow (24 h). Even if these data does not take into account the lag time, steady-state period and skin adsorption, it is coherent with the mean flow values and is used even for industrial purposes in regulatory studies to compare individual data.

The threshold between slow and rapid diffusion was selected as the skin flow of caffeine (2.011 µg cm⁻² h⁻¹) in our model). The use of caffeine as a reference active cosmetic ingredient in Franz cells permeation experiments is widely documented (Kim *et al.*, 2002; Trauer *et al.*, 2009). For each molecule tested, a specific analytical procedure was then developed. Molecules were detected and assayed by HPLC/UV (Gilson 305 HPLC pump equipped with an ABI KRATOS Spectroflow 783 UV/Vis Absorbance Detector, Villiers-le-Bel, France) or CPG/MS (HP Hewlett Packard Agilent 6890 Plus GC System Gas Chromatograph with HP 5973 MSD Mass Selective Detector, Marcy L'Etoile, France). These detection techniques are presented in detail in Table 1.

Measure of the Haptentation Potential (Factor B)

The potential of cosmetic compounds to form stable covalent complexes was studied using HSA as a binding protein. HSA is present in the skin in large amounts, is more soluble, easy to use and cheaper than pure keratin or collagen. This protein appears as a good model to investigate the haptentation potential of skin sensitizers (Aleksic *et al.*, 2007). A solution of the test molecule (250 µl in phosphate-buffered saline 0.1 M, pH 7.4) was mixed with 250 µl of a solution of HSA (4 mg ml⁻¹ in phosphate-buffered saline). The same optimal molar ratio of the tested molecule and HSA (2.2 : 1) was used in all experiments. This solution was incubated for 4 h at 20 °C in PVC centrifugation tube. Urea powder was added to reach a final concentration of 8 M. The

Table 1. Analytical techniques used for detection of the 32 different molecules included into the present study

Molecule	CAS number	Chromatographic system	Analytical column	Elution program	Detection threshold ($\mu\text{g ml}^{-1}$)
2,4-dimethyl-3-cyclohexene-1-carboxaldehyde	68039-49-6	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	1
2-octyl-3(2H)-isothiazolone (pestanal)	26530-20-1	HPLC/UV	Inertsil C18 ODS2 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (80/20 v/v)	0.04
2-thiobenzimidazole = proxel active	2634-33-5	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.01% trifluoroacetic acid (30/70 v/v)	0.2
3,4 dihydrocoumarin	119-84-6	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.08
5-chloro-2-methyl-4-isothiazolin-3-one = kathon	26172-55-4	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (20/80 v/v)	0.02
Abietic acid	514-10-3	HPLC/UV	Supelco Kromasil C8 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (80/20 v/v)	0.04
Benzocaine	94-09-7	HPLC/UV	Lichrosphere 60 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.04
Benzoic acid	65-85-0	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.4
Benzyl alcohol	100-51-6	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	5
Benzyl benzoate	120-51-4	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (20/80 v/v)	0.04
Benzyl salicylate	118-58-1	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (90/10 v/v)	0.04
Bronopol	52-51-7	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (90/10 v/v)	0.4
Cinnamaldehyde	104-55-2	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (10/90 v/v)	0.02
Cinnamyl alcohol	104-54-1	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.04
Citral	5392-40-5	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.4
Coumarin	91-64-5	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.04
Dihydroeugenol	2785-87-7	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.08
Ethyl vanillin	121-32-4	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.04
Eugenol	97-53-0	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.01% trifluoroacetic acid (50/50 v/v)	0.04
Geraniol	106-24-1	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.4

Table 1. (Continued)

Molecule	CAS number	Chromatographic system	Analytical column	Elution program	Detection threshold ($\mu\text{g ml}^{-1}$)
Ethylene glycol dimethacrylate	97-90-5	HPLC/UV	Intertsil C18 ODS2 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (70/30 v/v)	0.2
Isoeugenol	97-54-1	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.04
Linalol	78-70-6	HPLC/UV	Lichrosphere 100 RP-select B 250 mm $\times$ 4 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (60/40 v/v)	0.4
Methylparaben	99-76-3	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (20/80 v/v)	0.2
Octanoic acid	124-07-2	GC/MS	UB-Wax 30 m $\times$ 0.25 mm $\times$ 0.25 μm	$T_0 = 60^\circ\text{C}$ – from 60 to 150°C by $20^\circ\text{C min}^{-1}$ – 15 min at 150°C from 150 to 250 at $30^\circ\text{C min}^{-1}$	5
Panthenol	16485-10-2	HPLC/UV	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (5/95 v/v)	0.4
Propyl gallate	121-79-9	HPLC/UV	Supelco Kromasil C8 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (30/70 v/v)	0.04
Saccharin	81-07-2	HPLC	Intertsil C18 ODS2 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.01% acetic acid (85/15 v/v)	0.4
Salicylic acid	69-72-7	HPLC	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	55/45 TFA 0.01%/ACN	0.2
Sodium benzoate	532-32-1	HPLC	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.4
Sodium dehydroacetate	4418-26-2	HPLC	Intertsil C18 ODS2 250 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.085% orthophosphoric acid (40/60 v/v)	0.02
Vanillin	121-33-5	HPLC	Hypersil C18 200 mm $\times$ 4.6 mm $\times$ 5 μm	Acetonitrile/water with 0.01% trifluoroacetic acid (20/80 v/v)	0.02
GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet.					

solution was subsequently incubated 1 h at 20 °C. Urea made it possible to destabilize low energy interactions keeping only covalently bound haptens. An aliquot of the solution (500 µl) was then layered on to a Microcon (Millipore, Molsheim, France) filtration unit (cutoff 10 000 Da) and centrifuged for 2 h at 10 000 g. The free unbound molecule recovered in the lower compartment containing the filtrate was then quantified by HPLC, CPG or CPG-MS.

Measure of Interleukin 18 Production (Factor C)

As indicated by Corsini *et al.* (2009), IL18 production was tested using the NCTC 2544 human keratinocyte cell line. Cells were grown at 80% confluence and IL18 production was measured at a cell death ratio of 75% as established by these authors. The technique was validated initially by reproducing the data of Corsini *et al.* (2009) using two sensitizers (cinnamaldehyde and paraphenylenediamine) and two non-sensitizers (salicylic acid and sodium dodecyl sulfate). IL18 was assayed using a commercial ELISA kit (R&D Systems Europe, Lille, France).

Determination of the MTT⁷⁵ Value (Factor D)

The cytotoxicity of the molecules was determined using an MTT assay based on the reduction of yellow dye MTT into blue formazan under the effect the living cell's mitochondrial respiratory activity (Mosmann, 1983). Pre-tests were conducted to verify that all studied molecules had no direct effect on MTT reduction and did not interfere in the assay. The measures were made on 96 well microplates layered with a mean of 2×10^4 cells per well. After 48 h of incubation (37 °C, 5% CO₂) the medium (RPMI medium supplemented with 10% fetal calf serum, glutamine and antibiotic mix penicillin/streptomycin/fungizone) was withdrawn and replaced with a medium containing the tested molecule. Triton X100 was used as a positive control. The plates were incubated for 24 h, rinsed with phosphate-buffered saline and MTT dissolved in RPMI medium (0.75 mg ml⁻¹) was added in each well. After 3 h of incubation, the MTT solution was removed and an acid solution of isopropanol (isopropanol 96%/HCl 1 N 4%, v/v) was distributed in each well. The OD_{570nm} of the solution was measured after 30 min incubation at room temperature. The molecules were tested at different concentrations to determine the value inducing 75% of cell death (MTT⁷⁵ value).

Data analysis and Definition of a Decision Grid

For each molecule, every factor was determined as the mean of a minimum of three independent experiments $\pm$ standard error of the mean (SEM). When one of the four factors was not possible to measure, the molecule was excluded from the final analysis. For each factor, a threshold between the response to

sensitizers and non-sensitizers or irritant molecules was determined. These values, summarized in the decision grid presented in Table 2, were selected to reach the maximum correlation with data from *in vivo* assays using by rank of priority results from LLNA, GPMT and HPT.

Results

Among the 400 molecules of the data bank, only 91 different molecules were tested. The causes that led to reject many molecules were insolubility, instability in solution, excessively rapid evaporation or absence of validated analytical methods. The value of factor A was then measured on 33 different molecules, the value of factor B on 72 molecules, and that of factors C and D on 68 molecules. The diversity of the molecules was preserved as, for each factor, compounds having high- or low-intensity reactions according to Table 2 were studied (Fig. 3). However, the choice of classifying the compounds into only two categories (sensitizers/non-sensitizers) led to a relative increase in the number of molecules considered as sensitizers.

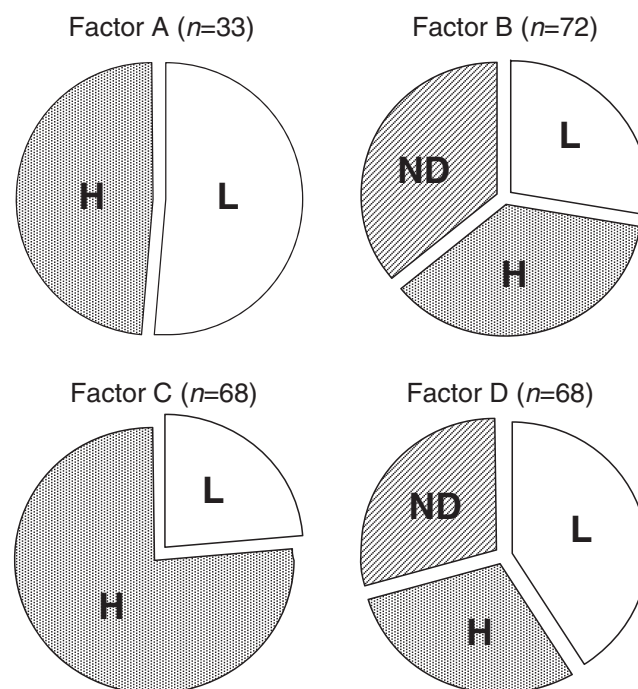


Figure 3. Distribution of the different molecules tested in regards to their response to the four selected factors following the thresholds presented in Table 2. H, high-intensity response; L, low-intensity response; ND, intermediate phenotype.

Table 2. Decision grid used to distinguish molecules inducing a high or low response to each of the four factors tested and interpretation in regards to the classification as non-sensitizer or sensitizer

	A Resorption ($\mu\text{g h}^{-1} \text{cm}^{-2}$)	B Haptentation (%)	C IL-18 (%)	D Acute cytotoxicity ($\mu\text{g ml}^{-1}$)
Non-sensitizer (No effect)	0	Low < 5	Low < 20	Low > 100
Non-sensitizer (irritant)	High > 2011	Low < 5	Low < 20	High < 100
Sensitizer	Low < 2011	High > 5	High > 20	Low > 100

This was particularly the case for factor C. For other factors, the proportion of sensitizers, non-sensitizers and in the case of factors B and D of molecules with intermediate behavior was stable. Finally, only 32 molecules were completely tested with the four selected factors (Table 3). When individual results for each factor were compared to data from GPMT, LLNA and HPT studies, the percentage of correlation was modest ($< 50\%$ for factor A and between 60 and 70% for factors B, C and D). However, the real correlation of this *in vitro* sensitization model with *in vivo* data can be only estimated after combining the four individual factors as presented in Table 4. In this case, the present *in vitro* sensitization model led us to consider the majority of tested molecules (18 molecules, i.e., 56.2% of the total) as sensitizers whereas non-sensitizers were only nine (28.1%). Five other molecules, namely 2,4-dimethyl-3-cyclohexene-1-carboxaldehyde, benzocaine, benzyl alcohol, sodium benzoate and vanillin, led to ambiguous results. As the study was based on the application of the safety principle, we decided to assimilate all unclassified molecules as potential sensitizers. In these conditions, the number of sensitizers reached 23 molecules and these molecules were over-represented in comparison to non-sensitizers (nine). However, considering that any molecule described *in vivo* as a weak to strong sensitizers was assimilated to a "sensitizer," the general coefficient of correlation between our *in vitro* approach and the reference *in vivo* data reached 81.2%. Only one false negative result was observed with sodium dehydroacetate (CAS 532-32-1), which in the GPMT is a moderate sensitizer whereas it appeared *in vitro* as a non-sensitizer. Other differences were one case of a false positive result and three cases where our *in vitro* model gave an ambiguous result whereas the molecules are non-sensitizers *in vivo*. If we assume that false negative results are the more critical cases in such a test, it appears that this *in vitro* model of skin sensitization led to safe results for 31 molecules of 32, i.e., in 96.8% of the cases.

Discussion

Since the definition of the 3R principle by Russell and Burch (1959), EU guidelines have promoted the development of alternative toxicological methods. These methods have evolved in two directions, i.e., *in silico* and *in vitro* approaches. In the case of *in silico* approaches, quantitative structure–activity relationships models have taken advantage of the rapid increase of calculator performances. Now, despite being able to perfectly integrate the 3D and thermodynamic variations of complex molecules, functional relationships of molecular descriptors remain initially dependent on the quality of experimental biological data (Chaudhry *et al.*, 2010; Liu *et al.*, 2008). The OpenTox project funded by the EU (Hardy *et al.*, 2010) was initiated to unify these data as they are often partial, obtained through very heterogeneous techniques and even sometimes contradictory. Consequently, *in vitro* approaches occupy an interesting intermediate position between *in vivo* experiments, whose legal application field is rapidly reducing, and *in silico* models. As mentioned in the introduction, the combination of these different models led to ITS, which are by themselves new promising approaches (Hartung *et al.*, 2013). In fact the present *in vitro* model is aimed at being combined with an *in silico* model that should be developed independently. An evolution since the initial conception of the project concerned the technique used to quantify a possible sensitization reaction. Indeed, we expected to employ reconstructed human skin explants such as RHE Skinethic[®], as

this model has been validated by ECVAM for the evaluation of skin irritation (Spielmann *et al.*, 2007). In addition, this model allows the use of suspensions of insoluble compounds. It has been shown that in this model, sensitizers are characterized by a high production of IL8 and a low production of IL1 α whereas non-sensitizers (irritants) generate a high amount of IL1 α and a low quantity of IL8 (Coquette *et al.*, 2003). However, skin explants appeared very sensitive to the conditions of surface application of the molecule and the dispersion of the results was excessively high. The origin of the tissue (foreskin or abdominal surgery) and the age of the donor have also certainly a large influence. This observation is consistent with that of a previous report (Coquette *et al.*, 1999) showing that the IL1 α /IL8 can lead to ambiguous results. As an alternative, it has been demonstrated that in human keratinocytes, allergens but not irritants, induce IL12 expression (Corsini *et al.*, 1999). However, more recently Antonopoulos *et al.* (2008) showed that IL18 should play a more central role in the cutaneous immune response and as this cytokine is constitutively expressed by human keratinocytes (Naik *et al.*, 1999) we decided to use it as a sensitization marker in our model.

Whereas simple local reactions, such as irritation or corrosion can be relatively easily reproduced *in vitro* using only one model (Kolle *et al.*, 2013), the challenge is much harder when we focus on complex mechanisms such as immune reactions and allergy. Nevertheless, the development of *in vitro* skin sensitization models is an immediate and major challenge for the cosmetic industry (Goebel *et al.*, 2012). As presented in the introduction chapter, in most cases, *in vitro* models developed until now have been focused on a key step of the sensitization process whereas the target is to describe the overall behavior of molecules, taking into consideration the whole pathway leading to the inflammatory response associated to sensitization. Defining an operational model also requires taking into consideration both scientific and economic constraints. In the present case, we decided to use simple techniques but to look at different key steps of the sensitization process. The interest of this strategy is clearly visible when we examine the individual coefficients of correlation of each factor with *in vitro* data. For instance, the correlation of skin permeation kinetics (factor A) with GPMT, LLNA or even HPT is low ($< 50\%$). However, this factor is only an endpoint measure of skin permeation and it does not by itself illustrate the sensitizing potential of a molecule although it was used in combination with multicompartamental modeling to generate a model of skin sensitization (Davies *et al.*, 2011). In fact, the combination of the four factors in the decision grid led to a final correlation between *in vivo* and *in vitro* assays of 81.2%. The measure of gene expression changes in human THP-1 monocytes, validated over the same number of chemicals as in the present study, led to 78% of results in agreement with *in vivo* data (Arkusz *et al.*, 2010). Compared only to the LLNA, the peptide reactivity assay measured on 38 chemicals presented accuracy values between 36.1 and 83.8% depending on the peptide used (Gerberick *et al.*, 2004). For this reason, our model presents a general gain over previously proposed models. In addition, in the present case, our results were not only compared to LLNA studies but also to data from GPMT and HPT experiments, which allow a more transversal comparison with these reference *in vivo* models. It is also important to note that these three *in vivo* tests are also frequently incoherent between each other. For instance, if we look only at the 32 molecules tested in the present study, discrepancies are observed with evaluations made using LLNS, GPMT and HPT in 25% of the cases.

Table 3. Experimental values of permeation (factor A), haptenation (factor B), IL-18 production (factor C) and MTT⁷⁵ value (factor D) measured for each of the 32 cosmetic compounds finally studied

Molecule	CAS number	Factor A Permeation ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Factor B Haptenation (%)	Factor C IL-18 (% control)	Factor D MTT ⁷⁵ value ($\mu\text{g ml}^{-1}$)
2,4-dimethyl-3-cyclohexene-1-carboxaldehyde	68039-49-6	0	3.7 ± 3	+ 31.4 ± 3	> 30
2-octyl-3(2H)-isothiazolone = kathon = pestanal	26530-20-1	0.469	5.5 ± 1	+ 29.2 ± 2	2.8 ± 1
2-thiobenzimide = proxel active	2634-33-5	3.019	13.1 ± 2	+ 27.8 ± 4	> 10
3,4 dihydrocoumarin	119-84-6	0	9.0 ± 9	+ 33.5 ± 11	> 30
5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	3.225	20.7 ± 1	+ 33.1 ± 7	0.6 ± 0.3
Abietic acid	514-10-3	0.087	31.5 ± 2	+ 21.8 ± 4	61 ± 16
Benzocaine	94-09-7	0.462	0.0 ± 0	+ 21.5 ± 8	> 100
Benzoic acid	65-85-0	0.467	6.7 ± 8	+ 26.6 ± 6	> 1000
Benzyl alcohol	100-51-6	6.636	6.7 ± 1	+ 21.4 ± 4	> 1000
Benzyl benzoate	120-51-4	0	5.8 ± 8	+ 48.0 ± 35	> 30
Benzyl salicylate	118-58-1	0.041	6.0 ± 3	+ 27.8 ± 17	16 ± 7
Bronopol	52-51-7	3.362	19.9 ± 1	+ 4.4 ± 2	10.5 ± 3
Cinnamaldehyde	104-55-2	2.061	7.4 ± 3	+ 40.2 ± 20	54 ± 7
Cinnamyl alcohol	104-54-1	12.143	1.8 ± 3	+ 21.8 ± 4	347 ± 27
Citral	5392-40-5	0.232	9.5 ± 7	+ 20.3 ± 5	19 ± 1
Coumarin	91-64-5	10.371	6.0 ± 4	+ 7.1 ± 6	> 30
Dihydroeugenol	2785-87-7	2.554	1.2 ± 1	+ 25.5 ± 4	> 10
Ethyl vanillin	121-32-4	6.281	3.6 ± 3	+ 21.7 ± 4	> 100
Eugenol	97-53-0	1.009	10.5 ± 4	+ 109.7 ± 57	10 ± 2
Geraniol	106-24-1	1.316	2.3 ± 2	+ 27.1 ± 10	> 30
Ethylene glycol dimethacrylate	97-90-5	0	24.6 ± 41	+ 22.5 ± 10	> 100
Isoeugenol	97-54-1	3.213	7.2 ± 4	+ 28.2 ± 8	92 ± 54
Linalool	78-70-6	2.449	2.2 ± 3	- 20.6 ± 12	> 30
Methylparaben	99-76-3	0.015	3.5 ± 1	+ 22.0 ± 3	17 ± 2
Octanoic acid	124-07-2	0	8.1 ± 3	- 9.8 ± 13	385 ± 108
Panthenol	16485-10-2	8.416	1.5 ± 1	- 3.2 ± 5	> 1000
Propyl gallate	121-79-9	0.096	4.6 ± 5	+ 38.4 ± 10	30 ± 8
Saccharin	81-07-2	1.261	2.5 ± 2	+ 27.9 ± 8	> 10
Salicylic acid	69-72-7	13.806	1.3 ± 1	- 32.4 ± 6	88 ± 6
Sodium benzoate	532-32-1	0.755	3.3 ± 2	- 5.3 ± 4	> 1000
Sodium dehydroacetate	4418-26-2	3.153	0.0 ± 0	+ 40.7 ± 9	578 ± 140
Vanillin	121-33-5	4.789	2.0 ± 1	- 7.7 ± 6	353 ± 33
IL, interleukin.					

Table 4. Final analysis of experimental values for the 32 cosmetic compounds tested after application of the decision grid (Table 2)

Molecule	CAS	Factor A	Factor B	Factor C	Factor D	Result	LLNA	GPMT	HPT	FIT
2,4-dimethyl-3-cyclohexene-1-carboxaldehyde	68039-49-6	L	L	H	+/-	+/-	M		St	(+)
2-octyl-3(2H)-isothiazolone = kathon	26530-20-1	L	H	H	H	S	M		S	+
2-thiobenzimidazole = proxel active	2634-33-5	H	H	H	+/-	S	M	St	S	+
3,4 dihydrocoumarin	119-84-6	L	H	H	+/-	S	M	St	S	+
5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	H	H	H	H	S	E	St	S	+
Abietic acid	514-10-3	L	H	H	H	S	W	M	S	+
Benzocaine	94-09-7	L	L	H	L	S	0	M	S	(+)
Benzoic acid	65-85-0	L	H	H	L	S	0	0	W	-
Benzyl alcohol	100-51-6	H	H	H	L	+/-			0	(+)
Benzyl benzoate	120-51-4	L	H	H	+/-	S	W	W	0	+
Benzyl salicylate	118-58-1	L	H	H	H	S		W	0	+
Bronopol	52-51-7	H	H	L	H	N		W	0	+
Cinnamaldehyde	104-55-2	H	H	H	H	S	M	St	S	+
Cinnamyl alcohol	104-54-1	H	L	H	L	S	W	M		+
Citral	5392-40-5	L	H	H	H	S	W	M	S	+
Coumarin	91-64-5	H	H	L	+/-	N	0	0	W	+
Dihydroeugenol	2785-87-7	H	L	H	+/-	+/-	M	St		-
Ethyl vanillin	121-32-4	H	L	H	L	N	0	0		+
Eugenol	97-53-0	L	H	H	H	S	W	M	St	+
Geraniol	106-24-1	L	L	H	+/-	S	W	0	S	+
Ethylene glycol dimethacrylate	97-90-5	L	L	H	L	S	W	0	S	+
Isoeugenol	97-54-1	H	H	H	H	S	M	St	S	+
Linalol	78-70-6	H	L	L	+/-	N	W		0	+
Methylparaben	99-76-3	L	L	H	H	+/-	0	0	W	-
Octanoic acid	124-07-2	L	H	L	L	S	0		^a	-
Panthenol	16485-10-2	H	L	L	L	N		0		+
Propyl gallate	121-79-9	L	H	H	H	S	S	St	S	+
Saccharin	81-07-2	L	L	H	+/-	+/-	0			-
Salicylic acid	69-72-7	H	L	L	H	N	0	0	0	+
Sodium benzoate	532-32-1	L	L	L	L	N		0	W	(+)
Sodium dehydroacetate	4418-26-2	H	L	H	L	N		M		-
Vanillin	121-33-5	H	L	L	L	N	0	W		(+)

^aR38 skin irritant.

Classification of individual responses to each factor is recorded as high (H), low (L) or intermediate (+/-). The final decision concerning the sensitizing (S), non-sensitizing (N) or eventually undetermined (+/-) activity of the molecules in our model is presented in the "Result" column. These results are compared to data from LLNA, GPMT and HPT studies where the degree of sensitizing activity was nul (0), weak (W), moderate (M), well demonstrated (S), strong (St) or extreme (E). The final correlation of the present *in vitro* model with these *in vivo* data is summarized as positive (+) or negative (-) in the column "FIT." For safety reasons, molecules presenting an undetermined phenotype were classified as sensitizer, if the result correlated positively with *in vivo* data it was noted (+) in the table.

In the list of chemicals tested in the present study, the number of sensitizers was over-represented but this is a consequence of our choice to separate the compounds into two classes to adapt to the needs of cosmetic molecule evaluation. There are advantages provided by this approach that, are mentioned by other authors (Aeby *et al.*, 2010; Basketter *et al.*, 2008), and this is at the basis of ITS. Covering different key points of the sensitization process, it is certainly safer for a predictive model when it is confronted with molecules of unknown activity.

This model certainly presents several limitations. Dendritic cells were not included and that is essential to note. These cells play a key role in the recognition and presentation of allergens to responsive T lymphocytes and, in this way, determine the intensity of skin sensitization (Ryan *et al.*, 2007). However,

dendritic cell culture is long and complex (Casati *et al.*, 2005; Ryan *et al.*, 2007) and was not used to keep the potential price of the test commercially viable. Another and more technical limitation was the difficulty to investigate insoluble molecules in aqueous buffer. This is a general limitation for all models based on cell culture but until now, all our attempts to realize the same work on reconstructed human skin were unsuccessful, essentially because of the interassay variability of the model. It should be possible to increase the series of tested molecules by adding a co-solvent to all weakly water-soluble compounds. The skin permeation kinetic values may then be biased as these vehicles act generally as passage enhancers (Asbill and Michniak, 2000). The use of such co-factors should have sense only if they are present in the final cosmetic formulation. Certain molecules

were also excluded from the study as they have an excessive partial gas pressure and evaporate rapidly. This makes it impossible to control the balance of mass at the end of the kinetics and haptentation studies. It should be possible to use gas-tight Franz cells to avoid the loss of tested molecules during the experiment. However, the same sublimation or evaporation process occurs naturally when the molecules rest on the skin. Although the results on these molecules should be less rigorously established as the balance of mass cannot be calculated, such molecules could be reintroduced into the list of tested compounds in further studies. The speed required to realize a whole sensitization evaluation is also a major parameter. According to ISO 10993-10 (2010), a GMPT cannot be realized within less than 3 weeks. In this *in vitro* model, the most time-consuming step is the development and validation of the analytical procedure necessary for permeation and haptentation tests. Micro-Franz cells can be now operated using automation (presently Franz Hanson Research Cells equipped with Microette plus Hanson Research 60-301-106, Corn switch Hanson Research 81-500-400 and Multifill Hanson Research 60-200-491) and we can postulate that the system can be operated without HPLC or CPG analysis. Indeed, in the permeation and haptentation tests, we are only looking at the distribution of a known molecule between physical or chemical compartments and we have no need to identify it again. In fact, we only need to determine the mass of free molecules in haptentation studies and those that reach the receptor compartment in the Franz cell studies. Such information can be obtained by non-analytical techniques. For instance, high throughput techniques such as surface plasmon resonance, which use chips grafted with high-affinity ligands or antibodies, leads to a rapid and high sensitivity detection of compounds in the pico- or even femo-molar range of concentration independently of any analytical molecular identification (Pattnaik, 2005). In these conditions, if we exclude the time necessary to grow the cells to subconfluence, a complete test as previously described should be possible within 3 days.

The present model is simple, well correlated to *in vivo* data, validated over cosmetic compounds of different structures and degrees of sensitization activity and presents a wide potential of evolution, which gives the opportunity to propose it for a wide use *in vitro* alternative to GPMT, LLNA or HPT.

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Conflict of Interest

The Authors did not report any conflict of interest.

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