28th July – 7th August 2019
Summer School of Science S3
Požega, Croatia

Do we really need animal testing to keep us safe?

Tania Capdevila, The British School of Barcelona, Barcelona, Spain
Felix Lahr, Berlin International School, Berlin, Germany
Lana Lukačević, I. Gymnasium, Zagreb, Croatia
Nora Marčetić, V. Gymnasium, Zagreb, Croatia
Nicoleta Spinu, Liverpool John Moores University, Liverpool, United Kingdom

Abstract

In 2009, the European Commission passed a Regulation (No. 1223/2009) that prohibited use of animal testing for cosmetic products. This is complemented by the REACH regulation (EC/1907/2006), according to which the animal testing of industrial chemicals should only be carried out as a last resort. As a result, alternative methods to animal testing have been developed to assess chemical toxicity in order to protect both users and environment. Herein, we aimed to understand the concept of 3Rs, legislative framework and existing *in vitro* and *in silico* methods, with an emphasis on computational approaches. Using available databases and software tools such as KNIME and Cytoscape, an adverse outcome pathway on skin sensitisation has been modelled including development of a structure-activity relationship for a chemical class. The project allowed to broaden our knowledge in regards to chemical safety assessment in the European Union along with the emerging research topics while applying analytical thinking to several proposed exercises.

Table of Contents

Introduction

Chemical testing assesses the efficacy of a chemical, as well as the safety of a user and the safety of our environment. For the longest time, tests on cosmetics and drugs have been carried out on animals. The Regulation (EC) N° 1223/2009 banned cosmetic tests on animals from March 2013. Drugs' safety and efficacy is still checked on animals as there aren't high-quality alternative tools to represent and predict possible effects of a drug. Nowadays, with the advancements in technology and scientific discovery, as well as the ethical concerns, alternative methods of testing chemicals have been proven to be more efficient than animal testing as briefly summarised below in Table 1.

Table 1. The use of animal testing and alternative methods for chemical assessment

Animal testing	**Alternative methods**
• Help avoid health disasters (e.g. a crisis caused by the chemical *sulfonylamide* in the 1930's) • Were used successfully in research (e.g. Pasteur's work on the anthrax vaccination, Pavlov's dog experiment pioneering classical conditioning and mirror neurons in our brains that are responsible for empathy tested on monkeys)	• Resemble humans' physiology and metabolism more closely than animal tests (e.g. a failure rate of 99.6% of drugs for Alzheimer's disease that had been tested on animals) • Differences between sexes are neglected (male mice are cheaper and used more often) • Open-up opportunities (e.g. while aspirin proved to be ineffective on animals, it proved to be effective in humans) • Several drugs/chemicals can be tested at once (e.g. high throughput techniques)

Alternative methods for animal testing are methods used for chemical testing instead of *in vivo* procedures. These methods include:

- ***in vitro* studies** - performed outside of the living organism on samples of isolated organs, tissues or cells (e.g. organoids, cell cultures, organ-on-a-chip)
- ***in silico* studies** - takes place on computers and involves the use of models and data to accurately predict the effects on a consumer and the interactions with other chemicals
- ***in chemico* studies -** does not include alive sample
- **Volunteer studies** - provide clinics and hospitals with a way to collect data about actual human physiology
- **Epidemiological studies -** held on population who share a common characteristics (e.g. sex, age, health condition).

Talking about *in silico*, several computational methods and tools are used in toxicology assessments. One of them is structure-activity relationship (SAR) modelling, which focuses on the interaction between the chemical structure and

the biological effects a chemical can evoke in the body. This model was refined to give quantitative SAR, which takes several other parameters than structure into account, such as physico-chemical properties, toxicological data etc. Property-activity relationship (PAR) and quantitative PAR functions along similar lines. Physiologically-based toxico- and pharmacokinetic models determine the fate of a drug in a living organism, with regards to its absorption, distribution, metabolism and excretion.

Often used synonym for alternative methods are 3Rs : **replacement** means the substitution of animal studies with other methods, **reduction** includes the reduction in the number of animals used to obtain information of a given amount and precision, and **refinement** stands for any decrease in the incidence or severity of inhumane procedures and minimising stress of testing animals.

Several important regulatory and non-governmental bodies are involved in the field of 3Rs. To mention a few:

- International Organisations:
    - International Conference on Harmonisation (ICH)
    - Organisation for Economic Co-operation and Development (OECD)
- European Agencies:
    - European Chemicals Agency (ECHA)
    - European Food Safety Authority (EFSA)
    - European Medicines Agency (EMA)
- European Institutions:
    - European Commission - Joint Research Centre (JRC) EU Reference Laboratory for alternatives to animal testing (EURL ECVAM)
- Industry/trade Organisations:
    - Cosmetics Europe
    - European Chemical Industry Council (Cefic)
    - European Federation of Pharmaceutical Industries and Associations (EFPIA)

In this project, we were especially interested in gaining knowledge about alternatives to animal testing of new chemicals in order to answer to the question: *Do we really need animal testing to keep us safe?* To formulate an answer we needed to get familiar with the concept of 3Rs, main actors and legislation in the European Union (EU), existing computational approaches and *in silico* modelling. The above mentioned aims were achieved by focusing on cosmetics, analysing the label of a cosmetic product at our choice, understanding skin sensitisation and related structural alerts causing this adverse effect by using the adverse outcome pathway concept, available databases and software tools. At the same time, during these steps, we were extending our vocabulary with scientific words and phrases used in this field.

Material and Methods

Since the project focused on analysing *in silico* procedures as alternatives to animal testing, a substantial amount of work on the project was carried out on computers.

We used computers to conduct research (documents, databases and websites of agencies), and software tools (KNIME version 4.0.0., https://www.knime.com/, last accessed on 05/08/2019 and Cytoscape version 3.7.1, https://cytoscape.org/, last accessed on 05/08/2019) to accurately create diagrams and networks to model and process data regarding the behaviour of chemicals, and to create spreadsheets to collect, display and evaluate information. In order to be able to use computers effectively, to know where to search for the information and how to use it, we first had several presentations by the project leader, which were interspersed with discussion rounds to test understanding and the development of one's own opinions regarding animal testing and chemical safety assessment. Also, we checked for the national regulatory bodies, centres involved in 3Rs using the JRC Inventory of the 3Rs knowledge sources (http://data.europa.eu/89h/jrc-eurl-ecvam-eurl-ecvam-3rs, last accessed on 05/08/2019), and national regulations on the use of animals for research purposes. This theoretical part was concluded after four days. The remaining four days were spent with analysing databases, acquainting oneself with the aforementioned software and carrying out analysis of aldehydes with regards to structure-activity relationship (SAR) and their role played in skin sensitisation. Five databases (PubChem, CosIng, COSMOS, Comptox, and AOP Wiki) were analysed for the type of information can be found using as an example *resorcinol*. A comparison between databases about chemical names, CASRN, SMILES, physico-chemical properties, ADME, toxicity tests, AOPs/mechanistic knowledge, uses/functions, cosmetics restrictions, SCCS opinions were performed. This exercise allowed to analyse the ingredients of the chosen cosmetic product, a shampoo/shower gel. The Cytoscape software was used to model our AOPs, and KNIME analytical platform to model *in silico* the chemicals behaviour.

Results and Discussions

The results of the project and research described in the "Material and Methods" section are thus listed by the general topics in which they are introduced. Detailed analysis of the implications of the results and the methods with which they were obtained, including our personal evaluation is presented below.

National Legislation, Regulatory Body and Centre for Alternative Methods

The European Union published the Cosmetic Directive in 1976 (https://ec.europa.eu/growth/sectors/cosmetics/legislation_en, last time accessed 06/08/2019). In 2009 it became the regulation which provided the official testing of cosmetics to ensure that the conditions concerning the composition of these products are satisfied. The directive manifests differently through the regulations as its role is to make recommendations to member states. The Regulation (EC) N° 1223/2009 was adopted in 2013 mainly as a result of the lobby. Therefore, testing cosmetics is

banned in European Union since 2013. According to a 2011 report by the European Commission on the number of animals used for scientific purposes, out of all 11.5 million animals used for testing in the member states, 8.75% were used for "toxicological and other safety assessment." This implies that, if these animal procedures were fully replaced with alternatives, up to approximately 1 million animals could be saved from animal testing yearly.

Table 2. Comparison between national countries of the Project's students on legislation, regulatory body and centre for alternative methods

Country	**Croatia**	**Germany**	**Spain**
National Regulation	*Pravilnik o zaštiti životinja koje se koriste u znanstvene svrhe* NN 25/13, 8/5/2013	*Tierschutzgestz (TierSchG)*	*Real Decreto 53/2013*
National Body	Ministarstvo poljoprivrede (Uprava za veterinarstvo i sigurnost hrane)	Bundesministerium fuer Ernaehrung und Landwirtschaft (Federal Ministry of Food and Agriculture)	Ministerio de agricultura, pesca y alimentación. Dirección general de producciones y mercados agrarios.Subdirección general de productos ganaderos
Centre(s) for Alternative Methods	*None*	German Centre for the Protection of Laboratory Animals (Bf3R), Berlin; CAAT-Europe, Konstanz	REMA-Madrid

The results from Germany, Croatia and Spain differed wildly. We found out that Croatia doesn't have a designated centre involved in supporting alternative methods to animal testing and that Germany and Spain both have a long history of animal welfare. Today, Germany has the Grade B and Spain has the Grade C, according to the Animal Protection Index published by the World Animal Protection; there was no data available for Croatia.

Data for Spain

The Spanish Network for the Development of Alternative Methods (REMA, http://www.remanet.net/) is a platform that coordinates initiatives of the industry, public administration, society and academia towards a reduction, refinement and replacement of animal experimentation (the three *R's*), promoting the development of alternative methods.

Real Decreto 53/2013, from February 1 (https://www.boe.es/boe/dias/2013/02/08/pdfs/BOE-A-2013-1337.pdf, last accessed 06/08/2019), establishes the basic rules for the protection of animals used in experimentation and other scientific purposes, including teaching.

Data for Germany

In Germany, the main policymaker and guiding force with regards to animal testing is the Federal Ministry of Food and Agriculture. It bases its action on the Tierschutzgesetz (Law for the Protection of Animals) from 2006. However, even before, in 1998, Germany had already implemented in its law that animal tests for cosmetics development should be banned. However, tests in other areas (for example, for scientific and toxicology research) is still legal. The ministry publishes a report annually since 2002 that mentions the amount of animals used for scientific purposes, the level of stress they have been subjected, the different species involved and information regarding the use of genetically modified organisms (with and without a pathological phenotype). Additionally, the ministry has set up a databank - Animaltestinfo, accessible to the public. It contains information about officially authorised animal tests with regards to the expected benefit to humans, what branch of science they are used for. Apart from that, the ministry also funds research in alternative methods to animal testing. One of these is an organisation called ZEBET and was founded to develop new methods for chemical testing and decrease use of animals. In cooperation with other institutions, such as the OECD, it aims to evaluate alternative methods and transform the advice it gives to institutions into legal words, guidelines and policies. The database of the organisation (called AnimAlt Zebet) contains portraits of alternative methods and is among one of the knowledge sources recognised by the European Commission for its promotion of the 3Rs. Non-governmental actors play a huge role in animal protection, too. The centre for alternative methods in Germany is German Centre for the Protection of Laboratory Animals (Bf3R) https://www.bfr.bund.de/en/german_centre_for_the_protection_of_laboratory_animals.html. The Center for Alternative to Animal Testing in Europe (CAAT-Europe) https://www.biologie.uni-konstanz.de/leist/caat-europe/ is hosted by the University of Konstanz.

Analysing Open-Access Databases Useful for *In Silico* Modelling in Cosmetics

Five data repositories were analysed for nine criteria which are detailed below. The results are compiled in the Table 3.

CASRN: A unique numeric identifier assigned by the Chemical Abstracts Service. The OECD AOP Wiki is the only database where you cannot find a CASRN.

SMILES: a specification in the form of line notation for describing the structure of a chemical using short ASCII strings, simplified molecular-input line-entry system. SMILES are only included in Pubchem and Comptox.

Physico-chemical properties (e.g. molecular weight, color, physical state, melting/boiling point, density, odor, solubility, flammability, logP and polarity). Included in Pubchem and Comptox.

ADME: absorption, distribution, metabolism and excretion. Pubchem and Comptox contain such details.

Toxicity tests: designed to generate data concerning the adverse effects of a substance on a human or animal health, or the environment. Could be *in vitro, in vivo* or *in silico* test. From all five databases all include toxicity tests apart from CosIng.

AOPs/Mechanistic knowledge: Adverse Outcome Pathway (we go into further detail later on). Only Comptox and OECD AOP Wiki have information about AOPs.

Uses/Functions of chemicals: OECD AOP Wiki is the only database which does not include them.

Cosmetic Restriction: Restrictions are conditions to protect human health and the environment from unacceptable risks posed by chemicals, therefore a cosmetic restriction does not allow certain chemicals to be used in cosmetic products unless under specific limitations such as concentration. Although many chemicals have cosmetic restriction, not all have it meaning they don't cause any harm/threat to us or the environment.

SCCS opinions: The Scientific Committee on Consumer Safety provides Opinions on health and safety risks (chemical, biological, mechanical and other physical risks) of non-food consumer products and services. Only CosIng has SCCS opinions mentioned.

Table 3. Comparison of five databases useful for analysing existing chemicals and *in silico* modelling

	Pubchem	CosIng	COSMOS	Comptox	OECD AOP Wiki
Chemical Name	Yes	Yes	Yes	Yes	Yes
CASRN	Yes	Yes	No	Yes	No
SMILES	Yes	No	No	Yes	No
Physico-chemical properties	Yes	No	No	Yes	No
ADME	Yes	No	No	Yes	No
Toxicity tests	Yes	No	Yes	Yes	Yes
AOPs/Mechanistic knowledge	No	No	No	Yes	Yes
Uses/Functions	Yes	Yes	Yes	Yes	No
Cosmetic Restriction	No	Yes	No	No	No
SCCS opinions	No	Yes	No	No	No

We discussed these criteria and came to the conclusion that doing it manually is very time consuming. Also, we noticed

little disagreements between databases, mainly in the numerical properties, but we agreed that what was important was to cite the database with the information is contained. Sometimes a database could have many information about one chemical whilst another database could have barely any, which is why it is helpful to compare and analyse two or more. Additionally, every database has its own structure and order to present their information. If there isn't any database which contain a chemical we are looking for we should not make speculations, we should research or even test it ourselves.

Analysing Ingredients of a Cosmetic Product

One part of our project involved the analysis of chemical ingredients in a certain product. We started off with choosing the product – the shower gel (shampoo) as shown in Figure 1, followed by database research of each chemical component, and finally data analysis. The results can be found in the Supplementary material with the collected data at the moment of the retrieval and availability.

Figure 1. The cosmetic product chosen to be analysed for its ingredients.

Firstly, we sorted them by their molecular weight, which is a very significant component for determination of chemical behavior. Also, it is important to note that almost all the chemicals had the molecular weight less than 500g/mol, proving their biocompatibility to skin and absorption and permeability capabilities. The chemical with the highest results was Hydroxypropyl Oxidized Starch, ant the lowest Propylene Glycol. After that, we conducted research regarding solubility, physical description and toxicity tests. Most of the ingredients had high water, ethanol and ester solubility (e.g. Panthenol, Citric acid, Menthol). The exception was Ethylene glycol distearate, which is almost insoluble in water. This has an impact in the preparation steps of a cosmetic product, the order of ingredients being critical to obtain the desired cosmetic product.

Regarding the physical description, the chemicals were either in the form of white crystals/powder (Sodium benzoate, Menthol) or white/transparent (almost) odorless viscous liquids (Propylene Glycol, Cocamidopropyl Betaine). Furthermore, we found out that almost all chemicals were tested on animals – rats, guinea pigs, mice and rabbits, while some of the chemicals have been tested on humans too (Citric acid, Ethylene glycol distearate).

Another thing we considered was the use/function of a certain chemical.

- PEGs are used in cosmetics and personal care products as solvents, thickeners, softeners and moisture carriers
- Cocamidopropyl Betaine and Disodium Cocoamphodiacetate are used for cleansing, foam boosting and hair and skin conditioning
- Citric Acid serves as a preservative and a buffering agent, masking agent
- Ethylene glycol distearate is an important emulsifier
- Hydroxypropyl Oxidized Starch is used for film forming
- Sodium benzoate acts as an anticorrosive, antimicrobial agent, flavoring agent or adjuvant, pH control agent.

Almost all the chemicals are skin and eye irritants, except for sodium benzoate which can lead to serious eye damage. Two of the ingredients have cosmetics restrictions – Phenoxyethanol (V/29) – concentration 1% and Sodium benzoate (V/1) – concentration of 0.5%, while Citric Acid is the only one that has SCSS opinions ("... the use of citric acid in cosmetic products, at a concentration up to 0.2%, as a preservative does not pose a risk to the health of the consumer. Oral care products and products intended to be used in the vicinity of the eyes are excluded. For deodorants and antiperspirants, it is also considered safe when used up to a total concentration of 0.2% as a preservative and/or an active ingredient").

Understanding Skin Sensitisation as One of Adverse Effects in Cosmetics

The areas in which important advances have taken place in the use of new alternative methods in cosmetics are: skin corrosion/irritation, eye irritation, skin sensitisation, skin absorption, UV-induced toxic effects (phototoxicity, photo - genotoxicity, photoallergy).

One way of understanding the mechanism of action of an adverse effect is by using the AOP concept. The AOP can be defined as an approach that establishes the linkages between a stressor (chemicals, non-chemicals) and the biological effects that lead to the adverse outcome, which is of interest in risk assessment. Risk assessment deals with the evaluation of a particular hazard. A hazard is anything that can cause harm; in this case, the chemical that is applied to the skin. A risk, on the other hand, is the likelihood that the hazard will cause harm. AOPs are helpful in this regard, as they detail any unwanted reactions associated with the use of the chemical. Skin sensitisation is a process, whereby an immune response is induced that is visible as the presence of red patches of skin. The official AOP of the OECD lists four key events that are involved in the elicitation of an immune response (https://aopwiki.org/aops/40, last accessed 06/08/2019).

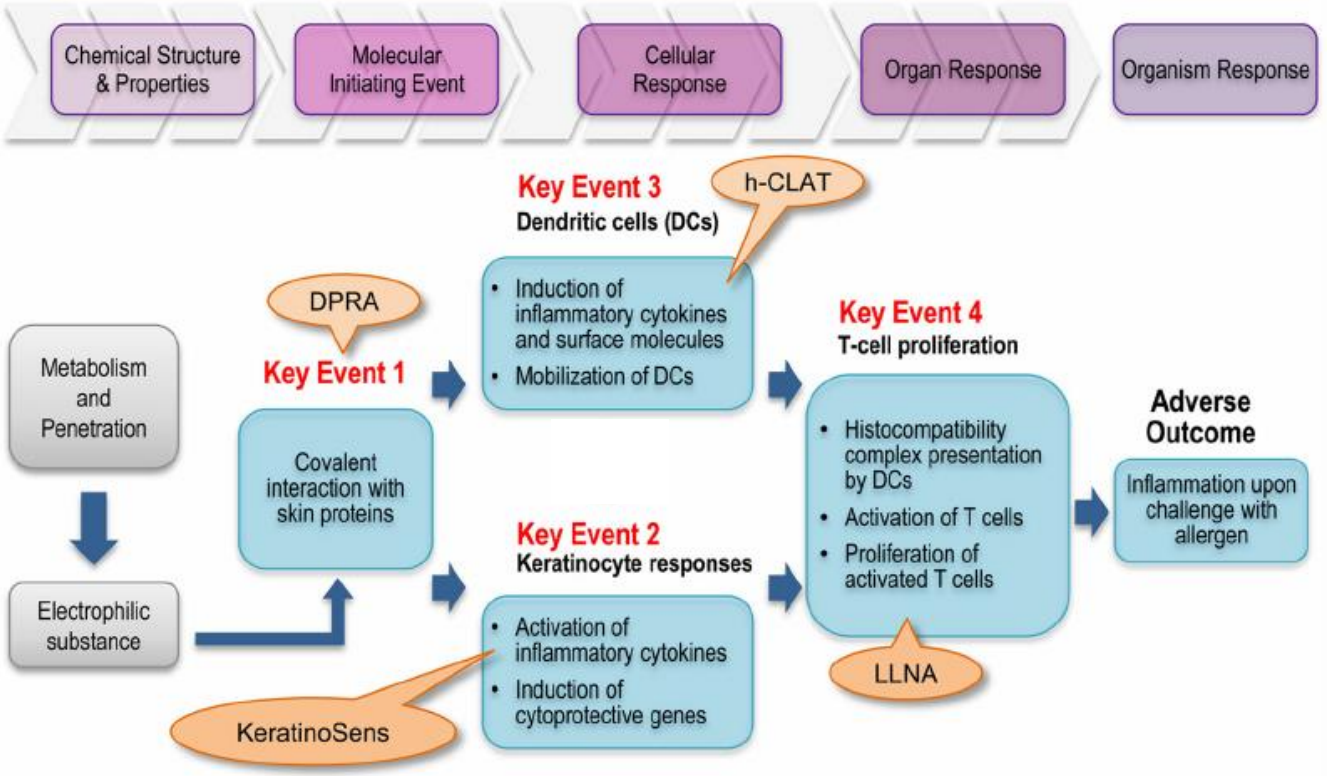

Figure 2. Adverse Outcome Pathway for skin sensitisation taken from https://doi.org/10.1002/jat.3281, last accessed 28/08/2019.

These events will be described with regards to immunology aspects of the event and the assays that are used to quantify the events.

1) Molecular Initiating Event: Covalent Binding to Skin Proteins

The first event in the AOP of skin sensitisation is the binding of the relevant hapten (in this case, an aldehyde) to proteins in the dermis layer of the skin. The hapten must satisfy several conditions in order to reach this structure. Firstly, it must be electrophilic, i.e. able to accept electrons. Naturally, the counterpart of the interaction, the proteins present in the skin (mainly glycine and lysine) will have to be of nucleophilic nature, i.e. able to donate electrons to the hapten. These interactions may occur via different pathways, such as Schiff Base formation, oxidation and Michael addition. Molecular weight is another major discriminant in assessing absorption patterns of a given chemical. In general, a lower molecular weight facilitates a readily uptake by the skin. In their paper from 2000, Bos et al. hypothesised that compounds with a molecular weight greater than 500 Daltons (Da; g/mol) are not able to pass through the skin. In fact, analysis of the 13 aldehydes studied in the course of the project reveals that the average molecular weight of the aldehydes is about 153 Da (min: 106 Da; max: 216 Da). There seems to exist no correlation between the weight and the sensitisation potential of the compounds. Finally, lipophilicity has been shown to favor skin absorption, due to their easy migration through the stratum corneum, the upper layer of the skin that is rich in lipids (such as free fatty acids and ceramides). However, due to the water saturation of the dermis, absorption of lipophilic compounds is inhibited. Absorbed haptens are absorbed into the dermis (and thus trigger the next key event) when slightly lipophilic. The

according physicochemical property is Kow, octanol/water partition constant, where a Kow >0 indicates lipophilicity and a Kow <0 indicates hydrophilicity. Database research of all 13 chemicals indicated an average Kow of ~2.22. In conclusion, the ideal hapten for skin absorption is electrophilic, has a low molecular weight and is slightly lipophilic. With these attributes identified, it is possible to predict the behaviour of the chemical in all steps of the pathway.

After traversing the stratum corneum, the aldehydes make their way to the epidermis, where they will bind to proteins. These proteins will be the scaffolds for the aldehydes to reach dendritic cells later on in the pathway. The assay used is an *in chemico* procedure, the Direct Peptide Reactivity Assay that measures the depletion of the peptide structures over a period of time. The method uses high-performance liquid chromatography to identify these changes. Depending on the depletion percentage, the chemical may be placed in one of four groups, with regards to their sensitisation potential.

2) Key Event 1: Keratinocyte Activation

Keratinocytes can be found in the epidermis, where they make up 90% of cells. They are involved in protection against fungal, bacterial, microbial infections, and serve an important part in the chemical immune system. The haptens bound to the proteins will activate keratinocytes and stimulate the secretion of cytokines that results in an inflammatory response of the skin. In addition to this release, an oxidation pathway in the cell, called ARE-Nrf 2, is induced. This pathway is responsible for the release of the enzyme luciferase, which catalyses the oxidation of the pigment luciferin to oxyluciferin, which exhibits bioluminescence. The assay involves the measurement of this light release to gauge luciferase activity and, thus, the degree to which keratinocyte activation has been brought about. The KeratinoSensTM method (an *in vitro* procedure) is recognised/validated by the OECD to identify luciferase activity.

3) Key Event 2: Dendritic Cell Activation

Apart from protecting the body from all kinds of infections and toxins, keratinocytes play an important role in the activation of dendritic cells (DCs). DCs are a part of the immune system and play a central role in the initiation of allergic responses. The internalisation of the hapten-protein cause the DCs to undergo structural changes, which will result in its ability to present the allergen-MHC-complex to naive T-cells. These cells allow the immune system to react to substances, which it has not yet encountered. In addition to the complex, dendritic cells also gain the ability to express certain surface markers. These molecules can be detected using the *in vitro* method of human Cell Line Activation Test (h-CLAT) that uses flow cytometry to assess the profile and number of cells expressed by dendritic cells.

4) Key Event 3: Activation/proliferation of T-cells

The aforementioned allergen-MHC-complex (which is an antigen-presenting structure) will reach T-cells, carrying with it a hapten-protein complex in a “groove.” If the T-cell doesn’t recognise the substance from a previous infection, an immune reaction will follow, the T-cells will differentiate into memory T-cells and proliferate. If the body is exposed to the chemical a second time, an immune reaction will follow and cause allergic contact dermatitis. The only OECD-validated assay for T-cell activation/proliferation is the Local Lymph Node Assay carried out in mice. It measures the increase of the number of lymphocytes (one of the cells in the immune system, of which T-cells are a member) upon

exposure to the chemical.

5) Adverse Outcome: Skin Sensitisation

The result of all of the pathways and reactions described above is skin sensitisation, which is characterised by the presence of a threshold number of T-cells at the point of sensitiser contact. Even though the initial reaction to the chemical (after it has first been applied), may take more than five weeks to become visible, following elicitations of the response usually occur within 1-2 days of application of the chemical.

A graphical representation of the AOP using Cytoscape v.3.7.1. software is shown below in Figure 3.

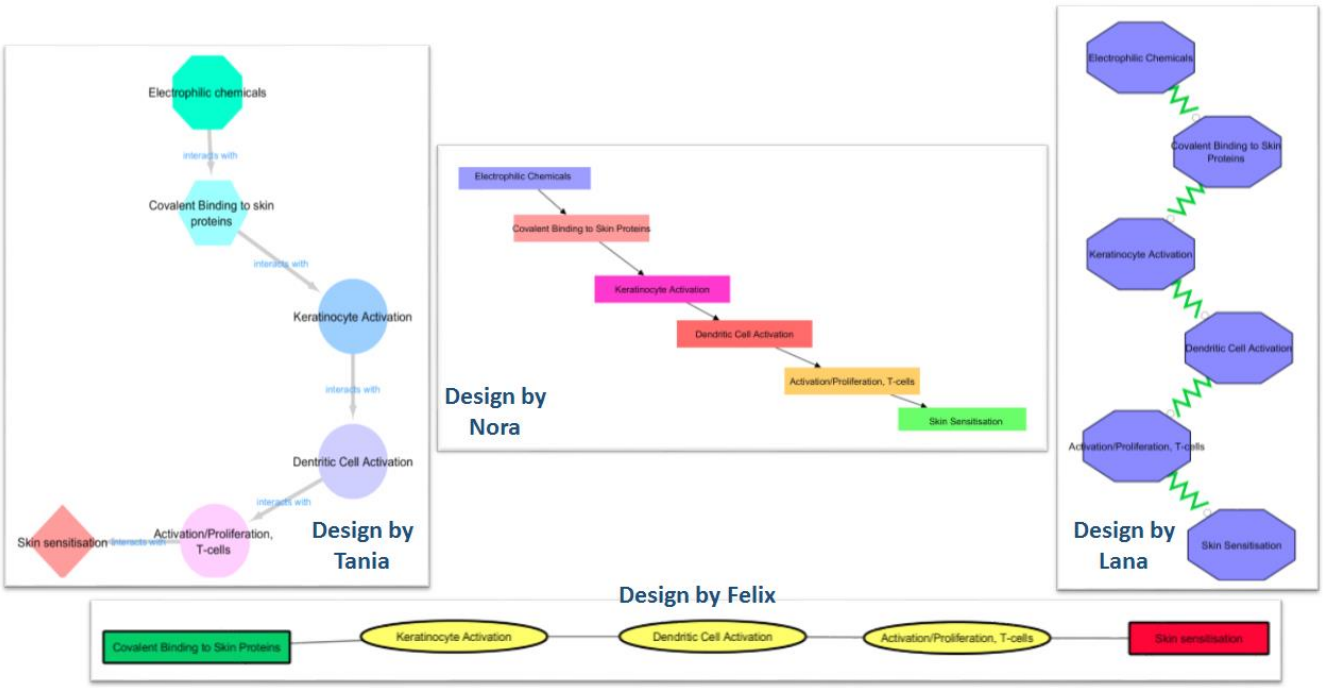

Figure 3. Modelling and design of the AOP on skin sensitisation using Cytoscape v.3.7.1 software.

Understanding Structural Alerts of a Skin Sensitiser

As part of our closer analysis of the AOP, we examined a group of chemicals that were likely to initiate a skin sensitisation response – aldehydes showed in Figure 4. They are characterised by the functional group -CHO, sometimes also called the formyl group. Aldehydes are also known for their smell, therefore they are widely used in cosmetic industry, e.g. fragrances. Depending on their structure, the severity of the allergic response to the exposure of the chemical could vary. Supported by database research, we concluded that the length of the side chain of the aldehyde was a key discriminant in assessing its toxicity. Our exercise on this topic was to group given aldehydes based on their structures in four groups. One of the groups was α, β-Unsaturated aldehydes. Similarity between cinnamaldehyde, α-methyl cinnamaldehyde, α-butyl cinnamaldehyde, α-amyl cinnamaldehyde and α-hexyl cinnamaldehyde is benzene ring. Difference is length of the chain - cinnamaldehyde has the shortest chain and as a consequence it is a strongest sensiter out of named ones. Others in the group are medium sensitisers. The results are represented in Figure 5.

We were confronted with some of the research that is being done in the fields of structure-activity and structure-property relationships (SAR and SPR, respectively) that are an important aspect of chemical safety assessment. It involves the extensive modeling of the interaction between the aldehyde and its target structure (i.e. the covalent proteins found in the skin) in a variety of media (*in silico, in vitro* and sometimes *in vivo*), which allows for the subsequent deduction of physical, biochemical and hence, toxicological data. In essence, this not only allows for the analysis of the chemical substance in hand but it is also important in other situations. When conducting a risk assessment for a chemical that has rarely been tested, the data of already tested chemicals can be used to predict the properties of the untested one. This extrapolative approach plays a crucial part in the discovery, where the similarities and differences in structure and property between *an old* and *a new* chemicals may be used (when explained why the comparison between these two substances is justified) to then make assumptions of the strengths and weaknesses of the new chemical.

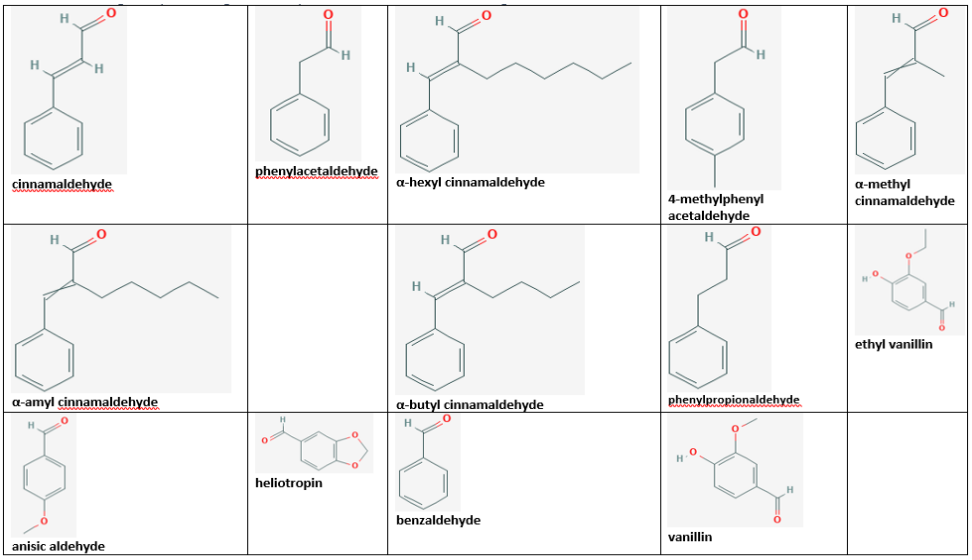

Figure 4. Aldehydes we needed to group based on their given structural similarities.

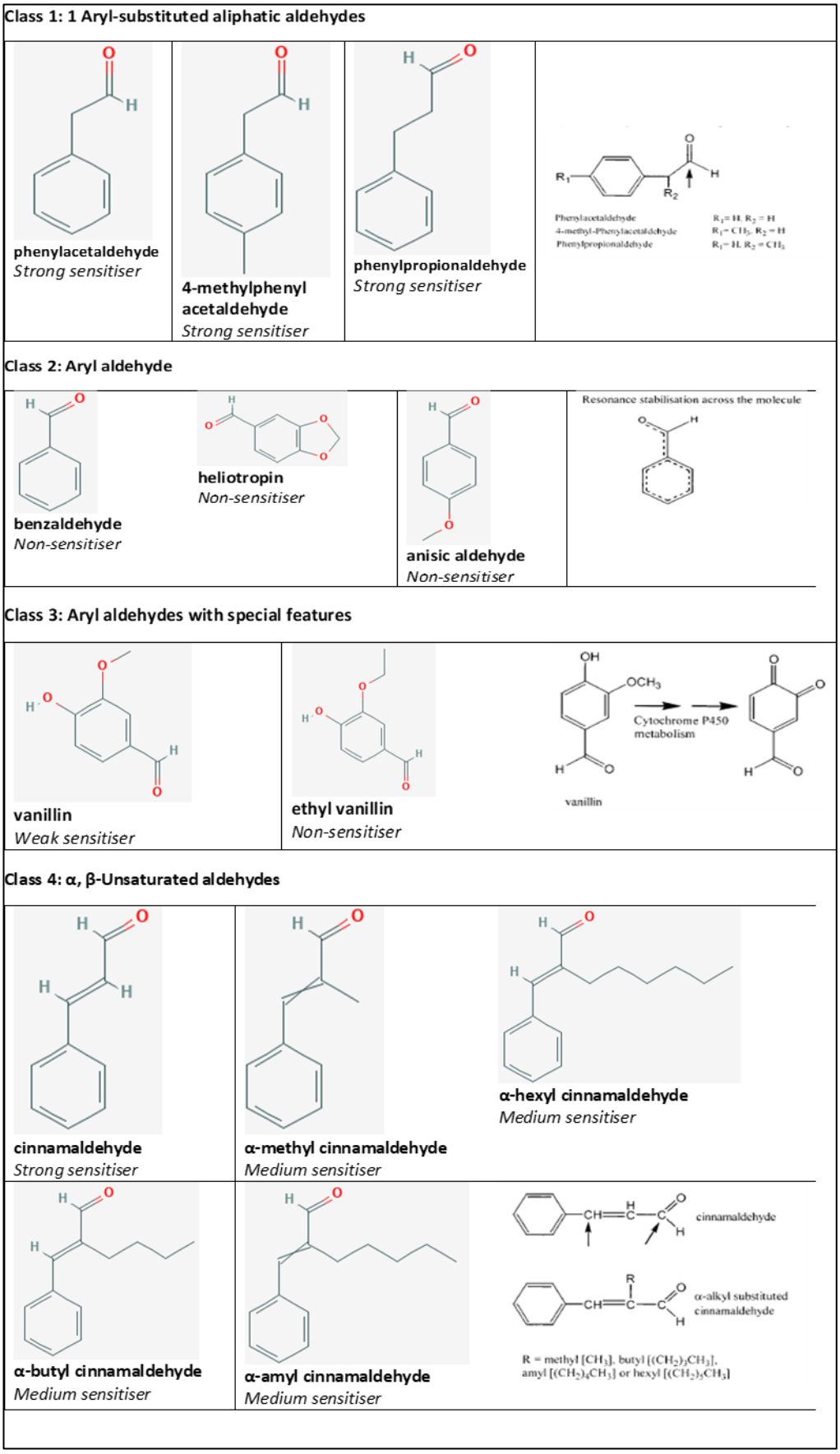

Figure 5. Grouping chemicals per their chemical structure and related potency.

Modelling in KNIME

Computational toxicologists have several options available to them to model the behaviour of a chemical. One of these is the use of data analysis platforms, which enable them to extract, process, manipulate and visualise data. In this project, the KNIME analytical platform was used. Workflows are based on the connection of so-called "nodes" to one another, creating a network. These nodes allow reading, transformation (i.e. grouping, filtering, sorting, concatenation), analysation and deployment of data.

The software was used in conjunction with our analysis of aldehyde structures and their importance to skin sensitisation. The aim of this part of the project was to create a spreadsheet or other type of graphical, tabular representation that contained several sets of quantifiable data.

The type of data we were most interested in was related to the physicochemical properties of the aldehydes, more specifically their LogP value (octanol/water partition constant) and molecular weight (in g/mol). Firstly, a table was created, only using the chemical's name and SMILES identifier. This data was then fed into the "XLogP" and "Molecular Properties" node that was able to derive the LogP and Molecular Weight value of the chemical using only the two specified criteria. The program then found the average of these two properties using the "Math Formula" node and the COL_MEAN function that averaged the values of the XLogP and Molecular Weights of the specified aldehydes. This resulted in two distinct tables that contained all information given, extracted from databases, and manipulated data (i.e. IUPAC name, SMILES, the name of the parameter, and the average of the parameter's value). Using the "Joiner" node these two data sets were combined to create a table containing all of the desired data. Finally, this table was converted to an Excel spreadsheet, fulfilling the aim of the project. As a further representation of the final set of data, the "Hierarchical Clustering" tool was used, such that the data was classified, only according to the numerical data we supplied the program with. The function clustered ten of the aldehydes in one cluster, and placed three in a separate category. Note: the user may adjust the number of clusters that the program returns. To give a more intuitive representation of the clustering, colour was added, the same colour representing a member of the same cluster. The resulted workflow is represented in Figure 6.

In conclusion, working with KNIME was uncomplicated, as the interface of the platform was very intuitive, the manipulation of data only involved numerical parameters and operations, and we used the tools of the software (instead of programming our own, which is possible using KNIME).

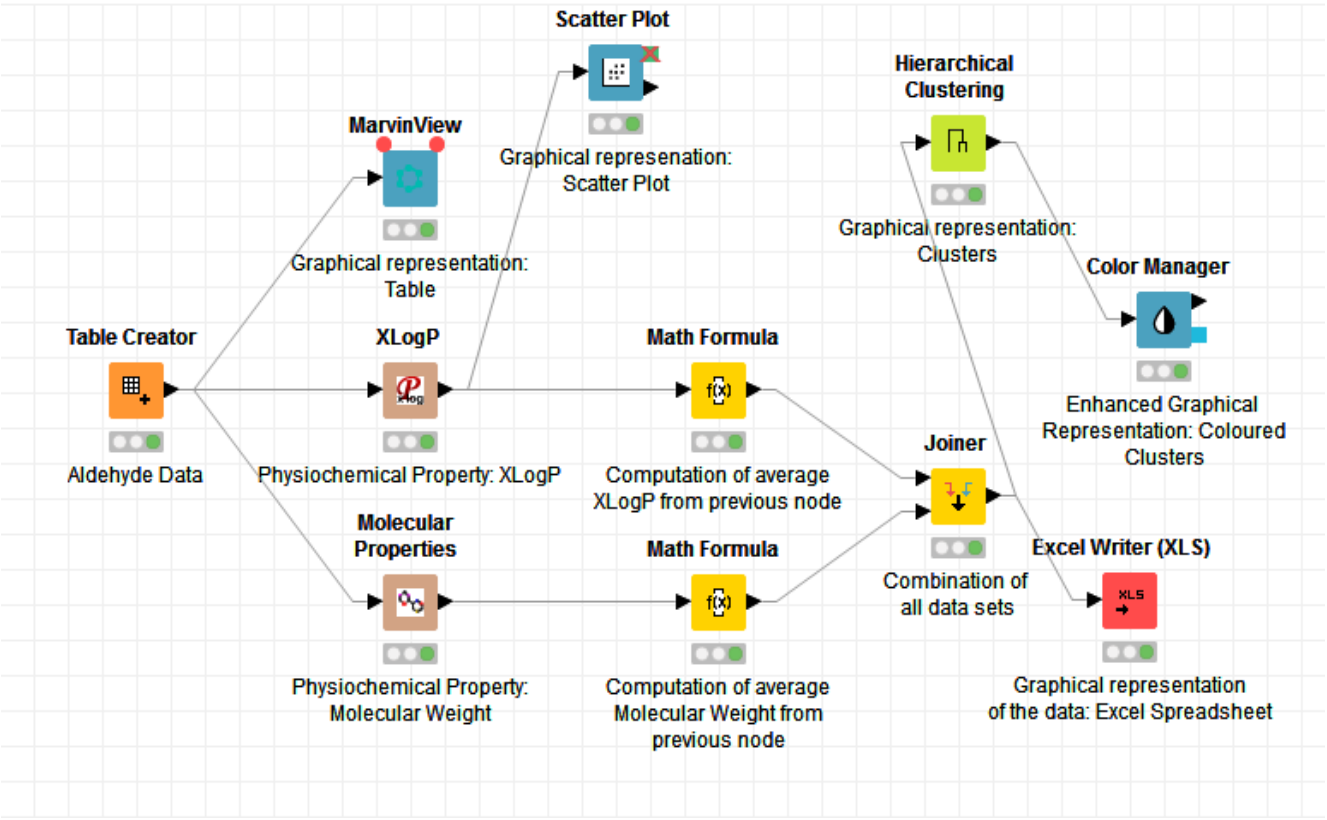

Figure 6. KNIME workflow of grouping aldehydes based on logP and molecular weight.

Conclusions

The aim of this project was to assess the question of whether we need animal testing to keep us safe. In doing so, we learnt about the various alternative methods, their strengths and limitations, their underlying principles and the results that can be obtained from them. We have considered various institutions and agencies that regulate and moderate the enforcement, development and dissemination of the alternative practices. Keystone agreements, such as the Cosmetics Regulation were considered and the necessity for change it brought about for a sizeable sector of the chemical industry.

During the second part of the project, we examined concrete, real-life examples, for which chemical risk assessments were necessary (during the shampoo/shower gel evaluation and the close analysis of the skin sensitisation AOP). Additionally, we were acquainted with some of the computational tools toxicologists use and refer to during testing, such as QSAR Toolbox. This allowed us to empathise with and relate to the work professionals in the field are carrying out.

We noticed that even though sophisticated alternatives to animal testing have been devised in response to the Regulation, they are often ineffective on their own. For example, organoids and organs-on-a-chip (*in vitro* procedures) both still aren't always adequate in simulating human biochemistry. The limits of computational methods lie in the fact that they are all based on models and are thus subject to the same weaknesses as models in any other field (such as meteorology or economics): their accuracy is inherently tied to the type and scope of data they are subjected to, the decision on how complex or simplistic to make the model, and how to use, assess and implement the data gained through computational methods.

Talking about data usage, during our database research especially, we were confronted with the challenge of how to decide if the data we retrieved was of high quality. This was genuinely difficult to gauge, as we found numerical properties in particular to vary from source to sources. Sometimes, extensive cross-referencing was necessary to come to the final decision what data we should use. At other time, certain descriptors of a molecule were not present at all. Apart from other factors, economic interests play a role in this withholding of information. No regulatory body forces the chemical industry to disclose information about their research on an open-access database, thus complicating the risk assessment process for toxicologists and highlighting the need for a certain degree of goodwill even in a competitive business framework.

Lessons Learned

We started off with the theory that animal testing is inefficient and unethical. We got an insight in the testing procedures and legal framework. From there on, we learned about different alternative methods and database research to prove that theory. Also, we got the chance to meet three scientists of the in3 Project and talk to them about the future of medicine and toxicology, which contributed to forming our own critical opinion on the current topics. The subjects tackled by the in3 team shared insights in the field of stem cell research. These cells appear to be promising not only in the field of toxicological assessment, but also as a way to improve tissue replacement and reconstruction (e.g. after a fire or in cancer treatment). Stem cells are undifferentiated and depending on how they are manipulated, can produce tissue that is not rejected by the body they were taking from, something that is not the case for transplant donor organs, which do not have a 100% success rate. Additionally, these cells also give rise to new possibilities for individualised medicine. Especially in combination with high-throughput technologies, they can be used to effectively model the biochemical responses of an area of exposure (or even many areas to simulate the behaviour of the body on a larger scale) to a chemical substance, thus paving new paths in chemical testing.

To summarise, we do arrive at the conclusion that there are effective alternative methods in place today already. All the models described in the report are not only more efficient, less time and cost effective, but are also taking into account more accurately the behaviour of the human body. International efforts have been made to promote and shape the future of alternative methods. These range from national bodies (e.g. the University of Konstanz in Germany) to “continental agencies” (such as the EURL ECVAM) to multilateral organisations (OECD). We do believe that the only way, in which better practices in the area of cosmetic industry (but also in many other areas of science) can only emerge if the interplay between all stakeholders (legal bodies, companies and independent research centres) results in a market situation that promotes the steady development of technologies, testing procedures, (market) research and product advertisement. Hopefully, in a way similar to cosmetics, other fields of research (especially fundamental research) will also develop and adopt alternative, validated and sustainable approaches for testing. The ECVAM concludes optimistically in their report from 2018: “(...) the advancement of the Three Rs across multiple sectors is being expedited through efficient and effective collaboration between multiple stakeholders engaging at an international level in a variety of fora.” We hope this trend continues in the future.

Acknowledgements

We would like to thank the S3 summer school coordinators, Nikolina, Dora and Mario, for planning, organising and managing the camp to make the research environment as pleasant as possible, all lecturers and workshop leaders, for improving our scientific literacy, raising questions about the developments of scientific innovation, and discussing the interaction of machines and humans in research.

Additionally, we would like to extend our warmest welcome to Nicoleta for not only teaching us the relevant material for the project in a visually affordable, exhaustive and unbiased way, but also for allowing us to pursue the topics we are most interested in.

Lastly, we would also like to thank the in3 young scientists Zahra, Cormac and Ivo, and everyone else involved in the preparation of this project.

References

Scientific Articles

- Bos JD, Meinardi MMHM (2000) The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp Dermatol 9:165-169
- MacKay C, Davies M, Summerfield V, Maxwell G (2013) From pathways to people: Applying the adverse outcome pathway (AOP) for skin sensitization to risk assessment. ALTEX 30(4):473-486 doi:10.14573/altex.2013.4.473
- Merkes M., *Better ways to do research*: http://www.humaneresearch.org.au/interview/better-ways-to-do-research, last accessed 06/08/2019
- López O, Cócera M, Wertz P W, López-Iglesias C, de la Maza A (2007) New arrangement of proteins and lipids in the stratum corneum cornified envelope. BBA 1768(3):521-529 doi:https://doi.org/10.1016/j.bbamem.2006.11.023
- Patlewicz G, Basketter D A, Smith C K, Hotchkiss S A M, Roberts D W (2001) Skin-sensitization structure-activity relationships for aldehydes. Contact Dermatitis 44(6):331-336 doi:https://doi.org/10.1034/j.1600-0536.2001.044006331.x
- Strickland J, Zang Q, Kleinstreuer N, Paris M, Lehmann D M, Choksi N, Matheson J, Jacobs A, Lowit A, Allen D, Casey W (2016) Integrated decision strategies for skin sensitization hazard. J Appl Toxicol 36(9):1150-1162 doi: https://doi.org/10.1002/jat.3281
- Vinken M (2013) The adverse outcome pathway concept: A pragmatic tool in toxicology. Toxicology 312(4): 158-165 doi: https://doi.org/10.1016/j.tox.2013.08.011
- Zhang B, Korolj A, Fook Lun Lai B, Radisic M, Advances in organ-on-a-chip engineering (2018) Nat. Rev. Mater. 3:257-278 doi: https://doi.org/10.1038/s41578-018-0034-7

Other Papers

- *US cosmetics are full of chemicals banned by Europe – why?*, https://www.theguardian.com/us-news/2019/may/22/chemicals-in-cosmetics-us-restricted-eu, last accessed 06/08/2019
- *Women Put an Average of 168 Chemicals on Their Bodies Daily*, https://articles.mercola.com/sites/articles/archive/2015/05/13/toxic-chemicals-cosmetics.aspx, last accessed 06/08/2019
- *Could banning sunscreen to help save coral do more harm than good?*, https://www.theguardian.com/fashion/2019/jun/19/could-banning-sunscreens-to-save-coral-do-more-harm-than-good, last accessed 06/08/2019
- *A refrigerator in your bathroom?,* https://cosmeticseurope.eu/blog/refrigerator-your-bathroom , last accessed 06/08/2019
- *Proposal to close UK mouse-research centre is 'major threat'*, https://www.nature.com/articles/d41586-019-02002-y, , last accessed 06/08/2019
- *Endocrine Disruptors: from Scientific Evidence to Human Health Protection Policy*, http://www.europarl.europa.eu/RegData/etudes/STUD/2019/608866/IPOL_STU(2019)608866_EN.pdf, last accessed 06/08/2019

Databases and Tools

Comptox v3.0.8, https://comptox.epa.gov/dashboard, last accessed 06/08/2019
CosIng, https://ec.europa.eu/growth/sectors/cosmetics/cosing_en, last accessed 06/08/2019
COSMOS DB, https://cosmosdb.eu/cosmosdb.v2/, last accessed 06/08/2019
OECD AOP Wiki v2.0, https://aopwiki.org/, last accessed 06/08/2019
PubChem, https://pubchem.ncbi.nlm.nih.gov/, last accessed 06/08/2019
QSAR Toolbox v4.3, https://qsartoolbox.org/, last accessed 06/08/2019
Cytoscape v3.7.1, https://cytoscape.org/, last accessed 06/08/2019
KNIME v4.0.0, https://www.knime.com/, last accessed 06/08/2019

Videos

- *Applying non-animal test methods for assessing skin sensitization*, https://youtu.be/n9z-h2XafW4, last accessed 05/08/2019
- *Cosmetics Europe*, https://www.youtube.com/user/Cosmeticseurope, last accessed 05/08/2019
- *EURL ECVAM and alternative methods*, https://youtu.be/jZHWTvUWSLE, last accessed 05/08/2019
- *Fighting Animal Testing: The Cosmetics Directive*, https://youtu.be/N2NbBXFskMc, last accessed 05/08/2019
- *Fighting Animal Testing: Cruelty Free Cosmetics*, https://youtu.be/Q37oCNq-vI0, last accessed 05/08/2019
- *It's Time to Think Outside the Cage | Charu Chandrasekera | TEDxUniversityofWindsor*, https://youtu.be/6O6kOcZH_mg, last accessed 05/08/2019
- *Sexy cells: Alternatives to animal experimentation | Judith Lechner | TEDxMCInnsbruck*, https://youtu.be/haKyOltVJMw, last accessed 05/08/2019
- *The EU decision-making process*, https://youtu.be/6xZM7Wfux_s, last accessed 05/08/2019
- *What is the difference between Directives, Regulations and Decisions?*, https://youtu.be/CZAC_uEJWfY, last accessed 05/08/2019

Supplementary Material

Assessment of the label of the chosen cosmetic product, shower gel (shampoo) using available and open-access databases.

	Cocamidopropyl betaine	Source	Citric acid	Source	PEG-3 Diststereate	Source	Ethylene glycol distearate	Source
CASRN	#4929-10-8	PubChem	77-92-9	PubChem			627-83-8	PubChem
IUPAC Name	2-[3-(dodecanoylamino)propyl-dimethylazaniumyl]acetate	PubChem	2-hydroxypropane-1,2,3-tricarboxylic acid	PubChem			2-octadecanoyloxyethyl octadecanoate	PubChem
Physical description	Viscous, pale yellow, transparent liquid	https://www.worldofchemicals.com/chemicals/chemical-properties/cocamidopropyl-betaine.html	A white or colourless, odorless, crystalline solid, having a strongly acid taste	PubChem			White flakes or beads at room temperature	https://raw-materials.mckinleyresources.com/item/misc-specialties/egds-ethylene-glycol-di-stearate/1083
Solubility	Used as a surfactant (is soluble in water and fat); solubility in other substances not tested	PubChem	Very soluble in water; freely soluble in ethanol; soluble in ether	PubChem			Almost insoluble in water, soluble in alcohol, acetone and ethyl ethers a	https://www.lookchem.com/Ethylene-glycol-distearate/
Molecular Weight	342.5 g/mol	PubChem	192.12 g/mol	PubChem			595 g/mol	PubChem
pKa			2.79	PubChem			-6.7	http://www.hmdb.ca/metabolites/HMDB0032260
LogP	5.40		-1.64	PubChem			14.41	http://www.hmdb.ca/metabolites/HMDB0032260
Half-life			73 years	PubChem				
Absorption			Ingested naturally through food	PubChem			Readily absorbed by skin and tissues	https://www.cir-safety.org/sites/default/files/PEG%20diesters.pdf
Distribution			All tissues	PubChem			Distributed to all tissues in the human body; can cross placental barrier	https://www.cir-safety.org/sites/default/files/PEG%20diesters.pdf
Metabolism			Citric acid in reaction with enzyme citratase /citrate lyase/ yields oxaloacetic acid & acetic acid.	PubChem				PubChem
Excretion			Urine	PubChem			Excreted unchanged in urine and faces	https://www.cir-safety.org/sites/default/files/PEG%20diesters.pdf
Toxicity Tests	Oral/rats, human lymphocytes, guinea pig, genetic toxicity in a bacterial reverse mutation assay using Salmonella typhimurium strains	https://www.cir-safety.org/sites/default/files/alkyl_betains.pdf	Eye /rabbit, skin /rabbit, *in vitro* /Human skin, inhalation/guinea pig	PubChem			rats, guinea pigs, humans	https://online.personalcarecouncil.org/ctfa-static/online/lists/cir-pdfs/pr237.PDF
Uses/ Functions	ANTISTATIC CLEANSING FOAM BOOSTING HAIR CONDITIONING SURFACTANT VISCOSITY CONTROLLING	PubChem, CosIng	Air freshener, preservative, buffering, chelating, masking	PubChem, CosIng	PEG Distearate ingredients clean the skin and hair by helping water to mix with oil and dirt so that they can be rinsed away. They also help to form emulsions by reducing the surface tension of the substances to be emulsified and help other ingredients to dissolve in a solvent in which they would not normally dissolve.	https://www.cosmeticsinfo.org/ingredient/peg-3-distearate	Emulsifying	CosIng
Reactive Functional Groups	Quaternary organic ammonium compounds	QSAR Toolbox	None	QSAR Toolbox				
Other Functional Groups	Carboxylic acid Organic amide and thioamide	QSAR Toolbox	Alcohol Alkane, branched with tertiary carbon Carboxylic acid	QSAR Toolbox				
Adverse Reactions	Skin irritation/corrosion	QSAR Toolbox	None	QSAR Toolbox	Minimal ocular irritation	https://www.cosmeticsinfo.org/ingredient/peg-3-distearate		
SCSS Opinions			Yes	CosIng				
Cosmetic Restriction								
GSH Classification	Corrosive, irritant, environmental hazard	PubChem	Corrosive, irritant	PubChem			Green circle - The chemical has been verified to be of low concern based on experimental and modeled data	PubChem, https://www.parchem.com/siteimages/Attachment/GHS%20Glycol%20Distearate%20MSDS.pdf

	Panthenol	Source	Hydroxypropyl Oxidized Starch	Source	PG-Trimonium Chloride	Pubchem	Menthol	Source
CASRN	16485-10-2	PubChem	68412-86-2	PubChem	53171-04-3	PubChem	1490-04-6	
IUPAC Name	2,4-dihydroxy-*N* -(3-hydroxypropyl)-3,3-dimethylbutanamide	PubChem	5-[6-[[3,4-dihydroxy-6-(hydroxymethyl)-5-methoxyoxan-2-yl]oxymethyl]-5-[3-(2,3-dihydroxy-2-methylpropoxy)-4-hydroxy-6-(hydroxymethyl)-5-methoxyoxan-2-yl]oxy-3,4-dihydroxyoxan-2-yl]oxy-4-hydroxy-6-(hydroxymethyl)-2-methyloxan-3-one	PubChem	(3-dodecanoyloxy-2-hydroxypropyl)-trimethylazanium;chloride	PubChem	5-methyl-2-propan-2-ylcyclohexan-1-ol	PubChem
Physical description	Panthenol is a viscous transparent liquid at room temperature, but salts of pantothenic acid (for example sodium pantothenate) are powders (typically white)	PubChem		PubChem		PubChem	white crystalline solid or granule	PubChem
Solubility	It is well soluble in water, alcohol and propylene glycol, soluble in ether and chloroform, and slightly soluble in glycerin.	PubChem		PubChem		PubChem	soluble in alcohol, chloroform, ether, petroleum ether, hexane, glacial acetic acid, liquid petrolatum and in mineral, fixed and volatile oils	PubChem
Molecular Weight	205.25 g/mol	PubChem	778.7 g/mol	PubChem	352 g/mol	PubChem	156.26 g/mol	PubChem
pKa	11.6.	ECHA		PubChem		PubChem		PubChem
LogP	−0.989	Wikipedia		PubChem		PubChem	3 logP	PubChem
Half-life		PubChem		PubChem		PubChem	fish biotrans - 0.06 days	PubChem
Absorption	oraly	PubChem		PubChem		PubChem	occured from topical use, well absorbed via the oral route of exposure, dermal absorption is slower than oral absorption	PubChem
Distribution	widely distributed into body tissues	PubChem		PubChem		PubChem		PubChem
Metabolism	converted to pantothenic acid	PubChem		PubChem		PubChem	In humans urinary elimination of menthol after oral application was almost complete within about 12 to 24 hours.	PubChem
Excretion	unchanged in urine and feces	PubChem		PubChem		PubChem	excreted into bile (glucuronides of menthol)	PubChem
Toxicity Tests	no hazard identified	ECHA		PubChem		PubChem	carried on 25 volonteers - abdominal pain, convulsions, nausea, vomiting, vertigo, ataxia, drowsiness and coma	PubChem
Uses/ Functions	a humectant, emollient and moisturizer	PubChem	FILM FORMING	CosIng		PubChem	cooling effects of menthol could facilitate the absorption of tobacco toxicants, denaturant	PubChem
Reactive Functional Groups		PubChem		PubChem		PubChem	lacks functional groups that hydrolyze under environmental conditions (pH 5 to 9)	PubChem
Other Functional Groups	Alcohol Alkane, branched with quaternary carbon Dihydroxyl derivatives Ether Organic amide and thioamide	QSAR Toolbox						
Adverse Reactions	Allergic reactions to dexpanthenol have been reported occasionally	PubChem			A skin and eye irritant	PubChem	Alcohol Alkane, branched with tertiary carbon Cycloalkane Isopropyl Terpenes	QSAR Toolbox
SCSS Opinions							causes serious eye damage and skin irritation, allergic reactions (e.g. contact dermatitis, flushing, and headache)	PubChem
Cosmetic Restriction	A skin and eye irritant	PubChem						
GSH Classification	Green half-circle - The chemical is expected to be of low concern based on experimental and modeled data. Additional data would strengthen EPA's confidence in the chemical's safer status.	PubChem			Corrosive and irritant	PubChem	Irritant	PubChem

	Phenoxyethanol	Source	Sodium Laureth Sulfate	Source	Disodium Cocoamphodiacetate	Source	Propylene Glycol	Source	Sodium benzoate	Source
CASRN	2-phenoxyethanol	PubChem	9004-82-4	Pubchem	68650-39-5	Cosmos	57-55-6	Pubchem	532-32-1	PubChem
IUPAC Name	colorless, clear, oily liquid with a faint aromatic odor	PubChem	sodium;2-dodecoxyethyl sulfate	Pubchem			propane-1,2-diol	Pubchem	sodium benzoate	PubChem
Physical description	soluble in alcohol, ether and low water solability	PubChem					thick, clear, colourless, hygroscopic, viscous liquid	Pubchem	A white, almost odourless, hygroscopic, crystalline powder or granules	PubChem
Solubility	138.16 g/mol	PubChem					greater than or equal to 100 mg/mL at 70° F	Pubchem	Freely soluble in water	PubChem
Molecular Weight	15.10 at 25 deg C	PubChem	332.43 g/mol	Pubchem			76.09 g/mol	Pubchem	144.1 g/mol	PubChem
pKa	1.2 LogP	PubChem						Pubchem	-2.27	PubChem
LogP	11.8 hours at an atmospheric concentration of 5X10+5 hydroxyl radicals per cu cm	PubChem					-0.9	Pubchem	-2.27	PubChem
Half-life	The rate in rats and humans : the rate of intestinal absorption was rapid, with 60-70% of the excreted (14)C detected at 3 hours and > 95% of the total 4-day urinary (14)C detected within the first 24 hr.	PubChem					Adults = 4 hours but it depends on concentration	Pubchem	4.3 days	PubChem
Absorption		PubChem					administered orally=gastrointestinal tract	Pubchem	oral and dermal	PubChem
Distribution		PubChem					throughout total body water.	Pubchem		PubChem
Metabolism		PubChem					undergoes metabolic oxidation to pyruvic acid, acetic acid, lactic acid, and propionaldehyde.	Pubchem	Hippuric acid	PubChem
Excretion	mouse/no effects on body weight, 3 died because of constant breeding	PubChem					urine	Pubchem	Urine	PubChem
Toxicity Tests	Agricultural, animals - (but non-veterinary) e.g., animal husbandry, farming of animals/animal production, raising of animals for food or fur, animal feed, products for household pets, air fresheners, fragrance	PubChem	oral/rat	PubChem			eye /rabbit,, skin/human, subcutaneous/mouse, intraperitoneal/mouse ,In Vitro/Human, leukemia cells,In Vitro/Human, lymphocyte	Pubchem	skin /human, lung/hamster, oral or intraperitoneal/rat , In Vitro/Rabbit, ocular, intraperitoneal/guinea pig, intramuscular/mouse	PubChem
Uses/ Functions		PubChem	For maintenance, repair and cleaning of automobiles(auto shampoo,polish/wax). Cleaning and washing products(detergents,soaps,dishwashing products, rinsing agents etc.). Related to food production(restaurants)		CLEANSING, FOAM BOOSTING, HAIR CONDITIONING, HYDROTROPE, SKIN CONDITIONING, SURFACTANT	CosIng	HUMECTANT SKIN CONDITIONING SOLVENT VISCOSITY CONTROLLING	CosIng	ANTICORROSIVE, ANTIMICROBIAL AGENT, FLAVORING AGENT OR ADJUVANT, PH CONTROL AGENT, MASKING, PRESERVATIVE	CosIng, COSMOS
Reactive Functional Groups			Esters of organic sulfonic or sulfuric esters	QSAR Toolbox						
Other Functional Groups			Ether Sulfate Surfactants - Anionic	QSAR Toolbox						
Adverse Reactions			Skin and eye irritation	PubChem			may irritate eyes and chemical is combustible	pubchem	Serious eye damage/eye irritation	PubChem
SCSS Opinions										
Cosmetic Restriction	V/29	CosIng							V/1	CosIng
GSH Classification	Irritant	PubChem	corrosive and irritant	PubChem			Irritant and health hazardous	PubChem	Irritant	PubChem