

**Development of Knowledge Within a Chemical-Toxicological Database
to Formulate Novel Computational Approaches for Predicting Repeated Dose
Toxicity of Cosmetics-Related Compounds**

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Abstract

The European Union (EU) Cosmetics Regulation established the ban on animal testing for cosmetics ingredients. This ban does not assume that all cosmetics ingredients are safe, but that the non-testing procedures (*in vitro* and *in silico*) have to be applied for their safety assessment. To this end, the SEURAT-1 cluster was funded by EU 7th Framework Programme and Cosmetics Europe. The COSMOS (*Integrated In Silico Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimize Safety*) project was initiated as one of the seven consortia of the cluster, with the purpose of facilitating the prediction of human repeated dose toxicity associated with exposure to cosmetics-related compounds through *in silico* approaches.

A critical objective of COSMOS was to address the paucity of publicly available data for cosmetics ingredients and related chemicals. Therefore a database was established containing (i) an inventory of cosmetics ingredients and related structures; (ii) skin permeability/absorption data (route of exposure relevant to cosmetics); and (iii) repeated dose toxicity data. This thesis describes the process of “knowledge discovery from the data”, including collation of the content of the COSMOS database and its subsequent application for developing tools to support the prediction of repeated dose toxicity of cosmetics and related compounds.

A rigorous strategy of curation and quality control of chemical records was applied in developing the database (as documented in the Standard Operating Procedure, chapter 2). The chemical space of the cosmetics-related compounds was compared to food-related compounds from the U.S. FDA CFSAN PAFA database using the novel approach combining the analysis of structural features (ToxPrint chemotypes) and physicochemical properties. The cosmetics- and food- specific structural classes related to particular use functions and manifested by distinct physicochemical properties were identified (chapter 3).

The novel COSMOS Skin Permeability Database containing *in vivo* and *in vitro* skin permeability/absorption data was developed by integrating existing databases and enriching them with new data for cosmetics harvested from regulatory documents and scientific literature (chapter 4). Compounds with available data on human *in vitro* maximal flux (J_{MAX}) were subsequently extracted from the developed database and analysed in terms of their structural features (ToxPrint chemotypes) and physicochemical properties. The profile of compounds exhibiting low or high skin permeability potential was determined. The results of this analysis can support rapid screening and classification of the compounds without experimental data (chapter 5).

The new COSMOS oral repeated dose toxicity database was established through consolidation of existing data sources and harvesting new regulatory documents and scientific literature. The unique data structure of the COSMOS oRepeatToxDB allows capturing all toxicological effects observed at particular dose levels and sites, which are hierarchically differentiated as organs, tissues, and cells (chapter 6). Such design of this database enabled the development of liver toxicity ontology, followed by mechanistic mining of *in vivo* data (chapter 7). As a result, compounds associated with liver steatosis, steatohepatitis and fibrosis phenotypic effects were identified and further analysed. The probable mechanistic reasoning for toxicity (Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) activation) was formulated for two hepatotoxicants, namely 1,3-bis-(2,4-diaminophenoxy)-propane and piperonyl butoxide.

Key outcomes of this thesis include an extensive curated database, Standard Operating Procedures, skin permeability potential classification rules, and the set of structural features associated with liver steatosis. Such knowledge is particularly important in the light of the 21st Century Toxicology (NRC, 2007) and the ongoing need to move away from animal toxicity testing to non-testing alternatives.

Abbreviations

ADI	Acceptable Daily Intake
ACS	American Chemical Society
ANN	Artificial Neural Network
AOP	Adverse Outcome Pathway
BAS	Bulgarian Academy of Science
CAS	Chemical Abstract Services
CERES	The U.S. FDA CFSAN Chemical Evaluation and Risk Estimation System
CFSAN	The U.S. FDA Center for Food Safety and Applied Nutrition
ChEBI	Chemical Entities of Biological Interest
CML	Chemical Mark-up Language
CMS ID	COSMOS database ID
COSING	The European Commission's Cosmetics Ingredients Database
COSMOS	Integrated <i>In Silico</i> Models for the Prediction of Human Repeated Dose Toxicity of Cosmetics to Optimise Safety
CSRML	Chemical Subgraphs and Reactions Markup Language
CTAB	The atom-bond connection table
DBMS	Database management system
DES	Data Entry System
DSSTox	The U.S. EPA Distributed Structure-Searchable Toxicity Database
EAFUS	Everything Added to Food in the United States
EC	The European Commission
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	The U.S. Environmental Protection Agency
ER	The entity relationship
EU	The European Union
EURL ECVAM	The European Union Reference Laboratory for Alternatives to Animal Testing
FCS	Food Contact Substances
FDA	The U.S. Food and Drug Administration
GLP	Good Laboratory Practice
GO	Gene Ontology
GRAS	Generally Recognised As Safe
ILSI	International Life Sciences Institute
InChI	IUPAC International Chemical Identifier
InChIKeys	IUPAC International Chemical Identifier Keys
INCI	International Nomenclature of Cosmetic Ingredients
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JRC	Joint Research Centre
KDD	Knowledge discovery from the data
LD proteins	Lipid droplet-associated proteins
LIMU	Liverpool John Moores University
LO(A)EL	The Lowest Observed (Adverse) Effect Level
MDB	Microsoft Access file format
MDL	Molecular Design Limited

MIE	Molecular Initiating Event
MINIS	MINIMUM Study inclusion criteria
MM	Molecular Modelling
MoA	Mode-of-Action
MOL	Molecule File format
MoS	Margin of Safety
NO(A)EL	The No Observed (Adverse) Effect Level
NTP	The U.S. National Toxicology Program
OCSPP	The U.S. EPA Office of Chemical Safety and Pollution Prevention
OECD	The Organisation for Economic Co-operation and Development
OpenTox	An Open Source Predictive Toxicology
OPPTS	The U.S. EPA Office of Pollution Prevention and Toxics
oRepeatToxDB	COSMOS oral repeated-dose toxicity database
PAFA	The U.S. FDA Priority-based Assessment of Food Additives
PBO	Piperonyl butoxide
PC	Principal Component
PCA	Principal Components Analysis
PCPC	The U.S. Personal Care Products Council
PPAR γ	Peroxisome Proliferator-Activated Receptor gamma
QA	Quality Assurance
QC	Quality Control
QSAR	Quantitative Structure-Activity Relationship
REFNUM	Reference Number from the EC COSING database
RN	Registry Number
SAR	Structure-Activity Relationship
SCCS	The European Commission Scientific Committee for Consumer Safety
SDF	Structure-Data file format
SED	Systemic Exposure Dosage
SEURAT	Safety Evaluation Ultimately Replacing Animal Testing
S-IN	Soluzioni-Informatiche
SMILES	The Simplified Molecular Line Input Entry System
SOM	Self-Organising Map
SOP	Standard Operating Procedure
TG	The OECD Test Guideline
Tox21	Toxicology in the 21st Century
ToxCast	The U.S. EPA Toxicity Forecaster
UVCB	Unknown or Variable Compositions, Complex Reaction Products and Biological Materials
WHO	World Health Organisation
XLS	Microsoft Excel file format
XML	Extensible Mark-up Language

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Chapter 1 Introduction

1.1. The EU COSMOS project as a response to the current European Cosmetics Regulation

Since the beginning of time, humans have applied various substances to the skin for multiple reasons: medicinal, religious, and to enhance beauty. Nowadays, the term “cosmetics” refers to a range of everyday hygiene and luxury products. According to the official definition of the European Union (EU), a cosmetic is “any substance or preparation intended to be placed in contact with the various external parts of the human body (...) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition”. The term “cosmetic product” refers to any cosmetic or mixture of cosmetics, as defined above. The final formulation of the cosmetic product, which is placed on the market and made available to the consumer, is named the “finished cosmetic product” (EC, 2003).

Cosmetics and cosmetic products are regulated at the EU level. The Cosmetics Regulation (which replaced the Cosmetics Directive as of 11 July 2013) established two bans on animal testing for cosmetics purposes, namely: the testing ban (referring to the testing of the finished cosmetic products and cosmetic ingredients on animals, completed as of 11 March 2009), and the marketing ban (related to the marketing in the EU of the finished cosmetic products and ingredients which have been tested on animals, completed as of 11 March 2013) (EC, 2003; EC, 2009). The ban on testing does not assume that all cosmetics ingredients are safe, but that non-testing procedures (*in vitro* and *in silico*) may have to be applied to assess their safety.

An EU cluster of seven projects, brought together under a Research Cluster entitled “Safety Evaluation Ultimately Replacing Animal Testing” (SEURAT-1), was formed as a direct response to this legislation, with the vision of the replacement of traditional animal-based experiments with predictive toxicology tools. Starting in January 2011, the five-year project “Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of Cosmetics to Optimise Safety” (COSMOS) was launched within the framework of SEURAT-1, as a collaboration between major international agencies (the European Commission (EC)

Joint Research Centre (JRC) and the U.S. Food and Drug Administration (FDA)), and a range of partners from industry and academia (including Altamira LLC, Columbus, OH, USA; Bulgarian Academy of Science (BAS), Sofia, Bulgaria; Molecular Networks GmbH, Nüremberg, Germany; Soluzioni Informatiche (S-IN), Vicenza, Italy), and was coordinated by Prof. M. Cronin from Liverpool John Moores University (LJMU, Liverpool, UK). The COSMOS project was funded jointly by the EC 7th Framework Programme and Cosmetics Europe (the industry's trade association for cosmetics, toiletries and perfumes, formerly Colipa), and was completed as of December 2015.

The main focus of the COSMOS project was the development of innovative non-testing (computational) tools and their subsequent integration into publicly available, transparent workflows to facilitate the complex process of predicting human repeated dose toxicity associated with exposure to cosmetics and related compounds. At that time, and still today, the SEURAT-1 cluster was the largest EU initiative undertaken to develop alternatives to animal-based toxicity testing for the safety assessment of chemicals. The current PhD program, conducted within the frame of the COSMOS project, supported these general efforts.

1.2. Computational alternatives to animal-based toxicity testing

In the field of predictive toxicology a range of diverse computational methods is applied in order to identify, characterise, and evaluate the hazards and risks posed by chemicals to human health and the environment (Yang et al., 2008; Yang et al., 2009; Hardy et al., 2012). These methods can be divided into two general categories, namely: prediction systems (statistical or knowledgebase expert ones) and data mining (Matthews & Contrera, 1998; Johnson et al., 2001; Greene, 2002; Benigni & Zito, 2004; Helma et al., 2004; Yang et al., 2006). The general principles of the methods relevant to the current thesis are introduced in the present section¹.

Generally, prediction systems are based on the premise that the physicochemical properties and biological activities of a chemical depend on its intrinsic nature and can be

¹ The provided description of the computational methods has been limited to those related to the research conducted within the current PhD program and does not cover all approaches utilised in the COSMOS project (e.g. Threshold of Toxicological Concerns, Physiologically Based Pharmacokinetic Modelling, etc.)

predicted directly from molecular structure or inferred from similar compounds whose properties and activities are known (Mostrag-Szlichtyng et al., 2010; Worth & Mostrag-Szlichtyng, 2010). These methods include a range of approaches, such as Structure-Activity Relationships (SARs), Quantitative Structure-Activity Relationships (QSARs), or chemical grouping and read-across.

The SAR-based approaches refer to the qualitative identification of the relationship between molecular structure (or a fragment thereof, i.e. an atom, or group of adjacently connected atoms in a molecule) and the presence of a particular biological activity, which may subsequently lead to the determination of structural alerts. SAR can also refer to the determination of the combination of steric and electronic features of the chemical compound considered necessary to ensure its intermolecular interaction with a specific biological target molecule, which results in the manifestation of a particular biological effect. In this case, SAR may be referred to as a “3-dimensional (3D) SAR” or “pharmacophore” (Worth & Mostrag-Szlichtyng, 2010).

QSAR-based methods express the relationship quantitatively (frequently in a form of a regression model) between a biological activity (which may be categorical or continuous) and one or more molecular descriptor(s), which describe chemical structure in numerical terms and serve as biological endpoint predictors (Worth & Mostrag-Szlichtyng, 2010; Todeschini & Consonni, 2009). The principles of the validation of QSAR models were published by the Organisation for Economic Co-operation and Development (OECD) (OECD, 2005). Guidance on the regulatory application of QSARs were published by the OECD and European Chemicals Agency (ECHA) providing a framework for using the data derived from the models as opposed to those derived experimentally (OECD, 2007a; ECHA, 2008).

It has to be highlighted that, traditionally, a range of statistically-based QSAR models were developed to describe the chemical information for the compounds investigated by employing a range of molecular descriptors representing relevant structural features. However, QSAR methods limit the information relating to the associated complex biology by collapsing it into a single value representing the predicted endpoint which, very often, imprecisely defines, or covers several different, mechanisms of action. As a consequence, the association between chemistry and biology, being the basis of the predictive toxicology, remains unclear and makes the resulting predictions difficult to interpret.

In order to overcome the limitations of traditional, chemistry-oriented QSAR modelling, identification of groups of chemicals with similar or related biological modes of action (MoA) can be performed, e.g. through chemical grouping/read-across, or systematic data-mining of the available biological data (both approaches have been discussed below). QSAR modelling within MoA-based categories of compounds (MoA QSAR) allows the linkage of the biological attributes underlying toxicity pathways with chemical structure frameworks, and addresses, in part at least, the limitations of statistical models.

In order to support the results of QSAR analysis, or to generate estimated data in the absence of suitable models, the chemical grouping and read-across approaches can be used. Guidance documents on the application of this method have been published by the OECD (OECD, 2007b) and ECHA (ECHA, 2008; ECHA, 2010). The term “read-across” refers to the use of endpoint information for (“source”) chemical(s) to make a prediction of the same endpoint for another (“target”) chemical, for which no, or inadequate, data may exist. With respect to the essential concept underlying predictive toxicology, i.e. that similar compounds (analogues) are expected to yield similar biological activity (Johnson & Maggiora, 1990), the source and target chemicals have to be considered similar according to (a set of) relevant characteristic(s) (e.g. structural, mechanistic, metabolic). Depending on the general data availability for a given endpoint, it may be possible that only a few suitable analogues can be identified, or, conversely, that a larger group of compounds can be found and used for a “chemical category” formation. The physicochemical properties and biological activities of the chemical category constituents are likely to be similar or follow a regular pattern as a result of relevant similarity characteristics and a common underlying mechanism or mode-of-action. In general, the application of read-across between analogues in a mechanistically supported, MoA-based chemical category is considered to be more reliable than the application of read-across in a smaller group of structural analogues based on a homologous series (Worth & Mostrag-Szlichtyng, 2010).

MoA-based predictive toxicology supports, and is supported by, the general Adverse Outcome Pathway (AOP) framework (OECD, 2013). AOPs are a predictive paradigm based on the upstream sequence of biological events that are determinants of the apical adverse outcome. An AOP typically starts from the Molecular Initiating Event (MIE), which triggers the progression of the pathway towards the higher level responses (Key Events, KEs), and

leads to the perturbations observed at the whole organism level. The MoA/AOP approach is increasingly applied to understand adverse health effects caused by repeated exposure to chemicals. The MIE delivers mechanistic information on chemical-biological interactions at the molecular level that can be further associated with structural and physicochemical characteristics of the chemical compound (Ankley et al., 2010).

The successful application of meaningful MoA-based tools, including chemical grouping, read-across, and QSAR models, is related to (and heavily relies on) systematic data-mining of the available biological data. Data mining (also termed “data/pattern analysis”, “data archaeology” or “data dredging”) can be regarded as a process of discovering patterns and retrieving knowledge from massive amounts of raw data, or “knowledge discovery from data” (KDD). This interdisciplinary process is situated at the crossroads of database technology, statistics, and artificial intelligence and is an iterative sequence of the following steps (Bramer, 2007; Han et al., 2012):

- Data preprocessing – preparation of the raw data for the actual data mining, involving:
 - Data cleaning – identification and removal of inconsistent data,
 - Data integration – merging multiple data sources,
 - Data selection – retrieval of the data relevant to the scientific question in hand,
 - Data transformation – data aggregation and summarisation;
- Data patterns discovery – actual data mining involving application of artificial intelligence techniques to uncover and extract hidden patterns in the data;
- Data patterns evaluation – identification of the patterns truly relevant to the investigated issue;
- Presentation of the knowledge developed by utilising visualisation and knowledge representation techniques.

Data mining allows for the identification of the concealed relationships and patterns in the data. Thus, it can be utilised as a predictive technique facilitating the objectives of computational toxicology (Yang et al., 2006).

The usefulness of QSAR- and predictive data mining- based tools to support regulatory safety assessments has been evaluated and demonstrated over the past decade (Yang et al., 2006; Yang et al., 2008; Benz, 2007; Mayer et al., 2008). Regardless of the method applied (i.e. predictive system or data mining), any computational technique relies to a great extent on the size, quality and availability of the biological data (Yang et al., 2006; Yang et al., 2009).

1.3. The role of a database in reaching the goals of predictive toxicology

In general, the term “database” refers to the collection of interrelated data. Together with a set of software programs for data access, management, organisation (defining the logical structure of the data, i.e. the data model), and update, it forms a database management system (DBMS) (Han et al., 2012). With respect to the database design, the commonly used data model is a relational model invented by Edgar F. Codd (Codd, 1970). The relational database can be defined as a collection of uniquely named, interrelated tables (relations), consisting of a set of attributes (columns, fields) and storing a set of tuples (rows, records). Each record (described by a set of field values) represents a database object identified by a unique key. Frequently, for relational databases semantic data models are also developed, e.g. the entity-relationship (ER) data model representing the database as a set of entities and their relationships (Chen, 2002; Han et al., 2012). The data in the relational database can be accessed *via* the database queries.

From a predictive toxicology standpoint, a database with a relational structure, capable of storing chemical structures and toxicity information which can be searched and retrieved, is a fundamental form of data for mining applications, computational model development, and read-across of diverse sources and endpoints. Connecting biological effects and chemicals involved in toxicity pathways can be performed exclusively after systematic data mining. This requires a database to be equipped with specifically designed ontologies and controlled vocabularies.

The term ontology refers to the explicit formal representation of a set of concepts and their relationships within particular domain, linking facts to the related terms in a causal order (Sowa, 1999; Noy & McGuinness, 2001). The iterative process of ontology development involves (and leads to) formulating and extending the domain knowledge by interactive

integration of knowledge from diverse domains. It requires (and allows for) abstracting and generalising information, extracting and formulating rules, and identifying associations with fundamental principles. An example of a successful mature ontology covering the cell biology area is the Gene Ontology (GO), widely used in biological databases, annotation projects, computational analyses for annotating newly sequenced genomes, text mining and modelling (Hardy et al., 2012).

A mature chemical-toxicological ontology is necessary for the KDD process in predictive toxicology. The toxic effects and underlying mechanisms can be identified through precisely categorised terms, which provide the rationale and the basis for further toxicity prediction (by chemical grouping and MoA QSAR modelling, for instance). Chemical-toxicological ontology supports existing knowledge applications (by sharing the common understanding of the information in the scientific community) and extensions (by providing a well-structured framework).

A chemical-toxicological database meeting the outlined requirements is a prerequisite to achieve any of the objectives of predictive toxicology in terms of modelling, knowledge creation and data management (Yang et al., 2006; Yang et al., 2008; Yang et al., 2009; Han et al., 2012; Hardy et al., 2012).

1.4. COSMOS database

One of the most critical considerations in reaching the objectives of the COSMOS project was the paucity of publicly available data for cosmetics and related chemicals. As such, the construction of a new, high quality chemical-toxicological database with a cosmetics-oriented domain was crucial.

The majority of publicly available repeated-dose toxicity data refer to the oral route of exposure and thus do not include cosmetics (and rarely cover cosmetics-related compounds), which are usually applied topically. However, since the scientific community has sufficient understanding of oral absorption and skin permeability processes, an approach using extrapolation (referred to as “oral-to-dermal extrapolation”) utilising this knowledge and oral repeated-dose toxicity data has been applied to realise the goals of the COSMOS project. There is an identifiable need to expand knowledge to cosmetics

ingredients and related substances. The content requirements for constructing the COSMOS database can therefore be summarised as follows²:

- An inventory of cosmetics and cosmetics-related compounds populated with high quality chemical structures and available regulatory information, such as daily intake estimates or regulation history;
- Skin permeability/absorption data for cosmetics and related compounds;
- Oral repeated dose toxicity data for cosmetics and related compounds.

The process of collating the required COSMOS database content, as well as the strategy of dealing with the difficulties and challenges associated with particular information types, are presented in the current thesis.

In order to serve as a foundation to develop computational tools to predict the repeated dose toxicity of cosmetics and related chemicals, the COSMOS database had to accommodate various types of biological data. The data model, capable of handling such a diverse information types, has been inherited from the risk assessment database of the Chemical Evaluation and Risk Estimation (CERES) project at the United States Food and Drug Administration (U.S. FDA) Center for Food Safety and Applied Nutrition (CFSAN). The CERES database houses the internal regulatory information of the CFSAN, as well as other toxicity databases, including the chemical records and toxicity data from the legacy U.S. FDA Priority-based Assessment of Food Additives (PAFA) database (Benz & Irausquin, 1991). The COSMOS and CERES databases share the same data model, technology, software programs, and a very similar user interface.

The COSMOS database data model consists of two interconnected data domains (Figure 1.1): a chemical domain and a biological domain. A very high-level overview of the main entities is provided in this section.

The central entity of the COSMOS database chemical domain is “Compound”, meaning that all other entities stored in this part of the database are Compound-related. An entity “Compound” (a chemical compound or substance) is identified by a unique system identifier, CMS ID, and represents a chemical composition which may consist of one or more

² The outlined data needs are limited to the data relevant for the presented PhD program, and do not exhaustively cover the requirements of the entire COSMOS project

molecules, i.e. “Structure” entities, referring to the molecular structures. Compound may thus be formed of multiple Structure entities, and, at the same time, a single Structure may appear in multiple Compounds (many-to-many relationship). Depending on its chemical composition, Compound may not be related to any Structure (this is discussed more in-depth in chapter 2). Regardless of the association of the Compound with Structure entities, multiple Compound-related information items can be stored in the COSMOS database, including Names, Identifiers, Use Functions, along with attributes further defining their source, type, etc.

The toxicological part of the data model reflects the diverse and heterogeneous layout of the COSMOS database content. At the very high level it defines the entity “Study”, corresponding to a toxicological study, which may consist of a various number of “Test” instances, representing a series of experiments applied to a “Test System” (which might be a series of animals, tissues, etc.). The Test System instances reflect all the peculiarities of the recorded Tests. Each Test entity references a Test Result, reporting the outcomes of the relevant toxicity experimental series. Finally, a Study references its own Study Result, aggregating the Test Results. The Study Result is a final outcome of the toxicity endpoint, which is based on all Test outcomes within a study. Such fine data granularity allows for the storage of the information regarding each single experimental series and observed toxicological effects on the one hand, and the summarised, higher-level information (final Study conclusion, e.g. compiled by the human expert) on the other. It also supports the development of the ontology sets facilitating the mechanistic data mining of the COSMOS database (which is discussed more in-depth in chapters 6 and 7).

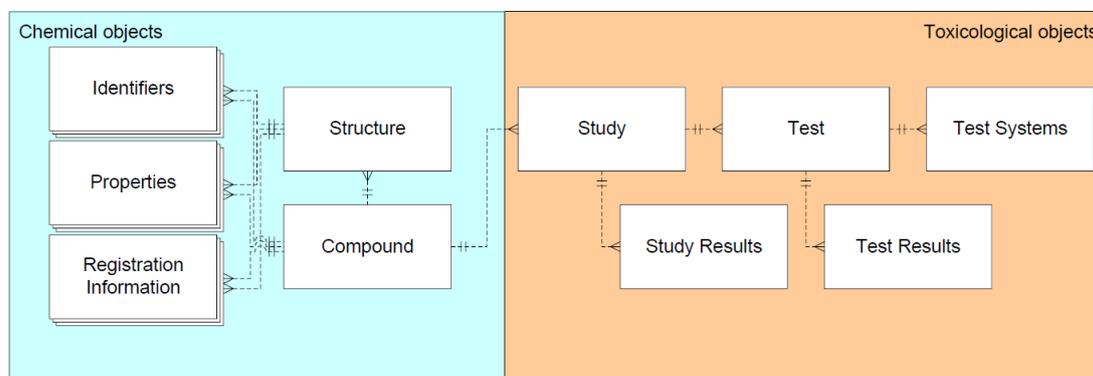


Figure 1.1
Simplified schematic diagram of the COSMOS database data model

The first version of the COSMOS database (COSMOS DB v1.0) was made publicly available in December 2013 and its dump is currently downloadable from <http://cosmospace.cosmostox.eu>. The final version of the COSMOS database (COSMOS DB v2.0) was released in April 2016 and can be accessed at: <https://cosmosdb.eu/cosmosdb.v2/>.

1.5. The present PhD program and its association with the COSMOS project

The main aim of the present PhD program, conducted in conjunction with the COSMOS project, was **the collation of the content, in terms of chemical structures, skin permeability and toxicological data, within a relational chemical-toxicological database, and its subsequent application for the development of knowledge to support the prediction of repeated dose toxicity of cosmetics and related compounds.**

This multi-faceted goal has been broken down into the following objectives, realised in collaboration with other COSMOS partners (please refer to Annex 1 for the detailed contribution of the author of present thesis), and discussed in depth in the subsequent chapters of the thesis:

Objective 1: The quality control of the COSMOS database chemical domain, with particular emphasis on cosmetics ingredients and related compounds

This objective (discussed in chapter 2) has been realised through:

- Development of the sets of controlled vocabularies for chemical compounds and structures annotations, with a specific goal to address the problematic issues related to the representation and identification of cosmetics related substances. It was a dynamic process associated with curation of part of the COSMOS database chemical domain (U.S. EPA DSSTox inventory);
- Preparation of the Standard Operating Procedure (SOP) for conducting the Quality Control/Quality Assurance (QC/QA) process of the COSMOS database chemical domain;
- Conducting the QC/QA process.

Objective 2: Characterising the chemical space occupied by cosmetics ingredients and related chemicals

This objective (discussed in chapter 3) was realised through:

- Performing structural features (ToxPrint chemotypes) and physicochemical properties space analysis of the COSMOS Cosmetics Inventory profiled for the most abundant use functions within cosmetics domain;
- Comparing the chemical space occupied by the cosmetics-related compounds with the food-related compounds from the U.S. FDA CFSAN PAFA database.

Objective 3: The development of a high quality COSMOS Skin Permeability Database enriched with cosmetics ingredients and related compounds

This objective (discussed in chapter 4) was realised through:

- Curation and integration of existing skin permeability/absorption data sources, namely the EDETOX and University of Kent databases;
- Development of the SOP for harvesting new data;
- Harvesting new skin permeability data for cosmetics ingredients and related compounds from the regulatory and literature sources according to the SOP developed;
- Integration of the newly harvested data with the EDETOX and University of Kent content;
- Preparation of a data entry tool for QC of the COSMOS Skin Permeability Database.

Objective 4: Classification of skin permeability potential following dermal exposure to chemicals to support the safety assessment of cosmetics related chemicals

This objective (discussed in chapter 5) was realised through:

- Data mining of the Skin Permeability Database that was constructed, leading to the collation of the set of compounds with available experimental data on maximal flux (J_{MAX});
- Structural (with ToxPrint chemotypes) profiling of the collated dataset combined with the analysis of the physicochemical properties of its compounds;
- Determination of a set of rules classifying a chemical compound into the category of low or high skin permeability potential.

Objective 5: Construction of a high quality database for oral repeated dose toxicity with dose/concentration level information for cosmetics and related compounds

This objective (discussed in chapter 6) was realised through:

- Harvesting new oral repeated dose toxicity data for cosmetics and related compounds from regulatory and literature sources according to the predefined SOP;
- Conducting the QC/QA process on the resultant COSMOS oRepeatToxDB that was constructed.

Objective 6: Mechanistic (ontology-based) liver toxicity data mining of the COSMOS oRepeatToxDB on the basis of the ontology developed from the collated data

This objective (discussed in chapter 7) was realised through:

- Validation of a liver toxicity ontology developed on the basis of the data collated when the COSMOS oRepeatToxDB was constructed;
- Ontology-based liver toxicity (steatosis) data mining of the COSMOS oRepeatToxDB;
- Structural (ToxPrint chemotype) analysis of the sets of chemical compounds obtained and identification of the structural fragments associated with the investigated endpoint;
- Formulation of the mechanistic reasoning for the toxicity of selected compounds on the basis of an evaluation of the published literature and the results of molecular modelling analysis (supplied by the collaborating COSMOS project partners from BAS).

As the current PhD program was undertaken within the auspices of the COSMOS project, it supported the general objectives of the Project to develop alternative (non-testing) tools to facilitate the safety assessment of cosmetics ingredients and related chemicals within the European Union. The outcomes of the current PhD program provide a solid foundation for further knowledge discovery.

Chapter 2

Quality Control of the COSMOS Database Chemical Domain

2.1. Background

2.1.1. General aspects of the quality of chemical information and structures

The COSMOS database is a chemo-centric system integrating data from various sources into a unified data model (please refer to chapter 1.4). For the development of a chemistry-aware database, the correct, unambiguous representation of chemical compounds encoded in a way enabling convenient storing, searching and integrating with other systems, is of major significance. The power of any cheminformatics tool depends greatly on the accuracy of the representation of molecular structures and related data, so that they can be understood by both human scientists and machines. The importance of the accurate identification of chemical structures for *in silico* modeling has been also recognised (Young et al., 2008; Fourches et al., 2010). It has been demonstrated that QSAR models developed with incorrect structures, or with the structures incorrectly handled by the computational tools, yield significantly poorer predictive accuracies when compared to the models developed on the basis of training sets with high quality structures.

Currently, several approaches aiming to address chemical identification and representation issues are utilised in the cheminformatics field. Their advantages and limitations are discussed below.

2.1.1.1. Chemical nomenclature

Chemical nomenclature refers to the set of formalised rules consistently applied to generate the names of chemical compounds within a particular convention, e.g. International Union of Pure and Applied Chemistry (IUPAC) names (IUPAC, 2016), or International Nomenclature of Cosmetic Ingredients (INCI) names (CIRS, 2016). In principle, chemical nomenclature should ensure unambiguous identification of a chemical compound, meaning that one chemical name should refer to a single substance. In practice, however, applying the nomenclature-imposed rules usually leads to very complex names, which cannot be commonly recognised or used to infer structural information without expertise. Thus, a variety of other names (trivial, trade, etc.) commonly recognised by the scientific

community is widely used. As they do not conform to any formal rules or system, they cannot directly serve as unambiguous identifiers of chemical compounds (Brecher, 1999; Fourches et al., 2010).

2.1.1.2. Chemical identification codes

Chemical identification codes are source-specific identifiers (e.g. digital), without any chemical significance *per se*, which may be utilised at a very local scale (e.g. within the laboratory or a corporate database to identify the compounds tested), or may be recognised more broadly. An example of internationally used compound identification numbers (albeit proprietary in their nature) are Registry Numbers (RNs), assigned by American Chemical Society (ACS) Chemical Abstract Services (CAS) (CAS, 2016). CAS RNs are intended to be unique numeric identifiers designating only one specific chemical substance and linking information to it (e.g. references, names, structures). They constitute from up to ten digits, divided by hyphens into three parts. The right-most digit is a “check digit” used to confirm the legitimacy and uniqueness of the entire identifier. CAS RNs are not related to any system of chemical nomenclature, and, as such, can provide a common link between various nomenclatures used to describe substances. However, it should be noted that a single chemical compound can be associated with multiple CAS RNs, as several types of CAS RNs are currently in use. They include alternate, deleted, and generic RNs. An Alternate Registry Number refers to the second RN generated for a less preferred structural representation of a substance. A deleted Registry Number is a RN once assigned to a substance, but later changed to another RN. Such cases may refer to the compounds that once appeared in the literature with a trade name, but without associated structural information, and which are later associated with a substance that has been already registered (Stanford University Libraries, 2016). A generic Registry Number is a RN representing the whole class of compounds (e.g. CAS RN “1330-20-7” for “xylene”) rather than pointing to the individual structure (e.g. 1,2-xylene (CAS RN “95-47-6”), or 1,3-xylene (CAS RN “108-38-3”).

2.1.1.3. Line notations

Line notations refer to the representation of chemical structures as linear strings of characters. The simplest example of a line notation would be the empirical molecular formula.

The most commonly used line notation system, the Simplified Molecular Line Input Entry System (SMILES) (Weininger, 1988), is based on a set of rules for converting the chemical structures into SMILES strings, which are accepted as an input format by the majority of chemistry software tools. The conversion process is fully automated. Both, stereochemistry and double bond geometry of molecular structures can be correctly handled by SMILES, however there are many errors involved in this format, due to the reality that some tools do not process SMILES correctly, or that many users are not sufficiently experienced to use them correctly. The other drawback of the SMILES representation is that multiple strings can be written for a single chemical. This limitation can be addressed by applying an algorithm for canonical atoms numbering, however it is successful only when used consistently as a single algorithm. In reality, different software tools utilise different canonical numbering algorithms, thus, the SMILES strings generated by them cannot be considered unique.

Another line notation system is the IUPAC International Chemical Identifier (InChI) codes, developed to provide a standardised format for a formalised version of IUPAC names, which could be interpreted by humans and conveniently used for searching the chemical databases. In order to represent a chemical compound, an InChI code contains layers of information on the atoms, bonds, connectivity, tautomeric forms, isotopes, stereochemistry and charge (as appropriate to individual chemicals). InChI codes provide truly unique string identifiers of chemicals. However, interpretation of InChI codes by human scientists requires a lot of expertise, and InChI codes are currently not accepted by the majority of software tools. InChIKeys are a version of InChI codes hashed into keys, i.e. strings of characters, in order to further support the storage and searching in large chemical databases. InChIKeys comprise of 27 characters, and are not interpretable by humans (Heller et al., 2013). For InChIKeys, there is a theoretical (albeit statistically unlikely) possibility of duplicates.

2.1.1.4. Coding constitutions

Coding constitutions represent the constitutions of chemical structures explicitly. The atom-bond connection table (CTAB) is one of the forms of chemical structure representation, describing the structural relationships and properties of a collection of atoms. Molecular structure is presented as a topological graph with nodes representing the atoms linked by edges representing bonds. The atoms in the CTAB may be wholly or partially

connected by bonds. The atom block of the CTAB specifies the atom coordinates (2- or 3-dimensional), atomic symbols, any mass difference (from mass in periodic table), charge (including radical state), stereochemistry and associated hydrogens. The bond block specifies the two atoms connected by the bond, the bond type (single, double, triple, aromatic), any bond stereochemistry and topology (chain or ring properties). The connection table is a fundamental part of the Molecular Design Limited (MDL) file format for the representation and communication of chemical information, including the Molecule (MOL) and Structure-Data (SD) files (MDL, 2005).

Chemical Mark-up Language (CML) provides a general means to represent chemical compounds using the Extensible Mark-up Language (XML) schema, allowing for the storage of the annotations and properties for the chemical compound (CMLC, 2016).

Of the various representation methods, the SD file is one of the most accurate and reliable for storing tautomer and stereochemistry information. In addition, the 3D chemical structures required to specify certain preferred conformations can be represented only by the xyz-coordinates in the connection table of the SD file or in CML atom blocks.

2.1.2. Compilation of the COSMOS database chemical domain

As described in section 1.4, the COSMOS database includes two interconnected parts: a “Compound”-centred part, referred to herein as “chemical domain”, and “Study”-centred biological/toxicological part. The chemical domain of the COSMOS database can be therefore regarded as a collection of compounds (CMS IDs) with specific attributes: registry numbers, names, structures (and their attributes) and use functions.

2.1.2.1. Chemical structure sources in COSMOS database

The chemistry part of the COSMOS database has been built through integration of several inventories of compounds (Table 2.1), donated for the COSMOS project by the U.S. FDA CFSAN CERES, the U.S. EPA DSSTox (*ca.* 12,000 records), and businesses related to the COSMOS project (e.g. Procter and Gamble contributed *ca.* 25,000 structures as a result of its membership of the Scientific Advisory Board). Multiple structures have been also retrieved manually by COSMOS consortium partners. At the time of data integration, each available connection table was assigned a “quality score” with respect to its origin. The values of the quality scores ranged from 100 (for the highest quality structures from respectable, curated

sources, e.g. the U.S. FDA CFSAN) to 5 (for the structures retrieved from publicly available, non-curated sources, e.g. the internet). The CAS structures donated from the U.S. FDA CFSAN CERES have been considered a “gold standard”.

Table 2.1

The source inventories of the COSMOS database chemical domain

Inventory (Owner, Name, Reference)	Inventory content
The U.S. FDA CFSAN CERES, including the Priority-Based Assessment of Food Additives Database (PAFA) (Benz & Irausquin, 1991)	<p>The U.S. FDA donation of about 70,000 public records from CERES, including the chemical part of the PAFA database.</p> <p>PAFA is a legacy database of regulatory-relevant chemical records, containing administrative, chemical and toxicological information on direct and indirect food additives, colour additives, and Generally Recognised As Safe (GRAS) and prior-sanctioned substances, as well as over 3,000 substances in an inventory called Everything Added to Food in the United States (EAFUS), being the list of ingredients added directly to food (FDA-approved as food additives), or listed or affirmed as GRAS.</p> <p>It is noteworthy that the PAFA Chemical Information includes historical data on: population exposure to chemicals, human consumption of the chemical, Acceptable Daily Intake (ADI) values set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and a “Technical Effect” descriptor to define chemical use categories.</p>
The U.S. EPA Distributed Structure-Searchable Toxicity Database (EPA DSSTox, 2016)	Repository of publicly available chemical structures, accurately mapped to the associated bioassay and physicochemical property data. About 12,000 DSSTox structures were donated to the COSMOS project by the U.S. EPA.
The U.S. EPA ToxCast Inventory (EPA ToxCast, 2016)	Toxicology in the 21st Century (Tox21) is a collaborative project among the U.S. EPA, NIH and FDA, aiming to develop enhanced methods for toxicity assessment. The Toxicity Forecaster (ToxCast) is one of the EPA’s contributions to Tox21 and refers to the chemical screening results for over 2,000 chemicals conducted in two research phases. The ToxCast inventory has been donated to the COSMOS project by the U.S. EPA.

2.1.2.2. COSMOS Cosmetics Inventory

A special emphasis was placed on cosmetics ingredients and cosmetics-related chemicals. The repository of them was compiled by merging the EC COSING database (COSING, 2016), and the U.S. PCPC list (Bailey, 2011) (Table 2.2) and is referred to as the “COSMOS Cosmetics Inventory”, a fundamental part of the COSMOS database.

Table 2.2

The source inventories of the COSMOS Cosmetics Inventory. The specified counts refer to the status of original, not curated inventories

Inventory (Owner, Name, Reference)	Inventory content
The European Commission COSING Database	<p>Database of information on cosmetic substances and ingredients contained in the EC Cosmetics Regulation (EC, 2009), Cosmetics Directive (EC, 2003), and Inventory of Cosmetic Ingredients (EC, 2006), as well as covered by the opinions on cosmetic ingredients of the Scientific Committee for Consumer Safety, SCCS (SCCS, 2016). The COSING database was downloaded in April 2011 from the EC COSING database website. The inventory file included:</p> <ul style="list-style-type: none"> ○ 19,391 COSING identifiers (REFNUMs), encoding the chemical compound together with its use functions (the single compound with multiple use functions has been represented by multiple REFNUMs) ○ 9,286 CAS RNs ○ 19,397 INCI names used in the EU <p>The International Nomenclature of Cosmetic Ingredients (INCI) system was established in the early 1970s by the PCPC. The INCI names are assigned according to the defined standards by the PCPC and are used in the USA, the EU, China, Japan, and many other countries for listing ingredients on cosmetic product labels. With few exceptions, the INCI labeling names in all countries should remain the same. The current (as of April 2016) list of INCI names is maintained by the PCPC, and includes over 16,000 ingredients (CIRS, 2016).</p> <ul style="list-style-type: none"> ○ 66 chemical use functions <p>The extensive list of possible functions of ingredients used in cosmetic products and their definitions from the COSING database has been provided in Annex 1.</p>
The U.S. Personal Care Products Council (PCPC) List	<p>The U.S. PCPC inventory has been compiled from a book (Bailey, 2011) published from the PCPC containing a list of cosmetics ingredients available in U.S. market. The inventory contained:</p> <ul style="list-style-type: none"> ○ 3,713 CAS RNs ○ 3,512 INCI names used in the U.S

2.1.2.3. Curation needs and integration of the source inventories

The integration of chemical inventories (Tables 2.1 and 2.2) required curating original records (and chemical structures), identifying and removing duplicate ones, and joining the inventories on the basis of the common identifier(s). Any of the chemical representation methods outlined in section 2.1.1 can potentially become a source of errors. With respect to the chemical structures, the errors may occur either due to the implicit limitations of line notations and coded constitutions, or due to their incorrect handling by the software tools, or humans lacking sufficient expertise.

For the COSMOS database, a range of additional quality-related issues had to be considered. The Cosmetics Inventory largely comprises botanical extracts, oils, mixtures, dyes, etc., which translate chemically into macromolecules (polymers, peptides), inorganic compounds, coordination and transition metal complexes, etc. Such compounds frequently require the Markush type of representation, which is not handled well by currently available cheminformatics tools. Particularly challenging (if not impossible) is assigning structures to Unknown or Variable Compositions, Complex Reaction Products and Biological Materials (UVCB) (EPA, 2016a). A number of such substances could not be represented by CTABs (or other line notations), and were considered “non-structurable”. Frequently, many of these types of compounds have not been yet registered in the CAS Registry Database, making the task of their accurate representation and identification even more complicated.

Due to the reasons outlined, the curation of records from the source inventories was demanding and required various types of processing. For instance, in case of the U.S. EPA DSSTox inventory (please refer to Annex 1), the main focus was placed on adopting the original annotations to the controlled vocabulary of the COSMOS database (dynamically developed during the curation efforts, please refer to sections 2.3.1 and 2.4.1).

Considering the COSING and PCPC lists contributing to the COSMOS Cosmetics Inventory, additional processing was necessary. For instance, both INCI names and CAS RNs should, in principle, uniquely identify the chemical compounds between (and within) the COSING and PCPC. In practice, due to the generic representations (please refer to section 2.1.1.2) of cosmetics ingredients and related chemicals, single compounds have been frequently associated with multiple CAS RNs, and conversely, single CAS RNs have been related to multiple compounds. Actual examples of many-to-many relationships between CAS RNs and INCI names in original records are presented in Table 2.3. Also, other nomenclature-related issues were commonly found, e.g. the lack of conformance between INCI names from COSING or PCPC lists with CAS Index Names, or differences between INCI names used in the U.S. and EU e.g. both INCI names “*oryza sativa bran cera*” (COSING) and “*oryza sativa (rice) bran wax*” (PCPC) refer to the same botanical compound.

Whilst some inventories (e.g. the U.S. FDA CFSAN CERES, or EPA DSSTox) were processed into the COSMOS database in a relatively straightforward manner, the COSMOS

Cosmetics Inventory required the development of a specific integration procedure, presented schematically in Figure 2.1.

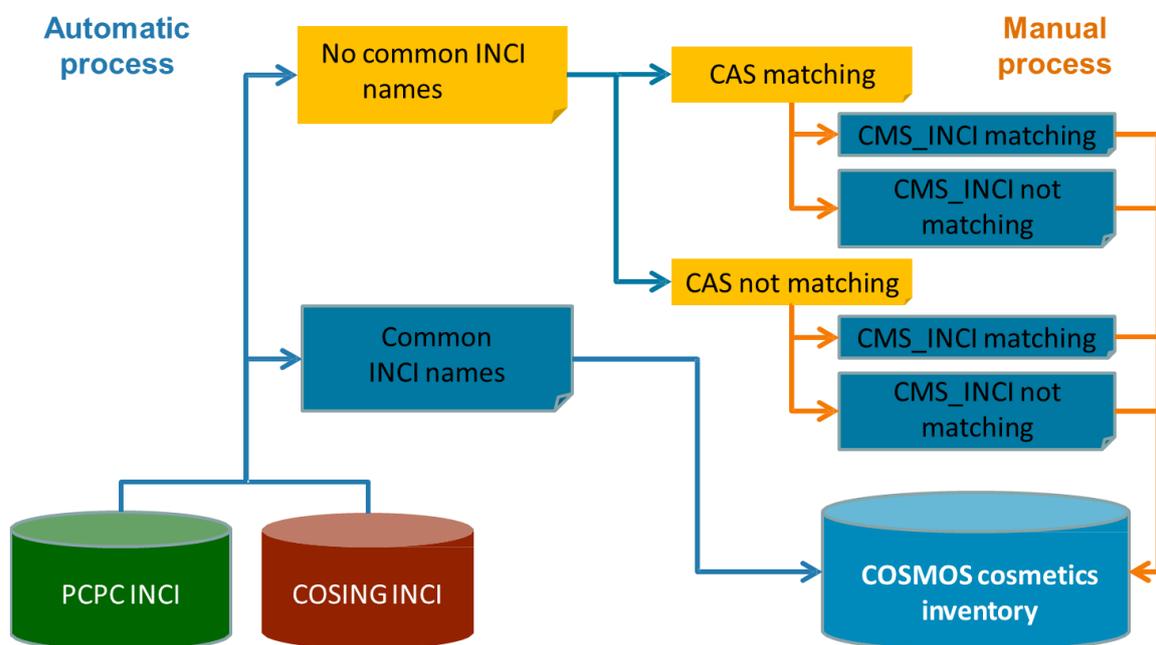


Figure 2.1

Integration of the EC COSING and the U.S. PCPC inventories leading to the construction of the COSMOS Cosmetics Inventory. The two lists were combined by two indices: INCI Names and CAS Registry Numbers. The compounds with common INCI Names were assigned a CMS ID in a fully automated process. For the compounds without common INCI Names, CAS RNs were analysed and used to text-mine the name nests. The INCI Names of these nests have been further examined and controlled. Due to many-to-many relationships, abundant between INCI Names and CAS RNs from the source lists, detections of duplicates in overlaps between the two inventories have been performed by direct comparison of InChIKeys for the compounds with available connection tables

Table 2.3

An example of many-to-many relationships between CAS registry numbers and INCI Names found for the compounds from the surfactants class in unprocessed COSING and PCPC inventories*

Source inventory	INCI Name (from source inventory)	CAS RN (from source inventory)	Molecular formula (from source inventory)	CAS index name (for CAS reported in the source inventory)	CAS molecular formula (for CAS reported in the source inventory)
COSING	Sodium laureth-12 sulfate	9004-82-4	Not given	Poly(oxy-1,2-ethanediyl), α -sulfo- ω -(dodecyloxy)-, sodium salt (1:1)	(C ₂ H ₄ O) _n C ₁₂ H ₂₆ O ₄ S.Na
PCPC	Sodium laureth-12 sulfate	66161-57-7	C ₃₆ H ₇₄ O ₁₆ S.Na	3,6,9,12,15,18,21,24,27,30,33,36-Dodecaoxaocatetracontan-1-ol, 1-(hydrogen sulfate), sodium salt (1:1)	C ₃₆ H ₇₄ O ₁₆ S.Na
PCPC	Sodium laureth-12 sulfate	9004-82-4 (generic)	C ₃₆ H ₇₄ O ₁₆ S.Na	Poly(oxy-1,2-ethanediyl), α -sulfo- ω -(dodecyloxy)-, sodium salt (1:1)	(C ₂ H ₄ O) _n C ₁₂ H ₂₆ O ₄ S.Na
COSING	Sodium laureth-7 sulfate	9004-82-4	Not given	Poly(oxy-1,2-ethanediyl), α -sulfo- ω -(dodecyloxy)-, sodium salt (1:1)	(C ₂ H ₄ O) _n C ₁₂ H ₂₆ O ₄ S.Na
PCPC	Sodium laureth-7 sulfate	9004-82-4 (generic)	C ₂₆ H ₅₄ O ₁₁ S.Na	Poly(oxy-1,2-ethanediyl), α -sulfo- ω -(dodecyloxy)-, sodium salt (1:1)	(C ₂ H ₄ O) _n C ₁₂ H ₂₆ O ₄ S.Na

*Inspecting the three records available for sodium laureth-12 sulfate, CAS RN “9004-82-4” (provided in both, COSING and PCPC inventories) is a generic CAS, representing the whole class of polymeric surfactants with dodecyl or C10-C16 range of alkyl chain length with varying number of ethoxy ether groups. This “generic representation” is clearly indicated in the CAS molecular formula by ill-defined number of repeating units (n), and in the CAS index name. On the contrary, CAS RN “66161-57-7” is a specific registry number, referring to the polymeric surfactant with dodecyl alkyl chain and 12 ethoxy ether groups, i.e. sodium laureth-12 sulfate. Whilst a compound with varying composition (such as a polymeric material having a distribution of chain lengths) can be represented by a generic CAS, a compound with specific composition/configuration within such a class should be associated with a specific CAS RN. Considering the example of sodium laureth-7 sulfate with the defined molecular formula: “C₂₆H₅₄O₁₁S.Na”, the specific CAS would be: “66197-75-9” and the corresponding CAS index name: “3,6,9,12,15,18,21-Heptaoxatritriacontan-1-ol, 1-(hydrogen sulfate), sodium salt (1:1)”

2.1.3. Final content of the COSMOS database chemical domain

As a result of merging the inventories identified in Tables 2.1-2.2, the entire COSMOS v.2.0 database (<https://cosmosdb.eu/cosmosdb.v2/accounts/login/?next=/cosmosdb.v2/>) consists of 81,604 chemical records. Connection tables are available for 46,791 (48%) compounds. The remaining ones largely consist of natural products (biological macromolecules, botanical oils, extracts, mixtures, etc., minerals) and other non-structurable substances. The InChI Keys analysis performed on 46,791 COSMOS structures indicated 44,773 unique CTABs. Structural duplicates in the COSMOS database were allowed in multiple cases requiring the use of representative structures (please refer also to Table 2.4). Over 72% of all available connection tables were assigned the highest quality score of 100 (section 2.1.2.1). The lowest quality structures, with a score of 5, comprise *ca.* 8% of all COSMOS CTABs.

2.1.3.1. COSMOS Cosmetics Inventory

The COSMOS Cosmetics Inventory, compiled from the EU COSING and U.S. PCPC list (Table 2.2; Figure 2.2), consists of 17,100 unique chemical records (by CMS IDs), associated with 9,278 unique CAS RNs and 16,111 unique INCI Names. The connection tables are available for 5,562 Cosmetics Inventory compounds.

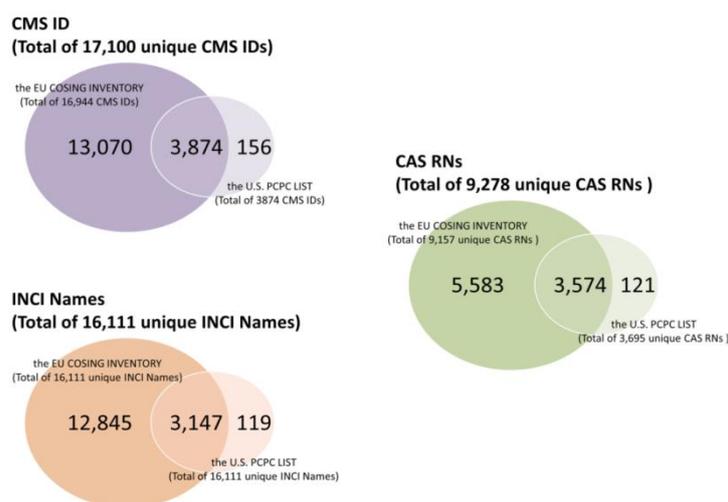


Figure 2.2
Venn Diagrams showing the number of chemical records (by assigned CMS IDs, curated CAS RNs or INCI Names) present in both (EU COSING and U.S. PCPC) or only one (EU COSING or U. S. PCPC) of the source inventories

2.2. The aims of chapter 2

As outlined, properly identified chemical compounds represented by high quality structures are crucial for the successful development of a chemical-biological relational database and for facilitating the development of any *in silico* tool for toxicity prediction.

The aims of the present chapter realised in collaboration with the COSMOS consortium partners (please refer to Annex 1) relate to the objective 1 of the current PhD program (section 1.5), and include:

- Development of the sets of controlled vocabularies for chemical compounds and structures annotations, with a specific goal to address the problematic issues related to the representation and identification of cosmetics related substances. It was a dynamic process associated with curation of part of the COSMOS database chemical domain (U.S. EPA DSSTox inventory);
- Development of a Standard Operating Procedure (SOP) to conduct the Quality Control/Quality Assurance (QC/QA) process of the COSMOS database chemical domain;
- Conducting the QC/QA process.

2.3. Materials and methods

2.3.1. Development of a controlled vocabulary for compounds and structures annotations

In every case of information exchange and integration, it is fundamental to have a set of common standards with respect to both the format and content of the data. According to the COSMOS database data model (chapter 1.4), the chemical compounds registered in the COSMOS database (uniquely identified with CMS IDs) can be associated with no (for non-structurable compounds), or one or more (for structurable compounds) connection tables. For each unique CMS ID multiple, non-unique information items may be stored (e.g. names, use functions, external identifiers). The many-to-many relationships between compounds and structures have been allowed, e.g. between parent and related compounds, such as components of mixtures, monomers of polymers, representative structures, etc. Controlled vocabularies were designed as an integral part of the COSMOS database data model, providing the terminology and foundation to correctly organise and

handle the complex relationships within the content of the COSMOS database chemical domain, as well as to deal with a range of problematic cosmetics-related compounds, e.g. those that are non-structurable and could not be represented by empirical molecular formulae, other line notations, or connection tables (section 2.1.2.3).

The process of the development of a controlled vocabulary was dynamic. The preliminary terminology was designed in collaboration with the U.S. FDA, the U.S. EPA, and Altamira LLC (please refer to Annex 1) at the initial stage of curating and integrating the source inventories. As the chemistry content curation progressed, the controlled vocabularies were refined and updated, as needed. The sets of the controlled vocabularies have been designed for the following compound- and structure-related elements: stereochemistry, double bond geometry, material type and composition type.

2.3.2. Development of the Standard Operating Procedure for the Quality Control (QC) of the COSMOS database chemical domain

In order to perform the Quality Control of the COSMOS database by several COSMOS partners with maintained consistency, it was necessary to develop a Standard Operating Procedure (SOP). It was compiled by Altamira LLC (Annex 1), and covered all the tasks planned to be conducted during the QC process (including the verification of correctness of chemical names, registry numbers, structures, and compound and structures annotations). The initial SOP was systematically updated during the ongoing QC procedure to cover newly identified issues.

2.3.3. Conducting the Quality Control (QC) and Quality Assurance (QA) Processes of the COSMOS database chemical domain

The Quality Control (QC) process can be defined as the verification of the accuracy of the database content with respect to the predefined standards, resolving the eventual deviations and modifying the process as needed. The QC of the COSMOS v.1.0 database chemistry content was led by LJMU, and has been completed with the effort of five COSMOS partners (Annex 1). Approximately 1% of structurable (i.e. containing connection tables) compounds included in the COSMOS database were sampled randomly and evenly distributed between the QC participants. QC was performed *via* the COSMOS database Data Entry System (DES), according to the previously developed SOP (section 2.3.2).

The Quality Assurance (QA) process maintains the quality standards of the database by sampling the observations at a given confidence level, such that the relevant statistics (error rates, i.e. the ratios of incorrect records to the total number of sampled records) can be reported. During the Quality Control process, the main emphasis was placed on assuring the connection tables, registry numbers and names correctness, as these elements were subsequently used for the calculation of the QA statistics. The QA statistics have been derived from the COSMOS DB DES audit trail and by comparing all the QC-ed records to the original, not QC-ed ones.

2.4. Results

2.4.1. Controlled vocabularies for chemical compounds and structures annotation

Sets of controlled vocabularies were developed to annotate the following compound- and structure- related elements: stereochemistry, double bond geometry, material type and composition type.

2.4.1.1. Stereochemistry

Stereochemistry annotations refer to the isomerism of the structure resulting from the differences in the spatial arrangement of atoms without accompanying differences in connectivity or bond multiplicity (IUPAC, 2016). The following controlled vocabulary was designed for the COSMOS database:

- **Absolute stereochemistry**, referring to the chemical structures for which the absolute configuration of the chiral centre(s) is provided. The structures with a known, specific, single configuration have the direction of rotation specified, i.e. they were annotated as “Absolute stereochemistry, rotation (-)”, or “Absolute stereochemistry, rotation (+)”.
- **Relative stereochemistry**, applicable to the structures for which the relative configuration of two or more chiral centres was provided (i.e. the relationship to two or more centres was specified), but the absolute value in their relationship is unknown.
- **Relative stereochemistry, racemic mixture**: The compound is a racemic mixture of the structure as drawn, and its identical mirror image.

- **No stereochemistry**, meaning that no stereochemistry is associated with the compound (i.e. the stereochemistry annotation was not applicable).

2.4.1.2. Double bond geometry

Double bond geometry annotations include:

- **Double bond geometry (E-)**, referring to structures with single or multiple double bonds(s), all with *trans*- geometry.
- **Double bond geometry (Z-)**, referring to structures with single or multiple double bonds(s), all with *cis*- geometry.
- **Double bond geometry (E-,Z-)**, referring to structures with multiple double bonds(s) representing both (*trans*-, *cis*-) types of geometric isomerism.
- **Double bond geometry unspecified**, referring to structures with double bond(s), but without geometric isomerism specified.
- **No double bond geometry**, relevant for structures that are not associated with any double bond geometry (i.e. the double bond geometry annotation is not applicable).

2.4.1.3. Material type

Material type refers to the chemical nature of the compound. The following material types have been recognised in the COSMOS database:

- **Biological**, referring to macromolecules of biological importance, e.g. protein or nucleic acid sequences, lipids, enzymes, etc. for which it is (usually) difficult to define a structure, or they are non-structurable.
- **Botanical**, referring to compounds of natural/plant origin, largely comprising of complex, structurally difficult to define (or non-structurable) mixtures, extracts, oils, etc.
- **IOM**, denoting the entire category of compounds including:
 - **Inorganic**: Chemical structures without organic carbon atoms; elements including metal atoms and metalloid atoms (boron (B), silicon (Si), germanium (Ge), arsenic (As), antimony (Sb), tellurium (Te)); ions (e.g. borate, chromate); minerals.
 - **Organometallic**: Chemical structures containing organic carbon(s) directly bonded to any metal atom other than alkali (I) or alkaline earth (II) metals.

- **Organometalloid:** Chemical structures containing organic carbon directly bonded to any metalloid atom.
- **Metal complex:** Chemical structures containing a central metallic atom covalently bonded to the total number of ligands, either larger or smaller than indicated by the central metal atom's oxidation state.
- **Metalloid complex:** Chemical structures containing a central metalloid atom covalently bonded to the total number of ligands, either larger or smaller than indicated by the central metalloid atom's oxidation state.
- **Organic,** to annotate the chemical compounds containing organic carbon atom(s) (i.e. not carbon monoxide, carbon dioxide, carbonates and cyanides), but not being classified as organometallics or organometalloids.
- **Polymer,** referring to the chemical compounds with polydispersed composition, i.e. constituted by regularly or irregularly repeated units.
- **Unspecified,** relevant in cases when little or no information was available for a compound (e.g. exhaust gases, complex reaction products obtained in industrial processes).

2.4.1.4. Composition type

Composition type corresponds to the chemical constitution of the compound, i.e. the elements listed in its molecular formula. In the majority of cases, the composition type can be inferred solely from the molecular formula. However, the information on the configuration of the chemical compound (e.g. geometric or positional isomers, stereoisomers) cannot be derived from the molecular formula and has to be inferred from the compound name or connection table, if applicable. The composition type has been controlled by the following vocabulary:

- **Defined formula,** referring to chemical compounds with chemical structure fully represented in the molecular formula (except configuration information, please refer to the descripton above and to the annotation "**varying isomers**" below)
- **Ill-defined formula,** relevant in many cases when chemical structure is only partially represented in the molecular formula. This composition type can include **varying compositions** and/or **varying number of repeating units (polydispersion)**

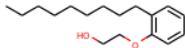
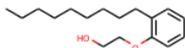
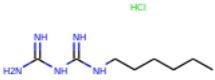
- **Varying composition**, refers to chemical structures in which one or more components are unknown (unspecified), or the stoichiometric ratios (e.g. in salts) are unknown.
- **Polydispersion**, refers to the varying number of repeating units. This term is relevant for polymers and is usually reflected in the molecular formula ($(/n)$), and/or in the name of the compound (*poly-*). For instance: C_8H_8 is a molecular formula of styrene, whereas $(C_8H_8)_n$ represents *polystyrene*.
- **Formulation**, for annotating well-defined (usually commercial, possibly proprietary) compositions of two or more substances.
- **Unspecified**, applicable in cases when little or nothing is known about the composition of the chemical and, as such, no molecular formula can be derived (e.g. complex reaction products).
- **Varying isomers**, referring to any type of relevant isomerism (may be geometric, positional, or stereoisomerism) and is applicable to both, defined and ill-defined compositions. The information on the isomerism cannot be inferred from the molecular formula and has to be decided upon after inspection of the name or the structure of the chemical compound.

2.4.1.5. Application of the annotations scheme

Examples of application of the above controlled vocabularies in the COSMOS database are presented in Table 2.4. The COSMOS annotations facilitate the compilation of computational datasets (i.e. the datasets that can be used for the computational analysis; described in more details in chapter 3).

Table 2.4

An example of application of composition and material type annotations in the COSMOS database. The records 1-2 and 3-5 refer to cases where the composition type of the compound cannot be inferred from the molecular formula alone and the positional isomerism has to be inferred from the compound name. Record 6 demonstrates the case when annotations can be assigned from the molecular formula alone. Cases where the representative structures have been used are also presented

Rec #	Molecular Formula	CAS RN	CAS Index Name	Composition Type	Material Type	Structure Image	Structure note
1	$(C_2H_4O)_n C_{15}H_{24}O$	26027-38-3	Poly(oxy-1,2-ethanediyl), α -(4-nonylphenyl)- ω -hydroxy-	III-defined formula – polydispersed	Organic - polymer		Representative structure for polymer (n=1)
2	$(C_2H_4O)_n C_{15}H_{24}O$	9016-45-9	Poly(oxy-1,2-ethanediyl), α -(nonylphenyl)- ω -hydroxy-	III-defined formula – polydispersed – varying isomers	Organic - polymer		Representative structure for polymer (n=1) and positional isomer (as 4-nonylphenyl)
3	C_8H_{10}	1330-20-7	Benzene, dimethyl-	Defined formula – varying isomers	Organic		Representative structure for positional isomer (as 1,3-dimethyl)
4	C_8H_{10}	108-38-3	Benzene, 1,3-dimethyl-	Defined formula	Organic		Actual structure
5	C_8H_{10}	95-47-6	Benzene, 1,2-dimethyl-	Defined formula	Organic		Actual structure
6	$(C_8H_{17}N_5)_n \cdot xClH$	32289-58-0	Poly[iminocarbonimidoyliminocarbonimidoylimino-1,6-hexanediyl], hydrochloride	III-defined formula – polydispersed – varying composition	Organic - polymer		Representative structure for polymer (n=1) and composition (x=1)

2.4.2. Standard Operating Procedure for the Quality Control (QC) process

The final SOP (updated and finalised after the QC) is provided in Annex 2. The entire QC procedure consisted of several steps, as presented in Figure 2.3.



Figure 2.3

An overview of the Quality Control procedure conducted of the COSMOS database chemical domain through Data Entry System. The QC procedure consisted of the following steps:

- (1) Logging-in to the COSMOS database Data Entry System with the personal access credentials;
- (2) Accessing the Data Entry System;
- (3) Selecting the “Review and QC Existing Structures and Data” option under the “Chemistry” section of Data Entry System;
- (4) Searching for the query compound (i.e. the one subjected to the QC) in the COSMOS database chemical domain;
- (5) Selecting the compound for QC from the search results list (the outmost right “QC-ed” box is not checked yet);
- (6) Selecting the tab corresponding to the QC-ed element: “Structure Annotation”, “Compound Annotation”, “Registry Numbers and IDs”, or “Names”, and performing the actual quality control tasks. Subsequently saving the results;
- (7) Receiving the confirmation of the successful completion of the QC process on the final screen (the outmost right “QC-ed” box is now marked)

The actual tasks performed during the QC of particular elements are discussed in the following paragraphs.

2.4.2.1. Structure annotation

The Quality Control of “Structure Annotation” (Figure 2.4A) covered the verification (and correction, if needed) of the following elements:

- Connection table (and depiction of the structure’s image). For each connection table the COSMOS database automatically calculates the InChIKey using the RDKit cheminformatics library (RDKit, 2016). For the purpose of the QC process, the “corrected connection table” has been defined as a connection table which has been sufficiently modified in the QC process, as to result the creation of a new InChIKey;
- Stereochemistry annotation (with respect to the developed controlled vocabularies);
- Double bond geometry annotation (with respect to the developed controlled vocabularies);
- The source provided for each structure. This element was subject to correction in addition to making any changes in the connection table during the QC procedure.

2.4.2.2. Compound annotation

The Quality Control of the “Compound Annotation” (Figure 2.4B) focused on checking of the correctness of the following elements:

- Molecular formula;
- Material type annotation (with respect to the developed controlled vocabularies);
- Composition type annotation (with respect to the developed controlled vocabularies).

2.4.2.3. Registry numbers and IDs

The QC of “Registry Numbers and IDs” (Figure 2.4C) focused on the verification (and resolving any conflicts, if necessary) of:

- The correctness of Registry Number(s) assigned to the compound (structure/name pair correctness);
- The correctness of specified RN types (active, alternate, deleted, generic, etc.) (please refer to section 2.1.1.2);

- The correctness of other (external) identifiers, e.g. the EC COSING REFNUMs, the DSSTox CIDs, etc., by querying them in the source inventories.

2.4.2.4. Names

The Quality Control of the “Names” (Figure 2.4D) covered the verification (and correction, if needed) of:

- The correctness of the chemical names provided for the query compound (also with respect to the structure, Registry Numbers, identifiers);
- The correctness of “Name Type”, as specified, e.g. INCI Name. The “Preferred Name” in the COSMOS database was not subjected to any changes;
- The correctness of “Name Source”, as specified. The “Name Source” should correctly correspond to the “Name Type”, e.g. for the INCI Name type, the correct sources could include “COSING” or “PCPC”.

(A) Structure Annotation

(B) Compound Annotation

(C) Registry Numbers and IDs

(D) Names

Figure 2.4
Quality Control of the COSMOS database chemical domain through the COSMOS DES: (A) Structure Annotation; (B) Compound Annotation; (C) Registry Numbers and IDs; (D) Names

2.4.3. Results of the QC/QA of the COSMOS database chemical domain

The QC/QA process of the COSMOS v.1.0 database chemistry content was performed for *ca.* 1% of structurable (i.e. containing connection tables) compounds randomly sampled from the database, giving a total number of 442 structures subjected to the QC. The QC tasks were accomplished through the COSMOS DB Data Entry System (DES), with particular emphasis placed on the QC of connection tables, Registry Numbers, and compound names, as these elements were used for the QA statistics determination.

2.4.3.1. Connection tables quality control

The QC of the 442 structures led to the correction of a total of 43 connection tables, giving an overall error rate of 9.7%. Three specific types of corrections were performed:

- Correction of the connectivity and stereochemistry, or protonation state, of the structure;
- Correction of the stereochemistry and protonation state (connectivity unchanged);
- Correction of the protonation state (connectivity and stereochemistry unchanged).

Overall, 16 connectivity changes have been made (giving the connectivity error rate of 3.6%), 24 stereochemistry changes (the error rate of 5.4%) and 3 changes for protonation states (the error rate of 0.7%).

2.4.3.2. Registry numbers quality control

During the QC cycle only two (out of 442) CAS RNs were identified as being incorrectly assigned and were thus corrected, giving an error rate of 0.45%. Additionally, 151 new CAS RNs were added to the compounds inspected.

2.4.3.3. Chemical names quality control

The QC process has resulted in correcting ten (out of 442) chemical names, giving an error rate of 2.2%. Additionally, 732 new names were added to the databases.

Based on the results of the QA on chemical structures, the approximate percentage of inaccurate structures in COSMOS v.1.0 is 4.3% (if stereochemistry is ignored), and 9.7% (if stereochemistry is considered). Approximately 2.2% of the names may contain errors and 0.5% of the records may have incorrect registry numbers. The COSMOS database is, by far,

the only publically available database, which chemistry content has been carefully QC-ed and for which the QA statistics are available.

2.5. Discussion

This chapter describes the process of collation of the COSMOS database chemistry content, including the COSMOS Cosmetics Inventory. It involved integration of several chemical inventories into a unified data model, and systematic curation and quality control of chemical records.

The need for a strategy addressing concerns over the quality of chemical information and structures was clearly demonstrated. As such, a novel set of controlled vocabularies was developed with respect to the specific features of cosmetics and related compounds. It provides the unified terminology systematising the complex relationships within the cosmetics domain and the framework to deal with a range of non-structurable (or difficult to structure) compounds. The designed COSMOS annotations may facilitate the compilation of computational datasets (i.e. the datasets that can be used for the computational analysis) by enabling fast and convenient identification of non-structurable compounds (e.g. unspecified compositions or material types), or compounds that are not handled well by the computational tools (inorganics, metal complexes, organometalloids, polymers, mixtures, etc.).

The systematic quality control procedure established during the COSMOS database development was captured as the Standard Operating Procedure (SOP) document (Annex 2), which can serve as a point of reference during future efforts of chemical structures collation.

The precise identification of chemical compounds within COSMOS Database supports the populating of the associated toxicological content by enabling accurate referencing of chemical and biological records. This, in turn, supports the development of meaningful *in silico* tools, as their performance is dependent on the accuracy of the representation of molecular structures and related data.

The COSMOS Database contains 81,604 chemical records. The COSMOS Cosmetics Inventory is particularly important part of the COSMOS database. It consists of 17,100

unique chemical records, associated with 5,567 chemical structures and is, by far, the largest publicly available inventory of cosmetics and related compounds. During the COSMOS project it served as a foundation to realise multiple objectives. After the COSMOS project, it still has numerous scientific and regulatory applications. As a publicly available, ready-to-use repository of cosmetics-related compounds with associated toxicological data it can support data mining, *in silico* methods development, risk assessment and read-across tasks (by, for example, enabling identification of structural analogs within cosmetics domain). The COSMOS Cosmetics Inventory was used in the analysis described in chapter 3, including the determination of structural features and physicochemical properties associated with different types of cosmetics and comparison of these characteristics with food-related compounds.

Chapter 3

Chemical Space Analysis of the COSMOS Cosmetics Inventory

3.1. Background

The chemical space of an inventory of chemicals (or a toxicity database) can be regarded as the ranges of physicochemical properties and structural features covered by its constituents. It is an important piece of information for many reasons (Yang et al., 2008; Worth & Mostrag-Szlichtyng, 2010):

- Analysis of chemical space may be a first step towards the development of meaningful computational methods accurately predicting toxicity by verifying and assuring sufficient coverage of the relevant chemical space. Utilising relevant means of describing the physicochemical and structural characteristics of the chemicals across various inventories ensures that the basic concept underlying predictive chemistry and toxicology that similar molecules are expected to exhibit similar properties and biological activities is fulfilled (Johnson and Maggiora, 1990);
- The structural and physicochemical properties space of a dataset, analysed together with the biological activity of its members, may provide insights improving the understanding of complex toxicological phenomena. It may lead to the identification of the chemistry-biology associations and facilitate the discovery of structural features linked to toxicity. Thus, it supports the development of mode-of-action knowledge from the data;
- Information on the chemical space covered by the inventory or a dataset is also particularly important for the successful application of existing computational tools. *In silico* models should be applied only to the chemical compounds situated within their applicability domains, since outside this they are unlikely to give reliable predictions. When the predictive performance of model is assessed by challenging it with an independent (external) validation (test) set, it is useful to compare the chemical space of the test set with that of the training set. When the predictive performance of a model is assessed against a limited test set, and the conclusions are generalised to a wider dataset (or chemical inventory), it is important to compare the chemical space of the test set with that of the wider inventory;

- The comparison of the chemical space occupied by diverse inventories representing various types of compounds (e.g. cosmetics- vs food-related, as in the research discussed in the present chapter) enables the investigation of the physicochemical and structural features of the molecules with respect to their specific use types in specific environments. It allows for the context of where and how the particular molecular characteristics have been manifested to be investigated.

Performing computational analysis of the chemical space of an inventory of compounds or a dataset requires the following steps: (i) preparing molecular structures for the purpose of computational analysis; (ii) calculating relevant molecular descriptors; (iii) applying appropriate statistical techniques to make the results of calculations interpretable. Each of these steps is discussed below.

3.1.1. Curation of chemical structures

The importance of appropriate preparation of molecular structures for computational analysis, so that they can be correctly interpreted and handled by the software used to compute molecular descriptors, has been widely recognised (Young et al., 2008; Fourches et al., 2010; Waldman et al., 2015) and outlined in chapter 2. No standardised sets of procedures that should be applied to curate structures have so far been formulated, as the computational generation of structures should be always performed with regard to the aim of the analysis. For instance, the investigation of general structural classes represented in a given inventory or a dataset requires a different approach than the calculation of quantum mechanical descriptors for individual compounds. In the first case, 2D structures may be sufficient, whereas in the second one, 3D coordinates of the investigated molecules have to be provided. General aspects related to the preparation of computational datasets are listed and discussed in depth in Table 3.1.

Table 3.1

General aspects associated with preparation of molecular structures for computational analysis (Young et al., 2008; Fourches et al., 2010)

Task	Description
Identification of IOM compounds (please refer to chapter 2)	The majority of molecular descriptors were designed for organic compounds for which valence-bond structures can be formulated and, as such, cannot be calculated for inorganics. For organometallics and organometalloids, calculations may be also not possible, due to the common lack of necessary calibration for rare earth metals.
Identification of salts	The majority of software tools cannot process organic salts, which contain metal counter ions. The properties of salts can vary from the properties of parent compounds. However, in cases when the organic part of the molecule is responsible for its biological activity, the metal counter ions should be removed and the remaining carbo- cations or anions should be neutralised.
Identification and processing of mixtures	Although some approaches to deal with mixtures have been suggested, the majority of available software tools are not capable of handling these types of compounds (consisting of multiple structures) in a transparent and appropriate way. The most common approach is based on automated removal of the mixture constituent(s) with the lowest molecular weights or the smallest number of atoms. However, in cases when the entire mixture has been tested for toxicity, the associated data do not necessarily correspond to the toxicity of individual components. The “smallest fragment” should be therefore removed only in cases when it is clear that the “largest fragment” is responsible for the biological activity of the whole mixture. Such cases refer, for example, to hydrates and hydrochlorides consisting of a large biologically active organic molecule and small inorganic one(s). In case of mixtures consisting of several organic constituents with similar molecular weight, the decision on the fragment removal should be made after careful review.
Validation of chemical structures with respect to the representation of mesomeric (resonance) forms, tautomeric forms and aromatic rings	Resonance structure and aromaticity should be represented in a consistent way as any differences in representation may significantly change the values of calculated properties. For tautomers, the mechanism of action (if known) should be considered prior deciding on the appropriate representation of the compound.
Detection and removal (if desired) of duplicates	Due to nomenclatures, digital identifiers, or SMILES strings limitations repetitions of the same compound (and hence the same structure) in large inventories are relatively common (chapter 2). To detect duplicate structures, InChIKeys comparison can be successfully applied (section 2.1.1.3). However, it should be stressed that duplicate structures may also appear as a result of performing some of the above mentioned structure processing steps. For instance, counter ion(s) removal, and subsequent neutralisation of the remaining organic part of the molecule, or small fragments removal, may both lead to a single “computational” structure, represented by a single InChIKey, which actually is a (neutralised) part or a substructure of several tested compounds. On occasions, the experimental results for those records may vary. This could mean that the structure curation procedure had led to the removal of a significant molecular fragment which may be responsible for the activity of the compound tested. Such cases should be reviewed manually by human experts. Generally, the process of the removal of duplicates should be controlled and it should be always possible to relate the resulting computational structure back to the original compound tested.

3.1.2. Calculation of molecular descriptors

“The molecular descriptor is the final result of a logic and mathematical procedure which transforms chemical information encoded within a symbolic representation of a molecule into a useful number or the result of some standardised experiment” (Todeschini & Consonni, 2009).

The selection of relevant molecular descriptors with regard to the aim(s) of the analysis is essential. In case of chemical space analysis, the selected molecular descriptors should be capable of capturing the variability of investigated compounds in as broad as possible manner. As such, several types of descriptors should, ideally, be utilised. Various descriptors generated by different computational algorithms are, in principle, expected to derive diverse types of information for the compared sets of molecules. The selected descriptors should be also transparent and easy to interpret.

Molecular descriptors can be categorised in a range of different ways, for example with regard to the molecular representation used for the calculations (into zero- to three-dimensional descriptors), or to the nature (and complexity) of the calculations (into constitutional, topological, geometrical, electronic, or quantum-mechanical approaches) (Fara & Oprea, 2016). The example of generic categories of molecular descriptors is provided in Table 3.2.

Table 3.2
General categories of molecular descriptors

Molecular descriptor category	Description
Zero-Dimensional (0D)	Descriptors derived directly from the molecular formula of the compound (e.g. molecular weight, number of atoms in the molecule, etc.)
One-Dimensional (1D)	Fragment counts (e.g. number of H-bond donor and acceptor atoms)
Two-Dimensional (2D)	Topological descriptors, referring to the manner in which the atoms are connected in the molecule and calculated from mathematical graph theory (e.g. topological polar surface area)
Three-Dimensional (3D)	Geometrical, electronic and quantum-mechanical descriptors derived from the results of empirical schemes or molecular orbital calculations requiring the 3D structure of the molecule (e.g. dipole moment)

Structural fingerprints comprise another type of descriptors, encoding the molecular structure in a form of binary digits (bit-strings) representing the presence or absence of

particular substructures in the molecule (Fara & Oprea, 2016). The molecular fragments for fingerprints generation are, in principle, designed to encode particular chemical information, which can be interpreted by human scientists. The molecules can be thus compared through the comparison of their fingerprints.

The 0- to 3-D molecular descriptors, along with the structural fingerprints, provide a large portion of the information about the molecule. They are well-defined by the available cheminformatics tools and have been broadly applied throughout the present thesis (in chapters 3, 5 and 7).

3.1.3. Application of relevant statistical methods

The subsequent step of chemical space analysis requires applying relevant statistical method(s) to transform the multidimensional space occupied by the studied inventories into a lower dimensional space, and to visualise it, so it can be interpreted by a human scientist. Multiple statistical approaches have been successfully applied to this end, including Cluster Analysis (Cattell, 1943), Artificial Neural Networks (ANN) (Kohonen, 1982; Zupan & Gasteiger, 1999), or Principal Components Analysis (PCA). The technique broadly utilised in the present thesis is PCA (this chapter and chapter 5), which is discussed below.

Principal Components Analysis (Pearson, 1901; Wold et al., 1987; Jolliffe, 2002; Begam & Kumar, 2014) is a multivariate method belonging to the group of statistical multidimensional factorial methods (Cordella, 2012) that provides a compact view of variation in a data matrix by defining the orthogonal directions of maximum variance. The Principal Components-based decomposition of the original data matrix is presented schematically in Figure 3.1. Briefly, PCA can be performed by eigenvalue decomposition of the covariance (or correlation) matrix of the data and the first Principal Component (PC) is the eigenvector (showing the “direction” of the highest variance in the data) with the highest eigenvalue (specifying the amount of variance in the data in this direction). The following Principal Components are the directions maximising variance among all directions orthogonal to the previous Principal Components. Frequently, PCA results are interpreted in terms of the loadings (the covariance/correlation between the particular Principal Components and the original variables, providing information on how much of the variation in a variable is explained by the PC) and scores (defining the positions of each observation in

the new space of the Principal Components). Generally, a large proportion of the total variability in a dataset can be explained by a small number of the Principal Components. PCA is one of the most frequently employed techniques for visualising and exploring the space occupied by large chemical/toxicological datasets.

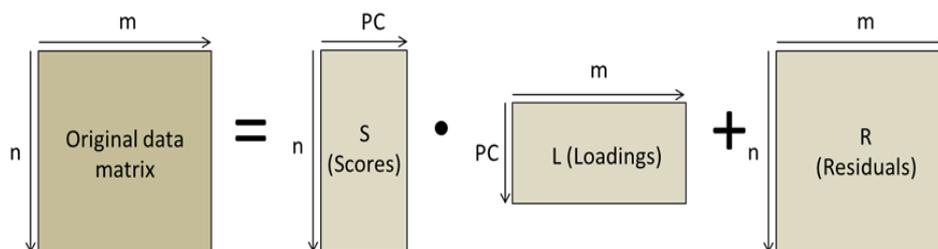


Figure 3.1

Linear decomposition of original data matrix (consisting of n compounds and m variables) by Principal Components Analysis. The columns in matrix S correspond to the score vectors of the Principal Components (PCs), whereas the columns in the matrix L to the loading vectors of the PCs. The matrix R represents any residual information not included in the S and L decomposition. PCA orders the loading and score vectors in decreasing order of variance (on the basis of (Burger & Gowen, 2011; Begam & Kumar, 2014))

3.2. The aims of chapter 3

The process of compiling the chemistry part of the COSMOS database and COSMOS Cosmetics Inventory was described in detail in chapter 2. The aims of the present chapter are related to the objective 2 of the current PhD program (section 1.5), and include:

- Analysis of the chemical space covered by the COSMOS Cosmetics Inventory in terms of the structural features (ToxPrint chemotypes) and physicochemical properties, with particular focus put on the most abundant use functions within the cosmetics domain;
- Comparison of chemical space occupied by cosmetics- to the food- related compounds from the U.S. FDA CFSAN PAFA.

3.3. Materials and methods

3.3.1. Analysed inventories

The chemical space analysis was performed for the two following inventories:

- COSMOS Cosmetics Inventory containing 5,562 structures (the collation of the COSMOS Cosmetics Inventory was described in chapter 2);
- Food-related compounds from the U.S. FDA CFSAN PAFA database (Table 2.1) including 4,337 structures.

The structures comprising both inventories were obtained from the COSMOS database as 2D SD files (referred to herein as “2D-tested” SDFs). The chemical space analysis was performed with respect to both, structural features and physicochemical properties. Each part of the analysis was based on a different methodology, thus required a different type of original SD files pre-processing.

3.3.2. Use functions of cosmetics-related compounds

Prior to the chemical space analysis, the COSMOS Cosmetics Inventory was characterised with respect to the 66 chemical use functions associated with cosmetics-related compounds from the EU COSING database (Table 2.2). The entire list of COSING use functions along with their definitions is provided in Annex 3. It was expected that specific use functions will be reflected in the structural characteristics and physicochemical properties of individual molecules.

3.3.3. Structural features (chemotypes) analysis

The analysis of general structural features represented by cosmetics and food related compounds was performed using novel approach based on the ToxPrint chemotypes (Yang et al., 2015), belonging to the structural fingerprints type of descriptors. The term “chemotype” refers to a means of representing the chemical entities as structural fragments encoded for connectivity (which may extend beyond a single connected fragment). If needed, chemotypes may be also encoded for physicochemical properties of atoms, bonds, fragments, electron systems and even whole molecules. The conceptual graph of the chemotype is presented in Figure 3.2.

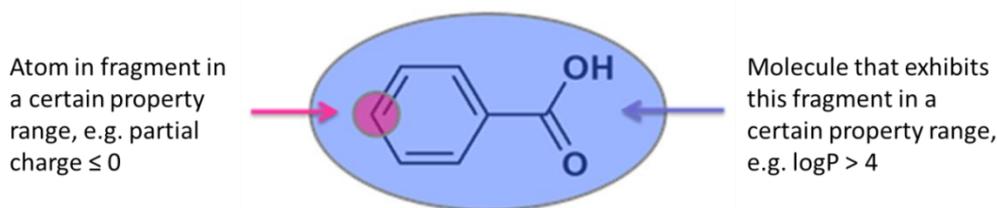


Figure 3.2
Schematic definition of a chemotype

The ToxPrint chemotypes (Yang et al., 2015), which were developed from over 100,000 chemicals present in publicly available chemical inventories and toxicity databases, cover a range of hierarchically organised structural classes (Table 3.3) and capture broad chemical spaces of food ingredients, food direct additives, food-contact substances, pharmaceuticals, agrochemicals, cosmetics ingredients and industrial chemicals. The library of 729 ToxPrint chemotypes is publicly available at www.toxprint.org and can be downloaded as a .csml (Chemical Subgraphs and Reactions Markup Language) file (Yang et al., 2015).

Table 3.3
Classes of ToxPrint chemotypes (from Yang et al., 2015)

Top class	1 st level classes	Total # of chemotypes
Atom	main group element, metal (group I, II, III, transition metals, metalloid, poor metals)	
Bond	C#N, C(~Z)~C~Q, C(=O)N, C(=O)O, C=N, C=O, C=S, CC(=O)C, CN, CNO, COC, COH, CS, CX, metal, N(=O), N[!C], N=[N+]=[N-], N=C=O, N=N, N=O, NC=O, NN, NN=N, NO, OZ, P(=O)N, P=C, P=O, PC, PO, QQ(Q~O_S), quaternaryN, quaternaryP, quaternaryS, S(=O)N, S(=O)O, S(=O)X, S=O, Se~Q, X(any), X[(any)_!C], X~Z	411
Chain	alkaneBranch, alkaneCyclic, alkaneLinear, alkeneBranch, alkeneCyclic, alkeneLinear, alkyne, aromaticAlkane, aromaticAlkene, oxy-alkaneBranch, oxy-alkaneLinear	95
Group	aminoAcid, carbohydrate, ligand, nucleobase	69
Ring	aromatic, fused, hetero, polycycle	144

In the current analysis, the ToxPrint chemotypes were used to explore and compare the structural space occupied by cosmetics- and food- related compounds. For comprehensive investigation, it was particularly important to preserve all chemical classes

occurring in both inventories, including inorganics, organometallics, organometals and metal complexes. Generating the fingerprints using ToxPrint chemotypes in this analysis did not require 3D computational structures. The salts and IOM compounds were therefore retained, and “2D-tested” SD files were used as input.

The analysis was performed with the publicly available (www.chemotyper.org) software application, ChemoTyper (Altamira LLC, Columbus, OH, USA; Molecular Networks GmbH, Nürnberg, Germany). Using the ChemoTyper, the input structures (imported from the “2D-tested” SD files) were mapped against the predefined ToxPrint library (toxprint_V2.0_r711.xml file downloaded at www.toxprint.org). The features describing the query compounds were subsequently exported in the form of a table of binary chemical fingerprints. The rows in this data matrix corresponded to particular molecules (structures) and the columns to particular ToxPrint chemotypes: a value of 1 indicated that the compound contained a given feature, whereas a value of 0 that it did not.

The frequencies of particular ToxPrint chemotypes (i.e. the number of “matches” between ToxPrint chemotypes and the query structures) have been investigated. The structural domains of the analysed inventories were presented in terms of distributions of the compounds across specified generic structural classes.

3.3.4. Analysis of physicochemical properties

The physicochemical properties space covered by the two inventories was investigated using diverse types of molecular descriptors. Therefore, this part of the analysis required pre-processing of original “2D-tested” files and generating 3D-computational structures. All these steps, as well as descriptors calculations, were conducted in the Corina Symphony software tool (Molecular Networks GmbH, Nürnberg, Germany).

The salts and IOM compounds identified through the material type and composition type annotations (section 2.4.1) have been excluded from investigation. The input files containing only organic compounds were pre-processed with the following options: desalting and small fragments removal, neutralising charged compounds, detecting and removing duplicate structures. Subsequently, the 3D coordinates have been generated and oriented according to their maximal moments of inertia. The resulting SD files (referred to

herein as “3D-computational” SD files) were used to calculate the “global molecular” and “size and shape” descriptors in Corina Symphony.

The data matrix containing (n) rows corresponding to the chemical compounds and (m) columns corresponding to the calculated descriptors was subsequently used as input in the Principal Components Analysis (PCA) performed in the JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.). For m standardised original variables (transformed to have zero mean and unit variance), m PCs were calculated. The first PC was the linear combination of the standardised original variables that had the greatest possible variance. Each subsequent PC was the linear combination of the variables that had the greatest possible variance and was uncorrelated with all previously defined components. The PCs explaining the large portion of variance in the analysed datasets and having the eigenvalues >1 were considered in the final analysis. Their scores were projected into 3D plots to visualise the physicochemical properties space. The most influential properties were identified.

3.4. Results

3.4.1. COSMOS Cosmetics Inventory – use functions analysis

The COSMOS Cosmetics Inventory was characterised with respect to the 66 chemical use functions from the EU COSING database. The Cosmetics Inventory is very diverse, with the majority of its constituents associated with multiple (up to 18) functions (for 72% of Cosmetics Inventory compounds up to three functions have been reported). The most populated use functions include skin protection and skin conditioning agents, surface-active agents (surfactants, emulsifiers, emulsion stabilisers, foaming agents and foam boosters), perfuming agents, and hair fixing and conditioning substances (Figure 3.3). The most populated use functions in the cosmetics domain have been further characterised with respect to their structural features and physicochemical properties in the following sections of this chapter.

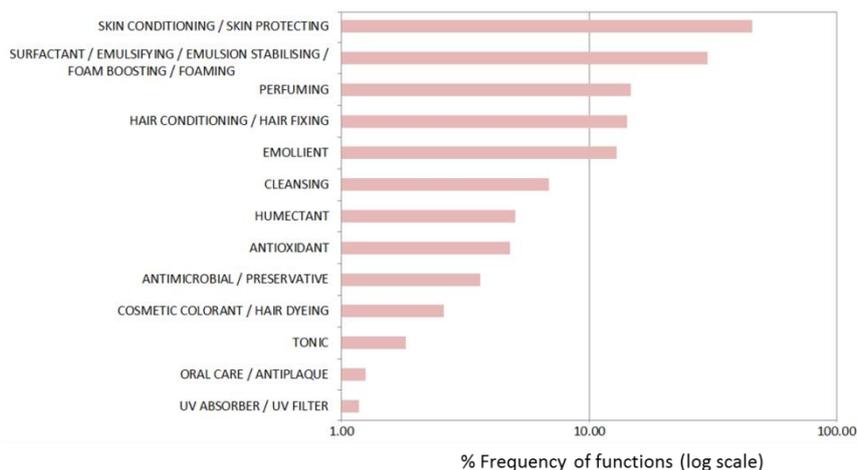


Figure 3.3
The most populated COSING use functions in the COSMOS Cosmetics Inventory

3.4.2. Structural (chemotypes) space analysis

The structural features (ToxPrint chemotypes) analysis was performed for cosmetics related compounds from the COSMOS Cosmetics Inventory (5,562 compounds) and food related compounds from the U.S. FDA CFSAN PAFA (4,337 compounds). The overlap between the two inventories (on the basis of the CMS IDs and InChI Keys generated for “2D-tested” structures) is presented in Figure 3.4.

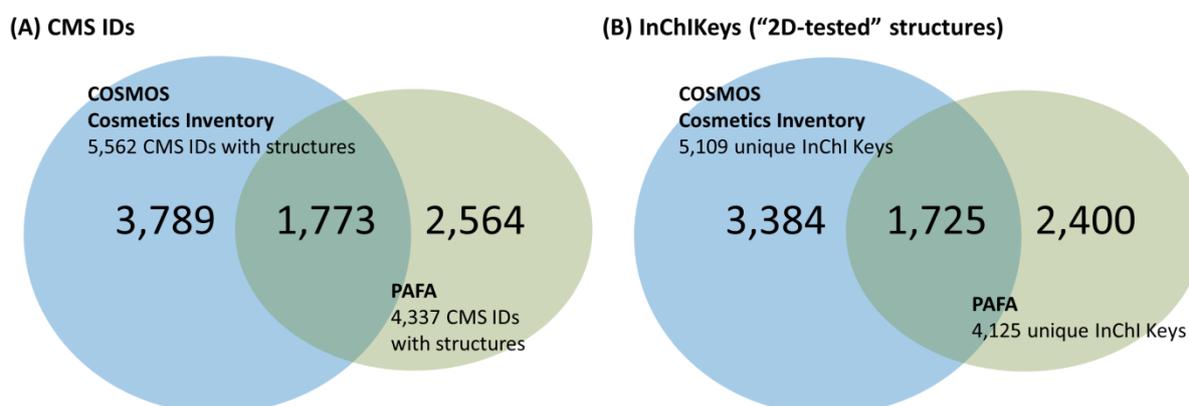


Figure 3.4
Venn Diagrams demonstrating the overlap between the COSMOS Cosmetics Inventory and food related compounds from the U.S. FDA CFSAN PAFA:

- (A) The inventories were compared by CMS IDs of compounds with available structures; 1,773 (out of 8,126) compounds appeared in both inventories; 3,552 was found only in COSMOS Cosmetics Inventory; 2,429 – only among PAFA members;
- (B) The original (not processed) 2D structures from both inventories were compared by the InChI Keys. A total of 7,509 unique InChI Keys was present in both inventories (the 617 structural duplicates resulted from using representative structures; please refer also to Table 2.4); 1,725 InChI Keys were common in both inventories

The structural domain of both inventories was covered by a total number of 553 (out of 729) ToxPrint chemotypes indicating a very sparse structural space. The overlap between structural features identified in each individual inventory (Figure 3.5) is large: 455 out of 553 ToxPrint chemotypes appeared in both the COSMOS Cosmetics Inventory and PAFA. However, due to distinctive use functions of cosmetics- and food-related compounds, several features appearing exclusively in only one of the inventories were also observed (Figure 3.5). For instance, organotin compounds (appearing in food contact materials) and dithiocarbamates (food contact substances, antimicrobials, food direct additives) were found only in PAFA, whereas several transition metals (used as preservatives, buffers or antimicrobials), metals from group III of periodic table (cosmetics colorants, hair dyes, antiperspirants, deodorants, also buffering and chelating agents), certain carbohydrates (skin conditioning, surface active agents, antioxidants), purine/adenine (skin conditioning), or polyethylene glycols with >10 ethylene oxide (EO) units (surface-active agents) were present only in the inventory of cosmetics related compounds.

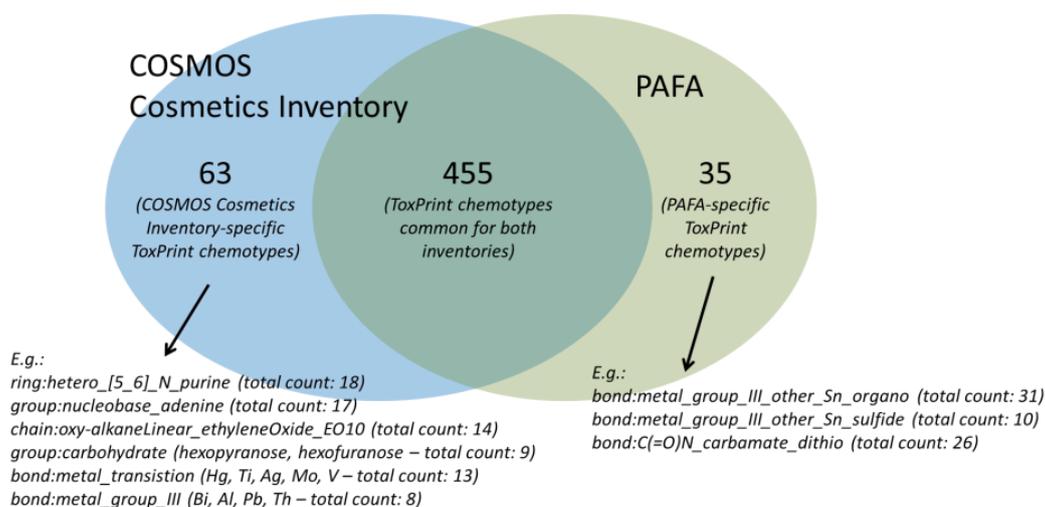


Figure 3.5 Venn diagram showing the overlap of ToxPrint chemotypes between the two investigated inventories

Both analysed inventories had similar proportions of acyclic carboxylic esters, aromatic carboxylic acids, halides, aromatic aldehydes, aliphatic ketones, benzyl alcohols, and heterocyclic rings (Figure 3.6).

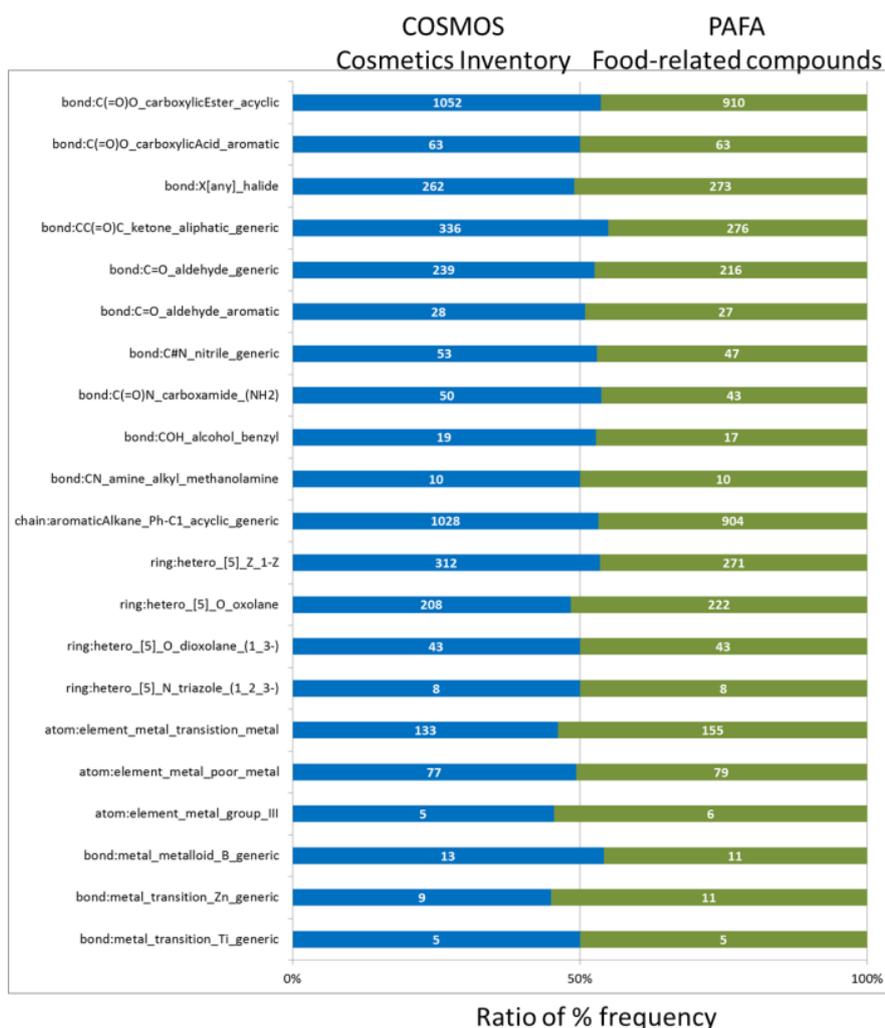


Figure 3.6

The structural classes (ToxPrint chemotypes) uniformly distributed between the two investigated inventories expressed as ratios of % frequencies (the actual counts of each feature are given on the plot's bars)

The structural classes appearing more frequently among cosmetics- than food-related compounds are presented in Figure 3.7. They include: polyethylene glycols, quaternary ammonium salts, organosilicons, long chain linear and branched alkanes and diethanolamines (i.e. the classes characteristic for surface active agents), primary, secondary, and tertiary aromatic amines, nitro- and azo- aromatic compounds (specific for cosmetics colorants and hair dyes, i.e. relatively reactive compounds that would not be associated with food), cyclic/heterocyclic and fused rings (present predominantly in perfuming agents).

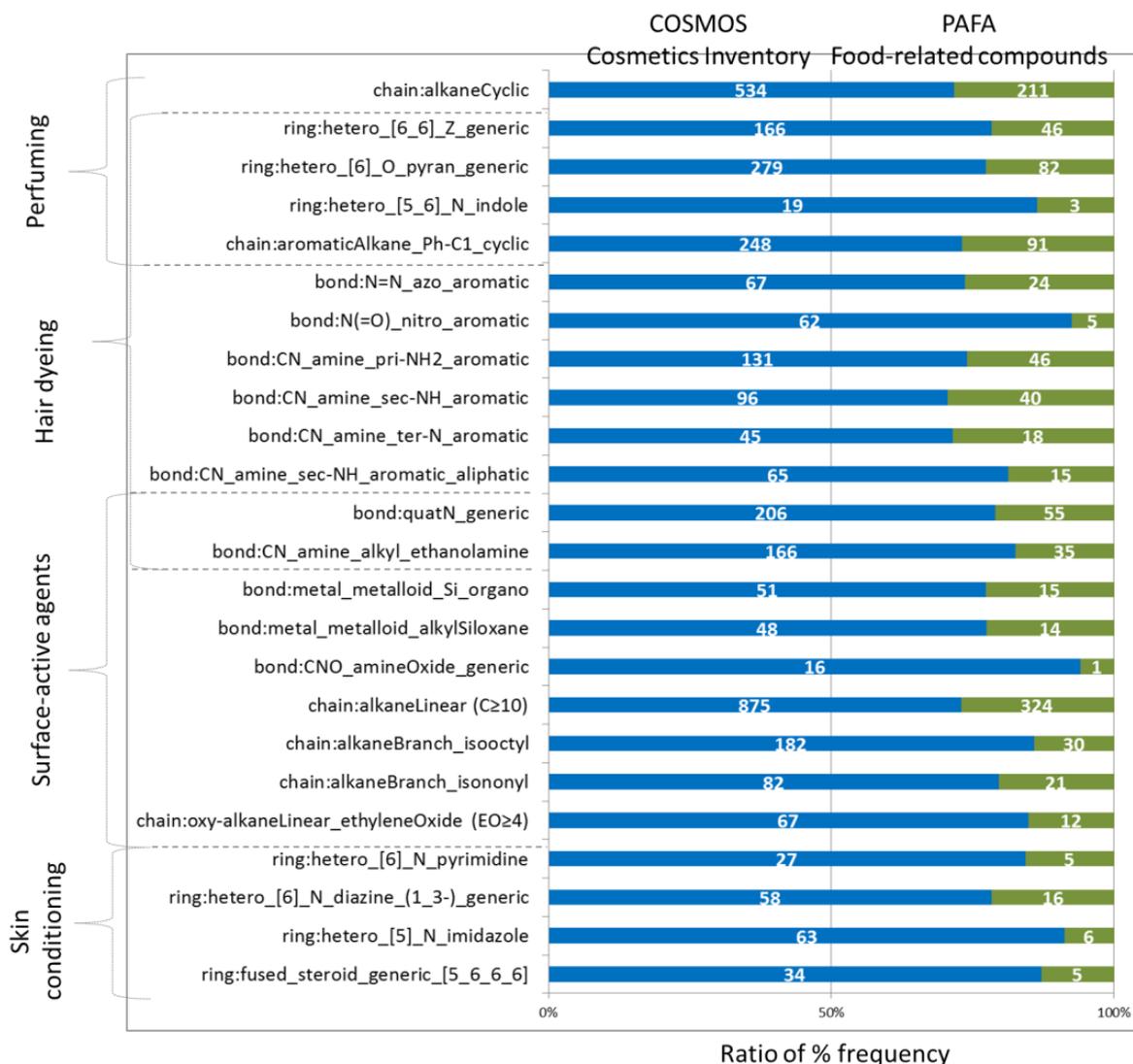


Figure 3.7

The general structural classes (presented as ratios of the total frequencies of ToxPrint chemotypes) identified more frequently in COSMOS Cosmetics Inventory than in PAFA inventory of food related compounds (the actual counts of each feature are given on the plot's bars)

The structural features more characteristic of food-related compounds include sulfides, disulfides, sulfhydrides, thio- carboxylic esters, thiophenes, furans and pyrazines (flavoring agents and food direct additives), as well as phosphites, phosphine oxides, acyl and alkyl halides, isocyanates and acid anhydrides (food contact materials) (Figure 3.8).

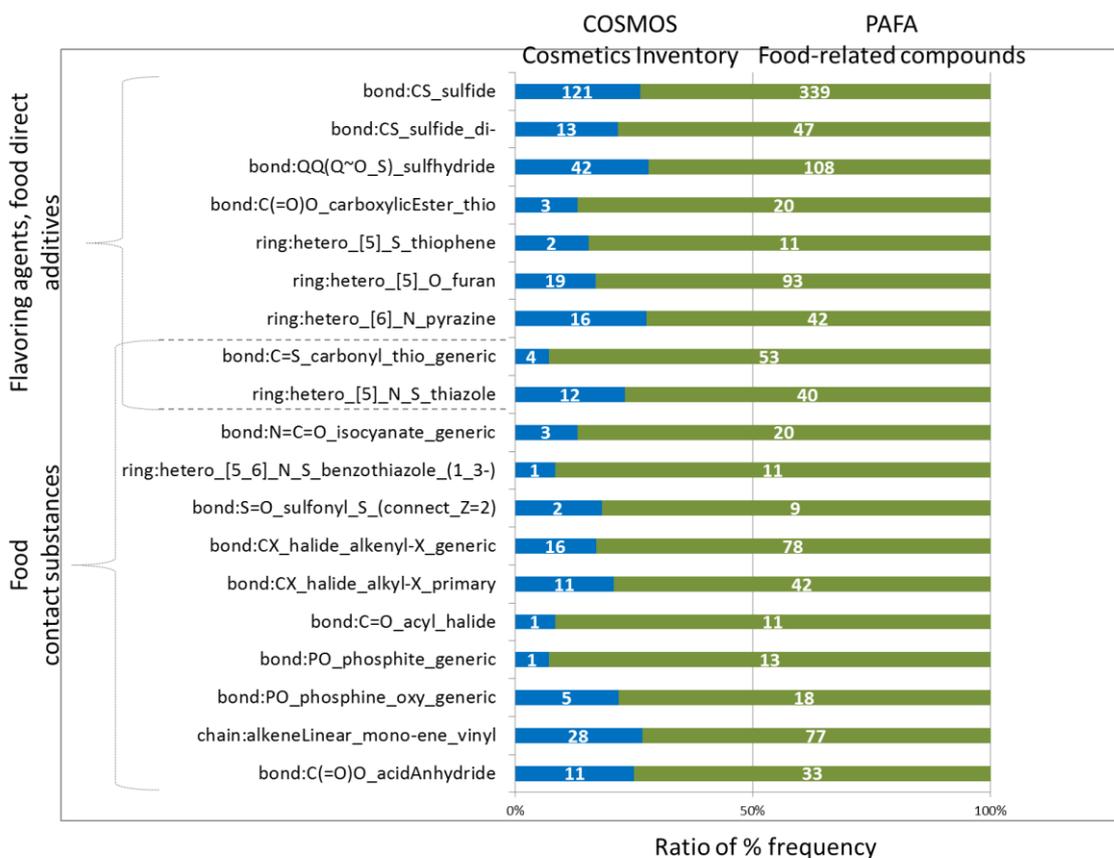


Figure 3.8

The general structural classes (presented as ratios of the total frequencies of ToxPrint chemotypes) identified more frequently in PAFA inventory of food related compounds than in the COSMOS Cosmetics Inventory (the actual counts of each feature are given on the plot's bars)

3.4.3. Physicochemical properties space analysis

The physicochemical properties space occupied by COSMOS Cosmetics Inventory and food-related compounds from PAFA was analysed with 14 (Table 3.4) Corina Symphony “global molecular” and “size and shape” descriptors (Molecular Networks, Nüremberg, Germany). They were calculated for 5,664 3-dimensional computational structures (3,935 cosmetics- and 3,022 food-related compounds) generated after:

- Removing IOM compounds (997 from COSMOS Cosmetics Inventory and 1,068 from PAFA);
- Removing small fragments (in 705 compounds from COSMOS Cosmetics Inventory and 309 from PAFA);
- Neutralising charged fragments (in 343 compounds from COSMOS Cosmetics Inventory and 157 from PAFA);

- Removing duplicates (630 from COSMOS Cosmetics Inventory and 247 from PAFA).

The overlap between the two final structure files is presented in Figure 3.9.

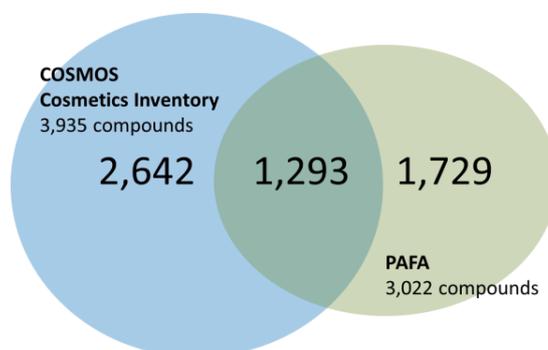


Figure 3.9

Venn Diagram demonstrating the overlap between COSMOS Cosmetics Inventory and food related compounds from the U.S. FDA CFSAN PAFA – comparison by unique InChI Keys generated for 3D-computational structures

Table 3.4

CORINA Symphony (Molecular Networks GmbH, Nüremberg, Germany) descriptors utilised in chemical space analysis.

GLOBAL MOLECULAR PROPERTIES
Number of open-chain, single rotatable bonds (BondsRot) [unitless]
Number of hydrogen bonding acceptors derived from the sum of nitrogen and oxygen atoms in the molecule (H-Acc) [unitless]
Molecular weight derived from the gross formula (MW) [Da]
Octanol/water partition coefficient of the molecule following the XlogP approach (Log P) [unitless]
McGowan molecular volume approximated by fragment contributions (McGowan) [mL/mol]
Topological polar surface area of the molecule derived from polar 2D fragments (TPSA) [Å ²]
Mean molecular polarisability of the molecule (Polariz) [Å ³]
SHAPE DESCRIPTORS
Molecular diameter – maximum distance between two atoms in the molecule (Diameter) [Å]
Molecular eccentricity (Eccentric) [unitless]
Molecular radius of gyration (Rgyr) [Å]
Molecular span – radius of the smallest sphere centred at the centre of mass which completely encloses all atoms in the molecule (Span) [Å]
Principal moment of inertia of 1st principal axis (InertiaX) [Da·Å ²]
Principal moment of inertia of 2nd principal axis (InertiaY) [Da·Å ²]
Principal moment of inertia of 3rd principal axis (InertiaZ) [Da·Å ²]

The Principal Components Analysis reduced the dimensions of 14 molecular descriptors to 3 representative PCs. The three PCs described a total of 89.73% of the variance in the defined chemical space. The scree plot (i.e. the plot of eigenvalues vs number of PCs) and variance explained by each PC are presented in Figure 3.10)

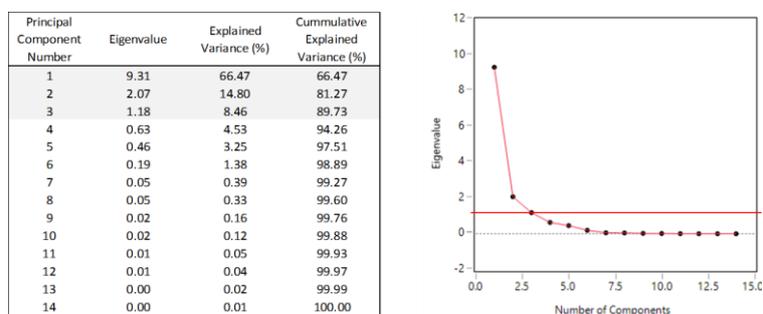


Figure 3.10
Scree plot and % variance explained by each calculated Principal Component. The PCs with eigenvalues >1 were considered. The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

The scores of the 3 PCs were used to generate 3D score plots (Figure 3.11) visualising the physicochemical properties space occupied by the compounds investigated.

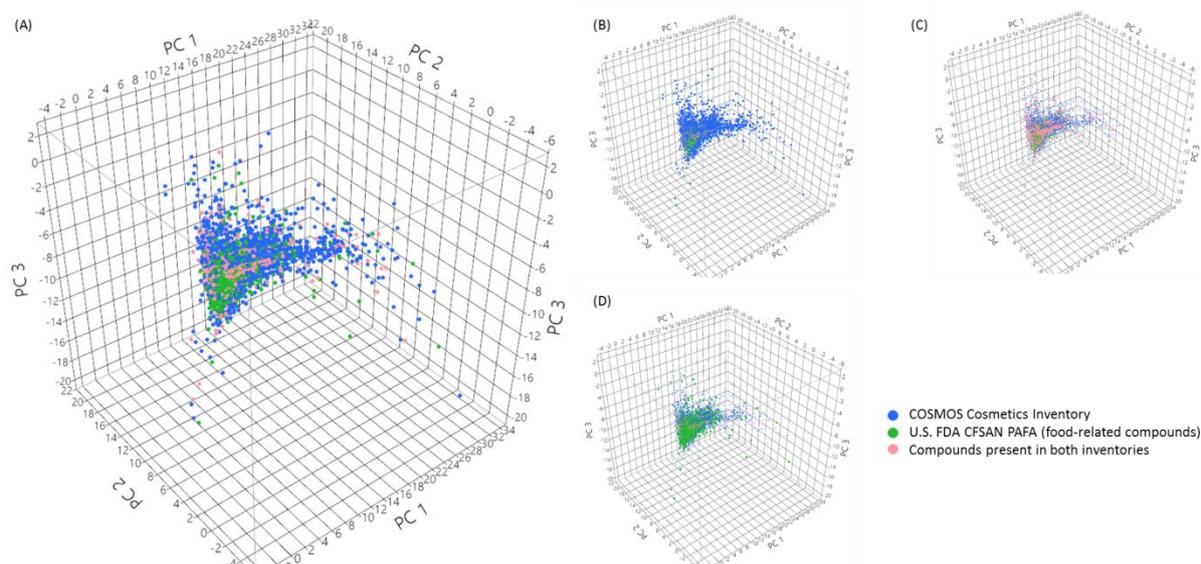


Figure 3.11
The 3D score plots visualising the physicochemical properties space of COSMOS Cosmetics Inventory and the U.S. FDA CFSAN PAFA database covered by the three PCs explaining 89.7% of the cumulative variance in both inventories (A).

Due to the large number of plotted compounds, additional plots were included: (B) Highlighted COSMOS Cosmetics Inventory compounds; (C) Highlighted compounds present in both, cosmetics inventory and PAFA; (D) Highlighted food-related compounds from the U.S. FDA CFSAN PAFA database. The plots were prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

The most influential properties associated with each PC are presented in Figure 3.12. The descriptors related to the flexibility of the molecules (BondsRot), their size and shape (MW, McGowan Diameter, Inertia, Rgyr, Span), as well as to the hydrophobicity/lipophilicity (logP) had the most significant impact on PC1. Topological polar surface area and the hydrogen-bonding (H-Acc) related properties had the highest loadings on the PC2. The descriptors related to the size and shape of the molecules (Eccentric, InertiaX) were the most significant in the PC3 (Figure 3.12).

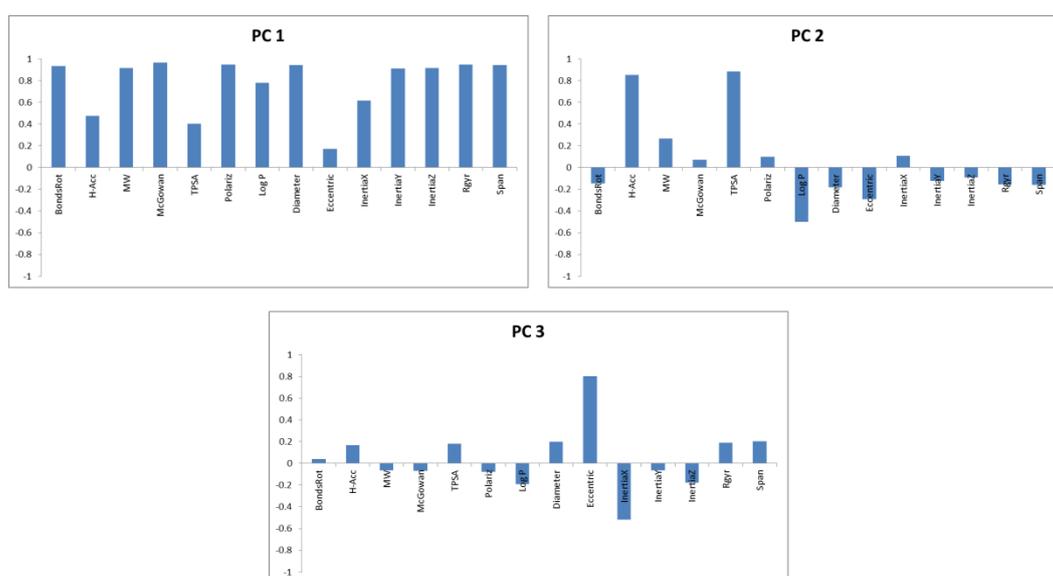


Figure 3.12

The loading bar plots for the three Principal Components characterising physicochemical space occupied by the cosmetics- and food- related compounds. The properties with loadings above (or close to) 0.7 were considered the most influential

The 3D PCs score plot (Figure 3.11) demonstrates that physicochemical properties space covered by cosmetics- and food- related compounds is very sparse and similar in shape. It indicates that both inventories cover a broad and similar range of values of utilised descriptors. This is not unexpected, considering a diversity of structural classes identified (3.4.2). The results of this analysis however suggest that the physicochemical properties utilised do not have sufficient power to discriminate between both inventories when applied alone and that structural features analysis is necessary to identify the differences between cosmetics- and food- related compounds.

As discussed in sections 3.4.1 and 3.4.2, the COSMOS Cosmetics Inventory is very diverse and includes cosmetics ingredients associated with multiple use functions. The most

populated use categories were therefore projected in the physicochemical properties space defined by PC1-PC3 (Figure 3.13). It became apparent that the compounds representing different use functions occupy very distinct regions represented by unique properties and are associated with specific structural features (section 3.4.2).

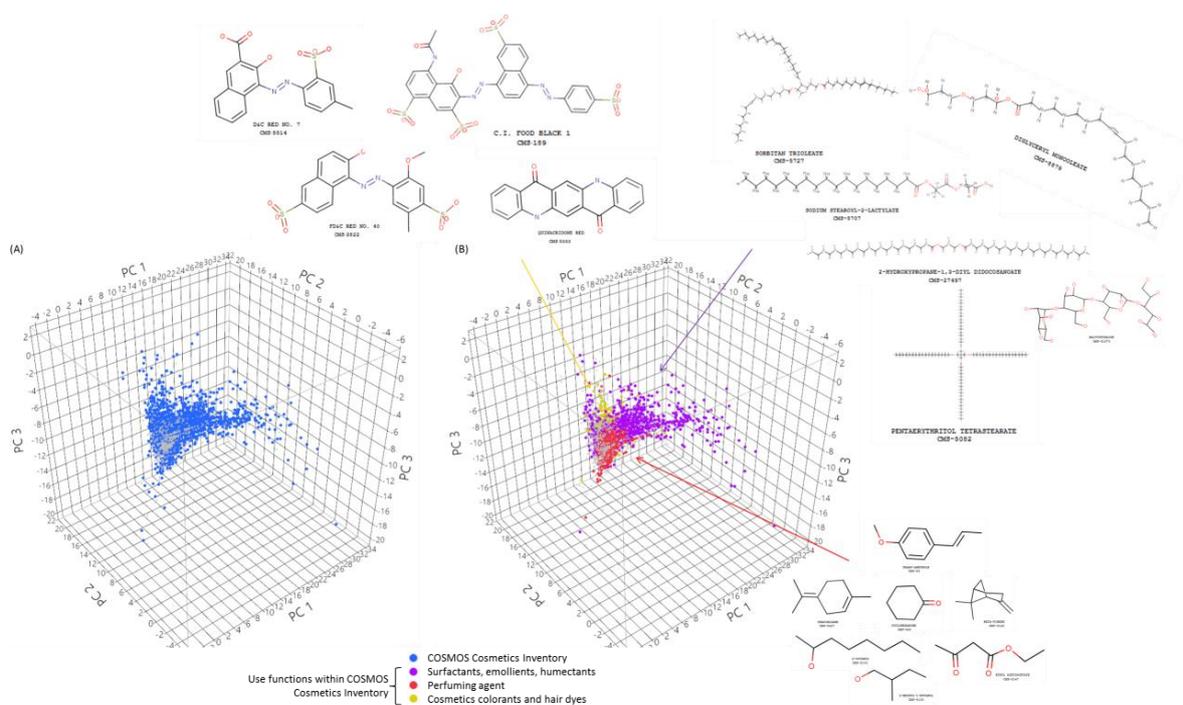


Figure 3.13

Projection of the most populated COSING use functions on the 3D score plot visualising the physicochemical properties space of analysed inventories of compounds. The plots were prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

3.5. Discussion

The chemical space of the COSMOS Cosmetics Inventory was analysed and compared with the chemical space of food-related compounds from the U.S. FDA CFSAN PAFA database. The novel approach of the ToxPrint chemotypes was used to determine the structural domains of each inventory. Subsequently, the associations between structural features, physicochemical properties, and use functions (with detailed applications within cosmetics domain) were identified.

It was demonstrated that cosmetics- and food- related chemicals cover sparse, but similar in shape, chemical space. The structural domain of both inventories was captured by a large number of 553 (out of 729) ToxPrint chemotypes, indicating a large variety of the

occurring structural classes. This diversity was reflected also in the broad ranges of the values covered by investigated physicochemical properties.

Both inventories analysed are broadly represented by carboxylic esters, aromatic carboxylic acids, halides, aromatic aldehydes, aliphatic ketones, benzyl alcohols, and heterocyclic rings. Several differentiating characteristics were also identified. The structural classes dominating among cosmetics-related compounds were associated with specific use functions and manifested through distinct physicochemical properties. Surfactants, emollients (softening and smoothing the skin) and humectants (holding and retaining the moisture) were particularly rich in carboxylic esters, polyethylene glycol esters, ethoxylated aliphatic alcohols, diethanolamines (DEA) and carboxamides (specific for nonionic surfactants), carboxylates and alkyl sulfates (anionic surfactants), quaternary ammonium salts (cationic surfactants), carbohydrate-based structures and long, linear and branched alkanes and alkenes. Perfuming agents included mostly aliphatic, cyclic and aromatic ketones, aldehydes, alcohols, diols, ethers and carboxylic acids, alkanes (aromatic with predominantly short C2-C4 chains attached to phenyl rings and aliphatic with short C2-C4 chains), compounds with polycyclic rings, and heterocyclic compounds with 3- (e.g. epoxides), 5- (e.g. oxolanes, pyrroles), 6- (e.g. pyranes) and 7-membered (e.g. oxepins) rings. The compounds used as cosmetics colorants and hair dyes were widely represented by aromatic and aliphatic amines, azo- compounds, nitro- compounds (multiple nitro- aromatic amines and nitro- benzenes), compounds with fused (naphthalene, indane, phenanthren, pyrene) and hetero- aromatic rings. The structural features characteristic for the food related compounds included sulfides, disulfides, sulfhydrides, thio- carboxylic esters, thiophenes, furans and pyrazines, occurring mostly in in flavoring agents and food direct additives, as well as phosphites, phosphine oxides, acyl and alkyl halides, isocyanates and acid anhydrides abundant among food contact substances.

The results of the present analysis demonstrate the utility and relevance of the novel methodology (ToxPrint chemotypes- and physicochemical properties-based) for the analysis of the chemical space occupied by large inventories of compounds. It allowed for identification of the differences between various use classes in the cosmetics domain, suggesting the need to treat them separately during computational modeling (i.e. development of different models and/or using different modelling approaches for different

groups). The fact that the analysis performed revealed differences between different use functions of cosmetics ingredients indicates the usefulness of this approach for chemical design and toxicity prediction as well as for read-across and category formation.

Chapter 4

The COSMOS Skin Permeability Database: Harvesting, Curating and Quality Control of the Data

4.1. Background

4.1.1. Measurement of skin permeability

Skin permeability is a complex biological and physicochemical process involving the transport of chemicals from the outer surface of the skin to the systemic circulation. The amount of a chemical penetrating the skin at a given time determines whether the chemical can potentially be bioavailable (i.e. absorbed into the systemic circulation) and, as such, exert toxicity after topical exposure (it should be noted that systemic absorption can be preceded by metabolic transformation(s) of a substance within the deeper layers of the viable skin). The mechanistic and physicochemical basis of skin permeability/absorption as well as multiple factors influencing its rate is described in more detail in chapter 5.

Experimental measurement of dermal penetration/absorption can be conducted both *in vivo* and *in vitro*. It should be noted that since the implementation of recent EU legislation (EC, 2009), the testing of new cosmetic ingredients *in vivo* has been prohibited. Historically, no standardised experimental protocols for any type of dermal penetration testing were formulated. Early *in vitro* models were not associated with any formal validation program (numerous in-house procedures were used in industry and academia). It took several decades to publish formally agreed guidelines, however the documents (Table 4.1) remain broad in nature and do not endorse specific protocols or protocol components (Brain et al., 2005).

Due to the historical lack of standard testing procedures to measure skin permeability, and the plethora of factors influencing the permeability/absorption process (e.g. species, sex, application site, dosage regimen, vehicle, occlusion, etc.; please refer also to chapter 5), experimental results are highly variable (Brain et al., 1995; Walters et al., 1997; van de Sandt et al., 2004). This presents a major challenge to the development of computational tools, as data variability is related to a decrease in the statistical validity of the models developed. The lack of accuracy of the experimental values together with errors

across different datasets contributes to the paucity of high quality data for skin permeability/absorption modelling.

Table 4.1

Overview of the test guidelines and other relevant documents available for the measurement of skin permeability/absorption

Test method	Available guidelines and documents
<i>In vivo</i>	<ul style="list-style-type: none"> ○ EC Test Method B.44: Skin Absorption: <i>In Vivo</i> Method (EC, 2008) ○ OECD Test Guideline 427: Skin Absorption: <i>In Vivo</i> Method (OECD, 2004a) ○ WHO Environmental Health Criteria 235: Dermal Absorption (WHO, 2006) ○ EFSA Guidance on Dermal Absorption (EFSA, 2012)
<i>In vitro</i>	<ul style="list-style-type: none"> ○ EC Test Method B.45: Skin Absorption: <i>In Vitro</i> Method (EC, 2008) ○ OECD Test Guideline 248: Skin Absorption: <i>In Vitro</i> Method (OECD, 2004b) ○ WHO Environmental Health Criteria 235: Dermal Absorption (WHO, 2006) ○ Basic Criteria for the <i>In Vitro</i> Assessment of Dermal Absorption of Cosmetic Ingredients (SCCS, 2010) ○ EFSA Guidance on Dermal Absorption (EFSA, 2012)

4.1.2. The COSMOS Skin Permeability Database

For over a decade the necessity of construction of an accurate, well-curated skin permeability database has been recognised. The European Union project “Evaluations and Predictions of Dermal Absorption of Toxic Chemicals” (EDETUX), coordinated by the University of Newcastle, UK (Project Number: QLKA-2000-00196) was a three-year initiative launched in 2000, which aimed to address the need for a comprehensive database of skin permeability values. One of the outcomes of this project was the EDETUX database (publicly available at: <http://edetox.ncl.ac.uk/>), containing *in vitro* and *in vivo* percutaneous absorption/penetration studies recorded for 320 chemicals from the published literature. The EDETUX database provides information on the following skin absorption/permeability parameters: percentage of dose absorbed, percentage of dose recovered, maximal flux, permeability coefficient, and lag time, wherever available (please refer to chapter 5 for definitions of these parameters). Although being a valuable resource of experimental data on skin permeability/absorption, the EDETUX database alone did not contain sufficient information on cosmetics ingredients and related chemicals to meet the needs of modellers in this area (specifically in the COSMOS project). Only about 35% of the substances in the

EDETOX database could be considered cosmetics-related, as they have been also found in the COSMOS Cosmetics Inventory.

A database was donated to the current data gathering initiative by Dr Taravat Ghafourian from the Medway School of Pharmacy, University of Kent, Chatham, UK, whose contribution is gratefully acknowledged. This database is referred to herein simply as “the Kent database”. It was compiled as an update of the EDETOX database after the exhaustive survey of over 1,800 recent (2001-2010) literature publications (Samaras et al., 2012). Although it includes human skin absorption/permeability data from *in vitro* experiments for a total number of 254 compounds, it again lacks information on cosmetics ingredients and related compounds, as its focus was placed mostly on pharmaceuticals.

The COSMOS Skin Permeability Database was therefore developed to address all of the above issues (please refer to Annex 1). On one hand, a database consolidation approach was applied for existing data i.e. the merging of the EDETOX and Kent databases. On the other, new data were harvested from a range of regulatory and literature sources. In particular, the main emphasis was placed on harvesting skin permeability data published after the completion of the EDETOX database. Concerns over the accuracy of data were minimised by establishing a consistent procedure for data curation and quality control.

4.1.3. General aspects of the quality of biological data

The quality of any biological data incorporated into a structured database depends on many complex aspects, but the most essential are the reliability of the data record and the data acceptance, both of which are described below.

4.1.3.1. The reliability of the data record

The data reliability refers to the accuracy of database records with respect to the original data and to the completeness of the original data with regard to the relevant guidelines. Guideline studies, i.e. the studies that have been performed in compliance with the protocols specified by internationally accepted documents, e.g. OECD Guidelines for the Testing of Chemicals, are usually conducted according to the Good Laboratory Practice (GLP) Regulations (e.g. OECD, 1998) (the exceptions are the guideline studies pre-dating the GLP regulations). GLP defines a set of principles for planning, performing, monitoring, recording,

reporting and archiving laboratory studies. Guideline, GLP-compliant studies are usually considered to be of high reliability.

The reliability of the data record can be assessed algorithmically at the time of the data entry. Each toxicity database defines a set of specific study inclusion criteria, i.e. the characteristics that the toxicological study must have in order to be included in the database (accordingly, the exclusion criteria would be the characteristics disqualifying the study from being included in the database, e.g. missing information on the test species). Compliance of the data records with the database inclusion criteria can be used to score the data for reliability. The study inclusion criteria play a particularly significant role in case of toxicity studies which have not followed any formal guideline document(s), i.e. non-guideline studies (e.g. old studies preceding the guideline documents). Often, when qualified according to the database-specific inclusion criteria (usually less stringent than the formal guideline requirements), such studies can be considered as a usable source of information.

Further, data accuracy can be assessed by the database Quality Control (QC) process and evaluated statistically by the Quality Assurance (QA) process (as defined in chapter 2).

4.1.3.2. Data acceptance

Data acceptance addresses the issues of the interpretability and relevance of data with respect to a particular purpose. The accuracy and completeness of recorded information does not implicitly ensure that this information leads to appropriate conclusions. Thus, acceptance is a more subjective, and a more difficult to quantify, aspect of data quality as it has to be evaluated by toxicologists with regard to a specific scientific question.

4.1.3.3. The Klimisch scoring system for data quality assessment

An example of a broadly applied (e.g. by ECHA) method of ascertaining the quality of biological data is the scoring system devised by Klimisch et al. (Klimisch et al., 1997). The Klimisch scheme, originally devised to evaluate the quality of fish acute toxicity data has been expanded and is currently applied to all toxicity endpoints. Klimisch and co-workers recognise three aspects of biological data quality: reliability, relevance and adequacy. With respect to the data reliability, the Klimisch score system has been developed to assign toxicity data into one of four categories (Table 4.2).

Table 4.2
The Klimisch score system (from Klimisch et al., 1997)

Data quality aspect	Definition	
Reliability	Evaluating the inherent quality of a test report or publication relating to preferably standardised methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings. The following categories can be distinguished:	
	(1) reliable without restrictions	“Studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a method.”
	(2) reliable with restrictions	“Studies or data from the literature, reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.”
	(3) not reliable	“Studies or data from the literature/reports in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g. unphysiologic pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgment.”
	(4) not assignable	“Studies or data from the literature, which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.).”
Relevance	Covering the extent to which data and/or tests are appropriate for particular hazard identification or risk characterisation.	
Adequacy	Defining the usefulness of data for hazard/risk assessment purposes. When there is more than one set of data for each effect, the greatest weight is attached to the most reliable and relevant one.	

The controversial point of the Klimisch scoring system is the term “restriction” in the categories of data quality, as it mixes the paradigms of data record reliability and acceptance by requiring human expert judgements.

4.1.4. Data record reliability in the COSMOS skin permeability database

The study inclusion criteria (please refer to section 4.1.3.1 for the definition of the term inclusion criteria) of the COSMOS Skin Permeability Database were established with

respect to its data model, which was defined by the experimental protocols of the *in vitro* and *in vivo* dermal permeability/absorption tests available from the EDETOX database and described by the OECD test guidelines (OECD, 2004a; OECD, 2004b). The study inclusion criteria of the EDETOX database required the following information types to be clearly provided in the source publications: chemical concentration (concentration of chemical applied), dose volume (volume of chemical applied to the skin), loading (amount of chemical added per unit area), area (area of skin to which the chemical was applied), vehicle (application medium), species (species of animal used in the study), exposure time (length of time the chemical was left on the skin), analytical method (method by which the results were determined), receptor fluid (medium that bathes the underside of the skin) and temperature (temperature of the receptor fluid/skin/water bath during *in vitro* experiments).

The COSMOS Skin Permeability Database data model (Figure 4.1) was designed with respect to both *in vitro* and *in vivo* percutaneous penetration/absorption studies. The following information types have been specified for *in vitro* experiments:

- The test system details on skin donor (species, strain, sex, age/weight at the time of skin harvesting, number of donors) and skin membrane (membrane type, thickness, size and storage, site from which the skin was obtained);
- The test conditions, including the dosage details (vehicle, receptor solutions, dose levels, exposure time, etc.) and diffusion parameters (diffusion cell types and size, bath and mounted membrane temperature, flow value, etc.);
- The study results for skin permeability/absorption parameters (lag time, steady-state flux, absorption – as total amount and/or % of dose absorbed permeability coefficient) and recovery (i.e. amounts and/or % of dose recovered from particular skin layers and receptor fluid, also washed from the skin surface and remaining on the diffusion cell material).

The criteria relevant to *in vivo* experiments include:

- The test system details on the test animals (species, strain, sex, age/weight at the study initiation, number of animals) and dosed sites (site and site area);
- The test condition details on dosage regimen (dose application method, dose delivery type, e.g. single or repeated dose, vehicle, dose levels, exposure duration, etc.);

- The results for skin permeability/absorption parameters (lag times, maximal flux, total amount (and %) of test substance absorbed, permeability coefficient), distribution/elimination of the test material (i.e. the amount or % of dose identified in particular skin layers, blood, excreta, carcass, etc. after euthanising the test animals), and recovery (the amount or % of dose recovered).

The majority of the study parameters have been represented by the controlled vocabulary.

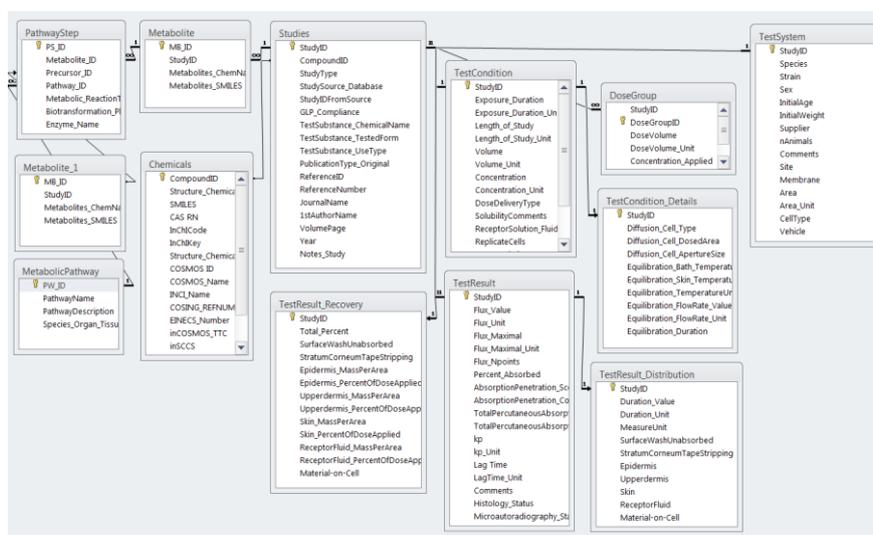


Figure 4.1
The entities and their relationships of the Skin Permeability Database data model

4.2. The aims of chapter 4

The biological data needs to achieve the goals of the COSMOS project were discussed in 1.4. Skin permeability data were identified as one of the essential elements of the COSMOS database. The aims of the present chapter realised in collaboration with the partners of the COSMOS project (please refer to Annex 1) are related to the objective 3 of the current PhD program (section 1.5) and include:

- Curation and integration of existing skin permeability/absorption data sources, namely the EDETOX and Kent databases;
- Development of the SOP for the harvesting of new data;
- Harvesting new skin permeability data for cosmetics ingredients and related compounds from regulatory and literature sources according to the developed SOP;

- Integration of the newly harvested data with the EDETOX and Kent content;
- Preparation of data entry tool for QC of the COSMOS Skin Permeability Database.

4.3. Materials and methods

4.3.1. Data sources

New data harvesting was performed by six COSMOS consortium partners (Altamira, BAS, U.S. FDA, S-IN, LJMU, and Molecular Networks; Please refer also to Annex 1). The focus was placed on high priority compounds from the COSMOS Cosmetics Inventory, i.e. the compounds for which oral repeated dose toxicity data had been identified, but for which the information on dermal permeability/absorption was missing. The following data sources were used:

- The European Commission Scientific Committee on Consumer Safety (EU SCCS) opinions on cosmetic ingredients, available at:
https://ec.europa.eu/health/scientific_committees/consumer_safety_en;
- The European Chemicals Agency (ECHA) Registered Substances database:
<https://echa.europa.eu>;
- Other relevant data sources: For cases where ECHA or SCCS opinions referenced an unpublished report as the original data source, the information provided in the ECHA or SCCS opinion was harvested. However, if the original source of data was a scientific publication or a book chapter, the original data source was consulted.

4.3.2. Curation and integration of existing databases

The EDETOX content was imported into a Microsoft Access database for further use as the COSMOS Skin Permeability Database. In order to merge it with the Kent database, curation and quality control of original Kent records was necessary. Curation was performed by Altamira/LJMU (Annex 1).

4.3.3. New data harvesting: data entry tool and data entry process

The data harvesters were provided with a data entry tool in the form of .xls file with several tables corresponding to the COSMOS Skin Permeability Data Model (section 4.1.4), prepared by Altamira/LJMU (Annex 1). In order to test the relevance of the .xls data entry

tables, two cycles of the pilot data harvesting were conducted prior to the launch of the final procedure. The pilot studies were accomplished successfully and provided a plethora of valuable information on how to improve the .xls tables to make the data entry process more convenient (for instance, the fields for recording specific activity value and unit of radiolabeled test substances were added).

The final data harvesting was performed according to the Standard Operating Procedure (SOP) which was prepared by Altamira/LJMU (Annex 1). The data entry tool consisted of eleven .xls tables. Controlled vocabularies were distributed in the form of drop-down pick lists and, along with the full SOP document, are provided in Annex 4. The details of the data entry procedure are summarised in Figures 4.2-4.3, using 1,3-phenylenediamine (CMS-1149, CAS RN: 108-45-2) as an example. This compound has been tested *in vitro* (the EU ECHA database has been harvested) and *in vivo* (the data from the scientific publication were harvested).

After finalising the data harvesting process, the results from each participating COSMOS partner were combined and integrated with the content of EDETOX and Kent databases by Altamira LLC (Annex 1).

Chemistry_Row#	COSMOS ID	CAS	INCI_Name	Other NAME	Data Entry Institution & Name	Links/Comments
1	CMS-1149	108-45-2	M-PHENYLENEDIAMINE	1,3-PHENYLENEDIAMINE	MIRA, AMS	

StudyInfo_Row#	StudyID	COSMOS ID	StudyType	StudyDescription	GLP_Compliance	ECHA Klimish Score	StudyInformation_Comment
1	AMS-1	CMS-1149	In vitro percutaneous penetration	Exp Supporting Dermal absorption.001	No data	2 (reliable with restrictions)	
4	AMS-46	CMS-1149	In vivo percutaneous penetration				

StudyReference_Row#	StudyID	HarvestedStudySource_Type	HarvestedStudySource_DatabaseName	OriginalReference_Type	OriginalReference_Title	OriginalReference_JournalName	OriginalReference_1stAuthorName	OriginalReference_Volume(Issue)	OriginalReference_Page-Page	OriginalReference_Year	OriginalReference_OtherInfo	StudyReference_Comment
1	AMS-1	Secondary or tertiary database	ECHA	Original publication	Percutaneous absorption, biotransformation, retention and excretion of 1,3-diaminobenzene	Fd Chem Toxic	Lam HR	11	741-749	1989		Results reported in ECHA - checked with original publication. In vivo study not reported in ECHA found in original paper and harvested (AMS-46)
4	AMS-46	Primary literature publication		Original publication	Percutaneous absorption, biotransformation, retention and excretion of 1,3-diaminobenzene	Fd Chem Toxic	Lam HR	11	741-749	1989		

StudySubstance_Row#	StudyID	TestSubstance_Name	TestSubstance_TestForm	MetaboliteID	SpecificActivity_Value	SpecificActivity_Unit	StudySubstance_Comment
1	AMS-1	1,3-Diaminobenzene	Parent-Neutral-Radiolabelled		85.4	micro-Ci/mmol	
4	AMS-46	1,3-Diaminobenzene (MPD)	Parent-Neutral-Radiolabelled		85.4	micro-Ci/mmol	

Metabolites_Row#	StudyID	MetaboliteID	Metabolite_Name	Metabolite_S MILES	Metabolic_PathwayStep_Precursor_Name	Metabolic_PathwayStep_MetabolicPathway_Name	Metabolic_PathwayStep_Biotransformation_Phase	Metabolic_PathwayStep_ReactionType	Metabolic_PathwayStep_Enzyme_Name	MetabolicPathway_Description	MetabolicPathway_Species	MetabolicPathway_OrganTissue	Metabolites_Comment

TestAnimal_Row#	StudyID	Species	Strain	Sex	Initial Age	Initial Weight	Supplier	Number of animals	TestAnimal_Comment
1	AMS-1	Rat	Wistar	Male	6 weeks		Møllegaard Breeding Center (Ejby, Denmark)	7	
4	AMS-46	Rat	Wistar	Male	6 weeks	200 g	Mollegaard Breeding Centre (Ejby, Denmark)	7	

TestSkin_Row#	StudyID	SkinMembrane_Type	SkinMembrane_Thickness_Value	SkinMembrane_Thickness_Unit	SkinMembrane_DiskSize_Value	SkinMembrane_DiskSize_Unit	SkinMembrane_Storage	Skin_Site	Site_Area_Value	Site_Area_Unit	TestSkin_Comment
1	AMS-1	Epidermis (NaBr-separated)			1.8	cm2	Excised skin wrapped at aluminium foil and stored at -20 degC	Back			
4	AMS-46							Back	16	cm2	

Figure 4.2

An illustration of the .xls data entry tables for skin permeability/absorption data harvesting (part 1): Chemistry (identification of the compound in the COSMOS database); StudySubstance (information on the tested substance, e.g. radiolabeling, tested form); StudyInfo (data quality related information), StudyReference (data origin); Metabolites (metabolism information, if available); and two tables related to the test system, namely TestAnimal and TestSkin. The fields marked in red refer to those controlled by vocabulary that have been not subjected to any changes, whereas the fields marked in green are those that were extended during the data harvesting

TestDose_Row#	StudyID	DoseGroupID	DoseDeliveryType	SolventVehicle	SolubilityComments	ReceptorSolution	AssayTechnique	DoseVolume	DoseConcentration_Value	DoseConcentration_Unit	DoseApplication_Solution(Formulation)_Value	DoseApplication_Solution(Formulation)_Unit	DoseApplication_Substance_Value	DoseApplication_Substance_Unit	ScoringTechnique	Exposure_Duration	Length_of_Study	TestConditions_Comment
1	AMS-1	AMS-DG-1	Single Dose	Saline (0.9%)		Saline (0.9%, 3 ml)			4	% (w/v)			556	micro-mol	Liquid Scintillation Counting		48 hours	
2	AMS-1	AMS-DG-2	Single Dose	Hydrogen peroxide (4%)		Saline (0.9%, 3 ml)			4	% (w/v)			556	micro-mol	Liquid Scintillation Counting		48 hours	
7	AMS-46	AMS-DG-113	Single Dose	Water				1.5 ml	4	% (w/v)			556	micro-mol	Liquid Scintillation Counting	24 hours	7 days	
8	AMS-46	AMS-DG-114	Single Dose	Hydrogen peroxide (4%)				1.5 ml	4	% (w/v)			556	micro-mol	Liquid Scintillation Counting	24 hours	7 days	

TestDiff_Row#	StudyID	DoseGroupID	Diffusion_Cell_Type	Diffusion_Cell_DosedArea_Value	Diffusion_Cell_DosedArea_Unit	Diffusion_Cell_ApertureSize_Value	Diffusion_Cell_ApertureSize_Unit	Equilibration_Bath_Temperature	Equilibration_Skin_Temperature	Equilibration_Temperature_Unit	Equilibration_FlowRate_Value	Equilibration_FlowRate_Unit	Equilibration_Duration	Diffusion_Comment
1	AMS-1	AMS-DG-1		1.8	cm ²			30 +/- 1	30 +/- 1	degC				Two-chambered glass diffusion cells
2	AMS-1	AMS-DG-2		1.8	cm ²			30 +/- 1	30 +/- 1	degC				Two-chambered glass diffusion cells
7	AMS-46	AMS-DG-113												
8	AMS-46	AMS-DG-114												

TestRes_Row#	StudyID	DoseGroupID	LagTime_Value	LagTime_Unit	Flux_Value	Flux_Unit	Flux_Maximal_Value	Flux_Maximal_Unit	Flux_Minimal_Value	Flux_Minimal_Unit	TotalPercentAbsorbed	TotalAmountAbsorbed_Value	TotalAmountAbsorbed_Unit	kp_Value	kp_Unit	AbsorptionPenetration_Score	AbsorptionPenetration_Comments	Histology_Status	Microautoradiography_Status	TimePoint_Information	Distribution_OtherMedia_Information	Elimination_Information	Results_Comment
1	AMS-1	AMS-DG-1			0.77	micro-mol/cm ² /hour								2.28E-03	cm/hour					not provided			
2	AMS-1	AMS-DG-2															Disruption of the membrane			not provided			
7	AMS-46	AMS-DG-113			0.26	micro-g/cm ² /hour					99.9		micro-mol							not provided	Carcass	Urine; Faeces	
8	AMS-46	AMS-DG-114									36.5		micro-mol							not provided	Carcass	Urine; Faeces	

TestRec_Row#	StudyID	DoseGroupID	TotalPercentRecovery	TotalRecovery_Value	TotalRecovery_Unit	Recovery_Measure_Unit	Recovery_SurfaceWashUnabsorbed	Recovery_StratumCorneumTapeStripping	Recovery_Epidermis	Recovery_UpperDermis	Recovery_Epidermis&Dermis	Recovery_TotalSkin	Recovery_ReceptorFluid	Recovery_Material-On_Cell	Recovery_Comment
1	AMS-1	AMS-DG-1													
2	AMS-1	AMS-DG-2													
7	AMS-46	AMS-DG-113				% of applied dose						0.92			
8	AMS-46	AMS-DG-114				% of applied dose						1.51			

Figure 4.3 An illustration of the .xls data entry tables for skin permeability/absorption data harvesting (part 2): TestDose (dosing conditions for each study – one study may be associated with multiple dose groups); TestDiffusion (details about the diffusion system utilised – applicable only for *in vitro* studies); TestResults (permeability/absorption parameters and distribution in blood/excreta/carcass, etc., in case of *in vivo* studies), TestRecovery (dose recovery information). The fields marked in red refer to those controlled by vocabulary that have been not subjected to any changes, whereas the fields marked in green refer to those that were extended during the data harvesting

4.3.4. The quality control of COSMOS Skin Permeability Database

During the data harvesting procedure, the data quality issues were addressed by recording the Klimisch scores and information about the compliance of the studies with the OECD guidelines (and GLP). This information was retrieved from the original data sources, i.e. EU ECHA and SCCS. As a first step, the record reliability and accuracy of the data harvested were reviewed manually (without any tools) at the stage of integrating new data with the EDETOX/Kent content.

Further, the QC of the COSMOS Skin Permeability Database content was performed by the COSMOS Expert Group (EG) coordinated by the International Life Sciences Institute-Europe (ILSI-Europe), including experts in this area: Prof. Mark Cronin (LJMU, Liverpool, UK), Dr Chihae Yang (Altamira LLC), Prof. Faith Williams (University of Newcastle, Newcastle, UK), Dr Nancy Monteiro-Riviere (Kansas State University, Manhattan, KS), Dr Gordon Barrett (Health Canada), Dr Miriam Verwei (TNO), Dr James Plautz (DSM Nutritional Products, Ciba, Inc., Switzerland) whose contribution is acknowledged. During this process, the experts also contributed additional information relating to skin metabolism to the database. Approximately 5% of compounds comprising the COSMOS Skin Permeability Database were subjected to the QC. The experts were provided with the .xls QC entry tables prepared by Altamira/ LJMU (with collaboration with the U.S. FDA; Please refer to Annex 1). The template for the COSMOS Skin Permeability QC tables is presented in Figure 4.4. The QC comments from the Expert Group were processed and incorporated into the final database.

Table	Field Name	COSMOS Skin Permeability Data	ENTER YOUR QC COMMENTS
Chemistry	Chemical Name		
Study Info	STUDY ID		
	Data Source URL		
	STUDY TYPE		
	GLP Compliance		
	ECHA Klimish Score (FROM SOURCE)		
	StudyInformation Comment		
	TIME HARVESTED		
Study Reference	Harvested StudySource Type		
	Harvested Study Source Database Name		
	Harvested Study Source Details		
	OriginalReference(Citation)_Type		
	Original Citation Title		
	Original Citation: Journal Title		
	Original Citation: 1st Author Name		
	Original Citation: Volume(Issue)		
	Original Citation: Page-Page		
	Original Citation: Year		
	Original Citation: OtherInfo		
	StudyReference Comment		
Test Substance	Test Substance Name		
	Tested Form		
	MetaboliteID		
	Specific Activity Value		
	Specific Activity Unit		
	Study Substance Comment		
Metabolites	Metabolites Comment		
	Metabolic PathwayStep Enzyme Name		
	Metabolic Pathway Description		
	Metabolic Pathway Species		
	Metabolic Pathway OrganTissue		
	Metabolic PathwayStep Precursor Name		
	Metabolic Pathway Step: Metabolic Pathway Name		
	Metabolic PathwayStep Biotransformation Phase		
	Metabolic PathwayStep ReactionType		
Test Animal and Skin	Species		
	Strain		
	Sex		
	Initial Age		
	Initial Weight		
	Supplier (usually not needed)		
	Number of animals		
	Skin Site		
	Skin Membrane Type		
	Skin Membrane Thickness Value		
	Skin Membrane Thickness Unit		
	Skin Membrane Disk Size Value		
	Skin Membrane Disk Size Unit		
	Site Area Value		
	Site Area Unit		
	Skin Membrane Storage		
	Test Skin Comment		
	TestAnimal Comment		
Test Condition Dose Group	Dose Delivery Type		
	DOSE APPLICATION MATERIAL TYPE		
	Dose Application Amount		
	Dose Application Amount Unit		
	Dose Volume Value		
	Dose Volume Unit		
	Dose Concentration Value		
	Dose Concentration Unit		
	Solvent Vehicle		
	Vehicle Category		
	Receptor Solution		
	Receptor Solution Category		
	SolubilityComments		
	Assay Technique		
	Scoring Technique		
	Exposure Duration		
	Length of Study		
	TestConditions Comment		
Test Condition Diffusion	APPLICATION METHOD		
	Diffusion Cell Type		
	Diffusion Cell Dosed Area Value		
	Diffusion Cell DosedArea Unit		
	Diffusion Cell Aperture Size Value		
	Diffusion Cell Aperture Size Unit		
	Equilibration Bath Temperature		
	Equilibration Skin Temperature		
	Equilibration Temperature Unit		
	Equilibration Flow Rate Value		
	Equilibration Flow Rate Unit		
	Equilibration Duration		
	Diffusion Comment		
Results	Log Time		
	Flux Value		
	Flux Unit		
	Flux Maximal Value		
	Flux Maximal Unit		
	Flux Npoints		
	Total Percent Absorbed		
	Total Absorbed Amount		
	Total Absorbed Amount Unit		
	kp Value		
	kp Unit		
	Absorption Penetration Comments		
	Histology Status		
	Microautoradiography Status		
	TimePoints Information		
	Distribution Other Media Information		
	Elimination Information		
	Results Comment		
Results Recovery	Total Percent Recovery		
	Total Recovery Value		
	Total Recovery Unit		
	Recovery MeasureUnit		
	Recovery Surface Wash Unabsorbed		
	Recovery Stratum Corneum Tape Stripping		
	Recovery Epidermis		
	Recovery Upper Dermis		
	Recovery Epidermis&Dermis		
	Recovery Total Skin		
	Recovery Receptor Fluid		
	Recovery Material-On-Cell		
	Recovery Comment		

Figure 4.4
The template of the COSMOS Skin Permeability QC tables

4.4. Results

In order to address the general lack of publically available, good quality skin permeability/absorption data for cosmetics and related compounds, the COSMOS Skin Permeability Database was developed. Existing data (EDETUX and Kent databases) were integrated with newly harvested ones.

The EDETUX database provided a total number of 1,657 records for *in vitro* studies (244 unique chemicals) and 844 records for *in vivo* studies (156 unique chemicals) for 297 unique chemicals (126 cosmetics-related). It was combined with 999 records from the curated Kent database containing:

- 436 updated studies for 156 compounds already present in EDETOX;
- 563 new studies for 128 compounds (88 new compounds and 40 already included in EDETOX. Out of those 88 new chemicals, only 16 were cosmetics-related).

The COSMOS data harvesting effort contributed 268 *in vitro* studies (for 85 compounds) and 159 *in vivo* studies (for 48 compounds) to the Skin Permeability Database. In total, 103 cosmetics-related compounds were harvested (99 new compounds and 4 already present in the database).

Thus, the final COSMOS Skin Permeability Database contains 484 unique chemical compounds for which 2488 *in vitro* and 1003 *in vivo* studies have been recorded (Figure 4.5).

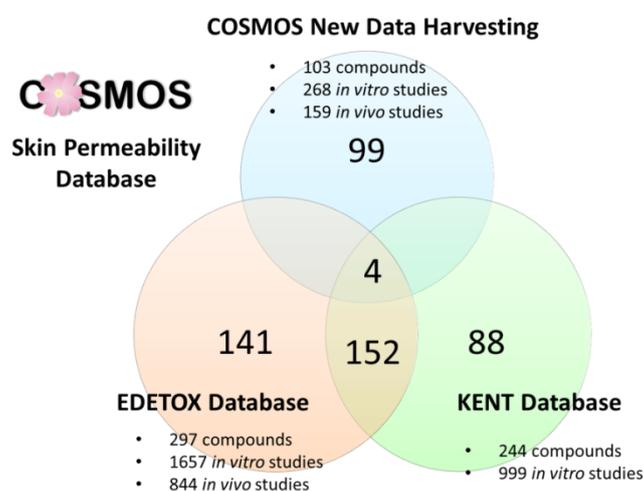


Figure 4.5
COSMOS Skin Permeability Database: Final content with respect to the data origin

The COSMOS Skin Permeability Database mostly contains defined organic compounds (only 24 do not fall into this category, please refer to the Table 4.3). Approximately 46% of its content was in common with the COSMOS Cosmetics Inventory. With regard to the EU COSING database use functions (Annex 3), the most populated cosmetics-related chemicals in the Skin Permeability Database include perfuming agents, antimicrobials, preservatives, antioxidants, colorants and hair dyes, skin and hair conditioning agents, surfactants and emulsifiers, moisturisers, emollients and humectants, and UV filters/absorbers. They cover a broad range of chemical classes, mostly alcohols, carboxylic esters, glycol ethers and parabens.

Table 4.3
COSMOS Skin Permeability Database constituents: composition and material types

Composition type	Material type	# compounds
Defined formula	IOM	13
Defined formula	Organic	452
Defined formula – varying isomers	IOM	2
Defined formula – varying isomers	Organic	8
Ill-defined formula	Organic	3
Ill-defined formula – polydispersed	Organic - polymer	5
Ill-defined formula – polydispersed – varying isomers	Organic - polymer	1
TOTAL		484

As far as experimental data content is concerned, a range of species (human, pig, rat, mouse, rabbit, minipig, guinea pig, dog, monkey, marmoset, snake), *in vitro* membrane types (dermis, epidermis, *stratum corneum*, full- and split-thickness skin and artificial membranes), *in vivo* application sites (abdomen, breast, back, scalp, leg, arm, etc.), and vehicles were included. The results recorded included flux, absorption, permeability coefficient and recovery data. The detailed statistics are presented in Table 4.4 and Figures 4.6-4.9.

Information on the distribution (in blood, plasma, carcass, or other organs and tissues) and elimination (*via* urine, faeces, exhaled air, etc.) was retrieved for over 40 compounds tested in more than 100 *in vivo* studies. In addition, information regarding skin metabolism was captured for 12 cosmetics-related compounds, including information on the metabolites identified in skin or eliminated after dermal applications, enzymes and probable pathways.

Table 4.4
Statistics of the COSMOS Skin Permeability Database for human, pig, rat and mouse studies

Species	Membrane Type	Main Application Site	Main Vehicle for reported species
<i>In vitro</i> studies (2488)			
Human (1206)	Epidermis (516)	Abdomen; Back; Breast (432)	Water (264); Neat (149); Formulation (68)
	Split-Thickness Skin (404)	Abdomen; Arm; Breast; Leg (302)	
	Full-Thickness Skin (223)	Abdomen; Breast (194)	
	Stratum Corneum (19)	Abdomen (18)	
Pig (226)	Split-Thickness Skin (186)	Back (145)	Ethanol (69); Formulation (25); Acetone (20)
	Full-Thickness Skin (28)	Ear (9); Back (5)	
Rat (502)	Full-Thickness Skin (240)	Abdomen; Back (199)	Acetone (76); Ethanol (52); Water (45); Hydrocarbon (41)
	Split-Thickness Skin (195)	Back (176)	
	Epidermis (47)	Back (19)	
Mouse (306)	Full-Thickness Skin (284)	Abdomen; Back (259)	Saline (115); Saline/ Aqueous Alcohol (45); Acetone (28)
<i>In vivo</i> studies (1003)			
Human (300)	Not applicable	Arm/Axilla/Hand/Palm (210); Abdomen (21); Back (12)	Acetone (129); Ethylene glycol/ Surfactant (57); Aqueous Ethanol (34)
Pig (48)	Not applicable	Back (27); Abdomen (14)	Ethanol (31)
Rat (394)	Not applicable	Back (340); Abdomen (13)	Acetone (120); Aqueous Ethanol (79); Formulation (38); Water (35); Neat (31)
Mouse (43)	Not applicable	Back (26)	Ethanol (11); Neat (9)

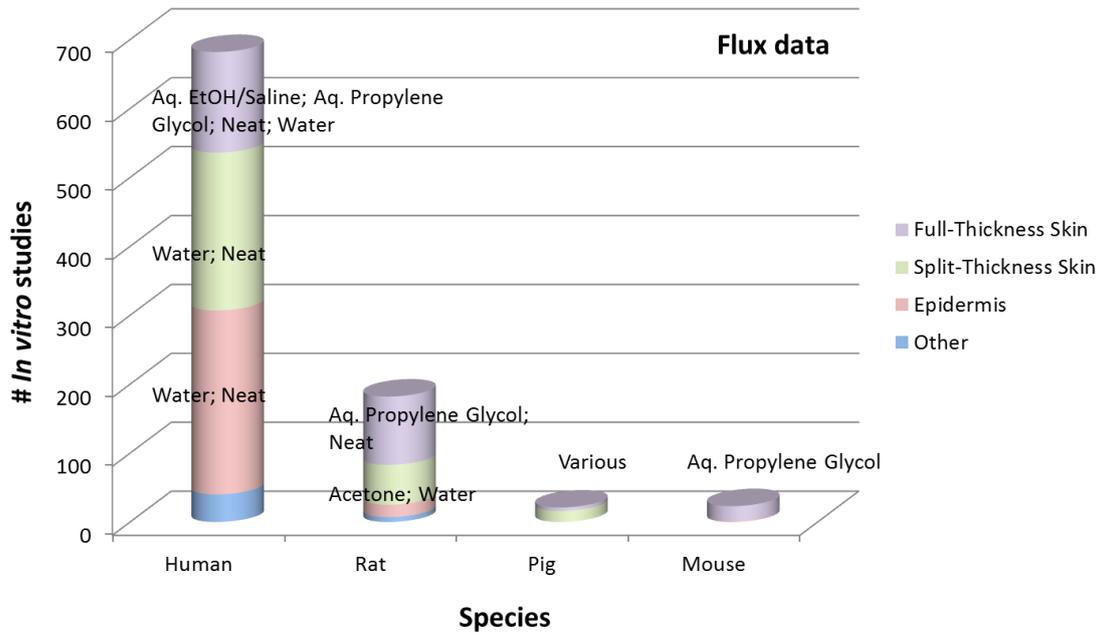


Figure 4.6 The *in vitro* flux data available in the COSMOS Skin Permeability Database, profiled with respect to the species, membrane types, and dominating vehicle types

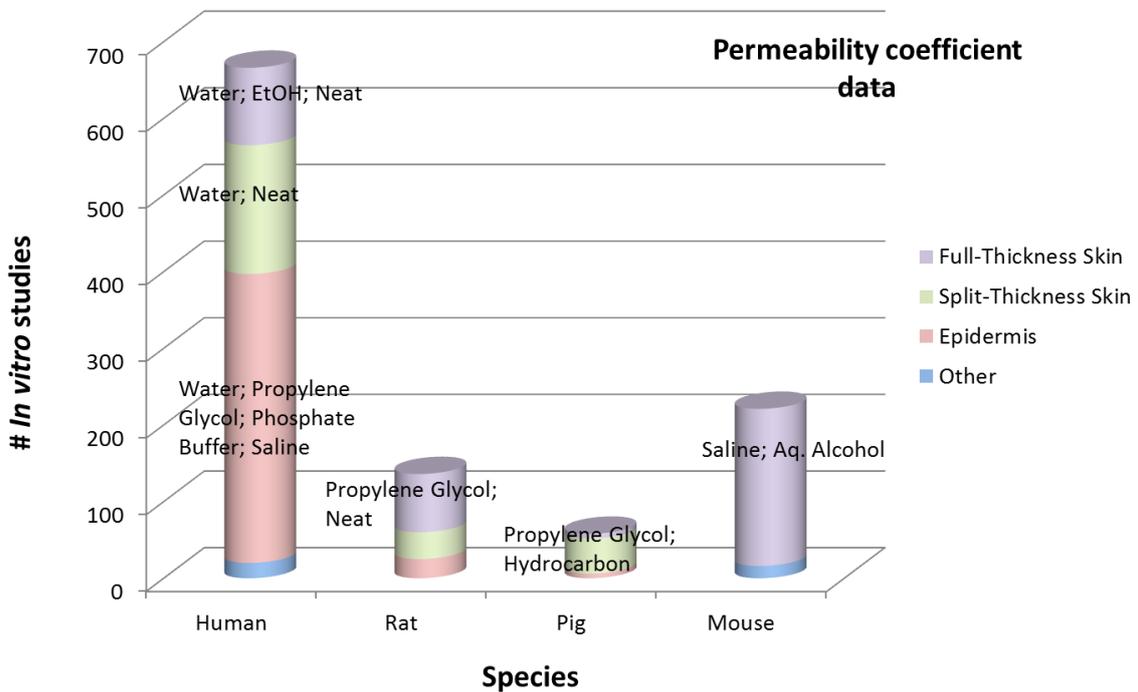


Figure 4.7 The *in vitro* permeability coefficient data available in the COSMOS Skin Permeability Database, profiled with respect to the species, membrane types, and dominating vehicle types

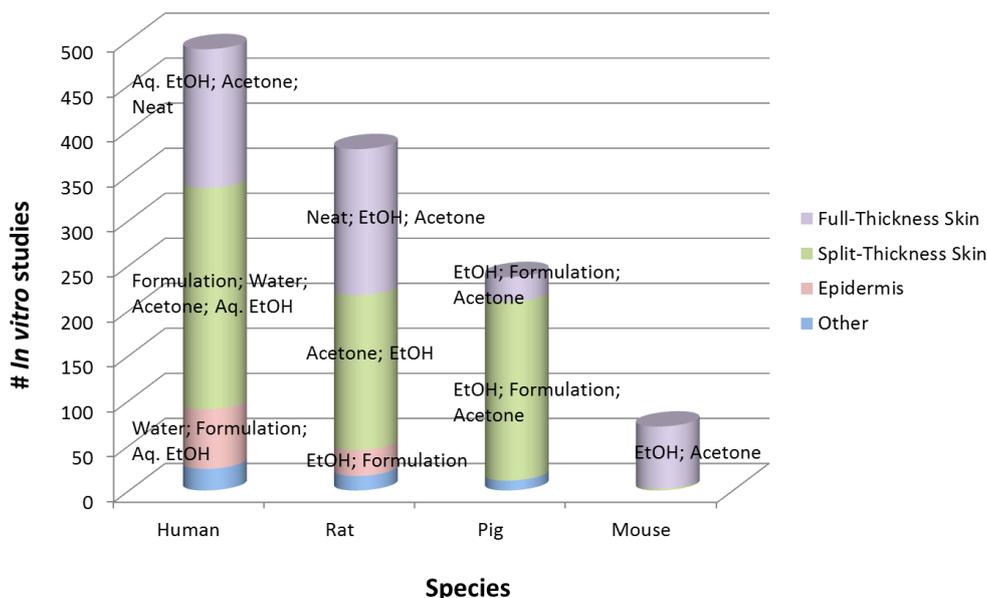


Figure 4.8 The *in vitro* absorption data (including the percentage and amount of the dose absorbed, whichever was provided) available in the COSMOS Skin Permeability Database. The data were profiled with respect to the species, membrane types and vehicles

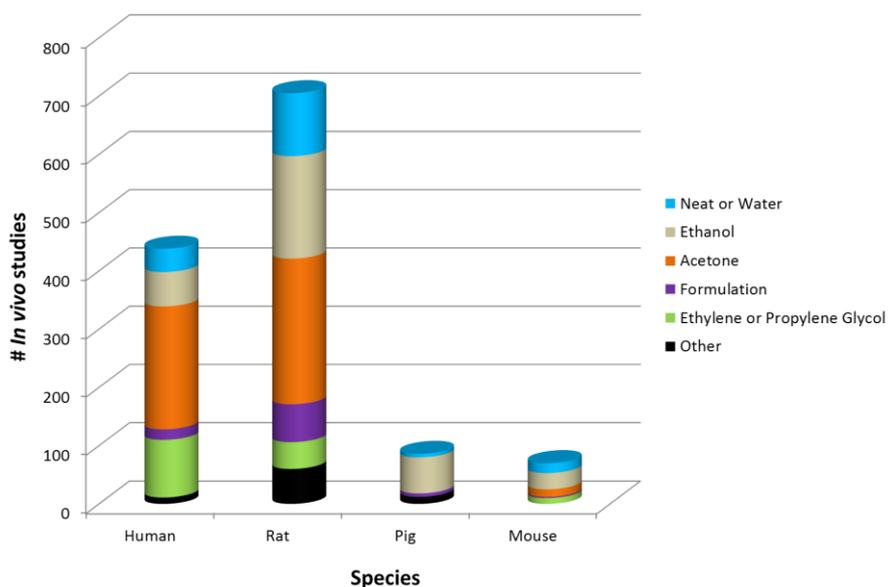


Figure 4.9 The *in vivo* absorption data (including the percentage and amount of the dose absorbed, whichever was provided) available in the COSMOS Skin Permeability Database. The data has been profiled with respect to the species and vehicles

4.5. Discussion

This chapter demonstrates the current demand for a database containing high quality skin permeability/absorption data for cosmetics and related compounds, and describes the collation of COSMOS Skin Permeability Database satisfying this need. The novel developed database, containing 2488 *in vitro* and 1003 *in vivo* studies for 484 compounds (46% of cosmetics-related ones), can be considered a milestone towards estimating the human exposure to chemicals *via* dermal exposure for several reasons.

The data curated from the EDETOX and Kent databases were enriched with the studies harvested manually from literature and regulatory sources, including SCCS opinions, which were not previously used for this purpose. The data harvesting procedure was conducted according to rigorous rules, iteratively developed and refined, as part of this work, ensuring consistency was maintained across the data harvesters and addressing the concerns with regard to data quality. These rules were captured in the SOP document (Annex 4), which may be consulted in the future and used to harvest additional data.

The COSMOS Skin Permeability Database provides information on the test system details (skin donors, skin membranes, or test animals) and test conditions (including diffusion experiment parameters of *in vitro* studies). The available results include lag times, flux, absorption (total amounts and %), permeability coefficients and recovery or distribution/elimination information (for *in vivo* studies) for the test materials. The unique data model of the skin permeability database enables efficient data mining and fast identification of the studies available for particular species, membrane types, conducted with (or without) certain vehicles, etc. This data structure supports the computational modelling activities.

The COSMOS Skin Permeability database has undergone a QC procedure performed by the experts from the COSMOS ILSI Expert Group, who added the information on the skin metabolism (pathways/steps/enzymes) for 12 cosmetics-related compounds.

Overall, the collated COSMOS skin permeability database is the largest database for this endpoint, with a range of high quality data for diverse types of chemicals (including cosmetics), that may serve as a solid basis for computational modelling and support the

development of *in silico* tools for safety/risk assessment of cosmetics and related compounds. It was a foundation of the analysis discussed in the subsequent chapter.

Chapter 5

Classification of Skin Permeability Potential Following Dermal Exposure to Support the Prediction of Repeated Dose Toxicity of Cosmetics-Related Compounds

5.1. Background

The safety evaluation of chemicals includes hazard characterisation and the assessment of potential human exposure. Human exposure to cosmetics and related compounds occurs primarily *via* the topical route. Thus, the rate and extent of the transfer of chemicals across the skin are of particular interest for the safety evaluation of cosmetics and related compounds.

The skin is a metabolically active, living organ, which can be penetrated by chemicals through complex biological and physicochemical processes. The skin is also the largest organ of the body and, as such, may act as a main route of entry of the compound into the systemic circulation. The multiple factors which influence the rate of percutaneous permeability have been broadly reported in the literature (Elias et al., 1981; Southwell et al., 1984; Rougier et al., 1987; Williams et al., 1992; Liu et al., 1993; Cua et al., 1995; Tsai et al., 2003; Otberg et al., 2004; Chilcott et al., 2005; Akomeah et al., 2007). In general, they are associated with:

- The variability of the structure of the skin, including inter-species differences (varying lipid content, thickness of the *stratum corneum*, number of appendageal shunts, etc.) and individual variations within species (age, sex, race), as well as anatomical site, skin condition, hydration, temperature and blood flow rate;
- The physicochemical nature of the penetrants, i.e. the compound and its vehicle, including the physical state, size (molecular weight), binding properties, etc.;
- The external conditions (experimental design, dosage regimen, etc., in case of laboratory studies).

5.1.1. The structure of the skin

The skin consists of several heterogenic structural compartments: epidermis (outer layer), dermis (inner layer), basement membrane (the multilayered structure at the junction

between epidermis and dermis), subcutis (the layer of fat and connective tissue below the dermis) and multiple appendages (sweat glands, hair follicles, etc.). The epidermis comprises about 5% of the thickness of the skin. The majority of the epidermal cells (keratinocytes) are formed by differentiation from one layer of mitotic basal cells (*stratum basale*). Nevertheless, several layers of epidermis (Figure 5.1) can be distinguished according to their cellular characteristics.

The outermost layer of the epidermis, the *stratum corneum*, is the actual barrier protecting the body against external influences and excessive water loss. It consists of multiple layers of non-viable cells, corneocytes, located in the lipid matrix, essential for the lipid skin barrier function. The structure of the *stratum corneum* resembles a “brick and mortar system” (Michaels et al., 1975).

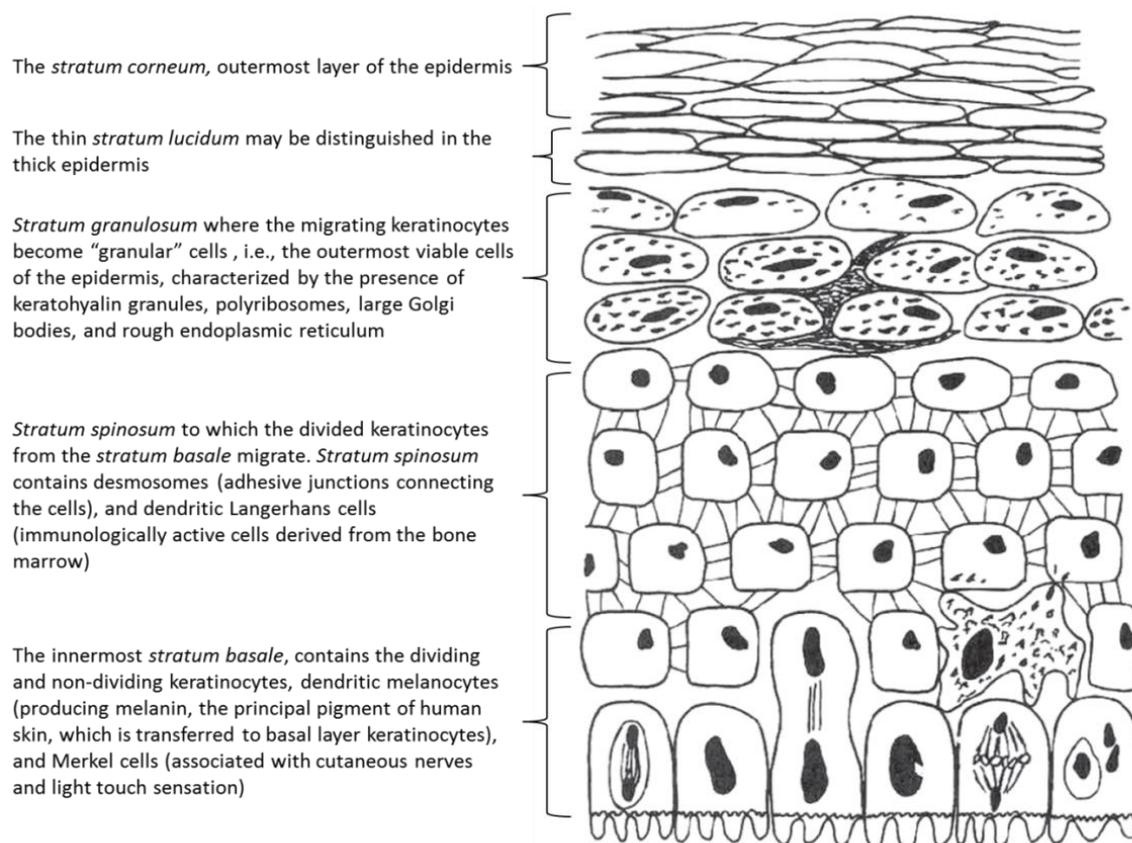


Figure 5.1
The structure of epidermis (adapted from WHO, 2006; Bensouilah & Buck, 2006; Menon et al., 2012; Van Smeden et al., 2014)

The dermis is a tissue of variable thickness (from 0.6 mm on the eyelids to 3.0 mm on the back, soles and palms), providing the nutritional support for the avascular epidermis. It consists of a tough, supportive fibrous protein matrix, composed of two layers: papillary (connecting with epidermis and build from thin collagen fibres) and reticular (including thicker collagen bundles). The dermis is built up from fibroblasts, producing collagen, elastin, structural proteoglycans and immunocompetent mast cells and macrophages. The blood vessels, sensory nerves (pressure, temperature, and pain) and lymphatic cells are located in the dermis, along with the inner segments of the sweat glands and pilosebaceous units. The dermis has multiple functions: it provides flexibility, strength, protection from infections as well as serving as a water storage organ (Bensouilah & Buck, 2006; Dancik et al., 2012). Derivative structures of the skin include hair, nails, sebaceous and sweat glands.

5.1.2. Transport of chemicals through the skin

The transport of chemicals through the skin can occur *via* intra- or inter-cellular transepidermal routes (i.e. across the *stratum corneum*) or through skin appendages. The intracellular (transcellular) route refers to the partitioning of chemicals into and out of the cell membrane of keratin-rich corneocytes. The intercellular process includes transfer of the chemicals through the lipid-rich matrix in which the corneocytes are embedded. The appendageal route involves the movements of chemicals through the skin appendages (hair follicles, sweat glands, and sebaceous glands), bypassing the corneocytes. Appendageal transport may be significant in particular regions of the skin, e.g. the hair-rich scalp, where the number of appendageal openings per unit of surface area is relatively large. In addition, it has been demonstrated that sebaceous glands may serve as a drug reservoir for some substances (Scheuplein, 1967). The transport of a chemical through the epidermis is followed by its uptake by the capillary network at the basement membrane (dermal/epidermal junction) into the cutaneous blood and lymphatic system (resorption). However, the chemicals can be retained in the viable epidermis, dermis, or in the subcutis if the blood flow is insufficient (WHO, 2006).

The permeability of a chemical through the skin is considered to be a process of passive diffusion. The rate of permeability is limited by the layer within the skin with the highest resistance to diffusion. The *stratum corneum* is, most commonly, the primary rate-limiting barrier. However, for very lipophilic substances the diffusion through the

hydrophilic epidermis and dermis can be rate limiting. Diffusion of compounds across a membrane is described by Fick's first law (Crank, 1975). For the individual (pseudo-homogeneous) layers of the skin Fick's law can be expressed by Equations 5.1-5.7 (WHO, 2006).

$$J = -D \frac{\partial C}{\partial x}$$

Equation 5.1

Where: J Flux (the rate of transfer of a chemical per unit area in particular time)
 D Diffusion coefficient
 $\frac{\partial C}{\partial x}$ Concentration gradient

Over a specific time (the so-called lag time), flux reaches a steady-state value and the cumulative amount penetrating the skin increases:

$$J_{ss} = \frac{D(C_1 - C_2)}{h}$$

Equation 5.2

Where: J_{ss} Flux at steady-state
 h Membrane thickness
 C_1, C_2 Chemical concentrations at interface of the two membranes (at $x_1=0$ and $x_2=h$, respectively)

Assuming a local equilibrium with the vehicle and that the *stratum corneum* controls the dermal diffusion process:

$$C_1 = K_m \cdot C_v$$

Equation 5.3

Where: C_1 Chemical concentration before the diffusion process (at $x_1=0$)
 K_m *Stratum corneum*-to-vehicle partition coefficient
 C_v Concentration of the vehicle

Assuming that after passing through the membrane ($x=h$) the chemical concentration is close to zero (so-called sink conditions):

$$J_{ss} = \frac{D \cdot K_m \cdot C_v}{h}$$

Equation 5.4

If:

$$K_P = \frac{K_m \cdot D}{h}$$

Equation 5.5

Where: K_P Permeability coefficient

Then:

$$J_{SS} = K_P \cdot C_v$$

Equation 5.6

The maximum steady-state flux through the membrane can be thus be expressed as:

$$J_{MAX} = \frac{D \cdot S_{SC}}{h} = K_{PV} \cdot S_V$$

Equation 5.7

Where: J_{MAX} Maximum steady-state flux
 S_{SC} The saturated concentration of solute in the *stratum corneum*
 K_{PV} The permeability coefficient of a solute in a vehicle
 S_V The solubility of the solute in that vehicle

The maximum steady-state flux (J_{MAX}) is a valuable parameter to determine the percutaneous penetration/absorption of a chemical.

5.1.3. Modelling of skin permeability

5.1.3.1. General overview of the tools and approaches

The computational modelling of skin permeability has been investigated for over two decades resulting in a plethora of *in silico* models and tools. A number of comprehensive reviews have been published in this area (Moss et al., 2002; Geinoz et al., 2004; Degim, 2006; WHO, 2006; Mitragotri et al., 2011; Jepps et al., 2013; Naegel et al., 2013) along with the comparative studies evaluating the available models (Bouwman et al., 2008; Lian et al., 2008; Farahmand & Maibach, 2009; Brown et al., 2012).

One of the most significant skin permeability (K_P) models was published by Potts and Guy (Potts & Guy, 1992) for 93 historical permeability coefficients compiled from the literature by Flynn (Flynn, 1990). The Potts and Guy model accounts solely for the size and hydrophobicity of the permeant (Eq. 5.8), with a resulting correlation coefficient of 0.67.

$$\text{Log}K_p = \frac{D \cdot S_{sc}}{h} = 0.71\text{Log}P - 0.061\text{MW} - 6.3$$

Equation 5.8

Where: Log K_p Logarithm of the permeability coefficient
 Log P Logarithm of the n-octanol/water partition coefficient
 MW Molecular weight

The main limitation of this model is the over-prediction of the K_p for highly lipophilic compounds. In order to address this drawback, a modification was proposed (Cleek & Bunge, 1993; Bunge & Cleek, 1995) that split components representing the *stratum corneum partitioning* and diffusivity. The Potts and Guy model has also been further extended by a number of other workers, e.g. Moss and Cronin used an updated and revised set of 116 compounds obtaining a correlation coefficient of 0.82 (Moss & Cronin, 2002).

Following the Potts and Guy model, a plethora of multi-parameter QSAR models have been developed for skin permeability. Diverse types and numbers of descriptors have been proposed (e.g. over 1,600 descriptors were used by Baert et al., 2007). The most frequently applied include lipid/water partition coefficients, water solubility, melting point, molecular size and hydrogen bonding (Abraham et al., 1999; Roberts & Sloan, 1999; Roberts & Sloan, 2000; Abraham & Martins, 2004; Zhang et al., 2009). The majority of the models developed were based on the assumption that inter-cellular pathway (i.e. through the lipid matrix) is the dominant route of transport for a compound and that the corneocytes are not permeable. Such models, however, tend to under-predict the skin permeability of hydrophilic compounds.

A further alternative approach to predict skin permeability is based on the use of mechanistic models. These are derived from the Fick's laws of diffusion and mass balance equations, taking into account various diffusion pathways, not being limited to the lipid-based inter-cellular pathway. A four-pathway model considering the aqueous pores in the *stratum corneum* lipid matrix and the appendageal openings as the hydrophilic pathway in addition to the impermeable corneocytes has been proposed (Mitragotri, 2003). The model yields good results for both hydrophilic and hydrophobic permeants. Another type of mechanistic model is based on the "brick and mortar" framework of the *stratum corneum*. The two-dimensional (biphasic) microtransport model was developed by Kasting and co-

workers (Wang et al., 2006; Wang et al., 2007) and further extended (Dancik et al., 2012). This model provides the maximal fluxes, concentrations and distribution details for the permeant, as well as the amount of permeant prone to removal/evaporation.

5.1.3.2. Computational modelling of maximal flux (J_{MAX})

As mentioned in section 5.1.2, J_{MAX} is a relevant measure of the skin permeation of chemical. Relatively few studies, however, have used J_{MAX} as a dependent variable in the QSPR models. Of these studies, predicting the maximal flux on the basis of the molecular volume and solubility of the permeant in octanol was proposed for a small dataset of 35 drugs (Kasting et al., 1987). Molecular weight-based simplistic regression models for J_{MAX} prediction have been developed for the aqueous solutes by Magnusson and co-workers (Magnusson et al., 2004a). Their model, which used the largest number of compounds (279) of any of the models, yielded a correlation coefficient of 0.688:

$$J_{MAX} = 0.041MW - 4.52$$

Equation 5.9

Where: MW Molecular weight

An alternative approach to predict J_{MAX} is based on the determination of the “rules of thumb” to identify compounds exhibiting high or low skin permeability – a type of “pass/fail” test for rapid screening of compounds without experimental data (Magnusson et al., 2004b). “Rules of thumb” for intestinal absorption have been successfully formulated by Lipinski and coworkers (Lipinski et al., 1997). With respect to skin permeability, this approach was utilised by Magnusson et al. (Magnusson et al., 2004b). In order to identify compounds with extreme (high or low) J_{MAX} values, they analysed the following molecular descriptors: molecular weight (MW), melting point (MP), octanol/water partition coefficient (Log K), aqueous solubility (Log S) and number of atoms available for hydrogen bonding (HB), divided into H-bond donors (HB-d) and acceptors (HB-a). The boundary values reported in their study are presented in Table 5.1. The properties with the highest predictive power were: MW, HB-a, and Log S. Using the combination of 2 or 3 predictors yielded the best results.

Table 5.1

The boundary values for “good” (high J_{MAX}) and “bad” (low J_{MAX}) skin permeants (taken from Magnusson et al., 2004b)

J_{MAX}	Descriptors					
	MW	MP	HB-d	HB-a	Log K	Log S
Low (“bad” penetrants)	> 213	≥ 223	≥ 0	≥ 3	> 1.2	> -1.6
High (“good” penetrants)	≤ 152	≤ 432	≤ 2	≤ 3	< 2.6	≥ -2.3

The work of Magnusson et al., was elaborated further by Xu et al. (Xu et al., 2013) who reduced the rules to MW and Log P only. The permeants with $MW \geq 400$, $\log P \leq 1$, or $\log P \geq 4$ have been considered “bad” penetrants.

The method of estimating dermal absorption (when the experimental data are not available) on the basis of calculated J_{MAX} values was proposed by Kroes and co-workers (Kroes et al., 2007). For J_{MAX} values between 0.1 and 10 $\mu\text{g}/\text{cm}^2/\text{h}$, they recommended assuming 40% absorption of the applied dose (per 24 hour exposure). For $J_{MAX} < 0.1$, or $> 10 \mu\text{g}/\text{cm}^2/\text{h}$, the percent dose absorbed was suggested to be 10% or 80%, respectively. For non-reactive chemicals with molecular weights greater than 1000, negligible absorption was assumed.

5.2. The aims of chapter 5

As maximal flux (J_{MAX}) is a significant parameter in the assessment of the dermal delivery of the chemical compounds (defining their maximal toxic or systemic effects resulting from the topical exposure), it has been selected as the endpoint of the present research. The aims of chapter 5 are related to the objective 4 of the current PhD program (please refer to section 1.5) and include:

- Data mining of the Skin Permeability Database (its development was described in chapter 4) leading to the collation of a set of compounds with available experimental maximal flux (J_{MAX}) data;

- Structural (ToxPrint chemotypes) profiling of the collated dataset followed by the analysis of the physicochemical properties of its constituents;
- Determination of a set of rules classifying a chemical as having low or high potential to permeate the skin.

5.3. Materials and methods

5.3.1. Dataset for analysis

The final content of the COSMOS Skin Permeability Database was described in chapter 4. The database contains measured *in vitro* J_{MAX} values for over 200 compounds. Many chemicals were tested in multiple experiments (e.g. as many as 46 studies are available for diethyltoluamide or salicylic acid), resulting in the total number of data points for *in vitro* J_{MAX} exceeding 900. The experiments were performed with a range of skin membranes (e.g. whole skin, epidermis, *stratum corneum*, etc.) obtained from various species (human, pig, mouse, rat, rabbit) and sampling sites (abdomen, breast, back, leg, neck) using diverse diffusion cells types (static or flow-through) and dose application regimes (occluded, non-occluded or semi-occluded conditions).

As mentioned previously, experimental measures of skin permeability are highly variable. The variability of *in vitro* J_{MAX} measurement results was reported in the scientific literature (Howes et al., 1996; Benech-Keiffer et al., 2000; Akomeah et al., 2007). It was demonstrated that for some compounds (e.g. methyl paraben) the variability is as high as 35%, and a difference between the highest and lowest flux values as high as 4-fold can be obtained, even when the same experimental protocol was applied (Chilcott et al., 2005; Akomeah et al., 2007). The underlying reasons were attributed to a variety of factors including human error, differences in experimental design (e.g. receptor chamber volume and surface area, the type of diffusion cell used), skin morphology (e.g. samples of variable origin) or the physicochemical properties of tested substance (Elias et al., 1981; Southwell et al., 1984; Rougier et al., 1987; Williams et al., 1992; Liu et al., 1993; Cua et al., 1995; Tsai et al., 2003; Otberg et al., 2004; Chilcott et al., 2005; Akomeah et al., 2007).

For the purpose of the present analysis the J_{MAX} values measured *in vitro* with human skin samples were utilised. The COSMOS Skin Permeability Database contains a total of 640 *in vitro* human studies (for 184 compounds) with J_{MAX} provided. The variability of the data

was analysed for all compounds with multiple results from more than a single study. When J_{MAX} values were highly variable and it was not feasible to make a conclusion regarding the skin permeability category, all the records for a compound were dropped from the analysis. For the compounds with multiple studies but low range of J_{MAX} values the means were calculated. The IOM compounds and mixtures were excluded from the investigation. It should be noted that only *in vitro* human data were used, but all membrane types (epidermis, split-thickness skin, full-thickness skin, etc.) and vehicles were considered.

Once the set of compounds with low variability of *in vitro* J_{MAX} values was identified, three initial categories, corresponding to low, medium and high skin permeability potential were defined using 33.3% and 66.7% quantiles.

5.3.2. Structural features and physicochemical properties analysis

The structures of compounds analysed were prepared for computational analysis in the Corina Symphony software tool (Molecular Networks GmbH, Nüremberg, Germany) following the procedure described in detail in chapter 3. Subsequently, the dataset was analysed with respect to the structural features and molecular properties.

The structural domain of the dataset was characterised by the ToxPrint chemotypes calculated in the ChemoTyper software tool (Altamira LLC, Columbus, OH; Molecular Networks GmbH, Nüremberg, Germany) and presented in terms of their distributions across different skin permeability categories. The in depth description of this methodology is provided in chapter 3.

The physicochemical profile of the dataset was determined with the “global molecular” and “size and shape” descriptors calculated in Corina Symphony software tool (Molecular Networks GmbH, Nüremberg, Germany). For the purpose of the current analysis, fifteen properties were selected *a priori* on the basis of knowledge available in the scientific literature (Table 5.2). The standardised values of the calculated descriptors were used as variables in the Principal Component Analysis conducted in JMP Pro 12.2.0 software (SAS Institute Inc.). This technique was discussed in-depth in chapter 3.

Table 5.2

CORINA Symphony (Molecular Networks GmbH, Nuremberg, Germany) descriptors utilized in the present analysis

GLOBAL MOLECULAR PROPERTIES
Number of open-chain, single rotatable bonds (BondsRot) [unitless]
Number of hydrogen bonding acceptors derived from the sum of nitrogen and oxygen atoms in the molecule (H-Acc) [unitless]
Number of hydrogen bonding donors derived from the sum of N-H and O-H groups in the molecule (H-Don) [unitless]
Number of tetrahedral stereocentres in the molecule (Stereo) [unitless]
Solubility of the molecule in water (Log S) [mol/L]
Mean molecular polarisability of the molecule (Polariz) [\AA^3]
Molecular complexity (Complex) [unitless]
Ring complexity (ComplexRing) [unitless]
Molecular weight derived from the gross formula (MW) [Da]
Octanol/water partition coefficient of the molecule following the XlogP approach (Log P) [unitless]
McGowan molecular volume approximated by fragment contributions (McGowan) [mL/mol]
Topological polar surface area of the molecule derived from polar 2D fragments (TPSA) [\AA^2]
SHAPE DESCRIPTORS
Molecular diameter – maximum distance between two atoms in the molecule (Diameter) [\AA]
Molecular radius of gyration (Rgyr) [\AA]
Molecular span – radius of the smallest sphere centred at the centre of mass which completely encloses all atoms in the molecule (Span) [\AA]

5.3.3. Defining the classification rules

On the basis of the structural and physicochemical profiles of the dataset, sets of rules were proposed for skin permeability categories to support the classification of query compounds without experimental data.

5.4. Results

5.4.1. Dataset for analysis

The dataset compiled consisted of 112 organic compounds (after removing compounds with high variability of reported J_{MAX} values and compounds which are not handled well by computational tools used for calculating molecular descriptors). The full

dataset is reported in Annex 5. The summary statistics and distribution of experimental J_{MAX} values in the entire dataset of 112 compounds analysed are presented in Figure 5.2A. Based on the 33.3% and 66.7% quantiles, the dataset was divided into the following skin permeability potential categories (the summary statistics and distributions of $\log J_{MAX}$ values within each category are presented in Figure 5.2B):

- Low (36 compounds): $J_{MAX} < 0.75 \mu\text{g}/\text{cm}^2/\text{h}$ ($\log J_{MAX} < -0.12$);
- Medium (38 compounds): J_{MAX} between $0.75\text{-}10 \mu\text{g}/\text{cm}^2/\text{h}$ ($\log J_{MAX} < -0.12, 1$);
- High (38 compounds): $J_{MAX} > 10 \mu\text{g}/\text{cm}^2/\text{h}$ ($\log J_{MAX} > 1$).

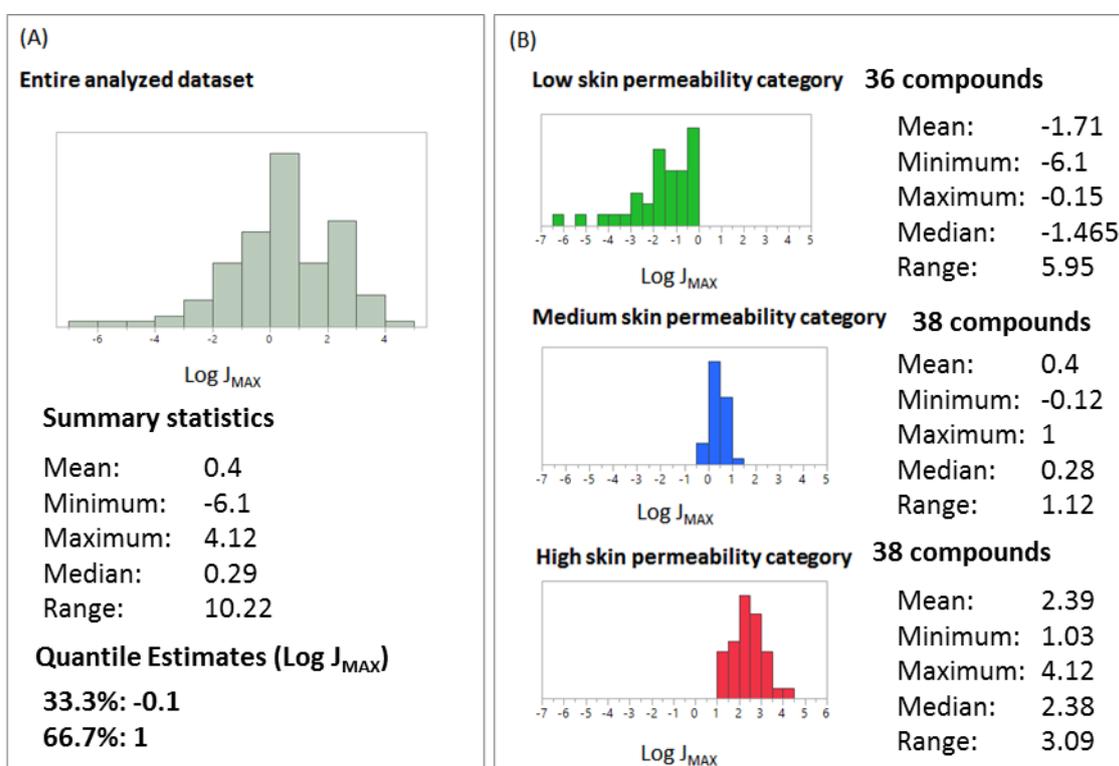


Figure 5.2

(A) The summary statistics, quantile analysis estimates (mean confidence interval $(1-\alpha)$ of 0.95) and histogram of $\log J_{MAX}$ values distribution for the set of 112 compounds subjected into analysis;

(B) The summary statistics and histograms of $\log J_{MAX}$ values distribution for the low, medium, and high skin permeability categories

The plots were prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

5.4.2. Chemotype analysis

The structural profile of compounds belonging to the low, medium and high skin permeability categories was analysed with the ToxPrint chemotypes (Figure 5.3). Fused rings

(e.g. steroids), cyclic alkenes and alkanes (C5 and C6), secondary alkyl alcohols, ketones, aliphatic amines, and neopentyl groups were found in abundance in the low skin permeability category of compounds. Aliphatic ethers and ethylene oxides were identified predominantly in compounds with high skin permeability potential. The medium skin permeability compounds were rich in aromatic rings with hetero atoms, aromatic alkanes, cyclic alkanes (C3), halides (especially alkyl halides) and halocarbonyls, tertiary alkyl alcohols, and carboxylic acids.

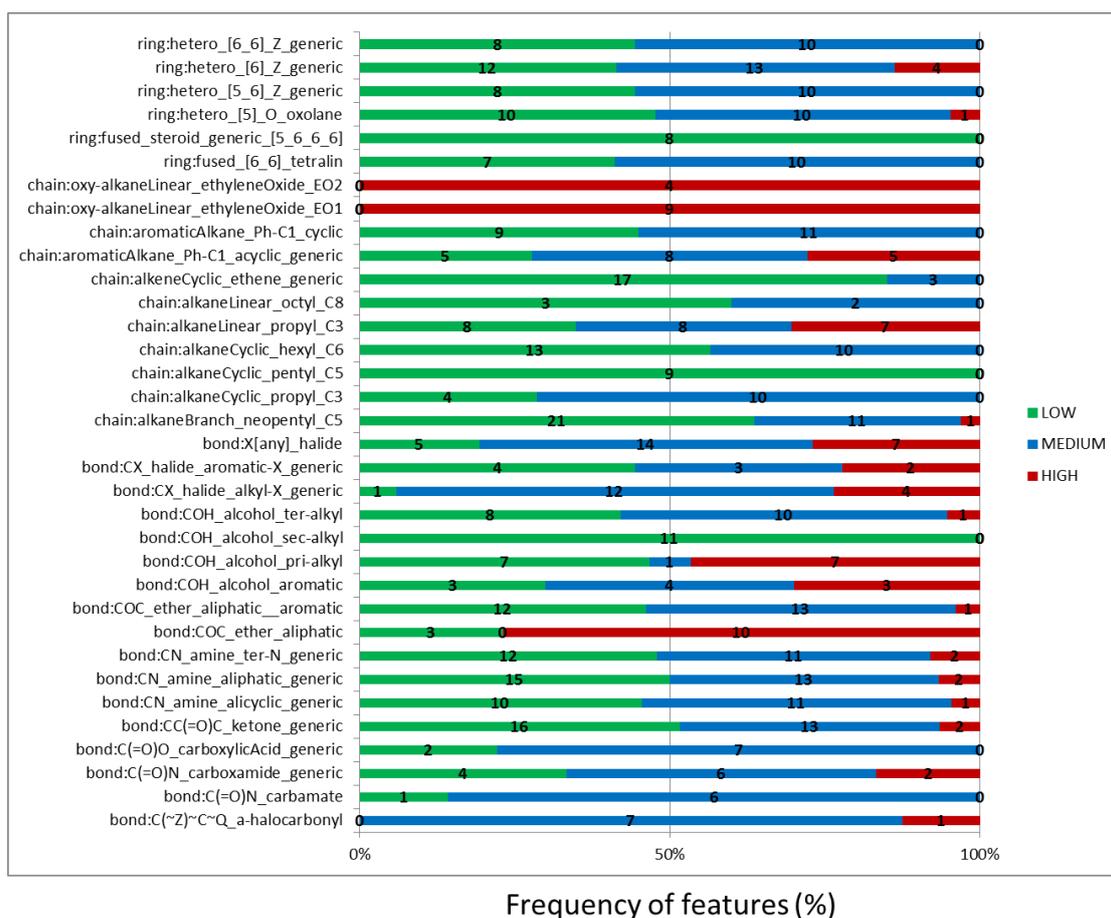


Figure 5.3

The structural profile of compounds from low, medium and high skin permeability categories, expressed as % frequency of features. The actual counts of each structural feature are provided on the plot's bars

5.4.3. Physicochemical properties analysis

The physicochemical properties space of a dataset of 112 compounds was investigated in terms of Principal Components Analysis on the basis of 15 physicochemical descriptors (Annex 7) selected *a priori* (as described in 5.3.3) and calculated in Corina

Symphony (Molecular Networks GmbH, Nüremberg, Germany). The scree plot and variance explained by each PC are presented in Figure 5.4.

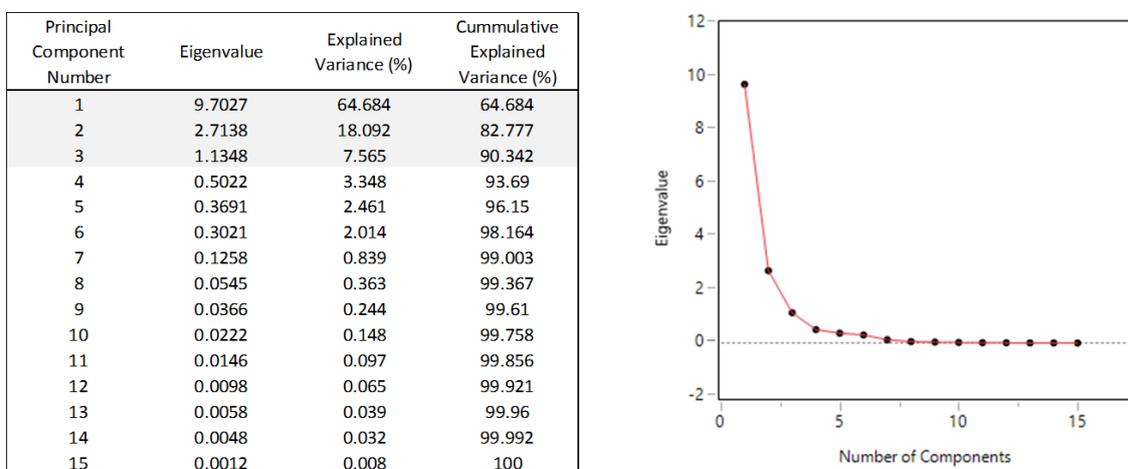


Figure 5.4

Scree plot and % variance explained by each calculated Principal Component. The PCs with eigenvalues >1 were considered. The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

The three first Principal Components (with eigenvalues >1) cumulatively explaining 90.3% variance in the dataset were considered. Their scores (Annex 7) were used to generate 3D score plot visualising the physicochemical properties space covered by the investigated dataset (Figure 5.5). The empirical categories of low, medium and high skin permeability potential were projected onto the PCs score plot.

The most influential properties associated with each considered PC are presented in Figure 5.6. The first Principal Component was most significantly influenced by descriptors related to the size, shape, and volume of the molecules (MW, Diameter, Complex, Span, Rgyr, McGowan), their hydrophilicity/lipophobicity (Log S, TPSA), mean molecular polarizability (Polariz), and the number of hydrogen bond acceptors (H-Acc). The octanol/water partition coefficient (Log P) had the highest loading on the PC2. The number of rotational bonds reflecting the flexibility of the molecule (BondsRot) and ring complexity (ComplexRing) were the most significant in the PC3.

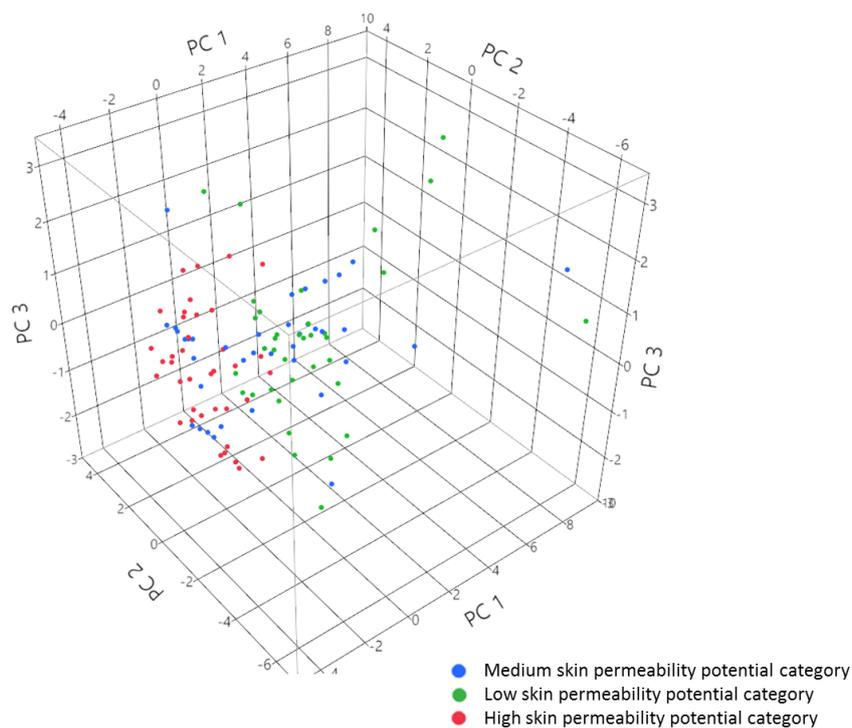


Figure 5.5

The 3D score plot of the three first Principal Components (PC1-PC3) defining the properties space of the dataset analysed (cumulatively explaining 90.3% of the variance in the dataset). The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

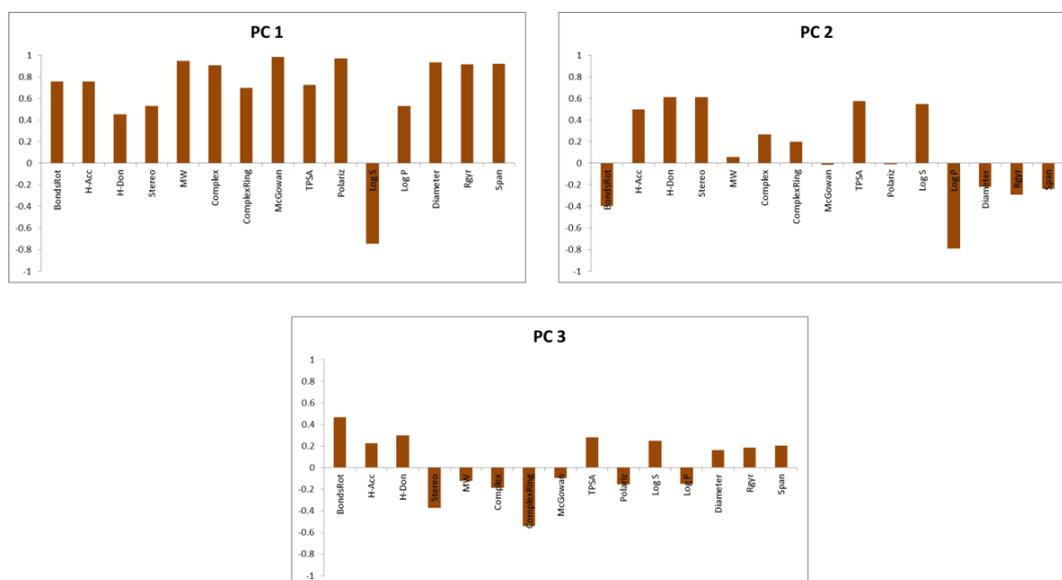


Figure 5.6

The loading bar plots for the three Principal Components characterising physicochemical properties of investigated dataset of 112 compounds. The properties with loadings above 0.7 (or close to) were considered to be the most influential

As presented in Figure 5.5, the regions rich in compounds with high or low skin permeability potential could be clearly distinguished in the investigated physicochemical properties space, whereas the compounds from the medium skin permeability category (evenly distributed across the entire space) did not form a separate cluster. This result indicates that the set of molecular descriptors utilised was relevant to identify the differences between low and high skin permeability compounds. The separation of the medium category compounds was not obtained as they exhibit a range of “mixed” features, not sufficiently discriminative to be captured solely by the properties used. Thus, the structural characteristics of “medium” category compounds were projected into the 2D score plot (PC1 vs PC2) of their physicochemical properties space (Figure 5.7).

It can be observed that the “medium” category compounds with distinctive structural features (identified in section 5.4.3) form individual clusters in the physicochemical properties space: a group of naltrexone derivatives, containing fused rings and cyclopropane moieties is located in the region richer in low skin permeability compounds, while the cluster abundant in halocarbonyls, alkyl halides, aromatic halides and medium-length chain aromatic alkanes is situated in the region occupied mostly by high skin permeability compounds. Therefore, to characterise medium skin permeability category fully, the structural features have to be considered together with physicochemical descriptors. The compounds from “low” and “high” skin permeability categories exhibit such different structural characteristics that the physicochemical properties alone are able to clearly reflect them (Figure 5.8). Complex molecules containing fused rings, cyclic alkenes and alkanes (C5 and C6), secondary alkyl alcohols, ketones and aliphatic amines are located on the right-hand side of the PC1 vs PC2 plot (Figure 5.8), whereas polyethylene glycol ethers and short chain alkanes are situated on the left-hand side. For these classes of compounds, belonging (respectively) to “low” and “high” skin permeability categories, the boundary values of descriptors were determined in the next step of the analysis.

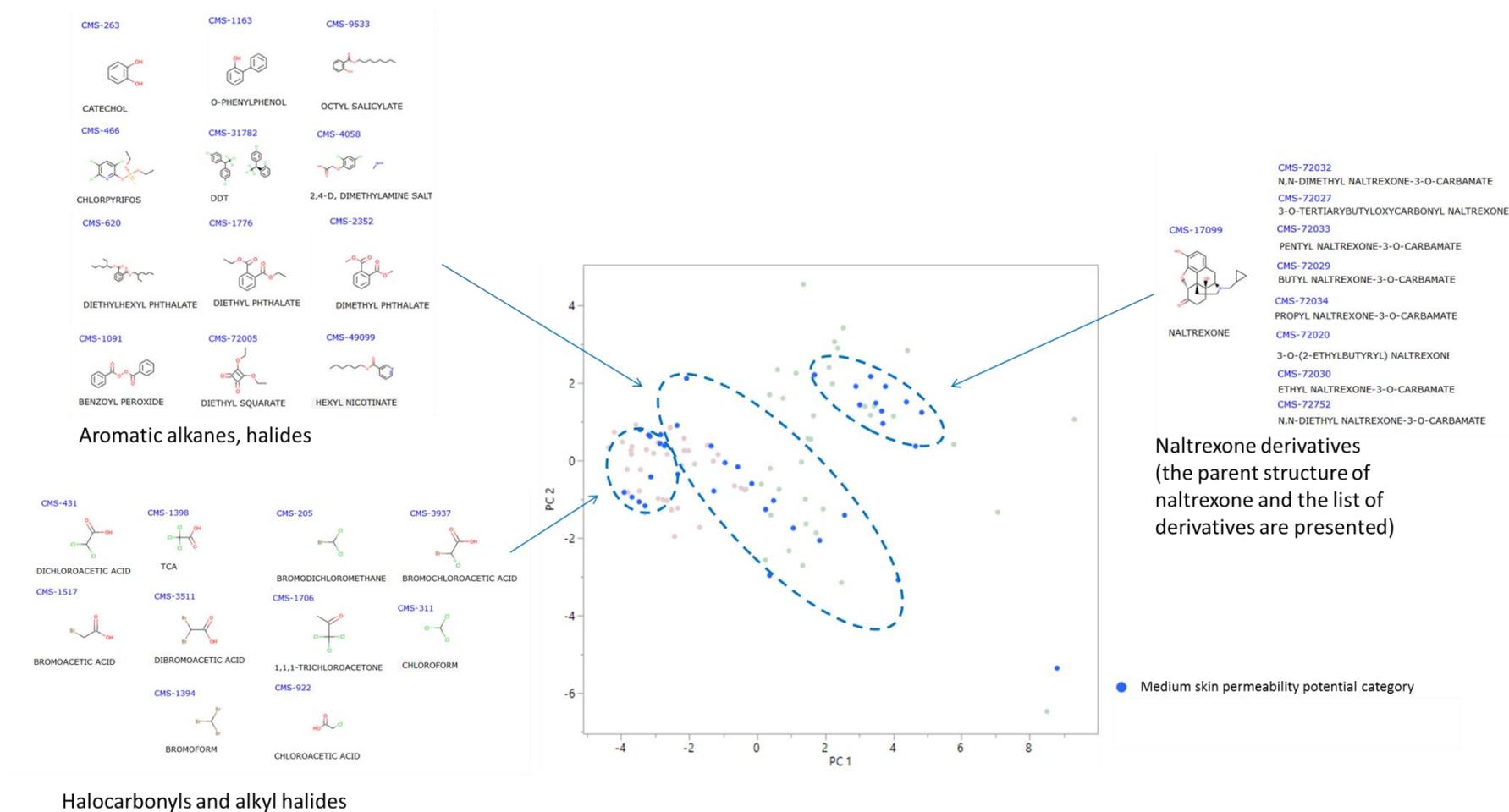


Figure 5.7

The 2D PCs score plot demonstrating the location of the compounds from the medium skin permeability potential category in the considered physicochemical properties space and their distinctive structural features (grey dots denote the compounds from the “low” and “high” categories). The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

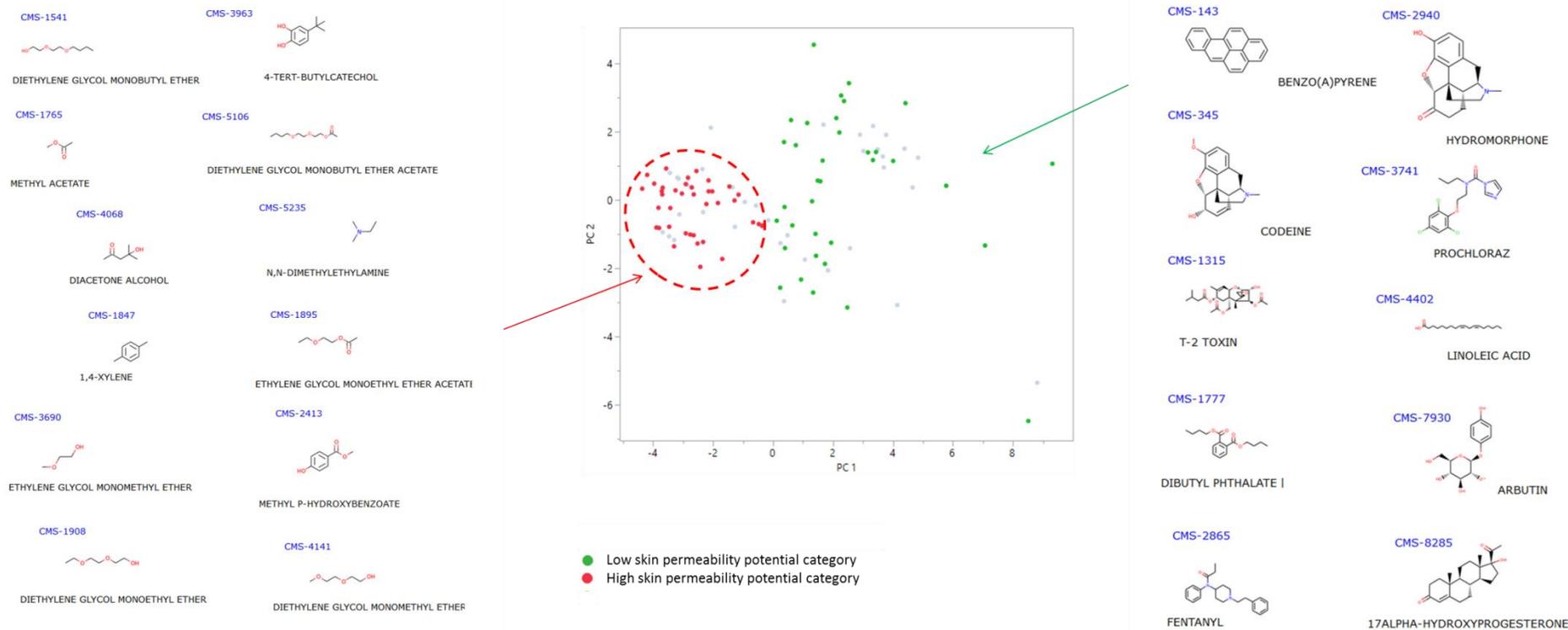


Figure 5.8

The 2D PCs score plot demonstrating the good separation between low and high skin permeability potential category compounds in the considered physicochemical properties space and their distinctive structural features (grey dots denote the compounds from the "medium" category). The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

5.4.4. Defining the classification rules

The distributions and ranges of values of the most significant physicochemical properties (as identified by loadings, Figure 5.6) were analysed for compounds assigned into the low and high skin permeability potential categories (Figure 5.9). In general, skin permeability decreased with increasing size, volume, and flexibility of molecules (molecular weight, diameter, complexity, span, radius of gyration, McGowan volume, the number of rotational bonds), as well as with the numbers of hydrogen bond acceptors and donors, topological polar surface area and molecular polarisability. The Log P and Log S values associated with “low” and “high” permeability categories were also identified.

The scatterplot matrix of correlations between the 12 physicochemical properties is presented in Figure 5.10. It visualises the capability of the descriptors to discriminate between low and high skin permeability categories.

The full set of defined boundaries of physicochemical properties’ values for particular structural classes is presented in Table 5.3. The interpretation of these results is provided in the following section.

Table 5.3

The set of rules for assigning the compounds without experimental data into the low or high skin permeability category on the basis of structural features and calculated properties. The structural features characteristic for medium skin permeability category compounds are also provided

Skin permeability category (Structural features)	Physicochemical properties ranges											
	MW	Complex	McGowan	BondsRot	Log P	Rgyr	H-Acc	TPSA	Polariz	Log S	Diameter	Span
HIGH (aliphatic ethers and ethylene oxides)	≤ 213	≤ 224	< 157	≤ 10	< -0.5	< 2	≤ 4	< 47	< 14	> -0.9	< 8.8	< 5
LOW (fused rings, multiple aromatic rings, cyclic alkenes and alkanes (C5 and C6), secondary alkyl alcohols, ketones, aliphatic amines with long aliphatic chains)	≥ 226	≥ 245	> 171	> 10	> 4	> 4	> 4	> 47	> 24	< -4	> 12.3	> 7.3
MEDIUM (halocarbonyls, alkyl halides, aromatic halides, medium-length chain aromatic alkanes)	Properties indicating "high" skin permeability											
MEDIUM (fused rings, cyclic alkanes (C3))	Properties indicating "low" skin permeability											

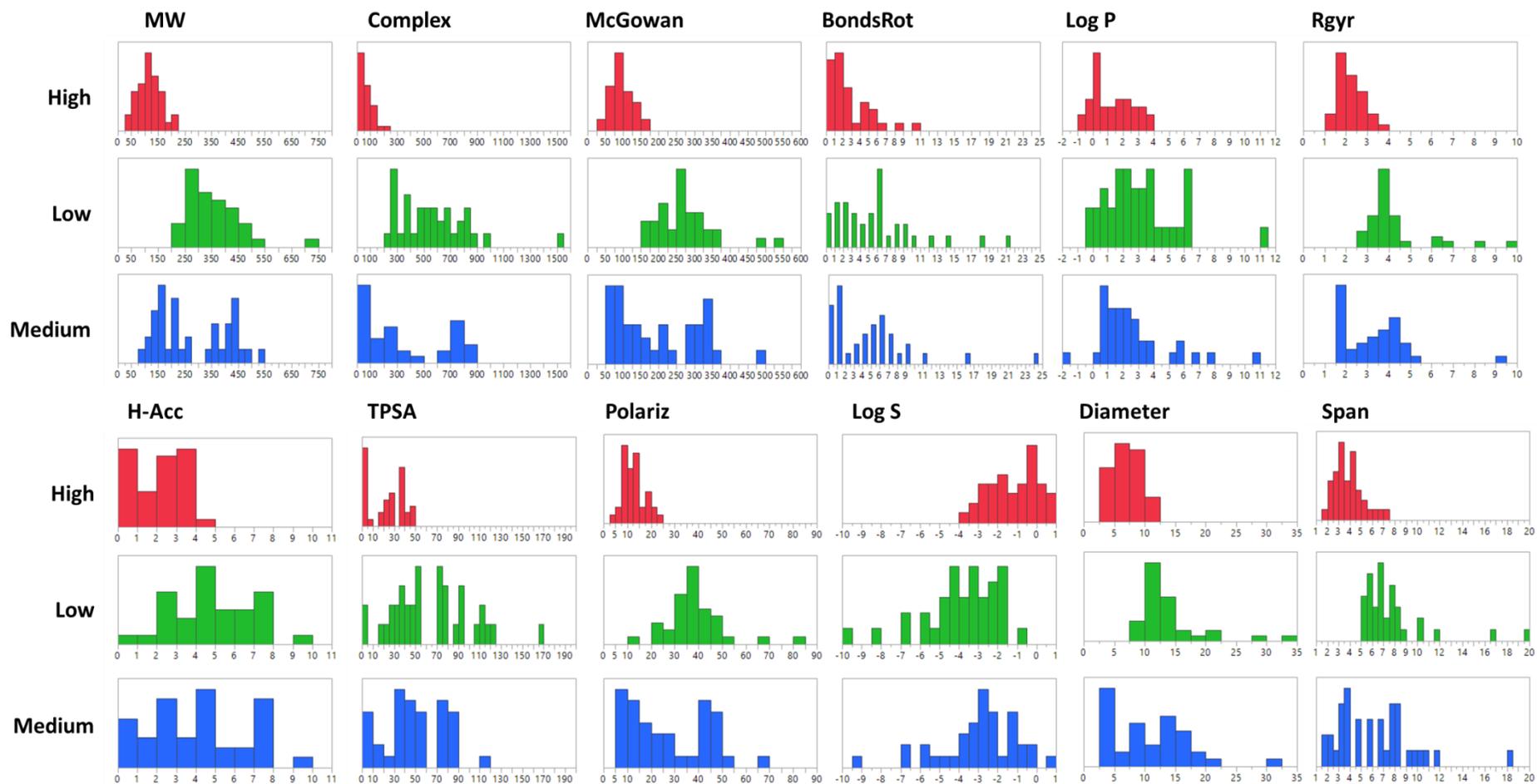


Figure 5.9

Histograms of distribution of 12 individual molecular properties within particular skin permeability potential categories. The boundary values of physicochemical properties ranges were defined for the “high” and “low” categories; the histograms of “medium” category were depicted to illustrate that the compounds from this group have the “mixed” values of analysed properties. The ranges and mean values for all descriptors are provided in Annex 7. The plots were prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

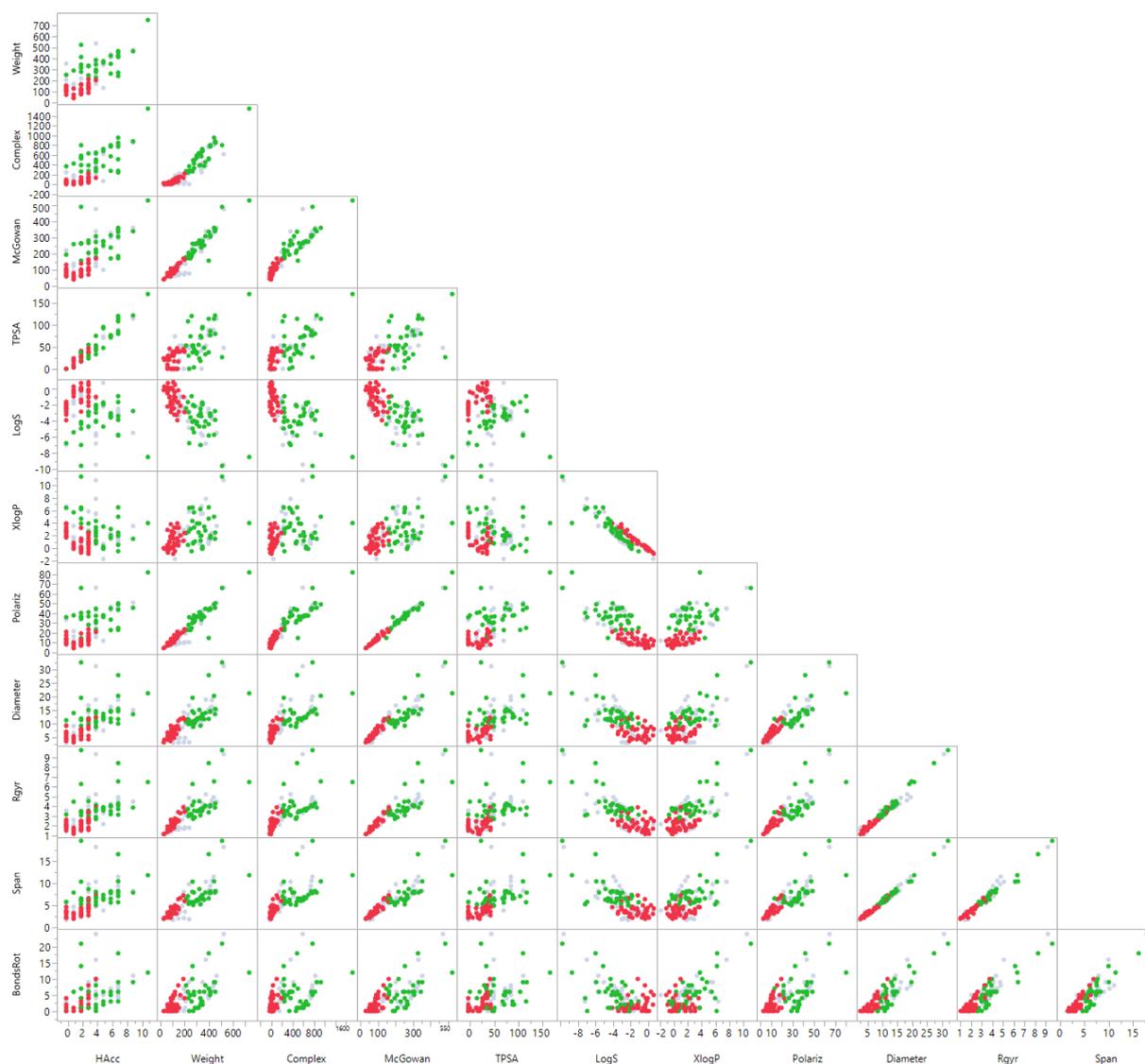


Figure 5.10

The scatterplot matrix presenting the correlations between individual molecular properties used to discriminate the categories of low (green) and high (red) skin permeability potential. The plot was prepared in JMP Pro 12.2.0 software tool (JMP, SAS Institute Inc.)

5.5. Discussion

The main function of the skin is to act as a physicochemical barrier, and, as such, to resist the penetration of chemicals. Many molecules, however, can pass this barrier in a passive diffusion process. As outlined above, the permeability of a chemical through the skin is limited by the skin layer with the highest resistance to diffusion (*stratum corneum*, or hydrophilic epidermis and dermis for very lipophilic substances). In the current analysis, the structural features and physicochemical properties of compounds exhibiting high and low skin permeability potential were analysed. The main molecular characteristics determining

the skin permeability potential identified were associated with the molecular size (including volume, dimensions, and complexity of the molecules), hydrophobicity and hydrogen bonding ability. These findings remain in agreement with the findings of other researchers (e.g. Cronin et al., 1999, Walters, 2002; Magnusson et al., 2004b; Abraham and Martins, 2004; Zhang et al., 2009; Kupczewska-Dobecka et al., 2010). The molecular size (molecular weight and diameter), volume (McGowan), complexity (Complex, ComplexRing), dimensions, and spatial arrangement of atoms in the molecules (Span, Rgyr, Stereo) are all negatively correlated with the skin permeability potential. Larger and more complex molecules have more difficulty in passing the corneal layer. This can be related to the steric hindrance – multiple aromatic rings, or fused rings in complex molecules (e.g. steroids) may slow diffusion by steric effects. In case of very polar solutes, the polar pathway with a defined radius for transport is utilised (Walters, 2002). The increasing molecular weight/size is generally positively correlated with a range of other features, e.g. the number of rotational bonds, or hydrogen-bonding capabilities of the molecules. High molecular flexibility (reflected in the number of rotational bonds) is associated with lower permeability potential. Similarly, the large number of hydrogen bond acceptors and/or donors may slow diffusion by hydrogen bonding effects. The topological polar surface area (TPSA) (the surface of polar fragments present in the molecule), octanol/water partition coefficient (Log P), aqueous solubility (Log S), and polarisability (Polariz) provide information on the hydrophobicity of the solutes and their partitioning between lipophilic and hydrophilic regions of the skin membrane. The compounds with large TPSA and high polarizability tend to be poor skin permeants. Poor skin permeability potential was observed for compounds with Log P >4 and Log S <-4.

In summary, in the present analysis it was demonstrated that the complex process of skin permeability expressed as the maximal flux, is governed by molecular size-, hydrophobicity- and hydrogen bonding-related properties, reflecting the structural features of the solutes. For the chemical classes identified with the ToxPrint chemotypes, the boundary values of physicochemical properties capable of distinguishing the groups of compounds with high and low skin permeability potential was identified (Table 5.3).

The analysis performed utilises a novel methodology (combination of ToxPrint chemotypes with physicochemical properties) and demonstrates its capability for

performing a simultaneous analysis for different groups of chemicals (cosmetics, drugs, pesticides), exhibiting various properties (e.g. highly lipophilic or highly hydrophilic) and structural diversity. The approach proposed can be further elaborated and used for the development of computational models utilising both types of descriptors at once. The determined profiles of bad and good skin penetrants can be used for rapid screening of chemicals without experimental data and support assessment of potential human exposure to chemicals *via* the topical route. This, in the broader scale, can facilitate the process of safety evaluation of chemicals, including cosmetics ingredients and related compounds.

Chapter 6

COSMOS Oral Repeated Dose Toxicity Database (oRepeatToxDB): Harvesting, Curating and Quality Control of the Data

6.1. Background

6.1.1. *In vivo* oral repeated dose toxicity tests

Repeated dose toxicity is a broad term referring to the general, adverse toxicological effects resulting from the repeated daily exposure to (or dosing with) a substance for a specified period of time, up to the expected lifespan of the test species. The main goal of repeated dose toxicity studies is the characterisation of the toxicological profile of the test substance including the identification of: the potential target organs of toxicity, the dose-response relationships for each toxicity endpoint (and margin between toxic and non-toxic dose), the delayed responses, cumulative effects (and, eventually, their reversibility) and the responses to toxic metabolites formed in the organism. The information delivered from repeated dose toxicity testing is essential for safety and risk assessment of any substance, including industrial chemicals, pharmaceuticals, biocides, and cosmetics ingredients (EMA, 2010; EURL ECVAM, 2016).

The major outcomes of a repeated dose toxicity study are: the No Observed (Adverse) Effect Level (NO(A)EL) value, referring to the highest dosage of the test substance for which no (adverse) treatment-related effects have been observed, and the related Lowest Observed (Adverse) Effect Level (LO(A)EL), i.e. the lowest dosage of the test substance where (adverse) treatment-related findings have been observed (SCCS, 2012).

The NO(A)EL value is used for systemic toxicity safety assessment, including, for example, the calculation of the Margin of Safety (MoS)³ for cosmetic substances by the EU SCCS. For systemic toxicity risk assessment, particularly significant is the “critical toxic effect”, defined as the first adverse effect (or its known precursor), that occurs at the

³ Margin of Safety (MoS) – uncertainty factor applied at the last stage of the safety evaluation of cosmetic substance, calculated by dividing the lowest NO(A)EL identified for this substance in repeated dose toxicity study by the Systemic Exposure Dosage (SED) value. Generally, minimum MoS value of 100 is necessary to conclude that a substance is safe for use (SCCS, 2012)

LO(A)EL. It may vary from lethality to a minor toxic effect, but in general is associated with the particular “critical” target organ in which it has been observed (EPA, 2016b).

As far as experimental protocols are concerned, several types of *in vivo* studies with repeated dosage regimen, and an oral route of exposure, can provide the above information. They include: short-term studies in rodents (28 days), subchronic studies in rodents and non-rodents (90 days), chronic studies in rodents (usually 12 months, however either shorter, e.g. 6 or 9 months, or a longer, e.g. 18 or 24 months, study duration is also applied) and reproductive/developmental/neurotoxicity studies. The list of the relevant guideline documents used in various regulatory agencies is summarised in Table 6.1. The guideline documents from the OECD, U.S. FDA and EPA for oral repeated dose toxicity studies (including chronic, subchronic, reproductive and developmental tests) require similar study parameters of design and result findings.

Table 6.1
Types of toxicity studies with oral repeated dosage and corresponding test guidelines

Test method	The EC Council Regulation Test Method (EC, 2008)	The OECD Test Guideline (TG)	The U.S. FDA Redbook 2000 Regulation (FDA, 2007)	The U.S. EPA OCSPP (previous OPPT) Guideline
Short-term, rodents	B.7	407 (OECD, 2008)	IV.C.3.a	870.3050 (EPA, 2000)
Subchronic, rodents	B.26	408 (OECD, 1998a)	IV.C.4.a	870.3100 (EPA, 1998a)
Subchronic, non-rodents	B.27	409 (OECD, 1998b)	IV.C.4.b	870.3150 (EPA, 1998b)
Chronic, rodents	B.30	452 (OECD, 2009)	IV.C.5.a	870.4100 (EPA, 1998c)
Chronic, non-rodents			IV.C.5.b	
Developmental (prenatal)	B.31	414 (OECD, 2001)	IV.C.9.b	870.3700 (EPA, 1998d)
Reproductive	B.34 (1GEN*) B.35 (2GEN**)	415 (1GEN), 416 (2GEN) (OECD, 1983; OECD, 2001a)	IV.C.9.a	N/A
Neurotoxicity	B.43	424 (OECD, 1997)	IV.C.10	OPPTS 870.6200 (EPA, 1998e)

* 1GEN: 1-generation study; ** 2GEN: 2-generation study

6.1.2. COSMOS oRepeatToxDB

For the purpose of constructing the COSMOS Oral Repeated Dose Toxicity Database, and in order to facilitate the development of *in silico tools* for target organ toxicity prediction, detailed information on critical target organ(s), critical toxic effect(s) and associated LO(A)EL values were absolutely essential. Although a plethora of repeated dose toxicity databases exist (extensive reviews of these have been published recently, e.g. by Madden, 2013), there is a general lack of publicly available databases providing organ level toxicity effect data associated with the LO(A)EL values. Moreover there is a lack of databases which link this information with accurately identified chemical compounds, and fewer or no databases that focus on cosmetics and their ingredients at the same time. There is also a general paucity of mature chemical-toxicological ontologies for target organs toxicity in the available databases (the importance of the ontologies for data mining has been described in chapter 1.3). The COSMOS Oral Repeated Dose Toxicity Database (oRepeatToxDB) was constructed to address these issues (the design of ontologies has been discussed more in-depth in chapter 7; please refer to Annex 1).

The process of the database development included consolidation of available data sources and harvesting of new ones. As far as the existing data sources are concerned, two publicly available databases were found that were relevant for the needs of COSMOS oRepeatToxDB, namely the U.S. EPA ToxRefDB (EPA DSSTox, 2016) and the public part of the U.S. FDA CFSAN CERES, including the PAFA database (Benz & Irausquin, 1991).

The majority of ToxRefDB content refers to agrochemicals (pesticides), since the U.S. EPA does not regulate cosmetics. However, about 15% of the content of ToxRefDB was found to be in common with the COSMOS Cosmetics Inventory. ToxRefDB content, along with NOEL/LOEL values, was provided by the U.S. EPA and used as received.

The U.S. FDA CFSAN PAFA (please refer also to Table 2.1) provides oral repeated-dose toxicity information for several hundred compounds, referencing (as original data sources) internal U.S. FDA documents, U.S. National Toxicology Program (NTP) study reports and the scientific literature. Approximately 30% of those chemicals were found to be common to the COSMOS Cosmetics Inventory. As the PAFA is the legacy database of the U.S. FDA, in many cases (mostly due to the technical limitations existing at the time of its construction) it does not provide sufficient level of detail for the toxicity data recorded (e.g.

information on the target organs). Thus, PAFA served more as the repository of cosmetics-related chemicals for which oral repeated dose toxicity studies have been performed. For several PAFA compounds the referenced materials have been consulted and either re-harvested or exchanged with more recent studies.

The main focus of the COSMOS oRepeatToxDB was placed on cosmetics and cosmetics ingredients. The only exception to this rule was the inclusion of Food Contact Substances (FCS) and impurities from the U.S. FDA Food Contact Notification program through collaboration with the CERES project. This was to enable the consideration of compounds which may be unintentionally present in the final cosmetic products (as a result of migration from packaging materials, for example).

The new oral repeated dose toxicity data were harvested for the cosmetics and related compounds from a range of regulatory and literature sources. The concerns over the accuracy of data incorporated into COSMOS oRepeatToxDB were minimised by establishing a consistent procedure for data curation and quality control, as described below.

6.1.3. Data record reliability in COSMOS oRepeatToxDB

The general aspects of the quality of biological data in toxicity databases have been described in detail in chapter 4.1.3. As far as the existing sources of the oral repeated dose toxicity data are concerned, both the U.S. FDA PAFA and the U.S. EPA ToxRefDB have their own sets of minimal study inclusion criteria specified for particular study types (chronic, subchronic, developmental toxicity, etc.). For instance, the U.S. FDA PAFA classifies the studies for “completeness” using regulatory guidelines from the U.S. FDA Redbook (FDA, 2007) along with its own “core standards” (minimum inclusion criteria). If the study satisfies the guideline, it is deemed to “meet the current standards” (PAFA completeness score “A”). When the study is not compliant with the guideline but acceptable to the PAFA inclusion criteria, it is classified as “not meeting the current standards, but meeting the core standards” (PAFA completeness score “B”). When the study does not meet the minimum standard of the database, it is considered “unacceptable by not meeting the core standards” (PAFA completeness score “C”). For example, the PAFA database allows the entry of a rat oral subchronic toxicity study using 5 animals at 2 dose levels without the full scale histopathology descriptions, whereas the FDA Redbook requires the use of 20 animals at

minimum 3 dose levels in a rat oral study and resulting full scale histopathology. Similarly, the U.S. EPA ToxRefDB “data usability score” classifies studies with respect to the U.S. EPA OPPT (OCSP) guidelines and ToxRefDB inclusion criteria. The guideline-compliant studies are deemed to be either “Acceptable Guideline (post-1998)” (following the GLP regulations) or “Acceptable Guideline (pre-1998)” (pre-dating the GLP regulations). The non-guideline studies are classified as “acceptable non-guideline” (when meeting the ToxRefDB standards) or “unacceptable, deficient evaluation”.

The COSMOS database data model has been described in chapter 1.4. At a very high level, for each toxicity study recorded for a given compound in COSMOS oRepeatToxDB, a particular toxicological effect occurring at a particular dose level and at a particular site is represented precisely. The study design captures all the details for species, sex, routes of exposure, dose groups (levels and number of animals), control group information and references. The effects are described by sets of controlled vocabulary and qualified by the time of the findings, severity, statistical significance, and treatment-relatedness. The sites are differentiated for organ (system), tissue (segment) and cells (organelles).

In order to represent the study data at this detailed level, the set of MINIMUM Study inclusion criteria (COSMOS MINIS) have been established for subchronic, chronic, reproductive, and developmental toxicity studies. Generally (Table 6.2), the requirements for the study parameters included specification of the following elements: study duration, animal species, route of exposure, control group, dose groups, dosage regimen, clinical signs, water/food consumption, hematology, clinical chemistry, urinalysis, organ weight, general necropsy/macro pathology and histopathology. Most of the study parameters have been represented by the controlled vocabularies. With regard to the COSMOS MINIS criteria, the COSMOS MINIS grade can be calculated algorithmically at the time of entering data into the database (Table 6.3).

Table 6.2
General overview of the study inclusion criteria established for the COSMOS oRepeatToxDB

Parameters	The Study Inclusion Criteria
Study type	Subchronic, chronic, carcinogenicity (non-neoplastic effects only), reproductive, developmental, neurotoxicity, immunotoxicity
Species	Rat and mouse (all studies); monkey and dog (all studies); rabbit (reproductive/developmental studies)
Duration	Greater than or equal to 28 days for subacute and subchronic studies; The requirement of “duration days” was not applied for reproductive, developmental or multi-generation studies
Route of exposure	Oral, including dietary, drinking water, gavage (or intubation)
Dose levels and range	Repeated dosage (the studies with single dose were not included). All studies with dose level and regimen information are included. At least one control group was required
Effects	All effects were recorded according to controlled vocabulary
Reference	Regulatory submissions, study reports, published literature (traceable citations)

Table 6.3
The COSMOS MINIS grade specifications

COSMOS MINIS Grade	Description
A	Study meets the current standards of the guidelines: OECD, U.S. FDA, or U.S. EPA and the information for all the required COSMOS MINIS criteria is provided
B	Study is non-guideline, but meets all COSMOS MINIS criteria
F	Study does not meet COSMOS MINIS Criteria
S	Assigned when the study data have been harvested from a summarised memo or opinions. Although the data source claims the guideline- or GLP-compliance, only summary data are available, which may lack detailed information

6.2. The aims of chapter 6

The aims of the present chapter realised in collaboration with the COSMOS consortium partners (please refer to Annex 1) are related to the objective 5 of the current PhD program (section 1.5) and include:

- Harvesting new oral repeated dose toxicity data for cosmetics and related compounds from the regulatory and literature sources according to the predefined SOP;
- Conducting the QC/QA process of the constructed COSMOS oRepeatToxDB.

6.3. Materials and methods

6.3.1. Harvesting new oral repeated dose toxicity data for cosmetics-related compounds

The oral repeated dose toxicity data for over 150 cosmetics-related chemicals were harvested by Altamira LLC, LJMU and the U.S. FDA (please refer also to Annex 1). The following data sources were used:

- The opinions of the EC SCCS, providing the safety assessments of substances used in cosmetics and consumer products. For many cosmetics and related compounds (e.g. hair dyes), SCCS is the only publicly available source of the repeated-dose toxicity information;
- The ECHA Registered Substances database, including the data used in a regulatory setting;
- Other relevant data sources: When the SCCS opinions or ECHA database referenced U.S. National Toxicology Program (NTP) studies or published literature as the data origin, the full level of detailed toxicity information was captured from the original sources. In cases where unpublished and not available study reports were used by ECHA or SCCS, the information presented in the Registered Substances database or in the opinion has been used.

6.3.2. Data entry tool and data entry process

The data harvesting procedure was performed with respect to the established COSMOS MINIS criteria, representing the highly detailed data model of the COSMOS oRepeatToxDB (please refer to section 6.1.3). Only those studies fully compliant with GLP or Guidelines (or ECHA studies with Klimisch score of 2) and with the COSMOS MINIS criteria have been harvested (the “unacceptable” ones were not considered).

Data entry was performed by adapting the U.S. EPA ToxRefDB forms to the COSMOS oRepeatToxDB needs. The data entry tool was delivered to each of the harvesters as a Microsoft Access (MDB) file, along with an additional XLS file for the treatment group

information upload. The data harvesting was conducted according to the Standard Operating Procedure document, prepared by Altamira LLC and a toxicologist from the U.S. FDA CFSAN. Prior to the actual data entry, the following steps were conducted for each harvested compound:

- Identification of the most recent data source providing the safety assessment for a query compound (e.g. SCCS opinion);
- Identification of the NO(A)EL value used to calculate the safety assessment metric (e.g. the Margin of Safety (MoS) from the SCCS opinion) (please refer to chapter 6.1.1);
- Identification of the original toxicity study with oral repeated dosage regimen (“critical study”), from which the NO(A)EL value (used for the safety assessment) was derived.

Subsequently, the relevant information was entered *via* the data entry tool. The data entry procedure is presented in Figures 6.1-6.2, using the example compound, a hair dye, HC Yellow No. 10 (CMS-43601, CAS RN: 109023-83-8), for which the SCCS opinion has been harvested (SCCP, 2007).

In general, several categories of toxicological effects have been recorded (with respect to the harvested study type) using the available controlled vocabulary lists to specify the effects-related details and corresponding target sites (please refer to the Study Effect List in Figure 6.2). The harvested studies were mostly for subchronic and chronic toxicity. However, several reproductive and/or developmental studies were recorded as well. The recorded effect types include:

- **In-Life Observations:** Observations recorded at the beginning of the treatment, during the treatment period and at the end of the treatment, before necropsy. They included clinical signs (any signs of changes in behaviour, locomotion, appearance, salivation, lacrimation, etc., that are associated with the treatment), mortality (associated with the compound), body weight/body weight gain, food consumption/efficacy, water consumption.
- **Pathology (Clinical):** Blood and urine test results, corresponding to clinical chemistry (biochemical parameters evaluated from the blood tests, e.g. enzyme activities, lipid

profile, electrolyte levels, etc.), haematology (all the blood parameters) and urinalysis (all urine tests and parameters).

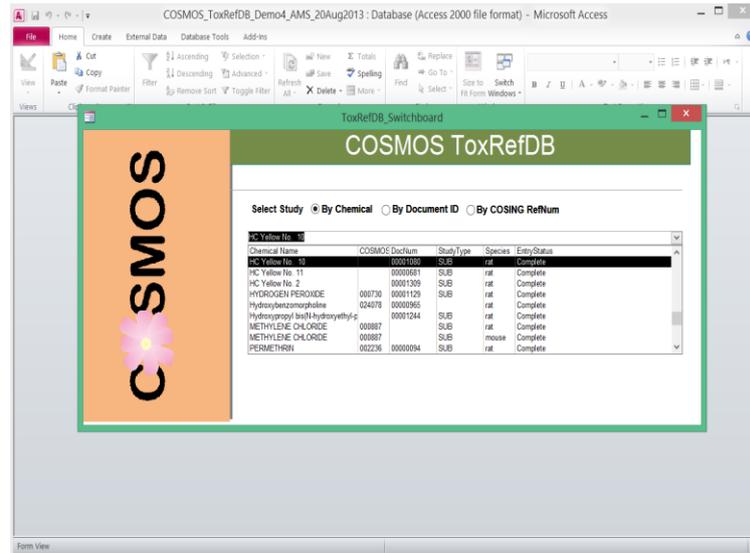
- **Pathology (Gross):** Gross examination of all organs after the treatment period referring to the changes in their appearance e.g. discoloration, pigmentation, enlargement, etc.
- **Organ Weight:** Post-treatment observations including absolute organ weight and relative (to the body weight and/or to the brain weight) organ weight.
- **Pathology (Non-neoplastic):** Post-treatment observations corresponding to the plethora of detailed histopathological changes observed in different organs. Each histopathological effect was recorded for particular target organ (e.g. liver), and further specified target sites (e.g. centrilobular hepatocytes).

For reproductive/developmental toxicity studies, the effect types refer to:

- **Maternal toxicity**, including in-life observations and necropsy findings (gross pathology, organ weights (uterine weight, especially), non-neoplastic pathology).
- **Reproductive toxicity**, including the information on the oestrus cycle (number of *corpora lutea*), reproductive performance (number of implantations, pre- and post-implantation losses, resorptions, birth index, gestation index, etc.), offspring survival (number of live and dead fetuses; litter size, litter viability), reproductive outcome (sex ratio), foetal/litter weight.
- **Developmental toxicity**, including skeletal malformations or alterations.

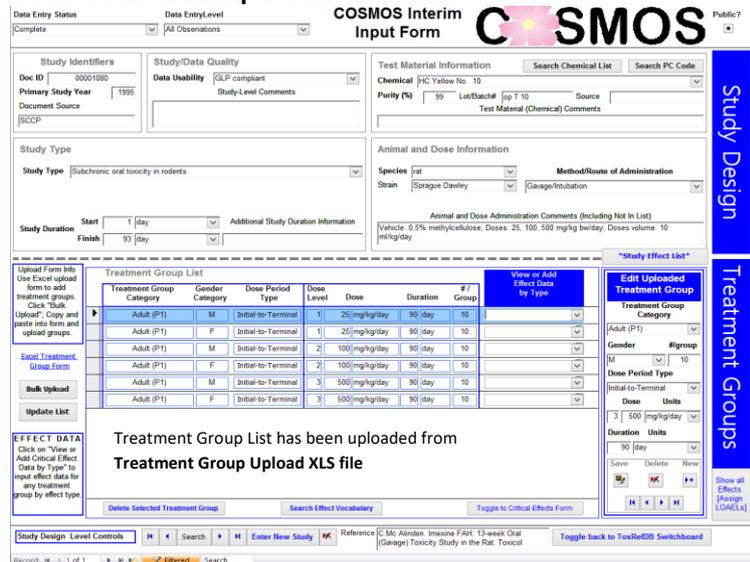
Newly harvested data underwent a manual curation process (conducted by Altamira LLC), prior to being ported into the COSMOS oRepeatToxDB.

Main screen of the COSMOS ToxRefDB



Selecting the harvested compound from the drop-down pick list.

COSMOS Interim Input Form



Entering the information on:

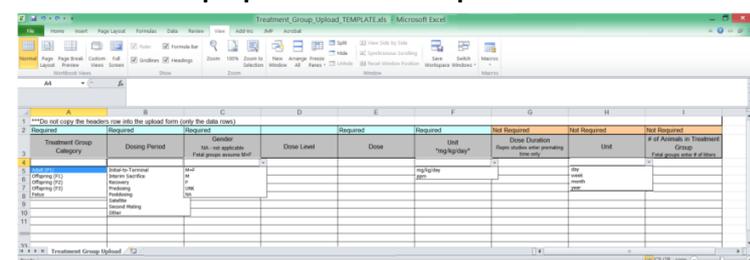
Data entry status (e.g.: complete) and **level** (e.g.: all observations or summary);

Harvested document (number, source, included references, etc.);

Study design: year; study/data quality; study type; study duration; test animals (species, strain); dosage regimen (route of administration, etc.);

Test material (purity, batch/lot, etc.).

Treatment Group Upload XLS file template



Filling-in the **Treatment Group Upload template** file using the drop-down pick lists.

Required fields in blue; Non-obligatory ones in orange

Figure 6.1

Screenshots from the COSMOS ToxRefDB data entry tool for oral repeated dose toxicity data harvesting (part 1): Main screen and COSMOS Interim Input Form

6.3.3. The QC/QA of the COSMOS oRepeatToxDB content

During the data harvesting procedure the Klimisch scores and the information on the study compliance with GLP or guidelines were captured from original data sources, i.e. from the EC ECHA database and from the SCCS opinions. The data record reliability was addressed by the COSMOS MINIS grade (please refer to section 6.1.3).

Prior to the public release of the oRepeatToxDB, the COSMOS ToxRefDB entry form tables were queried from the database for review and QC/QA process (Table 6.4). Approximately 2% of the toxicity records included in the COSMOS oRepeatToxDB were selected randomly. In total, 2722 records were checked for correctness/completeness against the original data sources. The outcome was used to calculate the error rates.

Table 6.4

The tables, fields, and total number of records subjected to the QC/QA procedure of the COSMOS oRepeatToxDB. During the QC/QA the number of incorrect records in particular tables have been recorded and used to provide the QA statistics

Table	Fields subjected to the QC/QA procedure	Total QC-ed records
Studies	Test Substance Name; % Purity; % Active; Tested Form; Test Substance Comments; Study Type; Duration; Route Of Exposure; Species; Strain; Animal Weight Or Age Range; Dose Or Conc. Levels; Dosage Regimen; Vehicle; Dose Comments; Study Design Comments; Data Record Reliability [Source]; Document Source; Document Number; Study Source Type; Study Report; Source; Study Report Number; Study Conducted Year; Journal Publication Citation; Study/Article Title; Year (Report/ Citation)	104
Survival	Sex; # Animals; Concentration Or Dose; Dosing Comments; Assay Type; Sites; Effects; Effects Description; Time Of Findings; Statistical Significance; Treatment Relatedness; Effects Severity; Comments	102
Food/Water consumption		238
Body weight		578
Clinical signs		578
Clinical chemistry		204
Haematology		408
Organ weight		204
Gross pathology		0
Pathology-micro		306
Total in QA		

6.4. Results

6.4.1. COSMOS oRepeatToxDB content

The final COSMOS oRepeatToxDB contained 340 *in vivo* oral repeated dose toxicity studies for 228 chemicals: 186 cosmetics-related studies (including 100 hair dyes) and 42 for impurities (originating from the U.S. FDA CFSAN CERES).

As far as the experimental data content are concerned, the COSMOS oRepeatToxDB was profiled for the species and study types (Figure 6.3).

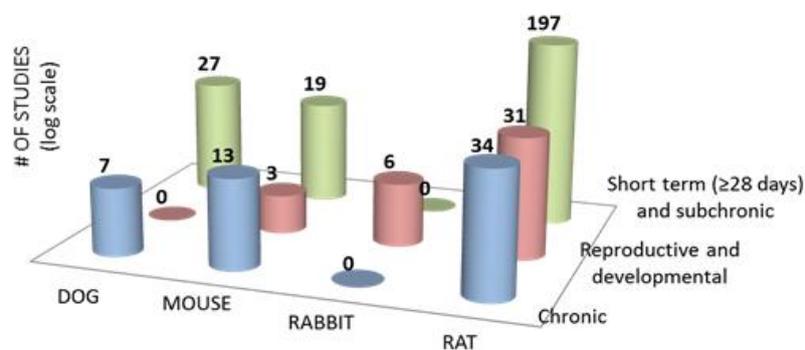


Figure 6.3
Number of studies per species per study type included in COSMOS oRepeatToxDB

The toxicological effects recorded included: clinical parameters (e.g. body weight gain, clinical signs, clinical chemistry, mortality, urinalysis), post-sacrifice measurements (organ weight, necropsy, histopathology) and parental, reproductive and developmental (pre-natal) toxicity. For each effect recorded the “direction” (increase/decrease/no change) and “severity” of finding was specified, as well as “treatment-relatedness”, “dose-dependency”, “statistical significance” and (where applicable) “biological/toxicological relevance”.

As far as the target organ toxicity was concerned, the organ weight, gross pathology and/or histopathology effects were recorded for a total number of 41 organs. The ten most frequently affected (in these three assays) are presented in Figure 6.4.

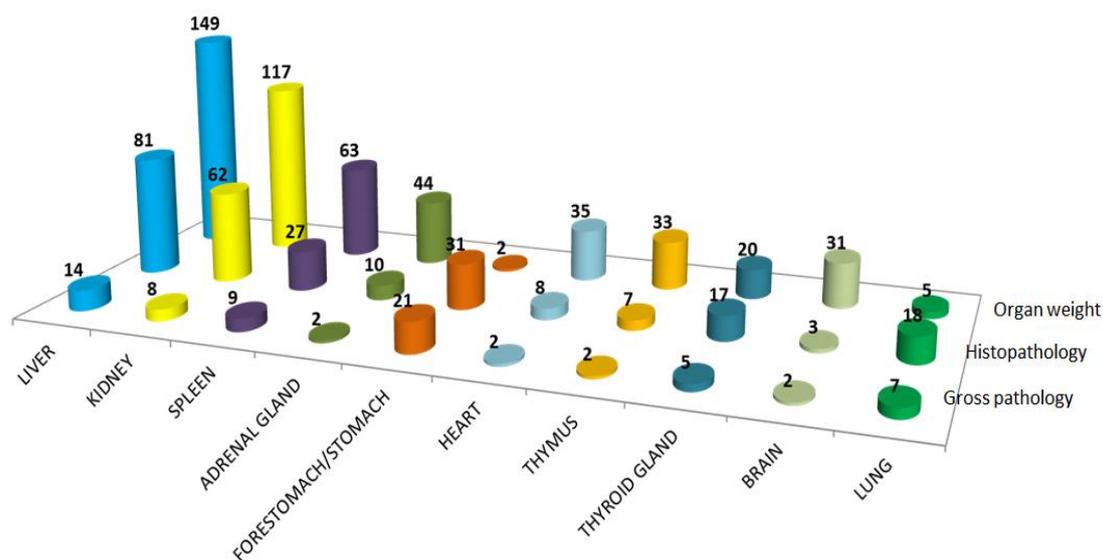


Figure 6.4
The ten most frequently affected target organs in organ weight, gross pathology and histopathology assays

The organ weight observations (including “decrease”, “increase” or “no change” of absolute organ weight, and/or relative to brain or body weight organ weight) were recorded for 18 different organs for the total number of 161 compounds tested in 201 studies. The gross pathological findings include macroscopic lesions and changes in appearance (mostly discoloration and size alterations) of 28 organs observed in 65 studies performed for 64 tested compounds. The histopathological lesions were recorded for 36 target organs and further profiled for the species. It turned out that the most sensitive organs are liver and kidney, whereas the most sensitive species is rat (Figure 6.5).

The histopathological lesions were recorded with respect to the sites hierarchically differentiated to the organs (systems), tissues (segments), and cells (organelles). This way of data recording enabled the further development of ontologies for particular target organs toxicity (this process is discussed in more depth in chapter 7). Overall, the COSMOS oRepeatToxDB includes histopathological data for the total number of 118 “organ (system)-tissue (segment)-cell” sites. The example of sites available for the most sensitive target organ (i.e. liver) are presented in Figure 6.6.

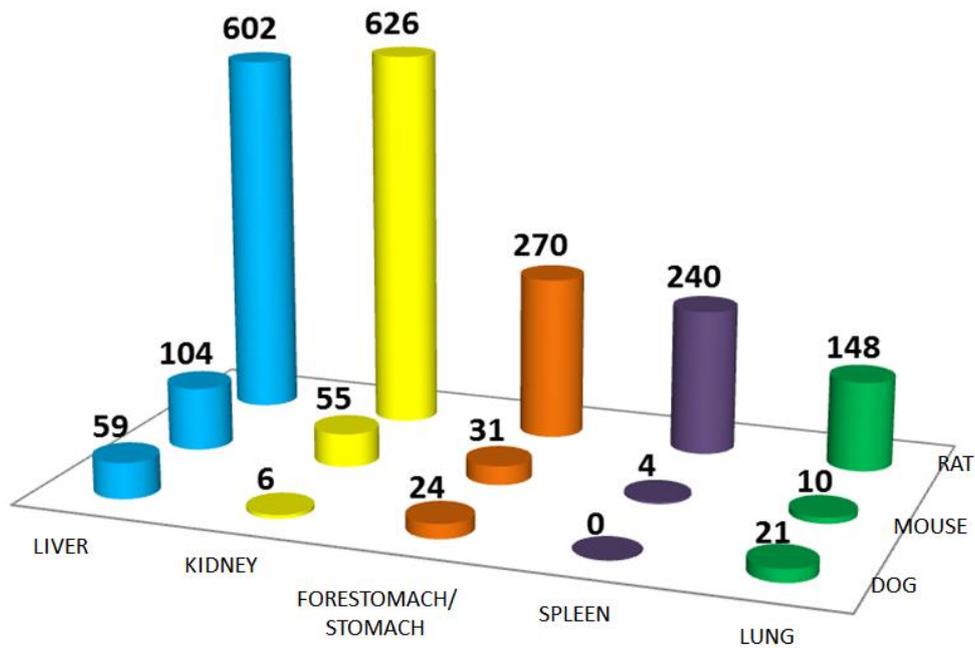


Figure 6.5

The five most sensitive target organs in COSMOS oRepeatToxDB, as indicated by the number of recorded histopathological lesions. They are followed by: thyroid, skeletal muscle, lymph node, bone marrow, heart, adrenal, pancreas and large intestine (not presented here). COSMOS oRepeatToxDB includes histopathological effects for the total number of 36 target organs

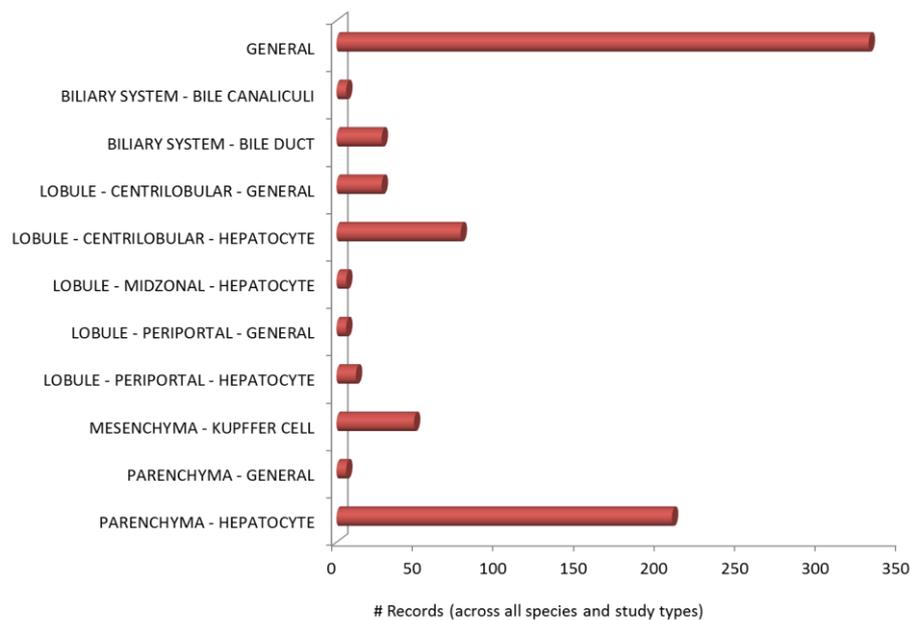


Figure 6.6

The examples of hierarchical “organ (system)-tissue (segment)-cell” sites available for the liver in oRepeatToxDB and corresponding total numbers of records with toxicological effects (across all species, study types and dose levels)

6.4.2. The QC/QA of COSMOS oRepeatToxDB content

The results of the QA on the COSMOS oRepeatToxDB indicated 0.57% erroneous records (e.g. mistake in animal counts or incorrectly inserted effects), and 5.2% missing records (e.g. effect descriptions).

6.5. Discussion

This chapter outlines the current requirements for publicly available, cosmetics-enriched, repeated dose toxicity database. In order to fulfil this need, the new oral repeated dose toxicity data were harvested from a range of scientific literature and regulatory sources (including SCCS opinions), and incorporated into novel COSMOS oRepeatToxDB. The high quality of the incorporated data was assured by consistent application of the established MINimum Study inclusion criteria (COSMOS MINIS) during the data collation, and rigorous procedure for the data curation and quality control.

The COSMOS oRepeatToxDB includes 340 toxicity studies for 228 chemicals (186 cosmetics-related, including 100 hair dyes and 42 impurities). This is (by far) the largest publicly available database in which the toxicological effects occurring at particular dose levels and at given sites are precisely represented for each recorded toxicity study. The study design captures all of the details for species, sex, routes of exposure, dose groups (levels and number of animals), control group information, and references. The effects are described by sets of controlled vocabularies and qualified by the time of findings, severity, statistical significance, and treatment-relatedness. The sites are hierarchically differentiated for organs (system), tissues (segment), and cells (organelles). For each relevant numeric endpoint recorded (e.g. LO(A)EL), the detailed information on the critical effects observed is included.

The unique data model of the COSMOS oRepeatToxDB and the level of details of the recorded data enable *in vivo* data mining and development of chemical-toxicological ontologies (discussed more in-depth in chapter 7). COSMOS oRepeatToxDB supports the development of *in silico* tools for predicting target organ toxicity. As revealed by the analysis of the database content, the most sensitive target organs (for which the highest number of toxicological effects was recorded) include liver and kidney, which indicates that future

research will be focused on these areas. The COSMOS oRepeatToxDB provided the foundation for the analysis described in chapter 7.

Chapter 7

Mechanistic, Ontology-based Liver Toxicity Data Mining in COSMOS oRepeatToxDB

7.1. Background

The importance of mechanistic (mode-of-action-based) data mining in predictive toxicology was discussed in-depth in chapter 1.2. The fundamental significance of mature chemical-toxicological ontologies for the successful application of data mining has been also outlined (chapter 1.3 and chapter 6). Briefly, the ontologies identifying the toxic effects observed at particular organism levels (organs, tissues, or cells) through precisely categorised terms can provide the rationale for the MoA-based toxicity prediction.

In general, chemicals exhibiting systemic toxicity do not cause a similar degree of effects in all organs, but elicit the major toxicity in one or two of them, which are termed “the target organ(s) for toxicity” (UNL, 2002). The analysis of the COSMOS oRepeatToxDB content presented in chapter 6.4.1 identified the liver as the most sensitive target organ for which the highest number of histopathological lesions has been recorded. The liver, as the largest internal organ and gland in the human body, is highly exposed to a range of potentially toxic substances entering an organism. Therefore, with regard to the prediction of repeated-dose toxicity, hepatotoxicity and the mode(s) of action of its potential chemical inducers are of particular interest.

7.1.1. Overview of the structure and functions of the liver

The liver is located in the right upper quadrant of the abdominal cavity, right of the stomach and overlying the gallbladder. Gross anatomy divides it into the two lobes, left and right, separated by the falciform ligament and visible from the parietal surface. The two additional lobes, the caudate lobe and the quadrate lobe, are visible between the right and left lobes from the visceral surface (Abdel-Misih & Bloomston, 2010).

The liver is a metabolically active organ associated with multiple functions. It regulates the levels of most chemicals in the blood and produces and excretes bile. Physiological functions of the liver cells (hepatocytes) also include synthesis and storage of protein, synthesis of cholesterol and phospholipids, transformation of carbohydrates,

storage of glycogen and detoxification. Hepatocytes are the primary target of many liver injuries, including excessive fat accumulation and drug induced liver damage. In order to understand the hepatotoxic effects, it is necessary to understand the distinctive features of hepatic vascular and biliary systems and their associations with liver cells' architecture (Bowen, 2003; Abdel-Misih & Bloomston, 2010; Krishna, 2013).

The liver vascular system is based on a dual blood supply: hepatic artery providing arterial oxygenated blood (approx. 25% of liver blood supply), and portal vein, directing the nutrient-rich venous blood from the stomach, small intestine, pancreas and spleen (75% of liver blood supply). Terminal branches of the hepatic artery and portal vein mix and drain as they enter liver sinusoids, i.e. vascular capillaries which are lined with endothelial cells separating the hepatocytes from the blood flowing through the sinusoids. Sinusoidal endothelial cells play an important role in hepatic microcirculation. The space between hepatocytes and sinusoidal endothelial cells contains hepatic stellate (Ito) cells, playing an essential role in liver regeneration. Stellate cells secrete growth factors and the main constituent materials of the matrix (e.g. collagen and reticulin) to replace the damaged hepatocytes and form scar tissue. The sinusoidal lumen contains liver-specific macrophages (Kupffer cell), which remove debris or aged/damaged erythrocytes in the blood flow and stimulate the immune system through the secretion of numerous factors and cytokines. The blood from the sinusoids reaches the central vein of each hepatic lobule (structural unit of the liver, please see below). Central veins fuse into hepatic veins, which leave the liver and drain into the *vena cava* (Bowen, 2003).

The biliary system (biliary tree) of the liver consists of canaliculi (intercellular spaces between adjacent hepatocytes surrounding the sinusoids) and ducts which transfer the bile from the liver into the small intestine. Hepatocytes secrete bile into the canaliculi. The bile flows parallel to the sinusoids, but in the opposite direction to the blood flow. Canaliculi coalesce into bile ducts lined with epithelial cells, ultimately draining into the common hepatic duct. It fuses with the cystic duct from the gallbladder and forms the common bile duct, running to the duodenum, the first section of the small intestine. The distinctive arrangement of the bile duct, hepatic artery and portal venule in the liver is called a portal triad (Bowen, 2003).

The liver is covered with a connective tissue (*septae*), dividing the parenchyma into very small, structural units – lobules. The hepatic lobule consists of hexagonal plates of hepatocytes with a central vein (hepatic venule) located in the middle and portal triads located peripherally. The lobule is divided into the following parts: centrilobular, midzonal, and periportal. The functional unit of the liver is hepatic acinus, roughly oval mass of hepatocytes around the hepatic arterioles and portal venules. The hepatic acinus is divided into the three zones reflecting the distance from the arterial blood supply. Zone 1 (corresponding to the periportal part of the hepatic lobule; surrounding the portal tract and located closest to the arterioles) consists of the best oxygenated hepatocytes. Acinar zone 2 (corresponding to the midzonal part of the hepatic lobule) and zone 3 (corresponding to the centrilobular part of the hepatic lobule; surrounding the hepatic venule) contain the poorer oxygenated hepatocytes, as blood from the portal tract flows through the acinar zones to the venule being deoxygenated (Bowen, 2003; Abdel-Misih & Bloomston, 2010; Krishna, 2013).

The toxic effect of many drugs on the liver has been linked to the circulatory dysfunction (Lautt, 2009), and the lesions caused by a range of pathologic processes are frequently reflected in the acinar structure (Bowen, 2003).

7.1.2. Toxicity categories of liver injury: steatosis, steatohepatitis and fibrosis

Liver steatosis, i.e. excessive accumulation of fatty acids in hepatocytes due to the impairment of the process of synthesis and elimination of triglycerides, may be a cause of a chronic liver injury. The pathological changes associated with steatosis include formation of lipid droplets in the cytoplasm of hepatocytes, lipid vacuolisation, hepatomegaly (hypertrophy, enlargement of the liver) and lipid deposition (Vitcheva et al., 2013).

When associated with inflammation, the fatty liver progresses to steatohepatitis. The pathological changes characterising steatohepatitis mostly involve the acinar zone 3 and include hepatocellular injury and inflammation in the lobular parenchyma. Hepatocellular injury is manifested by ballooning degeneration of hepatocytes, associated with formation of Mallory's hyalines (clumps of damaged material in the hepatocyte cytoplasm), apoptosis/necrosis, associated with increased hepatocellular expression of the membrane receptor Fas and megamitochondria in hepatocytes. The cell necrosis evokes an inflammatory reaction morphologically manifested by the appearance of inflammatory cells

(neutrophils, lymphocytes, macrophages), oedema and congestion around parenchymal cells (Vitcheva et al., 2013).

The chronic inflammation leads to liver fibrosis, associated with tissue destruction and simultaneous repair processes, resulting in a lack of balance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition. It can be characterised by thickening of the liver capsule (due to the excessive accumulation of ECM proteins, including collagen) and hyperplasia (due to the increased nuclear diameter and volume and cytoplasmic volume of the hepatocytes) (Bataller & Brenner, 2005; Vitcheva et al., 2013, Landesmann, 2016). The Adverse Outcome Pathway (Figure 7.1) for liver fibrosis has been recently proposed (Landesmann, 2016).

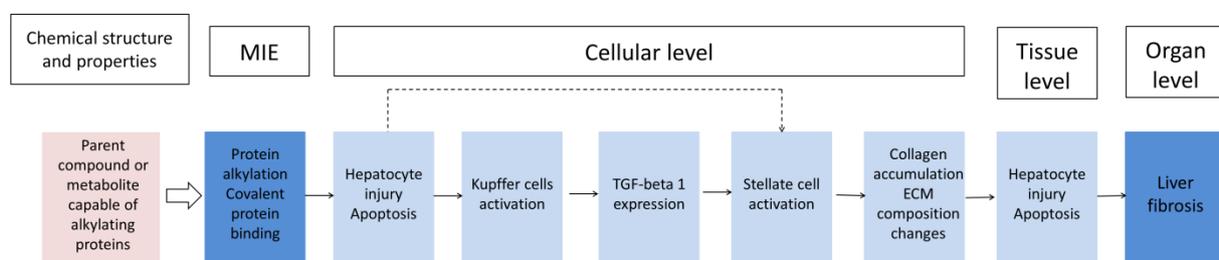


Figure 7.1

Schematic representation of the Adverse Outcome Pathway for liver fibrosis (from Landesmann, 2016). The Molecular Initiating Event (MIE) of this AOP is protein alkylation, leading to the following Key Events (KEs):

- Cell injury and death;
- Activation of Kupffer cells, due to engulfment of apoptotic bodies, i.e. fragmented DNA from apoptotic hepatocytes;
- Expression of fibrogenic cytokines, Transforming Growth Factor- β (TGF- β 1);
- Activation of hepatic stellate cells (HSC), which change from vitamin A-rich phenotype to a myofibroblastic phenotype, exhibiting fibrogenic properties (secretion of pro-inflammatory cytokines and chemokines, synthesis of matrix proteins and of inhibitors of matrix degeneration);
- Progressive collagen accumulation and changes in Extracellular Matrix (ECM) composition.

The excessive accumulation of ECM proteins progressively affects the whole organ and alters its normal functioning, which corresponds to liver fibrosis, the adverse outcome (AO). The scheme does not include two Key Events, namely inflammation and oxidative stress, which are considered to be present throughout the entire AOP

Liver fibrosis occurring in the majority of types of chronic liver diseases results in derangement of the liver architecture, portal hypertension and may produce irreversible circulation changes leading to cirrhosis and, eventually, liver failure and death.

7.2. The aims of chapter 7

The aims of the present chapter realised in collaboration with the COSMOS consortium partners (please refer to Annex 1) are related to the objective 6 of the current PhD program (section 1.5), and include:

- Validation of the liver toxicity ontology developed on the basis of the data collated at the stage of the construction of COSMOS oRepeatToxDB;
- Ontology-based liver toxicity (steatosis) data mining of COSMOS oRepeatToxDB;
- Structural (ToxPrint chemotype) analysis of the chemical compounds obtained as a result of data mining and identification of the structural fragments associated with the endpoint investigated;
- Formulating the mechanistic reasoning for the selected compounds on the basis of the literature search and results of molecular modelling analysis delivered by collaborating COSMOS consortium partners from BAS and S-IN.

7.3. Materials and methods

7.3.1. The development and validation of target organ toxicity ontologies

As mentioned in chapter 6.1.2, mature chemical-toxicological ontologies for target organ toxicity are lacking in the existing databases. The currently available resources are limited to the taxonomies, e.g. OpenTox (An Open Source Predictive Toxicology Framework, available at: <http://www.opentox.net/>) project dictionaries, describing the relations between chemical and toxicological data and experiments, or the taxonomy of biologically relevant chemical entities and their activities proposed in the ChEBI dictionary (Smith et al., 2007; Hardy et al., 2012).

As discussed in chapter 6, observed toxicity effects have been recorded in COSMOS oRepeatToxDB with respect to the corresponding target sites structured hierarchically (as organs/tissues/cells). Thus, it was possible to link the phenotypic effects occurring at very high (whole organism) level, with the pathological changes observed at various lower levels: organs, tissues and finally cells. The conceptual scheme representing this hierarchy is shown in Figure 7.2 (Vitcheva et al., 2013).

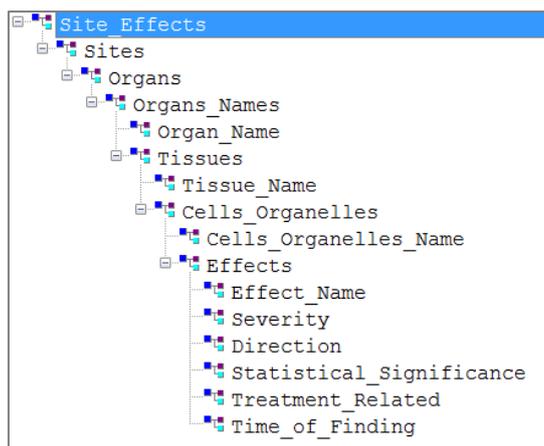


Figure 7.2

Conceptual scheme of the COSMOS oRepeatToxDB hierarchical ontology, enabling mapping of the observed pathological changes with high-level phenotypic effects (Vitcheva et al., 2013)

Within this concept, the cell, in which the majority of the biological and chemical processes occur, can be regarded as the bottom-level intersection where biology, chemistry and toxicity meet. It is in the information for the cell that interactions can be investigated by accounting for the proteins and genes involved on one hand and the chemical and biological roles of the chemical compounds (determined by their physicochemical, electronic, etc., properties) on the other. In order to enable linkage of the biological effects with chemicals involved in toxicity pathways, such ontologies (along with related controlled vocabularies) have been designed within COSMOS oRepeatToxDB.

The dynamic and iterative process of the development of ontologies actually began at the data harvesting stage, when the detailed domains and scopes of the ontologies were defined. The simultaneous inspection and mining of the data collated has led to the formulation of the initial ontologies. Subsequently, a range of iterative steps were performed, including:

- Further data mining;
- Consultations with trained toxicologists;
- Enumeration of the important terms in the ontologies;
- Re-defining the classes and hierarchies;
- Validation and updating of the ontologies.

The focus of the present chapter was placed on the validation of the ontology developed for liver toxicity. With respect to the molecular mechanisms involved in the

development of chronic liver disease (steatosis, steatohepatitis and fibrosis, discussed in section 7.1.2), the formulation of the reasoning can be presented schematically as shown in Figure 7.3 (Vitcheva et al., 2013; Mostrag-Szlichtyng et al., 2014). The liver toxicity ontology developed may be perceived as the computer science representation of this concept.

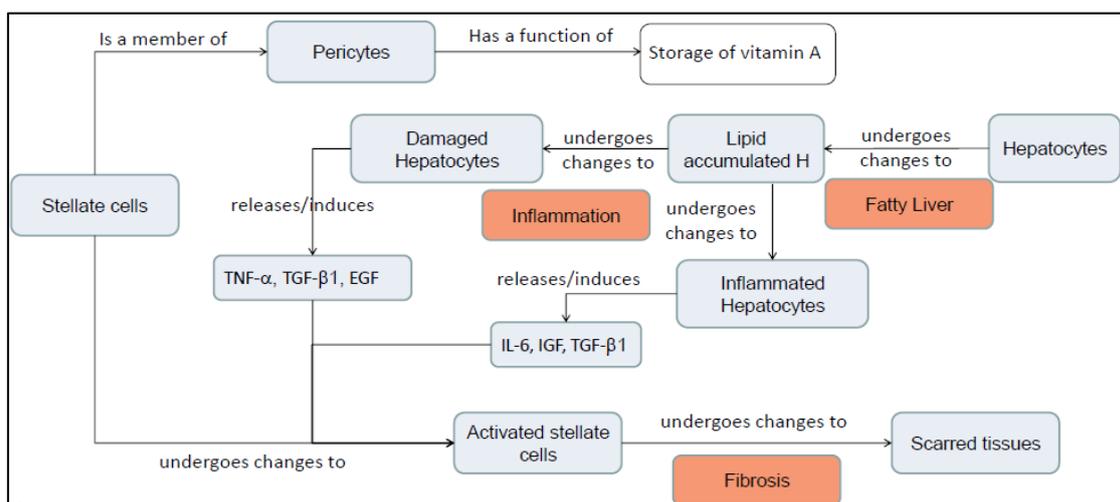


Figure 7.3
The molecular mechanisms involved in the development of chronic liver disease (Vitcheva et al., 2013)

7.3.2. Ontology-based liver toxicity data mining of the COSMOS oRepeatToxDB

The final COSMOS oRepeatToxDB was queried for compounds associated with liver toxicity. The extracted dataset was subjected to ontology-based data mining in order to identify the chemicals associated with the liver steatosis/steatohepatitis/fibrosis endpoints.

7.3.3. Structural analysis

The chemicals identified which were associated with the endpoints investigated were subjected to the chemotype analysis. The ToxPrint chemotypes library (www.toxprint.org) and the ChemoTyper software tool (www.chemotyper.org) were utilised (this methodology was described in-depth in chapter 3).

7.3.4. Mechanistic reasoning formulation

For the compounds selected the mechanism of toxic action was proposed on the basis of a literature search (NIH PubMed, <http://www.ncbi.nlm.nih.gov/pubmed>) and the

results of a molecular modelling analysis delivered by collaborating COSMOS consortium partners from BAS and S-IN.

7.4. Results

7.4.1. Ontology-based liver toxicity data mining

The ontology developed for liver toxicity covers a range of hierarchical sites and over 30 effects/pathological changes, giving the total number of 53 unique “site-effect” pairs (Table 7.1).

Querying COSMOS oRepeatToxDB for all histopathological effects recorded for the liver resulted in the retrieval of a total of 70 compounds with such effects. The terms related to liver steatosis/steatohepatitis/fibrosis were used to extract the final set of compounds of interest (please refer to section 7.1.2 for the description of the associated pathological changes). This analysis led to the identification of 13 relevant combinations of “tissue-cell” sites and effects (Figure 7.4) covered by 59 chemicals (Table 7.2) (Mostrag-Szlichtyng et al., 2014).

As an example of the type of compound identified, for allyl hexanoate (CMS-13529, compound #17 in Table 7.2), oRepeatToxDB contains information on a subchronic study in rat with the observed effect “fibrosis” and associated site “liver – general”, for tetrakis(hydroxymethyl)phosphonium sulfate (CMS-1349, compound #8 in Table 7.2) “cytoplasmic vacuolisation” has been recorded for “lobule – periportal – hepatocyte” in subchronic studies in rat, etc.

Table 7.1

The “liver-tissue-cell” sites with corresponding effects covered by the ontology in the COSMOS oRepeatToxDB (Vitcheva et al., 2013)

TISSUE	CELLS/ ORGANELLES	EFFECT NAMES
LIVER GENERAL		Congestion; Cytological Alterations; Fatty Change; Fibrosis; Firm Areas; Focal Cellular Change; Granuloma; Haematopoiesis; Hepatitis; Hypertrophy; Inflammation; Lesions (Nos); Lipid Deposition; Necrosis; Pigmentation; Proliferation
BILIARY SYSTEM – BILE CANALICULI		Pigmentation
BILIARY SYSTEM - BILE DUCT		Hyperplasia; Proliferation; Vacuolisation
ACINUS – PERIACINAR ZONE	ACINAR CELLS	Fibrosis; Hypertrophy; Necrosis
	CYTOPLASM	Vacuolisation
LOBULE – CENTRIOLOBULAR	HEPATOCYTES	Cytoplasmic Alteration; Distention; Enlargement; Glycogen Loss; Hypertrophy; Necrosis; Vacuolisation
LOBULE – MIDZONAL	HEPATOCYTES	Hypertrophy; Hyperplasia
LOBULE – PERIPORTAL	HEPATOCYTES	Cellular Infiltration; Hypertrophy
MESENCHYMA	STELLATE CELLS	Hypertrophy; Lipid Accumulation
	KUPFFER CELLS	Distention; Hyperplasia; Discoloration; Pigmentation
	CYTOPLASM	Vacuolisation
PARENCHYMA	HEPATOCYTES	Cellular Infiltration; Degeneration; Fatty Changes; Hypertrophy; Inflammation; Necrosis; Pigmentation; Proliferation; Vacuolisation

Table 7.2

The 59 compounds for which the histopathological changes related to the liver steatosis/steatohepatitis/fibrosis have been found in COSMOS oRepeatToxDB (Mostrag-Szlichtyng et al., 2014)

#	CMS ID	CAS RN	PREFERRED NAME	#	CMS ID	CAS RN	PREFERRED NAME
1	365	108-94-1	CYCLOHEXANONE	16	11422	106990-43-6	N,N''-1,2-ETHANEDIYLBIS[N-[3-[[4,6-BIS[BUTYL(1,2,2,6,6-PENTAMETHYL-4-PIPERIDINYL)AMINO]-1,3,5-TRIAZIN-2-YL]AMINO]PROPYL]-N',N''-DIBUTYL-N',N''-BIS(1,2,2,6,6-PENTAMETHYL-4-PIPERIDINYL)-1,3,5-TRIAZINE-2,4,6-DIAMINE
2	609	107-21-1	ETHYLENE GLYCOL	17	13529	123-68-2	ALLYL HEXANOATE
3	618	104-76-7	2-ETHYL-1-HEXANOL	18	13782	70624-18-9	POLY(CYANURIC CHLORIDE-CO-TERT-OCTYLAMINE-CO-1,6-BIS(2,2,6,6-TETRAMETHYL-4-PIPERIDYLAMINO)HEXANE)
4	663	98-01-1	FURFURAL	19	14793	7128-64-5	2,2'-(2,5-THIOPHENEDIYL)-BIS (5-TERT-BUTYLBENZOXAZOLE)
5	780	67-63-0	ISOPROPYL ALCOHOL	20	15009	135861-56-2	DIMETHYLDIBENZYLIDENE SORBITOL
6	887	75-09-2	METHYLENE CHLORIDE	21	15014	134701-20-5	2,4-DIMETHYL-6-(1-METHYLPENTADECYL)PHENOL
7	1178	51-03-6	PIPERONYL BUTOXIDE	22	15070	161717-32-4	PHOSPHOROUS ACID, CYCLIC BUTYLETHYL PROPANEDIOL, 2,4,6-TRI-TERT-BUTYLPHENYL ESTER
8	1349	55566-30-8	TETRAKIS(HYDROXYMETHYL)PHOSPHONIUM SULFATE	23	25576	56216-28-5	3,5-DIAMINO-2,6-DIMETHOXPYRIDINE DIHYDROCHLORIDE
9	2321	131-57-7	OXYBENZONE	24	26324	68391-30-0	C.I. BASIC RED 76
10	3598	108-91-8	CYCLOHEXYLAMINE	25	26325	68391-31-1	C.I. BASIC YELLOW 57
11	3639	111-76-2	ETHYLENE GLYCOL MONOBUTYL ETHER	26	26704	74578-10-2	2,6-XYLIDINE, 4-(2,6-DICHLORO-ALPHA-(4-IMINO-3,5-DIMETHYL-2,5-
12	3681	108-31-6	MALEIC ANHYDRIDE	27	26998	81892-72-0	1,3-BIS-(2,4-DIAMINOPHENOXY)PROPANE
13	3855	96-91-3	PICRAMIC ACID	28	27489	94158-13-1	2,2'-((4-AMINO-3-NITROPHENYL)IMINO)BIETHANOL HYDROCHLORIDE
14	4141	111-77-3	DIETHYLENE GLYCOL MONOMETHYL ETHER	29	27490	94158-14-2	2-(1,3-BENZODIOXOL-5-YLAMINO)ETHANOL HYDROCHLORIDE
15	4291	6197-30-4	OCTOCRYLENE	30	27591	97404-02-9	2-((4-AMINOPHENYL)AZO)-1,3-DIMETHYL-1H-IMIDAZOLIUM CHLORIDE

Continued on the following page

Table 7.2, continued

The 59 compounds for which the histopathological changes related to the liver steatosis/steatohepatitis/fibrosis have been found in COSMOS oRepeatToxDB (Mostrag-Szlichtyng et al., 2014)

#	CMS ID	CAS RN	PREFERRED NAME	#	CMS ID	CAS RN	PREFERRED NAME
31	4464	93-15-2	EUGENYL METHYL ETHER	46	28451	149591-38-8	BISHYDROXYETHYL BISCETYL MALONAMIDE
32	4878	77-90-7	TRIBUTYL ACETYLCITRATE	47	34877	71786-60-2	N,N-BIS(2-HYDROXYETHYL)ALKYL(C12-C18)AMIDE
33	4879	77-99-6	TRIMETHYLOLPROPANE	48	42308	26125-40-6	POLY(P-DICHLOROBENZENE-CO-SODIUM SULFIDE)
34	4997	107-88-0	1,3-BUTYLENE GLYCOL	49	44734	128729-28-2	HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HYDROCHLORIDE
35	5629	16470-24-9	C.I. FLUORESCENT BRIGHTENER 220	50	46977	16867-03-1	2-AMINO-3-HYDROXYPYRIDINE
36	5805	32687-78-8	1,2-BIS(3,5-DI-TERT-BUTYL-4-HYDROXYHYDROCINNAMOYL)HYDRAZINE	51	50942	3248-91-7	C.I. BASIC VIOLET 2
37	5856	52829-07-9	BIS(2,2,6,6-TETRAMETHYL-4-PIPERIDINYL) SEBACATE	52	56289	59820-63-2	3-METHYLAMINO-4-NITROPHENOXYETHANOL
38	7013	2682-20-4	2-METHYL-4-ISOTHIAZOLIN-3-ONE	53	56847	61693-43-4	3-AMINO-2,4-DICHLOROPHENOL HYDROCHLORIDE
39	8572	128-95-0	C.I. DISPERSE VIOLET 1	54	59879	77061-58-6	2-((4-(DIMETHYLAMINO)PHENYL)AZO)-1,3-DIMETHYL-1H-IMIDAZOLIUM CHLORIDE
40	8676	1777-82-8	2,4-DICHLOROBENZYL ALCOHOL	55	60520	82576-75-8	2-((4-AMINO-2-METHYL-5-NITROPHENYL)AMINO)ETHANOL
41	8750	75-85-4	AMYLENE HYDRATE	56	60750	84540-50-1	5-AMINO-6-CHLORO-O-CRESOL
42	9026	846-70-8	EXT. D&C YELLOW NO. 7	57	67443	78301-43-6	POLY(2,2,4,4-TETRAMETHYL-20-(OXIRANYLMETHYL)-7-OXA-3,20-DIAZADISPIRO(5.1.11.2)HENEICOSAN-21-ONE)
43	10280	125643-61-0	ALKYL(C7-9-BRANCHED) 3,5-DI-TERT-BUTYL-4-HYDROXYHYDROCINNAMATE	58	72013	68391-32-2	BASIC BROWN 17
44	10707	4368-56-3	C.I. ACID BLUE 62	59	72054	23920-15-2	HC BLUE NO. 11
45	10786	142-19-8	ALLYL HEPTANOATE				

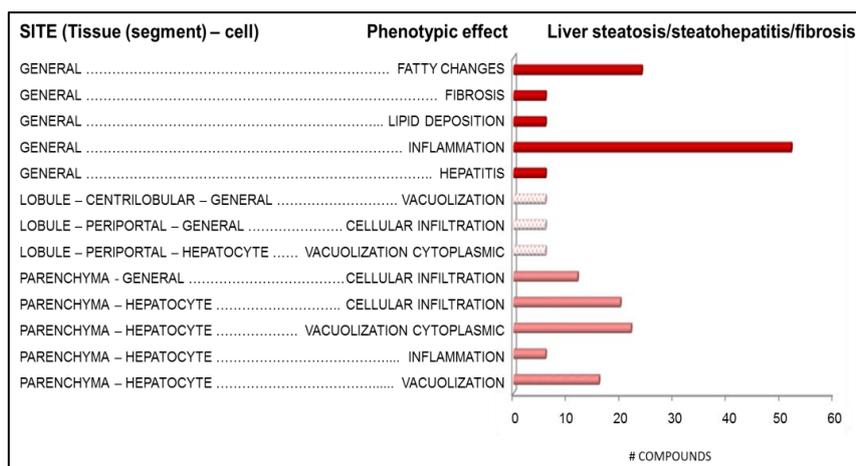


Figure 7.4
Example of liver “site-phenotypic effect” pairs related to the steatosis/steatohepatitis/fibrosis in COSMOS oRepeatToxDB

7.4.2. Structural analysis

The ToxPrint chemotypes analysis of 59 compounds obtained as a result of data mining revealed that the most abundant structural classes included alcohols (aliphatic and aromatic), amines (aromatic and aliphatic), ethanolamines, oxy- alkanes, ethers and compounds with azo- and nitro- aromatic groups (Figure 7.5) (Mostrag-Szlichtyng et al., 2014). The example compounds representing identified distinctive structural features are presented in Table 7.3

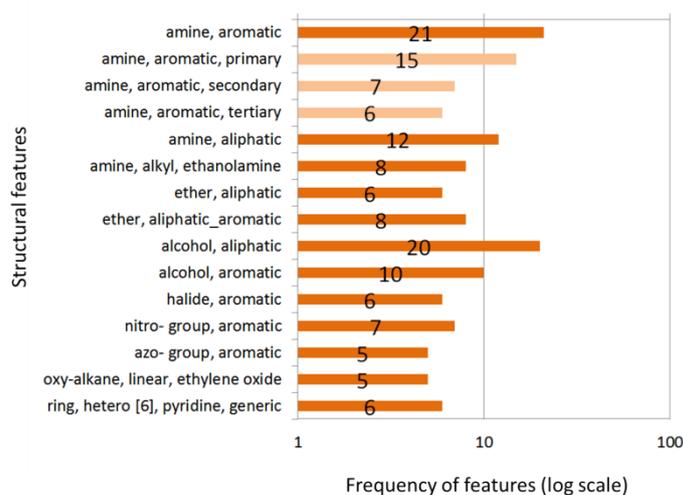
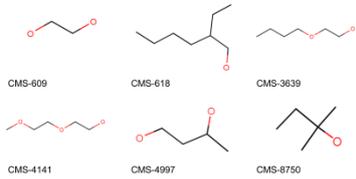
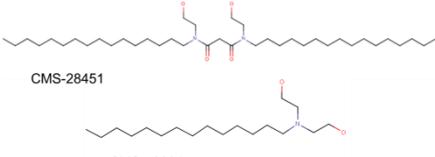
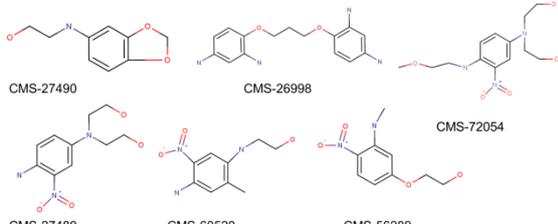
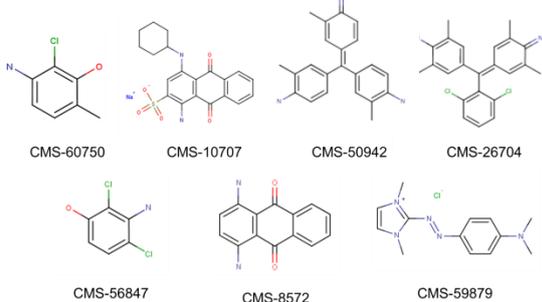
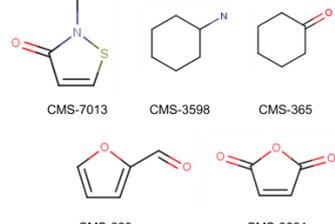
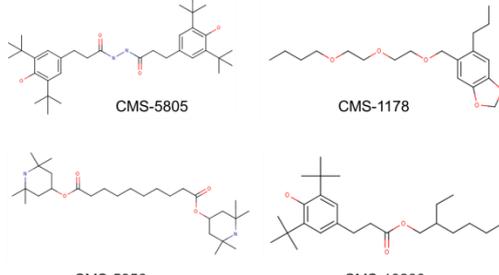


Figure 7.5
The structural profile of 59 compounds associated with liver steatosis/steatohepatitis/fibrosis expressed as the frequency of ToxPrint features (the actual counts were provided on the plot's bars)

Table 7.3

Distinct structural features identified in the set of 59 compounds associated with liver steatosis/steatohepatitis/fibrosis

Structural characteristics	Example compounds
Aliphatic alcohols/glycols, glycol ethers	 <p>CMS-609 CMS-618 CMS-3639 CMS-4141 CMS-4997 CMS-8750</p>
(Di)Ethanolamines, long aliphatic chains	 <p>CMS-28451 CMS-10894</p>
Aromatic nitro- groups, aromatic amines, (di)ethanolamines	 <p>CMS-27490 CMS-26998 CMS-72054 CMS-27489 CMS-60520 CMS-56289</p>
Aromatic halides, compounds with azo- aromatic groups, anthraquinones	 <p>CMS-60750 CMS-10707 CMS-50942 CMS-26704 CMS-56847 CMS-8572 CMS-59879</p>
Furans, thiazoles, cyclohexane derivatives	 <p>CMS-7013 CMS-3598 CMS-365 CMS-663 CMS-3681</p>
Rigid rings/cyclic fragments and long flexible chains	 <p>CMS-5805 CMS-1178 CMS-5856 CMS-10280</p>

7.4.3. Mechanistic reasoning formulation

As outlined in 1.2, mode-of-action-based predictive toxicology approaches support the Adverse Outcome Pathway (AOP) framework (OECD, 2013). In order to facilitate the understanding and formulation of the MoA/AOPs involved in the development of chronic liver disease, ontology based data mining and subsequent structural analysis was combined with the results of molecular modelling (MM), as performed by the collaborating COSMOS partners from BAS and S-IN (Mostrag-Szlichtyng et al., 2014; Al Sharif et al., 2016). The conceptual scheme summarising this approach is shown in Figure 7.6 (Mostrag-Szlichtyng et al., 2014).

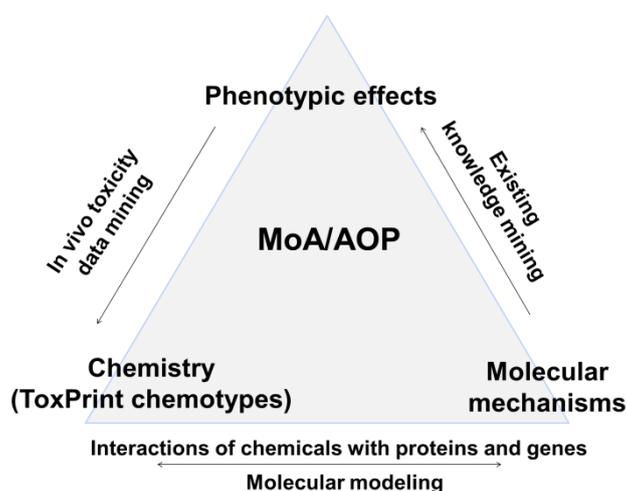


Figure 7.6

Conceptual scheme demonstrating the process of “knowledge discovery from data” (KDD) applied to liver steatosis. The possible associations between phenotypic effects, structural features and molecular mechanisms were identified through *in vivo* data and existing knowledge mining, combined with molecular modelling

The AOP leading to the liver steatosis was recently proposed (Al Sharif et al., 2014; Al Sharif et al., 2016), and the Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) has been suggested as one of the receptors involved in the MIE. PPAR γ is responsible for the regulation of adipogenesis (adipocyte proliferation and differentiation), lipid and glucose homeostasis, inflammatory responses, vascular functions and placental development. The MoA/AOP proposed by Al Sharif and co-workers, leading from tissue-specific ligand-dependent PPAR γ dysregulation to liver steatosis, is shown in Figure 7.7. Within this AOP the MIE induces up-regulation of target genes for lipid transport/binding proteins, fatty acid and triglyceride synthesizing enzymes, and lipid droplet-associated proteins (LD proteins).

This AOP covers the entire cascade of key molecular events and the subsequent cytological and histopathological manifestations of liver steatosis, namely increased number or size of lipid droplets, deposition of triglycerides in hepatic tissue (instead of in adipose one) and hepatomegaly (Al Sharif et al., 2014; Al Sharif et al., 2016).

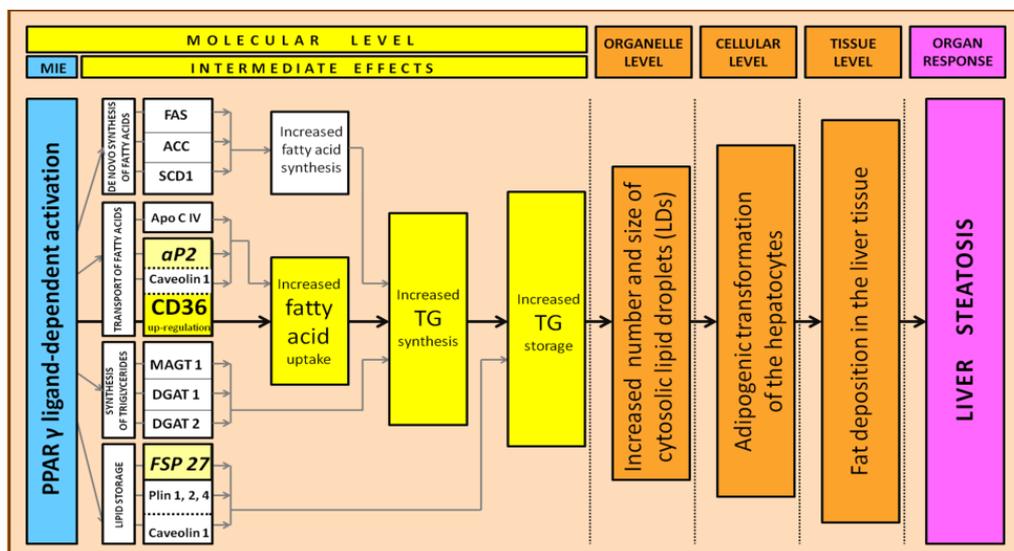


Figure 7.7
Proposed MoA/AOP from tissue-specific ligand-dependent PPAR γ dysregulation to liver steatosis (from Al Sharif et al., 2014)

A pharmacophore model for full PPAR γ agonists (Figure 7.8), indicating that hydrogen bonding and hydrophobic and aromatic structural elements are the most significant aspects, has also been proposed recently, as a result of a molecular modelling procedure (Al Sharif et al., 2016).

The structural analysis revealed that the set of chemicals identified contained potential PPAR γ agonists, i.e. compounds with rigid hydrophobic structural fragments and flexible aliphatic chains (refer to Table 7.3). In order to verify the hypothesis arising from the *in vivo* toxicity data mining/structural analysis, the virtual screening procedure developed by the COSMOS partners (Al Sharif et al., 2016) was applied to the set of 59 compounds with liver steatosis-associated phenotypic effects (Mostrag-Szlichtyng et al., 2014; Al Sharif et al., 2016). This procedure involved docking query structures in the binding pocket of PPAR γ . As a result, CMS-26998 (1,3-bis-(2,4-diaminophenoxy)propane; Compound #27 from Table 7.2) was identified as a partial PPAR γ agonist and CMS-1178 (piperonyl butoxide; Compound #7

from Table 7.2) as a full PPAR γ agonist (Figure 7.9) (Mostrag-Szlichtyng et al., 2014; Al Sharif et al., 2016).

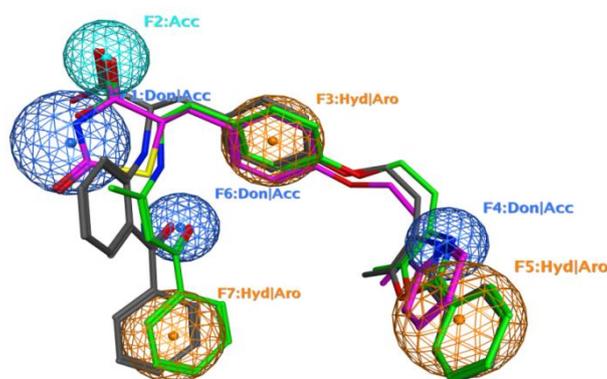


Figure 7.8
Pharmacophore model of full PPAR γ agonists: Rosiglitazone (in magenta), Compound 544 (in green) and Farglitazar (in gray) – extracted from the PDB database (PDB, 2015). Obligatory pharmacophore points (F1 or F2 and F3), and optional pharmacophore points (F4-F7) are related to the hydrogen bonding properties (Don|Acc) and hydrophobicity/aromaticity (Hyd|Aro) (from Al Sharif et al., 2016; Mostrag-Szlichtyng et al., 2014)

CMS-26998 (1,3-bis-(2,4-diaminophenoxy)-propane), in form of the hydrochloride salt, is used as a precursor for hair colouring, reacting with primary intermediates to form the final hair dye. The findings of a subchronic oral (gavage) repeated dose toxicity study in rat, with dose levels of 0, 40, 120 and 360 mg/kg bw/day, are recorded for this compound in COSMOS oRepeatToxDB (original data source: SCCP, 2007a). The SCCS concluded that the effects observed in this study may be indicators for organ toxicity (especially for the kidney). With regard to the liver, several observations related to the test substance were reported for the 120 mg/kg and the 360 mg/kg dose groups, including changes in clinical chemistry parameters (higher albumin, lower total protein levels, increased cholesterol and alkaline phosphatase activity) and higher liver weights in combination with increased incidence of inflammatory cell foci in the liver and intimal proliferation of the large veins. This compound was included in the list of potential steatosis inducers published by Vinken et al. (2012). Further insights regarding its possible mechanism of action are, however, not available in the scientific literature. In the present study, 1,3-bis-(2,4-diaminophenoxy)-propane was identified as a partial PPAR γ agonist, whereas the indications exist that the prosteatotic activity of PPAR γ is triggered specifically by full, and not partial, agonists (Chigurupati et al., 2015; Al Sharif et al., 2016). Further investigation would be, therefore, required.

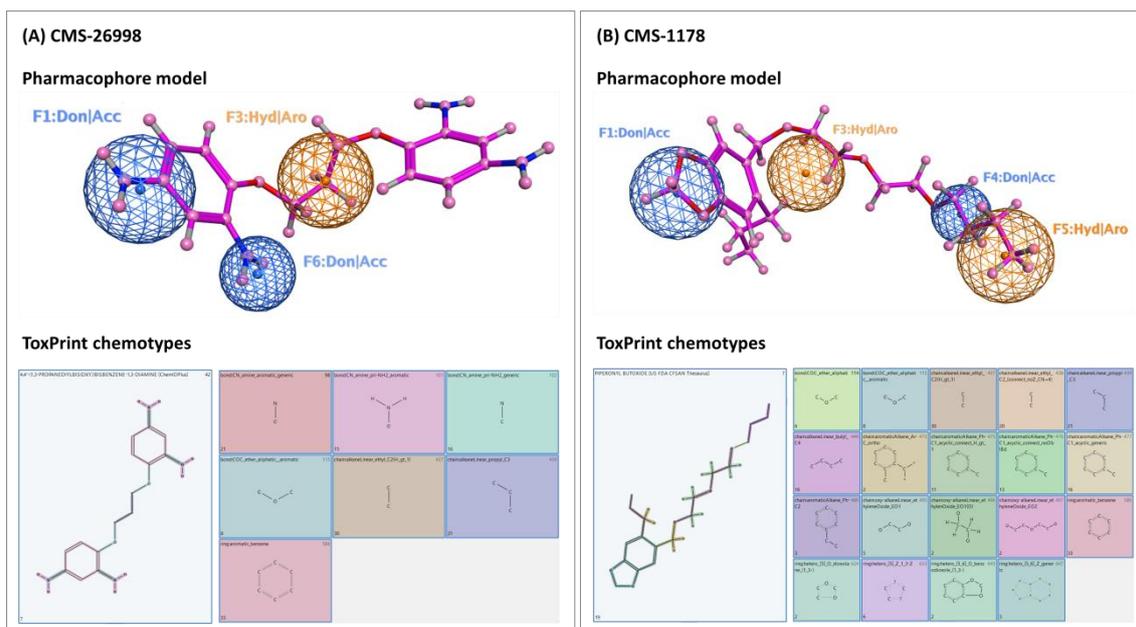


Figure 7.9

The structures, mapping onto the pharmacophore and ToxPrint chemotypes of two compounds identified in the virtual screening procedure involving docking the structures in the binding pocket of PPAR γ (Mostrag-Szlichtyng et al., 2014):

- (A) Partial PPAR γ agonist: CMS-26998 (1,3-bis-(2,4-diaminophenoxy)propane) with two obligatory pharmacophore points: F1 (Don/Acc) and F3 (Hyd/Aro) and one additional pharmacophore point: F6 (Don/Acc);
- (B) Full PPAR γ agonist: CMS-1178 (piperonyl butoxide) with two obligatory pharmacophore points: F1 (Don/Acc) and F3 (Hyd/Aro) and two additional points: F4 (Don/Acc) and F5 (Hyd/Aro)

CMS-1178 (piperonyl butoxide, PBO) is an insecticide synergist co-applied with pyrethrins and pyrethroids-like pesticides. It is used as an ingredient in anti-lice shampoos and is also classified as a skin protectant according to EU COSING use functions. The toxicity of PBO has been investigated in animal studies and liver was identified as the main target organ. Two studies for this compound are available in oRepeatToxDB: a 1-year oral (feed) study in dog (dose levels tested up to 53 mg/kg for males and 71 mg/kg for females), in which mild changes including liver steatosis and enlargement (hepatocyte hypertrophy) were observed, and a 90-day oral (feed) study in mouse (high dose levels tested of 10, 30, 100, 300, 1000 mg/kg bw/day), in which more severe effects, including necrosis and liver cancer, were recorded. The available toxicity studies indicated that severity and type of hepatotoxic effects depend on the duration of exposure to PBO and/or on the dose levels tested.

PBO was predicted to be a PPAR γ full agonist by the molecular modelling analysis and PPAR γ activation by PBO binding has been suggested as a possible MIE leading to the liver steatosis upon short-term (low dosage) exposure (observed in the dog study). This has been attributed specifically to the structural characteristics of the glycol side chain of PBO.

Noteworthy in our research regarding the hepatocarcinogenic effect of PBO observed in rodents (the mode of action of which remains unclear), was the suggestion that the mechanism is based on the potential of PBO to generate reactive oxygen species *via* metabolic pathways (opening of the methylenedioxy ring) and to induce oxidative stress, including oxidative DNA damage (Vitcheva et al., 2015).

The integrated application of ontology-based *in vivo* toxicity data mining and structural analysis enabled the identification of the set of chemicals associated with liver steatosis, steatohepatitis, and fibrosis phenotypic effects and containing specific ToxPrint chemotypes. The molecular modelling provided insights into possible modes of toxic action, creating the basis for further investigations.

7.5. Discussion

The importance of, and the need for, mature chemical-toxicological ontologies (still lacking in the current databases) were demonstrated throughout the present thesis (chapter 1.3, 6 and present chapter). This chapter describes the novel ontology developed for liver toxicity, including 53 unique “site-effect” pairs linking the phenotypic effects observed at the whole organism level with pathological changes occurring at lower levels (organs, tissues, cells).

The ontology developed was applied for mechanistic *in vivo* data mining, which demonstrated its utility for identifying the chemical compounds associated with liver steatosis, steatohepatitis, and fibrosis phenotypic effects; 13 relevant combinations of “tissue-cell” sites and effects from the ontology led to the determination of 59 hepatotoxicants. Their structural characteristics were investigated and the features associated with liver toxicity were found.

The mechanistic reasoning was discussed for two identified compounds, 1,3-bis-(2,4-diaminophenoxy)propane and piperonyl butoxide, and the activation of Peroxisome

Proliferator-Activated Receptor gamma (PPAR γ) was suggested as a mechanism involved in the MIE. The studies on the hepatocarcinogenic effect of PBO observed in rodents (the mode of action of which remains unclear), suggested the mechanism based on the potential of PBO to generate reactive oxygen species *via* metabolic pathways (opening of the methylenedioxy ring) and to induce oxidative stress, including oxidative DNA damage.

It is envisaged that the knowledge developed in this analysis will be further elaborated and will contribute to the development of structural alerts for liver steatosis/steatohepatitis/fibrosis. In the present research it was demonstrated that:

- Grouping of chemicals according to the precisely described phenotypic effects and structural analysis can be applied successfully to the discovery of associated chemotypes;
- Interactive application of molecular modelling methods can be used to support the outcome of ontology-based data mining and to provide insights into possible underlying molecular mechanisms;
- Combined approaches of the phenotype classification and molecular mechanisms investigation can successfully facilitate the process of MoA/AOP development.

Chapter 8 Discussion

8.1. Summary of work with respect to the objectives

As outlined in chapter 1, the three essential building blocks of the COSMOS database include: the Cosmetics Inventory, Skin Permeability Database and oRepeatToxDB (oral repeated dose toxicity database). In this thesis, undertaken in collaboration with COSMOS partners, the entire process of knowledge development has been conducted within each of these data domains. It covered:

- Harvesting, curating and integrating the data into a new relational chemical-toxicological database;
- Realising the scientific objectives formulated through data selection, exploration and pattern recognition;
- Evaluating and presenting the knowledge developed.

Each part of the work described has resulted in measurable outcomes and knowledge (the curated content of the database, Standard Operating Procedures, skin permeability potential classification rules, validated liver toxicity ontology, structural features associated with liver steatosis), supporting the development of computational methods for toxicity prediction and creating a solid foundation for further improvements in this area. The achievements of the realised PhD program are particularly significant in light of the current 21st Century Toxicology approaches (NRC, 2007) and the principles of the “3Rs” (Replacement, Reduction and Refinement) in toxicology (Russell & Burch, 1959), aiming to reduce the use of animals in toxicity testing.

8.1.1. COSMOS Cosmetics Inventory

Chapter 2 describes the current demand for a publicly available chemical inventory containing accurately identified cosmetics ingredients and related compounds and populated with good quality chemical structures. The need for a strategy addressing concerns over the quality of chemical information and structures was also highlighted. In order to satisfy these requirements, the collation of the COSMOS database and COSMOS

Cosmetics Inventory was associated with the establishment of a consistent procedure for chemical records' curation and quality control. It resulted in the development of novel controlled vocabularies for the compounds and structure annotation (stereochemistry, double bond geometry, material type and composition type) and the SOP document (Annex 2) describing the systematic QC approach (as applied during the collation of the COSMOS database content). The procedure designed with respect to the characteristic features of cosmetics-related compounds provided the standardised terminology to organise the complex relationships within this domain and the framework to deal with a range of non-structurable substances. The annotation schemes were constructed to enable convenient identification of chemicals not handled well by cheminformatics methods (inorganics, compounds containing metal or metalloid atoms, botanicals, biological macromolecules, polymers, and mixtures), thus facilitating the compilation of computational datasets and enhancing the development of *in silico* predictive toxicology tools. The precisely identified compounds allowed accurate referencing of the associated biological data.

The resulting publicly available (at: <https://cosmosdb.eu/cosmosdb.v2>) COSMOS database (81,406 chemical records) includes the largest (by far), quality-controlled inventory of cosmetics-related compounds (COSMOS Cosmetics Inventory with 17,100 records), which was necessary for conducting the novel analysis described in the chapter 3 of the present thesis, as well as for realising the objectives of chapters 4-7.

The chemical space of the collated Cosmetics Inventory was analysed and compared with the chemical space occupied by food-related compounds from the U.S. FDA CFSAN PAFA database (chapter 3). The novel approach, combining the ToxPrint chemotypes structural features and physicochemical properties analysis was successfully applied to identify the structural classes better represented by cosmetics- and food- related compounds, and demonstrated their relation to the physicochemical properties and specific use functions within the cosmetics domain. For instance, COSMOS Cosmetics Inventory was richer in polyethylene glycols, quaternary ammonium salts, organosilicons and diethanolamines (characteristic for surfactants, emollients and humectants), and aromatic amines, nitro- and azo- aromatic groups, specific for cosmetics colorants and hair dyes. The physicochemical properties specific for particular cosmetic use categories included "global molecular" and "size and shape" descriptors. The structural classes more abundant among

PAFA food-related compounds included sulfides, disulfides, sulfhydrydes, thio- carboxylic esters, thiophenes, furans and pyrazines (flavouring agents and food direct additives), and phosphites, phosphine oxides, acyl and alkyl halides, isocyanates and acid anhydrides (food contact materials). In terms of physicochemical properties space, the cosmetics- and food-related compounds occupied a very broad region of similar shape. It indicated that physicochemical properties utilised alone do not have sufficient power to discriminate between large inventories of cosmetics- and food- related compounds, and that structural features analysis is necessary to identify the differences between them. The analysis conducted may support further computational modelling efforts within the cosmetics domain. Large differences in structural and physicochemical features exhibited by different use categories of cosmetics indicated that, most probably, they should be investigated independently with different models and/or computational approaches.

The work summarised here achieved the aims of objective 1 (“The quality control of the COSMOS database chemical domain, with particular emphasis on cosmetics and related compounds”) and objective 2 (“Characterising the chemical space occupied by cosmetics and related chemicals”) of this PhD program.

8.1.2. COSMOS Skin Permeability Database

The new COSMOS Skin Permeability Database (chapter 4) was developed in order to address the need for a database containing high quality skin permeability/absorption data for cosmetics and related compounds. The existing data sources (EDETTOX and Kent databases) were consolidated with newly data harvested from regulatory documents and scientific literature according to the specifically developed, and deliberately rigorous, procedure. This procedure was documented in an SOP (Annex 4) and can support skin permeability/absorption data harvesting efforts in the future.

The final COSMOS Skin Permeability Database provides *in vivo* and *in vitro* skin data for over 230 cosmetics-related compounds (484 chemical compounds in total), covering a range of use functions and chemical classes. The quality-controlled content of this database, along with its unique data structure, enables efficient data mining and supports computational modelling activities. This database served as a foundation for the research described in chapter 5. Compiling the set of compounds with available human *in vitro* J_{MAX}

data was followed by the exploration of their structural features (ToxPrint chemotypes) and molecular properties (global, size and shape). This analysis led to the determination of profiles characteristic of compounds exhibiting low and high skin permeability potential. The obtained results may be applied for rapid screening and classification of compounds without experimental data.

The work summarised here achieved the aims of objective 3 (“The development of a high quality COSMOS Skin Permeability Database enriched in cosmetics and related compounds”) and objective 4 (“Classification of skin permeability potential following dermal exposure to chemicals to support the safety assessment of cosmetics related chemicals”) of this PhD program.

8.1.3. COSMOS oRepeatToxDB

The new COSMOS oRepeatToxDB (chapter 6) was compiled after enriching (integrated) existing data sources (including the U.S. EPA DSSTox and FDA CFSAN PAFA) with newly data harvested for cosmetics from regulatory documents and scientific literature. The data harvesting process, conducted with respect to the established MINIMUM Study inclusion criteria (COSMOS MINIS) and combined with a consistent strategy of data curation and quality control, minimised concerns over the data accuracy.

The final COSMOS oRepeatToxDB consists of 340 toxicity studies for 228 chemical compounds (186 cosmetics-related compounds, including 100 hair dyes and 42 impurities) and can be considered a milestone in terms of fulfilling the database-related needs of the 21st Century (predictive) Toxicology. Its unique data model enables the recording of the full level of details on toxicity studies: the toxicological effects occurring at particular dose levels and at specific, hierarchically represented sites (differentiated as organs, tissues, cells), NO(A)EL and LO(A)EL values with associated critical effects and target sites, the study design details (species, sex, routes of exposure, dose group levels, number of animals, control group information) and references. The effects were recorded with the sets of controlled vocabulary and qualified by time of findings, severity, statistical significance and treatment-relatedness. The rigorous curation of the COSMOS database chemistry content (chapter 2) ensured accurate identification of tested compounds.

The COSMOS oRepeatToxDB is a major improvement in the landscape of the currently available public databases, with multiple potential applications in safety/risk assessment, computational models development, and read-across. It was essential for conducting the research described in chapter 7.

The unique and novel design of the COSMOS oRepeatToxDB enabled the development of liver toxicity ontology (chapter 7), which was subsequently applied for mechanistic *in vivo* data mining, leading to the identification of compounds associated with liver steatosis, steatohepatitis and fibrosis phenotypic effects. The novel ToxPrint chemotype-based analysis of these hepatotoxicants led to the identification of associated structural classes. The probable mechanistic reasoning for toxicity was formulated for two compounds, namely 1,3-bis-(2,4-diaminophenoxy)-propane and piperonyl butoxide, that were (respectively) identified as being partial and full agonists of Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) in an interactively applied molecular modelling procedure. It was demonstrated that combined approaches of the phenotype- and structure-based classification and molecular mechanisms investigation can facilitate the process of Mode-of-Action/Adverse Outcome Pathway development.

The work summarised here achieved the aims of objective 5 (“Construction of a high quality database for oral repeated dose toxicity with dose/concentration level information for cosmetics and related compounds”) and objective 6 (“Mechanistic (ontology-based) liver toxicity data mining of the COSMOS oRepeatToxDB on the basis of the ontology developed from the collated data”) of this PhD program.

8.2. Final conclusions and perspectives

Realising the particular objectives within the present thesis contributed to the development of the content-related part of the COSMOS database: Cosmos Cosmetics Inventory (chapter 2), Skin Permeability Database (chapter 4) and oRepeatToxDB (chapter 6), comprising the largest quality-controlled, publicly available (<https://cosmosdb.eu/cosmosdb.v2/>) resource with chemical information and data for cosmetics ingredients and related compounds. The COSMOS database, delivered as a final outcome of the collaboration of consortium partners, is fundamental for the development of alternative (non-testing) methods for predicting repeated dose toxicity of cosmetics-

related compounds. It meets the database-related requirements necessary for reaching the goals of modern predictive toxicology (common with the goals of the 21st Century Toxicology), as it has a relational design, stores high quality, curated chemical structures and toxicity information (which can be searched and retrieved), is equipped with controlled vocabularies and ontologies, and through utilising open-source technology, addresses the lack of cosmetics-related chemical information and biological data in the public domain.

The particular data domains, constructed within this PhD program as a part of the COSMOS database, were used to develop knowledge that can be elaborated to facilitate the further development of *in silico* tools for risk/safety assessment of cosmetics related compounds:

- The chemical space analysis of the Cosmetics Inventory (chapter 3) provides insights into the features of cosmetics substances, delivering valuable information that can be utilised for the development of computational models within this domain. This knowledge can be used, for example, to select the descriptors appropriate for model building or for distinguishing structural classes that should be modelled individually;
- The rules formulated for the classification of compounds into low and high skin permeability potential (chapter 5) can support the estimation of bioavailability of chemicals after topical exposure (and, thus, their potential ability to exert systemic toxicity). This crucial knowledge for the risk and safety assessment of cosmetics related compounds can be further implemented into software applications, which can support rapid screening of compounds without experimental data and further modelling of their dermal permeability potential;
- The COSMOS oRepeatToxDB (chapter 6) by itself can serve as an excellent predictive toxicology tool for further knowledge development, as it provides toxicity effect data for “organ-tissue-cell” levels associated with the LO(A)EL values and links this information with accurately identified chemical compounds. It can be used for mechanistic data mining and development of ontologies for other (than liver) target organs. In the COSMOS project it served as a foundation for the compilation of the new non-cancer Threshold of Toxicological Concern (TTC) database, addressing the repeated-dose toxicity of cosmetics-related chemicals;

- The formulated knowledge on structural characteristics of compounds associated with liver steatosis, steatohepatitis and fibrosis, and on their potential modes-of-action (chapter 7), can be elaborated further and used for the development of the liver toxicity knowledgebase (including the rules and chemotype alerts).

The current PhD project is particularly significant in the light of the current European Union legislation, i.e. the banning of animal testing for cosmetics purposes, as it contributes to providing the necessary alternative (non-animal) tools for the risk and safety assessment associated with the repeated dose exposure to cosmetic ingredients.

Noteworthy, as of March 2016, the COSMOS DataShare Point, a web-based system for exchanging safety evaluation and toxicity data, has been launched (<https://www.mn-am.com/projects/cosmosdatasharepoint>). The COSMOS database will continue to be developed, maintained and made available to the public, creating a resource for toxicological data collection and a means to share data with the scientific community.

In conclusion, the main aim of the present PhD program, namely “the collation of the content within relational chemical-toxicological database and its subsequent application for development of knowledge to support the prediction of repeated dose toxicity of cosmetics and related compounds” has been fully achieved.

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