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The practical implications of the new testing system of chemicals

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Abstract

The thesis deals with the issue of registration of chemical substances in compliance with the requirements of European legislation. All substances manufactured in and imported to the European Union must have their hazardous properties examined. For the purpose of the thesis, an example of the registration of a chemical substance used for surface treatment was performed, with research information on the properties of this substance which was processed with the use of a data processing tool used in real registration.

Abstrakt

Práce je věnována problematice registrace chemické látky podle požadavků evropské legislativy. Všechny látky vyráběné a dovážené do Evropské unie musí mít prozkoumány nebezpečné vlastnosti. V rámci práce byla provedena ukázková registrace látky používané k povrchovým úpravám, využity byly rešeršní informace o vlastnostech této látky, které byly zpracovány v oficiálním nástroji pro zpracování dat, který je využíván při skutečné registraci.

KEYWORDS

registration, REACH, properties, tests, IUCLID, data

KLÍČOVÁ SLOVA

registrace, REACH, vlastnosti, zkoušky, IUCLID, data

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1 INTRODUCTION

At the end of the 1990s the Ministers for the Environment of the EU Member States discussed and evaluated the then valid conditions for placing chemical substances and products on the market. It was concluded that there was a large imbalance in the knowledge of properties of approximately 2,500 new chemical substances which had to be registered under Directive 67/548/EEC prior to placement on the market and approximately 30,000 old substances on whose properties the administrative authorities had virtually no information.

The Ministers for the Environment tasked the EU Commission with the preparation of a concept of new chemicals policy that would ensure the primary objective, i.e. to make sure that, approximately by 2020, only substances with well-known properties, used in a manner whose safety would be verified, are produced and placed on the market of the European Union. At the same time, the competitiveness of the European chemical industry should not be jeopardized and the principle of preventive safety should apply.

In 2001, the principles of the new chemicals policy were published in the White Paper. At the end of 2003 the Commission published the first draft Regulation that was then subjected to public review in an internet discussion; after that the draft was modified and submitted to the Council and the European Parliament for discussion. Additional modifications to the draft Regulation were made between the 1st and 2nd readings in the Parliament in 2005 and 2006. Before Christmas 2006 the draft was approved by the Parliament and the Council and on 30 December 2006 the resulting text of Regulation (EC) No. 1907/2006, known under the shortened name REACH Regulation (1) Regulation of the European Parliament and Council (EC) No. 1907/2006 (REACH), was published in the Official Journal. The text of the Regulation currently in force, after the correction of errors, was published in the Official Journal at the end of May 2007.

The final text of the Regulation was published in December 2006 as a legislative amendment applicable directly in all EU Member States without any further approval.

The aim of the REACH Regulation is to ensure that only substances with well-known properties in terms of health and environmental effects, substances with evaluated risks of use, are found on the territory of the EU. This will ensure safe handling of such substances.

One of the basic obligations of manufacturers and importers of chemical substances is the registration of substances in case of tonnage exceeding 1 tonne a year. As part of registration, each registrant collects data on the substance to be registered. To identify such data, they carry out the necessary tests of properties as required by the Regulation for the respective tonnage level. The registrant then processes the identified information into a document, called a dossier, and prepares a Chemical Safety Report (CSR) on it; as part of this Report they evaluate the risks of use in a document called an exposure scenario. Of course, there are multiple registrants of one substance and each individual registrant does not process the documentation by themselves. That is not the purpose of the Regulation. The one substance, one registration principle applies. The registrants of a specific substance therefore come together, make an agreement and financial settlement and appoint the lead registrant, who organizes all the necessary matters and submits the completed documentation to ECHA (European Chemicals Agency). They get the lead registrant number and access keys for the documentation previously submitted, on the basis of which other registrants of the specific substance also sign in for the registration. Each individual manufacturer or importer therefore obtains a registration number.

The registration process is very costly in terms of virtually all parameters. However, the procedure shared among the registrants distributes the costs among them even though the entire process is sometimes accompanied by a difficult procedure of negotiating cooperation.

Registration takes place in three rounds depending on the tonnage manufactured or imported per calendar year; the first round in 2010 concerned substances above 1,000 tonnes, the second round in 2013 for substances from 100 to 1,000 tonnes and the third round in 2018 for substances from 1 to 100 tonnes. Substances manufactured and imported at under 1 tonne a year do not have to be registered.

In the first two rounds, registration was associated with negotiations among individual registrants of a single substance; the third round already assumes that most small registrants in terms of tonnage purchase access to the data and the registration will be then easier for them. The ECHA set the fair price conditions for such cases in one of the amendments to the Regulation.

This Regulation applies to all types of chemical products, including chemicals for use in surface finishes and protective products.

2 BRIEF OVERVIEW OF EUROPEAN CHEMICALS LEGISLATION

The following section briefly summarizes the development of the chemicals legislation (i.e. legislation principally concerning chemistry and products of the chemical industry) in the EU, including the Czech Republic.

Council Directive 67/548/EEC of 27 June 1967

Harmonized system of classification, packaging and identification of chemical substances established, list of chemicals classified as hazardous issued (first version of Annex I).

Council Directive 76/769/EEC of 27 July 1976

Foundations laid for the system of prohibitions and restrictions of high-risk chemical substances and products.

Council Directive 79/831/EEC (6th amendment of Council Directive 67/548/EEC)

Notification ("registration" in Czech law) of new chemical substances, EINECS list (100,106 old substances for which registration is not obligatory), ELINCS list (so far over 4,000 substances registered under the Directive), systematic assessment of risks of the notified substances by the competent authorities of the Member States.

Council Directive 88/379/EEC of 07 June 1988

Classification, packaging and identification of hazardous products.

Directive 91/155/EEC of 5 March 1991

Uniform requirements for the safety data sheet.

Council Directive 92/69/EEC (17th adaptation to technical progress) of 31 July 1992

Hazard for the environment, polymer definition specified in detail, NLP (No Longer Polymer) List set up.

Council Regulation (EC) No. 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances

Inventory of existing chemicals placed on the market in tonnages higher than 10 tonnes per year.

Evaluation of existing priority substances by the competent authorities of the Member States.

White Paper on the Strategy for a Future Chemicals Policy (adopted by the European Commission in February 2001)

Policy goal specified: apply a new chemicals control system, ensuring that only chemicals with known properties are used no later than in 2020 in a manner that does not harm health and the environment.

I.9. Regulation (EC) No. 1907/2006 of the European Parliament and the Council (REACH) of 18 December 2006

Legislative instrument that should fulfil the policy goals specified in the White Paper.

3 KEY PRINCIPLES OF THE CHEMICALS POLICY APPLIED IN THE REACH REGULATION

The main instruments of the new chemicals policy on the way to achieving the declared objectives of protection of the health and safety of people and the environment are:

a) registration of chemical substances (**R**egistration),

b) evaluation of registration documents and evaluation of substances (**E**valuation),

c) Authorization of high-risk substances (**A**uthorization),

d) Restrictions and prohibitions of high-risk substances (Restriction of **Ch**emicals).

The unified approach to the application of new principles of chemical substances management should be achieved by the fact that the key tasks arising from the application of the REACH Regulation for administrative authorities are fulfilled by the European Chemicals Agency (ECHA), seated in Helsinki. The Agency was formally established by the REACH Regulation. Its managing and working bodies combine the principle of expertise with the principle of proportional representation of Member States, which should ensure professional decision-making as well as the essential principles of shared democratic decisions of all EU Member States on the most pressing social issues. The Agency is financed partly from the Union budget and partly from the revenues from fees paid for transactions it provides with respect to liable parties in industry and trade.

The tasks of authorized national authorities is limited to cooperation requested by the Agency and participation in the evaluation of priority substances included in the coordinated plan of substance evaluation by the Agency.

The REACH Regulation is rather extensive by itself. In addition, however, it is supported by a number of related legislative documents, such as Technical Guidelines (TGD), which lay down the methods of performing individual prescribed tests to obtain the required data and the method of preparing the Chemical Safety Report, and many others. Several electronic tools have been prepared for the registration as such; one of the main tools is the IUCLID 5 registration documentation, adopted from the OECD agenda as a unified IT tool for the registration of chemicals, biocides, etc.

4 MANDATORY DATA ON PROPERTIES

A key step in the preparation of registration documentation for chemical substances is to obtain data on the properties of the specific chemical. Such properties can be divided into the following groups:

- physical,
- physico-chemical,
- ecotoxicological,
- toxicological.

Each of these groups includes a number of data which must be obtained in any of the procedures described below. A list of these tests is given in Annex No. 1 hereto.

REACH is the first "registration" regulation to govern the use of laboratory animals (specifically vertebrates) for determining substance properties. It prohibits animal testing in case that existing data can be used or such data can be obtained in a different way (computational methods, alternative testing procedures,…). A strategy for obtaining data has been set up for this purpose:

- literature search,
- *in silico* methods (computational),
- use of similarity (Read Across methods),
- alternative testing methods (cell cultures,…),
- indicative tests with a limited number of animals,
- full test with the use of animals.

Long-term medical records of workers from the production of the respective substance and, where applicable, workers who have been in contact with it for a long time are considered a very precious source of information on substance properties. These are not conventional epidemiological studies (they are very hard to perform because of the usually combined exposure and their high costs) but actually only data on the sickness rate, difficulties, etc., implying e.g. allergy potential, ability to influence blood formation, etc.

It is necessary to point out that all experiments shall be conducted under good laboratory practice (GLP) as the highest and most complex level of work quality management, which significantly increases the cost of the entire data collection process. Physical and physico-chemical properties are the only exception out of this rule; in their case it is possible to perform tests outside the GLP regime (even though it is preferred); however, at least a quality management system is recommended (accreditation,…).

The validity of the obtained data is evaluated on the basis of the Klimisch score of one to four, where the lower the reliability, the higher the number. Data marked with a one have the highest quality.

1 – data obtained in the GLP system based on the prescribed guidelines (TGD),

2 – data obtained in the GLP system based on guidelines other than prescribed, but similar,

3 – data obtained outside the GLP system based on the prescribed or similar guidelines,

4 – other data that can only be used for support.

4.1 LITERATURE SEARCH

It is not enough to merely state the data obtained in this manner in the registration documentation (e.g. acute toxicity is 250 mg/kg). The original work must be obtained and carefully studied for rating based on the aforementioned Klimisch score (the method with which the data were obtained, GLP yes/no,…). It is a common problem that for many substances, very different values of the same property can be found for the same substance. Cooperation with an expert in the specific area is also necessary (toxicologist, ecotoxicologist, analyst,…). The rating based on Klimisch, from 1 to 4, is based on the determined test conditions.

4.2 *IN SILICO* METHODS (COMPUTATIONAL)

These procedures stem from complex mathematical modelling. They are divided into two basic groups: QSAR (Quantitative Structure Activity Relationship) and SAR (Structure Activity Relationship). Closer details are to be described. The Klimisch score of 2 is usually assigned to these results.

4.3 USE OF SIMILARITY (READ ACROSS METHODS)

This involves the transfer of well-known properties (or estimate of a specific property) from one substance to another for which the specific property is unknown. To follow this procedure it is necessary to comply with a number of conditions, in particular the similarity of the compared substances. This technique is described as the Human Expert System in section 5, which applies to computational methods. The Klimisch score here is 2, too.

4.4 ALTERNATIVE TEST METHODS

This involves experimental methods which are currently undergoing a dramatic development. Instead of laboratory animals, these methods use suitable models (cell cultures, bacteria,…) or procedures that limit the number of animals in experiments or reduce their potential suffering. Closer details are to be specified. These tests are performed in the GLP system in accordance with TGD and is rated by the Klimisch score of 1.

4.5 INDICATIVE TESTS WITH A LIMITED NUMBER OF ANIMALS

These tests are carried out before each "full" test (see 4.6) on one or two animals (depends on the type of the established property). If the result of the pre-test is absolutely clear, the test is discontinued.

4.6 FULL TEST WITH THE USE OF ANIMALS

This involves an experimental finding based on the prescribed guideline (TGD) in the GLP system; this result is then rated under Klimisch as 1. The principles of these tests are specified in OECD Testing Guidelines of Chemicals as well as in the REACH Regulation. The basic principles are summarized in Annex No. 1 hereto.

5 *IN SILICO* METHODS (COMPUTATIONAL)

Essential information on computational methods (Q)SAR

(Q)SAR is an abbreviation of (Quantitative) Structure – Activity Relationship. Other equivalent abbreviations: QSAA ("Quantitative Structure – Activity Analysis"), QPAR ("Quantitative Property – Activity Relationship") are rather historical and cannot be almost encountered these days.

The (Q)SAR analysis can be (easily) defined as the analysis of data on biological and chemical properties of substances, mostly using the methods of mathematical statistics. The physico-chemical, toxicological and ecotoxicological data of selected (well-known) substances (= core database, training set) are therefore generalized to apply to all substances similar to the training set. The concept of similarity is very general in this case and shall be discussed further. The goal of (Q)SAR methods is, in other words, the quantitative or qualitative estimate of the defined property of the chemical substance on the basis of its similarity with other, well-known substances. The quantity of the collected experimental data on individual substances results in efforts to generalize the obtained learnings. (Q)SAR methods try to convey this generalization from an empirical to a formal scientific level using mathematical – statistical analysis, logic and algorithmization.

The development of these methods is integrally associated with the use of information technology, that is why the term of *in-silico* testing of chemical substances has been used. This concept is used primarily in the context of biological activity testing (determination of toxicological and ecotoxicological properties of substance), as an alternative to *in-vitro* methods (tests on tissue, cell cultures, biological products) and *in-vivo* (tests on – higher – living organisms).

The only input into (Q)SAR methods (algorithms) is the structural formula of the tested substance (molecule). Therefore, this involves the search for a context between the structure of the substance and its physico-chemical, toxic or ecotoxic properties, including also the biokinetics or distribution and degradability in the environment.

The output is an end-point: an accurately defined property of the chemical substance (e.g. *Daphnia magna* LC50, 48 hours, etc.)

Based on the used algorithm, (Q)SAR computational systems can be divided into QSAR and SAR (expert). QSAR algorithms provide a quantitative output of the measurable property of the substance (such as physico-chemical parameters, LC50, LD50, NOEL ("No Observed Effect Level"),…, or the likelihood of non-quantifiable toxicological effect (mutagenicity,…). Expert systems provide qualitative information, such as classification of the monitored toxicological effect (highly mutagenic, little carcinogenic,…); in principle that are unable to provide quantitative information.

There are a number of approaches using advanced mathematical methods (3D-(Q)SAR, neural networks, classification algorithms,…) but as yet they are not in a condition fit for legislative purposes.

Essential information on computational methods SAR

SAR systems attempt at computer simulation of evaluation of unknown substances performed by humans on the basis of their experience and knowledge.

For this purpose they use computers as tools for evaluating a highly varied set of rules reflecting the presence of certain (marked) substituents or structural fragments (alerts, toxicofors) with respect to reactivity, activity, metabolism, etc. of molecules containing these alerts.

Human expert systems are based on rules created by humans – expert analysis. Artificial systems automate the process of finding and generalizing these rules, they are self-learning algorithms: generic algorithms, artificial neural networks, decision trees,…

As the first step it is necessary to create a defined set of "suspicious" structural fragments and substituents (alerts, toxicofors) and the conditions and rules for their action on the behaviour of the molecule. For human expert systems, this step is carried out by humans, while in case of artificial systems it is automated. This set is then algorithmized and implemented by software. It results in a database of toxicofors and a knowledge database.
As regards the human systems, these database remain unchanged until the next outside intervention.
In case of artificial systems, a learning process is usually built; it is possible to enter new molecules with a known toxicological effect using again the algorithm for creating such databases.

When an unknown substance is evaluated, known alerts and their number and combination are identified by the software in its structure and, applying the knowledge database – the ease of penetration of the substance to the point of action is also taken into account (using the same software mechanism), e.g. the likelihood of penetration through skin, etc. – an output is obtained in the form of evaluation of the likelihood of toxicological effects of the molecule under study. The output is therefore qualitative information.

Expert systems are often used to estimate the mutagenicity and carcinogenicity as well as reactivity, metabolism, degradability and distribution in the environment. 3D-SAR models are also based on them – studying the pharmaceutical efficacy of drugs depending of the substituents present, help for searching for new medicaments.

QSAR principles

QSAR systems are based on the concept that individual partial properties of the molecule contribute to the end point monitored. This can be expressed with the general formula:

$$\boldsymbol{Q = f(X_i)}$$

where *Q* is the monitored *end point* and *X* are the indicated physico-chemical or structural properties of the molecule. Search for the suitable function *F* is the subject of theoretical research but the model used almost exclusively today is the linear model, i.e. an additive contribution of individual partial properties to the monitored property is anticipated:

$$\boldsymbol{Q = \sum \alpha_i A_i}$$

Q is a (usually logarithmic) value of the *end point* α are regressive coefficients and A are individual physico-chemical or structural properties (descriptors) of the molecule. The values α can be positive or negative. Insignificant (α=0) members are excluded during the development of the model and statistical testing.

It seems to be suitable to express the linear model on a logarithmic scale, which is explained by the thermodynamic nature of the issue (chemical potential of the substance in the organism and logarithmic dependence of the chemical potential on the activity of the substance). (*Q* is therefore e.g. log(LC50) etc.)

Some QSAR models (Free – Wilson analysis) are a generalization of expert systems and, just like these expert systems, they use the presence of indicated molecular fragments; however, they replace expert analysis (performed by humans) with statistical analysis, also numerical. Correlation between the presence of the specific *alert* in the molecule and its efficacy is searched with respect to the determined *end point*. Each *alert* is statistically assigned an "impact factor" (weight) – regressive coefficient. In that case the symbol *A* in the formula above indicates individual *alerts*, the symbol α the weight of these *alerts*. These systems therefore work with molecular fragments but do not have a knowledge database dependent on previous expert analysis.

Other models use descriptors (*A*) of other measurable physico-chemical properties of the studied substance. Many such models were and are developed depending on the choice and number of descriptors (Hansch formula, Hammet formulas, …). One of the essential descriptors is, however, log(P), which is the logarithm of the partition coefficient n-octanol/water, which is explained by the context of this parameter with the distribution of the substance in the organism. The values of some descriptors can be obtained again numerically, e.g. using other QSAR formulas or quantum chemical calculations (energy HOMO, LUMO,…).

Compared with QSAR, SAR methods are considered as algorithms with a clearly defined essence, transparency and interpretability and are deemed to be more easily modifiable – the database of toxicofors and the knowledge database can be extended only as the input data for the algorithm (with no interference in the algorithm as such). A disadvantage of SAR systems is their dependence on the quality of the expert analysis – for human systems there is a higher likelihood of human error, for artificial systems biological efficacy can be more easily associated with incorrect fragments. As regards QSAR systems, the ability to provide a quantitative estimate of the monitored property is appreciated, including an estimate of uncertainty of such determination. The quality of the QSAR predictive model depends, however, to a large extent on the choice of descriptors and the amount of input data – unlike SAR systems, an increase in the amount of input data does not always lead to an increase in the quality of the model, but this could be vice versa.

Issue of chemical similarity

The accuracy of prediction of the selected end point by the (Q)SAR model depends in particular on the "similarity" of the monitored molecule to the molecules of the basic database of the predictive model. The assessment of this similarity is very difficult and even molecules very similar structurally may have absolutely different biological effects, considering their different mechanism of action. On the other hand, molecules with very different structures may have a similar activity. In addition, it is not easy to define the characteristics of similarity of individual molecules.

In a single predictive model each molecule is fully described by the set of its descriptors (structural or physico-chemical). As part of the specific predictive model, this defines the "characteristics of similarity" of individual substances that constitute the n-dimensional space (n = number of descriptors). Based on those characteristics, substances can be analysed and classified.

A model using certain input parameters is not often suitable for a whole range of existing molecules (e.g. because of different mechanisms of action). As part of the same software there may be several sub-models used for estimating the same *end point* – separate QSAR formulas – each suitable for a different type (group) of molecules (e.g. sub-model for aliphatic hydrocarbons; (organic) acids; aromatic hydrocarbons;...) Algorithms allowing the evaluation of similarity make it then possible to choose automatically a suitable sub-model for a specific evaluated substance. In genera, however, (Q)SAR formulas/algorithms are not able to evaluate inorganic compounds and organo-metal compounds. The following general rule also applies: "the simpler the evaluated molecule – the likelier the predicted result can be expected to be correct."

One of the legislative requirements for QSAR systems is the ability to evaluate the "suitability" of the tested molecule for the model used on the basis of the "similarity" criterion. In this respect the term "predictability domain" is often used – as an imaginary n-dimensional space defined by the specific QSAR model – into which the evaluated molecule falls (in that case the model is deemed as suitable for its evaluation) or does not fall.

However, it is necessary to emphasize that whether or not the specific molecule falls into a *predictability domain* of the specific model has ultimately no effect on the assessment of reliability of the obtained prediction. A substance outside the *predictability domain* can be evaluated correctly by the model while a substance within the *predictability domain* can be evaluated incorrectly.

Validation

The validation of software is a key and required process for implementing (Q)SAR systems into (legislative) practice.

Internal validation (cross-validation) is carried out, using the targeted regrouping of input data to monitor the effects of such changes on the model's critical parameters. Of course, a disadvantage of *cross-validation* is that it is closed (evaluation "by itself").

More valuable data can be obtained by external validation – monitoring of reliability of the results predicted for the unknown molecule model. However, there is a dilemma here: "It is more suitable to use a molecule with a known effect for creating the model or for the purposes of external validation?"

In OECD, activities aimed at selecting usable models / software and their "recognition as suitable" have been developed for many years. The *ad-hoc expert group on (Q)SARs* has been established, tasked to increase the acceptability of (Q)SAR systems for legislative purposes in the area of chemical substance control; this group was then extended by other experts from the Member States (including the Czech Republic). Another milestone of these activities was the definition of "Setubal principles", defining (ideal) conditions of acceptability of (Q)SAR systems. An OECD Guideline defining the conditions and procedures of validation of predictive systems was then prepared. The outcome of the efforts was the document ENV/JM/MONO(2007)2: GUIDANCE DOCUMENT ON THE VALIDATION OF (QUANTITATIVE) STRUCTURE-ACTIVITY RELATIONSHIPS [(Q)SAR] MODELS.

Legislative framework

It is already obvious today that the implementation of *in-silico* methods is seriously considered as serious testing methods for toxicological effects and parameters for new

substances as well as within a REACH programme. The main benefit is seen from an economic point of view (impact studies of EU (2005) mentioned savings of up to EUR 1 billion as part of REACH), the time demands of existing (in vivo and in vitro) tests and in terms of handling of living organisms by humans - the 3R principle (Replace, Reduce, Refine) (the impact studies envisaged about 1.5 million animals to be saved - as part of REACH).

In connection with the REACH Regulation it is suitable to mention Articles 13, 138, Annex VI, "STEP 1" and "STEP 4", also the "requirements for assessment of all available data" applicable to all tonnages and in particular Annex XI, clause 1.3 – the purpose of using (Q)SAR estimates as sources of information is clearly implied by all these parts of regulations.

In practice we encounter requirements for using *in-silico* methods more and more, in particular where the required information cannot be obtained in a different manner. Some European testing guidelines (e.g. A.8- "Partition coefficient", A.4- "Vapour pressure") also directly contain recommendations involving the use of QSAR estimates – and they are fulfilled in practice.

The use of *in-silico* methods therefore seems to be unavoidable and the need for a quick solution of this problem is obvious, which contributes to political pressure for the fastest possible introduction of alternative methods in (clinical) and chemical practice (2) Dubský Pavel et al.: Report on Investigation of Project VaV MŽP, VÚOS a.s. 2008.

Many expert groups under individual authorities worldwide deal with the introduction of predictive systems and the corresponding legislative framework. The most important ones include ECB (European Chemical Bureau), EC – JRC – IHCP – ECVAM (European Commission – Joint Research Centre – Institute for Health and Consumer Protection – European Centre for the Validation of Alternative Methods), OECD (Organization for Economic Cooperation and Development), US EPA (Environmental Protection Agency):

- ECB supports the development of (Q)SAR tools that could be potentially suitable for legislative purposes: ToxTree is a programme for the hazard classification of substances on the basis of their chemical structure. Danish (Q)SAR database is a database of information on about 166,000 organic substances obtained from (Q)SAR models. ECB Inventory of (Q)SAR models is an activity developed directly for the purposes of the REACH Regulation and constitutes a collection of information on potentially usable models and "products" of (Q)SAR.

- For many years OECD has developed significant activity in this area, involving the formulation of principles of software validation, draft Guidelines for the development, validation and use of (Q)SAR or collection of learnings on the possibilities of practical use of (Q)SAR methods.

  http://www.oecd.org/document/23/0,2340,en_2649_34373_33957015_1_1_1_1,00.html#

- US EPA provides the (Q)SAR product EPI Suit for evaluating the physico-chemical and (bio)degradation properties of substances.

  http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm

6 ALTERNATIVE TEST METHODS

Alternative methods are constantly being developed and verified for use in practice. These methods may often provide the same level of information as existing tests on animals and at the same time they use a smaller number of animals, causing less suffering or doing fully without the use of animals in accordance with the 3R concept.

In compliance with the applicable legislation governing animal protection, it is necessary to verify, in the registers of internationally verified and recognized methods, whether there is an alternative method to the planned experiment in which an animal does not have to be used. If such methods are available, they shall be taken into account in risk characteristics, subsequent classification and designation of the chemical substance based on hazard.

Alternative methods are a huge benefit especially for the testing of cosmetic products. In the Czech Republic, testing of cosmetic products on animals is prohibited by legislation. In this area they fully replace animal testing.

3R CONCEPT

In 1959 Russell and Burch published their book "The principles of humane experimental technique". The central issue of the publication is whether the inhuman aspects of experiments on animals can be limited or removed. The proposal resulted in the 3R concept which has already become established and is among the key principles of responsible and reasonable use of animals in tests. 3R is also accepted by many protection organization and certainly creates a platform on which bilateral dialogue can start. This reason as well as the accurate and understandable formulation of principles for using animals in experiments was a big benefit for the formulation of legislation in many countries, the Czech Republic included. 3R stands for Replacement, Reduction, Refinement (3) Basketter D.A., Scholes E.W., Chamberlain A., Barratt M.D.: An Alternative strategy to the Use of Guinea Pigs for the Identification of Skin Sensitization Hazard; Chem.Toxic. Vol. 33, No. 12, pp. 1051-1056, 1995, (4) Basketter D.A., Lea L.J., Cooper K., Stocks J., Dickens A., Pate I., Dearmam R.J., Kimber I.; Treshold for Classification as a Skin Sensitizer in the Local lymph Node Assay: a Statistical Evaluation; Food and Chemical Toxicology 37 (1999) 1167-1174, (5) Ehling G., Hecht M., Heusener A., Huesler J., Gamer A.O., H. van Loveren, Maurer Th., Riecke K., Ullmann L., Ulrich P., Vandebriel R., Vohr H.-W.: An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: First round; Toxicology 212 (2005) 60-68.*Replacement*

This involves replacement of animals in experiments. Any experimental method that does not require the use of an entire, living animal can be considered a replacement alternative. Some of these methods will make possible only partial replacement because they allow human killing of animals for collecting cells, tissues or organs for subsequent *in vitro* studies. Other methods are replaced in full since they require no biological materials obtained from a fully developed vertebrate. In some cases the studies may only include replacing methods. In other cases they complement animal experiments and contribute to the reduction of their number used in the entire project.

Possibilities of replacing animal experiments with alternative methods:

- physico-chemical methods,
- mathematical and computer models,

- use of lower organisms (invertebrates, plants, micro-organisms),
- use of early development stages of vertebrates,
- *in vitro* techniques.

Reduction

The concept of reduction includes any procedure that results in the achievement of at least the same amount of information using a lower number of animals or in the maximization of the information obtained from one animal, and then in a limitation or avoidance of the use of additional or other animals. This involves in particular the choice of a suitable method and layout of the experiment; equally important is the choice of a suitable animal model on the basis of knowledge of the biology of the selected species to avoid unnecessary errors and, in terms of the physiology and behaviour of the respective animal species, to suppress or fully exclude undesirable variables from the experiment. This is closely associated with the monitoring of environmental aspects for animals in user establishments and at farms as such. The quality of animals used in experiments also results in a major reduction.

Refinement

This refers to efforts to reduce or completely avoid painful and stressful approaches and experimental procedures. It is based on the biological needs of the animal and the corresponding technology of breeding and the environmental conditions so that all physiological requirements for the respective species could be met and the homoeostasis of the organism maintained. What is important during the experiment, in addition to the handling of the animal, is the choice of the most animal-friendly method, with the administration of local or total anaesthesia if it prevents pain or inadequate stress. Post-operation stage with simultaneous administration of analgesics, if necessary, is an integral part of the experiment.

To bring the content of 3R to life, a large degree of knowledge and practical experience obtained through corresponding education and practice is required. This creates conditions not only for an adequate quality of the research performed as well as for the responsible use of laboratory animals.

Typical examples of alternative procedures include:

IRE - The Isolated Rabbit Eye (IRE) Test Method

Principle of the method: the IRE method uses rabbit eyes obtained from slaughter rabbits at the slaughter site.

Prior to use, the eye is examined by means of a slit lamp and fluorescein; damaged eyes shall be discarded. The eye is removed and attached to a special mounting so that the cornea is placed vertically. The eye is then placed in a special chamber where the prescribed temperature is maintained and once a minute the eye is moistened by one drop of saline. This maintains the eye in an activated condition. The tested substances as well as the negative and positive controls are applied directly onto the cornea. Three eyes are used for each substance. Damage to the cornea is identified by an examination using a slit lamp at different time intervals. Corneal oedema is then determined using in-depth measuring accessories or an ultrasonic pachymeter (also at different time intervals) and fluorescein passage is determined as the last parameter. Individual parameters are evaluated separately. The final calculation is then carried out using those parameters to determine the irritation potential for each substance.

HET-CAM - The Hen's Egg Test - Chorionallantoic Membrane (HET-CAM) Test Method

Principle of the method: the potential irritancy of substances is detected by observing changes to the chorioallantoic membrane of a hen’s egg after exposure to the tested chemical.

The substance is applied directly on the chorioallantoic membrane of the hen's egg for 5 minutes. The time necessary to damage the membrane is monitored for that period of time. After the end of exposure, the membrane is tested for vascular damage. The irritancy of the tested substance is derived from the intensity and speed of occurrence of the damage. The method is very demanding for the standardization of egg quality – fertility and ability of hatching (10 – 15% of eggs must be discarded due to infertility, 20 – 30% of eggs for random lesions). The subjective nature of the evaluation is a disadvantage.

Test of eye irritancy on a three-dimensional model of human tissue (EpiOcularTM)

EpiOcular^{TM} – artificial human skin tissue based on human skin cells, cultivated on plastic matrix.

Principle of the method: the tested material is applied on the surface of a three-dimensional model of the cornea comprising normal epidermal keratinocytes cultured to form layered scaly epithelium, similarly to the cornea. The effect of the substance on the lifetime of the cells is then identified. Cytotoxicity is expressed as a reduction of mitochondrial dehydrogenase activity by measuring the activity of formazan formation from MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The tested substances are placed on the stratum corneum of the epidermal model at 3 different exposure times. Exposure is finished by washing with the use of phosphate buffer. After that the tissues are incubated with MTT (yellow tetrazolium salt). If the cells remain alive, MTT is reduced in the mitochodria by succinate dehydrogenase to a blue-and-violet formazan precipitate. It is then extracted from the cells using isopropanol and quantified spectrophotometrically (at 540 or 570 nm). The result is a numerical value - cell viability.

ICE – The Isolated Chicken Eye (ICE) Test Method

OECD Test Guideline No. 438 Isolated Chicken eye (ICE) Test Method for identifying Ocular Corrosives and severe irritants . Adopted September 7, 2009.

Principle of the method: the test on an isolated chicken's eye is an *in vitro* method that can be used to classify chemical substances or eye toxicity/irritancy. The ICE method uses eyes from slaughter chickens obtained at the slaughter site. The eye is removed and attached to a mounting so that the cornea is placed horizontally. The tested substances as well as the negative and positive controls are applied directly onto the cornea. Corneal damage is measured as a qualitative determination of opacity, qualitative determination of epithelial damage based on the preservation of fluorescein, quantitative measurement of reinforcement (of the oedema) and qualitative evaluation of the macroscopic damage to the surface. The results are evaluated separately and form ICE classes for each result, and are then combined to determine the irritation potential for each substance.

BCOP – The Bovine Corneal Opacity and Permeability (BCOP) Test Method

OECD Test Guideline No. 437 Bovine Corneal Opacity and Permeability Test Method for identifying Ocular Corrosives and severe irritants . Adopted September 7, 2009.

Principle of the method: the BCOP test is an organotypic model that provides short-term retention of normal physiological and biochemical functions of the bovine cornea *in vitro*. In this test, corneal damage by the tested substance is determined using quantitative measurement of changes in permeability (for fluorescein - UV/VIS by means of a spectrophotometer) and in opacity (i.e. permeability for light – measured by an opacitometer) of the isolated bovine cornea. Both measurements are then used to calculate the IVIS (*in vitro* irritancy score), used to allocate an irritancy category for an *in vivo* estimate of the tested substance's eye irritation potential (6) OECD Test Guideline No. 437 - Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosive and Severe Irritants, Adopted 7th September 2009, (7) OECD DRAFT PROPOSAL FOR A NEW GUIDELINE 437: Bovine Corneal Opacity and Permeability (BCOP) Test Method for Identifying Ocular Corrosives and Severe Irritants, May 2009, (8) INVITTOX Protocol 124: The Bovinne Corneal Opacity and Permeability (BCOP) Assay – SOP of Microbiological Associates Ltd., UK.So far, dozens of similar methods have been developed for a number of properties; the problem is, however, that many of them are not still

validated and included in the portfolio of recognized testing methods. Another problem is that in many cases these are highly demanding methods technologically, requiring professions uncommon at regular toxicological sites as well as necessitating the acquisition of new, often very complicated and costly equipment. This is why the mentioned tests are not widely available in practice, e.g. only one site performs them under the mandatory GLP regime in the Czech Republic.

PRACTICAL SECTION

The practical section of the paper deals with the model registration of methylethyl ketone oxime (butanone oxime).

GENERAL INTRODUCTION ON THE PROPOSED SUBSTANCE REGISTRATION

Essential information on the substance:

Name: Methylethyl ketone oxime (butanone oxime)

Substance identification:

Chemical Abstracts (CAS) No.: 96-29-7

EINECS No.: 202-496-6

Identification No.: 616-014-00-0

Chemical formula: C_4H_9NO

Molecular mass: 87

When work on this paper started, methylethyl ketone oxime was not registered. It was chosen as a substance used in paints; production in the fictitious tonnage of 1 - 100 tonnes per year, i.e. the basic scope of data for registration, was selected as a demonstration of registration.

When preparing the model registration documentation, literature search was carried out first concerning the availability of data on this substance in the area of physico-chemical, toxicological and ecological properties. The goal of the search is to facilitate data acquisition for the registration dossier.

The search involves data available on the specific substance, along with the source of the information in case the literature data are used in the registration documentation.

This is all older information which can be expected not to have been obtained in the GLP system for tests, as preferentially required by REACH. Therefore, an expert opinion would be needed in case of its application.

The outcome of the search is a “data need” table which provides an overview of whether data are available and, if so, whether they constitute literature data or test data, and which data are not available at all and the possibility of testing needs to be considered.

It was found out in the survey of and search for available data that there is an IUCLID 4 dataset for the substance, which means that the substance has already been registered under Directive 67/548/EEC.

This means that the original registrant has already collected and processed the data and has submitted them to its national authority and that there are study summaries on the end points required for registration under Directive 67/548/EEC.

The IUCLID dataset was issued on 19 February 2000. In the old system the substance was registered more than 10 years ago so it is possible to obtain the data by inquiring at the ECHA; they will be freely available and usable. Otherwise, if the term has not expired yet, the Agency shall provide contact information of the main registrant to the potential registrant; it is then possible to agree the price with the lead registrant upon submission of tests with vertebrates, or agree the price of an access key for the substance already registered.

Pre-registration had to be performed within the prescribed term so the substance could be registered. Each registrant who has carried out the pre-registration shall obtain access to SIEF (association of pre-registrants) a can determine which companies intend to register the specific substance, who the registration process coordinator (facilitator) is and, where applicable, whether the lead registrant has already been appointed.

For the purposes of the paper it is not possible to determine such data because it is not possible to log into the REACH IT system (electronic system for communication with the ECHA) for training purposes; only actual manufacturers and importers may do so.

The model registration assumes that the registrant prepares the documentation on their own. Data from the freely accessible IUCLID 4 were used for this purpose; at the time of its use, it did not have as extensive requirements for data as IUCLID 6 used at present.

If the registrant wanted to use data from registration based on the original system (Directive 67/548/EEC), they would have to ask the ECHA, which would mediate the submission of the originally tests, now freely accessible. This was not possible in the model registration in this paper, the author is not a registrant and cannot request official primary data from the Agency, so only freely accessible data were used in the sample dossier.

The registration documentation for the tonnage of 1 - 100 tonnes also includes the Chemical Safety Report, which cannot be prepared for the model registration because it is informed by the fully completed dossier and a number of data not freely accessible, which would be subject to financial settlement in case of an actual registration.

It is also very difficult to estimate the financial demands of the substance registration at the given tonnage. The price will be influenced by many factors. In particular cooperation of registrants, sharing of test costs (the more registrants, the lower the share of each of them). If the data from the studies were obtained for free in this case because the time of protection under the copyright has already expired, it would be necessary to count on costs of expert assessment; old tests do not have to meet all the required criteria.

The paper was started when it was possible to file a separate registration, i.e. unless our hypothetical registrant agreed with others (the price could be the reason for failing to agree), they could do everything themselves, except for tests on vertebrates, or could commission the necessary tests themselves.
In case of tests on vertebrates it is necessary to reach an agreement with the lead registrant and these tests or access to them would have to be purchased because they cannot be repeated in accordance with REACH rules.

During the preparation of the practical section there were several major changes in legislation. Filing a separate substance registration has not been possible since 2016. And the environment in which such data are processed has also developed and the currently valid database version is IUCLID 6.

The practical section of the paper was completed at the time of preparation of the model registration documentation in IUCLID 5. After reviewing the conditions of transition to the higher version IUCLID 6, I decided to retain the original version of the documentation. IUCLID 6 has different requirements for data processing inside the database and this would virtually mean reworking the whole paper.

Should a substance be currently registered, the process would be as follows:

1. If the substance were pre-registered, the potential registrant would have to determine who the lead registrant of that substance is. This can be determined from the list of lead registrants at the ECHA webpage. It is then necessary to determine from the lead registrant the conditions for identity of the substance to be registered with the substance already registered. If both substances are identical, it is possible to buy LoA (access to the submitted registration data) from the lead registrant. The pre-registration number is important in this case, no registration is possible without it,
2. If the substance has not been pre-registered, it is first necessary to perform analytical tests of the substance as required. These tests are then used to compile the inquiry dossier, i.e. an inquiry on the ECHA webpage to establish whether the substance has already been registered. If so, the ECHA shall assign an inquiry number to the ECHA and notify the identity of the lead registrant. The LoA agreement of purchase follows. Registration is not possible without the inquiry number even if the registrant knew the identity of the lead registrant in advance.
3. In both cases, as soon as the registrant obtains the LoA, it shall process the data applicable to the filing of the documentation of a co-registrant (small dossier) in the IUCLID 6 database and submit registration with the LoA access code to the ECHA.
4. As part of complete documentation, it is possible to purchase access to the Chemical Safety Report.
5. After adoption of the document by the ECHA, the registrant shall receive the registration number.

When work on this paper is over, the substance is already registered. A total of 14 registrants are listed in the documentation on the ECHA webpage. (One lead registrant and 13 co-registrants).

The substance is already registered for the following specified applications:

- manufacture of the substance,
- use as an intermediate product,
- manufacture of liquid paints,
- manufacture of mixtures in general (paints and coats, solvents, paint removers),
- industrial application of paints,
- manufacture of special chemicals,
- manufacture of polymer products and substances.

The author of the paper is not a registrant, so sample registration of methylethyl ketone oxime is just a model and not usable for actual registration, which would be settled by purchasing LoA access.

For the model demonstration it was not possible to use the registration data already published on ECHA's webpage; they are the property of the registrants and cannot be handled freely. The Agency expressly states that they shall not be used for new registration without any financial settlement with the data owners.

7 CONCLUSION

The new principles for placing chemicals on the European market are much more demanding than just the notification under the previous legislation. On the one hand, they should provide much better protection of the health of population and of the environment against the negative effects of chemical products, subject to strict compliance with all rules, but on the other hand they have several risks. One of them is the clear reduction of the competitiveness of the European chemical industry in the world, another one is an increase in the production costs of chemicals manufacturers and the resulting potential increase in prices of not only chemicals, but also any follow-up production that uses chemicals. In the social area, there is also the risk of job cuts. One of the causes is the immediate threat to small and medium enterprises because they have no funds to finance their higher costs of registration; another serious cause is the shift of production facilities of large companies outside the EU, i.e. where the REACH Regulation does not apply, so that such production is not burdened by the associated costs. One of the hazards is also true in the Czech Republic which has a rather developed chemical industry but not too strong in terms of capital. For illustrative purposes, the European chemical industry directly employs 1.2 million people and indirectly over 4 million people, which constitutes about 3% of the population capable of working. In the Czech Republic it directly employs 110,000 workers and related sectors provide jobs for over 300,000 people, which constitutes about 10% of the population capable of working. Obviously, failure to apply the Regulation could have major social and ensuing economic consequences. For informative purposes, the impact studies performed show that the estimable costs of chemicals registration incurred to Czech companies are higher than CZK 10 billion.

The REACH Regulation by itself is a very complex and extensive document and compliance with its registration conditions is not simple for manufacturers and importers even if they can help each other with agreements and joint work on the registration dossier.

The prepared document uses data published in literature and the compiled dossier shows what the registration documentation would look like and what the registrant has to establish so the documentation could be compiled.

Although work on the paper started what little was known about registration and ends at a time when there is a lot of information on it, it is not a useless paper because registration is not over. There are very realistic deliberations to reduce the annual tonnage for registration so that new registrants would be added, new substance properties are added (endocrine disruptors) along with new substances (e.g. pure substances in nano form) which need to be assessed and will be subject to registration.

And this paper shows that registration is not a simple process.

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In addition to the literature above, study materials were also used which were prepared for the purposes of training in the REACH Regulation, organized by the Union of the Chemical Industry of the Czech Republic and the Research Institute of Organic Synthesis in Rybitví.

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