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Applicability of *in silico* genotoxicity models on food and feed ingredients

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ABSTRACT

Evaluation of the genotoxic potential of food and feed ingredients is required in the development of new substances and for their registration. In addition to *in vitro* and *in vivo* assays, *in silico* tools such as expert alert-based and statistical models can be used for data generation. These *in silico* models are commonly used among the pharmaceutical industry, whereas the food industry has not widely adopted them. In this study, the applicability of *in silico* tools for predicting genotoxicity was evaluated, with a focus on bacterial mutagenicity, *in vitro* and *in vivo* chromosome damage assays. For this purpose, a test set of 27 food and feed ingredients including vitamins, carotenoids, and nutraceuticals with experimental genotoxicity data was constructed from proprietary data. This dataset was run through multiple models and the model applicability was analyzed. The compounds were generally within the applicability domain of the models and the models predicted the compounds correctly in most of the cases. Although the regulatory acceptance of *in silico* tools as single data source is still limited, the models are applicable and can be used in the safety evaluation of food and feed ingredients.

1. Introduction

Intake of micronutrients such as vitamins is essential for human health but in addition other bioactive compounds, nutraceuticals, are consumed for additional health benefits. Those micronutrients are either consumed as dietary supplements or in fortified foods. Also, animals receive feed with added micronutrients to promote better wellbeing. Another reason for the intentional addition of chemical compounds to food and feed is for coloring purposes, examples of this include farmed salmon and egg yolks, which receive their pink and yellow/orange color through addition of certain carotenoids into animal's feed.

To ensure food safety, regulatory bodies in the respective countries are mandated to regulate the use of food and feed ingredients. In the US, the United States Food and Drug Administration (US FDA) is responsible for the registration of food additives: Under the Federal Food, Drug, and Cosmetic Act (FFDCA), any substance that is intentionally added to food or animal feed is considered a food additive and subject to premarket review and approval by FDA, unless the substance is generally recognized as safe (GRAS) (FDA, 2017). In Europe, Regulation (EC) 178/2002 (European Parliament, 2002b) lays down the general principles and requirements of food law and procedures in matters of food safety. The European Food Safety Authority (EFSA) was established for scientific evaluation, advice, and communication of risks associated with the food chain. The authorization procedure for food and feed ingredients in Europe is established by three documents, handling the authorization of food additives, enzymes, and flavorings (Regulation EC 1331/2008) (European Parliament, 2008), feed additives (Regulation (EC) 429/2008 (European Commission, 2008), and dietary supplements (Directive 2002/46/EC) (European Parliament, 2002a).

One of the endpoints that needs to be covered in the registration of any new food/feed ingredient is genotoxicity. Usually, a core test battery of *in vitro* tests is recommended: these comprise of at least a bacterial mutagenicity test and a test for chromosomal damage (*in vitro* micronucleus test (MNT), chromosomal aberration test (CAb), or mouse lymphoma assay (MLA)) (EFSA, 2011; FDA, July 2000). With this test battery, the three kinds of genotoxicity

Abbreviations: CAb, chromosome aberration; CHO, Chinese hamster ovary; CHL, Chinese hamster lung; FP, false positive; EXP, experimental data; IND, indeterminate; MNT, micronucleus test; (Q)SAR, (quantitative) structure activity relationship; TN, true negative; TP, true positive.

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endpoints - gene mutations, structural and numerical chromosomal aberrations - should be detected. For substances with an expected higher exposure, an *in vivo* MNT is additionally required in the US (FDA, July 2000) whereas this assay – in the light of animal welfare – is only considered as follow-up assay in Europe (EFSA, 2011). Genotoxicity is usually one of the first toxicological endpoints evaluated in the development of new food/feed ingredients; a positive result in such a test usually leads to termination of a compound in the development process.

For the assessment of food/feed ingredient safety, *in silico* tools such as (quantitative) structure-activity relationship ((Q)SAR) models are accepted as indicator for hazard assessment and as prioritization tool (EFSA, 2014; Valerio, 2009, 2011). Furthermore, they can be used as part of a weight-of-evidence or read-across analysis after being backed up by other (Q)SAR models, structural alerts, or experimental data. For further applications, the acceptance of *in silico* models is rare; this encompasses especially the use of *in silico* predictions as replacement for experimental data. Positive predictions are more easily accepted by regulatory authorities applying the precautionary principle (Leist et al., 2008), and there is increasing regulatory acceptance for negative predictions, even without accompanying experimental data. Currently, negative *in silico* predictions are accepted in the evaluation of mutagenic potential of impurities of pharmaceuticals (ICH M7) (ICH, June 2014) and in excluding genotoxicity in residue definition of plant protection products (EFSA, 2016).

If the *in silico* tools are used for regulatory submissions, they should be validated according to the respective OECD guidance document ensuring that the model is transparent with known limitations of applicability, and represent an endpoint that can be measured (OECD, 2007). In addition, the developer of the model should provide the validation information, so that the user, who is often dependent on the choices of the developer, is able to justify whether the model can be used reliably or not. Further validation exercises, both internal and external, will play an important role in gaining regulatory acceptance of the routine use of *in silico* models (Roy et al., 2017). In case the model is used for compound prioritizing purposes, such a rigorous validation can be omitted, although it is sensible to know the model performance in the chemical space the model is applied.

In silico tools are a cost-effective and a rapid way to analyze compounds against many toxicological endpoints especially for compound prioritization purposes in the early development phase (Merlot, 2010; Modi et al., 2012; Valerio, 2009). The demand is high in the pharmaceutical industry and *in silico* tools are used in many stages of the drug development process (Dobo et al., 2012; Müller et al., 2006; Muster et al., 2008; Valerio, 2009). Workflows describing the use of *in silico* methods in risk assessment of food ingredients have been developed (Blaauboer et al., 2016; Schilter et al., 2014). Despite this, the use of *in silico* tools is not very widespread in the food industry, perhaps due to the lack of regulatory acceptance and also lack of expertise in the use of the tools and in the result analysis (Lo Piparo et al., 2011). Many *in silico* models have been developed with the help of pharmaceutical companies and regulatory authorities. In addition to the data from the public domain, the models may contain also proprietary compounds such as pharmaceutical impurities, that are not disclosed by the tool. Because the full training data is rarely available, evaluating the predictive power can not be fully assessed and the models may lack predictive power outside the chemical space of small, drug-like molecules.

In this study, the applicability and performance of selected computational tools for the genotoxicity assessment of food and feed ingredients was evaluated. The focus was set on vitamins, nutraceuticals, and carotenoids: vitamins and nutraceuticals are a heterogeneous group of chemicals containing natural compounds as well as drug-like small molecules, whereas carotenoids represent a group of structurally similar compounds that differ from pharmaceuticals in their structure (Fig. 1, section 2.1.) and physicochemical properties (e.g. high log P and molecular weight) (Tables 1 and 2, section 2.1.).

2. Materials and methods

2.1. Test set compounds

The focus on the dataset collection was set on common food and feed ingredients from the in-house chemicals: vitamins, coloring agents, nutraceuticals, and different flavoring compounds. In total, 27 compounds with experimental genetic toxicity data (Ames bacterial mutagenicity assay, *in vivo* or *in vitro* MNT, or *in vitro* CAb) in the in-house archive were available for this evaluation (Fig. 1). Two groups from these compounds were formed: one for bacterial mutagenicity model evaluation (Table 1) and the other for chromosome damage model evaluation (Table 2).

Bacterial mutagenicity tests were performed in five strains according to OECD 471 guideline (OECD, 1997). Chromosome damage can be tested in various test systems,but in this study, we focused on chromosome aberration test and micronucleus tests *in vitro* and *in vivo*. The i*n vitro* CAb or MNT assays were mostly performed using human lymphocytes, and *in vivo* MNT assay using mice or rats. The *in vitro* and *in vivo* chromosome damage tests were considered to be equal independent of the test, cell line, or species. The decision to combine the tests into *in vitro* or *in vivo* groups was based on the intention to use the data for evaluating chromosome damage *in vitro* or *in vivo* in general rather than focusing on the individual tests or species. Furthermore, OECD guideline 473 for the CAb assay *in vitro* does not discriminate between commonly used cell lines and the results are considered equal (OECD, 2016). Similarly, for the *in vivo* MNT, all rodents can be used and are seen equally according to the respective OECD guideline, but the most commonly used are mouse and rat (OECD, 2014). EFSA also considers the MNT and CAb tests as equal for the prediction of chromosome damage (EFSA, 2011).

Because the compound selection was based on marketed food and feed ingredients, the resulting test set contained almost exclusively negative compounds. The only exception was resveratrol that showed a positive effect in an *in vitro* CAb test, which is overruled by a negative *in vivo* MNT. For crystalline lycopene and 8′-Apo-beta-carotenoic acid ethyl ester, both positive and negative Ames results were available. However, the positive results were expected to be a response to reactive degradation products formed when the crystalline carotenoid is exposed to air and light (Boon et al., 2010; McClain and Bausch, 2003). If the substances were protected from degradation during the experiment, the Ames test was negative, indicating that the pure substance is not mutagenic.

2.2. Software

Bacterial mutagenicity was evaluated with seven different computational tools: Derek Nexus, Sarah Nexus (Lhasa Limited, www.lhasalimited.org), Toxtree (Ideaconsult, http://toxtree.sourceforge.net/), CASE Ultra (MultiCASE Inc., http://multicase.com), T.E.S.T (US EPA, https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test), Leadscope Model Applier (LSMA, www.leadscope.com), and the CAESAR mutagenicity model running in Vega (www.vega-qsar.eu). For chromosome damage, the tools evaluated were Derek Nexus, Toxtree, LSMA and CASE Ultra. From these tools, five models predicting the chromosome damage *in vitro* and five predicting the *in vivo* results were chosen. The