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Identification and evaluation of potentially mutagenic and carcinogenic food contaminants

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Abstract

Heat processing of food gives rise to a plethora of chemical compounds whose toxicological effects are largely unknown. Due to a general lack of experimental toxicological data, assessing the risks associated with the consumption of these substances remains a challenge. Computer models that allow for an *in silico* prediction of physicochemical and toxicological characteristics, may be able to fill current data gaps and facilitate the risk assessment of toxicologically uncharacterised chemicals, their transformation products and their biological metabolites. The overall aims of the present project were for the fellow: (i) to get acquainted with the application of computational toxicological analyses tools in risk assessment based on results and experiences from previous research performed at the German Federal Institute for Risk Assessment (BfR); and (ii) to apply the newly gained skills on historic and novel data using updated and additional *in silico* tools. The project contributed to the continuous further education of the fellow in the use of computational toxicology tools, corroborated findings related to the safety of heat-induced contaminants and laid the foundations for future collaborations between the fellow's home institution, the Institute of Marine Research (IMR) in Norway, and the BfR in Germany.

Keywords: *in silico* toxicology, QSAR, data mining, R

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1. Introduction

Heat processing gives rise to a plethora of substances whose toxicological effects are largely unknown. Particularly, the presence of chemical compounds that are considered possibly or probably carcinogenic to humans has attracted the attention of the public and initiated a debate on the healthiness of heated foods and beverages (Wenzl et al., 2007). Many of these undesired compounds are products of the Maillard reaction or lipid oxidation reactions and include, among others, legacy contaminants such as acrylamide, furan, acrolein, 5-hydroxymethyl furan. Contaminants of recent concern include 3-chloro-1,2,-propanediol (3-MCPD), 2-chloro-1,3,-propanediol (2-MCPD), glycidol and their fatty acid esters. Risk assessment has been performed for some of these substances but for many of the hundreds of heat-induced substances known to date, data on their toxicological properties are still lacking (Frenzel et al., 2017).

Conventionally, for single compounds, *in vivo* experiments are the method of choice for identifying the toxic effects of xenobiotics (Pradeep et al., 2016). However, time, costs and ethical constraints render these assays ineffective when the high throughput hazard assessment of large numbers of novel contaminants is required. In particular, the envisaged reduction in the use of animals in research, as outlined in the European Union (EU) Directive 2010/63/EU (Directive 2010/63/EU 2010), calls for a further development and wider application of alternative testing methods that are able to predict or estimate inherent toxic properties of chemical substances without the need for animal testing. In this context, computer-based (*in silico*) predictions such as quantitative structure–activity relationship models (QSAR) are gaining increased importance, especially as screening tools for prioritisation purposes as they provide faster, non-animal-based alternatives for the prediction of complex toxicological endpoints (Maunz et al., 2013; Pradeep et al., 2016).

In the risk assessment of chemicals, *in silico* tools are typically used in combination with other non-testing methods such as read-across in the context of Integrated Testing Strategies (ITS) and Weight-of-Evidence (WoE) approaches (EFSA 2014). Several QSAR models have been used and validated by US regulatory agencies and a set of internationally agreed upon validation principles for regulatory acceptance were laid out by the Organisation for Economic Co-operation and Development (OECD) (Pradeep et al., 2016). Also in the EU, QSAR are gaining acceptance in the prediction of toxicity reference values and the classification of thresholds for human and environmental risk assessments (Benfenati et al., 2017).

However, despite the recent advances in computational toxicology, challenges do remain that still hamper the use of QSAR in safety assessment decisions and reports. For example, different *in silico* tools using different mathematical algorithms and different training data sets were found to provide conflicting predictions (Gleeson et al., 2012). Therefore, regulators may compile predictions from different QSAR tools to come to a decision. Research is currently ongoing to investigate how to best combine the outputs of these tools to gain improved predictive performances for various toxic endpoints (Pradeep et al., 2016; Frenzel et al., 2017).

2. Description of work programme

At the Department of Food Safety of the German Federal Institute for Risk Assessment (BfR), recently a combine and conquer strategy for QSAR analyses was developed and applied to ~ 800 heat-induced food contaminants (Frenzel et al., 2017) as well as to ~ 600 secondary plant compounds (Glück et al., 2018). In the course of the EU-FORA programme (Bronzwaer et al., 2016), the fellow, Dr Josef D Rasinger from the Institute of Marine Research (IMR)[1] in Norway, was placed at the BfR, to become familiar with the *in silico* tools and approaches described in Frenzel et al. (2017) and to corroborate findings related to the safety of heat-induced contaminants through re-analyses of the already published data using updated and additional *in silico* tools and approaches.

Dr Rasinger is a nutritional toxicologist whose research at the IMR is focused on the development and implementation of molecular ('omics), biostatistical and bioinformatics tools for use in food and feed safety assessments (Rasinger et al., 2014, 2017; Reffatto et al., 2018; Nøstbakken et al., accepted). During his placement at the BfR, Dr Rasinger was introduced to modern *in silico* methodologies for hazard assessment, namely QSAR and further developed his knowledge and experience in risk assessment.

[1] In January 2018, the IMR was merged with The National Institute of Nutrition and Seafood Research (NIFES). The new institute will be a leading supplier of knowledge on the sustainable management of resources in marine ecosystems and the whole food chain from the sea to the fork: http://www.imr.no/en

The project work at the BfR took place in close collaboration with senior scientists of the Unit 51, 'Effect-based Analytics and Toxicogenomics Unit', who as part of the BfR Department of Food Safety (Department 5), examine effects of food ingredients and contaminants. This involves the analysis of the intake, distribution, metabolism and excretion of these substances (toxicokinetics) using *in vitro* and molecular biological methods including *in silico* tools, 'omics techniques, as well as classic chemical analytical methods. The fellow was supervised by Dr Albert Braeuning, the head of Unit 51, and Dr Falko Frenzel, a post-doctoral researcher in Unit 51 and principal investigator in the project around which the fellow's work is centred.

2.1. Aims

The aims of the present project were for the fellow: (i) to become familiar with the application of computational *in silico* toxicological analysis tools in risk assessment based on results and experiences from previous research performed at the BfR; (ii) to obtain transferable skills increasing the scientific capacity of the fellow's home institute; and (iii) to assess the possibility of and set up further networking and bilateral cooperation between the fellow's home and host institutions; the IMR in Norway and the BfR in Germany.

2.2. Activities/Methods

2.2.1. Application of computational *in silico* toxicological analyses tools in risk assessment

For a successful completion of this project, the fellow became familiar with commonly used open source QSAR tools and software tools for data management and data mining. The computer models employed included VEGA, T.E.S.T. and lazar. According to Frenzel et al. (2017), the fellow used five different prediction tools for mutagenicity and three prediction tools for carcinogenicity and two relevant toxicological endpoints for heat-induced food contaminants.

The VEGA platform[2] (v1.1.4) provides several different software tools to predict physicochemical, ecotoxicological and toxicological properties for compounds of interest. In addition, VEGA includes tools for read-across (ToxRead) and prioritisation (JANUS), and allows the integration of results using a weight-of-evidence approach (ToxWeight). Within VEGA, four models are available to predict carcinogenicity and mutagenicity and one mutagenicity consensus model is provided. The Toxicity Estimation Software Tool (T.E.S.T.[3]; v4.2.1) provided by the United States Environmental Protection Agency (US EPA) predicts mutagenicity using three different QSAR methodologies based on either hierarchical clustering, a so-called FDA approach as applied by the US Food and Drug Administration (FDA), a nearest-neighbour approach, and a consensus model. lazar[4] (lazy, structure–activity relationship) (Maunz et al., 2013) comprises two models for mutagenicity and six models for carcinogenicity. In addition to VEGA, T.E.S.T. and lazar, the OECD QSAR Toolbox[5] (v4.1) was used to retrieve information on experimental data on mutagenicity and carcinogenicity of the test compounds.

As described in Frenzel et al. (2017), a list of 814 substances comprising approximately 600 products of the Maillard reaction and 200 lipid oxidation products prepared within the HEATOX project[6] and 24 compounds of current interest in food safety risk assessment was subjected to VEGA, T.E.S.T. and lazar. All settings and parameters within each software tool were set as previously described (Frenzel et al., 2017).

For both handling of the input files and the combination of the output files, the fellow was introduced to R[7] (The R Project for Statistical Computing) and Python[8] (Python Software Foundation, version 2.7) based in-house scripts and KNIME[9] workflows. These tools allowed for the generation of Simplified Molecular Input Line Entry System (SMILES), International Chemical Identifier Keys (InChIKeys), MDL Mol files and structured data files (SDF); common file formats, needed for the translation of a test compound's chemical structure into a computer readable format. In addition,

[2] https://www.vegahub.eu/
[3] https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test
[4] http://lazar.in-silico.de/predict
[5] http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm
[6] https://cordis.europa.eu/publication/rcn/12731_en.html
[7] https://www.r-project.org/
[8] http://www.python.org
[9] https://www.knime.com/

scripts and snippets adapted from Frenzel et al. (2017) were deployed that allowed for the harmonisation of the heterogeneous output styles of the different QSAR software tools used.

During the re-evaluation of the data published in Frenzel et al. (2017), it became apparent that no major improvements could be achieved applying updated versions of the QSAR tools listed above. In the present EU-FORA project, the data set and QSAR workflow published in Frenzel et al. (2017) were therefore solely used as the training material to teach the fellow best practice approaches for QSAR-based data analysis, data preparation approaches and the proper use and output interpretation of the freely available *in silico* software tools. The dataset also was used to highlight and discuss the benefits and limitations of *in silico* approaches in risk assessment.

2.2.2. Acquiring transferable skills increasing the scientific capacity of the home institution

At the fellow's home institution currently efforts are ongoing to assess the safety of ethoxyquin (EQ; 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline). EQ is a synthetic antioxidant that is used as a technological additive to protect against lipid peroxidation in feed for pets, livestock and farmed fish. The use of EQ may result in residues of EQ and its transformation products (TP) being detected in edible tissues of animal origin, so humans can be exposed to these compounds through their diet and safety limits are to be set up.

Concerns on the safety of EQ and its TP and an overall lack of data led to a suspension of the authorisation of EQ as a feed additive for all animal species and categories (Commission Implementing Regulation EU 2017/962). A re-consideration of this assessment by the European Commission (EC) is possible if supplementary data on the safety of use and the efficacy of this additive are brought forward and current data gaps in the assessment of the exposure and the safety of EQ and its TP for animals, consumers and the environment are filled.

Based on novel data, which recently became available at the fellow's home institution, the fellow applied the newly gained computational *in silico* toxicology skills to predict toxicities and develop a prioritisation strategy for the risk assessment of the transformation TP of EQ. At the IMR, using a travelling-wave ion mobility spectrometry (TWIMS) coupled to quadrupole time-of-flight mass spectrometry (QTOFMS), 27 EQ TP were identified in oxidation experiments (Negreira et al., 2017). In subsequent experiments, 25 of those TP were detected in fish feed and 24 in fish from EQ exposure experiments (unpublished data). Using the *in silico* toxicology workflow outlined above, the fellow translated the list of EQ TP into machine readable chemical structure files and set out to predict *in silico* the toxicity of EQ TPs in both fish and mammals.

Work on this data set is currently ongoing. Similar to the prioritisation of the heat-induced contaminants performed in Frenzel et al. (2017), the output of this *in silico* analysis will allow a prioritisation of EQ TP according to their theoretical toxicities and highlight the compounds of most concern for consumers and farmed fish that need to be analysed further using *in vitro* or *in vivo* models of toxicity. The results of this work will be presented at the EFSA 2018 conference in Parma[10] and it is envisaged that a manuscript will be submitted for publication in a peer-reviewed journal before the end of the fellowship.

In addition to computational *in silico* toxicology competencies, the fellow also took part in the following activities that further developed his knowledge and experience in risk assessment:

- Three weeks of induction training in chemical and microbiological risk assessment at the EFSA premises in Parma (September 2017), and three 1-week modules focusing on different aspects of risk assessment and risk communications at the Austrian Agency for Health and Food Safety (AGES) in Vienna (December 2017), the BfR in Berlin (March 2018) and the Hellenic Food Authority (EFET) in Greece (June 2018).
- A 1-day seminar at the BfR on systematic literature search and review by a member of staff from the BfR library (November 2017).
- A 1-day risk assessment workshop at the BfR on food contamination by plasticisers (November 2017).
- A 1-day workshop at the BfR on mathematical modelling of metabolism and contaminant transfer in farm animals (December 2017).
- A 1-day introductory course to the KNIME-based BfR Food Chain-Lab software tools (PMM) laboratory at the BfR (March 2018).

[10] https://conference.efsa.europa.eu/

- The 24th EFSA colloquium: "Omics in risk assessment: state-of-the-art and next steps' held in Berlin (April 2018).
- A 2-day workshop at the BfR on risk assessment and risk management of genetically modified organisms (GMO; May 2018).

Throughout the year, the fellow also was given the opportunity to gain insight into other food-related research of the 'Department of Food Safety' (Department 5) by attending weekly seminars and through individual introductions to current projects related to risk assessment, food toxicology, novel foods and GMO. These meetings provided the opportunity to obtain an overview of the scientific work performed at Department 5, to discuss current issues in food safety risk assessment and to extend the fellow's network at the BfR. The fellow was also given the opportunity to present and discuss work accomplished under the auspices of the EU-FORA project at a departmental seminar in June 2018.

2.2.3. Establishment of further networking and bilateral cooperation between the fellow's home and host institutions

The placement of the fellow at the BfR in the course of the EU-FORA fellowship programme provided a unique opportunity to lay the foundations for future collaborations in the assessment of the applicability of modern methodologies in risk assessment research.

The 'Effect-based Analytics and Toxicogenomics Unit' at the BfR hosts the 'National Reference Laboratory for Animal protein in Feed (NRL-AP)'. The NRL-AP performs research on the prevention of food and feed fraud, allergen detection and risk assessment with the overall aim to provide farmed animals and food consumers trustful, healthy and low risk feed and food along the whole chain from farm to fork. Safe feed and food are also areas of research that are central to the fellow's home institution, the IMR.[1]

Currently, researchers at both institutes are working independently to develop molecular tools for food and feed safety risk assessment. At the IMR, in Norwegian Research Council (NRC) funded projects (NRC: 227387[11] and NRC: 268344[12]), in collaboration with the European Reference Laboratory for Animal Proteins in Feedstuffs (CRA-W), the University of Namur (UN) and the Leiden University Medical Centre (LUMC), global spectral library-based mass spectrometry methods are being developed for species- and tissue-specific differentiation of processed animal proteins (PAP) (Rasinger et al., 2016). In the course of this work, databases were created that are suitable for large-scale data mining for tissue- and species-specific peptide markers and the *in silico* prediction of potentially bioactive peptides such as allergens. Research at the BfR in projects funded by the German Federal Ministry of Food and Agriculture currently also focuses on mass spectrometry methods for detection of terrestrial animal species in highly processed feed. Unlike the global spectral library-based methods developed at the IMR, researchers at the BfR and its collaboration partner, the Natural and Medical Sciences Institute at the University of Tübingen (NMI), focus on targeted mass spectrometry analysis (Steinhilber et al., 2018). This approach allows a more accurate estimation of the abundance of selected PAP animal species in unknown samples, but strongly relies on the discovery of suitable marker peptides, which the IMR database can provide. In other words, combining these two complementary approaches will allow a more comprehensive proteomics-based screening and characterisation of proteic material.

Based on the common interest and complementary experience in the application of 'omics tools for PAP detection, the fellow and his home institute were named key collaborators in the Unit 51's EU-FORA hosting site application for the second cycle starting in autumn 2018. The application was successful and the Unit's next EU-FORA fellow will be working on a project entitled 'The use of novel DNA- and mass spectrometry-based detection methods for the identification of potential allergenic species and food authentication'; the mass spectrometry-based work will be conducted in close cooperation with the former fellow from the IMR.

In addition to the EU-FORA hosting site application, the fellow and his supervisor also drafted a joint application for funding under the NRC INTPART (International Partnerships for Excellent Education, Research and Innovation) programme.[13] The objective of the INTPART programme is to develop world-class research and education in Norway through long-term international cooperation and will provide funding for the establishment and further development of the institutional cooperation on

[11] https://www.forskningsradet.no/prosjektbanken/#/project/NFR/227387/Sprak=en
[12] https://www.forskningsradet.no/prosjektbanken/#/project/NFR/268344/Sprak=en
[13] https://www.forskningsradet.no/en/Funding/INTPART/1254007331831

research and higher education. The project proposal was entitled 'Food and feed risk assessment in a circular economy' and aims to set up a long-term research and education partnership between the IMR, a globally recognised institute for aquaculture and fisheries research, and the BfR, a leading European centre for risk assessment. At the time of writing, the project proposal was under review by the NRC.

3. Conclusions

In the course of the EU-FORA fellowship programme, the fellow was introduced to and gained experience in the use of modern *in silico* tools for chemical hazard assessment. In this process, the fellow learned best practice approaches for QSAR-based data analysis principles and strengthened personal skills related to the refinement of computational tools for data management and data mining. The EU-FORA project at the BfR contributed to the continuous further education of the fellow in the use of *in silico* toxicology tools, corroborated findings related to the safety of food contaminants and laid the foundations for future collaboration between the fellow's home institution, the IMR in Norway, and the BfR in Germany.

In addition to the practical 'learning by doing' education, the EU-FORA programme also provided for a rich social experience both during the training modules and the placement at the BfR. During the theoretical models close ties and valuable personal and professional networks were formed with other fellows, course tutors and course organisers. At the BfR, under the lead of Dr Gollnick, the very competent and helpful International Affairs team organised an introductory workshop to the BfR and various international events throughout the year. These fora proved a very valuable way to quickly become familiar with the institute and the city of Berlin and to establish close links and friendships with international guest scientists and resident researchers and staff at the BfR.

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Abbreviations

2-MCPD	2-chloro-1,3,-propanediol
3-MCPD	3-chloro-1,2,-propanediol
AGES	Austrian Agency for Health and Food Safety
BfR	German Federal Institute for Risk Assessment
CRA-W	European Reference Laboratory for Animal Proteins in Feedstuffs
EFET	Hellenic Food Authority
EPA	Environmental Protection Agency
EQ	6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline
EU-FORA	The European Food Risk Assessment Fellowship Programme
FDA	Food and Drug Administration
GMO	genetically modified organisms
IMR	Institute of Marine Research
InChIKeys	International Chemical Identifier Keys
INTPART	International Partnerships for Excellent Education, Research and Innovation
ITS	Integrated Testing Strategies
lazar	lazy structure–activity relationships
LUMC	Leiden University Medical Centre
NMI	Natural and Medical Sciences Institute at the University of Tübingen
NRC	Norwegian Research Council
NRL-AP	National Reference Laboratory for Animal protein in Feed
OECD	Organisation for Economic Co-operation and Development
PAP	processed animal proteins
QSAR	quantitative structure–activity relationship
QTOFMS	quadrupole time-of-flight mass spectrometry
SDF	structured data files
SMILES	simplified molecular input line entry system
T.E.S.T	Toxicity Estimation Software Tool
TP	transformation products
TWIMS	travelling-wave ion mobility spectrometry
UN	University of Namur
WoE	Weight-of-Evidence