UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
"Scienze degli alimenti"

CICLO: XXXII

New developments in harmonised risk assessment of emerging chemical hazards: "Chemical mixtures"

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Anni 2016-2019

Summary

Multiple chemicals known as “chemical mixtures” are ubiquitous, in our body and in the environment through food, the air, drinking water, while our skin can also be exposed to contaminants in soil and dust. Toxins can enter an organism via single or combined oral, dermal and inhalation routes. Hence, the number of chemicals and their combinations is practically infinite, rendering their assessment a very challenging task. There are numerous studies in the literature demonstrating that chemicals, when administered together, mostly induce additive combined toxicity while antagonistic, potentiation or even synergistic effects are less common; depending on composition, exposure and mode of action (MoA). Thus, combined toxicity should be taken into account when a risk assessment is performed particularly during the risk characterisation step to avoid underestimation of the risk. In recent years, the European Commission has requested further development of methods for the risk assessment of chemical mixtures and has highlighted the importance of identifying data gaps and related uncertainties in our current knowledge. In addition, risk assessment of chemical mixtures is included in recent food safety regulations. Over the years, various national and international scientific advisory bodies as well as regulatory agencies have published reports and guidance documents, addressing the risk assessment of chemical mixtures for human health, animal health and the environment.

The present thesis is divided into six chapters, which discuss different aspects of the risk assessment of chemical mixtures. **Chapter 1** provides an overview of the methods dealing with risk assessment of combined exposure to chemicals. **Chapter 2** reviews available knowledge and data gaps on single and multiple mycotoxins including their co-occurrence in the field, pre- and

post-mitigation practices as well as their toxicokinetics in test species and farm animals. **Chapter 3** investigates the relevance of metabolic interactions between human pharmaceuticals and chemicals of relevance to food safety (i.e. pesticides and flavonoids) particularly, concerning enzymes involved in xenobiotic metabolism (phase I, phase II and transporters). This has been specifically addressed through *in vitro* data collection reporting quantitative inhibition parameters (K_i, IC_{50}, % of inhibition) and extrapolation from *in vitro* to *in vivo* kinetics. **Chapter 4** provides an analysis of combined toxicity data on binary pesticide mixtures in test species. The analysis focuses on the quantification of the magnitudes of interaction after acute, subacute, sub-chronic or chronic exposure and expressed as a mean effect ratio for single compounds and binary mixtures. Further, this provides a transparent reporting of measured toxicological endpoints as well as a mean to test of the dose dependency of toxicological interactions. In **Chapter 5**, human exposure to phthalates is estimated using a probabilistic approach and the outcome is then compared with measured phthalate concentrations resulting from a biomonitoring study conducted in Norway. Finally, risk characterisation is performed for single phthalates as well as their combined exposure. **Chapter 6** provides a discussion of the methodologies applied in this thesis in comparison to those available in the peer-reviewed literature and, underlines challenges for the risk assessment of combined exposure to chemical mixtures and highlights the need for further work in this area of high relevance to human health, animal health and the environment.

Acknowledgments

First and foremost, I would like to thank my parents. Their personal sacrifices all these years provided me with the necessary tools to go forward with my career and my life.

I would like to express my gratitude to my promotor Chiara Dall'Asta for giving me the opportunity to work on a great project and enhance my knowledge in the field of risk assessment. I want to thank you most sincerely for your guidance, prompt responding and feedback. Also, for granting me freedom and unlimited support during all these years!

I would like to acknowledge my co-promotor Jean-Lou Dorne. Thank you for contacting me 4 years ago and letting me know of this great project with EFSA. I cannot thank you enough for always being available (even at the expense of your private time) to help me, encourage me and guide me. Thank you for being a great mentor!

Luca, thank you for the excellent collaboration, your availability and your valuable assistance. I really enjoyed the talks we had on every possible topic! I wish you all the best and great success in your career and personal life.

Everyone in the department: Laura, Marco, Tito, Francesco, Giulia, Veronica and Alessandra; thank you all for creating a great atmosphere to work in. I hope each and every one of you achieves your professional and personal goals.

To my colleague in Piacenza, Roberta: many thanks for the excellent collaboration during the MYCHIF project. Also, for your accessibility and support over the past 3 years. Best of luck with everything you do in the future.

To the guys at ANSES. First, I would like to thank Camille for giving me the opportunity to work on a very interesting project and for being available and

eager to answer any question I had. To Keyvin and Leonie, many thanks for your time, continuous assistance and guidance during my time there. I would also like to thank Chris and Marinho for all their support and for being so efficient in making all administrative problems disappear. All my “lunch buddies”: Thomas, Frank, Mathias, Natacha, Marie (2x), Sonia, Fabrizio, Claire, Jocelyn and Romain, thank you for welcoming me and allowing me to easily integrate in the institute.

A big thank you also to all my colleagues at NIPH and especially to Trine Husøy for the excellent collaboration and guidance during my year in Oslo.

I would also like to thank José, Edoardo and Maryam for the great collaboration and assistance during the first year of the PhD.

Last but not least, Rodolfo and Daniele: I had a great time hanging out with you, thanks for all the nice memories. I wish you all the best in your personal and professional life.

Thank you all!

July 2020

Thanasis

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Abbreviations list

15-Ac-DON	15-acetyldeoxynivalenol
3-Ac-DON	3-acetyldeoxynivalenol
ADI	Acceptable daily intake
ADME	Absorption, Distribution, Metabolism, Excretion
AFB1	Aflatoxin B1
AFs	Aflatoxins
Ahr	aryl hydrocarbon receptor
AOP	Adverse outcome pathway
AUC	Area under the curve
BBP	Butyl-benzyl-phthalate
BEA	Beauvericin
BMDL	Benchmark dose modelling
BSE	bovine spongiform encephalopathy
bw	Body weight
CA	Concentration addition
CI	Combination index method
CIT	Citrinin
CL	Clearance
C_{max}	Maximum concentration of a toxin
CYP	Cytochrome P450
D3G	Deoxynivalenol-3-β-D-glucoside
DBP	Di-n-butyl phthalate
DEHP	Di-2-ethylhexyl phthalate
DEP	Diethyl phthalate
DINCH	Di(isononyl)cyclohexane-1,2-clicarboxylate
DINP	Di-iso-nonyl phthalate
DON	Deoxynivalenol
EDI	Estimated daily intake
EFSA	European Food Safety Authority
ELS	Extensive literature search
EU	European Union
EUROSTAT	European Statistical Office
FB1	Fumonisin B1
FBs	Fumonisins
FDA	Food and Drug Administration
FX	Fusarenon-X
GI	Gastrointestinal
H2020	Horizon 2020
HBGV	Health Base Guidance Values
HepG2	Liver hepatocellular carcinoma cells
HI	Hazard Index
HK2	Human kidney cells
HQ	Hazard quotient
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
IVIVE	*In vitro* to *in vivo* extrapolations
Ki	Constant of inhibition
Km	Michaelis constant

LB	Lower bound
LOD	Limit of detection
LOQ	Limit of quantification
MB	Middle bound
MoA	Mode of action
MOE	Margin of exposure
MRLs	Maximum residue levels
mRNA	Messenger RNA
MRP	Multiresistant drug protein
NAMs	New Alternative Methods
NDs	Non-detects
NIV	Nivalenol
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
OTA	Ochratoxin A
PBK	Physiologically based kinetic model
P-gp	P-glycoprotein
POPs	Persistent organic pollutants
PVC	Polyvinyl chloride
QSAR	Quantitative structure-activity relationship
RAM	Rate of metabolism
RASF	Rapid Alert System for Food
RPF	Relative potency factor
T1/2	Elimination half-time of a toxin
TD	Toxicodynamics
TDI	Tolerable daily intake
TDS	Total diet study
TK	Toxicokinetics
UB	Upper bound
UF	Uncertainty factor
UGTs	UDP-glucuronyl transferases
US EPA	United States Environmental Protection Agency
V_{max}	Maximal velocity of a reaction
VOCs	Volatile organic compounds
WHO	World Health Organisation
ZEN	Zearalenone
ZEN-14Glc	Zearalenone-14-glucoside
ZEN-14GlcA	Zearalenone-14-glucuronide
α-ZEL	α-zearalenol
β-ZEL	β-zearalenol

Chapter 1

Risk assessment methodologies for single and chemical mixtures

1.1 Background and importance

Over the 1990s, various food related crises broke out in the European Union (EU), alerting the public and undermining people's perception of food production and distribution safety. For example, the crises caused by bovine spongiform encephalopathy (BSE), dioxins and Salmonella led the EU to create in 2000 the White paper on food safety, and subsequently the General Food Law (178/2002) and the European Food Safety Authority (EFSA), with the ultimate goal of guaranteeing food safety in the Union. Nowadays, thanks to the broad and increasing use of the internet and the social media, information (and misinformation) is travelling rapidly, making the public aware of the risks and hazards involved in their food. People are trying to be more conscious of what they eat and often criticise scientific opinions as being biased and serving pro-industry purposes, as happened in the recent case of glyphosate and the assessments of the European Food Safety Authority and the European Chemicals Agency.

The safety of food commodities is tested daily since the "farm to fork" chain is becoming more and more complex, multiple stakeholders are involved and different kinds of hazards (chemical, microbiological and physical) are always present and must be dealt with in time, lest they become risks that would jeopardise public health.

According to the European Food Safety Authority, an emerging risk is "A risk resulting from a newly identified hazard to which a significant exposure may occur, or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard."

Emerging risks may arise from three types of hazard (Noteborn et al., 2005):

- an unidentified new form of a known hazard (i.e. an unidentified mycotoxin)
- a not well-known hazard (i.e.nanoparticles, endocrine disrupter)
- a well-known re-emerging hazard (i.e. food packaging residues)

According to (EC) No 178/2002, one of the tasks of the European Food Safety Authority is the identification and evaluation of emerging risks in the food chain with the aim of containing or eliminating them. Article 34 (EC., 178/2002) states that the authority should establish monitoring procedures and systematically search and analyse information on emerging risks. A monitoring network has since been established in close cooperation with the EU member states and other involved stakeholders. Chemical mixtures are one of the main issues that have been identified by EFSA since 2008 and its' Opinion on the identification of approaches for the assessment of cumulative risks from pesticides. In past years, EFSA produced a number of opinions on mixtures (EFSA, 2013a; EFSA, 2013a; Quignot et al., 2015) and more recently in 2019 (EFSA, 2019a; EFSA, 2019b). All this work demonstrates that EFSA regards mixtures as a highly important emerging issue.

1.1.1 Background on risk assessment of combined exposure to chemical mixtures

Humans and animal species are exposed daily to an intractable number of different combinations of chemicals via food, water, air, soil, product materials and consumer goods. The compounds can enter our body through oral, dermal or inhalation routes or through all of these routes combined. Moreover, our body is a chemical mixture in and of itself, and when interacting with the incoming compounds, it renders mixture assessment even more complex (via biotransformation). Consequently, the toxic effects occurring might be more serious than those that the parent compound would have caused (e.g. masked mycotoxins), while the amount of chemicals that remain unidentified is also vastly increased. Moreover, it is known that not all chemicals in our organism are toxic, but the combined effects of a flavonoid, for example, or other antioxidants with a toxin (i.e. genistein with vinclozolin) could lead to adverse health effects, and therefore this type of assessment should also be considered for a reliable risk analysis to be produced. All the above make the number of chemicals and their combinations practically infinite and their assessment a very challenging task for risk assessors, regulators and decision makers.

In 1986, the Environmental Protection Agency published guidelines for the health risk assessment of chemical mixtures, and various studies have been published since. They demonstrate that chemicals, when administered together, may cause additive, antagonistic, potentiating or in rare cases even synergistic effects, depending on their chemical composition, mode of action and dose of exposure (Rizzati et al., 2016; Cedergreen, 2014; Boobis et al., 2011; Kortenkamp et al., 2009 and Moser et al 2006). The European legislation included mixtures when addressing pesticides and dioxins (EC 396/2005; EC 315/93 and EC 1881/2006). In 2012, the European Commission published a

report about chemical mixtures, elaborating on the dangers they pose and highlighting both the importance of their assessment and the gaps in our knowledge. (EC, 2012). Various national and international authorities as well as legislative bodies have published reports and opinions over the years, addressing the great and increasing uncertainty created by the concurrent administration of chemicals, and they have also published various guidelines for their assessment (i.e. WHO, 2009; EFSA, 2008; EFSA, 2009; US EPA 1986; Reffstrup, 2002; VKM, 2008; Bopp et al., 2015; RIVM, 2017). Moreover, there are various collaborative projects running currently in Europe financed by the Horizon 2020 and the 7th Framework programmes (e.g. EUROMIX, MYCHIF, EDC-MixRisk, EUToxRisk, HBM4EU), whose aim is to generate additional exposure data, develop predictive tools and identify remaining gaps so as to offer an insight into this very hot topic. A very detailed review of the main ongoing EU projects was published by Bopp et al. (2018).

1.1.2 Principles of combined toxicity

When researching combined exposure from chemical mixtures, it is important to assess whether an interaction can take place or not, and whether that interaction can lead to an antagonistic or even a synergistic effect. These phenomena do not necessarily reflect real life, since we are simultaneously exposed to more than one of these concepts and to an infinite number of chemicals, thus making their assessment even more challenging. Additionally, more than one of these principles may apply at the same time: for example, in dose-dependent mixtures the final effect of an exposure to a chemical mixture may vary considerably depending on the different dosage of each chemical. In the literature, there are examples of dose-dependent interactions where the same chemicals have synergistic effects at some levels and additive or even

antagonistic effects at different concentrations (Jonker et al., 2005; Crofton et al., 2005; Moser et al., 2005).

Table 1.1 Basic concepts and terminology used to describe the combined actions of chemicals in mixtures (adapted from VKM, 2008).

	Similar mechanism	**Dissimilar mechanisms**
No interactions	Simple similar action (Loewe additivity, Dose addition)	Simple dissimilar action (Bliss independence, Response (effect) addition)
Interactions	Complex similar action (Loewe synergism or antagonism)	Complex dissimilar action (Bliss synergism or antagonism)

1.1.2.1 Combined toxicity, dose addition and response addition

Combined toxicity of chemical mixtures refers to simple similar or dissimilar mode of action for which the chemicals do not interact. A mechanism of action includes the specific molecular targets (enzyme or receptor) to which a chemical binds, while the mode of action describes the functional changes at a cellular level from exposure of an organism to a substance.

As can be seen in Table 1.1, these chemicals can be divided into chemicals with a similar and chemicals with a dissimilar mode of action. When the compounds are of a similar mechanism, they can be assessed by means of Lowe additivity (1.1) or dose addition. On the other hand, when they have a different mechanism of action, Bliss independence or response addition could be used.

In the case of simple similar action, the effect would be the result of the sum of the contributing dose of each chemical. This means that, even if the doses of the chemicals are below the dose threshold, the final effect of the mixture

may be above the dose threshold, thus causing an adverse effect to the organism.

Loewe additivity: $$\frac{d1}{De1} + \frac{d2}{De2} = 1 \quad (1.1)$$

d1= dose of compound 1

d2= dose of compound 2

De1= dose of compound 1 to produce the effect e on its own

De2= dose of compound 2 to produce the effect e on its own

In simple dissimilar action, the presence of one chemical will not affect the toxicity of another, since each chemical may have different target sites or affect different sites in the organism.

The Bliss independence model is based on the principle that the compound effects are the result of a probabilistic process whereby chemicals act independently, meaning that none of the chemicals interferes with any of the others.

Bliss probabilistic independence:

$$Ea + Eb\,(1 - Ea) = Ea + Eb - EaEb \quad (1.2)$$

Ea = effect of chemical a

Eb = effect of chemical b and $0 \leq Ea \leq 1$ and $0 \leq Eb \leq 1$

Response addition is not really used in human risk assessment, since the reference values (i.e. NOAELs, BMDL) are values below the occurrence of a response. For the prediction of the mixture effect, when dose addition is not present and independent action is assumed, EFSA recommends using the dose-response curve of Benchmark Dose Modelling (EFSA, 2019b).

1.1.2.2 Interactions

Interactions between chemicals may occur on a toxicokinetic level (influencing absorption, distribution, metabolism, excretion) and are of concern since they may affect the internal dose of the compounds when those are exposed in a mixture rather than individually. Moreover, there can be interactions on a toxicodynamic level where a change in the response can be observed. In this case, combined toxicity can be a) synergistic, when the combined effect of the compounds is greater than the sum of the effects of each chemical given alone, or b) antagonistic, when the combined effect of chemicals is smaller than the sum of the effects of each chemical given alone. Interactions can occur at the cellular receptor site or at different sites of the same molecule (Norwegian Scientific Committee for Food Safety, 2008). In the case that two compounds act on the same target receptor and have complex similar action, then the final effect will not be additive, but either antagonistic or synergistic – if, for example, the substances compete for the same hormonal or enzymatic receptor sites (antagonism). Complex dissimilar actions, on the other hand, can be observed when two chemicals are involved in enzyme induction or inhibition in the toxicokinetic phase. Changes in the biotransformation of the compounds resulting either in the production of bioactivated substances or in an increase in the detoxified agents, influencing the final toxicity of the chemicals (Dekant, 2009; Ioannides and Lewis 2004).

1.2. Principles of human risk assessment of chemicals

Risk assessment plays an integral part in risk analysis along with risk communication and risk management. Its development is due to the need for taking decisions that will protect public health. It consists of qualitative and quantitative scientific analyses of the likelihood of harm associated with exposure to a chemical (WHO, 2010). The process of risk assessment can be conceptualised in a model (Figure 1.1) and consists of four steps: i) hazard identification, ii) exposure assessment, iii) hazard characterisation and iv) risk characterisation. The ultimate goal is to examine whether the estimated daily intake exceeds the safety limit and to give advice useful for decision-making.

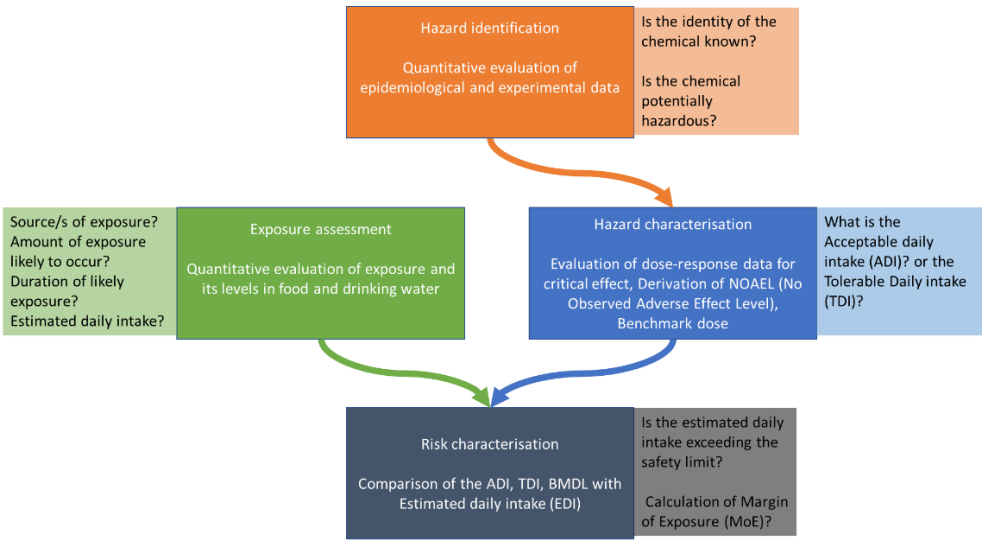


Figure 1.1 Conceptual model for the risk assessment process

1.2.1 Hazard identification

Hazard identification is defined as: *"The identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system, or (sub) population. Hazard identification is the first stage in hazard assessment and the first of four steps in risk assessment"* (WHO, 2004).

At this stage, two questions are addressed: i) the nature of any health hazard a chemical may inflict and ii) the conditions under which an identified hazard can be expressed. Analyses of scientific data range from observations in humans and farm animals to observations in laboratory animals, *in vitro* testing and structure-activity relationships. In this step of risk assessment, the adverse effects of the substance are described, including affected target organs and target tissues. Additionally, the extent of the population that may be at risk and the potential risks for specific population groups are discussed (WHO, 2009).

1.2.2 Hazard characterisation

The second stage of risk assessment is the hazard characterisation of a chemical. Hazard characterisation is the qualitative or quantitative description of the inherent properties of an agent that can potentially cause adverse effects. Where possible, it should include dose-response estimations, including uncertainties (WHO, 2004). In hazard characterisation, the relationship between the administered dose and the adverse effect is described. Moreover, the toxic threshold is calculated based on the type of the adverse effect. The aim of hazard characterisation is to establish health-based guidance values (HBGVs), i.e. the Acceptable Daily Intake (ADI), or the Tolerable Daily Intake (TDI), as well as the application of uncertainty factors (WHO, 2009). The

default uncertainty factor (100) is taking into account, toxicokinetic and toxicodynamic data for inter- and intraspecies differences. The 100-fold uncertainty factor has been widely used since its first introduction by Lehman and Fitszhugh (1954). More recently, it has been replaced by the framework proposed by Meek et al (2002) as shown in Figure 1.2

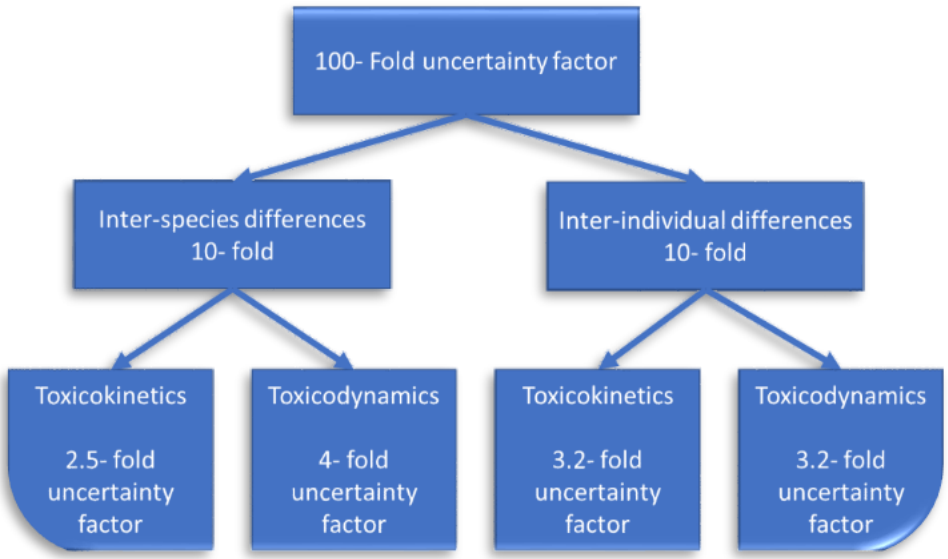


Figure 1.2 Sub-division of the 100-fold default uncertainty factor (adapted from Meek et al., 2002)

1.2.3 Exposure assessment

The Codex Alimentarius (FAO/WHO, 2008) defines exposure assessment as "the qualitative and/or quantitative evaluation of the likely intake of a chemical agent via food and other relevant sources". Dietary exposure is the combination of food consumption data (average and high consumers) with the concentration of the chemical of interest in food.

According to (WHO, 2009), the general equation (1.3) for both acute and chronic dietary exposure to a chemical (i.e. c) is:

$$\textit{Dietary exposure to chemical (c)} = \frac{\sum (food\ quantity\ x\ concentration\ of\ a\ substance)}{body\ weight\ (kg)} \qquad (1.3)$$

Exposure is an important element in risk analysis, since it is compared to the HBGVs in the risk characterisation step to determine if a xenobiotic does indeed pose a risk for the intended consumers. Occurrence data are combined with consumption data from across Europe and thus the exposure of the contaminants is calculated. The population can also be divided into specific groups according to high, medium or low consumption. Additionally, special risk groups should be identified, such as infants, toddlers, or people with different dietary habits (vegetarians, vegans, ethnic groups etc.). Depending on the nature of the toxin and the available data, acute and/or chronic exposure assessments are performed by means of deterministic or probabilistic modelling so a more reliable exposure estimate is produced (EFSA, 2012a). A deterministic model is a simple method of assessing dietary exposure by making use of data on one single virtual consumer (WHO, 1997). By contrast, probabilistic modelling accounts for the distribution of intakes amongst multiple individuals of a population, taking also into account intraspecies variability and the occurrence of residues in food commodities (EFSA, 2012b).

1.2.4 Risk Characterisation

Risk characterisation is the final step of the risk assessment process, whereby all the information gathered in the hazard assessment and the exposure assessment steps are integrated to answer the question whether, and if so, at what level the chemical poses a risk for the targeted population. The outcome of risk characterisation depends also on the nature of the compound in question. If the compound is an additive or a pesticide or any contaminant that can be avoided, then an Acceptable Daily Intake (ADI) can be established. If the contaminant is unavoidable (i.e. heavy metals, mycotoxins, heat treatment products), a Tolerable Daily Intake (TDI) can be calculated. In either case, a Hazard Quotient (HQ) is established to evaluate if the exposure level is acceptable/tolerable or not. The HQ is a simple ratio between the Estimated Daily Intake (EDI) and either the ADI or the TDI. The HQ (EDI/ADI or EDI/TDI) should be < 1 in order for the exposure to be considered acceptable/tolerable (EFSA, 2013a). A compound can also be a genotoxic carcinogen (carcinogen that directly interacts with the DNA) (e.g. benzo(α)pyrene, aflatoxin B1). If the chemical is avoidable, then tolerance is zero. If, on the other hand, the genotoxic carcinogen is unavoidable, then the following principles can be taken into account: i) as low as reasonably achievable (ALARA), and ii) margin of exposure (MOE). Of these two methods, the one preferred for the assessment of genotoxic carcinogens is the MOE, as the ALARA method does not take into account either human exposure or the potency of the carcinogen (WHO, 2009). The MOE was proposed by EFSA in 2005 as the appropriate method for the characterisation of compounds that are both genotoxic and carcinogenic. In order to calculate the MOE, the reference point on the dose-response curve (from animal experiments in the absence of human data) is divided by the EDI. The reference point used in the benchmark dose lower confidence limit 10% ($BMDL_{10}$),

which is the lower bound of the 95% confidence interval on a benchmark dose corresponding to a 10% response. An MOE of 10.000 or higher, based on $BMDL_{10}$ from animal studies and taking into account the uncertainties would be of low concern (EFSA, 2005).

Risk characterisation marks the end of risk assessment and its outcome can then be used by risk managers and decision makers in taking managerial decisions. In the process of risk management, the stakeholders involved need also to take into account political, social, economic and environmental factors, and reach a final decision based on the current available knowledge. The process of risk management is continuous, and food standards and related opinions should be reviewed regularly and updated, if necessary, to reflect the newest scientific knowledge available (Codex Alimentarius, 2007).

1.3. Principles of risk assessment for combined exposure to chemical mixtures

1.3.1 Problem formulation

Problem formulation is an iterative process between risk assessors and risk managers/decision makers. It is considered a prerequisite for risk assessment and can be especially useful for mixture risk assessment, which is a considerably more complex issue (EPA, 2007; Meek et al., 2011; Solomon et al., 2016; OECD, 2018; EFSA, 2019b). It was first introduced as guidance for environmental risk assessments (EPA, 1992). It is a systematic approach that takes into account initial research questions, includes an early identification of the possible chemicals (origin, composition, reactivity), and creates a conceptual model which describes the exposure sources, the pathway routes, the endpoints to be considered and their potential relationships (EFSA, 2019b; Solomon et al., 2016). Deciding on the approach (whole mixture or component-based assessment) and finally generation of an analysis plan that will include information on the data/methods/models to be used, tiering approaches, valuation of data gaps and uncertainties (OECD, 2018; EFSA, 2019b).

Problem formulation is one of the first steps of risk analysis (Figure 1.3) whereby the preliminary hypotheses are developed on the basis of possible occurrence and toxic effects of exposure to chemical mixtures simultaneously. It is of paramount importance that risk assessors and risk managers be in close collaboration with one another during this step in order that they guarantee the correct formulation of the research questions and assign realistic timeframes for the completion of the risk assessment so that the output is clear and relevant

to the question posed by the respective stakeholder (risk manager, regulatory authority, member state etc.).

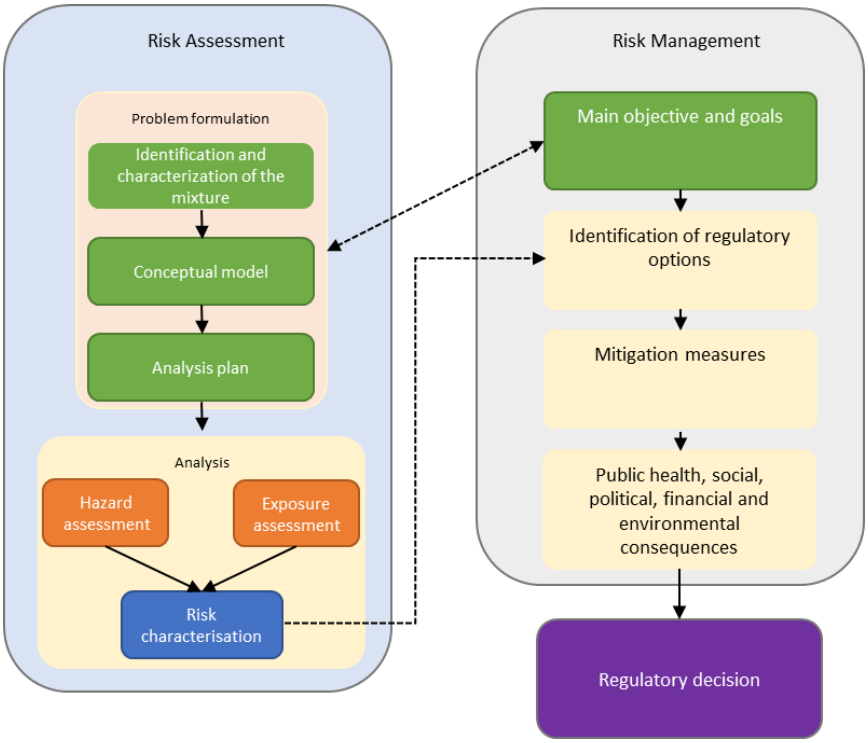


Figure 1.3 Representation of the risk assessment and risk management processes and their interactions (dotted arrows) (modified from Solomon et al., 2016).

1.3.2 Harmonised framework

Problem formulation is followed by the hazard and exposure assessment steps, which are taken either consecutively or in parallel. EFSA (2019) proposed a harmonised framework (Figure 1.4) which includes those factors that affect aspects of risk analysis such as exposure assessment, hazard assessment and risk characterisation. Each step is important in the context of combined exposure to chemical mixtures and the different steps of risk assessment are described in more detail in the following sections.

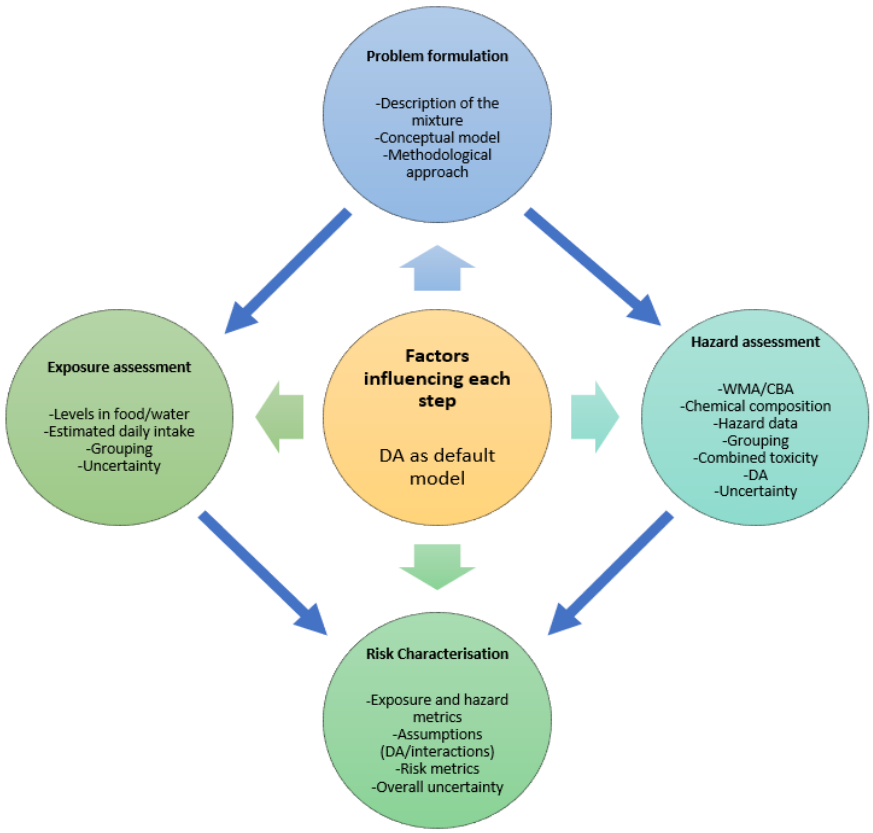


Figure 1.4 Overarching framework for human and animal risk assessment of combined chemicals (modified from EFSA, 2019b) WMA: whole mixture approach, CBA: component-based approach, DA: dose addition

1.3.3 Exposure assessment

Exposure assessment of chemical mixtures is one of the most challenging steps in risk analysis. An organism may be exposed to various chemicals from different sources (i.e. food, water, air). The tiered method (Figure 1.5) is the approach recommended by Meek et al. (2011) and also by other institutions (OECD, 2018; EFSA, 2019b).

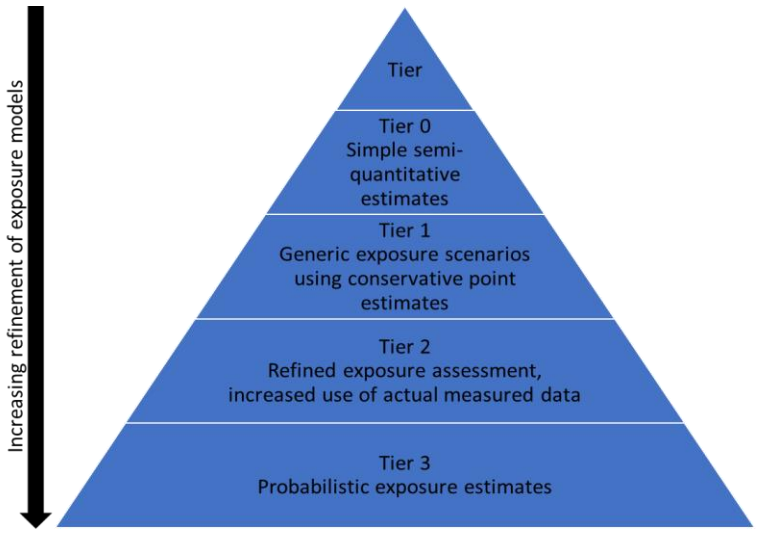


Figure 1.5 Exposure assessment, tiered approach (modified from Meek et al., 2011)

A more detailed representation of the tiered approach for the exposure assessment of mixtures, including the type of data needed depending on the nature of available data as well as the limitations of each tier, is presented (Table 1.2).

In the case of concurrent exposure to more than one chemical, the possible scenarios are as follows: i) acute exposure; a food consumption value can be selected and the concentration values for the compounds can be selected randomly from the concentration range of the chemicals (or from the high

percentile). ii) Chronic exposure; the mean consumed quantity per time of interest (days, weeks) and the mean concentrations of the compounds are selected. The low tiers are based on generic exposure scenarios following deterministic point estimates for both food consumption and chemical contaminant levels. In Tier 2 the exposure estimate is more refined and is determined by a deterministic/semi-probabilistic approach, taking into account i.e. EFSA consumption databases, including variability for the population in question. Finally, in Tier 3, a fully probabilistic assessment can be performed and the risk assessor can make use of distributions of chemical occurrence and individual consumption patterns. Moreover, in this tier, human biomonitoring data are available and can be utilised for the estimation of the internal dose and the toxicodynamics of each exposed chemical (EFSA, 2013a).

Table 1.2 Exposure assessment for chemical mixtures (modified from OECD, 2018)

	Exposure scenario	**Approach**	**Limitation**
Tier 0	Long term and widespread use across several sectors	Broad assumptions based on predictive information or hypothetical scenarios	Analysis of exposure limited to one or two disciplines (e.g. consumer or environmental exposure)
Tier 1	Conservative upper bounding estimates of total intake based on simultaneous use of multiple consumer products and use pattern	Conservative estimates of highest exposed groups Consideration of chemicals in groups or based on combined exposure	Individual differences in exposure, duration, bodyweight are not considered Inconsistency between biological half-life and predicted
Tier 2	Monitoring data for different subgroups	Deterministic sensitivity analysis (low, medium or high exposure values)	More data required
Tier 3	Considerations of differences in absorption among substances, monitoring data for exposure distribution	Probabilistic modelling	Extensive data requirements Considerable development work is necessary Considerable representative information and relevant exposure scenarios

1.3.3.1 Whole mixture approach

The whole mixture approach is used in the exposure assessment of mixtures when the mixture of chemicals is treated as a single element. Mixtures can be divided into simple mixtures (pesticide/biocide formulations), in which the compounds are known and there is usually information on their single dose toxicity, and complex mixtures such as mixtures produced by a refining process, process emissions, by-products and wastes (IGHRC, 2008). When the whole mixture approach is employed, the following hypotheses can be made; the single elements do not interact with each other, the components and concentrations do not vary significantly over time, and the toxicity studies are performed on the whole mixture, without any information on the toxicity of the individual components (OECD, 2018). Figure 1.6 presents the stepwise approach to the exposure assessment of a whole mixture as developed by EFSA in 2019.

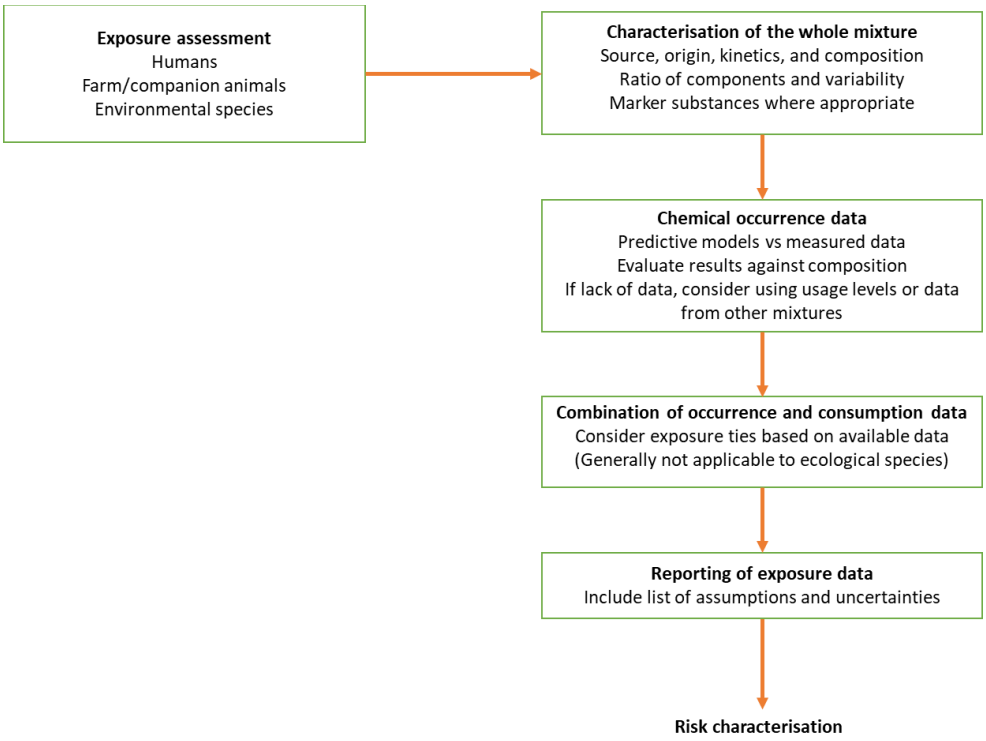


Figure 1.6 Exposure assessment using the whole mixture approach (adapted from EFSA, 2019b)

1.3.3.2 Component-based approach

In the component-based approach, the compounds of the mixture and their toxicological effects are considered as a group of individual substances. Thus, knowledge on the relative ratio of each component to the final toxicity is necessary. Additionally, possible chemical interactions need to be considered, although it is assumed that the components do not react chemically (OECD, 2018).

Therefore, when this approach is used for an exposure assessment of chemical mixtures, the exposure data for each individual compound are needed. Collecting data on the co-occurrence of the single constituents can provide a better understanding of the chemical relationship, including the

likelihood of co-occurrence during a set period. Moreover, the timescale should be determined in consultation with a toxicologist, so that important aspects such as the nature of toxicity and toxicokinetics be covered as well (EFSA, 2019b). Figure 1.7 below presents the stepwise approach to the exposure assessment of a compound-based mixture before risk characterisation, as developed by EFSA in 2019.

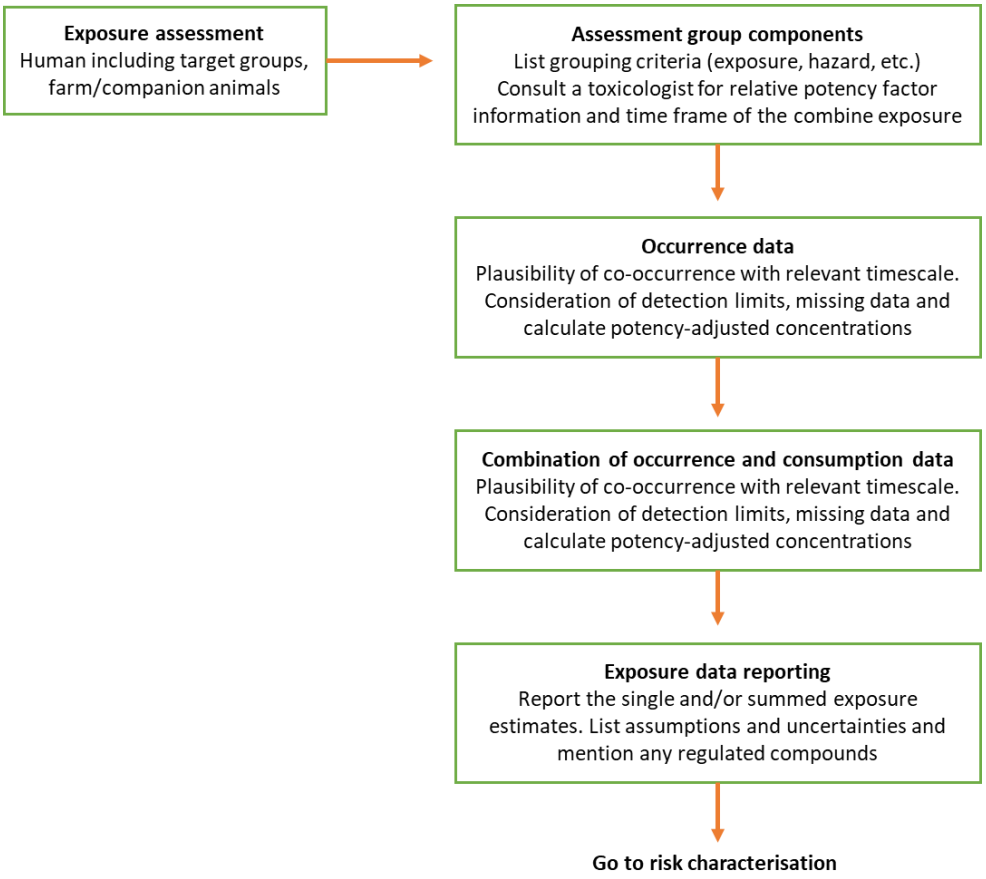


Figure 1.7 Exposure assessment using the component-based mixture approach (adapted from EFSA, 2019b)

1.3.4 Hazard assessment and risk characterisation of chemical mixtures

Hazard assessment consists of hazard identification and hazard characterisation. The hazard of a combined exposure to a chemical mixture can be assessed with the use of either the whole mixture or the component-based approach, depending on the available knowledge on the composition and the effects of the mixture. The outcome of the hazard assessment is then combined with the exposure data in order for the risk characterisation to be performed (EFSA, 2013a; WHO, 2009). If the composition is not well characterised, the whole mixture approach is used: the mixture is treated as a single chemical and dose-response data are used for its assessment. However, if the mixture compounds and their exposure are well defined, then the component-based approach could be used. The component-based approach employs the concepts of dose addition (similar action between chemicals) and response addition (independent action between chemicals). The default approach proposed by the European Food Safety Authority in the new guidelines for mixture risk assessment (2019) is dose addition. According to EFSA, this approach is the most conservative with regard to adverse effect prediction and can be more readily applied since it only requires data on exposure levels and references values (EFSA, 2019b). An important aspect of the component-based approach is that it examines whether the chemicals interact with one another, thus potentially causing either an antagonistic effect (less than additive, inhibition) or a synergistic effect (greater than additive). Interactions can occur either on the kinetic or on the dynamic aspects of the chemicals upon them entering the body. In toxicodynamics (TD), an interaction can occur between the toxicants on their mode of action, i.e. at a receptor site, a cell or an organ. On the toxicokinetic (TK) level, the interactions may occur at i.e. absorption, distribution, metabolism and excretion (Kinzler et al., 2014; EFSA, 2019b).

1.3.4.1 Whole mixture approach

The whole mixture approach is used in the hazard assessment of a chemical mixture when there are data for this mixture or when there are toxicological data for a mixture which is similar to the target mixture, both in its compounds and in the ratio of chemicals (EFSA, 2013a). This approach may not give an insight into the toxicity of the mixture, but it does provide the advantage of accounting for all the components, even for unidentified materials, as well as for any interactions among the chemical compounds. However, it is generally not a recommended approach (Boobis et al., 2011; EFSA 2012; EFSA, 2019b).

For the purposes of risk characterisation, the whole mixture is considered as a single chemical. If a reference value can be derived, then after the total uncertainty has been considered, the exposure is compared to the reference value, MOE or by HQ. If, on the other hand, a reference value cannot be established, then a MOE can be estimated from the exposure ratio with a reference point (i.e. NOAEL) (EFSA, 2019b).

1.3.4.2 Component-based approach

This approach is used for the hazard and risk characterisation of mixtures. It is based on the availability of information on the individual exposure and toxicity of each compound in the mixture (OECD, 2018).

For the hazard assessment of chemical mixtures, the chemicals can be grouped on the basis of different approaches (EFSA, 2019b) such as:

- weight of evidence (e.g. cumulative assessment groups) based on dose response information and knowledge on the mode of action
- dosimetry, use of toxicokinetics and kinetic modelling can be used for grouping refinement
- mechanistic data coming from OMIC techniques and *in vitro* models can be useful for group determination

With regard to risk characterisation, the default approach is dose addition, which includes the different methods summarised in Table 1.3.

Table 1.3 Risk characterisation methodologies when dose addition is assumed (OECD, 2018 and EFSA, 2019b)

Method	Calculation	Interpretation
Hazard Index (HI)	$\sum_{i=1}^{n} HQi$ with HQi = Exp_i/RV_i and $RV_i = RP_i/UF_i$	HI ≤ 1 acceptable risk HI ≥ 1 potential concern
Reference Point Index /Point of Departure	RPI = PODI = $\sum_{i=1}^{n} E\,xp_i/RP_i$	HI ≤ 1 acceptable risk HI ≥ 1 potential concern
Margin of exposure sum (MOET)	MOET= 1/[(1/MOE_i) +. . .+(1/MOE_n)]) Where $MOE_i = RP_i/Exp_i$ or $RP_{index}/(RPF_i * Exp_i)$	If MOET > 100 combined risk is acceptable
Relative Potency Factor (RPF)	RPF1 = TS1/TSi Mixture dose (Σ (Exposure * RPF)	Toxicity of the mixture is compared to the dose response curve of the index chemical
Toxic unit sum (ΣTU)	ΣTU = $\sum_{i=1}^{n} C_i$ / ECx_i Where TU = $[C_i]/[ECx_i]$	A ΣTU= 1 means that the mixture causes 50% effect (i.e. lethality)
Internal HI	IHQ_i = (Internal Exp_i/RV_i) IHI_i = ΣInternal Exp/ΣHQ	IHIi ≤ 1 acceptable risk IHIi ≥ 1 potential concern
Internal dose sum TU	IDTU = [Ci] * BAF/[Critical body residue] IDTUm = Σ IDU IDΣTU= $\sum_{i=1}^{n} IDTU_i$ with $IDTU_i = C_i * BAF_i/CBR_i$	If IDΣTU = 1 the mixture causes 50% effect

HI: Hazard Index; Expi: exposure of the individual chemical substances in the mixture; RVi: reference value of the individual chemical substance in the mixture (e.g. ADI or TDI); RI: Risk Index; PECi: predicted effect concentration of the individual chemical substance in the mixture; PNECi: predicted no effect concentration of the individual chemical substance in the mixture; RPI/PODI: Reference Point Index/Point of departure Index; RPi: reference point of the individual chemical substance in the mixture (e.g. NOAEL or BMDL); UF: uncertainty factor; MOE: Margin of Exposure; RPindex: reference point of the index chemical; MOET: Sum of margin of Exposures; MOEi: Margin of Exposure for chemical in the mixture; TU: Toxicity unit; Ci:concentration in media of chemical i in mixture; ECxi: Effect concentration of chemical substance i in the mixture (e.g. LD50,LC50, EC50, ECX); RPFi: relative potency factor of the individual chemical substance in the mixture; Internal HI: HI corrected for internal dose; Internal Exp: internal exposure for chemical i as a correction of the external dose (absorption, body burden, etc.); IHQi: Internal Hazard Quotient; Internal HI: Internal Hazard Index; HQ: Hazard Quotient; InternalSum TU: sum of TU corrected for internal dose; BAF: bioaccumulation factor.

1.3.5 Uncertainty analysis

Uncertainty analysis is one of the most challenging aspects of cumulative risk assessment. Uncertainty is defined as "lack of knowledge about specific factors, parameters or models" (EPA, 1997). It exists in every stage of the risk assessment process and gradually accumulates until its final stage.

Uncertainty should be taken into account and be explicitly reported at each step of the risk assessment. It should be documented with transparency and quantified to the degree that is scientifically possible (Codex, 2011).

When assessing chemical mixtures, there are multiple uncertainties such as:

- Type of the mixture (complex or simple)
- Inclusion of metabolites besides parent compounds
- Concurrent exposure or with a time-delay
- Chemical grouping assumption (dose addition)
- Lack of data on possible interactions
- Failure to consider multiple dosing of the single compounds in the mixture (dose dependent effects)
- Uncertainties concerning the modes of actions of chemicals
- Reference values and/or uncertainty factors
- Knowledge gaps and assumptions while performing predictive modelling, like physiologically based kinetic modelling (PBK), read-across, *in vitro* to *in vivo* extrapolations (IVIVE) and allometric scaling

Uncertainty can be characterised in different Tiers (1, 2 and 3) ranging from qualitative to deterministic and probabilistic respectively. More specifically, uncertainty can be characterised in qualitative terms without quantification– for example, there may be some text describing the magnitude, or standardised terms (Low, Medium, High) or scores (1-5, +/-) that were previously defined.

Additionally, uncertainty can be characterised quantitatively, with the use of one or more point-estimates i.e. a single representative as a conservative value, or two values demonstrating a range of uncertainty, or even a series of points representing estimates with varying levels of conservatism. The characterisation of uncertainty is considered probabilistic (Tier 3) when the outcome of uncertainty is characterised by a probabilistic distribution such as a Monte Carlo generated distribution (EFSA, 2006).

Uncertainties (qualitative and if possible quantitative) should be reported in a table at the end of the risk assessment, and finally it should be mentioned whether or not the overall uncertainty hinders the conclusion of the risk assessment (OECD, 2018; EFSA, 2019b). Guidelines including examples on how to report a risk assessment for chemical mixtures were published recently by EFSA, (2019). After reporting the uncertainties and the final uncertainty, the risk assessment is concluded and the evaluation of exposure/risk is determined as acceptable or not.

1.4 Aims of the thesis

There are growing concerns regarding exposure to chemical mixtures and the associated human health, animal health and environmental risks. Mixture toxicology has been characterised as the "holy grail" of toxicology because it gives a more realistic representation of the actual exposure, but also because there is an immense number of combinations between substances (Hartung, 2017). After exposure to only two substances, there are multiple variables that can influence the combined toxicity; to mention but some: various dosing, sequential exposure (timing of exposure), species differences and administration route. Reality is even more complex since exposure is not limited to only two chemicals or only to parent compounds (metabolism), but may also relate to a mixture of unknown composition, which amplifies the problem.

Having these considerations in mind, the following objectives have been set:

1. present the current state-of-the-art methodologies for mixture risk assessment;
2. investigate whether there are differences in a chemical's kinetics after exposure to food relevant chemical mixtures (binary);
3. investigate whether there are differences in a chemical's toxicity after concurrent exposure to chemical mixtures (binary);
4. evaluate, after mixture exposure, the potential influence of metabolic interactions on the single chemical's kinetics and subsequently on its toxicity;
5. perform probabilistic exposure estimates, compare with measured data and assess if mixture exposure will have an impact to the risk characterisation different to the one of the single chemicals.

Chapter 2

Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach – MYCHIF

2.1 Background and importance

MYCHIF was a project funded by EFSA (GP/EFSA/AFSCO/2016/01), started in January 2016 and concluded in September 2019. The main scope of MYCHIF was the development of modelling methods for assessing the risk of mycotoxin mixtures in food and feed, and identifying possible knowledge gaps. Literature searches were performed with the aim of collecting data on mycotoxin mixtures, ranging from occurrence in the primary production to health effects on animals and humans. Additionally, the data collected were used in the development of mycotoxin occurrence and toxicity models aimed to not only assess the chemical risk but also suggest mitigation measures. The project was divided into three different working packages (WPs), each of which produced a different scientific output. The task of WP1 was to identify multiple-mycotoxin occurrence data and mitigation practices performed in the primary production. It involved partners from the University of Sacred Heart in Piacenza (Italy) and the University of Minho (Portugal). The aim of WP2 was to collect toxicity data (toxicokinetic and toxicodynamic) on mycotoxins (single and mixtures) after their co-exposure to humans and animals. Moreover, in the same WP, other aspects of hazard identification were also

included, such as biomarkers (Italian National Research Institute of Health, ISS) and multi-mycotoxin analytical methods (University of Belfast, UK). The partners for this WP came from the University of Parma (Italy) and the French National Research Institute of Agronomy (INRA). Finally, the last WP (WP3) was responsible for data analysis including simulation techniques, modelling and sensitivity analysis. The main partner performing the above was the National Research Council, Institute of Biometeorology (IBIMET, Italy) in collaboration with all the aforementioned partners.

2.2 Occurrence and exposure data

Mycotoxin occurrence and co-occurrence may vary on an annual basis depending on climate and other environmental conditions (Ji et al., 2016; Eskola et al., 2018; Hojnik et al., 2019). Studies have shown that approximately 60% of the global food and feed samples are contaminated with mycotoxins (Pinotti et al., 2016; Streit et al., 2012; Gruber-Dorninger et al., 2019). For the year 2017, the European Rapid Alert System for Food (RASF) identified mycotoxins as one of the top 10 food hazards, taking into account the number of notifications in the European Union (EU), and as the second most significant food hazard coming from outside of the EU (RASF, 2018). Main mycotoxin-producing fungi include the genera *Aspergillus*, *Penicillium* and *Fusarium*. They are among the most potent natural toxins and their adverse effects range from acute toxicity including death (Azziz-Baumgartner et al., 2005) to chronic effects including alterations in tissues and organ functions such as in the hepatic, renal, intestinal, but also in the nervous, reproductive and immune systems (Bennett and Klich, 2003; Zain 2011; Marin et al., 2013; Oswald et al., 2005; Robert et al., 2017). In real life a fungus (i.e. *Fusarium graminearum*) can produce more than one parent compound (i.e. DON and

ZEN), and in addition, mycotoxins experience modification in the different pre- and post-harvest steps. While inside the plant, mycotoxins are metabolised, i.e. 3-Ac-DON, 15-Ac-DON, α-ZEL, β-ZEL, ZEN14Glc etc. Afterwards, when fed to animals, metabolites such as ZEN14GlcA, DON-3-glucuronide, α-ZEL 14GlcA, including the parent compounds and plant metabolites, can be detected. If heat degradation takes place, degradation metabolites are identified, i.e. 9-hydroxymethyl DON-lactone, DON-3-glucoside lactone (Figure 2.1). Humans, as final consumers, are exposed to a cocktail of chemicals that includes not only the mixture of the parent mycotoxins, but also all the metabolites from the pre- and post-harvest steps – making the task of their assessment even more challenging.

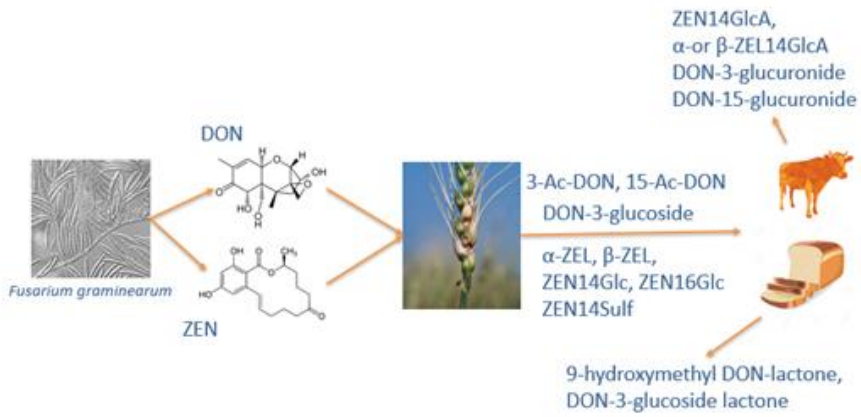


Figure 2.1 Example of the complexity of the total mycotoxin exposure count

2.2.1 Mycotoxin occurrence in maize and mitigation strategies

This section provides a summary of a data collection exercise relating to the occurrence of mycotoxin mixtures in maize as well as to key pre- and post-harvest mitigation measures that aim at reducing the co-occurrence of mycotoxins.

Maize is one of the principle cereals cultivated worldwide and is one of the main staple food and feed commodities. In 2017, the EU maize harvest reached 64.7 million tonnes, which is approximately 5% of global production. EU is also a major importer of maize, with 16 million tonnes coming from outside of the EU-28 countries, mainly originating from Ukraine, Brazil and Canada (EUROSTAT, 2018). Maize primary production has been linked to the exposure of various mycotoxins such as aflatoxins (AFs), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FBs), nivalenol (NIV), T-2 toxin (T2), HT-2 toxin (HT2). Besides economic losses (losses in the field, rejections, destruction of production), these mycotoxins may induce adverse health effects on the secondary production and/or eventually on humans. Consequently, the European Commission has set limits to their levels according to regulations EU/576/2006, EU/1881/2006 and amendments EU/574/2011 and EU/165/2013.

Because of their stability, mycotoxins can accumulate in the parts of a maize plant, including the fruits, during the different pre- and post-harvest production steps, when environmental conditions favour fungal growth. In Figure 2.2, the main strategies for managing mycotoxins are presented.

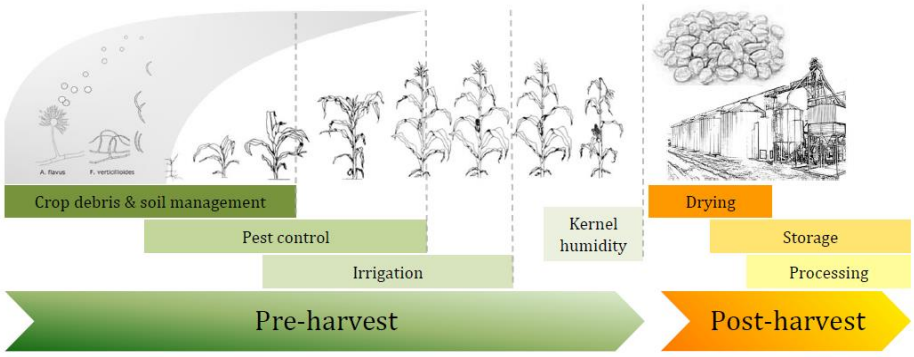


Figure 2.2 Management strategies for mycotoxin control from soil to processing (image taken from Palumbo et al., 2020)

As can be seen in Table 2.1, proper strategies have to be followed in order not only to mitigate, but also to prevent mycotoxin occurrence in maize. It is also clear that different strategies have various degrees of efficiency against different mycotoxins. Therefore, multi-techniques are recommended for mycotoxin decontamination/detoxification, although it should be mentioned that, once mycotoxins appear, their complete removal via processing is not usually feasible (Karlovsky et al., 2016).

Table 2.1 Mitigation processing techniques for mycotoxin occurrence in maize (information from Palumbo et al., 2020 and Karlovsky et al., 2016).

Prevention strategies	**Post-harvest strategies (% reduction in mycotoxins)**
Good Agricultural Practices including:	Physical methods
	-Drying (17-40% ochratoxin A)
-Proper hybrid selection	-Sorting
-Soil management	-Sieving (70-80% DON and ZEN)
-Crop rotation	-Flotation (53% DON)
-Water stress pest and disease control	-Washing (72-75% DON, 80-87% ZEN)
-Biological control agents	-De-hulling (93% AFs)
	-Steeping
	-Milling
	-Thermal processing (40-80% AFs, 65-83% ZEN)
	-UV radiation
	-γ radiation
	-Cold plasma
	-Alkaline treatment (75% AFs, 99% OTA)

Additionally, mitigation methods and their target (food or feed) should be treated with caution, since certain strategies can be country specific. Finally, it is important to take into consideration the fact that good agricultural practices, storage conditions, sanitation and monitoring can prevent high levels of mycotoxins. Processing, including chemical strategies, can also reduce the concentration of mycotoxins in food and feed.

2.2.2 Mycotoxin occurrence in rice and mitigation strategies

This section will provide a summary of the main findings from the extensive literature search on mycotoxin occurrence in rice and on effective pre- and post-harvest mitigation practices aiming at limiting mycotoxin contamination in this cereal.

Rice is one of the most consumed cereals in the world. Its consumption is especially high in Asia, which produces more than 90% of the total rice production (675 million tonnes out of a total 745 million tonnes) (EC, 2015). The occurrence of mycotoxins in rice is not as well documented as in other cereals, but high consumption and the risk associated with mycotoxin-producing fungi (even at lower levels when compared to other crops) make it a cereal of concern. Co-contamination of rice by multiple mycotoxins from different fungi at different stages of production has been recorded, which means that adequate pre- and post-harvest management practices are required (Gonçalves et al., 2019).

Table 2.2 Occurrence and co-occurrence of mycotoxins in rice and relevant products (adapted from Gonçalves et al., 2019)

Mycotoxins	Source	Co-occurrence observation	Reference
AFs, OTA	Rice and products	Co-occurrence: AFB1+AFB2, OTA+AFB1 in brown rice, AFs+OTA	Iqbal et al., 2016; Aydin et al., 2011 ; Buyukunal et al., 2010
AFs, FB1, DON	Rice	Co-occurrence: DON+FB1, AFs+FB1	Ortiz et al., 2013
AFB1, OTA, FB1	Rice	Co-occurrence: AFB1+OTA+FB1	Bansal et al., 2011
AFB1, OTA, DON, ZEN	Rice	Co-occurrence: DON+OTA, DON+ZEN, AFs+OTA+DON+ZEN	Dors et al., 2013
AFB1, OTA, DON, ZEN	Rice and products	Co-occurrence: AFs+ZEN, AFs+OTA	Almeida et al., 2012
AFs, OTA, FB1, DON, ZEN	Rice	Co-occurrence: AFs+OTA+FB1+DON+ZEN	Egbuta et al., 2015
AFs, OTA, DON, NIV, FBs, ZEN, HT-2	Rice	Co-occurrence: AFB1+AFB2, AFs+FBs	Majeed et al., 2018
AFs, OTA, CIT*	Red yeast rice	Co-occurrence: AFs+OTA+CIT	Samsudin and Abdullah, 2013

CIT: citrinin

As can be seen in Table 2.2, concurrent contamination of rice by multiple mycotoxins is possible, therefore attention should be paid to following good agricultural practices in the field in consultation with an agronomy specialist, while it should also be noted that, as with maize, in rice too mycotoxins cannot be completely eliminated even after applying the control measures summarised in Table 2.3. A tool that can assist in mycotoxin control and crop management

is predictive modelling, which has shown promising results in the literature so far (Battilani et al., 2016; Battilani and Camardo Leggieri, 2015 cited in Gonçalves et al., 2019).

Table 2.3 Mitigation processing techniques for mycotoxin occurrence in maize (information from Gonçalves et al., 2019).

Pre-harvest strategies	**Post-harvest strategies (% reduction in mycotoxins)**
Good Agricultural Practices including:	Storage
	-Controlled storage conditions
-Resistant rice varieties	-Modified atmosphere with CO_2
-Pest and disease control	Processing
-Crop rotation and lodged fields	-Hulls removal
-Biological control agents	-Milling
-Chemical control	-Washing (15%)
	-Cooking (41-51% AFB1, 50-80% BEA*)
	-Autoclaving (80% FB1)
	-Alkaline solution
	-Pulsed-light (75-90% AFB1)
	-γ-radiation (95% AFB1)
	-Ozon fumigation (70-94% AFB1)
	-Chemical control
	-Biological control

BEA: beauvericin

2.3 Toxicokinetic parameters of single and multiple mycotoxins in test and farm animal species

Possible toxicological interaction between multiple mycotoxins in living organisms are often overlooked, mostly because of relevant dose response *in vivo* data are lacking for test species let alone farm animals. Here, an extensive literature search was performed to review currently available data relevant to the toxicokinetics (TK) of single and multiple mycotoxins in test species (i.e. rat) and farm animals (i.e. pigs, chicken, cattle etc.). Inter-species differences in TK parameters between rats, chicken and pigs were assessed for markers of acute and chronic exposure and uncertainty factors allowing for interspecies differences in TK were derived. Finally, data for TK of multiple mycotoxins in those species are also presented and future challenges for hazard assessment of multiple mycotoxins taking into account both TK and toxicodynamic data for multiple co-occurring mycotoxins are discussed.

2.3.1 Introduction

Mycotoxins are natural toxins that are produced as secondary metabolites by fungi growing on plants during both pre and post-harvest conditions, with well over 400 structurally diverse congeners identified so far. Mycotoxins' occurrence and co-occurrence largely vary depending on yearly climate and other environmental conditions (Ji et al., 2016; Eskola et al., 2018; Hojnik et al., 2019). Over the years, it has been demonstrated that over 60% of global food and feed samples are contaminated with mycotoxins (Pinotti et al., 2016; Streit et al., 2012; Gruber-Dorninger et al., 2019). Consequently, the Rapid Alert System for Food (RASF) placed mycotoxins in the top 10 food hazards

with regards to the number of notifications in the Eurpopean Union (EU), as well as the second most important food hazard originating from outside the EU (RASF, 2018). In 2017, 529 notifications on products originating from countries outside the EU were highlighted, resulting in 464 border rejections (RASF, 2018). In terms of toxicity, mycotoxins and their metabolites are amongst the most potent natural toxins associated with acute and potentially lethal toxicity (Azziz-Baumgartner et al., 2005). Adverse effects associated with mycotoxins toxicity includes chronic disorders such as alterations in tissues and organ functions (hepatic, renal, intestinal, nervous, reproductive and immune systems) (Bennett and Klich, 2003; Zain 2011; Marin et al., 2013; Oswald et al., 2005; Robert et al., 2017). As an example, aflatoxin B1 (AFB1) has been classified as a genotoxic and carcinogenic compound to humans (as classified by IARC in Group 1 carcinogens) and recognised as one of the most potent known liver genotoxic carcinogens. Fumonisins (fumonisin B_1 and B_2) and ochratoxin A (OTA) have been classified as possibly carcinogenic to humans (IARC Group 2B) (Zain, 2011; Ostry et al., 2017; Voss et al., 2007). In the animal health field, exposure to mycotoxins causes a wide range of acute and chronic adverse effects on a range of species, resulting in large scale animal health concerns and huge financial losses in relation to animal production, the cost of which has been estimated in the billions of dollars range (Alshannaq and Yu, 2017; Smith, 2001; Zain, 2011).

A large body of evidence has concluded that humans and other animal species are constantly exposed to mycotoxins and that such exposure specifically involves multiple mycotoxins rather than single ones (De Ruyck et al., 2015; Grenier and Oswald, 2011; Solfrizzo et al., 2014; Stoev, 2015; Alassane-Kpembi et al., 2017a; Fremy et al., 2019). These mycotoxin mixtures may originate from different fungi species or strains but also from the modification that mycotoxins may go through particularly because of fungi-plant interaction

as well as food and feed processing, particularly depending on their specific formulation (Dall'Asta et al., 2016; Boevre et al., 2015; Freire and Sant'Ana, 2018; Bryla et al., 2018). Considering the above, it can be inferred that humans and animals can be exposed to a large combination of multiple mycotoxins.

Toxicokinetics (TK) studies show "what the body does to a chemical", in other words, how a substance enters an organism (absorption (A)) and goes through distribution, metabolism and excretion (DME): ADME. It can provide critical information related to hazard identification and characterisation particularly with regards to elimination patterns, persistence of chemicals (half-life, clearance) and the associated toxicological consequences of metabolism (bioactivation/detoxification) (Dorne and Fink-Gremmels, 2013). Indeed, the metabolism of mycotoxins such as AFB1 and zearalenone (ZEN) has been demonstrated to involve bioactivatation by cytochrome P450 (CYP) isoforms in key organs (i.e. liver) which subsequently result in the formation of metabolites with larger toxic potencies compared to the parental compounds i.e. AFB1-8,9-epoxide, α-zearalenol, α-zearalanol, (Gallagher et al., 1994; Bravin et al., 2009; Malekinejad et al., 2005). In farm animals, TK equally provides critical information for the hazard assessment of chemicals in animal species and supports the quantitative evaluation of the carry over and residues that are transferred to edible tissues and meat products (i.e. milk, eggs, etc.) (Daenicke and Brezina, 2013). Over the decade, the importance of TK data in risk assessment has been highlighted by the WHO, the OECD, the US EPA and the European Food Safety Authority (EFSA) to cite but a few, which all strongly recommend the integration of TK parameters in risk assessments of single and multiple chemicals (EFSA, 2013; EFSA, 2014a).

Assessing the TK of single and multiple mycotoxins is a challenging task, particularly for multiple mycotoxins because of the paucity of *in vitro* and *in*

vivo data from the peer-reviewed literature. Such information on the TK of multiple mycotoxins is important, since changes in TK after concomitant exposure (biotransformation, distribution, carry over) (Hassan et al., 2012; Han et al., 2012) may result in TK interactions (i.e. inhibition or induction of metabolism) as well as toxicodynamic (TD) interactions (i.e. antagonism, potentiation, synergism etc.) (Ji et al., 2017; Klaric, 2012; Dellafiora and Dall'Asta, 2017; Bouaziz et al., 2013; Alassane-Kpembi et al., 2013, 2014, 2015, 2017b). To account for inter- and intra-species variability in the human risk assessment of chemicals, the default 100-fold uncertainty factor has been used for the last 6 decades as two default 10-fold factors (interspecies and interindividual differences) (Dorne et al.,2005). Since then, these default values have been divided in default values for interspecies differences allowing for TK (4-fold factor), TD data (2.5-fold factor) and inter-individual differences in both TK (3.16) and TD (3.16) (Renwick 1993; Renwick and Lazarus, 1998). With regards to risk assessment of combined exposure to multiple chemicals, tiering principles are applied and allow to perform fit for purpose risk assessment based on the amount of information available and approaches range from simple deterministic and conservative approaches all the way to full probabilistic approaches (EFSA, 2019b). When *in vivo* toxicity data are lacking for the assessment, TK data can be integrated in low tier risk assessment using new approaches methodologies (NAMs) such as *in vitro/in vivo* extrapolations (IVIVE) models, and quantitative structure-activity relationship (QSAR) as computational models to predict hazard properties of chemicals. Another alternative approach to animal testing is read across, a method that enables the prediction of the toxicological endpoint of a compound by utilising already existing data on the same endpoint from one or more similar substances (Laroche et al., 2018). In contrast, physiologically-based kinetic (PBK) models for specific single chemicals and multiple chemicals are

often applied in higher tier assessments to quantify ADME parameters and ideally link internal dose to toxicity via a PBK-dynamic modelling. (Bois et al., 2010; Coecke et al., 2013; EFSA, 2014a, 2018; More et al., 2019; Mukherjee et al., 2014; Faeste et al., 2018; OECD, 2018). IVIVE approaches can be also coupled with reverse dosimetry (i.e. from a PBK) in order to estimate the *in vivo* exposure dose (Bell et al., 2018). In the past 35 years, various PBK models for mixtures of a range of compounds have been developed (Desalegn et al., 2019; Lu et al., 2016), the majority of which cover chemicals such as petroleum derivatives, volatile organic compounds, metals and a few on pesticides. PBK-PD modelling is one of the most high tier suitable tools for investigating interactions between chemicals and in a range of species organisms, and is the recommended approach by scientific advisory bodies such as WHO, US EPA and EFSA (WHO/IPCS, 2010; US EPA, 2006; EFSA, 2014a; EFSA, 2019b). TK and Metabolic interactions are important aspects of mixture risk assessment and QSAR methods can be used in modelling cytochrome P450 (CYP) enzyme substrates and inhibitors as key parameters to address metabolic interactions (Cherkasov et al., 2014). This work provides an overview on currently available TK data relevant to the hazard assessment of mycotoxins, with a focus on single and multiple mycotoxins, and provides new insights on the sensitivity ratios and uncertainty factors in interspecies differences. Future challenges for hazard assessment of multiple mycotoxins taking into account both TK and toxicodynamic data for multiple co-occurring mycotoxins are discussed.

2.3.2 Methods

2.3.2.1 Extensive literature searches

Extensive literature searches (ELS) were performed in June 2020 to identify *in vivo* toxicokinetic data on single and multiple mycotoxins and compute them in a database taking into account the EFSA systematic review guidance (2010). Keywords, databases, timeframes, number of hits and inclusion-exclusion criteria are presented in Annex A (Table A1). Searches from the grey literature were also performed i.e. google scholar and relevant EFSA opinions and their associated reference lists. Original articles published in other languages than English were excluded (i.e. Chinese, Japanese, Russian or Arabic) and patents, editorials and letters to editors were also excluded. The retrieved literature was screened based on the relevance of the papers (i.e. single and multiple mycotoxins, single compound or in mixture, reviews, ADME, bioavailability, carry over and residues) using a stepwise approach: 1. screening of titles and abstracts of all publications; 2. screening of full texts of a sub-selection of publications.

2.3.2.2 Comparative analysis of available toxicological studies on single and multiple mycotoxins

A separate literature search was performed in June 2020 in Web of Science (https://www.webofknowledge.com/) to provide an overview of the number of available in vitro and in vivo toxicological studies on single and multiple mycotoxins. The full details of the literature searches are provided in Annex A (Table A2) including search criteria and number of hits.

2.3.2.3 Comparative metabolism and toxicokinetics of single and multiple mycotoxins

Starting from the ELS detailed above (section 2.1), a particular focus was given to metabolism studies to identify the main metabolic pathways of mycotoxins in different animal species (i.e. pigs, rats, chickens, cows) with regards to phase I and Phase II enzymes as well as major metabolites in both. The resulting data were computed in a database for both single and multiple mycotoxins.

In vivo and in vitro toxicokinetic reporting markers of acute exposure (maximum measured toxicant concentration (c_{max}) and markers of chronic exposure; clearance (CL), area under the time-concentration curve (AUC) were collected and computed in a database for rats, pigs and chickens (Dorne et al., 2001). This database constituted the basis for the analysis of inter-species differences in TK of mycotoxins across these 3 species and for the derivation of uncertainty factors.

2.3.2.4 Interspecies differences in toxicokinetics and derivation of uncertainty factors

From the TK data collection, analysis of interspecies differences in TK was performed for acute (c_{max}) and chronic (CL and AUC) as calculated as the ratio between the reference species (rat) and the farm animals (chicken, pig) to quantify differences in internal dose. AUC and c_{max} values were normalised to the administrated dose in the animal and body weight. In contrast, elimination half-life was reported as such since they are independent of the amount of the chemical in the organism (Nnane, 2019).

The interspecies uncertainty factor (UF) was computed as the ratio (2.1) between the mean TK value (AUC, c_{max}, CL) for the species of interest (μs) against the mean TK value in the rat (μr).

$$UF = \frac{\mu s}{\mu r} \quad (2.1)$$

2.3.3 Results and Discussion

2.3.3.1 Extensive Literature Search

Figure 2.3, depicts the PRISMA flowchart for the ELS and the screening of the of the relevant metabolism and TK studies for further data collection. Initially, the peer reviewed literature was screened using predefined inclusion/exclusion criteria (Annex A, Table A1) and further assessed using abstract and full text. Overall, the pre-screening identified a total of 4170 papers for which 113 studies were included in the database while covering a range of animal species, most of which being available for rats (n=19), chicken (n=25) and pigs (n=23) while very few studies were available for other species (i.e., goat, monkey, turkey, donkey, trout) and are not presented here.

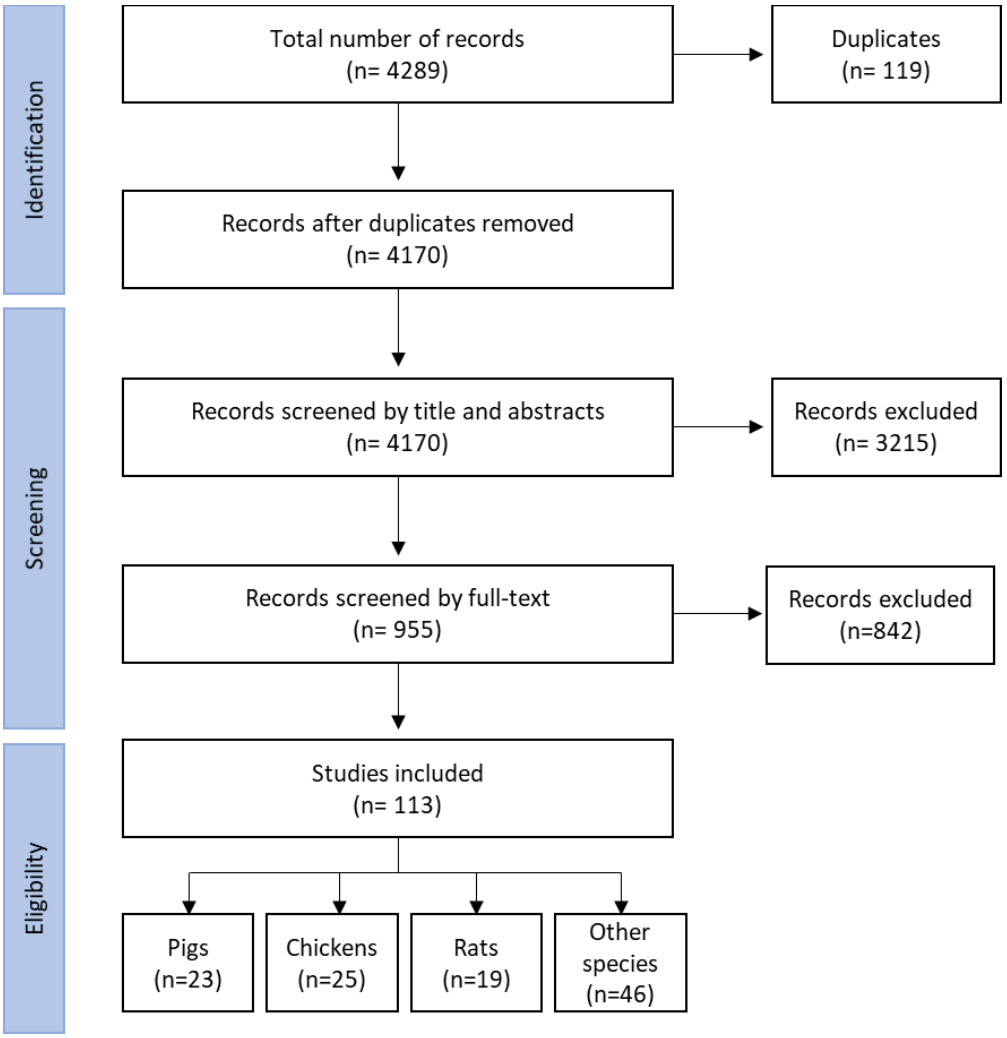


Figure 2.3 PRISMA flow chart for the extensive literature searches performed on with metabolism and toxicokinetic studies for single and multiple mycotoxins in test and farm animal species

2.3.3.2 Available toxicological studies for single and multiple mycotoxins

Available toxicological studies on single and multiple mycotoxins from the peer reviewed literature available over the past 30 years (1990-Dec 2019) were searched for and visualised using the Clarivate analytics algorithm from Web of Science. Overall, these studies are summarised in Table 1 and Table A2. Overall, a trend on an increase in published toxicological studies on single mycotoxins is observed while studies on multiple mycotoxins are much more limited (Figure 2.4). The presence of mycotoxins in human and animal samples has been reviewed by Escriva and colleagues (2017), for which the most common mycotoxins were OTA > ZEN (and metabolites) > DON (and metabolites) > enniatins > fumonisins > aflatoxins > T-2 and HT-2 toxins (Escriva et al., 2017).

Table 2.4 Available *in vitro and in vivo* toxicological studies from the peer-reviewed scientific literature for single and multiple mycotoxins

Toxicological studies	**1990**	**1995**	**2000**	**2005**	**2010**	**2015**	**2019**
Single mycotoxin	36	149	140	275	384	605	827
multiple mycotoxins	1	5	4	7	24	29	44

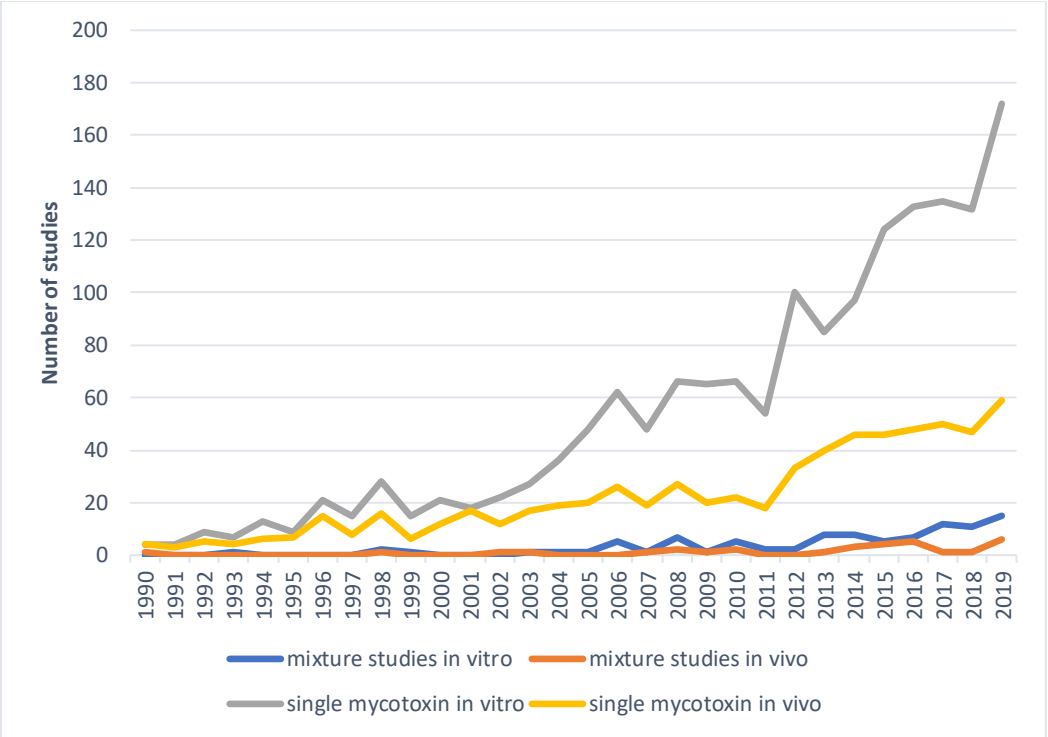


Figure 2.4 Number of *in vitro* and *in vivo* toxicological studies appearing in the literature on mycotoxin mixtures and on single mycotoxins

2.3.3.3 Comparative metabolism and toxicokinetics of single and multiple mycotoxins

2.3.3.3.1 Comparative metabolism of single mycotoxins

Table 2.5 summarises the available metabolism data for six mycotoxins namely DON, ZEN, T-2, AFB1, OTA and FB1 in rats, chickens and pigs. Wen et al. (2016) reviewed the biotransformation of AFB1, OTA, DON, T-2, FB1, and ZEN in animal cells and provided information on phase I (CYP isoforms) and phase II enzymes (UDP-glucuronyl transferases (UGTs) involved. Overall, biotransformation occurs mostly in the liver, the intestine and to a minor extent in the kidney, while excretion occurs in the urine and faeces.

Zearalenone

The biotransformation of ZEN in different animal species has been reviewed in the literature (Kuiper-Goodman et al., 1987; EFSA, 2011b, Wen et al., 2016). CYP1A2 and CYP3A1 are involved in the metabolism of ZEN in liver cells. Gut microbiome plays an important role in the metabolism of ZEN in the pig gastrointestinal (GI) tract through its reduction to α-zearalenol and the generation of other metabolites that have not yet been fully characterised (Kollarczik et al., 1994). Moreover, ZEN modified forms, such as glycoside and sulphated derivatives, have been found to be readily hydrolysed to ZEN in the pig GI tract and further transformed into other un-characterised metabolites (Binder et al. 2017). Hepatic oxidation of ZEN by CYP isoforms produces catechol metabolites which are subject to redox cycling and make such oxidation and/or the covalent modification of biological macromolecules possible including glutathione, proteins and nucleic acids (EFSA, 2011b). ZEN is hydroxylated either at the aromatic or aliphatic part in rats and it is also reduced to zearalenolsvia CYP isoforms CYP2B2 and CYP3A (Bravin et al., 2009; Duca et al., 2012). Metabolism of ZEN in rats and chickens produces more β-ZEL compared to pigs which produce more α-ZEL (5-fold) (EFSA, 2011; Devreese et al., 2015). To note that α-ZEL is a more potent xenoestrogen compared to β-ZEL. Therefore, the higher formation of α-ZEL over β-ZEL may significantly concur differentiate the sensitivity of animal species to ZEN stimulation, as found for pigs (particularly sensitive to ZEN stimulation) or chickens (more resistant to ZEN stimulation) (EFSA, 2017b). Additionally, ZEN and its phase I metabolites are readily conjugated in vivo with glucuronic acid (GlcA) and sulfate groups (EFSA, 2011b). Finally, few in vivo studies are available for the metabolism of conjugated forms of ZEN, where main catalysts for GlcA and sulphate are UGTs and SULTs respectively. In vitro evidence showed that the major ZEN glucuronide formed in the liver and GI

tract of animals is 14-O-glucuronide (EFSA, 2011b). In a pig study, the degree of glucuronidation of ZEN and its reduced metabolite was β-ZEL> ZEN>α-ZEL in urine and ZEN> β-ZEL> α-ZEL in the liver (Zollner et al., 2002).

Deoxynivalenol

DON is partially transformed in the gut to the less toxic de-epoxidised metabolite DOM-1 by gut bacteria prior to absorption both in experimental and farm animals (He et al., 1992; EFSA, 2017; Daenicke and Brenzina, 2013). The main biotransformation pathway of DON is conjugation with UGTs leading to the formation of mainly DON-3-glucuronide and DON-15-glucuronide whereas, DON-sulfonates were the major metabolites in rat faeces (EFSA, 2017). On the other hand, the major metabolite identified in chickens is DON-3α-sulfate (Guerre, 2013). Notably, DON is directly conjugated via phase II glucuronidation and in addition gut microbiota produces the de-epoxide form DOM-1 (He et al., 1992; Nagl et al., 2014) and in faeces, DON was largely metabolised to DOM-1. In rats and mice, 3-acetyl-DON (3-Ac-DON) and 15-acetyl-DON (15-Ac-DON) was mainly transformed to DON by gut microbiota prior to absorption and systemic distribution (EFSA, 2017). After transformation, 3-Ac-DON and 15-Ac-DON followed the kinetics of DON. In rats, DON-3-glucoside was also mainly transformed to DON in the gut prior to absorption and followed the kinetics as DON. However, its total bioavailability was considerably lower than for DON (EFSA, 2017). The cleavage of DON-3-glucoside in the gastrointestinal tract was species and strain specific.

Ochratoxin A

OTA has a low rate of metabolism with the major metabolic pathway of OTA is its hydrolysis to ochratoxin α (OTalpha) and glucuronic acid conjugation while oxidative metabolism is less important (EFSA, 2020). OTalpha is the

major OTA detected in rat's urine and faeces, with less important metabolites being OTA-hexose and OTA-pentose in urine (EFSA, 2006; Zepnik et al., 2003). There were no, 4- and 10-Hydroxy-OTA, lactone ring open OTA, glutathione and glucuronide conjugates present in urine, blood, liver or kidney above detectable limits (Zepnik et al., 2003). OTA is hydroxylated in the liver (by CYP450) resulting in the R- and S-isomers of 4-OH-OTA, the R- isomer is the main hydroxy product in rodents, while the S- is the major in pigs and chicken, where also the main metabolite was the 7-OH-OTA (Wu et al., 2011; Yang et al., 2015). Additionally, besides hepatic metabolism, bacterial metabolism in the gastrointestinal tract yields the cleavage product, OTalpha, which can be absorbed from the lower gastro-intestinal tract (Wu et al., 2011; EFSA, 2006).

Fumonisin B1

There are two main metabolic pathways for FB1; a) hydrolysis of the ester groups (HFB1) with a consecutive release of the two tricarballylic acid moieties, b) fatty acylation of the amino group (N-acyl-FB1 and N-acyl-HFB1). In rats, after oral gavage of FB1 the bile contained only unchanged FB1 and no evidence of any metabolites (EFSA, 2018). On the other hand, when rats were dosed with FB1 for three weeks, small amounts of partially hydrolysed FB1 (pHFB1) and HFB1 were detected in the faeces (Hahn et al., 2015), indicating that hydrolysis may occur at the colonic microbiome. Acylation has been shown to be meditated by ceramide synthase, as the fatty acylation of free sphingoid bases. N-fatty acyl FB1 was detected in the kidney and liver of rats after i.p. administration of FB1 for five consecutive days, while the FB1 metabolites in the kidney contained C16:0 acyl groups and C24 groups in the liver (Harrer et al., 2015). To our knowledge, there is no information on the metabolism of FB1 in chickens. On the other hand, FB1 is metabolised to

pHFB1 and HFB1 in the liver and the digestive track of pigs and can be found in the liver, kidneys and muscles of pigs (Guerre, 2013; EFSA, 2018).

Aflatoxin B1

AFB1 is mainly metabolised in the liver but there is also evidence of intestinal and lung metabolism (EFSA, 2020). The CYPs involved are CYP3A4, 3A5 and 1A2 (EFSA, 2007). CYP3A4 can catalyse the formation of the AFB1 exo 8,9-epoxide, which is able to bind to DNA. The predominant site for DNA adduct formation by AFB1-exo-8,9-epoxide is N7-guanine, resulting in trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-gua), which in turn can be transformed into the ring-opened, and more stable and therefore more persistent AFB1-formamidopyrimidine (AFB1-FAPY) adduct (EFSA, 2020). CYP2A6 leads to the formation of AFB1-exo-8,9-epoxide in chickens while CYP1A1 can lead to the formation of some exo-epoxide as well as a high proportion of endo-epoxide, which does not bind to DNA and AFM1 (EFSA, 2007; Deng et al., 2018). On the other hand, in pigs the main metabolites identified were AFM1, unchanged AFB1 and traces of aflatoxicol (Luthy et al., 1980). CYP1A2 is more efficient in producing 8,9-epoxide at the low AFB1 concentrations that may be found following dietary exposures. AFB1 is also metabolised by CYP3A5 to the exo-8,9 epoxide but is less efficient in forming aflatoxin Q1 (AFQ1) (EFSA, 2007). CYP3A4 and 1A2 oxidise AFB1 major to the hydroxylated metabolites AFM1 and AFQ1. AFB1 can be transformed to aflatoxicol in the liver by the reduction of AFB1 mediated by a NADPH-dependent reductase. A major detoxification pathway for detoxification of 8,9-epoxides is GST-mediated conjugation with GSH (Pottenger et al., 2014). The levels of individual GST expression in the liver relate to the GSH conjugation for AFB1 detoxification. There are two types of GSTs involved (GST-μ encoded by the GSTM1 gene and GST-θ encoded by

GSTT1. Finally, AFM1, AFP1, AFQ1 and aflatoxin-dialcohol is conjugated with glucuronic acid and excreted in faeces and urine (EFSA, 2020).

T-2 and HT-2 toxin

T-2 toxin is metabolised in the liver, intestine and other tissues, with its major metabolite being HT-2. The biotransformation of T-2 toxin includes acetylation, hydroxylation, de-epoxidation, deacetylation and glucuronide conjugation (EFSA, 2011a). In rats, major metabolites are found in the urine including 3-hydroxy-HT-2 toxin, T-2 tetraol and a number of uncharacterised metabolites. In the faeces, de-epoxy-T-2 tetraol, 3-hydroxy-HT-2 toxin and 3 unknown metabolites have been identified including de-epoxy 3-hydroxy-HT-2 toxin as major metabolites (EFSA, 2011a). Both CYP1A5 and CYP3A37 are responsible for the hydroxylation of T-2 to 3'-OH-T-2 in chickens (Antonissen et al., 2017). Additionally, in chickens the 3'-hydroxy-HT-2 toxin is the major metabolite, other metabolites such as 3'-hydroxy-T-2 toxin, 3-acetoxy-3'-hydroxy-HT-2 toxin, HT-2 toxin, 4-acetoxy-T-2 tetraol (TMR-1), 8-acetoxy-T-2 tetraol (TMR-2), 15-acetoxy-T-2 tetraol, T-2 triol, and T-2 tetraol are also found in excreta (Wu et al., 2011). While in pigs, the major metabolites found in the urine and bile are 3'-hydroxy-HT-2 and T-2 triol after T-2 toxins i.v administration. The main T-2 products found in the urine were glucuronide-conjugated products; HT-2, 3'-hydroxy-T-2, 3'-hydroxyHT-2, and T-2 toxins, while in the GI track 21 additional metabolites can be identified such as HT-2, 3'-hydroxy-T-2, and 3'-hydroxyHT-2 and de-epoxy metabolites (Wu et al., 2011).

Table 2.5 Biotransformation of mycotoxins *in vivo* in farm animals

Mycotoxin	Species	Phase I				Phase II				Other			
		CYP	Metabolite	Source	Reference	Enzyme	Metabolite	Source	Reference	Enzyme	Metabolite	Source	Reference
DON	rats	-	-	-	-	-	DON-3-sulfonate, DOM-3-sulfonate, DON-3-glucuronide	urine, faeces	EFSA, 2017	-	-	-	-
DON	pigs	-	-	-	-	UGT	DON-GlcA	liver	Goyarts and Daenicke, 2006	-	DOM-1	kidneys, liver, muscle, spleen fat, small intestine	Daenicke et al., 2004; Daenicke et al., 2010; Daenicke et al., 2013
DON	chickens	-	-	-	-	-	DON-3a-glucuronide DON-3a-sulfate	-	Guerre 2015	-	DOM-1	intestinal microflora	Guerre 2015
ZEN	rats	CYP1A2, CYP3A1, CYP3A2, CYP2A2, CYP2PC	-	-	Duca et al., 2012; Wen et al., 2016; Schelstraete et al., 2019	-	-	-	-	-	-	-	-
ZEN	pigs	-	Zeranol, α-zearalenol	muscle	Zollner et al 2002	-	-	-	-	-	-	-	-
ZEN	pigs	-	α-zearalenol, β-zearalenol, zeranol. taleranol, zearalanone	liver	Zollner et al 2002	-	-	-	-	-	-	-	-
ZEN	pigs	-	α-ZEL, β-ZEL	plasma	Daenicke and Winkler 2015	-	14-O-glucuronide	intestine, liver	EFSA, 2011b	-	-	-	-
ZEN	broiler chickens, hens	-	α-ZEL, β-ZEL	plasma	Devreese et al., 2015	UGT	ZEN-GlcA, ZEL-GlcA	-	Devreese et al., 2015	-	-	-	-

ZEN	chickens	-	α-ZEL, β-ZEL, 5-OH-ZEN, 9-OH-ZEN, 6-OH-ZEN, 15-OH-ZEN	plasma	Yang et al., 2017	-	α-ZEL-16-GlcA, α-ZEL-14-GlcA, β-ZEL-16-GlcA, β-ZEL-14-GlcA, ZEN-14-SO_3H, β-ZEL-14-SO_3H, α-ZEL-14-SO_3H	plasma and faeces	Yang et al., 2017	-	-	-	-
T-2	rats	-	4-hydroxy-HT-2, 4-hydroxy-HT-2,	urine, faeces	EFSA, 2011	-	-	-	-	-	-	-	-
T-2	pigs	-	deepoxy-HT-2, deepoxy-T-2 triol, deepoxy-T-2 tetraol	liver, bile	Wu et al., 2010	-	glucuronide conjugate 3'-hydroxy-T-2, neosolaniol, HT-2, 3'-hydroxy-HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol	bile, urine	Wu et al., 2010	-	-	-	-
T-2	chicken	CYP1A1, CYP1A5, CYP3A37	HT-2, 3'-hydroxy-HT-2 toxin, 3'-hydroxy-HT-2 toxin, 3'-hydroxy-T-2 toxin, 3-acetoxy-3'-hydroxy-HT-2 toxin, 4- acetoxy-T-2 tetraol, 8-acetoxy-T-2 tetraol, 15-acetoxy-T-2 tetraol, T-2 triol, and T-2 tetraol.	intestine	Wu et al., 2010; Antonissen et al., 2017	-	-	-	-	-	-	-	-
AFB1	rats	CYP1A1, CYP1A2, CYP3A1, CYP1B1	AFB1-exo-8,9-epoxide	liver	Nayak et al., 2010; Duca et al., 2012	UGT1A1, GSHT	AFB1-exo-8,9-epoxide	liver	Nayak et al., 2010				

AFB1	pigs		AFB1, AFM1, aflatoxicol	liver	Luthy et al., 1980	-	-	-	-	-	-	-	-
AFB1	chickens	CYP1A1, CYP2A6	-	-	-	-	exo-AFB1-8,9-epoxide	liver	Deng et al., 2018				
OTA	rats	CYP1A1, CYP1A2, CYP2B1	4(S)-OH-OTA, 4(R)-OH-OTA, 5′-OH-OTA,7′-OH-OTA, 9′-OH-OTA,OTB, 4(S)-OH-OTB4(R)-OH-OTBOTHQ Ochratoxin β (OTβ) ochratoxin B (OTB)	urine, kidney, spleen	Tao et al., 2018	-	-	-	-	-	-	-	-
OTA	pigs	-	OTα, OTβ,	intestine, urine, faeces,	Wu et al., 2011	-	-	-	-	-	-	-	-
OTA	pigs	-	4(R)- and 4(S)-OH-OTA,	liver microsomes	Wu et al., 2011	-	-	-	-	-	-	-	-
OTA	chicken	-	4(S)-OH-OTA, 4(R)-OH-OTA, 5′-OH-OTA, 7′-OH-OTA, 9′-OH-OTA, OTB	faeces	Yang et al., 2015	-	-	-	-	-	-	-	-
FB1	rats	CYP1A1, CYP4A1	HFB1	liver microsomes	Wen et al., 2016	-	-	-	-	-	-	-	-
FB1	pigs	-	pHFB1, HFB1	intestine cells	EFSA, 2018	-	-	-	-	-	-	-	-
FB1	pigs	-	pHFB1, HFB1	liver, kidney, muscles	EFSA, 2018	-	-	-	-	-	-	-	-

2.3.3.3.2 Comparative metabolism of multiple mycotoxins

Rats

Metabolic interactions between chemicals either through enzyme induction or inhibition may occur at the level of phase I reactions including oxidation, reduction (CYP) and hydrolysis (esterase's), phase II conjugation reactions (i.e. UGTs), glutathione-s-transferases (GST) or at the level of transporters (phase 0 or phase 3) (i.e. P-glycoprotein, BCRP2, Organic anion transporter proteins (OATP)). These interactions may impact on kinetic parameters in the organism reflecting chronic exposure such as $t_{1/2}$, AUC or clearance and may modulate toxicity (Kadar et al., 2017). However, the relevance of such interactions should be considered in the light of the inhibition or induction potency of the compound on the enzymes or transporters. In addition, environmentally relevant exposure should be considered when assessing these interactions since, often, experimental studies might involve doses that are order of magnitudes above relevant exposures, which may bias the assessment.

An interaction between ZEN and AFB1 may decrease the efficiency of the aflatoxin detoxification pathway compared to its activation, which may increase the genotoxicity of AFB1 (Duca et al., 2012). Additionally, co-exposure to DON and ZEN (2 mg/kg and 20 mg/kg respectively) in mice resulted in inhibition of liver metabolism. In particular, analysis of the metabolic pathway showed that co-exposure to DON and ZEN down-regulated valine, leucine and isoleucine biosynthesis, glycine, serine and threonine metabolism, and O-glycosyl compounds related to glucose metabolism in liver tissue. (Ji et al., 2017). Concurrent AFB1 and FB1 in vitro exposure in rat hepatoma cells (H4IIE cells) provided evidence for increase in transcriptional activity of CYP1A1 via aryl hydrocarbon receptor (Ahr)-dependent mechanisms. Such an increase in enzyme activity was significantly higher

compared to that from single mycotoxins and authors hypothesised that FB1 may increase the bioactivation of AFB1 which may result in an increase in toxicity (Mary et al., 2015).

Farm animals

There were no *in vivo* biotransformation data available in the literature for multiple mycotoxin co-administration to pigs. While in chickens, concurrent exposure of DON (4.22 mg/kg) and a mixture of FBs (17.87 mg/kg FB1, 6.48 mg/kg FB2, 1.65 mg/kg FB3) for 15 days, followed by mRNA expression profiling of hepatic CYP isoforms as well as multidrug resistance proteins (MDR) showed a significant upregulation of CYP1A4 compared to that with FBs alone (Antonissen et al., 2017).

2.3.3.4 Toxicokinetic parameters of mycotoxins in in rats

TK studies on mycotoxins account for 14% of the reported *in vivo* animal studies (Escriva et al., 2015). A summary of markers of acute (c_{max}) and chronic exposure ($t_{1/2,}$ CL, AUC) are presented in Table 2.6. The lowest clearance was observed for OTA followed by FB1, DON and ZEN, while the AUC was the highest in this respective order (OTA>FB1>DON>ZEN) and also was the c_{max}. Toxicokinetic data for multiple mycotoxins are more limited in the literature. These include studies investigating the impact of co-administration of AFB1 and OTA to F334 rats (0.25 mg/kg bw and 0.5 mg/kg bw respectively), and its consequences on TK including higher absorption and metabolism with a T_{max} of 10 hours for AFB1 and 2 hours for OTA while no changes observed in OTA levels in plasma, liver and kidney tissues compared to that with OTA alone (Vettorazzi et al., 2010; Vettorazzi et al., 2011). (Corcuera et al., 2012). Specifically, no significant differences in c_{max} for OTA alone (4148.9 μg/L) were observed compared to that resulting from OTA/AFB1 co-exposure (4326.2 μg/L) and T_{max} of OTA was unchanged (2

hours). The authors concluded that AFB1 does not influence the TK of OTA; however, more studies should be performed to further confirm such findings. (Corcuera et al., 2012). A TK study with AFB1and T-2 toxin (gavage 0.25

mg/kg bw of each) has been conducted by Han and co-workers (2012) in Male Sprague-Dawley rats. Results concluded on a T_{max} of 0.17 min for AFB1 compared to 2-3 hours for AFB1 alone and authors hypothesised that T-2 may increase the time-to-pick concentration of AFB1 in plasma, heart, liver, spleen, lung, kidney and brain.

Table 2.6 Markers of acute and chronic exposure for TK of mycotoxins in rats

Mycotoxin	Species	Exposure route	N	AUC (ng*h/mL) (SD)	c_{max} (µg/L) (SD)	$t_{1/2}$ (h) (SD)	CL (L/h kg) (SD)	Reference
OTA (0.5 mg/kg bw)	F 344 rats	Oral	18	-	-	224	-	Zepnik et al., 2003
OTA (0.05 mg/kg bw)	Wistar rats	Oral	5	24000	390	120	0.00091	Hagelberg and Hult, 1989
AFB1 (0.6 mg/kg bw)	Sprague-Dawley rats	Oral	5	-	-	91.8	-	Coulombe and Sharma, 1985
DON (0.1 mg/kg bw)	Sprague-Dawley rats	Oral	3	48.81	10.44 (5.87)	-	2.59[a]	Saint-Cyr et al., 2015
ZEN (8 mg/kg bw)	Sprague-Dawley rats	Oral	6	46.1 (16.5)	4.5 (1.3)	16.8 (8.4)	189.9	Shin et al., 2009
FB1 (10 mg/kg bw)	Wistar rats	Oral	72	970	180	3.15	0.072	Martinez-Larranaga et al., 1999
T-2 (na)	rats	Oral	-	-	-	0.33	-	Wu et al., 2010

2.3.3.5 Toxicokinetic parameters of mycotoxins in farm animals

Experimental in vivo TK parameters in farm animals exposed to a range of single and multiple mycotoxins are reported in Table 2.7. TK parameters for multiple mycotoxins were very limited for farm animals. Overall, elimination half-lives of mycotoxins (i.e. DON, ZEN) were lower in chickens (0.63-2.69 hours and 0.34-0.36 hours respectively) (Osselaere et al., 2013; Pralatnet et al., 2012; Devreese et al., 2015) compared to those in pigs. (1.75-6.05 hours and 5.3-25 hours respectively) (Goyarts et al., 2010; Paulick et al., 2015; Danicke and Winkler, 2015; Fleck et al., 2017). Such differences provide a TK rationale

for the differences in DON and ZEN's sensitivity between pigs and chicken (the former are more sensitive compared to the latter, see below).

Pigs

TK data for single mycotoxins were available in pigs. Absorption and bioavailability of fumonisins was low as it is known in both experimental and farm animals whereas absorption and distribution of DON in pigs was high with a rapid plasma elimination half-life (1-4 h). Elimination half-lives for ZEN was on average over 40-fold larger than that for DON (87 h and 1.2-3.19 h respectively) (EFSA, 2017a; Thanner et al., 2016). (EFSA, 2017; Bertero et al., 2018; EFSA, 2018). The limited *in vivo* TK studies on multiple mycotoxins included DON and FBs (FB1 and FB2) (3 mg DON/kg feed, 6mg FBs/kg feed) and an increase in combined liver toxicity was observed compared to the single compounds and the authors hypothesised a possible increased in the absorption of FBs in the presence of DON. However, no measurement confirmed this hypothesis, DON is known to affect the functional barrier of the intestine and FBs are poorly absorbed after single administration (Grenier et al., 2011). No data is available on the excretion of mycotoxins when administrated in a mixture in pigs in literature

Chickens

Generally, chickens have higher tolerances to DON compared with that in pigs. Poultry are characterised by lower absorption and bioavailability higher metabolism capacity and plasma clearance. TK data after exposure to multiple mycotoxins in chicken were also very limited and concurrent administration of FB1 and DON to broilers did not affect bioavailability and other TK parameters (Antonissen et al., 2015).

In addition, the tissue residues of OTA in liver and kidney after concurrent administration of OTA and AFB1to hens (5 mg/kg feed of each compound), and compared to OTA administered alone, resulted in a 10-fold and 2-fold decrease of OTA levels in the liver (2.21 ng/g vs 22.54 ng/g) and kidney (2.81 ng/g vs 4.22 n/g) respectively and 2-fold decrease of OTA levels in the kidney (Hassan et al., 2012). In addition, the authors also noted that both OTA and AFB1 residues were lower in edible tissues (breast muscle) and eggs compared to those with the single compounds (Hassan et al., 2012). No data on multiple mycotoxin excretion patterns were available for chickens.

Table 2.7 Main toxicokinetic parameters of mycotoxins in farm animals when administrated as single mycotoxins and as binary mixtures

Mycotoxin	Species	Exposure route	Sample size (N)	AUC (ng*h/mL) (SD)	c_{max} (µg/L) (SD)	$t_{1/2}$ (h) (SD)	CL (L/ h kg) (SD)	Reference
DON (0.15 mg/kg bw)	sows	Oral+IP	3	28.8	4.2	1.75	0.55	Goyarts et al., 2010
DON (0.05 mg/kg bw)	pigs	Oral	4	168.62 (22.68)	29.73 (6.8)	2.73 (0.48)	0.3	Devreese et al., 2014
DON (0.07 mg/kg bw)	pigs	Oral	6	343.04 (99.9)	28.76 (5.78)	6.05 (1.92)	0.75	Paulick et al., 2015
DON (0.1 mg/kg bw)	pigs	Oral	7	120.5 (29.88)	42.07 (8.48)	-	-	Saint-Cyr et al., 2015
DON (1.59 mg/kg bw)	pigs	Oral	18	132.5 (30.2)	17.2 (5.3)	3.74 (1.01)	0.34	Rohweder et al., 2013
DON (0.125 mg/kg bw)	pigs	Oral	6	335 (60)	35.7 (6.2)	3.8 (1)	-	Faeste et al., 2018
ZEN (1 mg/kg bw)	pigs	Oral	1	-	-	5.3	-	Danicke and Winkler 2015
ZEN (1 mg/kg bw)	pigs	Oral	6	-	1.15	25 (15)	-	Fleck et al., 2017
FB1 (0.5 mg/kg bw)	pigs	Oral	5	-	-	1.6	0.55	Prelusky et al., 1994
OTA (0.5 mg/kg bw)	pigs	Oral	4	-	1.74	88.8	0.35	Galtier et al., 1981
T-2 (0.15 mg/kg bw)	pigs	IV	2	-	-	1.5	-	Corley et al., 1986

T-2 (1 mg/kg bw)	pigs	IV	6	708 (76.6)	2736 (236.3)	0.58	1.5	Sun et al., 2014
DON (1 mg/kg bw)	broiler chicken	Oral	5	-	12.73 (4.06)	2.69 (0.96)	2.83 (0.74)	Pralatnet et al., 2015
DON (0.75 mg/kg bw)	broiler chicken	Oral	8	10.22 (3.15)	8.22 (2.69)	0.7 (0.27)	-	Devreese et al., 2012
DON (0.5 mg/kg bw)	broiler chickens	Oral	6	-	4.2(0.9)	-	6.12	Broekaert et al., 2015
DON (0.75 mg/kg bw)	broiler chickens	Oral	8	21.35 (8.02)	26.1 (14.64)	0.63 (0.19)	39	Osselaere et al., 2013
ZEN (1.2 mg/kg bw)	broiler chickens	Oral	5	-	15.9 (4.5)	-	-	Buranatragool et al., 2015
ZEN (3 mg/kg bw)	broiler chickens	Oral	6	21.87(12.12)	39.17 (18.67)	0.34(0.07)	9.06 (4.61)	Devreese et al., 2015
ZEN (3 mg/kg bw)	laying hens	Oral	6	29.31 (6.78)	54.12(22.9)	0.36	11.38 (3.03)	
FB1 (2.5 mg/kg bw)	broiler chickens	Oral	6	1510(901.16)	33(21.3)	1.77 (0.14)	56.67	Antonissen et al., 2015
OTA (2 mg/kg bw)	broiler chickens	Oral	6	5.57	0.78	4.15	358.7	Galtier et al., 1981
OTA (0.25 mg/kg bw)	broiler chickens	Oral	4	301.43 (42.71)	47.48 (18.07)	14.11 (6.45)	0.71 (0.17)	Devreese et al., 2018
T-2 (2 mg/kg bw)	broiler chickens	Oral	8	56 (13.88)	53.1 (10.42)	0.39 (0.049)	-	Sun et al., 2015
T-2 (0.02 mg/kg bw)	broiler chickens	IV	8	14.81 (6.05)	185.4 (79.79)	0.065	1.8	Osselaere et al., 2013

2.3.3.6 Interspecies differences in the toxicokinetics of single mycotoxins for markers of acute and chronic exposure and uncertainty factors

The current default factor (10-fold) allowing for inter-species differences in toxicokinetics used in animal health risk assessment allows extrapolation from the test species to the farm animal and has been split into a 4-fold factor for the TK dimension and a 2.5-fold factor for the TD dimension (Renwick 1993; Renwick and Lazarus, 1998; Dorne et al., 2005; EFSA, 2020).

From the TK data collected on different mycotoxins in different species, the variability in the sensitivity among different species in comparison to the rat model that is the species predominantly used for human risk assessment is presented. The toxicokinetic data assessed were; area under the curve (AUC), maximum concentration (c_{max}) and clearance (CL) of pigs and chickens in the mycotoxins DON, FB1, ZEN and OTA with the respective kinetic parameters of rats (Table 2.8). For AFB1 and T-2, there were not enough in vivo kinetic data in all three species to perform the calculations. The uncertainty factors (UF) were measured using equation (1) on the interspecies differences on the above-mentioned TK parameters.

Table 2.8 Interspecies differences in the toxicokinetics of mycotoxins: comparison between rats and farm animals and derivation of uncertainty factors

Mycotoxins	**Species**	**$T_{1/2}$ ratio[a]**	**$T_{1/2}$ (UF)[a]**	**AUC ratio[a]**	**AUC (UF)[a]**
DON	pig	14.06	21.7	16.73	24.09
	chicken	4.62	11.1	5.88	10.21
AFB1	pig	2.87	2.87	-	-
	chicken	1.33	1.33	-	-
FB1	pig	0.51	0.51	-	-
	chicken	0.56	0.65	-	-
ZEN	pig	1.48	3.24	0.17	0.34
	chicken	0.02	0.03	0.33	0.69
OTA	pig	0.49	0.49	-	-
	chicken	0.02	0.02	-	-

[a]species of interest/rat

The data in Table 2.8, illustrate a comparative analysis of kinetic differences between rats, pigs and chicken for markers of acute (c_{max}) and chronic exposure (AUC and CL) for four mycotoxins (DON, ZEN, FB1 and OTA) and the corresponding uncertainty factors as the internal dose ratios for each TK parameter and species. After oral administrations of 0.07- 0.1 mg/kg bw of DON to pigs, AUC and c_{max} was 4.5- and 2.2-fold larger respectively compared to that in rats (0.1 mg/kg bw) (Paulick et al., 2015; Saint-Cyr et al., 2015; Devreese et al., 2015; Osselaere et al., 2013; Buranatragool et al 2015). In contrast, AUC in chickens was 23-fold shorter compared to rats after an oral dose of 0.75 mg/kg bw. After oral administration of 5 mg/kg bw FB1 in pigs the cmax was higher (3-fold) than in the rat (10 mg/kg bw). On the other hand,

chickens showed a lower CL with ZEN in comparison with the rat (18.6-fold) (Devreese et al., 2015; Shin et al., 2009) and both chickens and pigs showed higher c_{max} against the rat (26.3 and 9.4 fold respectively) (Devreese et al., 2015; Buranatragool et al 2015; Daenicke and Winkler 2015). Finally, for OTA chickens showed a higher mean CL than pigs and rats namely, 0.71, 0.35, 0.0009 L/h kg respectively (Hagelberber and Hult 1989; Galtier et al., 1981; Devreese et al., 2018).

2.3.4 Conclusions and future perspectives

This Chapter provides a review of comparative ADME of 6 mycotoxins in rats, pigs and chicken with regards to phase I/ phase II enzymes and TK parameters reflecting markers of acute and chronic exposure. In addition, the literature on multiple mycotoxins was also assessed and revealed that comparison between rats, pigs and chicken after assessing the main TK parameters of mycotoxins in farm animals, it can be concluded that animal species have different levels of sensitivity to different mycotoxins. These differences provide a rational that kinetic parameters are an essential indicator to mycotoxin toxicity. The uncertainty factors for inter-individual differences were also compared against the default factors and it was clearly seen that there are differences in the sensitivity of experimental animals in comparison to farm animals. Thus the use of default factors may occasionally lead to an over or under-estimation of the risk.

In literature, there are limited papers addressing combined effects of mycotoxins and even fewer considering multiple doses including no effect data, which was the main limitation of our study. Due to the fact that real-life exposure to mycotoxins is in mixtures, a rational way to perform their risk

assessment is either by setting priorities based on the frequency of the co-occurring mycotoxins or by considering the potency of the final toxic effect, giving special attention to the possible synergistic effects.

Harmonised in vitro and in vivo methodologies are needed, addressing possible mycotoxin co-exposure, co-administration of multiple doses of mycotoxins, feeding animals with low doses (below dose-threshold) of multiple mycotoxins, investigation on the inter and intra species variability and recalculation of the default safety factors (Assuncao et al., 2016; Dorne et al., 2005). Additionally, it is very important to have comprehensive well-designed studies, able to address toxicodynamic and/or toxicokinetic parameters on mycotoxin co-occurrence (EFSA, 2017; Assuncao et al., 2016). As shown in this study, multiple CYP P450 are involved in the biotransformation of mycotoxins, which are responsible not only in the production of a wide range of metabolites including their bio-activated forms (i.e. 8,9 exo-epoxide of AFB1). Mycotoxins as it has been shown in the literature are also accountable for interfering with CYP P450 enzymes by either inducing or inhibiting their activity, influencing their biotransformation when administrated as a mixture, which in turn may alter their mixture toxicity (Alessane-Kpembi et al., 2017; Klaric et al., 2013; Mary et al., 2015). Availability of in vitro and in vivo metabolism data of mycotoxins will shed light on their interactions.

Moreover, there are many challenges when designing in vitro and/or in vivo studies, which will introduce a factor of uncertainty on the risk evaluation. To name a few; the nature of data extracted from different studies with diverse study designs, different experimental parameters (pH, digestion time, medium), dissimilar animal species, body weight, age, sex and different research aims (Dorne and Fink-Gremmels, 2013). These challenges had to be

taken into account in our study, including the mycotoxin administration route, which varied in various studies (i.e. oral, gavage, pure compound, in feed, natural contaminated feed, spiked samples). Studies on the kinetics and metabolic rate of substrates (i.e. CYP1A2) and renal clearance showed values of 5, 5.4 for rats and 10.6, 13 for mice respectively in comparison to humans' values, which are larger than the default value of 4 (Walton et al., 2001, 2004). These findings suggest that sensitivity of experimental animals (i.e. rats, mice) differs in comparison to other animal species; thus, utilising the default factors to assess interindividual differences may sometimes be over conservative or even not adequate for chemical risk assessment. The authors in both studies concluded that the way forward should be the replacement of default factors with compound-related values. The kinetic ratios used in this study to present interspecies differences were based on single mycotoxin studies and often only one study was available with the in vivo data required for the comparisons this is adding a degree of uncertainty to our estimations.

Availability of TK data is of paramount importance for the refinement of risk assessment of chemicals. In order to increase the predictability of kinetic modelling a multidisciplinary approach is needed, a crossover between in vitro testing with QSAR and PBK modelling can assist in performing accurate in vitro to in vitro extrapolations. In order to achieve this approach, besides harmonised testing it is important to have standardised and validated in vitro methods addressing kinetic parameters of mycotoxins such as; single Vmax, Km, intrinsic clearance and isoform-specific metabolic interactions (Ki), including possible metabolic interactions of mycotoxins (in phase I, II and transporters). In order to improve the accuracy of the in vitro kinetic methods

the EU funded the Predict IV FP7 Project (https://cordis.europa.eu/project/ rcn/ 86700_en.html).

In vitro assays enable the evaluation of substrate/inhibitor potential in different chemicals on CYP enzymes and transporters. Moreover, in vitro studies using i.e. human hepatocarcinoma (HepG2) cells can not only assess the toxicity of different mycotoxins (single and multiple) and their potential enzyme inhibition (Ki values) but also allow the estimation of their ADME parameters when coupled with in silico tools such as the SwissADME tool (Zhou et al., 2017; Taroncher et al., 2018).

Data retrieved via in vitro testing can be used for IVIVE estimations of hepatic clearance, volume of distribution (Vd) and potentially peak and plasma concentrations of compounds (Wambaugh et al., 2018). Additionally, besides IVIVE, allometric scaling can predict the TK parameters of mycotoxins in different animal species (Faeste et al., 2018; Shiran et al., 2006). When toxicokinetic data are missing, QSAR computational methods can assist in estimating not only i.e. partition coefficients of compounds or other kinetic parameters like V_{max} and K_m but also possible metabolic interactions of compounds based on their chemical structure (Peyret and Krishnan, 2011; Cherkasov et al., 2014). In the high assessment tiers, PBK models can be used to make use of TK information and calculation of the internal dose (fraction of exposure dose after ADME) of the compounds by a mass balance calculations (WHO, 2009). An increasing number of papers performing predictive modelling using kinetic data for mycotoxins is becoming available (Faeste et al., 2018; Zeng et al., 2019; Qian et al., 2013; Mukherjee et al., 2014). The challenge with using PBK models for mixtures is the vast amount of data needed. Besides the kinetic parameters of the parents and metabolites, also

physiological and biological data on the species of interest (body weight, organ weight, age, hepatic blood flow, body surface) and information on the expression of the relevant enzymes in the liver, gut, etc., in the test species of interest are needed. The model predictions can be validated from in vivo studies and ultimately, when predictive kinetic models are validated, read across can be used with more confidence in the cases of compounds and their mixtures on which there is currently little to no information (Laroche et al., 2018).

Furthermore, calculation of kinetic and dynamic inter- and intra-species variability including uncertainty will improve the quality of data and refine toxicological risk assessments (OECD 2018). There are various predictive models developed so far taking into account mixtures including their interactions and population variability (Grech et al., 2019; Tohon et al., 2019; Valcke and Haddad 2015; Bois et al., 2010; Cheng and Bois 2011).

This study identified that there are limited TK data for mycotoxin mixtures in the literature. In addition, it can be concluded that assessing the TK of mycotoxin mixtures is a complex subject, which should be studied using case-by-case approach. Various parameters may affect the kinetics of mixtures such as; mycotoxin dosage, exposure pathway, interspecies and intraspecies differences. Due to the limited availability of scientific papers on mixture effects in comparison with single compounds, there are limited available in vitro and in vivo data related to concurrent mycotoxin exposure. Moreover, the number of mycotoxins considered so far in TK studies only accounts for a few of those that are likely to occur in animal diet. Since testing of all mycotoxin mixture combinations is infeasible, focus should be given on the prioritisation of mycotoxin mixtures, creation of harmonised methods for generating in vitro

and if necessary in vivo TK data and finally making use of predictive kinetic modelling that include uncertainty and inter and intraspecies variability analysis. In particular, all those emerging mycotoxins prone to cause interactions with those consolidated, as affecting animals' health and welfare should be prioritised in their testing. All the above will assist in reducing the overall uncertainty and lead to a more robust risk assessment on multiple mycotoxins.

Chapter 3

Investigating the relevance of metabolic interactions in the food safety area

3.1 Background and importance

Chemicals may have common metabolic routes and metabolic interactions can be relevant, particularly through inhibition or induction of xenobiotic metabolising enzymes, and should be assessed. Such metabolic interactions may occur through inhibition or induction of phase I enzymes, including oxidation, reduction and hydrolysis (i.e. Cytochrome P450 (CYP), esterases), phase II conjugation enzymes (i.e. UDP-glucuronosyltransferases (UGT), glutathione-s-transferases (GST)) or transporters (phase 0 or phase 3) (e.g. P-glycoprotein) (Iyanagi, 2007). There is growing evidence in the peer-reviewed literature that, after concomitant xenobiotic exposure, compounds may interact again through either inhibition or induction of metabolism, and may interfere not only with bioactivation or detoxification rates and consequently with TK parameters such as area under the curve, clearance or half-life. Such TK interactions may also affect the toxicodynamics of the chemicals and should be considered in the risk characterisation step (Kadar et al., 2017). Toxicokinetic interactions may lead to a potentiation or an inhibition of the toxicity of a chemical. For example, a non-mutagen could potentiate the effects of a mutagenic compound due to the enhanced metabolic bioactivation of the mutagenic compound (Krishnan and Brodeur, 1994). Additionally, saturation, induction or inhibition may produce greater levels of toxicants or cause slower

detoxification and this may increase the risk of adverse health effects as bioactivated compounds may stay longer in the body i.e. slower elimination half-life (IGHRC, 2008; Hodgson, 1999). Metabolic interactions between chemicals has been studied in detail within the pharmaceutical field, since drug-to-drug interactions can change either the effectiveness or toxicity of drugs and is of great concern to the pharmaceutical industry (Yuan et al., 1999; Ansari, 2010; Sweeney and Bromilow, 2006; Galetin et al., 2007). Such knowledge can be transferred to the food safety field if the required data are available. The aim of the present chapter is to investigate the relevance of metabolic interactions for chemicals relevant to food safety and the human health such as pesticides and flavonoids.

3.2 Enzyme kinetics

Enzymes are single or multiple-chain proteins able to catalyse chemical reactions in organisms. They have the ability to bind to the active site of a substrate and can accelerate biochemical reactions by reducing the amount of energy needed for the reaction to occur (Essel and Osel, 2014). Enzyme kinetics have been studied since 1902 when Andrian Brown investigated the rate of hydrolysis during the catalysis of sucrose by β-fructofuranosidase (Brown, 1902)

$Sucrose + H2O \rightarrow glucose + fructose$

$$E + S \rightleftharpoons ES \rightarrow P + E \quad (3.1)$$

Where: E= enzyme, S= substrate, ES= enzyme-substrate complex and P= product.

One of the best-known and most widely used models for enzyme kinetics is the Michaelis-Menten model (Doran, 2013). The model describes the rate of enzymatic reactions by relating the reaction rate to the concentration of the substrate, and is presented by the following equation:

$$v = \frac{d[P]}{dt} = \frac{V_{max}[S]}{K_m + [S]} \quad (3.2)$$

Where: v = reaction rate, P = product, V_{max} = maximal velocity of a reaction, S = substrate and KM = Michaelis-Menten constant (substrate concentration at which the reaction rate is half of V_{max}).

Equation (3.2) has been used for the prediction of product formation in an enzymatic reaction, and it measures how well the substrate binds with an enzyme (binding affinity).

3.2.1 Phase I enzymes

Phase I is the first phase of the biotransformation of endogenous and xenobiotic compounds into more water-soluble ones so their elimination rate can be increased. For this to happen, the compounds are oxidised, hydrolysed or reduced, which leads to an increase of their hydrophilicity (Croom, 2012). The most important enzymes in this phase, responsible for most of the above mechanisms as well as for the chemical degradation and eventual elimination of xenobiotics, are the Cytochrome P450 (CYP) enzymes. They are heme-proteins, located mainly in the liver, but also in the intestines and kidneys as well as in other parts of the body (Guengerich, 2008).

Cytochrome P450 enzymes can biotransform not only thousands of naturally occurring chemicals such as hormones, sterols, bilirubin and vitamins, but also toxins (Guengerich, 2008; Chang, 2009; Li et al., 2019). They comprise several isoforms, which are classified into families and subfamilies according to their similarity in primary structures. This variability is due to genetic polymorphism, while their low substrate specificity also means that they are able to protect an individual from toxic effects (Jackson et al., 2010). In the diet there are unavoidable chemicals coming from various sources (food, drugs, food supplements, herbs) which can increase or decrease the activity of the CYP enzymes, by functioning either as inducers or as inhibitors of CYP enzymes (Hodges and Minich, 2015). In the literature there are many examples of inhibition molecularly based (competitive, non-competitive, mixed), reversible and irreversible (e.g. suicide inhibition) (Hodgson et al., 2003; Abass et al., 2012), Table 3.1 summarises some examples.

3.2.1.1 Inhibition

Competitive

Competitive inhibition occurs when the inhibitor is homologous to the substrate and competes for the enzymes' active site. In such a case, either the inhibitor or the substrate will bind to the enzyme (Copeland, 2000). Thus, for the competition with the inhibitor to be overcome, more substrate is needed.

$$\begin{array}{l} E + S \rightleftharpoons ES \rightarrow P + E \\ \quad + \\ \quad I \\ \quad \Downarrow \\ \quad Ki \\ \quad \rightarrow \\ \quad EI + S \rightarrow \text{No reaction} \end{array} \qquad (3.3)$$

Uncompetitive inhibition

Uncompetitive inhibition occurs when the inhibitor binds only to an already formed enzyme substrate complex (ES) and renders the complex inactive (Copeland, 2000).

$$\begin{array}{l} E + S \rightleftharpoons ES \rightarrow P + E \\ \qquad\qquad + \\ \qquad\qquad I \\ \qquad\qquad \Downarrow \\ \qquad\qquad K'i \\ \qquad\qquad \rightarrow \\ \qquad\qquad ESI \rightarrow \text{No reaction} \end{array} \qquad (3.4)$$

Mixed inhibition

This type of inhibition differs from the previous ones in that the inhibitor can now bind both to a free enzyme and to the already formed enzyme substrate complex. Thus, the inhibitor both hinders the binding of the substrate and decreases the turnover number of the enzyme (Berg et al., 2002). A special case of mixed inhibition is the non-competitive inhibition. In this type of inhibition, the inhibitor and the substrate bind to different sites in the enzyme, so both inhibitor and substrate can bind to the enzyme. In non-competitive inhibition the Michaelis constant (Km) remains the same, but maximum velocity (V_{max}) changes since, even if there is an increase in the substrate, the binding of the inhibitor (I) cannot be prevented (Copeland, 2000).

$$\begin{array}{ccc} E + S \rightleftharpoons ES \rightarrow P + E & & \\ + \qquad\qquad + & & \\ I \qquad\qquad I & & \\ \Downarrow \qquad\qquad \Downarrow & & \\ Ki \qquad\qquad K'i & & \\ \rightarrow \qquad\qquad \rightarrow & & \\ EI \qquad\qquad ESI \rightarrow \text{No reaction} & & (3.5) \end{array}$$

Table 3.1 Examples of xenobiotics as CYP isoform inhibitors

Compound	Compound type	Enzyme	Type of inhibition	Reference
curcumin	flavonoid	CYP2B6	non-competitive	Appiah-Opong et al., 2007
curcumin	flavonoid	CYP1A2	competitive	Appiah-Opong et al., 2007
curcumin	flavonoid	CYP2C9	non-competitive	Appiah-Opong et al., 2007
6-hydroxyflavone	flavonoid	CYP2C9	non-competitive	Si et al., 2008
tetrahydropalmatine	alkaloid	CYP1A2	competitive	Zhao et al., 2015
amentoflavone	flavonoid	CYP2C9	mixed	Kimura et al., 2010
silibinin	flavonoid	CYP2D6	competitive	Beckmann-Knopp et al., 2000
nuciferine	alkaloid	CYP2D6	competitive	Ye et al., 2014
St John's wort	medicinal plant	CYP2D6	uncompetitive	Hellum et al., 2007
piperine	alkaloid	CYP3A4	non-competitive	Volak et al., 2008
berberine	alkaloid	CYP3A4	competitive	Zhao et al., 2015
chlorpyrifos	pesticide	CYP3A4	non-competitive	Joo et al., 2007
trans-resveratrol	stilbenoid	CYP3A4/5	non-competitive	Hyrsova et al., 2019
cis-resveratrol	stilbenoid	CYP3A4/5	non-competitive	Hyrsova et al., 2019
oxyresveratrol	stilbenoid	CYP3A4/5	mixed	Hyrsova et al., 2019

3.2.1.2 Induction

In this type of activity, a molecule enhances the expression of an enzyme, so there is an increased rate of liver and intestine metabolism. Examples of CYP inducer chemicals are St John's Wort and the flavonoids tangeretin, α-Naphthoflavone (Zhou et al., 2003; Yu et al., 2011; Quignot et al., 2019).

3.2.2 Phase II enzymes

Phase 2 for xenobiotic metabolism, plays an important part in the biotransformation of both endogenous (i.e. bilirubin) and xenobiotic compounds. It is usually the main detoxifying step in the biotransformation of xenobiotic compounds to more easily excretable forms (Omiecinski et al., 2011). The most important reactions in this step are the conjugating reactions, such as glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation (Nanjappan et al., 2018). These reactions aim to produce more hydrophilic conjugate products. The major phase II enzymes are uridine 5'-diphospho-glucuronosyltransferases (UGTs), N-acetyltransferases, sulfotransferases and glutathione S-transferases, and are located mainly in the liver, the kidneys and the intestines (Jancova et al., 2010). In the human liver, ten UGTs are expressed, namely in order of expression level, UGT2B7 > UGT1A1 > UGT2B4 > UGT2B15 > UGT1A4 > UGT2B10 > UGT1A9 > UGT2B17 > UGT1A6 > UGT1A3. Various food relevant compounds (herbal extracts, fatty acids, flavonoids, quinones and other natural compounds) have been identified in the literature as inhibiting glucuronidation (Lv et al., 2019). Besides inhibition, compounds like polycyclic aromatic hydrocarbons (PAHs),

and pesticides like fipronil, indoxacarb etc. have been shown to be inducers of UGTs, which consequently promote adverse effects in the thyroid and the liver (damage and inflammation) (Kondraganti et al., 2005 and Roques et al., 2012). Below in Table 3.2 examples of xenobiotic glucuronide inhibitors and inducers are presented.

Table 3.2. Examples of xenobiotic as UGT isoform inhibitors and inducers (data originating from Lv et al., 2019; Kondraganti et al., 2005 and Xu et al., 2005)

Compound	Compound type	Enzyme	Effect	Reference
blueberry	plant extract	UGT1A1	competitive inhibition	Katoh et al., 2009
ginseng	plant extract	UGT1A1	inhibition	Katoh et al., 2009
3-methylcholanthrene	PAH	UGT1A6, GSTA1, GSTA2	induction	Xu et al., 2005
3-methylcholanthrene	PAH	UGT1A2, UGT1A6, UGT1A7	induction	Kondraganti et al., 2005
oleic acid	fatty acid	UGT1A1	non-competitive inhibition	Shan et al., 2014
licochalcone A	flavonoid	UGT1A1	competitive inhibition	Wang et al., 2017
silybin	lignan	UGT1A1	inhibition	Sridar et al., 2004
resveratrol	stilbenoid	UGT1A1	inhibition	Sun et al., 2015
capsaicin	plant extract	UGT1A1	inhibition	Sun et al., 2015

3.2.3 Transporters

This phase consists of active membrane transporters whose main role is the influx (phase 0) or efflux (phase 3) of xenobiotics from the cellular membranes. P-glycoprotein (P-gp) is an important efflux molecule mitigation transporter from a cell using ATP hydrolysis (Loscher and Potschka, 2005). P-gp is coded by the multiple drug resistance MDR1 gene and belongs to the ATP-binding cassette (ABC) which is involved in reducing the intracellular accumulation of xenobiotics (Chung et al., 2016). It plays a major role in the absorption, distribution and elimination of drugs and xenobiotics. It has been shown that flavonoids such as biochanin A and silymarin inhibit the P-gp facilitated efflux and increase the absorption of a drug i.e. digoxin, which was used as a substrate (Zhang et al., 2003). Additionally, there is evidence that pesticides such as organochlorine and organophosphate insecticides are inhibitors of human P-gp. Chedik et al. (2018) published a comprehensive review detailing interaction of pesticides with transporters. The authors highlighted the fact that various pesticides (i.e. organochlorines, pyrethroid and organophosporus pesticides) can inhibit the activity of ATP-binding cassette and P-gp are impacted by pesticides increasing their toxic potential (Chedik et al., 2018). Another family of ABC transporters is the multiresistant drug protein (MRP), particularly MRP2 and MRP3 are important in the efflux of conjugated xenobiotic metabolites. Finally the organic anion peptide transporters (OATP) which mediates the sodium-independent transport of xenobiotics and are involved in the hepatic uptake by OATP 1B1 and OATP1B3 (Clerbaux et al., 2019 as cited in Darney, 2020).

3.3 Metabolic interactions affecting chemical kinetics and dynamics

It has been shown in the literature that after concurrent exposure to a chemical mixture, interactions can occur at the metabolic level, which can consequently affect the individual chemical kinetics including AUC and Clint (Kadar et al., 2017; Grime and Riley, 2006). An increase in the AUC or changes in the clearance rate indicate that the toxin stays in the body for a longer period; thus, there is an increased possibility for expressing its adverse effect (Palleria et al., 2013). Moreover, after concurrent xenobiotic exposure, one compound may affect the uptake rate of another by competing at the biological ligands or by inhibiting the other compound's transport proteins (Cedergreen, 2014). In the case of an inhibition of P-gp, there is an increase in the bioavailability of a poorly absorbed chemical (Rehman et al., 2017). In general, a chemical can modify (increase/decrease) the biotransformation rate of another chemical. In the case of a decreased biotransformation rate, the toxicity is higher if the parent compound is more potent. On the other hand, if the toxicity of the metabolite is higher (bioactivation), then an increase in the biotransformation will lead to increased toxicity (Cedergreen, 2014). For instance, a mixture of pyrethroids with azole fungicides will cause synergistic adverse effects to animals and plants since the azoles will inhibit a range of CYP responsible for the biotransformation of pyrethroids (Cedergreen, 2014; Kretschmann et al., 2015).

3.4 Extensive literature search

An extensive literature search and meta-analysis was performed by Quignot et al., 2015, in order to collect data on metabolic interactions for, pharmaceuticals, hormones, food additives, natural products and various contaminants, with the aim of identifying relevant peer-reviewed publications. From this database, an extensive literature search on metabolic interactions for pharmaceuticals, pesticides and flavonoids, has been performed in April 2019 with additional searches in the grey literature. Details on both literature searches can be found in Annex B (Table B1).

Inclusion and Exclusion criteria were set for the identification of the relevant papers and data collection.

Inclusion criteria

- *In vitro* experimental laboratory studies using cells of human origin
- Data and information relating to Ki, IC_{50}, % of inhibition of pesticides and flavonoids related to CYPs, P-pg, UGTs, glutathione transferases

Exclusion criteria

- *In vitro* experimental studies using experimental animals' cell lines
- Duplicate studies
- Studies with only qualitative data

Data collection

Data were collected and computed into a standardised and structured database based on modified OECD Harmonised Templates (OHTs) and associated picklists (OECD Harmonised Templates for Reporting Chemical Test

Summaries, available online: http://www.oecd.org/ehs/templates/ harmonised-templates-health-effects.htm).

3.5 Relationship between IC_{50} and Ki

The two parameters that are most often used to measure the potency of an inhibitor are 1. the inhibitor constant (Ki) and 2. the half-maximal inhibitory concentration (IC_{50}). Ki is an indicator of the potency of an inhibitor and represents the concentration required to produce half of the maximum inhibition. The IC_{50} is the concentration of the compound required to cause 50% inhibition of the P450 enzyme. The relationship between the two depends on the type of enzyme inhibition. Cheng and Prusoff (1973) presented an equation of this relationship:

$$IC_{50}=Ki*(1+[S]/Km) \quad (3.6)$$

Where S: concentration of the substrate, and Km: Michaelis-Menten constant

In Table 3.4 the equations for calculating the Ki from IC_{50} in different inhibition types are summarised.

Table 3.4 Calculating Ki from IC_{50} (adapted from Cer et al., 2009; Haupt et al., 2015; Ramsay and Timpton, 2017)

Type of inhibition	**IC_{50} to Ki relationship**	**Equations when Km= [S]**	**Effect on V_{max}**
competitive inhibition	$K_i = \frac{IC50}{\frac{S}{Km}+1}$	$K_i = \frac{IC50}{2}$	None
uncompetitive inhibition	$K_i = \frac{IC50}{\frac{Km}{S}+1}$	$K_i = \frac{IC50}{2}$	↓ to $\frac{Km}{1+\frac{[I]}{Kis}}$
non competitive	$K_i = IC_{50}$	$K_i = IC_{50}$	↓ to $\frac{Km}{1+\frac{[I]}{Kis}}$
mixed	$IC_{50} = \frac{[S]+Km}{\frac{[S]}{Kis}+\frac{Km}{Ki}}$	IC_{50}=Ki to IC_{50}=2*Ki [a]	↓ to $\frac{Km}{1+\frac{[I]}{Kis}}$

[a] Depending on the ratio between the inhibitor binding to the enzyme and the inhibitor binding to the enzyme-substrate complex Ki= inhibitor constant representing the binding to the enzyme-substrate only

3.6 Extensive literature search results

A database was produced from selected studies resulting from the analysis of 179 peer reviewed publications. The database was organised for phase I (CYPs), phase II (UGTs) and trasnporters (P-pg) and by enzyme isoforms. A detailed overview of the outcome of the search can be found in Annex B. The inhibitors, which appear to be the most potent, according to their IC_{50}, are summarised in Table 3.5. The evaluation of the strength of the inhibition is based on the IC_{50}. An $IC_{50} > 10$ μM signifies a weak inhibition and a concurrent reduction in the clearance by 20-50 %. Marginal inhibitors are the inhibitors when 1 μM < IC_{50} < 10 μM and in this case clearance is reduced by 50-80 %. Strong inhibitors are defined as those with an IC_{50} close to 1μM and in this type of inhibition, the clearance is reduced by more than > 80 % (Bjornsson et al., 2003). The most potent inhibitor observed was amentoflavone, a biflavonoid constituent of several plants including Ginkgo biloba, which can also be found as a supplement which inhibits CYP3A4 involved in the biotransformation of the hormone testosterone on. In addition, co-administration of the pesticide fipronil with the drug ketoconazole demonstrated a potent inhibition of ketoconazole metabolism (IC_{50}=0.16 μM) on CYP3A4. For CYP1A2 from the various pesticides and flavonoids inhibitors (Table B2) the most potent were chlorpyrifos (IC_{50}=0.7 μM) and parathion (IC_{50}=0.8 μM). Penitrothion and chlorpyrifos were the most potent inhibitors of CYP2B6 (IC_{50}=2.5 μM and IC_{50}=2.8 μM respectively) from the pesticides and curcumin demonstrated the strongest inhibition from the flavonoids (IC_{50}=2.8 μM) reported (Table B4). Additionally, chlorpyrifos also appeared as the most potent inhibitor (while weak) of CYP2A6 with an IC_{50} of 16 μM (Table B5). Regarding UGTs, inhibition data were identified only for

UGT1A4, which are summarised in Table B18. The most potent inhibitors for UGT1A4 were ruscogenin (IC_{50}=0.2 μM), tigogenin (IC_{50}=0.54 μM) and disosgenin (IC_{50}=0.7 μM). After mixture administration of isorhamnetin with quercetin the permeability coefficient on P-gp increased (8 cm/s) in comparison with single quercetin administration (4.97 cm/s) (Lan et al., 2008). Similarly, quercetin, bergamottin and grapefruit showed an increase on P-gp permeability coefficient (8.04, 8.75, 26.01 $x10^{-7}$ cm/s) when co-administrated with saquinavir in comparison with single saquinavir administration (8.0 $x10^{-7}$ cm/s) (Eagling et al., 1999). Finally, silymarin and biochanin A, showed weak inhibition on P-pg (Ki= 30.7 and 32.7 μM respectively). Summary tables on P-pg interactions can be found in Tables B16 and B17.

Table 3.5 Summary of the most potent inhibitors identified

Compound	Compound type	Inhibitor	Dose of inhibitor (µg/kg)	Inhibitor type	CYP	IC_{50} (µM)	Reference
testosterone	hormone	amentoflavone	n/a	flavonoid	CYP3A4	0.027	Kimura et al., 2010
ketoconazole	drug	fipronil	0.87	pesticide	CYP3A4	0.16	Tang et al., 2004
bupropion	drug	fenitrothion	0.28	pesticide	CYP2B6	2.5	Abass and Pelkonen, 2013
bupropion	drug	curcumin	0.069 – 73.6	flavonoid	CYP2B6	9.4	Volak et al., 2008
imipramine	drug	parathion	0.15 -0.73	pesticide	CYP2C19	0.4	Di Consiglio et al., 2005
diclofenac 4'-hydroxylase	drug	galangin	n/a	flavonoid	CYP2C9	0.5	Si et al., 2008
tolbutamide	drug	malathion	0.33	pesticide	CYP2C9	2.5	Abass and Pelkonen, 2013
imipramine	drug	chlorpyrifos	0.088 – 0.88	pesticide	CYP1A2	0.7	Di Consiglio et al., 2005
dextromethorphan	drug	tetrahydropalmatine	n/a	alkaloid (supplement)	CYP2D6	1.17	Zhao et al., 2015
dextromethorphan	drug	phenthoate	1.6 – 32.04	pesticide	CYP2D6	3	Abass et al., 2009
testosterone	hormone	chlorpyrifos	3.5 – 70.1	pesticide	CYP2A6	16	Butler et al., 1997

3.7 Metabolic interactions in mixture risk assessment

The importance of metabolic interactions and their potential effect on combined toxicity of chemical mixtures has been described earlier (Section 3.1). Consequently, when performing a component-based risk assessment of a mixture, reliable information on the toxicokinetics of the compounds, including biotransformation, metabolic pathways and MoA/MeA for the components is necessary (IGHRC, 2008). Additionally, it is of great importance to have the above-mentioned information on the metabolic pathways, which relate to the activation or deactivation of the active form. In the case, that information on the interaction's possibility is missing, risk assessors may use a default approach and assume no interactions (Tier 1). It is documented that when mixtures are administered in doses below the threshold level dose, interactions do not usually occur (VKM, 2008). The enzymes involved in the xenobiotic biotransformation have low specificity and high capacity, so low levels of inhibition or induction will not cause an obvious toxicity effect (Dekant, 2009). Thus, the levels of exposure must be relatively high in order to influence the enzyme function and affect toxicity, a phenomenon more commonly present when assessing drug-to-drug interactions (high doses).

If data are available, showing that there is enough evidence to consider toxicokinetic interactions, then interaction hazard index (HI_I) and PBK modelling are the two major tools to assess the mixture interactions (Desalegn et al., 2019).

3.7.1 Interaction-based hazard index approach

The interaction-based hazard index approach is used for additive effects, but can also be used to account for interactions. The interaction-based HI (HI_I) is calculated with the following equation, whereby the HI is multiplied with uncertainty and the strength of evidence of the interaction-taking place (Sarigiannis et al., 2012):

$$HI_I = HI * UF_I^{WOEn} \quad (3.7)$$

Where UF_I: uncertainty factor for interactions

WOEn: numerical weight-of-evidence, which reflects the strength and consistency of evidence for interaction

The weak points of the above method were summarised by Reffstrup et al. (2012) and include among others:

- Lack of guidance on adequate uncertainty factors;
- The weighing factors lack empirical assessment support for the key experimental values;
- Magnitude of interaction is not included;
- Finally, the data used in this model are evaluated qualitatively, which increases the uncertainty and thus increasing the applicability of the model.

3.7.2 Physiologically based toxicokinetic modelling

Another approach used to predict the interactions and the overall toxicity of a mixture is the PBK model. According to WHO, the physiologically-based toxicokinetic modelling is "*a model that estimates the dose to target tissue by taking into account the rate of absorption into the body, distribution and storage in tissues, metabolism and excretion on the basis of interplay among critical physiological, physicochemical and biochemical determinants*" (WHO, 2010). This type of modelling provides information on the kinetic parameters (ADME) of a compound and helps predict the actual internal dose after a chemical is administered to an organism. Teorell and colleagues first applied this type of modelling in the pharma industry in 1937 and the concept flourished in the '60s and '70s in the fields of drug discovery, environmental pollutants and industrial chemicals (Reddy et al., 2005). The pharma industry has been implementing PBK simulations in the various steps of drug development. A list with various examples of approved drugs for which PBK was used can be found in Jamei (2016). In recent years, PBK modelling is usually coupled with pharmacodynamic data (PBK-PD), which can predict what the effect of the compound on the organism is and provide a reference value for the chemical. If a chemical reaches the organism (animal or human) unintentionally (contaminant) and has an adverse effect, then the modelling is called physiologically based toxicokinetic and toxicodynamic model respectively (PBTK, PBTK-TD). This type of modelling is used at the Tier III assessment level, meaning that a great number of data are required in order for it to be usable. Additionally, it provides a more realistic picture of system biology allowing interactions modelling. PBK-PD modelling is one of the most suitable tools for coupling interactions between chemicals and exposed

organisms, and there are various examples of its use in the literature (Paini et al., 2019 and Tan et al., 2018). Legislative agencies like WHO and US EPA recommend its use in assessing the risk of chemical mixtures, while in 2014 EFSA summarized the difficulties in its application i.e. a large number of data and detailed TK knowledge, detailed TD knowledge for PBTK-TD models, great expertise and resources, and model validation (EFSA, 2014a).

PBK application in mixtures

In the past 35 years, a huge endeavour towards the development of PBK models for mixtures of various chemical compounds has been made (Reddy et al., 2005; Lu et al., 2016). The majority of these models focus mainly on petroleum, volatile organic compounds and metals, while a few only focus on pesticides. More specifically, gasoline, VOCs, pesticides are assessed by using either a top-down (evaluation of chemical interactions starting from more complex mixtures to the individual compounds) or a bottom-up approach (binary mixture interactions linking PBK of single chemicals to predict their kinetics in a complex mixture) (Desalegn et al., 2019; Dennison et al., 2004). Another technique that has been used especially for hydrocarbon compounds is the "lumping analysis", whereby chemicals with similar characteristics are grouped together (Verhaar et al., 1997 and Yang et al., 1995).

The advantage of understanding and identifying the mechanisms underlying binary chemical interactions is mainly that the acquired knowledge can be used in the assessment of more complex mixtures too (Tardiff et al., 1997; Haddad et al., 1999; Haddad et al., 2000; Cheng and Bois, 2011). Thus, binary mixture information is important for formulating PBK models for complex mixtures

(Krishnan and Brodeur, 1994; Tardiff et al., 1997). A bottleneck for this type of modelling is that all interactions at the binary level should be defined both quantitatively and qualitatively (Hadddad et al., 2010).

Data needs

In order for researchers to start applying these models described above, an extensive literature review is required that would help find the necessary physiological and biological data for the species of interest (body weight, organ weight, age, hepatic blood flow, body surface). The next step should be to collect the necessary *in vitro* parameters (i.e. V_{max}, Km, Ki, intrinsic clearance), the *in vivo* parameters (elimination half-life, bioavailability, absorption, clearance) and finally, the information on the expression of the relevant enzymes in the liver, gut etc., in humans as well as in the relevant test species.

Moreover, when modelling a binary mixture, data on the mode of action of the chemicals are needed. When the chemicals do not interact, then they can be treated as single compounds (Haddad et al., 2010). For interacting chemicals, the PBK models for the individual compounds should be performed first and the type of interaction should be accounted for. Usually in the literature, the type of interaction that occurs most frequently is the metabolic inhibition (van Gestel et al., 2016), The rates of metabolism for compound 1 (RAM_1) and for compound 2 (RAM_2) are calculated with a modified Michaelis–Menten formula for the effect of the interaction (Haddad et al., 2010; Krishnan et al., 2002; Reffstrup et al., 2012).

$$RAM_1 = \frac{Vmax1 * C1}{C1 + Km1 * (1 + C2/Ki21} \quad (3.8)$$

$$RAM_2 = \frac{Vmax2 * C2}{C2 + Km2 * (1 + C1/Ki12} \quad (3.9)$$

Coupling human variability with PBK modelling

Over the past 30 years, various studies have been published, addressing the importance of adding human variability to the PBK model, mainly for drug development purposes in the pharma industry (Jones et al., 2015; Paini et al., 2017). From a food contaminant perspective, US EPA attempted in 2005 to develop a PBK model for a mixture of N-methyl carbamates, including population exposure distributions, with the aim of determining the cumulative risk. However, because of the absence of data, it ended up performing the assessment by using relative potency factors. EPA concludes that the complexity of the compounds means that it is necessary to consider the whole picture and include as much information as possible (exposure data, uncertainty factors, safety factors etc.) (US EPA, 2005).

The human variability factor is unavoidable and will always exist thus eliminating this factor is rather impossible. It can take various forms, relating as it does to genetic, population, age-dependent and ethnic/cultural differences (Burin and Saunders 1999; Zeise et al., 2013). There are many examples in the literature and various case studies have already been conducted on the importance to introduce human variability into a PK model (Caldwell et al., 2012; Huisinga et al., 2012; McNally and Loizou, 2015; Paini et al., 2019). In relevance to toxicology, relevant papers have already been published since the

early 1990s (e.g. Clewell and Andersen 1996; Dankovic and Bailer 1994). Frederic Bois and colleagues produced a comprehensive review in 2010, providing detailed information on the different sources of variability in kinetic modelling (ADME), and the way in which variability is taken into account by performing Monte Carlo simulations and Markov chain analysis. Coupling PBK models with the Monte Carlo approach allows the simulation of variation in the kinetics and the metabolism of chemicals. By using simulated distribution data on kinetics, physiological parameters and toxicodynamics, hence including variability in the model, uncertainty decreases and the reliability of the predictions of the model increases (Darney et al., 2018; Darney 2020; Kasteel et al., 2020). Figure 3.2 presents an overview of the workflow needed for the performance of a PBK-TD modelling for mixtures, including variability.

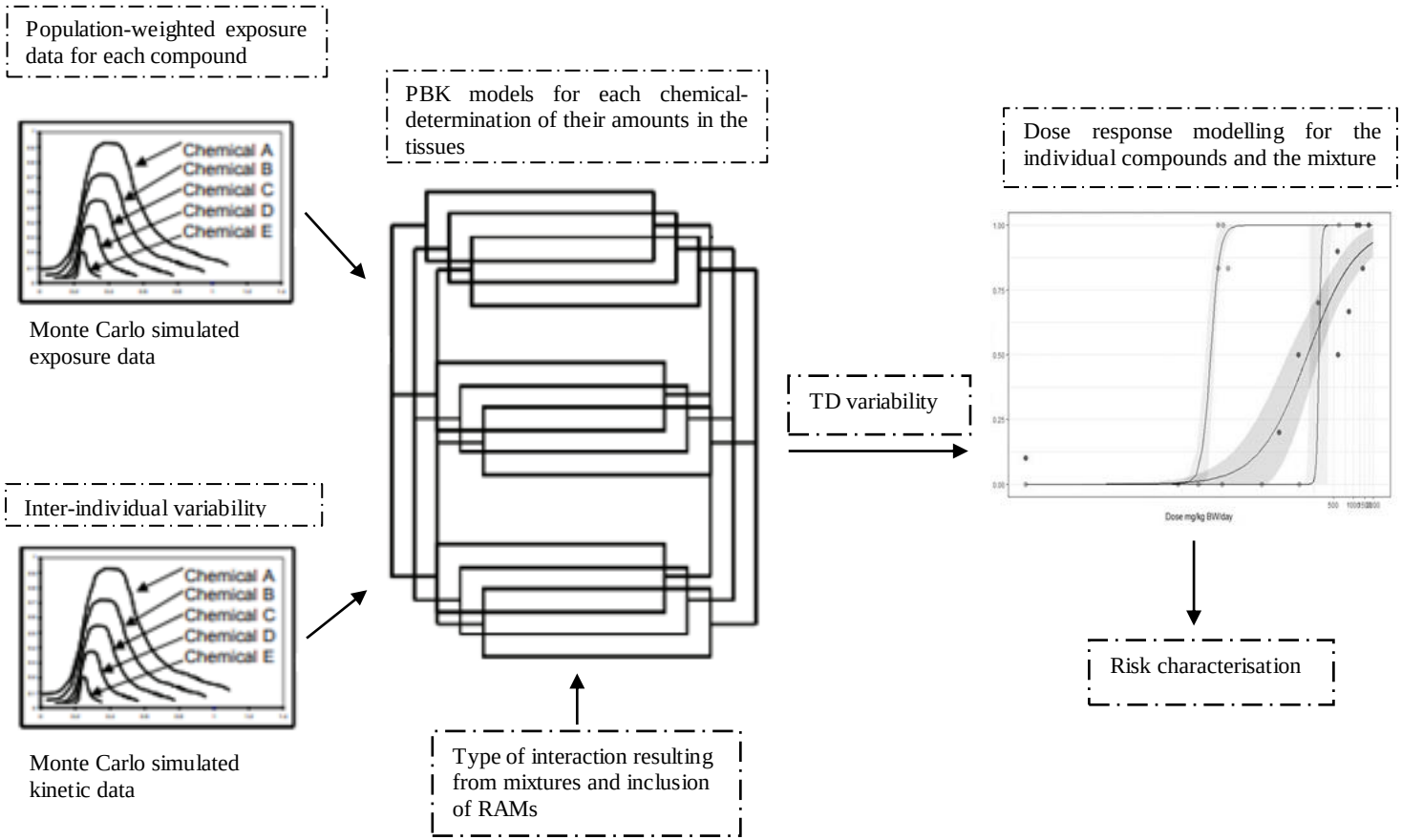


Figure 3.2 Workflow to perform PBK-TD modelling for mixtures including variability (Modified from US EPA, 2006)

These models can also be extrapolated to target different life stages (infants, the elderly etc.) or sexes for a further characterisation of the exposure (Haddad et al., 2006).

3.8 Uncertainty factors taking into account for metabolic interactions

In the absence of human toxicokinetic data, *in vitro* TK data can help derive UFs relating to metabolic interactions. Quignot et al. (2019), while investigating the inhibition and induction of grapefruit juice (GFJ) and St. John's wort (SJW) on CYP3A4 substrates in humans, derived CYP and Pgp-related UFs for single compounds and for binary mixtures, integrating CYP3A4 variability, bioavailability and the substrate fraction metabolised by CYP3A4. While the UFs for the single compounds were in the range of the default TK UF of 3.16, the CYP3A4/Pgp-related UFs for inhibition (GFJ) and induction (SJW) were six-fold and two-fold respectively (Quignot et al., 2019).

3.9 In vitro to in vivo extrapolation

For the extrapolation of the observed *in vitro* intrinsic clearance to *in vivo* clearance, the following equation was used (Nakamori et al., 2011)

$$CL = \frac{V_{max}}{Km} * MPPGL * \frac{LW}{BW} \quad (3.10)$$

V_{max}= Maximum reaction rate when the enzyme is fully saturated by the substrate, Km= Michaelis constant, substrate concentration at which the reaction rate is half of V_{max}, MPPGL = microsomal protein per gram of liver (18.2 for humans Abass, 2010), LW = liver weight and BW = body weight

The outcome of the IVIVE calculation can be found in Table 3.6. An interesting observation was the notable difference in the calculation of the clearance of curcumin as compared with the data observed. The main reason for this variation stems from the fact that curcumin does not behave in the same way when exposed *in vivo* and when exposed *in vitro*. After the *in vivo* exposure, its clearance is extremely fast in comparison with the *in vitro* administration.

Table 3.6 Calculated vs observed clearance

Reference	Chemical 1	Chemical 2	CYP	Cl Extrapolated in vivo (ml/min kg)	CL observed in vivo (ml/min kg)	Single compound	Reference Cl observed
Appiah et al., 2007	diclofenac	curcumin	CYP2C9	688.4	3.5	diclofenac	Obach et al., 2008
Beckmann-Knopp et al., 2000	dextromethorphan	silibinin	CYP2D6	20.5	2.14	dextromethorphan	Capon et al., 1996
Beckmann-Knopp et al., 2000	warfarin	silibinin	CYP2C9	0.17	0.06	warfarin	Obach et al., 2008
Luan et al., 2005	propranolol	-	total	2.83	12	propranolol	Obach et al., 2008
Ngui et al., 2001	warfarin	-	CYP3A4	0.09	0.06	warfarin	Ngui et al., 2001
Ngui et al., 2001	warfarin	quinidine	CYP3A4	0.09	0.06	warfarin	Obach et al., 2008
Tsujimoto et al., 2017	testosterone	cabbage	CYP3A4	2.58	24.5	testosterone	Obach et al., 2008

The differences between the observed and the calculated values can be explained on the basis of the following; substrate concentration affects clearance, data on unbound fraction of chemicals in microsomes were missing, i*n vivo* intrinsic clearance prediction in human microsomes average a 2.8 fold under-prediction (Wood et al., 2017).

3.10 Impact of enzyme inhibition intrinsic clearance

As was mentioned above various CYP enzymes are responsible for the biotransformation of xenobiotics. After co-exposure of chemicals, metabolic interactions may occur leading to inhibition on CYP activity, which in turn can slow metabolism and clearance of the co-administrated chemicals, leading to an increased toxic potential of the chemicals of interest. In Table 3.7, the main findings on the influence metabolic interactions can devise on intrinsic clearance are summarised. When data on intrinsic clearance were not available, the following equation was used for its estimation.

$$CI_{int} = \frac{V_{max}}{K_m(\frac{cinh}{K_i})} \quad (3.11)$$

Additionally by using equation (3.10) from above, the estimated intrinsic clearance was extrapolated to *in vivo* clearance and finally yet importantly for each inhibitor the ADI was retrieved, for its comparison with the respective doses used in the experiments.

Table 3.7 Comparative clearance values between single and chemical mixtures

Chemical 1 (µM)	**Chemical 2 (µM)**	**CYP450**	**Cl_{int} (ml/mg min)**	**CL *in vivo* (ml/mg min)**	**ADI (µM)**	**Reference**
naphthalene (0-500 µM)	-	CYP3A4	0.019	0.008	-	Cho et al., 2007
naphthalene (0-500 µM)	chlorpyrifos-oxon (5 µM)	CYP3A4	0.52	0.23	0.0029	Cho et al., 2007
naphthalene (0-500 µM)	chlorpyrifos-oxon (80 µM)	CYP3A4	0.9	0.39	0.0029	Cho et al., 2007
linuron (2 µM)	-	CYP1A2 CYP3A4 CYP2C19 CYP2B6	177	77.6	0.012	Kadar et al., 2017
linuron (2 µM)	ethion (4 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	176.4	77.4	0.005	Kadar et al., 2017
linuron (2 µM)	ethion (20 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	167.94	73.6	0.005	Kadar et al., 2017
linuron (2 µM)	chlorfenvinphos (4 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	169.2	74.2	0.0014	Kadar et al., 2017
linuron (2 µM)	chlorfenvinphos (20 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	141.6	62.1	0.0014	Kadar et al., 2017
chlorfenvinphos (4 µM)	-	CYP1A2 CYP3A4 CYP2C19 CYP2B6	315	138.2	0.0014	Kadar et al., 2017
chlorfenvinphos (4 µM)	linuron (2 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	304.8	133.7	0.012	Kadar et al., 2017
chlorfenvinphos (4 µM)	linuron (10 µM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	220.8	96.8	0.012	Kadar et al., 2017
chlorfenvinphos (4 µM)	ethion (4 µM)	CYP1A2 CYP3A4	312.6	137.1	0.005	Kadar et al., 2017

		CYP2C19 CYP2B6				
clorfenvinphos (4 μM)	ethion (20 μM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	250.2	109.7	0.005	Kadar et al., 2017
ethion (4 μM)	-	CYP1A2 CYP3A4 CYP2C19 CYP2B6	303.6	133.2	0.005	Kadar et al., 2017
ethion (4 μM)	linuron (2 μM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	288	126.3	0.012	Kadar et al., 2017
ethion (4 μM)	linuron (10 μM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	282	123.7	0.012	Kadar et al., 2017
ethion (4 μM)	chlorfenvinphos (4 μM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	283.2	124.2	0.0014	Kadar et al., 2017
ethion (4 μM)	chlorfenvinphos (20 μM)	CYP1A2 CYP3A4 CYP2C19 CYP2B6	233.4	102.4	0.0014	Kadar et al., 2017
methoxyresorufin (0.65-10 μM)	-	CYP1A2	0.49	0.21		Appiah et al., 2007
methoxyresorufin (0.65-10 μM)	curcumin (25 μM)	CYP1A2	0.13	0.056	8.14	Appiah et al., 2007
benzyloxyresorufin (0.65-10 μM)	-	CYP3A4	0.075	0.033		Appiah et al., 2007
benzyloxyresorufin (0.65-10 μM)	curcumin (2.5 μM)	CYP3A4	0.059	0.026	8.14	Appiah et al., 2007
methoxyresorufin (1.4-22.7 μM)	-	CYP2D6	0.53	0.23		Appiah et al., 2007
methoxyresorufin (1.4-22.7 μM)	curcumin (45 μM)	CYP2D6	0.02	0.01	8.14	Appiah et al., 2007
diclofenac (3-20 μM)		CYP2C9	1.28	0.56		Appiah et al., 2007
diclofenac (3-20 μM)	curcumin (6 μM)	CYP2C9	0.11	0.04	8.14	Appiah et al., 2007
benzyloxyresorufin (3.1-50 μM)	-	CYP2B6	0.004	0.0017		Appiah et al., 2007
benzyloxyresorufin (3.1-50 μM)	curcumin (50 μM)	CYP2B6	0.0004	0.0002	8.14	Appiah et al., 2007

phenacetin (80-800 μM)	-	CYP1A2	0.0007	0.0003	Zhao et al., 2015
phenacetin (80-800 μM)	tetrahydropalmatine (280 μM)	CYP1A2	0.0021	0.0009	Zhao et al., 2015
phenacetin (80-800 μM)	neferine (160 μM)	CYP1A2	0.0025	0.0011	Zhao et al., 2015
phenacetin (80-800 μM)	berberine (78 μM)	CYP1A2	0.0004	0.0002	Zhao et al., 2015
dextromethorphan (10-150 μM)	-	CYP2D6	0.0045	0.002	Zhao et al., 2015
dextromethorphan (10-150 μM)	tetrahydropalmatine (280 μM)	CYP2D6	0.0001	2.55734E-05	Zhao et al., 2015
dextromethorphan (10-150 μM)	neferine (160 μM)	CYP2D6	0.0008	0.0004	Zhao et al., 2015
dextromethorphan (10-150 μM)	berberine (78 μM)	CYP2D6	0.0002	8.26124E-05	Zhao et al., 2015
testosterone (160-1600 μM)	-	CYP3A4	0.0019	0.00083	Zhao et al., 2015
testosterone (160-1600 μM)	tetrahydropalmatine (280 μM)	CYP3A4	0.0003	0.00014	Zhao et al., 2015
testosterone (160-1600 μM)	berberine (78 μM)	CYP3A4	0.0005	0.00024	Zhao et al., 2015

For most of the compounds and enzymes investigated, a decrease in the metabolic clearance was observed after co-administration with an inhibitor. An exception to this observation, was in the co-administration chlorpyrifos-oxon with naphthalene for which the clearance increased due to the induction of naphthalene's metabolism due to chlorpyrifos-oxon (Cho et al., 2007), The addition of ethion, chlorfenvinphos, and linuron pesticides showed a decrease (1.5 fold) in the clearance after their co-administration in comparison with when each pesticide was given individually. Similarly, co-administration of cumarin and other flavonoids showed a decrease (3-fold) on intrinsic clearance. Human clearance was predicted within a two-fold of the estimated intrinsic clearance. CYP inhibition and reduction in the clearance of a chemical is a clear indication of an increase in the internal dose of the chemical and may produce adverse effects in the case of drug-drug co-administration. However, it should be noted that the doses of the inhibitors were 1000 times higher than their respective ADI.

3.11 Conclusion

In this chapter, the inhibition and/or induction of different plant extracts, flavonoids and pesticides to their respective enzymes was characterised during the different stages of metabolism. The importance of metabolic interactions during all phases of biotransformation was highlighted and attention was drawn to the fact that a change in the biotransformation of a xenobiotic (i.e. pesticide) can influence its final toxicity. The most potent CYP inhibitors identified were; fipronil, parathion, chlorpyrifos, fenitrothion, malathion, phenthoate for pesticides, while for flavonoids were; amentoflavone, galangin, tetrahydropalmatine and curcumin. For UGTs, flavonoids such as ruscogenin,

tigogenin and disosgenin showed strong inhibition on UGT1A4. Concerning P-gp, there were limited interaction data in the literature. Moreover, the intrinsic clearance of single and chemical mixtures was estimated and compared, showing that a CYP inhibition can increase the amount of time a chemical stays in the body causing overexposure. In the meta-analysis of the metabolic interaction data, most often only the inhibition parameters were mentioned (IC_{50}, Ki, % of inhibition) and no other kinetic parameters such as the K_m and the V_{max} were reported. Finally, the significance in the availability of interaction data was presented, in order to increase the reliability of NAMs such as IVIVE and PBK modelling for single and multiple compounds, which in turn will lead to more accurate risk assessments.

Chapter 4

Analysis of combined toxicity data for human risk assessment of chemical mixtures: Pesticides and persistent organic pollutants (POPs) case study to support methodological development

4.1 Background and relevance

Human and animal species are exposed daily to pesticide residues from various products. They can cause numerous different toxicological effects in different organs and functions in the organism such as; brain, nervous system, blood, liver, kidney, developmental, reproductive, hormone disruption and the immune system in test animals and humans (Perobelli et al., 2010; Gomez-Gimenez et al., 2018; Aroonvilairat et al., 2018; Bano and Mohanty 2020 ; Zhang et al., 2020).

Moreover, some pesticides such as organochlorines are characterised as POPs. These chemicals are of great concern all around the globe, due to their persistence in the environment and their ability to bio accumulate in the organisms. Due to their bioaccumulation, organisms at the top of the food chain (humans) if exposed to even low levels of POPs, can suffer from an increased risk for cancer, reproductive disorders, neurobehavioral impairment, endocrine disruption, genotoxicity and birth defects (WHO, 2010).

Pesticides generally occur more often as mixtures than as individual compounds, as a result their potential toxicological effects as a mixture is

increasingly important. On the contrary, their toxicological effects on human and animal health after low exposure is not well characterised (Hernandez et al., 2013). Additionally, the risk assessment of pesticide residues in food has been based on the toxicological evaluation of the single compounds (Reffstrup et al., 2010). However there is ample evidence in the literature that the toxicological effect produced by a mixture of pesticides can be additive, less than additive (antagonistic), or greater than additive (synergism) (Hernandez et al., 2013 and DeLorenzo and Serrano, 2003). From the above mixture concepts, the one most frequently occurring in the literature for most pesticide groups is the additivity concept (Thomson, 1996; Cedergreen, 2014).

Thus, it is of great importance to assess pesticide mixtures in order to avoid potential underestimation when performing a risk characterisation. Moreover, the European Commission has already published articles advising for the pesticide studies to be carried out on mixtures so that the possible cumulative, aggregate or synergistic effects be assessed (EC 396/2005). The US EPA published already in 1986 a guidance on how to conduct health risk assessment for chemical mixtures. Moreover, it published a supplementary guidance in 2000 and in 2003 a report on how to apply relative potency factors to pesticide mixtures. (US.EPA, 2000, 2003). Additionally, EU legislation on plant protection products and biocides advises the assessing authorities to evaluate and take into account "known cumulative and synergistic effects when the scientific methods are available" (EC 1107/2009; EU 528/2012).

EFSA in accordance with the (EC 396/2005) regulation has published a number of opinions on how to perform a mixture risk assessment on pesticides (EFSA, 2008, 2009, 2013c) and more recently, in 2019 two more opinions on cumulative assessment groups of pesticides for their effects on the thyroid and the nervous system (EFSA, 2020, 2019d). This work will also assist on the

characterisation of the mode of action (similar/dissimilar) for an accurate estimation of the adverse effect from the mixture.

This chapter aims to compute and analyse scientific evidence dealing with the combined toxicity of chemical mixtures in order to support the development of methodologies for human risk assessment. An extensive literature search was performed to gather relevant toxicity data from different animal species (i.e. rat, mouse, dog and rabbit) into excel databases. Combined toxicity data for binary mixtures were analysed for each study with the aim of quantifying magnitudes of interaction, as the mean effect ratio between single compounds and binary mixtures, following acute, subacute, sub-chronic or chronic exposure. These meta-analyses allow i) transparently reporting measured toxicological endpoints using a weight of evidence approach, ii) testing the dose dependency of toxicological interactions, and iii) illustrating how meta-analysis can support hazard characterisation of the combined toxicity of chemical mixtures. Additionally, the toxicological endpoints retrieved were compared with the toxicological data from EFSA's chemical hazards database "Openfoodtox" for the respective exposure times. When information was not available from the EFSA's online tool, other open databases were used, such as those of the US EPA, OECD e-chem portal and PPDB-pesticide properties.

4.2 Methodology

4.2.1 Extensive literature search

An extensive literature search (ELS) was performed by Quignot et al., 2015 on pesticide and POP mixtures in experimental animals from 1975 to 2014. Additionally using the same keywords (Table 4.1) the search was updated in June 2017, identifying papers relevant to the toxicity of pesticide and POP mixtures using PubMed and Web of Science databases.

Table 4.1 Keywords used for the ELS

Keywords
Pesticide OR Persistent Organic Pollutants OR POPs
AND
Mixture OR mixtures OR interaction OR interactions OR synergising OR synergism OR synergisms OR synergist OR synergistic OR synergistically OR synergists OR agonism OR agonisms OR agonist OR agonists OR antagonism OR antagonisms OR antagonist OR antagonists OR inhibitor OR inhibitors OR inhibitory OR induction OR inhibition OR "dose addition" OR "response addition"
AND
toxicity OR toxic OR toxicological OR pharmacodynamic OR toxicodynamic
NOT
"in vitro" OR "cell" OR "cells" OR "review"

After the search was concluded, the papers were collected in an EndNote file and the publications were screened. First, the papers were screened by title and abstract. This first screening was then followed by the screening of the full-

text of the publications. The studies that were deemed relevant were included in the database.

For a harmonised screening of the literature, inclusion and exclusion criteria were applied:

Inclusion criteria

- Timeframe: Studies published until 14.06.2017
- Review question:
  Does the publication provide toxicological results of combined exposure to chemical mixtures (priority given to binary mixtures studies)?
- Populations of interest: rats, mice, rabbits and dogs

Study design:

- *in vivo* experimental laboratory studies
- *in vivo* field/semi-field studies

Outcome of interest:

Data and information on the toxicodynamic effects of mixtures of chemicals (LD50, NOEL, NOAEL)

Exclusion criteria

- Studies providing only qualitative information on the relevant endpoint
- *In vitro* studies
- Duplicates

4.2.2 Methods for the statistical analysis

Most studies reported the toxicological effect as an arithmetic mean (X) and included the respective standard deviations (SD). For a harmonised analysis, the geometric mean (GM), the geometric standard deviation (GSD) and the coefficient of variation (CV) were calculated for the respective toxicological endpoints (Quignot et al., 2015).

The coefficient of variation for normal distribution CVN was calculated with the equation (4.1) below:

$$CV_N = \frac{SD}{X} \tag{4.1}$$

If the standard error (SE) is reported instead of the SD, then the SD, CV and 95% confidence interval of the mean are estimated according to equations (4.2), (4.3) and (4.4):

$$SD = \sqrt{n}\ SE \tag{4.2}$$

$$SD = CV\ X \tag{4.3}$$

$$SD = \frac{UCI - LCI}{2t_{0.975,n-1}}\sqrt{n} \tag{4.4}$$

Where UCI and LCI are the upper and lower bounds of the confidence interval respectively and $t_{0.975,n-1}$ is the 97.5 percentile of the t distribution with n – 1 degrees of freedom (assumed that for a symmetric confidence interval, the confidence interval is constructed in the common way $X \pm t \times SE$)). In order to transform the arithmetic values n to the log scale, the equations 5-6 were used (Dorne et al., 2002):

$$GM = \frac{X}{\sqrt{(1+CV_N^2)}} \tag{4.5}$$

$$GSD = exp\left\{\sqrt{ln(1 + CV_N^2)}\right\} \quad (4.6)$$

The equation for calculating the coefficient of variation for lognormal distributed data is:

$$CV_{LT} = \frac{ln(GSD)}{ln(GM)} \quad (4.7)$$

A comparative analysis was also performed between the dose applied for each toxicological endpoint and the respective reference values (NOAEL or BMDL) as found in EFSA's open source chemical hazards database "Openfoodtox" for acute, sub-chronic and/or chronic toxicity for all species. If the information was missing, other publicly available databases were utilised i.e. US EPA, OECD e-chem portal, PPDB-Pesticide Properties Database. In order to measure the ratio of difference between the two values, the DoseB was calculated:

$$Dose_B = \frac{Applied\ Dose_B}{Endpoint_B} \quad (4.8)$$

4.2.3 Mixture modelling using the BMD EFSA software

For the hazard assessment of non-genotoxic or carcinogenic food substances, the No-Observed-Adverse-Effect Level (NOAEL) is used as a point of departure for critical effects (Sand et al., 2017). As the name dictates, it is the dose at which there is no effect on the endpoint of interest, thus allowing the calculation of a reference point upon which to derive a health-based guidance value such as the ADI. In order to calculate the ADI, the following formula is used:

ADI = NOAEL/ Safety factor (i.e. 100) (4.9)

The disadvantages of this method are:

- It uses only qualitative and no quantitative information;
- It takes into consideration one dose only
- The experimental design is influencing the outcome

An alternative to the NOAEL is the Benchmark Dose (BMD), which makes use of the dose response data to estimate the shape of the overall dose-response relationship for the selected endpoint. From this curve, the BMD dose level is derived on the basis of a specific change in the response. The advantage of the BMD approach is the extended use of the dose-response data from both experimental and epidemiological studies for better risk quantification and characterisation. It makes use of all the dose-response data in order to estimate the shape of the curve (Figure 4.1) and not only single dose data. Additionally, it can be used for all toxicological endpoints.

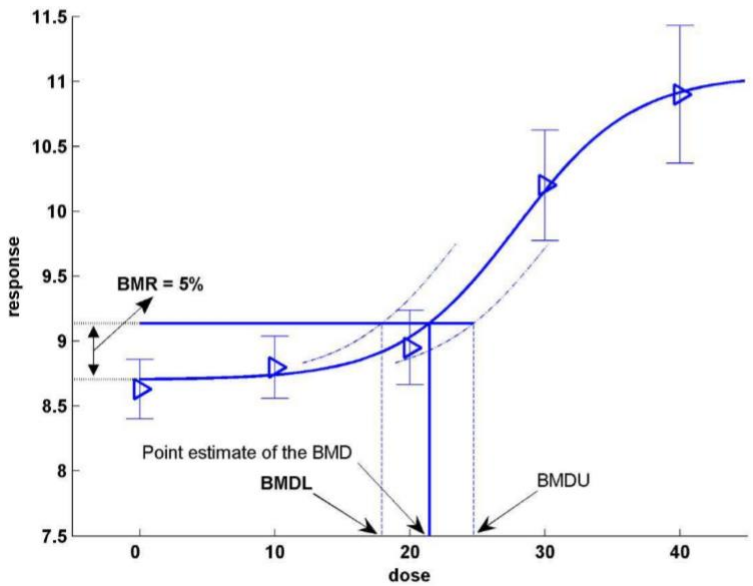


Figure 4.1 BMD modelling example (from EFSA, 2017d)

On the dose-response curve (EFSA, 2017d) the mean responses are marked by triangles along with the respective confidence intervals. The curve (solid line) is defined based on the BMD, which is the dose corresponding to a predetermined change in the response called benchmark response (BMR). The dotted lines represent the upper and lower 95% confidence bounds for the effect size and are defined on the basis of the lower and upper bounds of the BMD (BMDL and BMDU respectively). Therefore, the selection of BMR is an important part of the BMDL calculation and depends on the endpoint of interest, the type of available data (quantal, continuous) and their source (e.g. epidemiology) (EFSA, 2017d). For quantal data 10% extra risk is recommended and 5% for biological adverse effects i.e. reproductive or developmental. A 1% BMR can be used for quantal human data from epidemiology studies. For continuous data, it is important to also consider the biological endpoint of interest and the BMR change that is considered significant for the respective endpoint (i.e. 10% for bodyweight change) (US EPA, 2012).

The BMD approach can be used for all food chemicals and particularly for the determination of the Margin of Exposure (MOE), especially for compounds that are both genotoxic and carcinogenic, thus NOAEL cannot be applied (US EPA, 2012; EFSA, 2017d).

From the database, the papers containing dose response data were identified and then "cleaned" in order to fit the criteria required by the EFSA BMD software.

4.2.4 Mixture modelling using CA/IA approach

In order to perform the mixture modelling and determine if the relationship between the pesticides is synergistic/antagonistic or is better described by dose addition or is dose level dependent, the formulas used are derived from the study performed by Jonker et al. (2005).

For concentration addition, the following equation (4.10) was used:

$$\sum_{i=1}^{n} \frac{c_i}{f_i^{-1}(y_{c_i})} = \exp(G_{c_1,c_2,\ldots,c_n})$$

Where ci: the respective concentration of the chemicals, fi-1 (y): the inverse dose response relationship of the mixture, and G: the quantity of the chemical.

For independent action, the equation (4.11) was used:

$$y_{c_1,c_2,\ldots,c_n} = u_0 \Phi \left\{ \Phi^{-1} \left(\prod_{i=1}^{n} h(c_i) \right) + G_{c_1,c_2,\ldots,c_n} \right\}$$

Where Φ: the function for the standard cumulative normal distribution

h(ci): the cumulative distribution function related to the concentration (c) of the compound

Synergism or antagonism can be described with the following equation (4.12)

$$G(z_1,\ldots\ldots\ldots,z_n) = \alpha \prod_{i=1}^{n} z_i$$

Where z is the ratio between the toxic unit (TU) of a chemical (i) and the sum (Σ) of TU for a chemical j

The parameter α determines if there is antagonism (when it is positive) or synergism (when it is negative)

In the case of dose-ratio dependent action the following equation was used (4.13)

$$G(z1, z2) = (\alpha + b1z1)\, z1z2$$

Where the b parameter determines if there is antagonism when > 0, or synergism when < 0

For dose level dependent deviation the equation (4.14) was used;

$$G(z_1, \ldots\ldots\ldots, z_n) = \alpha\,(1 - b_{DL} \sum_{i=1}^{n} TU50_i) \prod_{i=1}^{n} z_i$$

Where if α is positive then $\sum_{i=1}^{n} TU50i = 1$ thus if bDL =1 and $G(z_1,..,z_n) = 0$, no deviation from the concentration addition is observed. If bDL= 0, then the equation takes the form of the equation (4.13) describing synergism or antagonism.

4.3 Results and discussion

4.3.1 Outcome of ELS

Quignot and colleagues (2015) identified 13256 papers in the literature covering years 1975-2014, while the search from 2014-2017 yielded 591 papers. After following abstract and full text filtering, 225 papers were included from both searches. The PRISMA graph below (Figure 4.2) summarises the outcome of both searches.

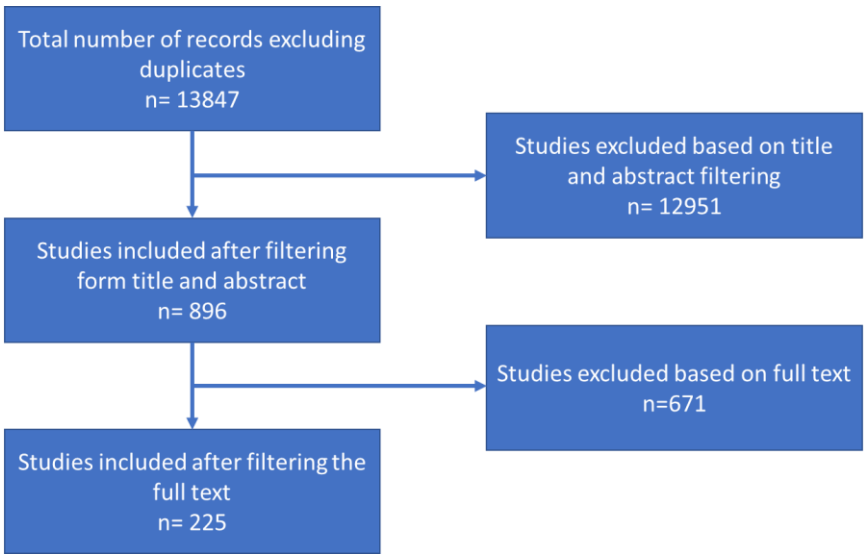


Figure 4.2. PRISMA flow-chart presenting the total number of papers from 1975-2017

From the 225 papers included in the database, 113 containded dose-response data. In rat *spp*. magnitudes of combined toxicity effects following intraperitoneal or oral exposure ranged from 1.02 to 92.5 (increased toxicity) and from 1.1 to 2.77 (decreased toxicity) for pesticides and POPs.

Magnitudes of interactions were expressed as a ratio between geometric means of effect with and without interaction. The ratio is expressed on a harmonised scale starting at one, in order to reflect changes in the different toxicological endpoints. These ratios were calculated for the following toxicological endpoints: general toxicity (i.e. body weight, sperm mobility), biochemical parameters (i.e. uric acid, creatinine), enzyme activity (such as liver glutathione reductase) and oxidative stress; thus, statistically significant ratios ranged from 1.1 to 92.55 for pesticides and POPs interactions in rat *spp*. Moreover, regarding the comparative analysis between the experimental administered dose of the second compound of the binary mixture (covariate) and the reference values from the literature ($Dose_B$) ranged from 0.007 to 0.25, thus, showing that in most of the studies the dose applied for the second compound of the mixture is well below the corresponding endpoint.

4.3.2 Mixture modelling results

Unfortunately, the EFSA open software was not able to perform the BMD calculations for mixtures and the results did not provide a clear outcome (dose addition/antagonism/synergism). Thus, it was decided that the toxicodynamic relationship should be investigated using a software developed by Jonker and colleagues in 2005.

For the modelling of pesticides and/or persistent organic pollutants, a template was used following the equations described in section (4.2.2). For the formulas to be used, the data had to follow some requirements, such as:

- Binary mixture
- Multiple doses (at least 2, or 3 including control)
- Control data (when the dose is 0)

The response model regarding concentration addition for the binary pesticide mixture of chlorpyrifos and diazinon is shown in Figure 4.3. The selected endpoint was the toxicity of these compounds on brain cholinesterase. The original data for this model were published by Timchalk et al. (2005). As it can be clearly seen, the dose addition model describes relatively well the dose response relationship of these compounds and their effect on brain cholinesterase both as individual compounds and as in a mixture.

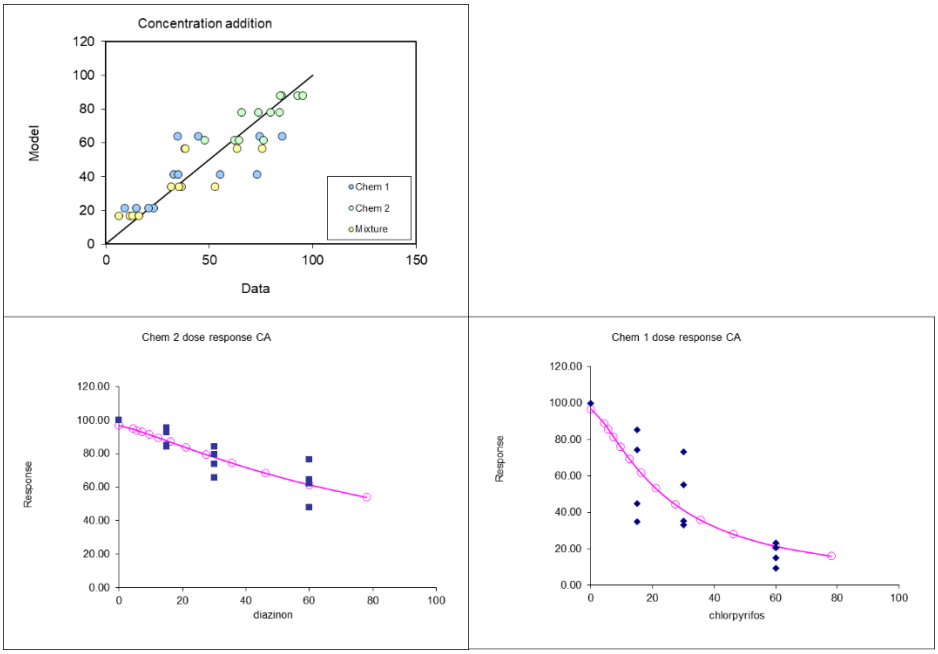


Figure 4.3 Modelling for chlorpyrifos (chem 1) and diazinon (chem 2) using data from Timchalk et al. (2008) by following concentration addition

On the other hand when the concepts of synergism and antagonism where modelled (Figure 4.4) the data points were not fitting the model not as single chemicals nor as in mixture.

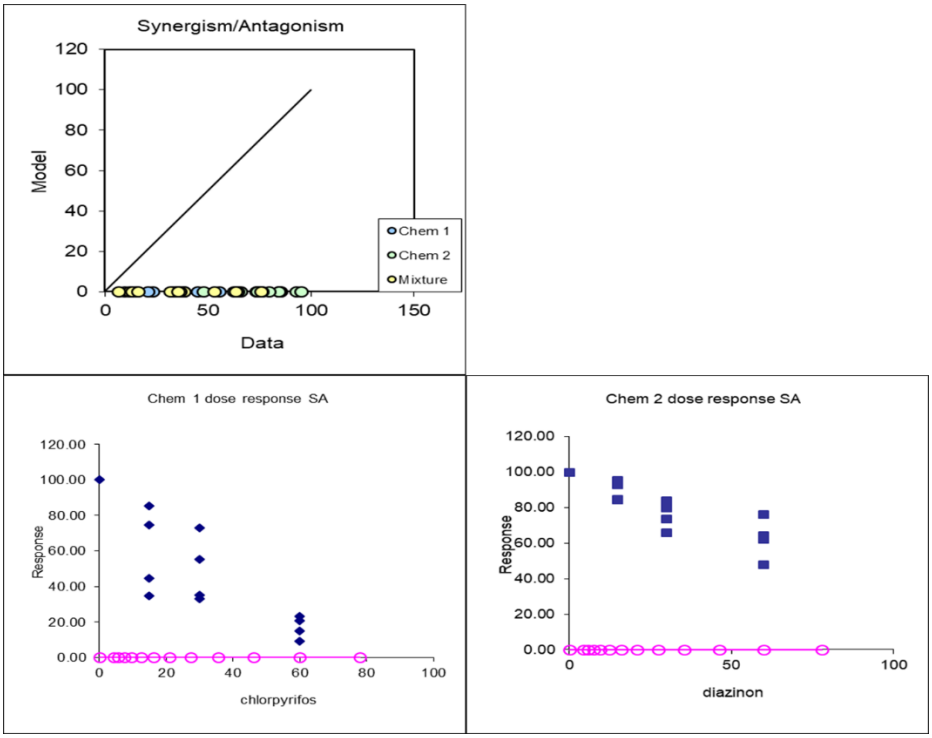


Figure 4.4 Modelling for chlorpyrifos (chem 1) and diazinon (chem 2) using data from Timchalk et al. (2008) by following synergism/antagonism.

When the dose response data were modelled regarding a dose-level dependent interaction (Figure 4.5) as above the outcome was a poor fit to the model for the individual pesticides and for their mixture.

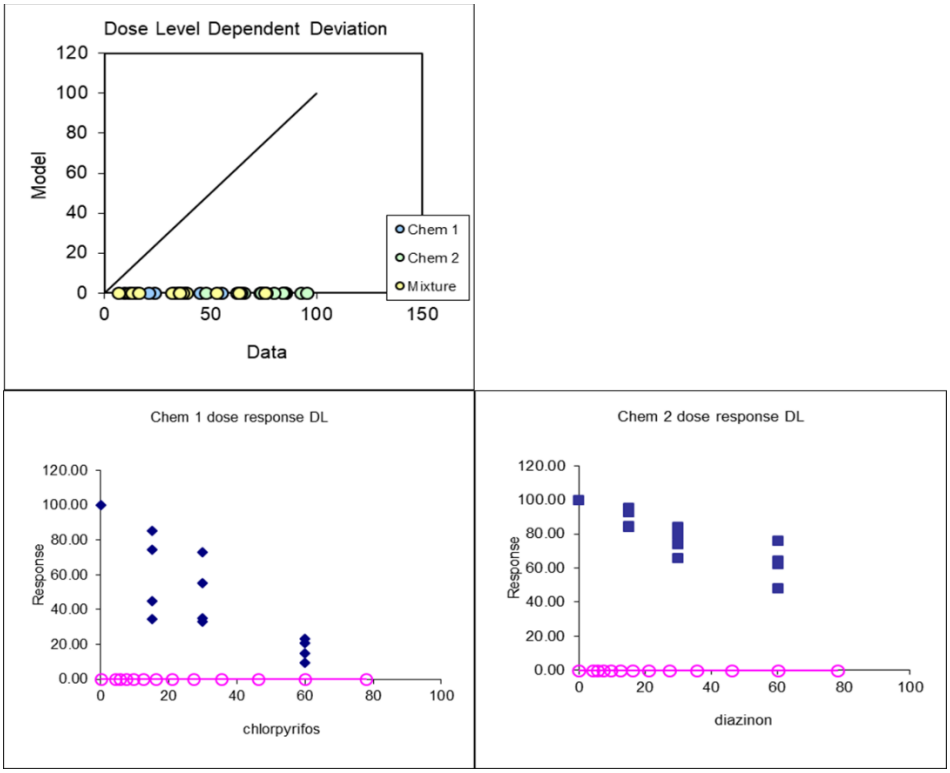


Figure 4.5 Modelling for chlorpyrifos (chem 1) and diazinon (chem 2) using data from Timchalk et al. (2008) by following dose-level addition

From the three figures, it we can see that the most appropriate approach, and the one better fitting the dose response data for single chemicals and for the mixture, is the concentration addition approach. This outcome matches the conclusion from the Timchalk et al. (2005) study. The modelling of all the dose-response data following either dose addition or synergism/antagonism can be found in Annex C. In all cases, the relationship better fitting the models was the concentration addition approach.

4.4 Conclusion

Exposure to pesticides has been associated with various toxic effects to humans and animal species. Usually, both are exposed to pesticide mixtures and most studies in the literature analyse the effects of the individual compounds. As was presented in this chapter this does not represent what commonly occurs in real life since exposure to pesticide mixtures even with individual concentrations below the MRLs, can lead to antagonistic, synergistic and more commonly to additive effects, thus the actual risk from pesticide exposure is underestimated. Finally, from the mixture studies analysed on pesticides and POPs, the concentration addition model is best describing the mixture effect.

Chapter 5

Probabilistic exposure assessment on phthalates in food

5.1 Background and importance

Phthalates are diesters of phthalic acid and have been widely used in the industry as plasticizers giving flexibility and durability to polyvinyl chloride (PVC) plastics. Commonly their uses range from plasticizers in plastics, including food contact materials and toys, as emulsifying agents and solvents in cosmetics, to excipients used in the pharmaceutical industry (Fan and Lin, 2011; US EPA, 2012; Kelley et al., 2012) Their widespread use leads to a ubiquitous, constant and virtually unavoidable exposure in humans. Currently, the global production volumes of phthalate plasticisers stand at approximate 5.5 million metric tonnes per year (OECD, 2018). The biggest market being the People's Republic of China accounting for 45% of all use, followed by Europe and the United States of America with a combined 25% use. There is substantial evidence that phthalates can induce disruption in estrogenic activity, reproductive, developmental and liver toxicity both in experimental animals and in humans (Gray et al., 2000; Heudorf et al., 2007; Lyche et al., 2009; Chen et al., 2014). Di-2-ethylhexyl phthalate (DEHP), one of the most widely used phthalates has been linked with liver carcinogenicity in rodents and has been classified by IARC as possibly carcinogenic to humans (Category 2B). Although, the exact mechanism of action involved (peroxisome proliferation), is under investigation for its relevance to humans (IARC, 2013). Phthalates were authorised for use as food contact materials in the EU market

in 2011 (EC 10/2011). Due to their toxicological potential in humans, the use of DBP, DEHP and DIBP was regulated as not to exceed concentrations equal or greater than 0.1% by weight of plasticised material, individually or in combination in the EU market after July 2020 (EU 2018/2005). Thus, various phthalate substitutes have emerged such as di(isononyl)cyclohexane-1,2-clicarboxylate (DINCH) tributyl O-acetylcitrate, triethyl 2-acetylcitrate, trihexyl O-acetylcitrate (Schutze et al., 2012; Kim et al., 2019). Phthalates can migrate into the air, water and foodstuff, and humans can be exposed via multiple pathways such as dermal, oral and inhalation.

Dietary exposure assessment is needed to evaluate qualitatively or quantitatively the likely human exposure to biological, chemical and physical agents via food. In exposure assessment the magnitude, frequency and duration of human exposure to an agent is measured and the different exposure pathways, including inhalation, ingestion of water or food and dermal contact are taken into account (Giovanoulis et al., 2018). Exposure is a crucial aspect in risk assessment as it informs on the transition of an identified hazard to a risk or a non-risk. In order to estimate human exposure to a chemical agent, concentration data in food products as well as food and drink consumption data are needed.

After obtaining data on the concentrations of the chemical agents in food and drinks and on dietary consumption, the dietary exposure assessment is conducted by using a deterministic (using point estimates) or a probabilistic analytical approach. Probabilistic analyses include more complicated modelling approaches than the deterministic (point estimates) and rely on distributions of data as input in place of single values. The outcome of probabilistic analyses is a distribution of possible exposure estimates, rather than one point estimate derived by the deterministic approach, and assists in

characterising variability and uncertainty within the population. By using a distribution of exposure estimates rather than point estimates, the likelihood of providing biased results is smaller. The use of statistical methods i.e. Monte-Carlo simulations also provide greater credibility in comparison with deterministic approaches and/or expert judgment, which may be led by subjectivity. Even though probabilistic methods can provide a more reliable exposure estimate, it should be mentioned that availability of consumption and exposure data is paramount and limited concentration data can lead to a higher uncertainty in the final exposure estimate.

A biomonitoring study (BM) was performed in Norway between September 2016 and November 2017 as part of the EuroMix project financed by H2020 programme. The study included males and females, who recorded their food consumption (including weights) and cosmetic use and collected all 24h urine for two non-consecutive days (Husøy et al., 2019). The consumption data from this study along with the concentration data from the literature were used for the probabilistic exposure estimates for males and females on both days.

The aim of this work is to assess the exposure of the study population to the most occurring phthalates (as recorded in the EuroMix biomonitoring study namely; di (2-ethylhexyl) phthalate (DEHP), di-iso-nonyl phthalate (DINP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP) butyl-benzyl-phthalate (BBP) and the phthalate substitute DINCH. Additionally, the estimated exposure is compared to the concentrations of phthalate metabolites measured in the urine collected during the biomonitoring study. Finally, a risk characterisation is performed on each phthalate individually and as a mixture.

5.2 Materials and Methods

5.2.1 Biomonitoring study

A biomonitoring study was performed in Norway between September 2016 and November 2017 as part of the EuroMix project financed by H2020 programme and is described in detail by Husøy et al. (2019). In brief, the study included 144 participants (44 males and 100 females) participating the first study day and of these 140 participants (43 males and 97 females) completed the second study day. There was a 2-3 weeks interval between the two study sessions. The participants were asked to report their food and drink intakes, in weighed food records and report the use of cosmetics and personal care products, for the two 24-h periods. In addition, 24-h urine was collected for both periods.

5.2.2 Systematic literature search

A systematic literature search was performed in October 2019 in order to collect concentration data on phthalates in foods. The search included DBP, BBP, DEHP, DEP, DINP and DINCH for the period 2010 to 2019. The databases used were Web of Science and PubMed. An additional search was performed by the end of November 2019 by a librarian at the Norwegian Institute of Public Health for the abovementioned compounds starting from 2008; including databases such as Embase, Cochrane, Medline and Web of Science. A PRISMA flow chart summarising the outcome of the literature search is presented in Figure 5.1. A detailed description of the search strategy used can be found in Annex C. The retrieved papers were organised in an EndNote 9 file to ensure traceability, and duplicates were removed. The quality of the extracted papers was evaluated considering the adequacy of sampling,

validated method and adequate instrumentation used. Finally, the phthalate concentrations and food item/category were extracted to an excel table (2267 data points), where information on the country of origin, type of analytical method, number of samples and the type of descriptive data (median, mean, minimum, maximum) were also collected.

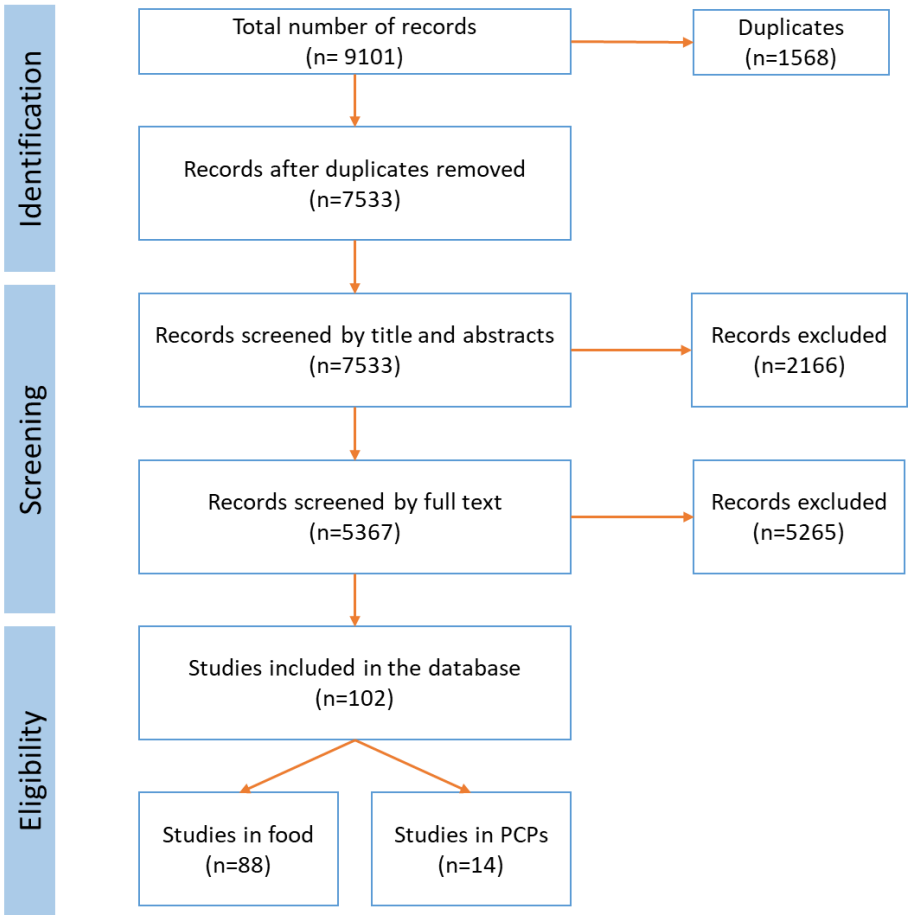


Figure 5.1 PRISMA flow diagram on the literature search performed

5.2.3 Exposure modelling from diet

From the 88 studies with food concentrations 56 studies contained data originating from countries around the world. Since the consumption data originated from a Norwegian population, only concentration studies coming from the European Union were selected. This provides information on phthalate concentrations in foods more representative to a Norwegian diet. Studies reporting minimum, maximum and median values were selected. In total, 14 studies reporting phthalate concertation's in foods were included. The collected concentrations were weighted by multiplying the phthalate concentrations with the number of samples tested in each respective study (26539 data points). Three different exposure estimates were calculated for each phthalate (with the exception of DINP) based on the concentration in food reported as lower-, medium- and upper-bound by taking into account minimum, median and maximum values, respectively. In respect to DINP there was a lack of data for minimum and maximum values, thus only medium bound was estimated. Moreover, in the lower-bound, the NDs were replaced by 0, while in the medium-bound, NDs were replaced with half of the limit of detection (LOD) or half of the limit of quantification (LOQ) (in case LOD was not available) and for the upper bound ND was replaced by its respective LOD/LOQ (Claeys et al., 2008; Sakhi et al., 2014).

Finally LB, MB, UB phthalate concentrations were calculated using R (3.6.4 version). Data were summarised by 50^{th} (P50), 5^{th} (P5), 95^{th} (P95) percentiles, mean, standard deviation, minimum, maximum and when possible the geometric mean and geometric standard deviation, for LB, MB and UB for

each phthalate. The P5, P50 and P95 were used for the probabilistic exposure estimates.

For the estimated exposure of the five phthalates, the consumption data from the EuroMix study were combined with the concentration data from the literature using the following equation (5.1).

$$Diet\ Exposure\ = \sum \frac{x \times C}{BW}\ \ [\frac{\mu g}{kg\ bw\ day}] \qquad (5.1)$$

Where C is the concentration of phthalates in foods (µg/g); x is the individual gram food eaten (g/day) as reported in the weighed food record for the same day as the urine collection, and BW is the individual body weight (kg).

The individual estimated exposure for each phthalate were modelled using 1000 Monte Carlo interactions, and the triangular type of distribution was based on the P5, P50 and P95 as parameters values. Triangular distributions were used due to the limited availability of concentration data in foods. Triangular distribution is a continuous probability shaped as a triangle and with the Pert distribution, can be used when minimum, maximum and the mode are available (Borek et al., 2014) and is being used in the phthalate exposure estimation (Martinez et al., 2017). A detailed description of the Monte-Carlo parameters for the LB, MB and UB exposure of phthalates to different food categories, including the respective data points, can be found in Table 5.1. The estimated exposure was calculated in R version 3.6.4.

Table 5.1 Monte-Carlo parameters for the phthalate exposure from food

Parameter	Symbol	Units	Type	P50 conc. (P5-P95)LB {data points}	P50 conc. (P5-P95) MB {data points}	P50 conc. (P5-P95) UB {data points}	References
DEHP conc. in	C	-					
Bread		µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {387}	71(46-71) {414}	2264(2264-2264) {377}	Sakhi et al., 2014; Biedermann et al., 2013; Skrbic et al., 2017; Fierens et al., 2012; Fierens et al., 2012b; Fierens et al., 2013; Guerranti et al., 2016; Dugo et al., 2011; Vavrous et al., 2019; Lo Turco et al., 2016; Amiridou and Voutsa 2011; Chatonnet et al., 2014; Del Carlo et al., 2008)
Cereals		µg/kg	T	$1e^{-5}$($1e^{-5}$-386) {129}	40 (5-130) {240}	1073(276.5-1628) {129}	
Cakes		µg/kg	T	32(32-32) {11}	61(56-85) {122}	165(165-165) {11}	
Fruits and vegetables		µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {69}	5(0.1-33) {143}	1413(361-1413) {69}	
Meat and meat products		µg/kg	T	10(10-10) {209}	37(5-117) {579}	850(433-850) {209}	
Fish and fish products		µg/kg	T	$1e^{-5}$($1e^{-5}$-17.7) {41}	12.5(5-86) {212}	5932(2596-5932) {41}	

Dairy	µg/kg	T	$1e^{-5}$($1e^{-5}$-312) {158}	24.5(9.3-463) {195}	260(19-743) {158}
Cheese	µg/kg	T	31(31-360) {43}	173(124-265) {152}	2385(2286.3-2385) {41}
Butter and different oils	µg/kg	T	$1e^{-5}$($1e^{-5}$-182) {253}	120(42.1-520) {170}	1827(1200-10110) {288}
Sweets	µg/kg	T	$1e^{-5}$ ($1e^{-5}$-32) {90}	9.5(5.6-191.3) {127}	243(243-483.1) {90}
Beverages	µg/kg	T	$1e^{-5}$ ($1e^{-5}$-0.1) {361}	0.7(0.005-353) {521}	133(0.09-1131.7) {361}
Snacks	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {29}	35(35-35) {29}	308(308-308) {29}
BBP conc. in					
Bread	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {387}	0.8(0.8-1.3) {424}	8.1(8.1-8.1) {387}
Cereals	µg/kg	T	$1e^{-5}$($1e^{-5}$-0.8) {129}	1.25(0.2-3.7) {240}	14(5.8-70) {129}
Cakes	µg/kg	T	0.2(0.2-0.2) {11}	3.75(1.25-3.75) {122}	14(14-14) {11}
Fruits and vegetables	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$)	0.25(0.05-0.25)	26(9-58)

			{69}	{126}	{69}
Meat and meat products	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {209}	2.5(0.25-78) {542}	12(12-18) {209}
Fish and fish products	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {41}	2.5(0.25-32) {226}	8(3-8) {41}
Dairy	µg/kg	T	$1e^{-5}$($1e^{-5}$-1.9) {158}	1.5(0.25-2.5) {195}	5(1.7-13) {158}
Cheese	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {43}	3.75(2.5-3.75) {152}	48(46.1-48) {41}
Butter and different oils	µg/kg	T	7.8($1e^{-5}$-99) {227}	10(1.65-29) {170}	1040(3.63-1210) {288}
Sweets	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {90}	0.2(0.2-0.25) {88}	23(23-23) {51}
Beverages	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {361}	2(0.005-9) {361}	96(0.1-269) {361}
Snacks	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {29}	0.6(0.6-0.6) {29}	14(14-14) {29}
DBP conc. in					
Bread	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {387}	3.8(2.8-3.8) {424}	106(106-106) {388}
Cereals	µg/kg	T	$1e^{-5}$($1e^{-5}$-80) {129}	4.6(1.3-16) {240}	61(17-133) {133}
Cakes	µg/kg	T	1.3(1.3-1.3)	5.1(2.5-7.1)	65(65-302.5)

			{11}	{122}	{12}
Fruits and vegetables	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {69}	1.2(0.25-1.7) {143}	17(5.6-480) {69}
Meat and meat products	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {209}	1.5(0.25-6) {579}	25(15-25) {209}
Fish and fish products	µg/kg	T	$1e^{-5}$($1e^{-5}$-0.21) {41}	2.5(0.75-12) {226}	12.5(12-13) {41}
Dairy	µg/kg	T	$1e^{-5}$($1e^{-5}$-15) {158}	1.9(0.25-15) {195}	6.5(0.8-54) {158}
Cheese	µg/kg	T	$1e^{-5}$($1e^{-5}$-15) {43}	4.6(2.5-31) {152}	54(52-54) {41}
Butter and different oils	µg/kg	T	$1e^{-5}$($1e^{-5}$-101) {253}	6(2.5-26) {170}	203(16-309) {288}
Sweets	µg/kg	T	$1e^{-5}$($1e^{-5}$-23.5) {90}	1.9(0.92-39.4) {127}	41(41-58.4) {90}
Beverages	µg/kg	T	$1e^{-5}$($1e^{-5}$-0.1) {381}	0.46(0.044-104) {498}	125(0.2-2212) {381}
Snacks	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {29}	3.2(3.2-3.2) {29}	65(65-65) {29}
DEP conc. in					
Bread	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {387}	1.6(0.75-1.6) {414}	23(23-23) {377}
Cereals	µg/kg	T	$1e^{-5}$($1e^{-5}$-4.7)	0.75(0.3-1.5)	558(5.37-558)

			{129}	{240}	{129}
Cakes	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {11}	1.5(1.5-2.1) {122}	5.3(5.3-5.3) {11}
Fruits and vegetables	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {69}	0.75(0.25-1.8) {143}	2.8(2-26) {69}
Meat and meat products	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {209}	1.7(0.75-4) {579}	11(1.4-11) {209}
Fish and fish products	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {41}	0.75(0.6-1.5) {189}	5(2.7-9.3) {41}
Dairy	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {158}	2.5(2.5-5) {147}	11(1-11) {147}
Cheese	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {39}	2.5(1.5-9.3) {150}	11(11-11) {39}
Butter and different oils	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {253}	4(2.5-6.3) {166}	198(4-230) {284}
Sweets	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {90}	0.75(0.25-5.8) {127}	2.4(2.4-25.2) {90}
Beverages	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {231}	0.067(0.005-7.5) {391}	0.3(0.01-15) {231}
Snacks	µg/kg	T	$1e^{-5}$($1e^{-5}$-$1e^{-5}$) {29}	0.1(0.1-0.1) {29}	5.3(5.3-5.3) {29}
DINP conc. in					
Bread	µg/kg	T	N/A	74(74-74)	N/A

				{38}	
Cereals	µg/kg	T	N/A	3.9(0.5-7.1) {112}	N/A
Cakes	µg/kg	T	N/A	362(88-734) {112}	N/A
Fruits and vegetables	µg/kg	T	N/A	6.15(2.9-9.4) {75}	N/A
Meat and meat products	µg/kg	T	N/A	43(0.5-275) {371}	N/A
Fish and fish products	µg/kg	T	N/A	38(2-55) {186}	N/A
Dairy	µg/kg	T	N/A	17(17-17) {38}	N/A
Cheese	µg/kg	T	N/A	81(6.8-166) {112}	N/A
Butter and different oils	µg/kg	T	N/A	15(4-360) {110}	N/A
Sweets	µg/kg	T	N/A	4(4-4) {38}	N/A
Beverages	µg/kg	T	N/A	0.4(0.4-3.2) {112}	N/A
Snacks	µg/kg	T	N/A	N/A	N/A

Consumption of foods		g/day	N	From diaries	From diaries	From diaries	Husøy et al., 2019
Body weight	BW	kg	LN	65.2±14.2	65.2±14.2	65.2±14.2	Husøy et al., 2019

LN = Log-normal; T = Triangular; N = Normal distribution. P50, P5 and P95 values were used for triangular distributions. The parameters were normalised taking into account the sample size, thus larger weight was given to analyses with bigger sample size.

5.2.4 Analyses of phthalates in urine

Eleven different phthalate metabolites (monoethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl 5-carboxypentyl phthalate (MECPP), mono-2-carboxymethyl hexyl phthalate (MMCHP), mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP), mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP), mono-4-methyl-7-carboxyoctyl phthalate (cx-MiNP), 6-hydroxy monopropylheptylphthalate (oh-MPHP) (Table 5.2) were determined in the three urine time pools and a 24-hour concentration of each metabolite was estimated by adding the amounts of the three time pools of urine from study days 1 and 2. On-line column switching liquid chromatography coupled to tandem mass spectrometry was used in order to determine the phthalate metabolites. Additionally, labelled internal standard solution and enzyme solution to deconjugate glucoronidates (betaglucuronidase in ammonium acetate buffer, pH 6.5) were added to the urine sample (300 μL). Incubation of samples for 1.5 hours at 37 °C, after an addition of 20% formic acid. The samples were centrifuged, and the supernatant was injected into the system. The limits of detection (LOD) were between 0.07 and 0.7 ng/mL. The accuracy of the method ranged from 70 % to 120 %. In-house pooled urine samples and standard reference material from National Institute of Standards and Technology (NIST) were simultaneously analysed with the samples and the precision for the phthalate metabolites was below 20 %.

Table 5.2 Overview of the phthalate metabolites measured in urine

Phthalate	**Metabolite**
Di(2-ethylhexyl) phthalate (DEHP)	Mono-2-ethylhexyl phthalate (MEHP)
	Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)
	Mono-2-ethyl-5-oxohexyl phthalate (MEOHP)
	Mono-2-ethyl 5-carboxypentyl phthalate (MECPP)
	Mono-2-carboxymethyl hexyl phthalate (MCMHP)
Butyl-benzyl-phthalate (BBP)	monobenzyl phthalate (MBzP)
Di-n-butyl phthalate (DBP)	monobutyl phthalate (MnBP)
Diethyl phthalate (DEP)	monoethyl phthalate (MEP)
Di-iso-nonyl phthalate (DINP)	mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP)
	mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP)
	mono-4-methyl-7-carboxyoctyl phthalate (cx-MiNP)

5.2.5 Measured vs estimated phthalate exposure

In order to compare the estimated exposure with the phthalate levels found in the urine, the individually measured phthalate metabolite concentration in the urine were back calculated (µg/kg bw) to external exposure (equation 5.2) of their respective parent compounds by taking into account toxicokinetic parameters such as absorption and percentage of the phthalate excreted in the urine (Table 5.3).

$$Parent\ concentration\ (x) = \frac{(\frac{y}{ap})}{de} \quad (5.2)$$

Where y is the total amount of phthalate metabolites in urine (ng/kg bw); ap is the percentage (%) of absorption for each respective phthalate and de is the % of the oral dose excreted as phthalate metabolites determined in urine, in order to correct for metabolites not analysed in this study

The oral absorption rate of DEHP in humans is 75% and the percentage of the sum of DEHP metabolites in urine, namely MEPH, MEHHP, MEOHP, MECPP, MMCHP, represent 67% of the oral dose of DEHP (Koch et al., 2005; EFSA, 2019c). A 70% of the DEP amount is absorbed in humans and the percentage of monoethyl phthalate (DEP metabolite) excreted in urine is 78% (Wang et al., 2019; Kawano, 1980). The absorption rate of DBP in humans is 100% (EFSA, 2019c) and a 70% of the oral dose is excreted as monobutyl phthalate (EFSA, 2019c). Similarly, BBP has also a 100% absorption rate and 73% of the oral dose is found as monobenzyl phthalate in urine (EFSA, 2019c). Finally, DINP has an absorption rate of 50% in humans and is found as mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP), mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP) and mono-4-methyl-7-carboxyoctyl phthalate (cx-MiNP). The amount of DINP that is excreted as these metabolites is up to 40% (INSERM, 2011). DEHP and DINP concentrations were calculated as, the sum of their respective metabolites, adjusted for molecular weight.

Table 5.3 Toxicokinetic parameters of phthalates

Phthalate	Absorption (%)	Elimination half-life (h)	(%) of oral dose excreted as analysed metabolites in urine	Reference
DEHP	75	5-24	67	EFSA, 2019c; Koch et al., 2005, 2006
DEP	70	52.8	78	Wang et al., 2019; Kawano 1980; WHO, 2003
DBP	100	6	70	EFSA, 2019c
BBP	100	6	73	EFSA, 2019c
DINP	50	18-36	40	INSERM, 2011

5.2.6 Statistical analysis

Further statistical analysis was performed by calculating the linear regression between middle bound and urine for males and females on both days. Two-way ANOVA tests were performed calculating any correlation between the sexes and the two days with the levels of phthalates found in the biomonitoring study. In addition, one-way ANOVA was used to test any significant within day correlation variations in the levels of phthalates for males and females on both days. For all calculations, R version 3.6.4 was used.

5.2.7 Phthalates "grouping" for risk characterisation

In order to calculate the phthalate exposure as a mixture, it was first necessary to group the five substances. According to EFSA (2018), a method of grouping substances in a mixture is calculating the Relative Potency Factor (RPF). After choosing reproductive toxicity as toxicological endpoint the most data rich compound- DEHP was selected as the "index compound". Afterwards the RPFs were defined then the phthalates mixture concentration was expressed as shown below in equation 5.3 (modified from EFSA, 2019c).

DEHP Equivalents µg/kg food) =

$$DEHP*1 + DEP*0.01 + DBP*5 + BBP*0.1 + DINP*0.3 \quad (5.3)$$

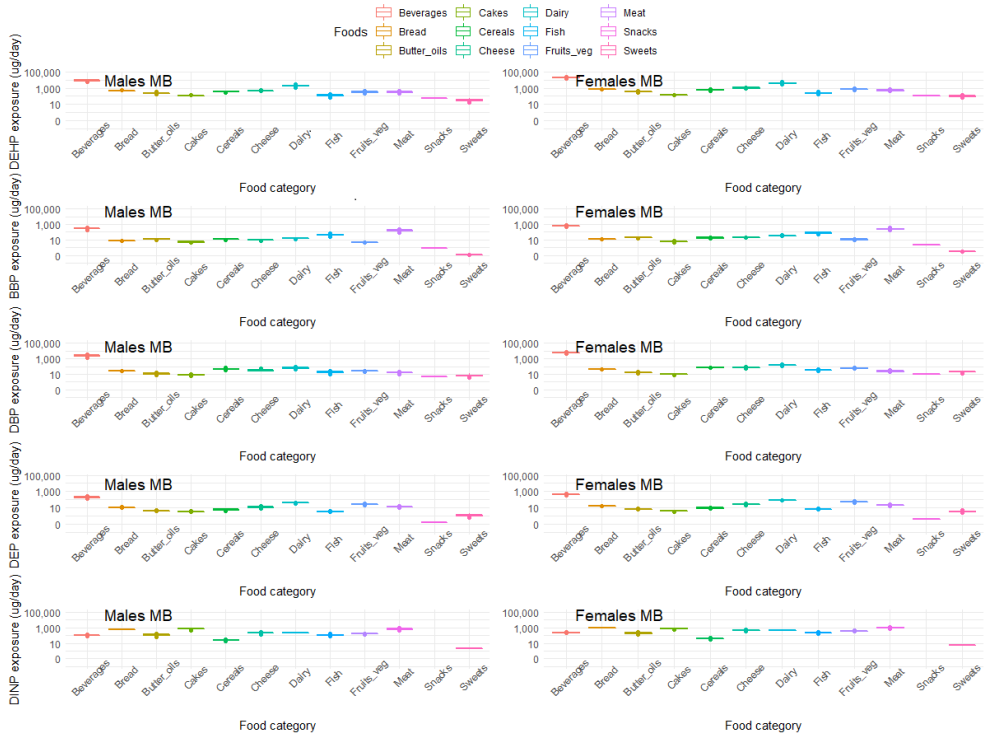
5.3 Results

5.3.1 Estimated exposure from food

The exposure was based on twelve food and drink groups, each contributing differently to the total exposure for each phthalate (Figure 2). The food groups were beverages (water, fruit juices, coffee, tea, alcoholic beverages and soft drinks), bread, butter including different types of oils, cakes, cereals, cheese, dairy, fish, fruits and vegetables, meat products, sweets and snacks. The probabilistic estimated exposure was simulated according to the equation 1 by performing Monte-Carlo modelling with 1000 iterations. The main food categories contributing to exposure were beverages, dairy products, meat, and vegetables.

As it can be seen in Figure 5.2, there were no statistically significant differences between the dietary sources of MB exposure between males and females for study day 1, with the exception of DEHP where females had a higher exposure compared to males. For females, the food categories attributing to the highest exposure were beverages meat and dairy products. For males, the highest estimated exposure was from beverages, meat, cheese and dairy products. Beverages were the food group that contributed the most to BBP, DBP and DEP exposure, irrespectively of gender, while meat contributed considerably to BBP and DiNP exposure. Dairy products also seem to an important source to all the phthalates. The results for day 2 did not differ significantly from day 1 and can be found in Annex D (Figure D3)

Figure 5.2 Phthalate exposure from different food groups, based on the 24-h weighed food record for Day 1



Phthalate exposure in food was estimated as described in section 2.3 and as it can be seen also in summary table (Table 5.4) there were no significant differences between males and females. From higher to lower the exposure from food was ranked as DEHP > DINP > DBP > BBP > DEP.

Table 5.4 Summary table showing the probabilistic exposure estimates (μg/kg bw) for the five phthalates

Phthalate	Scenario	P5 (males, females)	P50 (males, females)	P95 (males, females
DEHP	LB	0.23, 0.08	0.6, 0.6	2.23, 2.1
	MB	1.12, 1.0	2.3, 3.8	4.13, 10.3
	UB	21.9, 17.4	35, 35	56.0, 66.0
DEP	LB	0.0014, 0.0013	0.003, 0.003	0.013, 0.012
	MB	0.029, 0.031	0.07, 0.09	0.188, 0.228
	UB	0.239, 0.226	0.8, 0.7	2.55, 2.53
DBP	LB	0.01, 0.089	0.06, 0.059	0.23, 0.22
	MB	0.09, 0.11	0.6, 0.7	2.196, 2.675
	UB	3.078, 3.483	14, 18	47.5, 58.4
BBP	LB	0.0024, 0.0022	0.01, 0.008	0.041, 0.042
	MB	0.05, 0.055	0.1, 0.1	0.3, 0.34
	UB	0.88, 0.97	2.8, 3.5	6.78, 8.21
DINP	LB	N/A	N/A	N/A
	MB	0.3, 0.24	0.7, 0.6	1.62, 1.43
	UB	N/A	N/A	N/A

5.3.2 Exposure modelling and correlation with phthalate metabolites concentrations in the urine

After estimating the individual phthalate exposure from phthalate metabolite concentrations in the urine (Day 1), we presented it together with the probabilistic intake estimates (Day 1) under the three scenarios (Figure 5.3). The backward intake estimate based on urinary metabolite concentrations are between the LB and MB probabilistic intake estimates for all phthalates but DEP that is closer to the UB. Additionally the estimated exposure for day 2 was not significantly different to the one in day 1 with the exception of DBP

(p= 0.01) and can be found in Annex D (Figure D1). Finally, there were no significant differences estimated between males and females for both days.

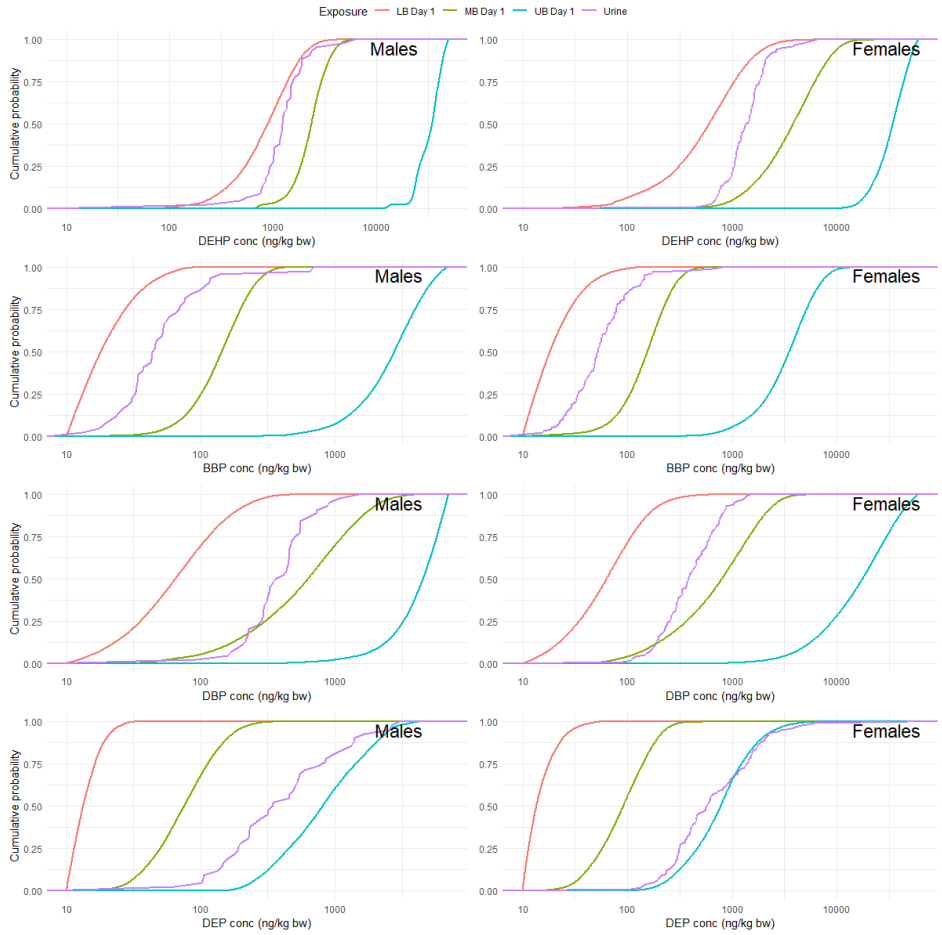


Figure 5.3 Cumulative phthalate exposure of males and females on day 1

Consumption time and urinary data measured were divided into three consumption and urinary pools respectively. Consumption and urine collected between 06:00-12:00 (pool 1), 12:00-18:00 (pool 2) and from 18:00-06:00 next day (pool 3). In the different time pools, the exposure was estimated and plotted against the phthalate metabolite levels measured in the urine of the respective time pools. Under a more detailed exploration of the dietary intake estimates by time zones within 24-h, we found that the specific probabilistic

intake estimates of the period 12:00-18:00 were closer to the exposure estimates based on urinary phthalate metabolite concentrations collected at 18:00-06:00 (Figure 5.4). Additionally there were no significant differences between males and females for the exposure from phthalate metabolite concentrations in urine for pool 2 nor in the phthalate measured in the urine from pools 1 and 3. The estimated exposure for day 2 was not significantly different to the ones reported for day 1 and can be found in Annex D (Figure D2).

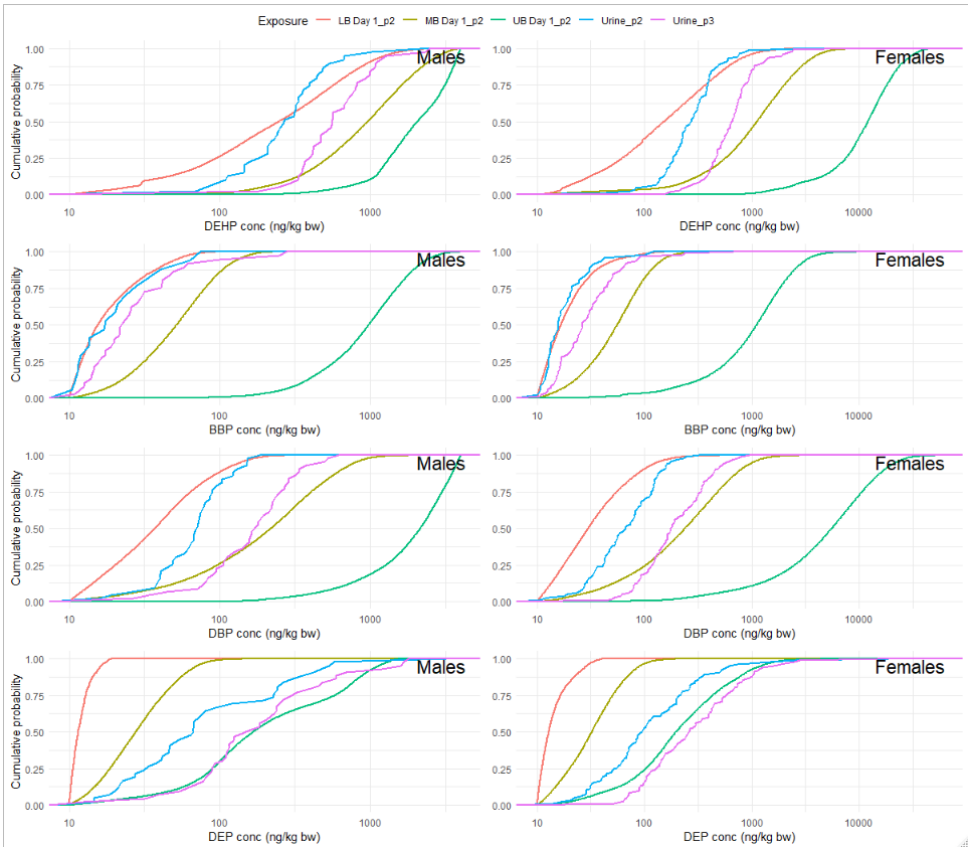


Figure 5.4 Cumulative phthalate exposure of males and females on pool 2 day 1 with urinary pools 2 and 3

The individual exposures estimates for MB and UB for both days were also plotted against the respective phthalate exposure measured in the urine. In Figure 5.5, the individual MB estimated exposure is plotted against each measured exposure from phthalate in the urine for days 1 and 2. The UB individual estimated exposure data (except DEP) were depicting a very poor correlation with the measured exposure and can be seen in the Annex D (Figure D4). In comparison with the cumulative estimated exposure, the individual estimated exposure does not fit to the exposure model with the same accuracy for most phthalates. As it can be seen from the correlation distributions below (Figure 5.5), the estimated exposure tends to overestimate the individual exposure when compared with the individual measured exposure for DEHP, BBP and DBP. For DEP the estimated exposure both underestimate and overestimate the measured exposure. On the other hand, in the case of DINP the estimated exposure underestimates the measured exposure since most of the individual values are on the left side of the correlation line. The most accurate estimates seem to be on the DBP for males and females since the individual exposure values are following closer 1:1 correlation with the measured DBP in the urine.

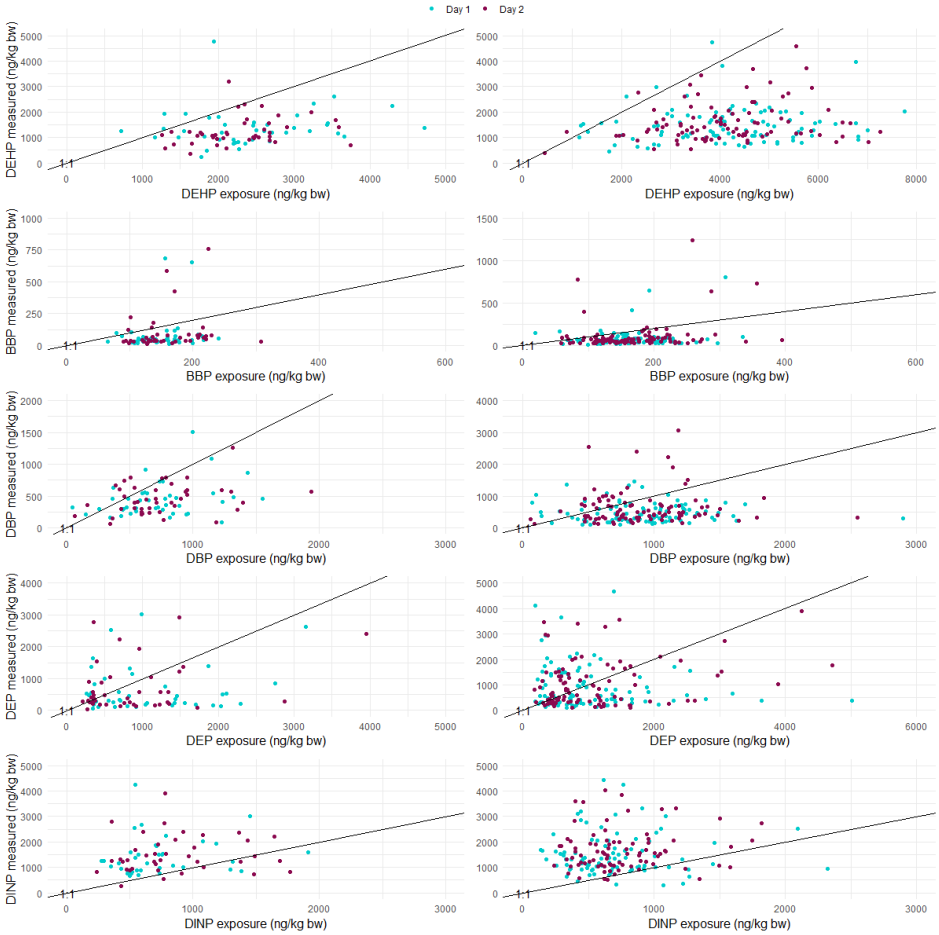


Figure 5.5 Middle bound individual exposure estimates for males (left) and females (right) correlated with measured urine, with the exception of DEP were the UB estimated exposure is presented.

5.3.3 Risk characterisation for the phthalate mixture with respect to reproductive toxicity

The estimation of exposure to phthalates using the food concentration and consumption data is described in section 3.2. Table 5.5 below, provides a summary of the median estimated exposure for males and females for day 1. The highest estimated median exposure was 35 μg/kg bw per day on the UB for DEHP and lowest was on the LB of DEP as 0.003 μg/kg bw per day.

Table 5.5 Estimated exposure (P50) for LB, MB and UB for males and females (μg/kg bw day)

Phthalate	Estimated LB exposure (males)	Estimated LB exposure (females)	Estimated MB exposure (males)	Estimated MB exposure (females)	Estimated UB exposure (males)	Estimated UB exposure (females)
DEHP	0.88	0.6	2.3	3.8	35	35
DEP	0.003	0.003	0.07	0.09	0.8	0.7
DBP	0.06	0.059	0.6	0.7	14	18
BBP	0.01	0.008	0.1	0.1	2.8	3.5
DINP	N/A	N/A	0.7	0.6	N/A	N/A

In order to proceed with the risk characterisation, the RPFs were estimated with equation 3 thus the potency-related exposure was calculated as shown in (Table 5.6) using the same approach followed by EFSA (2019). For most of the phthalates as Health Base Guidance Values (HBGV) the tolerable daily intakes (TDI) were selected from EFSA (2019), with the exception of DEP where the TDI was calculated by WHO (2003).

Table 5.6 Calculation of relative potency factors

	DEHP	**DEP**	**DBP**	**BBP**	**DINP**
N(L)OAEL (mg/kg bw per day)	4.8	1600	2.0	50	50
Uncertainty Factor	100	300	200	100	100
Additional assessment factor	N/A	N/A	N/A	N/A	3.3
HBGV (mg/kg bw per day)	0.05	5	0.01	0.5	0.15
HBGV (μg/kg bw per day)	50	5000	10	500	150
RPF	=1.0 (index compound)	0.01	5	0.1	0.3

The estimated exposure was converted to RPF-related exposure estimates by using equation (5.3). The individual and mixture phthalate estimates were subsequently compared to their TDI and the TDI of DEHP, respectively (Table 5.7). When the phthalates are assessed individually, their MB levels are lower than their respective TDI's, thus no risk is assumed. When comparing the estimated exposure for the UB, all phthalates with the exception of DBP are in lower amounts than their TDI. DBP's levels are slightly above the TDI for both males and females thus indicating that there may be a risk for reproductive toxicity. Once comparing the MB phthalate mixture exposure to its respective TDI (0.05 mg/kg bw day) for DEHP, the levels are lower for both males and females (0.0055 and 0.0075 mg/kg bw day respectively). On the other hand when taking into account the UB mixture exposure, then both males and females are exposed to higher amounts than the TDI (Table 5.7).

Table 5.7 Individual and mixture phthalate estimated exposure (MB) for males and females on study day 1 compared with TDI

Phthalate	TDI (mg/kg bw/day)	Estimated MB exposure (males) (mg/kg bw/day)	Estimated MB exposure (females) (mg/kg bw/day)	Estimated UB exposure (males) (mg/kg bw/day)	Estimated UB exposure (females) (mg/kg bw/day)
DEHP	0.05	0.0023	0.0038	0.035	0.035
DEP	5	0.00007	0.00009	0.0008	0.0007
DBP	0.01	0.0006	0.0007	**0.014**	**0.018**
BBP	0.5	0.0001	0.0001	0.0028	0.0035
DINP	0.15	0.0007	0.0006	N/A	N/A
Mixture	0.05	0.0055	0.0075	**0.105***	**0.125***

*The P95 of the DINP exposure (MB) was used for the mixture estimate

5.4 Discussion

5.4.1 Phthalate estimated exposure in food

Probabilistic estimated exposure was performed for five phthalates in food using 144 and 140 participants from the EuroMix study for days 1 and 2 respectively. In Table 5.8 the estimated exposure (P5, P50, P95) for all scenarios is compared against the estimated exposure from three TDS studies summarised by EFSA, (2019c) and from a study performed using Norwegian food products by Sakhi et al., in 2014. The total diet studies namely; Bradley et al., 2013; FSAI, 2016; ANSES, 2016a, b was performed in three different countries (UK, Ireland and France) with data referring to 2007, 2012 and 2011-12. The estimated exposure for the LB and the MB in most cases was in the same range as the estimates reported from the literature. On the other hand, our

estimates for the UB are significantly higher. This can be attributed to a variety of factors such as; limited number of concentration studies, cases where only a single concentration value was available (P50) thus driving to a high exposure, the addition of tap water to the beverages food group led to high consumption values and when combined with the maximum concentration values used for the calculation of the UB lead to very high exposure estimates. By taking into consideration the maximum concentration values, the estimated exposure for the UB were significantly higher than estimates reported in the literature (Table 5.8) and in most cases would lead to an overestimation of the risk. This is also supported with the poor correlation between the UB individual estimated exposure and the individual measured exposure in the urine. The exception was the DEP, where the UB estimate correlated better with the measured estimate in comparison with both LB and MB.

Table 5.8 P5, P50, P95 exposure estimates (µg/kg bw per day) compared with exposure estimates from the literature summarized by EFSA, 2019c and a study in Norwegian foods by Sakhi et al. (2014)

Phthalate	Scenario	P5 (males, females)	P50 (males, females)	P95 (males, females	Sakhi et al., 2014 (P50)	Sakhi et al., 2014 (P95)	EFSA 2019 mean (min-max)	EFSA 2019 P95 (min-max)
DEHP	LB	0.23, 0.08	0.6, 0.6	2.23, 2.1	0.366	0.751	0.446-3.459	0.902-6.148
	MB	1.12, 1.0	2.3, 3.8	4.13, 10.3	0.384	0.78		
	UB	21.9, 17.4	35, 35	**56.0, 66.0**	0.406	0.809		3.9-24.3
DEP	LB	0.0014, 0.0013	0.003, 0.003	0.013, 0.012	0.00151	0.00711	N/A	N/A
	MB	0.029, 0.031	0.07, 0.09	0.188, 0.228	0.0112	0.022		
	UB	0.239, 0.226	0.8, 0.7	2.55, 2.53	0.0203	0.0395		
DBP	LB	0.01, 0.089	0.06, 0.059	0.23, 0.22	0.024	0.052	0.042-0.769	0.099-1.503
	MB	0.09, 0.11	0.6, 0.7	2.196, 2.675	0.0296	0.0593		
	UB	3.078, 3.483	14, 18	**47.5, 58.4**	0.0352	0.0678		0.36-4.1
BBP	LB	0.0024, 0.0022	0.01, 0.008	0.041, 0.042	0.00581	0.15	0.009-0.207	0.021-0.442
	MB	0.05, 0.055	0.1, 0.1	0.3, 0.34	0.0184	0.16		
	UB	0.88, 0.97	2.8, 3.5	6.78, 8.21	0.0308	0.173		0.7-8.0
DINP	LB	N/A	N/A	N/A	0.392	1.08	0.232-4.27	0.446-7.071
	MB	0.3, 0.24	0.7, 0.6	1.62, 1.43	0.402	1.09		
	UB	N/A	N/A	N/A	0.412	1.10		20.3-155.6

For performing the probabilistic exposure estimates, triangular distributions were used due to the limited availability of concentration data in foods. After a careful observation at the distributions and performing a linear regression there were, no significant differences in the phthalate exposure were observed for males and females for both days, with the exception of DBP. The top four food groups with the highest contribution are shown in Tables 5.9 and 5.10 below.

Table 5.9 Top food groups contributing to the highest exposure for males

Phthalate	**First**	**Second**	**Third**	**Fourth**
DEHP	Beverages	Dairy	Cheese	Bread
DEP	Beverages	Dairy	Fruits	Meat
DBP	Beverages	Dairy	Cereals	Cheese
BBP	Beverages	Meat	Fish	Dairy
DINP	Meat	Cakes	Bread	Cheese

Table 5.10 Top food groups contributing to the highest exposure for females

Phthalate	**First**	**Second**	**Third**	**Fourth**
DEHP	Beverages	Dairy	Cheese	Bread
DEP	Beverages	Dairy	Fruits	Cheese
DBP	Beverages	Dairy	Cereals	Cheese
BBP	Beverages	Meat	Fish	Dairy
DINP	Meat	Bread	Cakes	Cheese

Sakhi and colleagues, (2014) estimated the food groups with the highest contribution to phthalate exposure as, milk and dairy (DEP), beverages (DBP), meat and meat products (BBP) and bread (DEHP, DINP). These categories appear often in the literature as the ones responsible for high phthalate concentrations. Serrano et al., (2014) reviewed several food monitoring

surveys from North America, Europe and Asia with data between 1990 and 2013. High phthalate concentrations (>300 μg/kg) were often observed for DEHP in different types of meat, oils and fatty products (butter, cooking oils, animal fat). DEHP values ranged from 404 to 5591.7 μg/kg. Additionally, high levels of DBP and BBP were observed in this food group namely 3287.5 and 11083 μg/kg respectively. On the other hand, low phthalate concentrations (<LOD) were reported for dairy, grain products and fruits. Fifteen different phthalates were determined in a TDS from UK with the most important being DEHP, DBP, DiBP, DEP and BBP. The most important food groups with the highest phthalate prevalence of DEHP were fish, poultry and other meat products. Nuts, bread, oils, fats and meat products for DBP and DEP and BBP were present only in cereal and bread respectively (Bradley et al., 2013). Our main findings show that beverages, dairy and cheese products, meat and meat products and fish products were the most important food groups. There were not many differences between the males and females on phthalate contributions from the different food groups. The fourth most important food category for DEP was meat for males, cheese for females and finally the second and third most important categories on DINP for males were cakes and bread, for females, these two categories are reversed. The reason beverages contribute to a great degree in this study is the high recorded consumption coupled with the addition of tap water in this food group leading to high total amounts of exposure. Since phthalates can be found also in tap water at concentrations 0.001-2.81 μg/kg (Domingues-Morueco et al., 2014; Blanchard et al., 2013; Gonzalez-Salamo et al., 2017).

5.4.2 Comparing estimated exposure with measured levels in urine

The measured exposure when plotted against the cumulative estimated exposure showed a rather good fit for the whole study population. The measured exposure distribution was in-between the LB and the MB for all phthalates that are most commonly found in food. The only exception was DEP that the measured exposure approached the UB of the estimated exposure. This is probably due to DEP exposure from other sources besides food such as PCPs where the prevalence of DEP is higher. Our estimated exposure was able to predict the potential phthalate exposure for this population making it a useful tool for the risk assessment of phthalates in humans.

After assessing the phthalate levels measured in urine within day, there were no significant differences within the Pools. Additionally the phthalate levels in the urine were not significantly different between the two days. Upon comparing the exposure of Pool 2 with the phthalates found in urine Pools 2 and 3, Pool 3 was fitting better to the exposure model and this is in accordance with the current knowledge on their short half-life (Table 5.3).

Plotting of the individual estimated exposure for phthalates showed an overestimation of estimated exposure when compared with the individual measured exposure, with the exception of DINP where our estimates underestimate the exposure. In case of DEP, the phthalate levels found in the urine for the pools 2 and 3 were close to the UB level and even higher (females) this can be explained due to fact that DEP is more commonly used in cosmetics and its levels found in the urine include other sources besides food. The correlation between measured and estimated phthalate levels ranged from zero (BBP) to low (±<0.30) for DINP to moderate (between 0.30 and 0.49) for DBP,

DEHP and DEP. This observation may have two main sources; availability of concentration data and back-calculation of phthalate metabolites to external dose. While the consumption database is well documented with low uncertainty, the concentration studies from the literature addressing the European market was rather limited, contributing with considerable uncertainty. In order to compare the phthalate measured in the urine with their respective exposure estimates, a back-calculation of the phthalate metabolites levels to the external dose of the parental compound was needed. This is a source of uncertainty since we considered only absorption and % of dose excreted in urine without taking into account other toxicokinetic factors such as metabolism distribution and faecal excretion.

5.4.3 Phthalate mixture exposure

Our results on the risk characterisation of the phthalate mixture showed that the estimated exposure of the study population was approximately 10-fold lower than the TDI of the mixture indicating no risk. For males the estimated mixture exposure was 0.0055 mg/kg bw, while for females 0.0075 mg/kg bw respectively. The risk characterisation on DBP, BBP, DEHP and DINP performed by EFSA showed mixture estimated exposure ranging from 0.0009 to 0.0072 mg/kg bw for mean consumers and 0.0016-0.0117 mg/kg bw for the high (P95) consumers (EFSA, 2019c). The worst-case estimates (UB) reported in this study, not only were 10 times higher (0.105 for males and 0.125 females) than the P95 estimates from EFSA but they were also higher than the TDI (0.05 mg/kg bw). This could be due to our approach on estimating the UB, which was based on the maximum concentrations reported in the literature for each food group and the treatment of the ND values (ND= LOD or LOQ).

5.4.4 Strengths and weaknesses

In the collection of phthalate concentration in food, the concentration data points were normalised with the number of samples analysed, giving more weight to the studies with a high number of samples thus reducing the power of some possible outliers. On similar thought for our statistical analyses, P5 and P95 were used instead of minimum and maximum values. It is well known in the literature that probabilistic estimates (Monte-Carlo distributions) are more reliable compared to simple deterministic approach, which uses only point estimates, and indeed our estimates for LB and MB were in accordance with previously published studies.

For the MB estimates, only medians were used as a statistical descriptor and studies including only mean were not included. The reason is that when these studies were included, the uncertainty in the final estimated exposure increased especially when comparing with the individual phthalate levels from the BM study. This can be attributed to the fact that a mean estimate can be more affected by maximum values thus making it a less reliable statistical descriptor. The consumption data used in this study may under- or over report the food consumption of the whole population. This limitation had an effect on the accuracy of results on the individual estimated exposure (Sioen et al., 2012). Using concentration data from different studies in the literature adds uncertainty to the estimated exposure. Additionally the concentration database was less detailed and less diverse in comparison with the consumption database where many food items are included.

5.5 Conclusion

This study presents the estimated exposure on five phthalates namely DEHP, BBP, DBP, DEP and DINP for two different days in food and beverages from an adult population in Norway. Median exposure estimates were significantly lower than their respective TDI values established by EFSA and WHO. Additionally, beverages, dairy and meat products were the major contributors of phthalate exposure to the adult Norwegian population. Finally, when assessing the phthalates as a mixture, the middle bound estimates were 10 times lower than the TDI. On the other hand, for the worst case estimates (UB) the exposure levels are almost double in comparison with the TDI, thus indicating a potential risk to the study population if exposed to very high concentration levels of phthalates.

Chapter 6

Discussion and conclusion

In the present thesis, different aspects of mixture risk assessment were presented. In the first chapter, the relevance and importance of assessing mixtures were described, including the state of the art on the methodologies for risk assessment of mixtures. The task of assessing chemical mixtures is more complex compared to single compounds. There are further steps in the risk assessment process; hazard assessment is more challenging (i.e. interactions, data gaps on MoA) and an infinite number of chemical combinations is adding to the exposure estimation and the final risk characterisation leads to an increased amount of uncertainty. On the other hand, methodologies and guidelines from different regulatory institutes were presented on how to perform the mixture risk assessment following a systematic approach. Different assessment methodologies for various scenarios and their limitations were presented in all steps of risk assessment and finally how to address mixture uncertainty was highlighted.

The second Chapter presented parts of the work performed for the MYCHIF project on mycotoxin mixtures. Multiple mycotoxins and fungal metabolites occur often in a wide range of corps and agriculture produce. Cereals such as wheat and maize are often reported having high concentrations of FBs, DON, AFs and ZEN while FBs often exceed the legal maximum levels in maize foods and feed (Palumbo et al., 2020). Additionally, maize and rice were found to be contaminated with AFs and in rice, there were also reports of it being highly contaminated with OTA (Goncalves et al., 2019; Palumbo, 2019). Regarding co-occurring mycotoxins, this project showed that the crops with the highest

probability of being contaminated with mycotoxin mixtures were wheat and maize. The mycotoxin DON appeared having the highest co-occurring potential with FBs in maize and with ZEN in wheat (Battilani et al., 2020; Palumbo, 2019). Unfortunately, there is a lack of data concerning mycotoxins co-occurrence and further studies need to be performed to identify co-occurrence patterns of mycotoxins mixtures (Palumbo, 2019). Additionally, there were several data gaps regarding *in vivo* toxicity after concurrent exposure to mycotoxin mixtures. This Chapter also summarised available TK data on single and multiple mycotoxins in lab and farm species, since knowledge on kinetics gives a better understanding on the interspecies differences and the sensitivity of each species to mycotoxins. Various parameters may affect the kinetics of mixtures such as; mycotoxin dosage, exposure pathway, interspecies and intraspecies differences. From the available studies in chickens and pigs, the elimination half-lives for DON and ZEN were lower in chickens compared to pigs, providing a TK rationale for the differences in DON and ZEN's sensitivity between pigs and chicken. Due to the limited availability of scientific papers on mixture effects in comparison with single compounds, there are limited available *in vitro* and *in vivo* data related to concurrent mycotoxin exposure. Availability of TK data is of paramount importance for the refinement of risk assessment of chemicals. In order to increase the predictability of kinetic modelling a multidisciplinary approach is needed. A crossover between *in vitro* testing with QSAR and PBK modelling can assist in performing accurate *in vitro* to *in vitro* extrapolations. To demonstrate that, an increasing number of papers performing predictive modelling using kinetic data for mycotoxins is becoming available (Faeste et al., 2018; Zeng et al., 2019; Qian et al., 2013; Mukherjee et al., 2014). In order to apply predictive modelling methods raw TK/TD data are still needed. Besides harmonised testing it is important to have standardised and validated

in vitro methods addressing kinetic parameters of mycotoxins such as; single V_{max}, K_m, intrinsic clearance and isoform-specific metabolic interactions (K_i), including possible metabolic interactions of mycotoxins (phase I, II and transporters). It was shown that there are multiple P450 enzymes responsible for the biotransformation of mycotoxins to their bioactivated forms. Mycotoxins are known to interfere with P450 enzymes (induction/inhibition) and influence their biotransformation after mixture co-administration, which in turn may alter their mixture toxicity (Duca et al., 2012; Alessane-Kpembi et al., 2017; Klaric et al., 2013; Mary et al., 2015). Finally, it was highlighted that there are limited TK data for mycotoxin mixtures in the literature and it can be concluded that assessing the TK of mycotoxin mixtures is a complex subject, which should be studied using a case-by-case approach.

Chapter 3, emphasised on the metabolic interactions between binary mixture compounds, using mainly drug-pesticide and drug-flavonoid combinations. Drug data were used since they are better characterised in the literature in comparison with food xenobiotics. Compounds inhibiting or inducing enzyme activity in all biotransformation phases were presented in detail (Annex A). The most potent CYP inhibitors identified were; fipronil, parathion, chlorpyrifos, fenitrothion, malathion, phenthoate for pesticides while, amentoflavone, galangin, tetrahydropalmatine and curcumin, for flavonoids. For UGTs mixture interactions, flavonoids such as ruscogenin, tigogenin and disosgenin showed strong inhibition on UGT1A4. Concerning P-gp, there were limited mixture interaction data in the literature. Additionally, the intrinsic clearance was linked with the enzyme inhibition rate (Ki or IC_{50}) of pesticides and flavonoids on drugs, demonstrating that enzyme inhibition leads to a decrease in the clearance (1.5 and 3 fold respectively) of the chemical, in comparison with single administration. Decreased clearance indicates that the amount of time a chemical stays in the body increases, causing overexposure

and an increase on its toxic potential. On the other hand, it should be noted that most of the doses of the inhibitors were significantly higher than their respective ADI.

The importance of metabolic interactions during all phases of biotransformation was highlighted and attention was drawn to the fact that a change in the biotransformation of a xenobiotic or a natural can influence its final toxicity. The intrinsic clearance of the mixture was not only compared with the respective clearance of the single compound but also it was extrapolated to *in vivo* (IVIVE) clearance with a 2-fold accuracy. Availability of *in vitro* kinetic data (V_{max}, K_m, Cl_{int}) as model inputs for IVIVE models is often highlighted in the literature (Lautz, 2020; Algharably et al., 2019). The database containing inhibition/induction data developed by Quignot et al., (2015) and updated in this Chapter for pesticides and flavonoids can be published and be made available as an open source database for the purposes of risk assessment of chemical mixtures. Open source databases systematically assessing inhibition/induction *in vitro* data can be used not only for IVIVE but also in other alternative methods including PBK modelling. There are various generic PBK models available in the literature for humans and animal species supported by data on the expression of CYP enzymes and transporters for risk assessment purposes (Lautz et al., 2020a; Lautz et al., 2020b; Lautz et al., 2020c; Dessalegn et al., 2019).

Chapter 4 investigated a different aspect on the mixture hazard assessment that of mixture toxicity *in vivo* in different lab animals. Our findings from the literature showed that indeed pesticides occur often as a mixture and can cause a range of toxic effects both to human and animal species. Additionally, exposure to pesticide mixtures even when the individual concentrations are below the MRLs, can lead to antagonistic, synergistic and more commonly to

additive effects, confirming the need for including the mixture effect when performing a risk assessment on pesticides. Finally, from the mixture studies analysed on pesticides and POPs, the concentration addition model is the one best describing the mixture effect, which is in agreement with most studies in the literature and the default approach proposed by multiple regulatory authorities (Thomson, 1996; Cedergreen, 2014).

In Chapter 5, focus was given on phthalates, chemicals with wide use in the industry and in consumer products. They have been associated with health effects such as reproductive, developmental toxicity and endocrine effects. The exposure from five different phthalates in food was estimated using probabilistic tools (Monte Carlo) and compared with their measured levels from a BM study with 144 participants in two 24-h days, part of the EuroMix project. The exposure estimates made for the LB and the MB were in the same range as estimates from the literature and in both scenarios, the exposure for all five phthalates was significantly lower than their respective TDI values established by EFSA and WHO. On the other hand, the UB estimates were significantly higher. This observation was attributed to a variety of factors such as; the maximum concentration values were used from the literature, limited number of concentration studies, cases where only a single concentration value was available (P50) thus driving to a high exposure, the addition of tap water to the beverages food group led to high consumption values and when combined with the maximum concentration values used for the calculation of the UB lead to very high exposure estimates. The food groups with the highest contribution (first and second) to phthalates were; beverages, dairy, bread and meat products, which is in accordance with the Sakhi et al., 2014, where milk and dairy, beverages, meat and bread were responsible for DEP, DBP, BBP, DEHP and DINP respectively. High phthalate levels (besides the

aforementioned food groups) have been also observed in butter, cooking oils, fish, and nuts (Serrano et al., 2014; Bradley et al., 2013).

The phthalate-estimated exposure was not influenced by gender, though females appeared to have slightly higher estimated exposure than males. Additionally, there were no significant differences on the exposure estimates between the two days. Finally, when assessing the phthalates as a mixture, the MB estimates were 10 times lower than the TDI indicating that there is low risk from phthalates to the study population. On the other hand, for the worst case estimates (UB) the exposure levels are approximately double in comparison with the TDI, thus indicating a potential risk to the study population if exposed to very high concentration levels of phthalates.

Weaknesses of the thesis approach

The approach adopted in this thesis for the investigation of mixtures was to examine the data, already available in the literature and then assess whether these same existing data can provide sufficient information. The PhD period having come to its end, it can now be concluded that this approach is not recommended since, as has been shown, the research on mixtures is still at its infancy, and as a result, the data required for the assessment of their effect are missing. Moreover, meta-analysing mixture data from the literature has been a rather challenging task since the studies were not necessarily performed with endpoints relevant to risk assessment. In the literature, there are limited number of papers addressing combined effects of mixtures and even fewer papers considering multiple doses including no effect data. Additionally, it is very important to have comprehensive well-designed studies which can address toxicodynamic and/or toxicokinetic parameters of co-occurrence. A different approach for this PhD would have been to prioritise one or two chemical mixtures relevant to food safety and perform laboratory experiments (*in vitro*)

on both kinetics and toxicity, using multiple doses. The generated data could then be combined in a PBK-TD model using QSAR or read-across for possible missing information. This might have solved the aforementioned issues and would have allowed a more holistic view of the mixture challenge. Moreover, by using simulated distribution data on kinetics, physiological parameters and toxicodynamics in the model, the uncertainty decreases and the reliability of the predictions of the model increases.

Strengths of the thesis approach

On the other hand, this thesis gave an overview on different methodologies to conduct a risk assessment for chemical mixtures. It highlighted the importance of addressing mixtures when assessing the risk from chemicals. Additionally, it underlined the importance on the availability of toxicokinetic data which play an important role in the assessment of the final mixture toxicity. Extensive and systematic literature searches were the basis for all the activities performed in this dissertation. Data on the different TK parameters (ADME) were used in the assessment of mycotoxin mixtures, helping in the understanding of interspecies differences and giving an insight on the TK influence to the final mixture toxicity. The collection of metabolic interactions data from the literature allowed their in depth characterisation and emphasised their importance in the different biotransformation phases of a chemical mixture. Moreover, it was demonstrated how an interaction (inhibiton/induction) may lead to kinetic changes, which in turn may influence the compounds toxicity. Additionally, information on TK parameters of phthalates, assisted in the back calculation of the measured phthalate metabolites in the urine to the oral dose levels of their parent compounds, thus allowing the comparison of our estimates with the measured exposure. By screening the available toxicity data in pesticides and POPs, this thesis highlighted that doses even below the

reference values can cause an adverse effect after concurrent administration. Finally, performing a systematic literature search on the concentrations of phthalates was crucial in order to perform their exposure assessment. Collection of TK data on phthalates also allowed the back calculation of the measured exposure and permitted its comparison with our exposure estimates.

Moving forward

Since it is impossible to measure experimentally the effects of all chemicals and their infinite combinations on our health, it is important to learn as much as possible about our body biology and the chemicals' effects on it. In order to achieve this goal, crucial steps are; systematic mapping of the effects of xenobiotics on our health in AOPs (more than 200 completed since 2010) and filling of current knowledge gaps.

Performing a risk assessment of chemical mixtures is a challenging task. In recent years, scientists have been developing more and more tests on chemical mixtures, but occasionally, the methods are neither consistent, nor relevant or comprehensive. A step forward was the more generic RA frameworks and guidelines developed by international institutes such as WHO (Meek et al., 2011), OECD (OECD, 2018) and EFSA (EFSA, 2019b).

Attention should be given to develop *in silico* and computational methods validated with reliable *in vitro* methods, as well as, integrating different NAMs to decrease the limitations of each individual technique. As an example, Hartung and colleagues (Luechtefeld et al., 2018a,b) are performing read-across QSAR coupled with machine learning in order to predict potential toxicological effects on different relevant endpoints. Chemical mixture risk assessment requires collaboration between scientists (i.e. chemists, toxicologists, informaticians, statisticians, biologists) and a multidisciplinary approach that would combine *in vitro* and *in vivo* models with computational

models such as PBK, QSAR, read-across and big data coming from "omics" and high content screening. Multidisciplinary approaches would help performing a more refined risk assessment. The main issue hindering its wide application, especially for the high Tiers, is lack of guidance, data and expertise (Kienzler et al., 2016). A great way forward is initiatives such as the Partnership for Assessment of Risk from Chemicals (PARC) under Horizon Europe starting in 2022 until 2028, aiming to fill knowledge gaps regarding hazards, occurrence, exposure to chemicals and chemical mixtures, by bringing together European risk assessment and regulatory entities.

A major factor influencing progress on the matter is the differences in governmental approaches and the respective regulatory policies. When regulatory bodies are convinced that alternative methods are sufficiently reliable and begin to depend less on *in vivo* experiments, scientific research will be "forced" to invest more, thus the corresponding research and competences will expand.

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Annexes

Annex A

Table A1. Keywords, databases and number of records retrieved from the ELS

Keywords
MYCOTOXIN*/TI,AB **OR** AFLATOXIN*/TI,AB **OR** FUMONISIN*/TI,AB **OR** DEOXYNIVALENOL/TI,AB **OR** OCHRATOXIN*/TI,AB **OR** ZEARALENONE/TI,AB **OR** PATULIN/TI,AB **OR** TRICHOTHECENE*/TI,AB **OR** ERGOT ALKALOID*/TI,AB **OR** T2 TOXIN*/TI,AB **OR** HT2 TOXIN*/TI,AB **OR** T2 **OR** TRIOL/TI,AB **OR** BEAUVERICIN/TI,AB **OR** ENNIATIN*/TI,AB **OR** MONILIFORMIN/TI,AB **OR** MYCOTOXINS+NT/CT **OR** AFLATOXINS+NT/CT **OR** FUMONISINS/BI **OR** DEOXYNIVALENOL/BI **OR** OCHRATOXINS/BI OR ZEARALENONE/BI **OR** PATULIN/BI **OR** TRICHOTHECENES+NT/CT **OR** ERGOT ALKALOIDS+NT/CT **OR** BEAUVERICIN/BI **OR** ENNIATINS/BI **OR** MONILIFORMIN/BI **OR** FUMONISIN/BI **OR** OCHRATOXIN/BI **OR** TRICHOTHECENE/BI **OR** BEAUVERICINS/BI **OR** ENNIATIN/BI **AND** TOXICOKINETICS **AND** (HUMANS/BI **OR** CHILD/BI **OR** INFANT/BI) **OR** (MAN **OR** MEN **OR** WOMAN **OR** WOMEN) **OR** (CHILD **OR** CHILDREN) **OR** (FOETUS **OR** FETUS **OR** TODDLER*) **OR** (INFANT* **OR** INFANCY **OR** ADOLESCENT*) **OR** (ADOLESCENCE **OR** ADULT*) **OR** ADOLESCENTS/BI **OR** (RAT **OR** RATS **OR** MOUSE **OR** MICE) **OR** (PIG* **OR** SWINE **OR** COW* **OR** CATTLE **OR** CHICKEN) **OR** (POULTRY **OR** TURKEY **OR** DUCK **OR** FISH*) **OR** (CAT **OR** CATS **OR** DOG **OR** DOGS) **OR** (RABBIT **OR** RABBITS) **OR** (RATS/BI **OR** DOGS/BI) **OR** (CHICKENS/BI **OR** CATTLE/BI) **OR** (POULTRY/BI **OR** TURKEY/BI) **OR** (DUCKS/BI **OR** FISHES/BI)

Literature databases examined	MEDLINE®, CABI: CAB Abstracts®, FSTA®, Food Science Technology, CAS-Chemical Abstracts Service, Biological Abstracts, Excerpta Medica, SCI-Science Citation Index
EFSA opinions taken into consideration for data collection	-Aflatoxins in feed and in food chain (EFSA, 2004; EFSA2007) -T2 and HT2 (EFSA, 2011a) -Ochratoxin A (EFSA, 2006) -Beauvericin and enniatins (EFSA, 2014b) -Deoxynivalenol (EFSA, 2017a) - zearalenone (EFSA, 2011b; EFSA, 2017b) -Fumonisins (EFSA, 2018)
Outcome of interest	Data and information relating to the toxicokinetic effects of single or mixtures of mycotoxins (ADME, bioavailability, carry over)
Inclusion criteria	The paper is addressing toxicokinetic data for single and/or co-occurring mycotoxins -*in vivo* experimental laboratory studies -*in vivo* field/semi-field studies
Exclusion criteria	-*in vitro* studies -Studies duplicated: the most updated is usually reported -Studies reporting data on mixtures having a potential protective or therapeutic effect on the toxicity (i.e. mycotoxin + quercetin)
Total number of records retrieved excluding duplicates	4170
Literature search timeframe	2010-06/2020

Table A2. Keywords and number of records retrieved for the comparative mixture vs single compound analysis

Keywords	Number of records
Mycotoxin* AND toxi*	9921
Mycotoxin* AND toxi* AND *in vitro*	1637
Mycotoxin* AND toxi* AND *in vivo*	672
Mycotoxin* AND mixture* AND toxi*	428
Mycotoxin* AND mixture* AND toxi* AND *in vitro*	96
Mycotoxin* AND mixture* AND toxi* AND *in vivo*	31
Single mycotoxin articles	8528
Single mycotoxin reviews	1001
Single mycotoxin *in vitro* articles	1412
Single mycotoxin *in vitro* reviews	190
Single mycotoxin *in vivo* articles	672
Single mycotoxin *in vivo* reviews	90
Mycotoxin mixtures articles	398
Mycotoxin mixtures reviews	21
Mycotoxin mixtures *in vitro* articles	89
Mycotoxin mixtures *in vitro* reviews	5
Mycotoxin mixtures *in vivo* articles	31
Mycotoxin mixtures *in vivo* reviews	1
Date of the literature search	06/06/2020

Annex B

Table B1. Extensive literature search keywords and outcome from Quignot et al., 2015 (1997-2013) and this study (2013-2019).

Compounds	Keywords	Search performed
Pharmaceutical, Food product, Pesticide, Hormone, Food additives, Food contact materials, Mycotoxins, Natural plant product Contaminants, Phytoestrogens	"Drug Interactions"[Mesh] OR drug interactions OR Drug Interaction OR Interaction, Drug OR Interactions, Drug OR Interaction* OR synergis* OR agonis* OR antagonis* OR potentiation* OR additivity OR augmentation* OR inhibition OR "antagonists and inhibitors"[Subheading] OR induction AND Metabolic network and pathways OR "Metabolic Networks and Pathways"[Mesh] OR Metabolic Pathways OR Metabolic Pathway OR Pathway, Metabolic OR Pathways, Metabolic OR Metabolic Networks OR Metabolic Network OR Network, Metabolic OR Networks, Metabolic OR Pharmacokinetics OR "Pharmacokinetics"[Mesh] OR Drug Kinetics OR Kinetics, Drug OR Toxicokinetics AND CYP2C9[All Fields] OR CYP2C19[All Fields] OR CYP2D6[All fields] OR CYP3A4[All fields] OR "pglycoproteins"[MeSH Terms] OR "pglycoproteins"[All Fields] OR "p glycoprotein"[All Fields] OR "pglycoprotein"[MeSH Terms] OR "p-glycoprotein"[All Fields]OR "p gp"[All Fields] OR pgp[All Fields] OR "glutathione transferase"[MeSH Terms] OR "glutathione transferase"[All Fields] OR	1997-2013 by Quignot et al., 2015

	"glutathione s transferase"[All Fields] OR Glucuronidation[All Fields] OR "Renal excretion"[All fields] OR "Half-life"[All Fields] OR "half-lives"[All Fields] OR "mean residence time"[All Fields] OR Cmax[All Fields] OR Tmax[All Fields] OR "total clearance"[All Fields] OR "renal clearance"[All Fields] OR "area under the curve"[All Fields] OR "AUC"[All Fields]	
Pesticide$	mixture* OR agonis* OR synergis* OR antagonis* OR potentiation* OR additivity OR interaction OR inhibition* OR induction AND CYP* OR UGT OR p-glycoprotein* OR p gp OR pgp OR glutathione transferase OR glutathione s transferase	2013 - April 2019, this study
Flavonoid$		2013 - April 2019, this study
Number of studies 1993-2013	10715	
Number of studies 2013-April 2019	1601	
Number of studies after exclusion criteria 1993-2013	89	
Number of studies after exclusion criteria 2013-April 2019	90	

Table B2. Summary table on CYP1A2 inhibition of pharmaceuticals

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
imipramine	drug	azinphos-methyl	pesticide	CYP1A2	IC_{50}	1.1	Di Consiglio et al., 2005
imipramine	drug	chlorpyrifos	pesticide	CYP1A2	IC_{50}	0.7	Di Consiglio et al., 2005
imipramine	drug	parathion	pesticide	CYP1A2	IC_{50}	0.8	Di Consiglio et al., 2005
melatonine	hormone	chlorpyrifos	pesticide	CYP1A2	IC_{50}	3	Abass et al., 2013
melatonine	hormone	fenitrothion	pesticide	CYP1A2	IC_{50}	2.9	Abass et al., 2013
melatonine	hormone	malathion	pesticide	CYP1A2	IC_{50}	74.3	Abass et al., 2013
melatonine	hormone	phenthoate	pesticide	CYP1A2	IC_{50}	69.8	Abass et al., 2013
melatonine	hormone	profenofos	pesticide	CYP1A2	IC_{50}	3.1	Abass et al., 2013
methoxyresorufin	drug	curcumin	flavonoid	CYP1A2	IC_{50}	25	Appiah et al., 2007
phenacetin	drug	curcumin	flavonoid	CYP1A2	IC_{50}	95.4	Volak et al., 2008
phenacetin	drug	piperine	flavonoid	CYP1A2	IC_{50}	29.8	Volak et al., 2008

duloxetine	drug	propolis	sesinous mixture	CYP1A2	IC_{50}	6.9*	Ryu et al., 2016
phenacetin	drug	tetrahydropalmatine	flavonoid	CYP1A2	Ki	244.8	Zhao et al., 2015
phenacetin	drug	neferine	alkaloid	CYP1A2	Ki	343.75	Zhao et al., 2015
phenacetin	drug	berberine	alkaloid	CYP1A2	Ki	19.15	Zhao et al., 2015

* In µg/ml

Table B3. Chlorpyrifos influencing the metabolic rate of naphthalene by CYP1A2

Compound	Compound type	Dose	Inhibitor	Inhibitor type	Dose	CYP	Metabolite	Metabolic rate (pmol/mg prot/min)	SD	Reference
naphthalene	pesticide	40	-	-	-	CYP1A2	1-naphthol	5.13	0.4	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	20	CYP1A2	1-naphthol	3.96	0.42	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	40	CYP1A2	1-naphthol	2.55	0.50	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	80	CYP1A2	1-naphthol	1.49	0.19	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	120	CYP1A2	1-naphthol	1.10	0.14	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	160	CYP1A2	1-naphthol	0.91	0.11	Cho et al., 2007

naphthalene	pesticide	40	-	-	-	CYP1A2	2-naphthol	0.15	0.01	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	20	CYP1A2	2-naphthol	0.10	0.01	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	40	CYP1A2	2-naphthol	0.05	0.01	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	80	CYP1A2	2-naphthol	0.04	0.00	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	120	CYP1A2	2-naphthol	0.03	0.00	Cho et al., 2007
naphthalene	pesticide	40	chlorpyrifos	pesticide	160	CYP1A2	2-naphthol	0.03	0.00	Cho et al., 2007

Table B4. Summary table on CYP2B6 inhibition of pharmaceuticals

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
bupropion	drug	chlorpyrifos	pesticide	CYP2B6	IC_{50}	2.8	Abass et al., 2013
bupropion	drug	fenitrothion	pesticide	CYP2B6	IC_{50}	2.5	Abass et al., 2013
bupropion	drug	malathion	pesticide	CYP2B6	IC_{50}	69.3	Abass et al., 2013
bupropion	drug	phenthoate	pesticide	CYP2B6	IC_{50}	77	Abass et al., 2013
bupropion	drug	profenofos	pesticide	CYP2B6	IC_{50}	18.1	Abass et al., 2013
benzyloxyresorufin	drug	curcumin	flavonoid	CYP2B6	IC_{50}	45.5	Appiah et al., 2007
benzyloxyresorufin	drug	curcumin	flavonoid	CYP2B6	IC_{50}	51	Appiah et al., 2007
bupropion	drug	curcuminoids	flavonoid	CYP2B6	IC_{50}	9.4	Volak et al., 2008
bupropion	drug	piperine	alkaloid	CYP2B6	IC_{50}	>50	Volak et al., 2008

Table B5. Summary table on CYP2A6 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
testosterone	hormone	chlorpyrifos	pesticide	CYP2A6	IC_{50}	16	Butler et al., 1997
coumarin	plant chemical	chlorpyrifos	pesticide	CYP2A6	IC_{50}	284	Abass et al., 2013
coumarin	plant chemical	fenitrothion	pesticide	CYP2A6	IC_{50}	95	Abass et al., 2013
coumarin	plant chemical	malathion	pesticide	CYP2A6	IC_{50}	126	Abass et al., 2013
coumarin	plant chemical	phenothoate	pesticide	CYP2A6	IC_{50}	97.7	Abass et al., 2013
coumarin	plant chemical	profenofos	pesticide	CYP2A6	IC_{50}	128	Abass et al., 2013

Table B6. Summary table on CYP2C19 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
omeprazole	drug	phenthoate	pesticide	CYP2C19	IC_{50}	35	Abass et al., 2013
omeprazole	drug	profenofos	pesticide	CYP2C19	IC_{50}	18.3	Abass et al., 2013
omeprazole	drug	λ-Cyhalothrin	pesticide	CYP2C19	IC_{50}	273.1	Abass et al., 2013
omeprazole	drug	α-Cypermethrin	pesticide	CYP2C19	IC_{50}	595.1	Abass et al., 2013
omeprazole	drug	cypermethrin	pesticide	CYP2C19	IC_{50}	1198	Abass et al., 2013
omeprazole	drug	deltamethrin	pesticide	CYP2C19	IC_{50}	336.9	Abass et al., 2013
omeprazole	drug	fenvalerate	pesticide	CYP2C19	IC_{50}	380.5	Abass et al., 2013
omeprazole	drug	chlorfluazuron	pesticide	CYP2C19	IC_{50}	7.5	Abass et al., 2013

omeprazole	drug	hexaflumuron	pesticide	CYP2C19	IC_{50}	6	Abass et al., 2013
omeprazole	drug	isoproturon	pesticide	CYP2C19	IC_{50}	127	Abass et al., 2013
omeprazole	drug	atrazine	pesticide	CYP2C19	IC_{50}	801	Abass et al., 2013
omeprazole	drug	glyphosate	pesticide	CYP2C19	IC_{50}	3.7	Abass et al., 2013
omeprazole	drug	carbaryl	pesticide	CYP2C19	IC_{50}	892.2	Abass et al., 2013
omeprazole	drug	abamectin	pesticide	CYP2C19	IC_{50}	81.9	Abass et al., 2013
omeprazole	drug	carbendazim	pesticide	CYP2C19	IC_{50}	979	Abass et al., 2013
omeprazole	drug	carbosulfan	pesticide	CYP2C19	IC_{50}	61.9	Abass et al., 2010
imipramine	drug	azinphos-methyl	pesticide	CYP2C19	IC_{50}	2	Di Consiglio et al., 2005
imipramine	drug	chlorpyrifos	pesticide	CYP2C19	IC_{50}	7	Di Consiglio et al., 2005
imipramine	drug	parathion	pesticide	CYP2C19	IC_{50}	0.4	Di Consiglio et al., 2005
omeprazole	drug	metalaxyl	pesticide	CYP2C19	IC_{50}	> 101	Abass et al., 2007
omeprazole	drug	profenofos	pesticide	CYP2C19	IC_{50}	75	Abass et al., 2007
omeprazole	drug	chlorpyrifos	pesticide	CYP2C19	IC_{50}	96	Abass et al., 2009
omeprazole	drug	fenitrothion	pesticide	CYP2C19	IC_{50}	58.6	Abass et al., 2009
omeprazole	drug	phenthoate	pesticide	CYP2C19	IC_{50}	36.2	Abass et al., 2009
omeprazole	drug	profenofos	pesticide	CYP2C19	IC_{50}	75	Abass et al., 2009
s-mephenytoin	drug	curcuminoids	plant chemical	CYP2C19	IC_{50}	7.4	Volak et al., 2008

s-mephenytoin	drug	piperine	alkaloid	CYP2C19	IC_{50}	>50	Volak et al., 2008
tolbutamide	drug	lotus leaf extract	plant extract (alcoholic)	CYP2C19	IC_{50}	77.38*	Ye et al., 2014
tolbutamide	drug	lotus leaf extract	plant extract (alkaloid)	CYP2C19	IC_{50}	40.79*	Ye et al., 2014
tolbutamide	drug	lotus leaf extract	plant extract (flavonoid)	CYP2C19	IC_{50}	93.04*	Ye et al., 2014

* In µg/ml

Table B7. Summary table on CYP2C8 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (µM)	Reference
amodiaquine	drug	chlorpyrifos	pesticide	CYP2C8	IC_{50}	212	Abass et al., 2013
amodiaquine	drug	fenitrothion	pesticide	CYP2C8	IC_{50}	102	Abass et al., 2013
amodiaquine	drug	malathion	pesticide	CYP2C8	IC_{50}	171	Abass et al., 2013
amodiaquine	drug	phenthoate	pesticide	CYP2C8	IC_{50}	78.4	Abass et al., 2013
amodiaquine	drug	profenofos	pesticide	CYP2C8	IC_{50}	52.3	Abass et al., 2013

Table B8. Summary table on CYP2C9 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
tolbutamide	drug	chlorpyrifos	pesticide	CYP2C9	IC_{50}	89.9	Abass et al., 2013
tolbutamide	drug	fenitrothion	pesticide	CYP2C9	IC_{50}	4.8	Abass et al., 2013
tolbutamide	drug	malathion	pesticide	CYP2C9	IC_{50}	2.5	Abass et al., 2013
tolbutamide	drug	phenthoate	pesticide	CYP2C9	IC_{50}	35	Abass et al., 2013
tolbutamide	drug	profenofos	pesticide	CYP2C9	IC_{50}	18.3	Abass et al., 2013
tolbutamide	drug	λ-cyhalothrin	pesticide	CYP2C9	IC_{50}	273.1	Abass et al., 2013
tolbutamide	drug	α-cypermethrin	pesticide	CYP2C9	IC_{50}	1595	Abass et al., 2013
tolbutamide	drug	cypermethrin	pesticide	CYP2C9	IC_{50}	1198	Abass et al., 2013
tolbutamide	drug	deltamethrin	pesticide	CYP2C9	IC_{50}	336.9	Abass et al., 2013
tolbutamide	drug	fenvalerate	pesticide	CYP2C9	IC_{50}	380.5	Abass et al., 2013
tolbutamide	drug	chlorfluazuron	pesticide	CYP2C9	IC_{50}	7.5	Abass et al., 2013
tolbutamide	drug	hexaflumuron	pesticide	CYP2C9	IC_{50}	6	Abass et al., 2013
tolbutamide	drug	isoproturon	pesticide	CYP2C9	IC_{50}	127	Abass et al., 2013
tolbutamide	drug	atrazine	pesticide	CYP2C9	IC_{50}	801	Abass et al., 2013
tolbutamide	drug	glyphosate	pesticide	CYP2C9	IC_{50}	3.7	Abass et al., 2013

tolbutamide	drug	carbaryl	pesticide	CYP2C9	IC_{50}	892.2	Abass et al., 2013
tolbutamide	drug	abamectin	pesticide	CYP2C9	IC_{50}	81.9	Abass et al., 2013
tolbutamide	drug	carbendazim	pesticide	CYP2C9	IC_{50}	979	Abass et al., 2013
tolbutamide	drug	carbosulfan	pesticide	CYP2C9	IC_{50}	> 100	Abass et al., 2010
warfarin	drug	silibinin	flavonoid	CYP2C9	IC_{50}	43	Beckmann-Knopp et al., 2000
warfarin	drug	silibinin	flavonoid	CYP2C9	IC_{50}	45	Beckmann-Knopp et al., 2000
warfarin	drug	silibinin	flavonoid	CYP2C9	Ki	18	Beckmann-Knopp et al., 2000
warfarin	drug	silibinin	flavonoid	CYP2C9	Ki	19	Beckmann-Knopp et al., 2000
warfarin	drug	silybin A	flavonoid	CYP2C9	IC_{50}	18	Beckmann-Knopp et al., 2000
warfarin	drug	silybin B	flavonoid	CYP2C9	IC_{50}	8.2	Brantley et al., 2010
warfarin	drug	isosilybin A	flavonoid	CYP2C9	IC_{50}	> 100	Brantley et al., 2010
warfarin	drug	isosilybin B	flavonoid	CYP2C9	IC_{50}	74	Brantley et al., 2010
warfarin	drug	silybin A	flavonoid	CYP2C9	Ki	10	Brantley et al., 2010
warfarin	drug	silybin B	flavonoid	CYP2C9	Ki	4.8	Brantley et al., 2010
tolbutamide	drug	metalaxyl	pesticide	CYP2C9	IC_{50}	> 100	Abass et al., 2007

tolbutamide	drug	profenofos	pesticide	CYP2C9	IC_{50}	18.3	Abass et al., 2007
tolbutamide	drug	chlorpyrifos	pesticide	CYP2C9	IC_{50}	89.9	Abass et al., 2009
tolbutamide	drug	fenitrothion	pesticide	CYP2C9	IC_{50}	4.8	Abass et al., 2009
tolbutamide	drug	malathion	pesticide	CYP2C9	IC_{50}	2.5	Abass et al., 2009
tolbutamide	drug	phenthoate	pesticide	CYP2C9	IC_{50}	35	Abass et al., 2009
tolbutamide	drug	profenofos	pesticide	CYP2C9	IC_{50}	18.3	Abass et al., 2009
tolbutamide	drug	chlorfluzuron	pesticide	CYP2C9	IC_{50}	7.5	Abass et al., 2009
tolbutamide	drug	hexaflumuron	pesticide	CYP2C9	IC_{50}	6	Abass et al., 2009
tolbutamide	drug	glyphosate	pesticide	CYP2C9	IC_{50}	3.7	Abass et al., 2009
diclofenac	drug	curcumin	plant chemical	CYP2C9	IC_{50}	6	Appiah et al., 2007
flurbiprofen	drug	curcuminoids	plant chemical	CYP2C9	Ki	10.6	Volak et al., 2008
flurbiprofen	drug	piperine	alkaloid	CYP2C9	IC_{50}	11.5	Volak et al., 2008
testosterone	hormone	lotus leaf extract	plant chemical (alcoholic)	CYP2C9	IC_{50}	83.46	Ye et al., 2014
testosterone	hormone	lotus leaf extract	plant chemical (alkaloid)	CYP2C9	IC_{50}	52.58	Ye et al., 2014
testosterone	hormone	lotus leaf extract	plant chemical (flavonoid)	CYP2C9	IC_{50}	123.21	Ye et al., 2014
diclofenac 4'-hydroxylase	drug	baicalein	flavonoid	CYP2C9	Ki	4	Si et al., 2008

diclofenac 4'-hydroxylase	drug	galangin	flavonoid	CYP2C9	Ki	0.73	Si et al., 2008
diclofenac 4'-hydroxylase	drug	6-hydroxyflavone	flavonoid	CYP2C9	Ki	11	Si et al., 2008
diclofenac 4'-hydroxylase	drug	baicalein	flavonoid	CYP2C9	Ki	1	Si et al., 2008
diclofenac 4'-hydroxylase	drug	galangin	flavonoid	CYP2C9	Ki	0.5	Si et al., 2008
diclofenac 4'-hydroxylase	drug	6-hydroxyflavone	flavonoid	CYP2C9	Ki	17	Si et al., 2008

Table B9. Summary table on CYP2D6 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
dextromethorphan	drug	chlorpyrifos	pesticide	CYP2D6	IC_{50}	45.9	Abass et al., 2013
dextromethorphan	drug	fenitrothion	pesticide	CYP2D6	IC_{50}	91.9	Abass et al., 2013
dextromethorphan	drug	malathion	pesticide	CYP2D6	IC_{50}	126	Abass et al., 2013
dextromethorphan	drug	phenthoate	pesticide	CYP2D6	IC_{50}	3	Abass et al., 2013
dextromethorphan	drug	profenofos	pesticide	CYP2D6	IC_{50}	72.7	Abass et al., 2013
dextromethorphan	drug	λ-cyhalothrin	pesticide	CYP2D6	IC_{50}	3.1	Abass et al., 2013
dextromethorphan	drug	α-cypermethrin	pesticide	CYP2D6	IC_{50}	537	Abass et al., 2013
dextromethorphan	drug	cypermethrin	pesticide	CYP2D6	IC_{50}	1289	Abass et al., 2013

dextromethorphan	drug	deltamethrin	pesticide	CYP2D6	IC_{50}	3.3	Abass et al., 2013
dextromethorphan	drug	fenvalerate	pesticide	CYP2D6	IC_{50}	3.1	Abass et al., 2013
dextromethorphan	drug	chlorfluazuron	pesticide	CYP2D6	IC_{50}	213	Abass et al., 2013
dextromethorphan	drug	hexaflumuron	pesticide	CYP2D6	IC_{50}	520	Abass et al., 2013
dextromethorphan	drug	isoproturon	pesticide	CYP2D6	IC_{50}	363	Abass et al., 2013
dextromethorphan	drug	atrazine	pesticide	CYP2D6	IC_{50}	1412	Abass et al., 2013
dextromethorphan	drug	glyphosate	pesticide	CYP2D6	IC_{50}	603	Abass et al., 2013
dextromethorphan	drug	carbaryl	pesticide	CYP2D6	IC_{50}	441	Abass et al., 2013
dextromethorphan	drug	abamectin	pesticide	CYP2D6	IC_{50}	184	Abass et al., 2013
dextromethorphan	drug	carbendazim	pesticide	CYP2D6	IC_{50}	12	Abass et al., 2013
dextromethorphan	drug	carbosulfan	pesticide	CYP2D6	IC_{50}	> 100	Abass et al., 2010
dextromethorphan	drug	silibinin	flavonoid	CYP2D6	IC_{50}	173	Beckmann-Knopp et al., 2000
dextromethorphan	drug	chlorpyrifos	pesticide	CYP2D6	IC_{50}	3.3	Abass et al., 2009
dextromethorphan	drug	fenitrothion	pesticide	CYP2D6	IC_{50}	91.9	Abass et al., 2009

dextromethorphan	drug	phenthoate	pesticide	CYP2D6	IC_{50}	3	Abass et al., 2009
dextromethorphan	drug	profenofos	pesticide	CYP2D6	IC_{50}	72.7	Abass et al., 2009
dextromethorphan	drug	lambda-cyhalothrin	pesticide	CYP2D6	IC_{50}	3.1	Abass et al., 2009
dextromethorphan	drug	deltamethrin	pesticide	CYP2D6	IC_{50}	3.3	Abass et al., 2009
dextromethorphan	drug	fenvalerate	pesticide	CYP2D6	IC_{50}	3.1	Abass et al., 2009
dextromethorphan	drug	carbendazim	pesticide	CYP2D6	IC_{50}	12	Abass et al., 2009
dextromethorphan	drug	curcumin	plant extract	CYP2D6	IC_{50}	50	Appiah et al., 2007
dextromethorphan	drug	piperine	alkaloid	CYP2D6	IC_{50}	>50	Volak et al., 2008
dextromethorphan	drug	tetrahydropalmatine	alkaloid	CYP2D6	Ki	1.17	Zhao et al., 2015
dextromethorphan	drug	neferine	alkaloid	CYP2D6	Ki	86.37	Zhao et al., 2015
dextromethorphan	drug	berberine	alkaloid	CYP2D6	Ki	3.17	Zhao et al., 2015
o-methyl-14 C-dextromethorphan	drug	piperamide-C7	alkaloid	CYP2D6	IC_{50}	3.16	Subehan et al., 2006
o-methyl-14 C-dextromethorphan	drug	nigramide R	alkaloid	CYP2D6	IC_{50}	0.32	Subehan et al., 2006

o-methyl-14 C-dextromethorphan	drug	ursolic acid	plant chemical	CYP2D6	IC_{50}	>100	Usia et al., 2005
o-methyl-14 C-dextromethorphan	drug	oleanolic acid	plant chemical	CYP2D6	IC_{50}	>100	Usia et al., 2005
o-methyl-14 C-dextromethorphan	drug	vindoline	alkaloid	CYP2D6	IC_{50}	15.9	Usia et al., 2005
dextromethorphan	drug	hyperoside	phenolic compound	CYP2D6	Ki	2.01	Song et al., 2013
dextromethorphan	drug	lotus leaf extract	plant extract (alcoholic)	CYP2D6	IC_{50}	12.05*	Ye et al., 2014
dextromethorphan	drug	lotus leaf extract	plant extract(alkaloid fraction)	CYP2D6	IC_{50}	0.96*	Ye et al., 2014
dextromethorphan	drug	lotus leaf extract	plant extract (flavonoid fraction)	CYP2D6	IC_{50}	139.4*	Ye et al., 2014
dextromethorphan	drug	nuciferine	alkaloid	CYP2D6	Ki	1.88	Ye et al., 2014
dextromethorphan	drug	N-nornuciferine	alkaloid	CYP2D6	Ki	2.34	Ye et al., 2014

dextromethorphan	drug	2-hydroxy-1-metho-xyaporphine	alkaloid	CYP2D6	Ki	1.56	Ye et al., 2014
dextromethorphan	drug	st john wort	plant	CYP2D6	Ki	50*	Hellum et al., 2007
dextromethorphan	drug	common valerian	plant	CYP2D6	Ki	2905000*	Hellum et al., 2007
dextromethorphan	drug	common sage	plant	CYP2D6	Ki	743000*	Hellum et al., 2007

* In µg/ml

Table B10. Summary table on CYP3A4 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
silybin	flavonoid	testosterone	hormone	CYP3A4	IC_{50}	49.8	Jancova et al., 2007
silybin	flavonoid	testosterone	hormone	CYP3A4	Ki	21	Jancova et al., 2007
midazolam	drug	chlorpyrifos	pesticide	CYP3A4	IC_{50}	4	Abass et al., 2013
omeprazole	drug	chlorpyrifos	pesticide	CYP3A4	IC_{50}	32.2	Abass et al., 2013
midazolam	drug	fenitrothion	pesticide	CYP3A4	IC_{50}	3.1	Abass et al., 2013
omeprazole	drug	fenitrothion	pesticide	CYP3A4	IC_{50}	4.2	Abass et al., 2013
midazolam	drug	malathion	pesticide	CYP3A4	IC_{50}	57	Abass et al., 2013
omeprazole	drug	malathion	pesticide	CYP3A4	IC_{50}	102	Abass et al., 2013
midazolam	drug	phenthoate	pesticide	CYP3A4	IC_{50}	3	Abass et al., 2013
omeprazole	drug	phenthoate	pesticide	CYP3A4	IC_{50}	66.8	Abass et al., 2013
midazolam	drug	profenofos	pesticide	CYP3A4	IC_{50}	72.7	Abass et al., 2013
omeprazole	drug	profenofos	pesticide	CYP3A4	IC_{50}	142	Abass et al., 2013
midazolam	drug	λ-cyhalothrin	pesticide	CYP3A4	IC_{50}	3.1	Abass et al., 2013
omeprazole	drug	λ-cyhalothrin	pesticide	CYP3A4	IC_{50}	3.9	Abass et al., 2013
midazolam	drug	α-cypermethrin	pesticide	CYP3A4	IC_{50}	1613	Abass et al., 2013
omeprazole	drug	α-cypermethrin	pesticide	CYP3A4	IC_{50}	148	Abass et al., 2013
midazolam	drug	cypermethrin	pesticide	CYP3A4	IC_{50}	70	Abass et al., 2013
omeprazole	drug	cypermethrin	pesticide	CYP3A4	IC_{50}	249	Abass et al., 2013
midazolam	drug	deltamethrin	pesticide	CYP3A4	IC_{50}	42.9	Abass et al., 2013
omeprazole	drug	deltamethrin	pesticide	CYP3A4	IC_{50}	18.6	Abass et al., 2013

midazolam	drug	fenvalerate	pesticide	CYP3A4	IC_{50}	19.3	Abass et al., 2013
omeprazole	drug	fenvalerate	pesticide	CYP3A4	IC_{50}	74.3	Abass et al., 2013
midazolam	drug	chlorfluazuron	pesticide	CYP3A4	IC_{50}	986	Abass et al., 2013
omeprazole	drug	chlorfluazuron	pesticide	CYP3A4	IC_{50}	170	Abass et al., 2013
midazolam	drug	hexaflumuron	pesticide	CYP3A4	IC_{50}	833	Abass et al., 2013
omeprazole	drug	hexaflumuron	pesticide	CYP3A4	IC_{50}	843	Abass et al., 2013
midazolam	drug	isoproturon	pesticide	CYP3A4	IC_{50}	618	Abass et al., 2013
omeprazole	drug	isoproturon	pesticide	CYP3A4	IC_{50}	1543	Abass et al., 2013
midazolam	drug	atrazine	pesticide	CYP3A4	IC_{50}	2.8	Abass et al., 2013
omeprazole	drug	atrazine	pesticide	CYP3A4	IC_{50}	618	Abass et al., 2013
midazolam	drug	glyphosate	pesticide	CYP3A4	IC_{50}	1080	Abass et al., 2013
omeprazole	drug	glyphosate	pesticide	CYP3A4	IC_{50}	1163	Abass et al., 2013
midazolam	drug	carbaryl	pesticide	CYP3A4	IC_{50}	933	Abass et al., 2013
omeprazole	drug	carbaryl	pesticide	CYP3A4	IC_{50}	941	Abass et al., 2013
midazolam	drug	abamectin	pesticide	CYP3A4	IC_{50}	117	Abass et al., 2013
omeprazole	drug	abamectin	pesticide	CYP3A4	IC_{50}	55	Abass et al., 2013
midazolam	drug	carbendazim	pesticide	CYP3A4	IC_{50}	1229	Abass et al., 2013
omeprazole	drug	carbendazim	pesticide	CYP3A4	IC_{50}	966	Abass et al., 2013
midazolam	drug	carbosulfan	pesticide	CYP3A4	IC_{50}	11.2	Abass et al., 2010
omeprazole	drug	carbosulfan	pesticide	CYP3A4	IC_{50}	23.8	Abass et al., 2010
erythromycin	drug	silibinin	flavonoid	CYP3A4	IC_{50}	> 200	Beckmann-Knopp et al., 2000
erythromycin	drug	silibinin	flavonoid	CYP3A4	IC_{50}	> 200	Beckmann-Knopp et al., 2000

testosterone	drug	parathion	pesticide	CYP3A4	IC_{50}	19	Butler et al., 1997
testosterone	drug	parathion	pesticide	CYP3A4	IC_{50}	7.3	Butler et al., 1997
imipramine	drug	azinphos-methyl	pesticide	CYP3A4	IC_{50}	11.6	Di Consiglio et al., 2005
imipramine	drug	chlorpyrifos	pesticide	CYP3A4	IC_{50}	7.7	Di Consiglio et al., 2005
imipramine	drug	parathion	pesticide	CYP3A4	IC_{50}	5	Di Consiglio et al., 2005
felodipine	drug	quercetin	flavonoid	CYP3A4	IC_{50}	208,65	Fasinu et al., 2013
felodipine	drug	quercetin	flavonoid	CYP3A4	Ki	346,82	Fasinu et al., 2013
saquinavir	drug	bergamottin	food product	CYP3A4	IC_{50}	0.74	Eagling et al., 1999
saquinavir	drug	bergamottin	food product	CYP3A4	IC_{50}	3.07	Eagling et al., 1999
saquinavir	drug	6'7'-dihydroxybergamottin	food product	CYP3A4	IC_{50}	0.33	Eagling et al., 1999
saquinavir	drug	6'7'-dihydroxybergamottin	food product	CYP3A4	IC_{50}	1.68	Eagling et al., 1999
terfenadine	drug	grapefruit (Naringenin)	food product	CYP3A4	Ki	22	Li et al., 1997
midazolam	drug	metalaxyl	pesticide	CYP3A4	IC_{50}	> 103	Abass et al., 2007
omeprazole	drug	metalaxyl	pesticide	CYP3A4	IC_{50}	> 104	Abass et al., 2007
midazolam	drug	profenofos	pesticide	CYP3A4	IC_{50}	72	Abass et al., 2007
omeprazole	drug	profenofos	pesticide	CYP3A4	IC_{50}	>100	Abass et al., 2009
midazolam	drug	chlorpyrifos	pesticide	CYP3A4	IC_{50}	4	Abass et al., 2009
omeprazole	drug	chlorpyrifos	pesticide	CYP3A4	IC_{50}	32.2	Abass et al., 2009
midazolam	drug	fenitrothion	pesticide	CYP3A4	IC_{50}	3.1	Abass et al., 2009
omeprazole	drug	fenitrothion	pesticide	CYP3A4	IC_{50}	4.2	Abass et al., 2009
midazolam	drug	malathion	pesticide	CYP3A4	IC_{50}	57	Abass et al., 2009

midazolam	drug	phenthoate	pesticide	CYP3A4	IC_{50}	3	Abass et al., 2009
omeprazole	drug	phenthoate	pesticide	CYP3A4	IC_{50}	66.8	Abass et al., 2009
midazolam	drug	profenofos	pesticide	CYP3A4	IC_{50}	72.7	Abass et al., 2009
midazolam	drug	lambda-cyhalothrin	pesticide	CYP3A4	IC_{50}	3.1	Abass et al., 2009
omeprazole	drug	lambda-cyhalothrin	pesticide	CYP3A4	IC_{50}	3.9	Abass et al., 2009
midazolam	drug	cypermethrin	pesticide	CYP3A4	IC_{50}	70	Abass et al., 2009
midazolam	drug	deltamethrin	pesticide	CYP3A4	IC_{50}	42.9	Abass et al., 2009
omeprazole	drug	deltamethrin	pesticide	CYP3A4	IC_{50}	18.6	Abass et al., 2009
midazolam	drug	fenvalerate	pesticide	CYP3A4	IC_{50}	19.3	Abass et al., 2009
omeprazole	drug	fenvalerate	pesticide	CYP3A4	IC_{50}	74.3	Abass et al., 2009
midazolam	drug	atrazine	pesticide	CYP3A4	IC_{50}	2.8	Abass et al., 2009
omeprazole	drug	abamectin	pesticide	CYP3A4	IC_{50}	55	Abass et al., 2009
testosterone	hormone	amentoflavone	flavonoid	CYP3A4	Ki	0.027	Kimura et al., 2010
testosterone	hormone	imperatorin	flavonoid	CYP3A4	Ki	0.64	Kimura et al., 2010
testosterone	hormone	apigentin	flavonoid	CYP3A4	Ki	0.67	Kimura et al., 2010
benzyloxyresorufin	drug	curcumin	flavonoid	CYP3A4	Ki	7.4	Appiah et al., 2007
dibenzylfluorescein	drug	aframomum cuspidatum	food plants	CYP3A4	% inhibition	73.68*	Angobon et al., 2010
dibenzylfluorescein	drug	aframomum melegueta	food plants	CYP3A4	% inhibition	99.88*	Angobon et al., 2010
dibenzylfluorescein	drug	corchorus olitorius	food plants	CYP3A4	% inhibition	100.3*	Angobon et al., 2010
dibenzylfluorescein	drug	harrisonia abyssinica	food plants	CYP3A4	% inhibition	12.4*	Angobon et al., 2010
dibenzylfluorescein	drug	jatropha curcas	food plants	CYP3A4	% inhibition	97.59*	Angobon et al., 2010

dibenzylfluorescein	drug	lippia multiflora	food plants	CYP3A4	% inhibition	75.16*	Angobon et al., 2010
dibenzylfluorescein	drug	lonchocarpus sericeus	food plants	CYP3A4	% inhibition	72.66*	Angobon et al., 2010
dibenzylfluorescein	drug	morinda lucida	food plants	CYP3A4	% inhibition	36.97*	Angobon et al., 2010
dibenzylfluorescein	drug	oxythenantera abyssinica	food plants	CYP3A4	% inhibition	-1.47*	Angobon et al., 2010
dibenzylfluorescein	drug	persea americana	food plants	CYP3A4	% inhibition	42.79*	Angobon et al., 2010
dibenzylfluorescein	drug	phyllanthus amarus	food plants	CYP3A4	% inhibition	73.64*	Angobon et al., 2010
dibenzylfluorescein	drug	piper guineense	food plants	CYP3A4	% inhibition	96.75*	Angobon et al., 2010
dibenzylfluorescein	drug	solanum macrocarpon	food plants	CYP3A4	% inhibition	98.27*	Angobon et al., 2010
dibenzylfluorescein	drug	talinum triangulare	food plants	CYP3A4	% inhibition	14.73*	Angobon et al., 2010
triazolam	drug	curcuminoids	plant chemical	CYP3A4	Ki	11	Volak et al., 2008
triazolam	drug	piperine	alkaloid	CYP3A4	Ki	5.4	Volak et al., 2008
testosterone	hormone	tetrahydropalmatine	alkaloid	CYP3A4	Ki	52.2	Zhao et al., 2015
testosterone	hormone	berberine	alkaloid	CYP3A4	Ki	18.47	Zhao et al., 2015
n-methyl-14C-erythromycin	drug	ursolic acid	plant chemical	CYP3A4	IC_{50}	>100	Usia et al., 2005
n-methyl-14C-erythromycin	drug	oleanolic acid	plant chemical	CYP3A4	IC_{50}	>100	Usia et al., 2005
n-methyl-14C-erythromycin	drug	vindoline	alkaloid	CYP3A4	IC_{50}	20.1	Usia et al., 2005

omeprazole	drug	lotus leaf extract	plant (alcoholic extract)	CYP3A4	IC_{50}	98.01[a]	Ye et al., 2014
omeprazole	drug	lotus leaf extract	plant (alkaloid extract)	CYP3A4	IC_{50}	44.87[a]	Ye et al., 2014
omeprazole	drug	lotus leaf extract	plant (flavonoid extract)	CYP3A4	IC_{50}	97.5[a]	Ye et al., 2014

* In per cent (%), [a] in µg/mL

Table B11. Inhibitors influencing the metabolic rate of pesticides on CYP3A4

Compound	Compound type	Dose (μM)	Inhibitor	Inhibitor type	Dose	CYP	Metabolite	Metabolic rate (nmol/mg prot/min)	SD	Reference
carbosulfan	pesticide	2.5	-	-	-	CYP3A4	-	1.560939	0.114613	Abass et al., 2010
carbosulfan	pesticide	5	-	-	-	CYP3A4	-	2.431577	0.038126	Abass et al., 2010
carbosulfan	pesticide	15	-	-	-	CYP3A4	-	6.702249	7.84E-05	Abass et al., 2010
carbosulfan	pesticide	50	-	-	-	CYP3A4	-	14.41147	0.993236	Abass et al., 2010
carbosulfan	pesticide	150	-	-	-	CYP3A4	-	14.97663	0.114535	Abass et al., 2010
carbosulfan	pesticide	300	-	-	-	CYP3A4	-	14.66301	1.604428	Abass et al., 2010
carbosulfan	pesticide	500	-	-	-	CYP3A4	-	15.15153	0.76401	Abass et al., 2010
carbosulfan	pesticide	2.5	ketoconazole	drug	100	CYP3A4	-	0.029945	0.152818	Abass et al., 2010
carbosulfan	pesticide	5	ketoconazole	drug	100	CYP3A4	-	0.212903	0.152739	Abass et al., 2010
carbosulfan	pesticide	15	ketoconazole	drug	100	CYP3A4	-	0.548679	0.114613	Abass et al., 2010
carbosulfan	pesticide	50	ketoconazole	drug	100	CYP3A4	-	0.769842	0.152739	Abass et al., 2010

carbosulfan	pesticide	150	ketoconazole	drug	100	CYP3A4	-	1.220309	0.190944	Abass et al., 2010
carbosulfan	pesticide	300	ketoconazole	drug	100	CYP3A4	-	1.250372	0.114613	Abass et al., 2010
carbosulfan	pesticide	500	ketoconazole	drug	100	CYP3A4	-	1.509817	0.573066	Abass et al., 2010
carbosulfan	pesticide	50	-	-	-	CYP3A4	carbosulfan sulfinamide	0.502696	0.030578	Abass et al., 2010
carbosulfan	pesticide	150	-	-	-	CYP3A4	carbosulfan sulfinamide	1.857032	0.045758	Abass et al., 2010
carbosulfan	pesticide	300	-	-	-	CYP3A4	carbosulfan sulfinamide	2.669341	0.076422	Abass et al., 2010
carbosulfan	pesticide	500	-	-	-	CYP3A4	carbosulfan sulfinamide	3.145858	0.160348	Abass et al., 2010
carbosulfan	pesticide	50	ketoconazole	drug	100	CYP3A4	carbosulfan sulfinamide	0.050847	0.068745	Abass et al., 2010
carbosulfan	pesticide	150	ketoconazole	drug	100	CYP3A4	carbosulfan sulfinamide	0.970069	0.045801	Abass et al., 2010
carbosulfan	pesticide	300	ketoconazole	drug	100	CYP3A4	carbosulfan sulfinamide	1.301462	0.030578	Abass et al., 2010
carbosulfan	pesticide	500	ketoconazole	drug	100	CYP3A4	carbosulfan sulfinamide	1.190193	0.114504	Abass et al., 2010
fipronil	pesticide	80	-	-	-	CYP3A4	fipronil sulfone	17.3	1.2	Joo et al., 2007
fipronil	pesticide	80	chlorpyrifos	pesticide	0.25	CYP3A4	fipronil sulfone	10.1	1.2	Joo et al., 2007
fipronil	pesticide	80	chlorpyrifos	pesticide	2.5	CYP3A4	fipronil sulfone	1.7	0.5	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	40	-	-	-	CYP3A4	N-ethyl-m-toluamide	1.33	0.16	Joo et al., 2007

N,N-diethyl-3-methylbenzamide	pesticide	40	chlorpyrifos	pesticide	40	CYP3A4	N-ethyl-m-toluamide	1.33	0.16	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	40	-	-	-	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	3.08	0.54	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	40	chlorpyrifos	pesticide	40	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	5.93	0.68	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	-	-	-	CYP3A4	N-ethyl-m-toluamide	2.08	0.13	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	5	CYP3A4	N-ethyl-m-toluamide	3.85	0.13	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	10	CYP3A4	N-ethyl-m-toluamide	4.69	0.50	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	20	CYP3A4	N-ethyl-m-toluamide	5.42	0.15	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	40	CYP3A4	N-ethyl-m-toluamide	7.93	0.54	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	80	CYP3A4	N-ethyl-m-toluamide	8.05	0.58	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	160	CYP3A4	N-ethyl-m-toluamide	5.42	0.56	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	300	CYP3A4	N-ethyl-m-toluamide	5.24	0.11	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	-		-	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	1.80	0.10	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	5	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	1.54	0.09	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	10	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	1.21	0.15	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	20	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	1.16	0.07	Joo et al., 2007

N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	40	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	0.97	0.06	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	80	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	0.73	0.05	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	160	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	0.41	0.04	Joo et al., 2007
N,N-diethyl-3-methylbenzamide	pesticide	100	chlorpyrifos	pesticide	300	CYP3A4	N,N-diethyl-m-hydroxymethylbenzamide	0.63	0.19	Joo et al., 2007

Table B12. Summary table on CYP3A4/A5 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
midazolam	drug	trans-resveratrol	stilbenoid	CYP3A4/A5	Ki	5.5	Hyrsova et al., 2019
testosterone	hormone	trans-resveratrol	stilbenoid	CYP3A4/A5	Ki	1.5	Hyrsova et al., 2019
testosterone	hormone	cis-resveratrol	stilbenoid	CYP3A4/A5	Ki	6.2	Hyrsova et al., 2019
testosterone	hormone	trans-piceatannol	stilbenoid	CYP3A4/A5	Ki	10.7	Hyrsova et al., 2019
testosterone	hormone	cis-piceatannol	stilbenoid	CYP3A4/A5	Ki	14	Hyrsova et al., 2019
testosterone	hormone	oxyresveratrol	stilbenoid	CYP3A4/A5	Ki	12.3	Hyrsova et al., 2019
midazolam	drug	oxyresveratrol	stilbenoid	CYP3A4/A5	Ki	19.9	Hyrsova et al., 2019
testosterone	hormone	pterostilbene	stilbenoid	CYP3A4/A5	Ki	8.7	Hyrsova et al., 2019
testosterone	hormone	pinostilbene	stilbenoid	CYP3A4/A5	Ki	6.7	Hyrsova et al., 2019
midazolam	drug	pinostilbene	stilbenoid	CYP3A4/A5	Ki	53.7	Hyrsova et al., 2019

Table B13. Summary table on CYP3A5 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
testosterone	hormone	methylparathion	pesticide	CYP3A5	IC50	31	Butler et al., 1997
dibenzylfluorescein	drug	aframomum cuspidatum	food plants	CYP3A5	% inhibition	100.27*	Angobon et al., 2010
dibenzylfluorescein	drug	aframomum melegueta	food plants	CYP3A5	% inhibition	99.87*	Angobon et al., 2010
dibenzylfluorescein	drug	corchorus olitorius	food plants	CYP3A5	% inhibition	2.32*	Angobon et al., 2010
dibenzylfluorescein	drug	harrisonia abyssinica	food plants	CYP3A5	% inhibition	96.98*	Angobon et al., 2010
dibenzylfluorescein	drug	jatropha curcas	food plants	CYP3A5	% inhibition	15.76*	Angobon et al., 2010
dibenzylfluorescein	drug	lippia multiflora	food plants	CYP3A5	% inhibition	71.14*	Angobon et al., 2010
dibenzylfluorescein	drug	lonchocarpus sericeus	food plants	CYP3A5	% inhibition	62.71*	Angobon et al., 2010
dibenzylfluorescein	drug	morinda lucida	food plants	CYP3A5	% inhibition	6.05*	Angobon et al., 2010
dibenzylfluorescein	drug	oxythenantera abyssinica	food plants	CYP3A5	% inhibition	33.58*	Angobon et al., 2010

dibenzylfluorescein	drug	persea americana	food plants	CYP3A5	% inhibition	46.44*	Angobon et al., 2010
dibenzylfluorescein	drug	phyllanthus amarus	food plants	CYP3A5	% inhibition	70.02*	Angobon et al., 2010
dibenzylfluorescein	drug	piper guineense	food plants	CYP3A5	% inhibition	98.3*	Angobon et al., 2010
dibenzylfluorescein	drug	solanum macrocarpon	food plants	CYP3A5	% inhibition	14.63*	Angobon et al., 2010
dibenzylfluorescein	drug	talinum triangulare	food plants	CYP3A5	% inhibition	6.59*	Angobon et al., 2010

* In percent (%)

Table B14. Summary table on CYP2E1 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (μM)	Reference
chlorzoxazone	drug	chlorpyrifos	pesticide	CYP2E1	IC_{50}	3.6	Abass et al., 2013
chlorzoxazone	drug	fenitrothion	pesticide	CYP2E1	IC_{50}	2.8	Abass et al., 2013
chlorzoxazone	drug	malathion	pesticide	CYP2E1	IC_{50}	4.4	Abass et al., 2013
chlorzoxazone	drug	phenthoate	pesticide	CYP2E1	IC_{50}	4.4	Abass et al., 2013
chlorzoxazone	drug	profenofos	pesticide	CYP2E1	IC_{50}	2.8	Abass et al., 2013
chlorzoxazone	drug	curcuminoids	plant chemical	CYP2E1	IC_{50}	>200	Volak et al., 2008
chlorzoxazone	drug	piperine	alkaloid	CYP2E1	IC_{50}	>50	Volak et al., 2008
chlorzoxazone	drug	lotus leaf extract	alcoholic plant extract	CYP2E1	IC_{50}	61.43*	Ye et al., 2014

chlorzoxazone	drug	lotus leaf extract	plant extract alkaloid fraction	CYP2E1	IC_{50}	63.84*	Ye et al., 2014
chlorzoxazone	drug	lotus leaf extract	plant extract flavonoid fraction	CYP2E1	IC_{50}	102.1*	Ye et al., 2014

*In µg/mL

Table B15. Summary table on CYP3A6-3A9 inhibition

Compound	Compound type	Inhibitor	Inhibitor type	CYP	Inhibition parameter	Value (µM)	Reference
testosterone	hormone	chlorpyrifos	pesticide	CYP3A6	IC_{50}	16	Butler et al., 1997
testosterone	hormone	malathion	pesticide	CYP3A7	IC_{50}	215	Butler et al., 1997
testosterone	hormone	fenitrothion	pesticide	CYP3A8	IC_{50}	18	Butler et al., 1997
testosterone	hormone	azinphos-methyl	pesticide	CYP3A9	IC_{50}	29	Butler et al., 1997
testosterone	hormone	chlorpyrifos	pesticide	CYP3A6	IC_{50}	7	Butler et al., 1997
testosterone	hormone	malathion	pesticide	CYP3A7	IC_{50}	110	Butler et al., 1997
testosterone	hormone	fenitrothion	pesticide	CYP3A8	IC_{50}	7.6	Butler et al., 1997
testosterone	hormone	azinphos-methyl	pesticide	CYP3A9	IC_{50}	9.7	Butler et al., 1997
dibenzylfluorescein	drug	aframomum cuspidatum	food plants	CYP3A7	% inhibition	105.5*	Angobon et al., 2010
dibenzylfluorescein	drug	aframomum melegueta	food plants	CYP3A7	% inhibition	97.24*	Angobon et al., 2010
dibenzylfluorescein	drug	corchorus olitorius	food plants	CYP3A7	% inhibition	30.29*	Angobon et al., 2010
dibenzylfluorescein	drug	harrisonia abyssinica	food plants	CYP3A7	% inhibition	107.42*	Angobon et al., 2010

dibenzylfluorescein	drug	jatropha curcas	food plants	CYP3A7	% inhibition	76.7*	Angobon et al., 2010
dibenzylfluorescein	drug	lippia multiflora	food plants	CYP3A7	% inhibition	81.2*	Angobon et al., 2010
dibenzylfluorescein	drug	lonchocarpus sericeus	food plants	CYP3A7	% inhibition	80.28*	Angobon et al., 2010
dibenzylfluorescein	drug	morinda lucida	food plants	CYP3A7	% inhibition	31.55*	Angobon et al., 2010
dibenzylfluorescein	drug	oxythenantera abyssinica	food plants	CYP3A7	% inhibition	63.22*	Angobon et al., 2010
dibenzylfluorescein	drug	persea americana	food plants	CYP3A7	% inhibition	91.35*	Angobon et al., 2010
dibenzylfluorescein	drug	phyllanthus amarus	food plants	CYP3A7	% inhibition	101.52*	Angobon et al., 2010
dibenzylfluorescein	drug	piper guineense	food plants	CYP3A7	% inhibition	95.34*	Angobon et al., 2010
dibenzylfluorescein	drug	solanum macrocarpon	food plants	CYP3A7	% inhibition	40.03*	Angobon et al., 2010
dibenzylfluorescein	drug	talinum triangulare	food plants	CYP3A7	% inhibition	60.07*	Angobon et al., 2010

* In per cent (%)

Table B16. Summary table on interactions on Pgp for pharmaceuticals

Compound	Compound type	Inhibitor	Inhibitor type	Kinetic parameter	Mean	Value	SD	Reference
saquinavir	drug	-	-	permeability coefficient	8	(x 10-7 (cm/s)	1.95	Eagling et al., 1999
saquinavir	drug	bergamottin	food product	permeability coefficient	8.75	(x 10-7 (cm/s)	1.84	Eagling et al., 1999
saquinavir	drug	quercetin	food product	permeability coefficient	8.04	(x 10-7 (cm/s)	1.25	Eagling et al., 1999
saquinavir	drug	grapefruit	food product	permeability coefficient	26.01	(x 10^{-7} (cm/s)	13.37	Eagling et al., 1999
digoxin	drug	-	-	permeability coefficient	0.29	(x 10^{-6} (cm/s)	0.049	Zhang et al 2003
digoxin	drug	biochanin A	flavonoid	permeability coefficient	0.435	(x 10^{-6} (cm/s)	0.118	Zhang et al 2003
digoxin	drug	silymarin	flavonoid	Ki	30.7	µM	-	Zhang et al 2003
digoxin	drug	biochanin A	flavonoid	Ki	32.7	µM	-	Zhang et al 2003
cyclosporine	drug	-	-	permeability coefficient	7.2	(x 10^{-6} (cm/s)	0.2	Rodriguez-Proteau et al., 2006
cyclosporine	drug	grapefruit	flavonoid	permeability coefficient	6	(x 10^{-6} (cm/s)	0.3	Rodriguez-Proteau et al., 2006
cyclosporine	drug	genistein	flavonoid	permeability coefficient	4.9	(x 10^{-6} (cm/s)	0.1	Rodriguez-Proteau et al., 2006

cyclosporine	drug	quercetin	flavonoid	permeability coefficient	4.4	(x 10^{-6} (cm/s)	0.2	Rodriguez-Proteau et al., 2006
cyclosporine	drug	-	-	permeability coefficient	3.1	(x 10^{-6} (cm/s)	0.1	Rodriguez-Proteau et al., 2006
cyclosporine	drug	xanthohumol	flavonoid	permeability coefficient	0.7	(x 10^{-6} (cm/s)	0.1	Rodriguez-Proteau et al., 2006
digoxin	drug	-	-	permeability coefficient	2.3	(x 10^{-6} (cm/s)	0.1	Rodriguez-Proteau et al., 2006
digoxin	drug	xanthohumol	flavonoid	permeability coefficient	2.5	(x 10^{-6} (cm/s)	0.1	Rodriguez-Proteau et al., 2006

Table B17. Summary table on interactions on Pgp for flavonoids

Compound	Compound type	Inhibitor	Inhibitor type	Kinetic parameter	Mean	Value	SD	Reference
quercetin	flavonoid	-	-	permeability coefficient	4.97	cm/s	0.25	Lan et al., 2008
quercetin	flavonoid	isorhamnetin	flavonoid	permeability coefficient	8	cm/s	0.57	Lan et al., 2008
isorhamnetin	flavonoid	-	-	permeability coefficient	2.28	cm/s	0.09	Lan et al., 2008
isorhamnetin	flavonoid	quercetin	flavonoid	permeability coefficient	4.61	cm/s	0.19	Lan et al., 2008

hesperetin 7-O-glucuronide	flavonoid	-	-	Pgp efflux	1.71	nmol	0.44	Brand et al., 2008
hesperetin 7-O-glucuronide	flavonoid	cyclosporine	drug	Pgp efflux	1.66	nmol	0.43	Brand et al., 2008

Table B18. Summary table on interactions on UGTs for pharmaceuticals

Compound	Compound type	Inhibitor	Inhibitor type	UGT	Kinetic parameter	Mean (μM)	Reference
tamoxifen	drug	diosgenin	plant product	UGT1A4	IC_{50}	3.7	Xu et al., 2016
midazolam	drug	diosgenin	plant product	UGT1A4	IC_{50}	6.8	Xu et al., 2016
olanzapine	drug	diosgenin	plant product	UGT1A4	IC_{50}	0.7	Xu et al., 2016
asenapine	drug	diosgenin	plant product	UGT1A4	IC_{50}	12.7	Xu et al., 2016
trifluoperazine	drug	diosgenin	plant product	UGT1A4	IC_{50}	1.9	Xu et al., 2016
tamoxifen	drug	ruscogenin	plant product	UGT1A4	IC_{50}	0.57	Xu et al., 2016
midazolam	drug	ruscogenin	plant product	UGT1A4	IC_{50}	1.08	Xu et al., 2016
olanzapine	drug	ruscogenin	plant product	UGT1A4	IC_{50}	1.7	Xu et al., 2016
asenapine	drug	ruscogenin	plant product	UGT1A4	IC_{50}	10	Xu et al., 2016
trifluoperazine	drug	ruscogenin	plant product	UGT1A4	IC_{50}	0.2	Xu et al., 2016

tamoxifen	drug	gitogenin	plant product	UGT1A4	IC_{50}	6.13	Xu et al., 2016
midazolam	drug	gitogenin	plant product	UGT1A4	IC_{50}	5.7	Xu et al., 2016
olanzapine	drug	gitogenin	plant product	UGT1A4	IC_{50}	6	Xu et al., 2016
asenapine	drug	gitogenin	plant product	UGT1A4	IC_{50}	22	Xu et al., 2016
trifluoperazine	drug	gitogenin	plant product	UGT1A4	IC_{50}	0.69	Xu et al., 2016
tamoxifen	drug	tigogenin	plant product	UGT1A4	IC_{50}	7.9	Xu et al., 2016
midazolam	drug	tigogenin	plant product	UGT1A4	IC_{50}	4.07	Xu et al., 2016
olanzapine	drug	tigogenin	plant product	UGT1A4	IC_{50}	3.99	Xu et al., 2016
asenapine	drug	tigogenin	plant product	UGT1A4	IC_{50}	15	Xu et al., 2016
trifluoperazine	drug	tigogenin	plant product	UGT1A4	IC_{50}	0.54	Xu et al., 2016
tamoxifen	drug	solasodine	plant product	UGT1A4	IC_{50}	4	Xu et al., 2016
midazolam	drug	solasodine	plant product	UGT1A4	IC_{50}	7.5	Xu et al., 2016
olanzapine	drug	solasodine	plant product	UGT1A4	IC_{50}	7.6	Xu et al., 2016
asenapine	drug	solasodine	plant product	UGT1A4	IC_{50}	17.7	Xu et al., 2016
trifluoperazine	drug	solasodine	plant product	UGT1A4	IC_{50}	4.3	Xu et al., 2016

Table B19. Summary table on interactions on UGTs for flavonoids

Compound	Compound type	Inhibitor	Inhibitor type	Metabolites	Kinetic parameter	Mean (μL/min/mg)	SD	Reference
baicalein	flavonoid	-	-	baicalin	Metabolic clearance	428	-	Li et al., 2012
baicalein	flavonoid	wogonin	flavonoid	baicalin	Metabolic clearance	71	-	Li et al., 2012
wogonin	flavonoid	-	-	wogoniside	Metabolic clearance	945	-	Li et al., 2012
wogonin	flavonoid	baicalein	flavonoid	wogoniside	Metabolic clearance	800	-	Li et al., 2012
oroxylin A	flavonoid	-	-	A-7-O-glucuronide	Metabolic clearance	2274	-	Li et al., 2012
oroxylin A	flavonoid	wogonin	flavonoid	A-7-O-glucuronide	Metabolic clearance	1277	-	Li et al., 2012
baicalein	flavonoid	epicatechin	flavonoid	-	IC50	32.5 (μM)	2.56	Fong et al., 2012
baicalein	flavonoid	-	-	-	Basolateral amount	418.3 (ng)	58.4	Fong et al., 2012
baicalein	flavonoid	epicatechin	flavonoid	-	Basolateral amount	661.6 (ng)	97.3	Fong et al., 2012

Table B20. Summary table on interactions on UGTs for pesticides

Compound	Compound type	Dose (μM)	Inhibitor	Inhibitor type	Dose (mM)	Metabolic clearance (μl/min/cm)	SD	Reference
α-naphthol	pesticide	250	-	-	-	2.5	0.58	Mizuma and Awazu 2004
α-naphthol	pesticide	250	baicalein	flavonoid	1	2.57	0.25	Mizuma and Awazu 2004
α-naphthol	pesticide	250	chrysin	flavonoid	0.5	1.84	0.27	Mizuma and Awazu 2004
α-naphthol	pesticide	250	EGCG	flavonoid	1	2.15	0.2	Mizuma and Awazu 2004
α-naphthol	pesticide	250	epicatechin	flavonoid	1	1.44	0.16	Mizuma and Awazu 2004
α-naphthol	pesticide	250	quercetin	flavonoid	0.5	1.74	0.18	Mizuma and Awazu 2004

Annex C

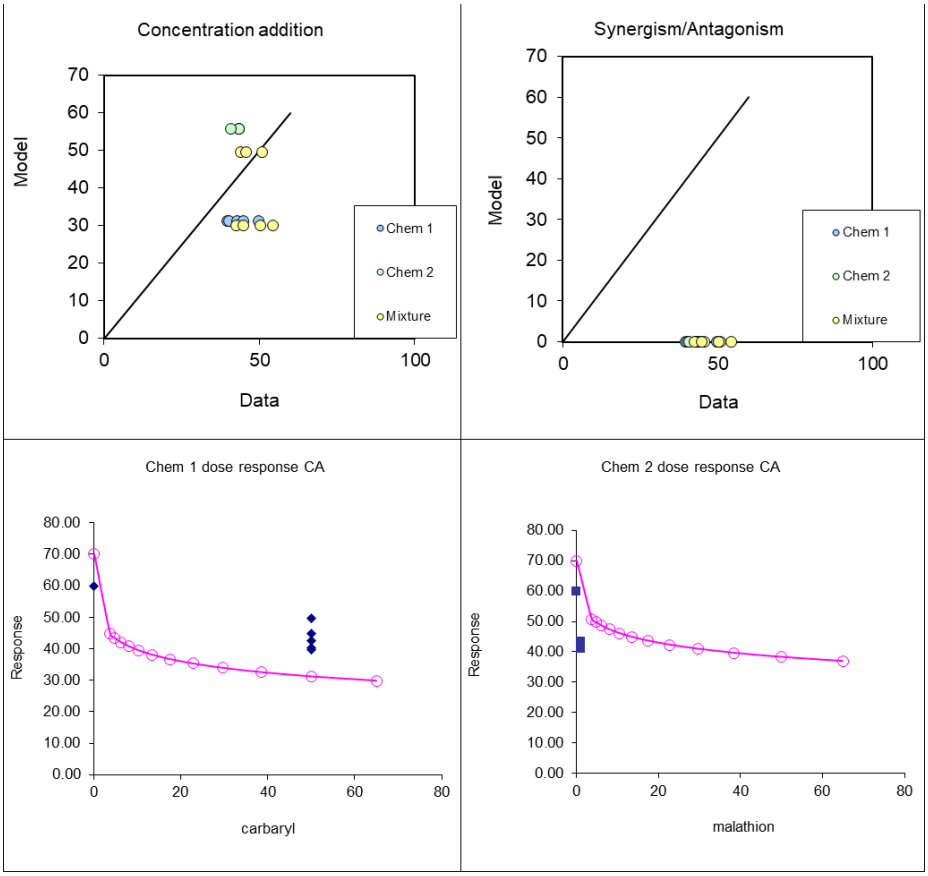

Figure C1. Modelling for carbaryl (chem 1) and malathion (chem 2) using data from Abdel-Raham et al. (1985), with the toxicological endpoint being the systemic enzyme glutathione.

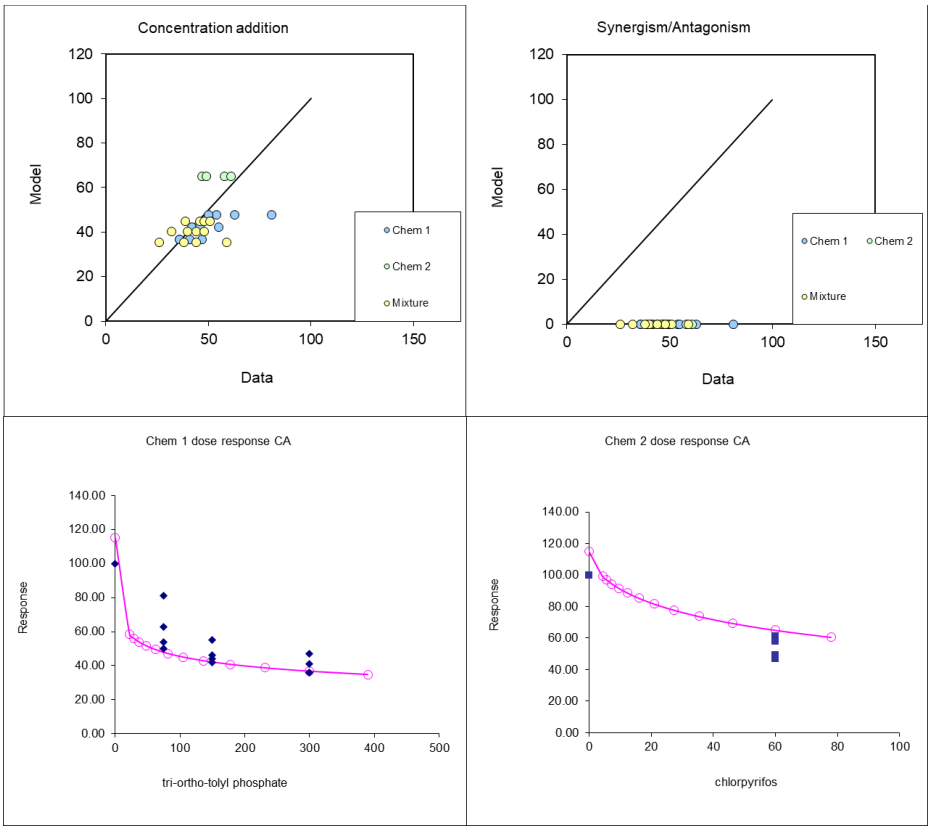

Figure C2. Modelling on tri-ortho-tolyl phosphate (chem 1) and chlorpyrifos (chem 2) using data from Elrich et al. (2004), with the toxicological endpoint being the effect on acetylcholinesterase

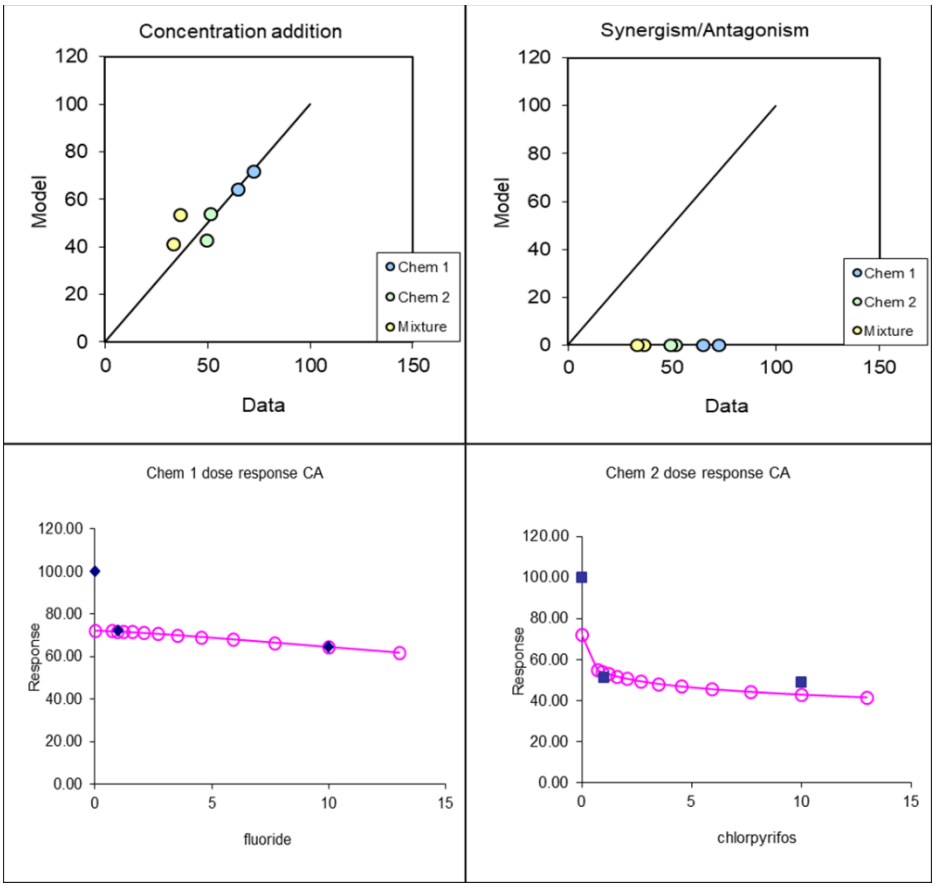

Figure C3. Modelling on fluoride (chem 1) and chlorpyrifos (chem 2) using data from Baba et al. (2013), on Superoxide dismutase enzyme.

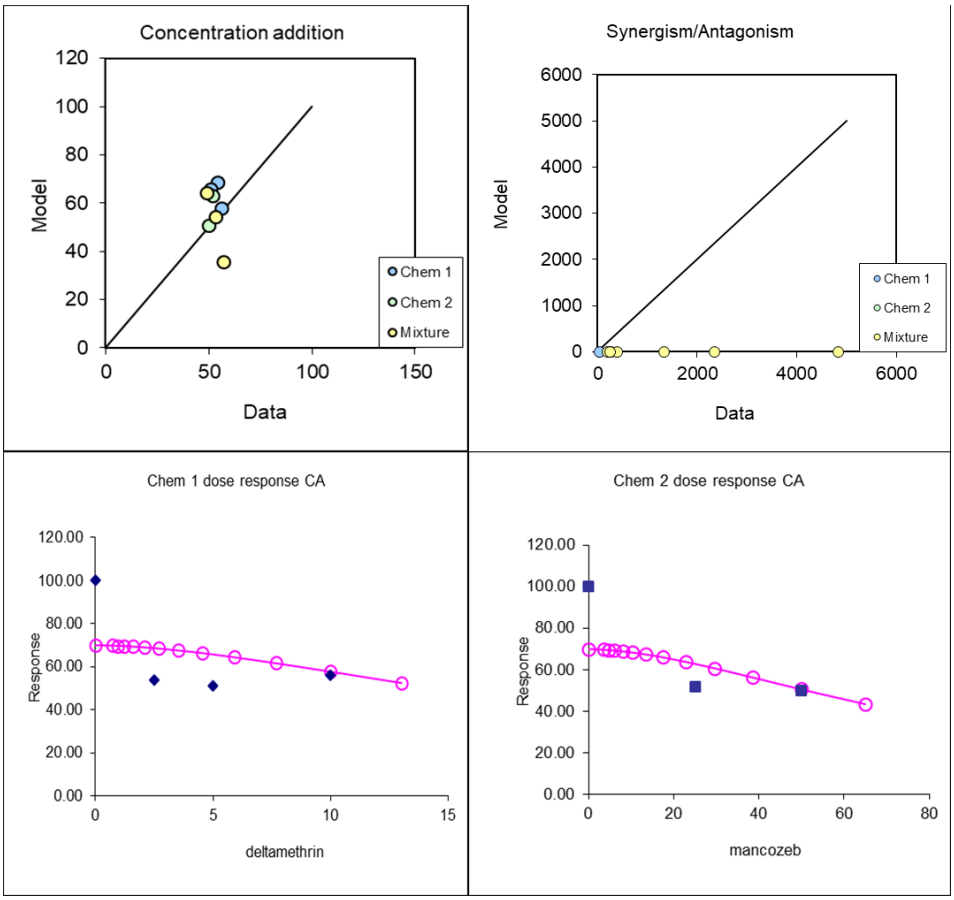

Figure C4. Modelling on deltamethrin (chem 1) and mancozeb (chem 2) mixture on hepatotoxicity (AST), with data originating from Szepvolgyi et al., (1988).

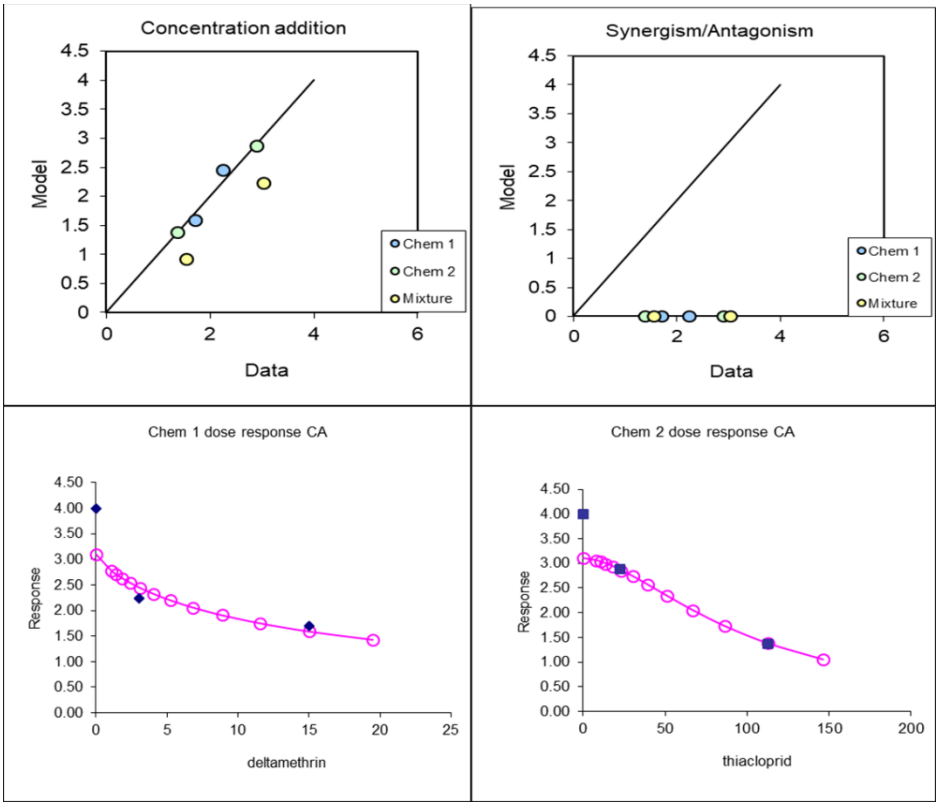

Figure C5. Modelling on the endocrine effect (T3) of a mixture of deltamethrin and thiacloprid pesticides. Data originating from Sekeroglu et al., (2014).

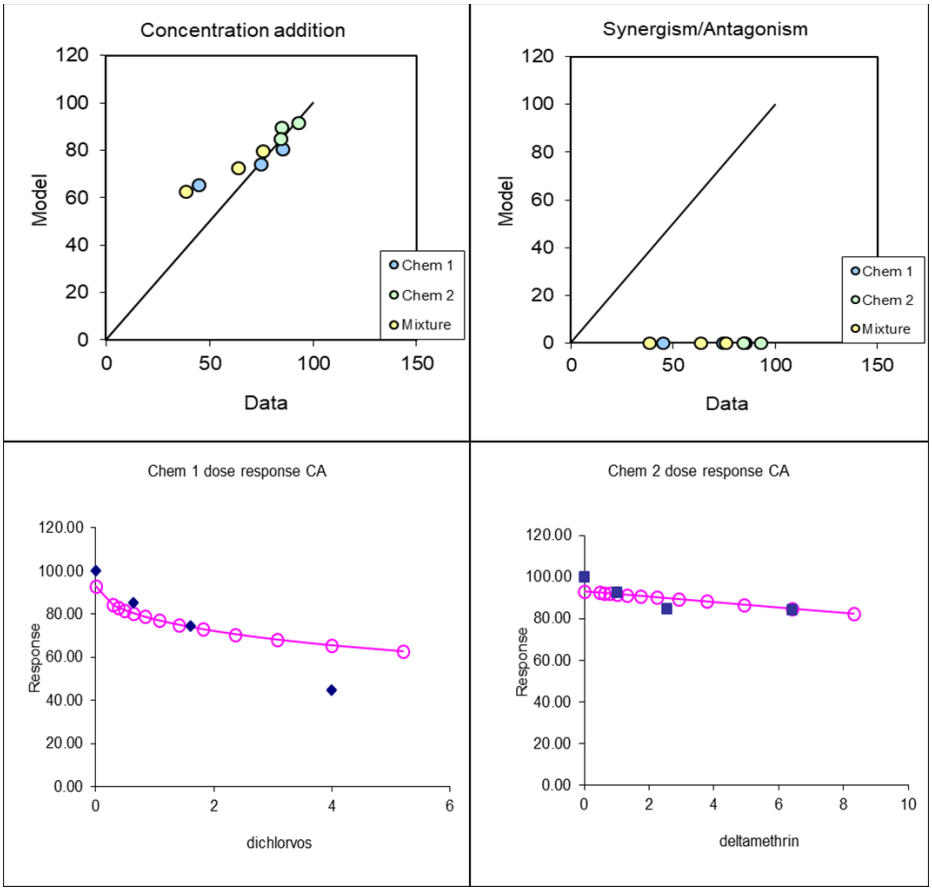

Figure C6. Modelling of the mixture dichlorvos (chem 1) and deltamethrin (chem 2) regarding hepatotoxicity (AST), using the data from Wang et al., (2013).

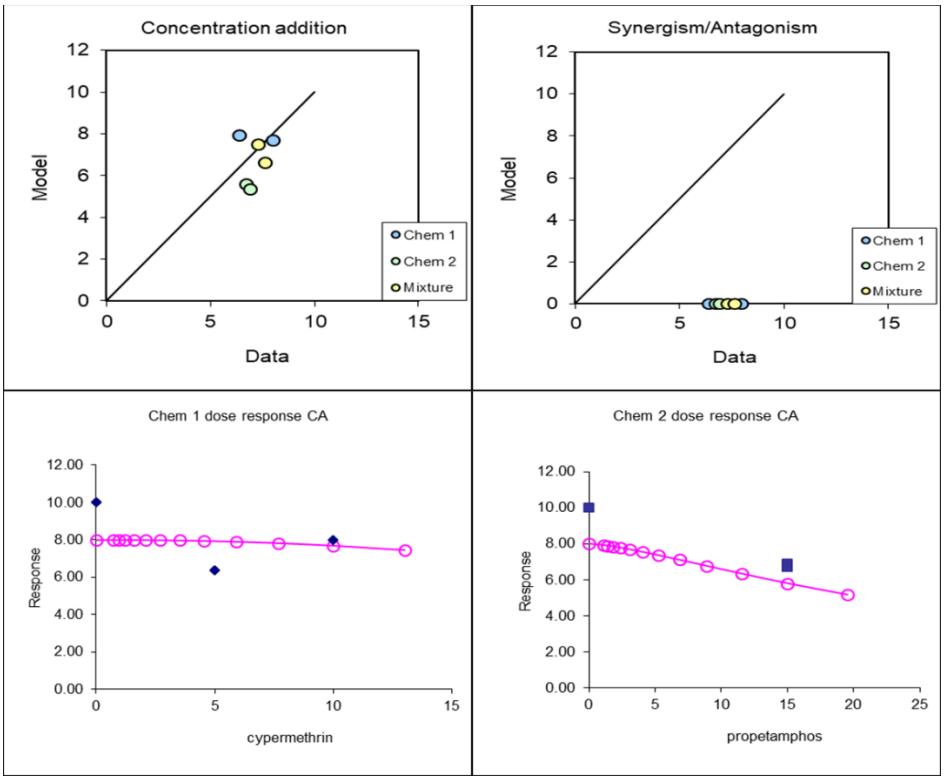

Figure C7. Modelling estimating the systemic enzyme MDA in mice after concurrent exposure to cypermethrin (chem 1) and propetamphos (chem 2) pesticide mixture. Data originating from (Kanbur et al., 2007).

Annex D

Table D1. Database: Ovid MEDLINE(R)

1	Diethylhexyl Phthalate/	2598
2	exp Phthalic Acids/	20296
3	Dibutyl Phthalate/	1095
4	Dicarboxylic Acids/	3577
5	((DEHP or "di 2-ethylhexyl phthalate" or "di 2ethylhexyl phthalate" or "Bis 2-ethylhexyl phthalate" or "Bis 2ethylhexyl phthalate" or "diethylhexyl phthalate" or "dioctyl phthalate" or DINP or "di-iso-nonyl phthalate" or "di-isononyl phthalate" or "diiso-nonyl phthalate" or "diisononyl phthalate" or "Bis7-methyloctyl" or "Bis7methyloctyl" or "benzene-1,2-dicarboxylate" or "benzene1,2-dicarboxylate" or "benzene-12-dicarboxylate" or "benzene-1,2dicarboxylate" or "benzene12-dicarboxylate" or "benzene-12dicarboxylate" or "benzene1,2dicarboxylate" or "benzene12dicarboxylate" or "di-isononyl phthalate" or "di-isononylphthalate" or "diisononyl phthalate" or diisononylphthalate or DEP or "diethyl phthalate" or "phthalic acid diethyl ester" or "Ethyl phthalate" or Anozol or DBP or "dibutyl phthalate" or "Di-n-butyl phthalate" or "Din-butyl phthalate" or "Di-nbutyl phthalate" or "Dinbutyl phthalate" or "Dinbutylphthalate" or "nButyl phthalate" or "Butyl phthalate" or BBP or "butyl-benzyl-phthalate" or "butyl-benzylphthalate" or "butylbenzyl-phthalate" or "butylbenzylphthalate" or BBPHT or "benzyl butyl phthalate" or "benzylbutyl phthalate" or benzylbutylphthalate or DINCH or "1,2-cyclohexane dicarboxylic acid" or "12-cyclohexane dicarboxylic acid" or "1,2cyclohexane dicarboxylic acid" or "12cyclohexane dicarboxylic acid" or "Diisononyl cyclohexane-1,2-dicarboxylate" or "Diisononyl cyclohexane1,2-dicarboxylate" or "Diisononyl cyclohexane-12-dicarboxylate" or "Diisononyl cyclohexane-1,2dicarboxylate" or "Diisononyl cyclohexane12-dicarboxylate" or "Diisononyl cyclohexane-12dicarboxylate" or "Diisononyl cyclohexane1,2dicarboxylate" or "Diisononyl cyclohexane12dicarboxylate" or "1,2-Cyclohexanedicarboxylic acid" or "12-Cyclohexanedicarboxylic acid" or "1,2Cyclohexanedicarboxylic acid" or "12Cyclohexanedicarboxylic acid" or "bis 7-methyloctyl ester" or "bis 7methyloctyl ester" or "Bis 7-methyloctyl Cyclohexane*"	3094

	or "Bis 7methyloctyl Cyclohexane*" or Hexamoll) adj5 (contamination or exposure or level?)).tw,kf	
6	or/1-5	25537
7	exp Food/	1250593
8	exp Cosmetics/	46149
9	(cosmetic? or toiletri* or "hygiene product?" or "hygiene item?" or "personal care product?" or "personalcare product?" or "beauty care product?").tw,kf.	48183
10	(makeup or shampoo or soap or balsam or shower gel or conditioner or lotion or deodorant or moisturizer or wrinkle cream or sunscreen or mouthwash or toothpaste or perfume or cologne or "lip gloss" or lipstick).tw.	25638
11	(bread or cake or potato or vegetable? or fruit? or meat or fish or seafood or shellfish or egg? or milk or dairy or butter or margarine or oil or mayonnaise or sweet? or sugar or beverages or drink? or water or cola or lemonade or coffee or tea or nutmeg or curry or "bay leaf" or "bay leaves" or pepper? or salt or spice or clove? or mustard or ketchup or dill or allspice? or nut? or almond? or walnut? or pecan? or cashew? or hazelnut? or peanut?).tw.	1628360
12	(food or nutrition or meat).tw,kw.	558783
13	or/7-12	2948927
14	6 and 13	4635
15	limit 14 to yr="2008 -Current"	2668
16	remove duplicates from 15	2661

Table D2. Database: Embase

1	"phthalic acid bis(2 ethylhexyl) ester"/	4007
2	phthalic acid/	4448
3	phthalic acid derivative/	3512
4	phthalic acid diethyl ester/	1331
5	phthalic acid benzyl butyl ester/	763
6	dicarboxylic acid derivative/	1297
7	((DEHP or "di 2-ethylhexyl phthalate" or "di 2ethylhexyl phthalate" or "Bis 2-ethylhexyl phthalate" or "Bis 2ethylhexyl phthalate" or "diethylhexyl phthalate" or "dioctyl phthalate" or DINP or "di-iso-nonyl phthalate" or "di-isononyl phthalate" or "diiso-nonyl phthalate" or "diisononyl phthalate" or "Bis7-methyloctyl" or "Bis7methyloctyl" or "benzene-1,2-dicarboxylate" or "benzene1,2-dicarboxylate" or "benzene-12-dicarboxylate" or "benzene-1,2dicarboxylate" or "benzene12-dicarboxylate" or "benzene-12dicarboxylate" or "benzene1,2dicarboxylate" or "benzene12dicarboxylate" or "di-isononyl phthalate" or "di-isononylphthalate" or "diisononyl phthalate" or diisononylphthalate or DEP or "diethyl phthalate" or "phthalic acid diethyl ester" or "Ethyl phthalate" or Anozol or DBP or "dibutyl phthalate" or "Di-n-butyl phthalate" or "Din-butyl phthalate" or "Di-nbutyl phthalate" or "Dinbutyl phthalate" or "Dinbutylphthalate" or "nButyl phthalate" or "Butyl phthalate" or BBP or "butyl-benzyl-phthalate" or "butyl-benzylphthalate" or "butylbenzyl-phthalate" or "butylbenzylphthalate" or BBPHT or "benzyl butyl phthalate" or "benzylbutyl phthalate" or benzylbutylphthalate or DINCH or "1,2-cyclohexane dicarboxylic acid" or "12-cyclohexane dicarboxylic acid" or "1,2cyclohexane dicarboxylic acid" or "12cyclohexane dicarboxylic acid" or "Diisononyl cyclohexane-1,2-dicarboxylate" or "Diisononyl cyclohexane1,2-dicarboxylate" or "Diisononyl cyclohexane-12-dicarboxylate" or "Diisononyl cyclohexane-1,2dicarboxylate" or "Diisononyl cyclohexane12-dicarboxylate" or "Diisononyl cyclohexane-12dicarboxylate" or "Diisononyl cyclohexane1,2dicarboxylate" or "Diisononyl cyclohexane12dicarboxylate" or	4189

	"1,2-Cyclohexanedicarboxylic acid" or "12-Cyclohexanedicarboxylic acid" or "1,2Cyclohexanedicarboxylic acid" or "12Cyclohexanedicarboxylic acid" or "bis 7-methyloctyl ester" or "bis 7methyloctyl ester" or "Bis 7-methyloctyl Cyclohexane*" or "Bis 7methyloctyl Cyclohexane*" or Hexamoll) adj5 (contamination or exposure or level?)).tw,kw.	
8	or/1-7	15030
9	exp food/	968397
10	exp cosmetic/	107533
11	(cosmetic? or toiletri* or "hygiene product?" or "hygiene item?" or "personal care product?" or "personalcare product?" or "beauty care product?").tw,kw.	67238
12	(makeup or shampoo or soap or balsam or shower gel or conditioner or lotion or deodorant or moisturizer or wrinkle cream or sunscreen or mouthwash or toothpaste or perfume or cologne or "lip gloss" or lipstick).tw.	32819
13	(bread or cake or potato or vegetable? or fruit? or meat or fish or seafood or shellfish or egg? or milk or dairy or butter or margarine or oil or mayonnaise or sweet$ or sugar or beverages or drink? or water or cola or lemonade or coffee or tea or nutmeg or curry or "bay leaf" or "bay leaves" or pepper? or salt or spice or clove? or mustard or ketchup or dill or allspice? or nut? or almond? or walnut? or pecan? or cashew? or hazelnut? or peanut?).tw.	1885578
14	(food or nutrition or meat).tw,kw.	705237
15	or/9-14	2896015
16	8 and 15	4258
17	limit 16 to (embase and yr="2008 -Current")	2176
18	remove duplicates from 17	2137

Table D3. Database: Cochrane Database of Systematic Reviews

1	MeSH descriptor: [Diethylhexyl Phthalate] this term only	10
2	MeSH descriptor: [Phthalic Acids] explode all trees	877
3	MeSH descriptor: [Dibutyl Phthalate] this term only	3
4	MeSH descriptor: [Dicarboxylic Acids] explode all trees	1990
5	((DEHP or "di 2-ethylhexyl phthalate" or "di 2ethylhexyl phthalate" or "Bis 2-ethylhexyl phthalate" or "Bis 2ethylhexyl phthalate" or "diethylhexyl phthalate" or "dioctyl phthalate" or DINP or "di-iso-nonyl phthalate" or "di-isononyl phthalate" or "diiso-nonyl phthalate" or "diisononyl phthalate" or "Bis7-methyloctyl" or "Bis7methyloctyl" or "benzene-1,2-dicarboxylate" or "benzene1,2-dicarboxylate" or "benzene-12-dicarboxylate" or "benzene-1,2dicarboxylate" or "benzene12-dicarboxylate" or "benzene-12dicarboxylate" or "benzene1,2dicarboxylate" or "benzene12dicarboxylate" or "di-isononyl phthalate" or "di-isononylphthalate" or "diisononyl phthalate" or diisononylphthalate or DEP or "diethyl phthalate" or "phthalic acid diethyl ester" or "Ethyl phthalate" or Anozol or DBP or "dibutyl phthalate" or "Di-n-butyl phthalate" or "Din-butyl phthalate" or "Di-nbutyl phthalate" or "Dinbutyl phthalate" or "Dinbutylphthalate" or "nButyl phthalate" or "Butyl phthalate" or BBP or "butyl-benzyl-phthalate" or "butyl-benzylphthalate" or "butylbenzyl-phthalate" or "butylbenzylphthalate" OR BBPHT or "benzyl butyl phthalate" or "benzylbutyl phthalate" or benzylbutylphthalate or DINCH or "1,2-cyclohexane dicarboxylic acid" or "12-cyclohexane dicarboxylic acid" or "1,2cyclohexane dicarboxylic acid" or "12cyclohexane dicarboxylic acid" or "Diisononyl cyclohexane-1,2-dicarboxylate" or "Diisononyl cyclohexane1,2-dicarboxylate" or "Diisononyl cyclohexane-12-dicarboxylate" or "Diisononyl cyclohexane-1,2dicarboxylate" or "Diisononyl cyclohexane12-dicarboxylate" or "Diisononyl cyclohexane-12dicarboxylate" or "Diisononyl cyclohexane1,2dicarboxylate" or "Diisononyl cyclohexane12dicarboxylate" or "1,2-Cyclohexanedicarboxylic acid" or "12-Cyclohexanedicarboxylic acid" or "1,2Cyclohexanedicarboxylic acid" or "12Cyclohexanedicarboxylic acid" or "bis 7-methyloctyl ester" or "bis 7methyloctyl ester" or "Bis 7-methyloctyl Cyclohexane*" or "Bis 7methyloctyl Cyclohexane*" or Hexamoll) NEAR/5 (contamination or exposure or level?)):ti,ab	224
6	1 OR 2 OR 3 OR 4 OR 5	3084
7	MeSH descriptor: [Food] explode all trees	31944
8	MeSH descriptor: [Cosmetics] explode all trees	3090
9	(cosmetic? or toiletri* or "hygiene product?" or "hygiene item?" or "personal care product?" or "personalcare product?" or "beauty care Product?"):ti,ab	3619
10	(makeup or shampoo or soap or balsam or "shower gel" or conditioner or lotion or deodorant or moisturizer or "wrinkle cream" or sunscreen or mouthwash or toothpaste or perfume or cologne or "lip gloss" or lipstick):ti,ab	6544
11	(bread or cake or potato or vegetable* or fruit* or meat or fish or seafood or shellfish or egg* or milk or dairy or butter or margarine or oil or mayonnaise or sweet* or sugar or beverages or drink* or water or cola or lemonade or coffee or tea or nutmeg or curry or "bay leaf" or "bay leaves" or pepper* or salt or spice or clove* or mustard or ketchup or dill or allspice* or nut* or almond* or walnut* or pecan* or cashew* or hazelnut* or peanut*):ti,ab	109517
12	(food or nutrition or meat):ti,ab	46358
13	7 OR 8 OR 9 OR 10 OR 11 OR 12	150537
14	6 AND 13 with Cochrane Library publication date from Jan 2008 to Nov 2019	221

Table D4. Database: Web of Science

1	6 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2008-2019*	726
2	5 OR 4 OR 3 OR 2 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	4,666,736
3	TS= ("food" or "nutrition" or "meat") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	915,047
4	TS=("bread" or "cake" or "potato" or "vegetable$" or "fruit$" or "meat" or "fish" or "seafood" or "shellfish" or "egg$" or "milk" or "dairy" or "butter" or "margarine" or "oil"or "mayonnaise" or "sweet$" or "sugar" or "beverages" or "drink$" or "water" or "cola" or "lemonade" or "coffee" or "tea" or "nutmeg" or "curry" or "bay leaf" or "bay leaves" or "pepper" or "salt" or "spice" or "clove" or "mustard" or "ketchup" or "dill" or "allspice$" or "nut$" or "almond$" or "walnut$" or "pecan$" or "cashew$" or "hazelnut$" or "peanut$") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	4,074,645
5	TS=("makeup" or "shampoo" or "soap" or "balsam" or "shower gel" or "conditioner" or "lotion" or "deodorant" or "moisturizer" or " face cream" or "sunscreen" or "mouthwash" or "toothpaste" or "perfume" or "cologne" or "lip gloss" or "lipstick") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	40,659
6	TS=("cosmetic$" or "toiletri*" or "hygiene product$" or "hygiene item$" OR "personal-care product$" or "personal care product$" or "personalcare product$" or "beauty care product$") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	53,889
7	TS(("DEHP" or "di 2-ethylhexyl phthalate" or "di 2ethylhexyl phthalate" or "Bis 2-ethylhexyl phthalate" or "Bis 2ethylhexyl phthalate" or "diethylhexyl phthalate" or "dioctyl phthalate" or "DINP" or "di-iso-nonyl phthalate" or "di-isononyl phthalate" or "diiso-nonyl phthalate" or "diisononyl phthalate" or "Bis7-methyloctyl" or "Bis7methyloctyl" or "benzene-1,2-dicarboxylate" or "benzene1,2-dicarboxylate" or "benzene-12-dicarboxylate" or "benzene-1,2dicarboxylate" or "benzene12-dicarboxylate" or "benzene-12dicarboxylate" or "benzene1,2dicarboxylate" or "benzene12dicarboxylate" or "di-isononyl phthalate" or "di-isononylphthalate" or "diisononyl phthalate" or "diisononylphthalate" or "DEP" or "diethyl phthalate" or "phthalic acid diethyl ester" or "Ethyl phthalate" or "Anozol" or "DBP" or "dibutyl phthalate" or "Di-n-butyl phthalate" or "Din-butyl phthalate" or "Di-nbutyl phthalate" or "Dinbutyl phthalate" or "Dinbutylphthalate" or "nButyl phthalate" or "Butyl phthalate" or "BBP" or "butyl-benzyl-phthalate" or "butyl-benzylphthalate" or "butylbenzyl-phthalate" or "butylbenzylphthalate" or "BBPHT" or "benzyl butyl phthalate" or "benzylbutyl phthalate" or "benzylbutylphthalate" or "DINCH" or "1,2-cyclohexane dicarboxylic acid" or "12-cyclohexane dicarboxylic acid" or "1,2cyclohexane dicarboxylic acid" or "12cyclohexane dicarboxylic acid" or "Diisononyl cyclohexane-1,2-dicarboxylate" or "Diisononyl cyclohexane1,2-dicarboxylate" or "Diisononyl cyclohexane-12-dicarboxylate" or "Diisononyl cyclohexane-1,2dicarboxylate" or "Diisononyl cyclohexane12-dicarboxylate" or "Diisononyl cyclohexane-12dicarboxylate" or "Diisononyl cyclohexane1,2dicarboxylate" or "Diisononyl cyclohexane12dicarboxylate" or "1,2-Cyclohexanedicarboxylic acid" or "12-Cyclohexanedicarboxylic acid" or "1,2Cyclohexanedicarboxylic acid" or "12Cyclohexanedicarboxylic acid" or "bis 7-methyloctyl ester" or "bis 7methyloctyl ester" or "Bis 7-methyloctyl Cyclohexane*" or "Bis 7methyloctyl Cyclohexane" or "Hexamoll") NEAR/5 ("contamination" or "exposure" or "level$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=1987-2019*	

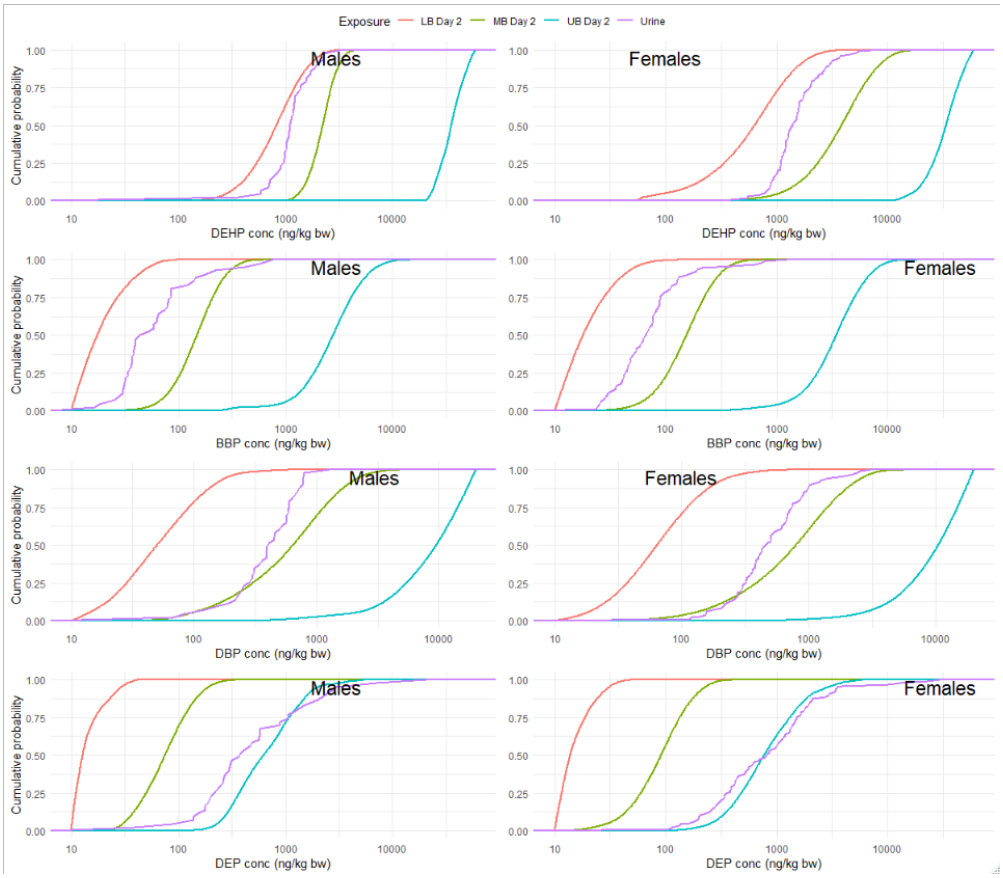

Figure D1. Cumulative phthalate exposure of males and females on day 2

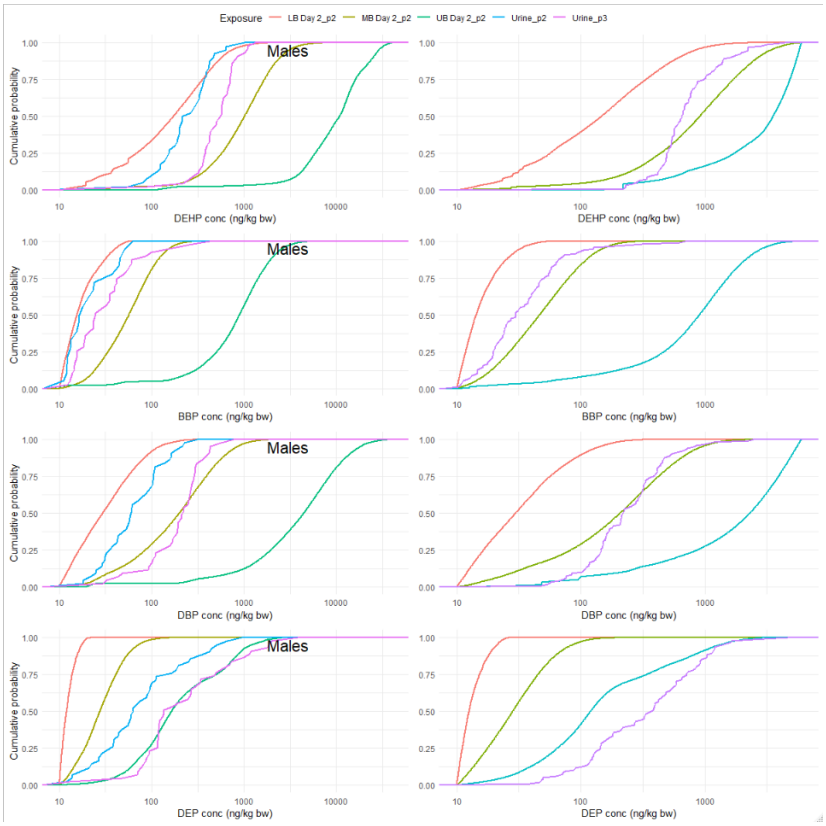

Figure D2. Cumulative phthalate exposure of males and females on pool 2 day 2 with urinary pools 2 and 3

Figure D3. Phthalate contribution from different food groups on day 2

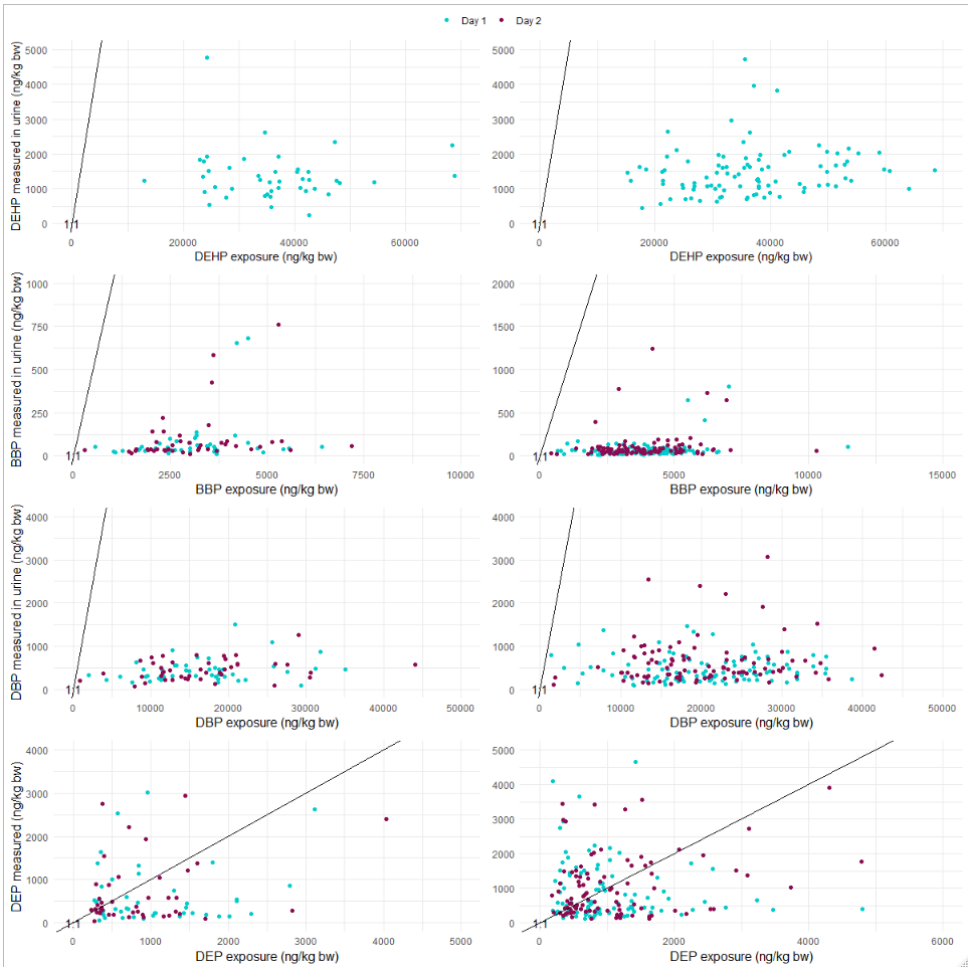

Figure D4. Upper bound individual exposure estimates correlated with measured urine

Graphs including papers with means

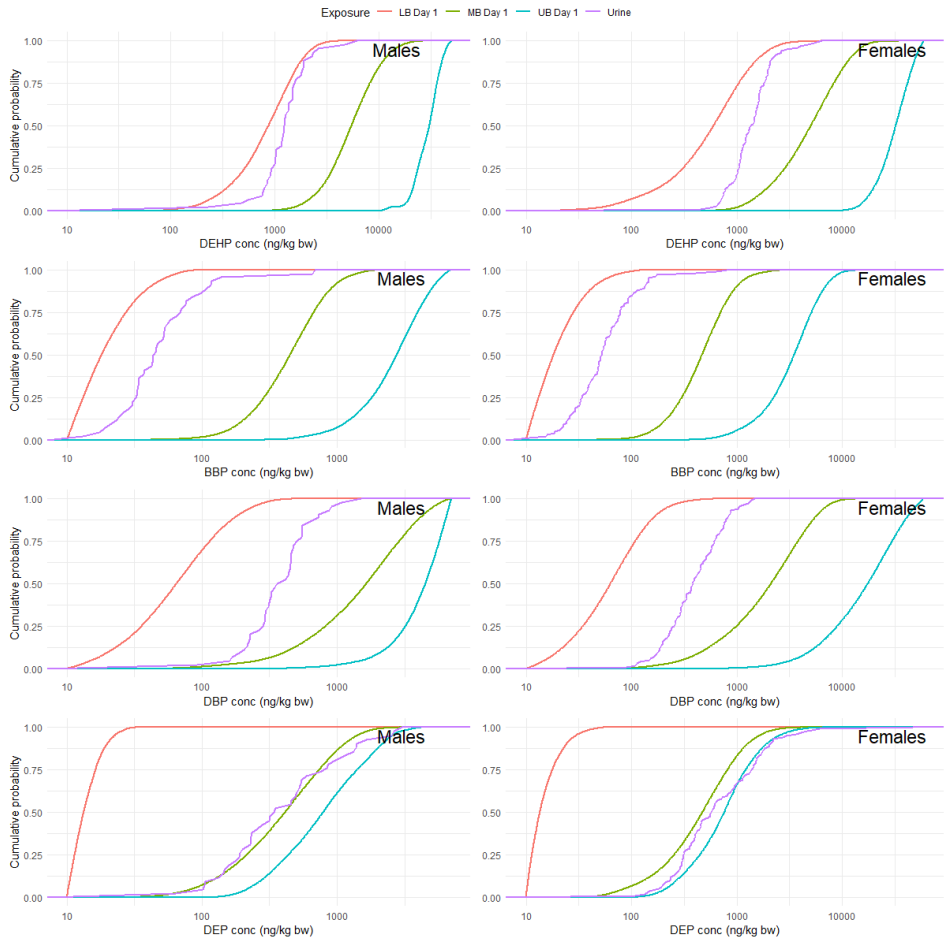

Figure D5. Cumulative phthalate exposure of males and females on day 1 (studies with means are included)

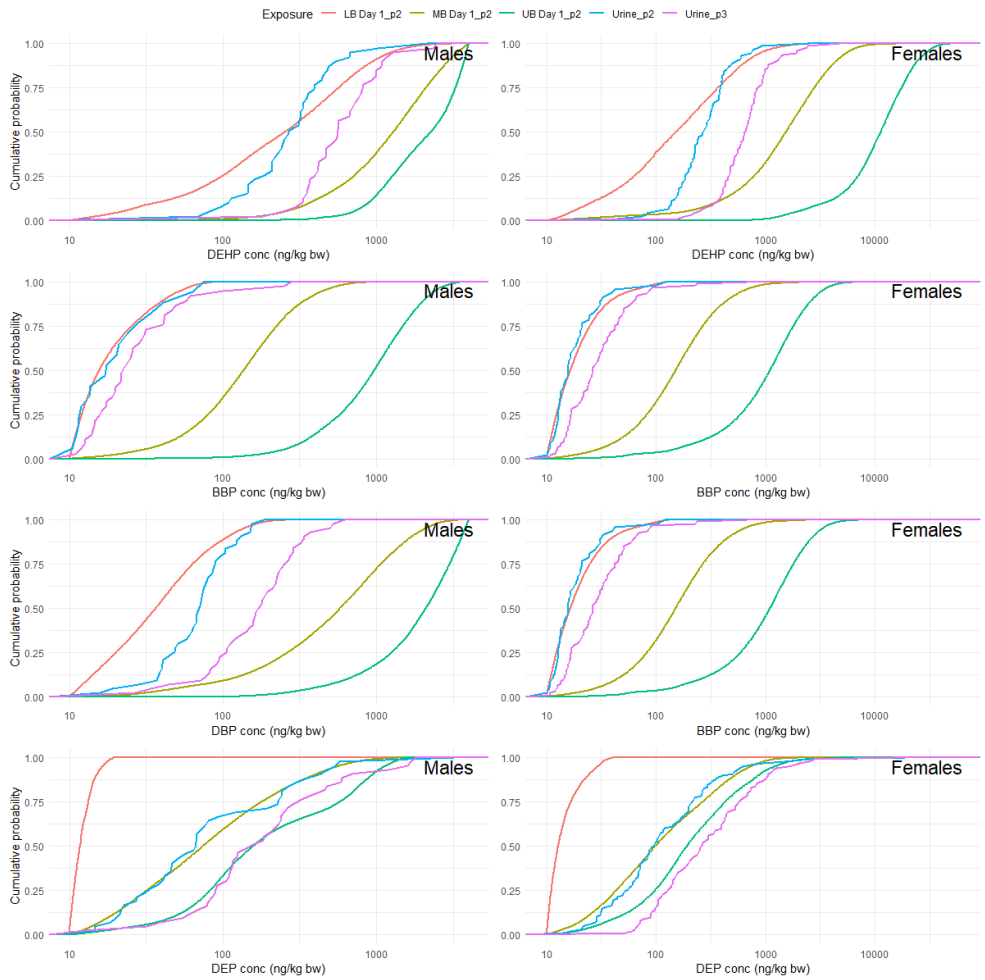

Figure D6. Cumulative phthalate exposure of males and females on pool 2 day 1 with urinary pools 2 and 3 (studies with means are included)

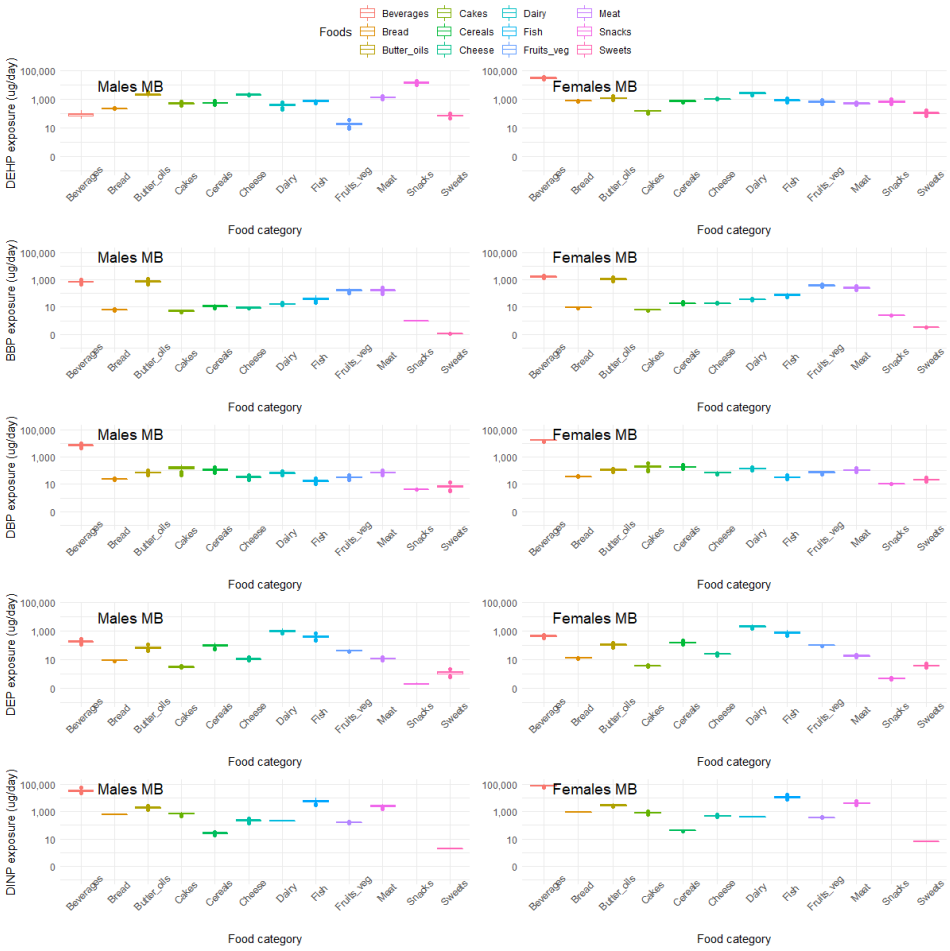

Figure D7. Phthalate contribution from different food groups on day 1 (studies with means are included)

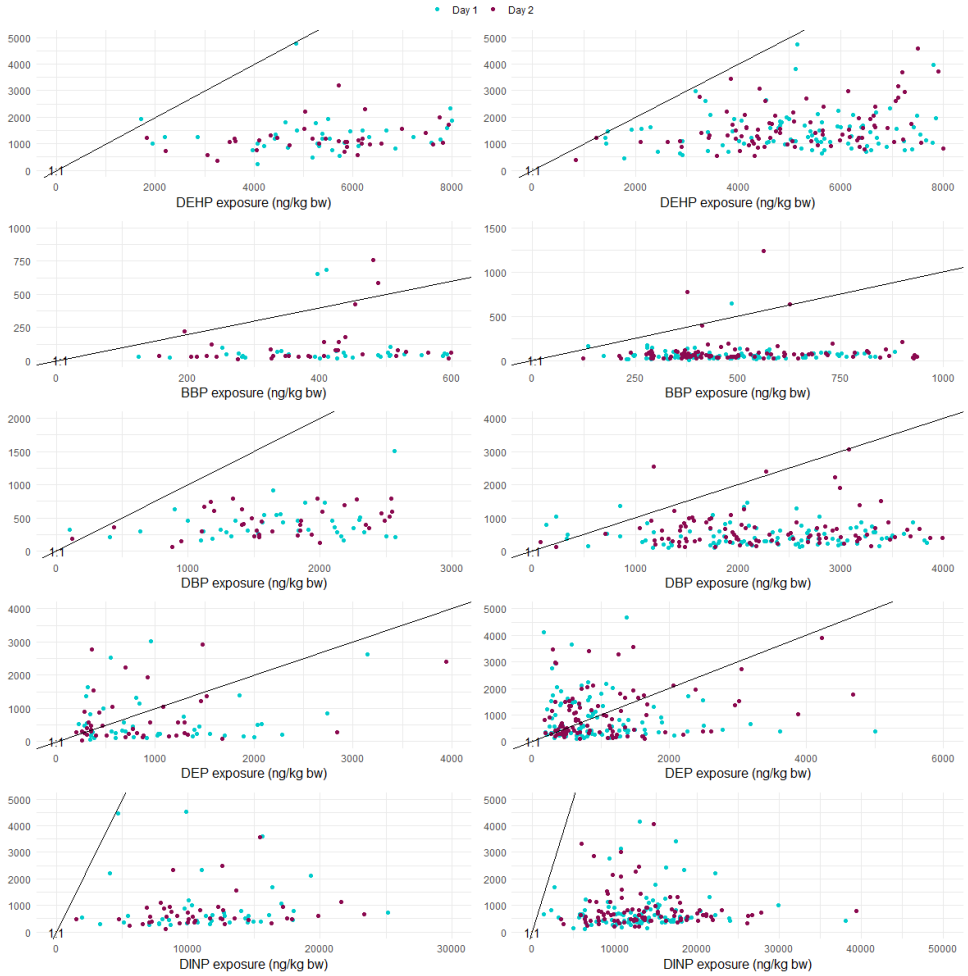

Figure D8. Middle bound individual exposure estimates correlated with measured urine, with the exception of DEP were the UB exposure estimate is presented (studies with means are included)

Curriculum vitae

Athanasios Gkrillas was born on November 17th, 1986 in Marousi, Greece. After finishing his high school studies in his hometown Patras in 2004, he enrolled for a Licentiate Degree in Agronomy, Plant Production and Rural Environment at the University of Thessaly in Volos Greece. After graduation, he completed his military service and relocated to the Netherlands for his MSc studies in Food Quality Management at Wageningen University. For his MSc end project, Athanasios worked under the supervision of Prof. Pieternel A. Luning, on the validation of HACCP in small and medium dairy companies, and as an intern he performed audits on the validation of HACCP in eight dairy companies in the southwest part of Greece. After the completion of his MSc studies (2013), Athanasios was employed by the European Food Safety Authority (EFSA) as a trainee at the unit of Biological Hazards and Contaminants for one year. Once the traineeship ended, he moved to Milton Keynes in the United Kingdom to work in the private sector as a quality control and quality assurance officer at Sensient Flavours and Merck Sharp & Dohme (MSD) respectively until December 2016. Subsequently, in January 2017, Athanasios moved to Parma in Italy and started his PhD in Food Sciences at the University of Parma under the supervision of Prof. Chiara Dall'Asta. His doctoral research aimed to support the development of methodologies for human risk assessment of chemical mixtures in collaboration with EFSA and the Catholic University of Sacred Heart of Piacenza. As part of his PhD, he carried out a short-term visit of eight months to the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in Paris, France, working on the modelling of human variability in toxicokinetics processes and on metabolic interactions of binary mixtures. From September 2019 to August 2020, he worked as a European Food Risk Assessment Fellow at the Norwegian Institute of Public Health (NIPH) as part of the EFSA fellowship for food risk assessment (EU-FORA). At the end of the fellowship, he was offered a position to continue working for NIPH from a different post.

Scientific activity

List of manuscripts

Manuscript 1

Pre- and Postharvest Strategies to Minimize Mycotoxin Contamination in the Rice Food Chain

Gonçalves A., Gkrillas A., Dorne J.L., Dall'Asta C., Palumbo R., Lima N., Battilani P., Venâncio A., Giorni P.

Comprehensive Reviews in Food Science and Food Safety, 2019. 18: 441-454. DOI:10.1111/1541-4337.12420

Manuscript 2

Comparative Toxicokinetics of single and multiple mycotoxins in test and farm animal species: a review

Gkrillas A., Terciolo C., Neves M., Palumbo R., Dorne J.L., Battilani P., Oswald I.P., Dall'Asta C.

Manuscript submitted to Food and Chemical Toxicology

Manuscript 3

Mycotoxins in maize: mitigation actions, with a chain management approach

Palumbo R., Gonçalves A., Gkrillas A., Dall'Asta C., Venâncio A., Logrieco A., Battilani P.

Phytopathologia Mediterranea, 2020. 59(1): 5-28. DOI:10.14601/Phyto-11142

Manuscript 4

The route of mycotoxins in the grape food chain

Gonçalves A., Palumbo R., Guimarães A., Gkrillas A., Dall'Asta C., Dorne J.L., Battilani P., Venâncio A.

American Journal of Oenology and Viticulture, 2020. 71:89-104. DOI: 10.5344/ajev.2019.19039

Manuscript 5

Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach - MYCHIF

Battilani P., Palumbo R., Giorni P., Dall'Asta C., Dellafiora L., Gkrillas A., Toscano P., Crisci A., Brera C., De Santis B, Campbell K., Venâncio A., Gonçalves A., Terciolo C., Oswald I.P.

EFSA Supporting Publications 17(1), EFSA journal, 2020. DOI: 10.2903/sp.efsa.2020.EN-1757

Manuscript 6

Exposure estimates of phthalates from foods and comparison with biomonitoring data in 24-hour urine for two non-consecutive days using data from the Norwegian biomonitoring study EuroMix.

Gkrillas A., Dirven H., Papadopoulou E., Andreassen M., Hjertholm H., Husøy T.

Manuscript pending submission

Fellowship

EU-FORA – The European Food Risk Assessment Fellowship Programme

2nd of September 2019 – 31st of August 2020

Workshops

EuroMix stakeholder training on Monte Carlo risk assessment, EFSA, Parma, 21st of February 2017

XXII Workshop on the Developments in the Italian PhD Research, Bolzano, 20th – 22nd of September 2017

Joint seminar UNIPR/Wageningen, Department of Food and Drug, UNIPR, Parma, 15th of March 2017

Quadram institute visit, UNIPR, Parma, 3rd – 4th of September 2018

ILSI food contaminants workshop, UNIPR, Parma, 18th of September 2018

Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach – MYCHIF, EFSA, Parma, 20th of September 2019

Courses

"R introduction", Hans Peter Stüger, Reinhard Fuchs, Austrian Agency for Health and Food Safety, EFSA, Parma, February 2017

"Inference statistics", Hans Peter Stüger, Antonia Griesbacher, Austrian Agency for Health and Food Safety, EFSA, Parma, January 2017

"Generalised Linear Models", Hans Peter Stüger, Lisa Stadlmüller, Austrian Agency for Health and Food Safety, EFSA, Parma, April 2017

"Statistics in food science and nutrition", Prof. Palla, Department of Food and Drug, UNIPR, Parma, May 2017

"Scientific Communication for PhD students", Prof. Bettini and Prof. Lodola, Department of Food and Drug, UNIPR, Parma, 29th of September 2017

"Fundamentals in food toxicology", Prof. Marko, University of Vienna, UNIPR, Parma, 6th -8th of March 2018

"Genetics of taste & Food Preferences", Prof. Gasparini, University of Trieste, UNIPR, Parma, 3rd of May 2018

"EU-FORA Induction training"

EFSA, Parma, 2nd – 20th of September 2019

"EU-FORA Module 1 training"

Arcotel Kaiserwasser, Vienna, 25th – 29th of November 2020

"EU-FORA Module 2 training"

Online course, 10th – 14th of August 2020

"EU-FORA Module 3 training"

Online course, 24th – 31st of August 2020

Poster presentations

"Development of harmonised methodologies for human and ecological risk assessment of multiple chemicals to support risk managers"
Athanasios Gkrillas
XXII Workshop on the Developments in the Italian PhD Research, Bolzano, 20th – 22nd of September 2017

"Meta-analysis of combined toxicity data for human and ecological risk assessment of multiple chemicals: Case studies to support methodological development"
Athanasios Gkrillas, Edoardo Carnesecchi, Maryam Zare Jeddi, Chiara Dall'Asta and Jean-Lou Dorne
Recent advances in food analysis (RAFA), Prague, 7th – 10th of November 2017

"Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach – MYCHIF"
Athanasios Gkrillas, Roberta Palumbo, Carlo Brera, Katrine Campbell, Armando Venancio, Isabelle P. Oswald, Piero Toscano, Jean Lou Dorne, Paola Battilani and Chiara Dall'Asta
World mycotoxin Forum (WMF), Amsterdam, 12th – 14th of March 2018

Oral presentations

"Identification and characterisation of emerging risks in the food production chain"
Athanasios Gkrillas
Quadram institute visit, UNIPR, Parma, 3rd – 4th of September 2018

"Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach – MYCHIF – Case study 3 – Toxicokinetics in chickens"
Athanasios Gkrillas, Luca Dellafiora and Chiara Dall'Asta
MYCHIF Workshop, EFSA, Parma, 20th of September 2019

Conferences and summer schools

- ILSI Europe Webinar on Mycotoxin Mitigation, 29th of November 2016
- Biocontrollo di Aspergillus flavus e gestione di filiera, un futuro per maiscoltura e "latte italiano di qualità", Piacenza, 9th of February 2017
- Food Integrity conference (Barilla), Parma, 10th – 11th of May 2017
- Metabolomics Seminar, Parma (Italy), Agilent, UNIPR, Parma, 16th of March 2017
- Joint Research Centre (JRC) Summer School on Alternative Approaches for Risk assessment, Ispra, 16th – 19th of May 2017
- Recent advances in food analysis (RAFA), Prague, 7th – 10th of November 2017
- World mycotoxin Forum (WMF), Amsterdam, 12th – 14th of March 2018
- UNIPR Summer School on "emerging risks", EFSA, Parma, 15th – 17th of May 2018
- European Food Safety Authority (EFSA) conference, Parma, 18th – 21st of September 2018
- Norwegian society for Pharmacology and Toxicology, Beitostølen, 23rd – 26th of January 2020