Food Contaminants and Human Health

Challenges in chemical mixtures

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Table of contents

Foreword 5

General Introduction 7

1. Analysis of multiple contaminants in food 9

1.1. Food contaminants and human health - challenges in analysis of multiple chemicals 11

1.2. Trends in analytical methods 17

1.3. Occurrence of food contaminants 27

2. Risk assessment of multiple contaminants in food 55

2.1. Multiple biomarker approach of mycotoxins and its contribution for the exposure assessment 57

2.2. OPEN platform for clinical nutrition - an online dietary assessment 63

2.3. Multifaceted approaches in risk assessment 67

3. Advances in bioaccessibility and toxicology of multiple contaminants in food 79

3.1. An overview of the models for simulating food digestion 81

3.2. *In vitro* tools for bioaccessibility studies 85

3.3. Toxicological interactions between mycotoxins 94

3.4. Predicting the toxicity of multiple chemicals by using conceptual mathematical models 99

3.5. Insights into individual and combined toxic effects 106

4. Screening and mitigation strategies for multiple contaminants in food 121

Concluding remarks 135

Foreword

Food safety is a critical component for sustainable development. Safer food contributes to less illness, and hence increased productivity and improved livelihood. Contaminants may however be present in food and their presence could promote several health threats. Chemical food contamination may cause non-communicable diseases, in particular cancer, and can also affect reproductive health and the immune system.

In recent years, there has been increasing recognition that humans are exposed to a multitude of contaminants and this exposure can have different health effects. Risk assessment of combined Human exposure to multiple chemicals (chemical mixtures) poses several challenges to scientists, risk assessors and risk managers, particularly the complexity of the terminology and problem formulation, the diversity of chemical entities, and the toxicological profiles and exposure patterns in test species and humans. The increasing complexity of questions in food safety now requires more innovative approaches to better prioritize the risks, taking into account the overall knowledge and allowing a rapid integration of new scientific developments.

In order to discuss and share knowledge on emerging issues such as the combined effects of multiple chemicals on human health, the National Institute of Health Doutor Ricardo Jorge (INSA) held on 13th and 14th April, in Lisbon, within the MYCOMIX project, an INSA scientific research project, the international conference "ICFC2015 - International Conference on Food Contaminants: challenges in chemical mixtures". Promoting events such as this one allows INSA to achieve its mission, contributing to the progress of food science and human health.

This book compiles the extended abstracts from some of the world-renowned researchers invited to give a lecture at the ICFC2015, in addition to the abstracts from oral and poster sessions. It is our hope that this publication could constitute a useful tool for your scientific research.

Fernando de Almeida

Chairman of the Executive Board of the National Institute of Health Doutor Ricardo Jorge

General Introduction

Chemicals from anthropogenic (persistent organic pollutants, maillard reaction products, phthalates, pharmaceuticals and pesticides residues, food additives) and natural (heavy metals, metalloids, marine biotoxins, mycotoxins) origins may be present in food either as undesirable contaminants or diet components. The presence of chemical contaminants in food is often unavoidable as it is the human exposure to contaminants. Therefore, it is crucial to develop effective exposure and risk assessment strategies in order to protect public health.

Quantitative exposure assessment is a methodology developed to analyze scientific information in order to evaluate the severity and probability of an adverse event. Risk exposure assessment can be used to estimate human exposure to chemical contaminants through the consumption of food and therefore to provide a link between possible hazards in the food chain and the risks reflected to human health. Risk assessment results may also offer the scientific grounds for risk management decisions and options. Two of the most important parameters affecting the risk assessment are the amount of food consumed during a specific period of time and the chemical contaminant concentration. As far as the evaluation of the toxicological properties of the chemical mixtures in foods is concerned, detailed information on the composition of the mixture and the mechanism of action of each specific chemical are required. Compared to the assessment of individual substances, additional complexity is introduced when the risk assessment concerns mixtures of substances.

Combined toxicity of chemicals is very hard to predict because it is influenced by several factors, including their chemistry, toxicokinetics (including bioaccessibility, given that only bioaccessible contaminants will be available for intestinal absortion), toxicodynamics and mechanism of action, as well as by aspects related to the experimental design, endpoints analysed and applied statistics. The development of an interdisciplinary work is crucial and should involve several scientific areas such as analytical methodologies to perform the analysis of multiple contaminants in food, exposure and risk assessment models developed for chemical mixtures as well as methodologies determining the bioacessibility and toxicity interactions of multiple contaminants.

MYCOMIX titled “Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact” (PTDC/DTP-FTO/0417/2012), is a two years national project (2013-15) funded by the Portuguese Foundation for Science and Technology (FCT). This project aims to

explore, for the first time, the toxic effects of multiple mycotoxins in infant food and its potential health impact promoting the development of important scientific areas such as the analytical methods, risk assessment, bioaccessibility and toxicology of multiple contaminants in food. These research domains had inspired the layout of “ICFC2015 - International Conference on Food Contaminants: challenges in chemical mixtures”, an international meeting held in April 2015, in Lisbon.

This book is organized in four chapters. The first chapter concerns an overview on the challenges related to the analysis of multiple contaminants and human health and reports new trends in analytical methods and occurrence data on multiple contaminants co-occurring in food, both crucial to perform exposure assessment. The second chapter concerns the risk assessment of multiple food contaminants including an overview on multibiomarkers approach and web-based dietary assessment tools and their contribution to exposure assessment studies. It also includes a set of multifacetated approaches used in risk assessment projects and studies. The third chapter reports the advances in bioaccessibility and toxicology of multiple contaminants in food and includes an overview on models for simulating food digestion (with reports using in vitro tools for bioaccessibility studies), toxicological interactions between food contaminants and preditive models used to analyse these interative effects (including a set of insights individual and combined toxic effects). Finally, a fourth and last chapter refers to screening and mitigation strategies to reduce hazard promoted by the presence of multiple contaminants in food.

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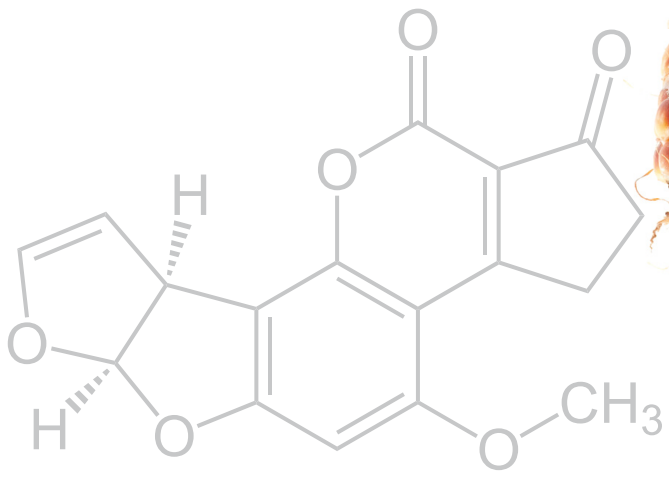
Analysis of multiple contaminants in food



1.1. Food contaminants and human health - challenges in analysis of multiple chemicals

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Abstract

The main aim of monitoring for food and feed contaminants is to provide evidence that food and feed is safe for the consumer and products meet legislative provisions. Often natural toxins, such as mycotoxins, co-occur in regulated product categories; therefore the determination of only a few does not always allow a reliable classification of a product.

In the case of pyrrolizidine alkaloids (PA), which are candidates for regulation, reliable monitoring seems an even more complex challenge. Next to the estimated +6000 plant species worldwide that produce these toxins, at least 600 different PA have been identified.

One strategy to solve the complexity of this task is to develop methods, which in the case of mycotoxins must be able to determine a rather wide class of substances ranging from rather polar to unpolar nature in one go or, such in the case of PA, to identify a reasonable number of markers or a sum parameter that might be used for a fit-for-purpose classification of commodities.

This paper will give, on the basis of practical examples, insight on the complexity to identify suitable analytical methods that need to consider the natural occurrence of myco- and plant toxins while taking into account the need for a good judgement on the safety of food and feed based on the results provided by the analytical method used.

Introduction

Mycotoxins are products of fungal secondary metabolism. Over 400 mycotoxins are known nowadays of which aflatoxins, trichothecenes, fumonisins, ochratoxin A, zearalenone and *Alternaria* toxins are the main representatives [1]. They can display a range of severe toxic effects in humans and animals. Aflatoxin B_1 (AFB_1) is the most potent natural carcinogen in animal experiments (rats), ochratoxin A (OTA) is nephrotoxic, fumonisins B_1 and B_2 (FB_1 and FB_2) exhibit neuro- or hepatotoxicity and carcinogenicity depending on the target species affected, deoxynivalenol (DON) shows immunotoxic effects, zearalenone (ZON) is an endocrine disruptor

binding to the oestrogen receptors; and T-2 and HT-2 toxins inhibit protein synthesis and are highly haematotoxic [2]. Nowadays, the issue of co-occurrence and combined toxicity is gaining increased relevance. Exposure to several classes of mycotoxins often results in an additive effect, not excluding also a possible synergistic interaction [3].

The first harmonized legislation at EU level was published in 1998 and established maximum limits for AFB_1 and total aflatoxins in several food commodities [4]. Since then, the range of mycotoxins and food categories covered by the legislation expanded considerably: AFs are regulated in 17 food categories, OTA in 12, patulin in 5, DON in 9, ZON in 10 and fumonisins in 6. Furthermore, this number is expected to increase in the coming years as modified mycotoxins (including masked/conjugated toxins and modified during processing) as well as new mycotoxins and plant toxins must gain the attention of regulators as potential health risks. Examples of potential candidates are Alternaria toxins, beauvericin/enniatins, ergot alkaloids, citrinin, DON conjugates, among others.

For a sound assessment of food and feed safety the full spectra of relevant analytes must be monitored.

The proposal of the scientists to face this challenge goes on the direction of developing multi-analyte methods combining a generic sample preparation protocol with a highly selective method exhibiting sufficient detection capacity, such as LC-MS [5,6].

The general insight into the contamination scenario depends very much on the analytical capabilities of the methods employed, namely in terms of limits of detection (LODs) and quantification (LOQs). Reported LOQs from different laboratories using comparable methods can vary an order of magnitude, as it was found in the determination of T-2 and HT-2 in cereals [7], or even two orders of magnitude in some extreme scenarios. As a result, efforts devoted to evaluate food contaminants by using various sources of monitoring results have shown to face a great proportion of results left-censored (<LOQ, e.g. 87-100% for Alternaria toxins, 65% for T-2 and HT-2) [7], therefore minimum detection capabilities need to be established in order to conduct meaningful surveys.

Examples of multi-analyte scenarios

The requirement of sufficient low detection capability have already been taken in consideration for efforts drafting a CEN standard method for the determination of regulated mycotoxins in feed and compound feed (CF) using a single LC-MS method. As a result, the aimed LOQs are 2.5 to 10-fold lower than the lowest regulated limit, while an additional challenge derives from

the fact that for some mycotoxins the working range of the method has to span 1 order of magnitude (AFB_1 and OTA) whereas for others it should span more than 2 orders of magnitude, with a superior limit at the mg kg^{-1} level. This is due to the highest regulated level in some feed categories, which must also be reliably monitored. As a result, effective methods allowing measurements starting at the aimed LOQ are privileged while an additional challenge is the linearity of the method proved by appropriate calibration over the whole working range, preferably combined in a single procedure.

In fact, the outcome of a proficiency test run by the EURL-mycotoxins in 2013 demonstrated that this can be a challenge given the fact that most laboratories monitored fumonisins with a focus on a range lower than 60 mg kg^{-1} which is the highest recommended level in the legislation [8]. The background for this challenge is that methods can include a methodological step that has limitations on the maximum level that can be accurately determined (methodology dependence of the results). A benefit might derive from "dilute & shoot" approaches for which the risk of limiting method steps is low, which however in return have other disadvantages as discussed later.

As a matter of fact, should the user not be aware of such limitations, the phenomenon can run simply unnoticed and nothing but a fraction of the target analyte is reported.

Despite sample preparation is a main source of such limiting effects, it can provide several benefits:

– obtain a cleaner extract reducing matrix effects in LC-MS analysis,
– concentrate the analyte prior chromatographic separation, therefore providing lower LOQs,
– protect the analytical column and ESI source from dirt co-extractives.

Nonetheless, as sample preparation is a source of considerable restrains, as discussed above, and accounts for about 2/3 of the analytical effort, it is worth to skip if not strictly required.

Use of IACs (immunoaffinity columns) is very popular in mycotoxin analysis. In an experiment it was demonstrated that the chromatographic resolution can be improved and preserved, if chromatographic separation is preceded by an IAC clean-up, especially for polar analytes as deoxynivalenol-3-glucoside. Based on these findings, an elegant method was developed for the determination of DON, 3-acetylDON, 15-acetylDON and DON-3-G in cereals, taking advantage of the cross-reactivity of an IAC for DON and its conjugates. The IAC clean-up was coupled with a hot water desorption and fluorescence detection [9] and is therefore a valid alternative to commonly used LC-MS methods for the purpose of monitoring DON and its conjugates.

Recent trends, supported by the development of sensitive mass selective detectors, focus towards the simplification of sample preparation with the aim to cover as many analytes as possible within a single sample preparation step [5]. Nevertheless, complex samples such as CF might benefit from sample preparation in the form of tailored clean-up procedures if certain aspects, such as detection capability, are an asset. As a matter of fact, LOQs up to 10-fold lower were obtained when sample clean-up was performed prior detection in one of our studies with rather little effort. Furthermore, suppliers of IACs have recently placed products on the market that support the multi-analyte capabilities of LC-MS methods allowing to monitor 8 regulated mycotoxins known to be found in cereals, yet allowing the determination of these mycotoxins in CF at levels 10-fold lower. This can be a viable option for risk assessment surveys where low LOQs are envisaged or when monitoring is carried out with lesser sensitive past generation LC-MS systems that are still in use.

As the number of relevant mycotoxins is expanding, new challenges in their analysis (in a single run) come from wider span in physico-chemical properties, either polarity or pKa. This is, indeed, the case of *Alternaria* toxins, which range from fairly neutral and apolar to very acidic compounds. The same can be mentioned for the modified toxins (e.g. DON conjugates) which are typically more polar than the parent substance. Therefore, sample preparation and analysis protocols must be developed which can cope with the character of the analytes.

The analysis of pyrrolizidine alkaloids (PA) is another challenge for the analyst, as more than 600 PA are known. The full PA characterization seems an overwhelming job not fit for purpose. Besides, it has been demonstrated in a proficiency test carried out in 2012 [10] that methods measuring the "total PA" by means of the necine base backbone found in PA (after reduction with LiAlH4) give higher values than methods attempting to determine relevant PA as single. The proficiency test organisers expressed the opinion that single analyte focussed approaches for PA must question if the selected single PA parameters meet the spectrum of present PA adequately. This assumption was recently underpinned in a study by These *et al.* [11] who reported that the number of PA in samples is likely to be higher than previously concluded with single parameter methods. Therefore, it seems worth to undertake efforts to identify representative/marker compounds which give a good estimation of the PA content or use grouped parameters as originally concluded by Kempf *et al*. [12].

Likewise, the EU legislation enforces the determination of AFB1 and the sum of four aflatoxins in several food commodities. On the basis of a collection of 1019 results taken from the RASFF database, where both parameters were determined in peanuts, it was shown that only

a rather small fraction of samples were compliant based on AFB1 but not compliant considering the sum of four AFs. Indeed, in a number of samples the sum of all four AFs was more than twice the value of AFB_1, but in the majority of cases both parameters, AFB_1 and the sum, exceeded the provisions and would have led to the rejection of the product in any case. This let us question if analytical efforts to determine all four aflatoxins, including all aspects, such as calibrant stability, accreditation, etc. are justified. Protection of consumer's health must be achieved this way or alternatives should at least be discussed? Saying this, it does not hamper that surveillance studies continue to encompass all the aflatoxin congeners to gather further knowledge.

While we have been discussing quantification issues arriving from the simultaneous determination of mycotoxins in complex food and feed samples, identification issues are very much lacking harmonization for this specific field. To tackle this issue a committee composed by experts from National Reference Laboratories for mycotoxins is working on a guidance document concerning the "Identification criteria for determination of mycotoxins in food and feed". Also, a presentation on the topic was recorded at the 2014 Integration and Enlargement workshop in Zagreb which is online available via the website of the EURL for mycotoxins [13].

Conclusions

As the number of known fungal and plant toxins continue to grow, the analytical load of control laboratories grows as well. Therefore evidence-based and fit-for-purpose strategies must be communicated to legislators allowing appropriate legislative enforcement to the control laboratories. The analytical equipment available and knowledge on effective sample preparation nowadays offers the possibility of multi-analyte screening and quantification. The analytical capability has in general improved leading to the possibility of determining simultaneously more toxins than in the past. However, proficiency tests in combination with questionnaires on methodological data and multiple samples also stressing the method scope, as carried out by the Institute for Reference Materials and Measurements (JRC-IRMM), have shown to be an invaluable asset improving the laboratories competence in this field.

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1.2. Trends in analytical methods

1.2.1. The new era of marine biotoxins analysis

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Marine biotoxins are natural contaminants of the marine environment produced by several phytoplankton species. Significant efforts have been devoted to investigate the presence of these toxins, as well as to isolate them, to evaluate their toxicity to establish limits for their regulation.

The analytical methods traditionally used for the analysis of marine biotoxins involved the use of mouse bioassays, which have been a very useful tool in the absence of other analytical alternatives. These mouse bioassays have been also a valuable tool for toxicological purposes. The development of analytical tools alternative to the mouse bioassays has been one of the main focus of scientists working in this field over the last few years and as a result of the work carried out in this area, new analytical methods have been proposed. The transition from mouse to chemistry has been one of the great advances in the marine biotoxins field, this has been an important challenge, mainly because of the limited standards and reference materials available, nevertheless significant achievements have been reached also in this area and all these efforts prompted to the accomplishment of the important goal of replacing the mouse bioassays by alternative methods and in particular by chemical methods. A very important step on this transition has been the recent replacement of the mouse bioassay by liquid chromatography coupled to tándem mass spectrometry, as the reference method to control lipophilic toxins in the European Union. Alternative methods have been also included in the EU Legislation for the control of paralytic shellfish toxins, being nowadays in a situation where the three groups of marine biotoxins included in the EU Legislation have chemical methods for their oficial control which made possible to define the new era on the analysis of marine biotoxins. An overview on the transition from mouse to chemistry, as well as the main challenges for this transition and the future perspectives, not only for the analysis of the toxins presently included in the EU Legislation, but also for the marine toxins emerging in the EU coasts, will be reviewed in this presentation.

1.2.2. Performance of modern liquid chromatography-tandem mass spectrometry based methods for the simultaneous analysis of several hundreds of fungal metabolites

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Objective: In the recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been applied for the analysis of multiple pesticides, pharmaceuticals, veterinary drugs and natural toxins. Most methods rely on a sample clean-up (e.g. QuEChERS) to deal with matrix effects that are regarded as the biggest problem in LC-MS(/MS) as they negatively affect the accuracy of the methods. However, every clean-up limits the number of analytes as some of the target substances might not be amenable to the chosen procedure. Therefore, our method targeting several hundreds of fungal metabolites and a few plant toxins is based on the direct injection of diluted crude extracts. Many authors have expressed their concern about the limited accuracy of this approach, as matrix effects might not be effectively under control. Therefore, this presentation aims to discuss the analytical performance of our method with special emphasis on the results obtained from proficiency testing. In addition, the merits of the method will be shown based on a survey in samples obtained from an indigenous tribe from Colombia.

Methodology: Samples are extracted with an acidic acetonitrile/water mixture and are directly injected after dilution. Analysis is performed using a conventional C18-HPLC column in connection with the QTrap®5500 LC-MS/MS. Quantification is performed using external calibration; results are corrected for apparent recoveries.

Results: All z-scores were in the satisfactory range in the multi-toxin proficiency tests on raw grains. In a regular proficiency testing scheme we participate in, 95% of all submitted results are satisfactory although very complex samples (e.g. compound feed, coffee, spices) were included. Citrinin occurred at very high levels > 10 mg/kg in more than half of the maize samples from Colombia, whereas regulatory limits were exceeded only in few samples.

Conclusion: The merits of our multitoxin method are not compromised by an insufficient accuracy.

1.2.3. Acrylamide determination in Portuguese food matrices by UPLC-PDA and UPLC-MS

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The aim of this study was the determination of acrylamide in Portuguese food matrices thought the development and optimization of a chromatographic method with two different detectors.

Acrylamide is classified by the International Agency for Research on Cancer (IARC) as a probable carcinogenic compound and the growing concern in human food is due to the fact that it was found in some foods when processed at high temperatures [1,2].

Samples were bought randomly in local supermarkets and correspond to foods that suspect to contain high levels of this compound and contribute significantly to human consumption such as bolo do caco, fries, breakfast cereals, biscuits, coffee, coffee substitutes and pastel de nata.

Sample preparation involved solid phase extraction. To quantify acrylamide were developed two chromatographic methods, UPLC-PDA and UPLC-MS/MS. The method who proved to be more suitable and quantify unequivocally acrylamide was UPLC-MS/MS.

The chosen foodstuff for acrylamide determination presented a dissimilar range of values. Bolo do caco values depends on the cocking procedures. The lowest cooking temperature yield a lower acrylamide content (669 µg/Kg) while with the two samples cooked with the highest temperature the acrylamide content was must higher (1653 µg/Kg). In fries the content of acrylamide found was approximately 365 µg/Kg, while for breakfast cereals it varies between 238 and 187 µg/Kg depending on chocolate content. The content found was 58 µg/Kg for crackers and 203 µg/Kg for gingerbread. Coffee substitutes presented a value 5 times more than the coffee which was the lowest value determined with an acrylamide value of 25 µg/Kg. According to EFSA acrylamide values for pastry are between 75 and 1044 µg/Kg and the content of pastel de nata was 331 µg/Kg.

The acrylamide content in all samples of Portuguese products analyzed were below the indicative values published by EFSA [2] and are not considered to be main hazards of concern.

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1.2.4 Applications of molecularly imprinted polymers for mycotoxin analysis in food samples

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Molecular imprinting (MI) is based on copolymerization of functionalized and cross-linkable monomers in the presence of a template molecule, namely the analyte or a surrogate of it. After template removal, the resulting cavities are tailor-made to be complementary in size, shape and functional groups to the target molecules. These engineered materials can be used as artificial sensing units, capable of replacing antibodies, enzymes or other biological receptors. In the last decade they have been broadly applied as recognition elements, for a single analyte or a group of determinands, in solid-phase extraction (MISPE), including mycotoxins analysis [1], affinity chromatography, binding assays or sensors [2].

This communication will discuss the synthesis of MIPs for selective extraction of mycotoxins such as zearalenone, alternariol (AOH) and its derivatives and their application to food analysis. Several mycotoxin surrogates have been synthesized and tested for polymer preparation to avoid toxin leakage problems during MISPE. Polymer composition (template molecule, functional monomer(s), cross-linker, porogenic solvent) has been optimized in each case using a combinatorial approach. The selectivity of the novel MIPs towards the mycotoxins has been characterized by equilibrium rebinding analysis. The application of these materials as SPE sorbents for the analysis of food samples will be presented. The novel MIPs have shown to be an excellent alternative to commercially available SPE sorbents usually applied to that end.

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1.2.5. Optical genosensors based on magnetic microbeads for the detection of mycotoxigenic fusarium species

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Early and accurate detection of fungal pathogens to species or strain level is of great interest to prevent food poisoning and in the implement disease management strategies. The genus Fusarium includes a variety of phytopathogenic fungi that represent a serious threat to agro-alimentary resources and causes significant losses in grain cereal yield and quality. The detection of *Fusarium* fungi is particularly difficult due to the genus diversity and the mold presence at low concentrations in clinical and natural environments. This genus includes *Fusarium verticillioides* and *Fusarium proliferatum*, widely distributedin wild and cultivated plant species, especially in warm climates, which produce a range of highly toxic mycotoxins, such as tricothecenes, zearalenone, beanverine or fumonisins.

This communication will discuss the development of optical genosensors for the detection of *F. verticillioides* and *F. proliferatum* in food samples based on the use of specific oligonucleotide capture probes designed on the bases of the sequences of the IGS region (Intergenic Spacer of rDNA), which is highly variable among species [1,2]. A sandwich hybridization assay has been implemented in which the target rDNA in the sample was hybridized with the capture probe immobilized on magnetic microspheres followed by a second hybridization with a biotin labelled detection probe. The resulting complex was incubated with streptavidin phycoerythrin

to generate the fluorescent signal. The genosensors have been applied to the detection of the selected fungi in maize samples.

Acknowledgements

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1.2.6. An environmentally friendly multi-extraction method for screening of mycotoxins

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Objective: Fast and simple extraction of mycotoxins from agricultural commodities is a crucial step in the development of rapid test systems. Extraction is usually performed with organic solvents e.g. methanol or acetonitrile, due to low solubility of most mycotoxins in aqueous solutions. Especially for on-site screening methods such as fast and easy Lateral Flow Devices (LFDs), untrained people are exposed to danger when using organic solvents. Moreover they are harmful to the environment when large amounts are used for sample extraction. The reduction of these substances or complete replacement is of great interest and importance for the future.

Methodology: A unique water-based multi-extraction method for mycotoxins was developed. One uniform extract can be used for rapid screening of the following mycotoxins: aflatoxins, deoxynivalenol, fumonisins, and zearalenone.

Therefore, an appropriate amount of milled grain is weighed in and extracted in a ratio of 1:3 using the water-based extraction solvent. The same extract can then be used for the determination of the above mentioned mycotoxins.

Within a few minutes, the simple extraction and the analysis can be performed, if necessary on-site at points of reception.

Results and Conclusion: The developed tests were successfully validated according to USDA/GIPSA guidelines. Accuracy and Precision were in required range, and stability is given up to 1 year when stored at room temperature.

Following quantitation ranges for detection in corn can be given:

Aflatoxins: 5-100 µg/kg
Deoxynivalenol: 250-5000 µg/kg
Fumonisins: 500-5000 µg/kg
Zearalenone: 40-1000 µg/kg

This presentation will demonstrate a multi-extraction method by employing a newly designed extraction buffer system on an aqueous basis for the detection of four different mycotoxins using Lateral Flow Devices.

1.2.7. Using stable isotope internal standard for the accurate quantification of cyclopiazonic acid with a HPLC-MS/MS method

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Objective: Cyclopiazonic acid (CPA) is an indole tetramic acid mycotoxin with tremorgenic, neurochemical and mutagenic toxicity. It is produced by certain *Penicillium* and *Aspergillus* spp., including two important industrial molds for the production of fermented foods (*Penicillium camemberti* and *Aspergillus oryzae*). By consuming contaminated feed, the animals accumulate CPA in their muscles, milk and eggs and humans are exposed to CPA by ingesting these products, as well as by direct consumption of contaminated agricultural products. Therefore, it is important to have accurate analytical methods for the detection and quantification of CPA in food and feed.

Methodology: We have developed and optimized an HPLC-MS/MS method for the detection and quantification of CPA in food and feed samples. To compensate the matrix effect in complex products and guarantee accurate quantification, fully carbon-13-labelled CPA was used as internal standard (IS). Cheese samples were extracted with 0.1% formic acid in acetonitrile. After centrifugation, the supernatant was spiked with the IS and directly injected into the HPLC-MS/MS, without any further clean-up or dilution step.

Results: A validation of the developed method showed for the matrix white mold cheese an LOD of 0.02 ng/mL (0.2 µg/kg) and an LOQ of about 0.05 ng/mL (0.5 µg/kg). The recoveries of spiked cheese samples were close to 90%. In some commercially available white mold cheeses, high amounts of CPA (up to 3.8 mg/kg) could be found.

Conclusion: The ^{13}C-labelled CPA as IS for an HPLC-MS method compensates matrix effects and other fluctuations and is a good tool to get more reliable results. The presented method is applicable for detection of CPA in difficult matrices like white mold cheese and does not need sophisticated clean-up.

1.2.8. Safety of food packaging plastic materials

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Packaging has become an indispensible element in the food manufacturing process, and different types of additives, such as antioxidants, stabilizers, lubricants, anti-static and anti-blocking agents, have also been developed to improve the performance of polymeric packaging materials.

Recently the packaging has been found to represent a source of contamination itself through the migration of substances from the packaging into food.

Various analytical methods have been developed to analyze the migrants in the foodstuff. Overall migration corresponds to the total components, identified or not, that migrate from the packaging material. The objective of the present work is the optimization and validation of the methodology to determine the overall migration from plastic materials to foodstuffs aiming the accreditation according ISO/IEC 17025 [1]. The chosen method consists in the total immersion of plastic materials intended to come in contact with foodstuffs into aqueous based food simulants. The selection of the simulating conditions was made in agreement with National Legislation [2,3,4] and NP EN 1186 [5]. The overall migration from plastic samples was determined as the mass of non-volatile residue after evaporation of the food simulant following immersion.

The validation of the analytical procedure consisted on the evaluation of the parameters: limit of detection (LOD), limit of quantification (LOQ), repeatability (ri) and intermediate precision (Pi). LOD and LOQ were determined through the analyses of a series of blank samples.

Repeatability (ri) and Intermediate Precision (Pi) were evaluated using real samples of different kinds of plastic with different levels of overall migration, according ISO 5725 [6]. The uncertainty estimation, was based on intralaboratory validation data.

We concluded the laboratory performance complies with the requirements of EN 1186 [5] and is able to evaluate the requirements laid down in Commission Regulation (EU) No 10/2011[7]

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[5] NP EN 1186:2002 – Materials and articles in contact with foodstuffs – Plastics – Part 1: Guide to the selection of conditions and test methods for overall migration; NP EN 1186:2002 – Materials and articles in contact with foodstuffs – Plastics – Part 3: Test methods for overall migration into aqueous food stimulants by total immersion.

[6] ISO 5725:1994 - Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions; Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method; Part 3: Intermediate measures of precision of a standard measurement method; Part 6: Use in practice of accuracy values.

[7] Commission Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food.

1.2.9. Assessment of plant food supplements adulteration with psychopharmaceutical drugs

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Objective: The purpose of this study was to compare three different extraction methods (two based on ethanol extraction and one on the Quick, Easy, Cheap, Effective, Rugged, and

Safe (QuEChERs) method) to assess the possible addition of psychopharmaceutical drugs (fluoxetine, sertraline, citalopram, venlafaxine, paroxetine, trazodone, and diazepam) as adulterants in St. John's wort (*Hypericum perforatum*) based plant food supplements (PFS).

Methodology: Analysis was performed in a Nexera Ultra-High Performance Liquid Chromatograph (UHPLC) coupled to a triple-quadrupole mass spectrometer (LCMS-8030 Shimadzu) with an electrospray ionization source (ESI), operating in positive ion mode, using a Kinetex C18 fused core column (150 × 2.10 mm i.d.; 1.7 µm) (Phenomenex). Multiple reaction monitoring mode (MRM) was selected and pharmaceuticals were quantified by internal standard calibration method. Calibration curves were constructed in the range 10 – 1000 µg/L. The three different extraction methods were compared based on the analysis of spiked samples.

Results: The QuEChERS method provided the best results in terms of recovery, although the different *Hipericum perforatum* based PFS showed distinct behaviours during extraction, probably due to differences in their composition since spiked samples included capsules and tablets. Average recovery values in the analysed samples were in the range 57.5 – 119.7%, reflecting matrix interference in some of them.

Conclusions: The methodology was applied to five St. John's wort based PFS commercially available in the Portuguese market, and none of the adulterants surveyed was detected.

Acknowledgements

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1.3. Occurrence of food contaminants

1.3.1. Determination of Brominated Flame Retardants in food using Ultra Performance Liquid Chromatography – Tandem Mass Spectrometry as a part of the monitoring campaign in Belgium

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Objective: The study was undertaken in order to respond to the Commission Recommendation 2014/118/EU on the monitoring of brominated flame retardants (BFRs) in food in Europe. BFRs are anthropogenic chemicals that are added to a wide variety of consumer products in order to improve their fire resistance. BFRs may slowly leak from the products into the environment. Due to their persistence and potential to bioaccumulation in the food chain, BFRs may cause adverse effects in humans and animals. There is a lack of information on the occurrence data of BFRs in food which has hampered accurate completion of intake assessment.

Methodology: Measurements of BFRs were performed using UPLC-MS/MS technique on ACQUITY UPLC system (Waters) coupled to Xevo-TQ-S mass spectrometer (Waters). The MS was operated in electrospray ionization mode in negative polarity. The target compounds included tetrabromobisphenol A (TBBPA) and analogues, brominated phenols (BrPh) and analogues, and hexabromocyclododecanes (HBCDs).

Results: The optimisation of sample preparation procedure was performed using fish (salmon) as matrix. Spiking experiments demonstrated good results applying a mixture of dichloromethane and hexane as extraction solvent. To increase the efficiency of the extraction process, an accelerated solvent extraction (ASE) system was utilized. The extract was further cleaned-up by gel permeation chromatography (GPC) followed by purification on a multi-layer silica column (including a layer of acidified silica). These steps were required to ensure elimination of lipids prior to injection into the UPLC-MS/MS system. The extraction solvent comparison as well as method performance characteristics will be reported.

Conclusions: An UPLC-MS/MS method was developed for determination of the BFRs in fish. The method will be applied and validated for other food matrices. Eventually, using this method, food samples collected in Belgium will be analysed for the presence of BFRs.

1.3.2. Metal contaminants in cinnamon samples marketed in Portugal

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Introduction: Cinnamon has long been used by man for medicinal purposes and as a spice in many traditions wordwhile. Regardless of its alleged healing properties, spices and herbs may be the source of undesirable toxic elements. Although the consumption of cinnamon and cinnamon-based supplements has increased in the last decades in Portugal, little is known about its elemental composition.

Objectives: The main goal of this study was to characterize the elemental composition of cinnamon samples (branded and bulk) available in the Portuguese market and quantify, by wavelength dispersive X-ray fluorescence spectroscopy, the most abundant toxic metals eventually present.

Materials and Methodology: After semi-quantitative analysis and system calibration, samples were analysed for Cu, Zn, Fe, Mn, and Al content, using a 4 kW commercial WDXRF system (Bruker S4 Pioneer).

Results and Conclusion: Semi-quantitative analysis revealed a common elemental pattern among all samples tested: the presence of approximately 16 elements, such as Ca, K, S, P, Si, Mg, Fe, Mn, Mo, Cl, Sr, Cu, Zn, Ru, Al, and Br, being Ca and K the most abundant. Nonetheless, a quantitative analysis by a validated calibration was preformed for Cu, Zn, Fe, Mn, and Al. Although the content of each element varied among brands and/or bulk cinnamon samples, high concentrations of toxic metals (Al, Fe, Mn, Zn, and Cu) were also found, ranging from 45-1353 ppm for Al, 33-534 ppm for Fe, 122-188 ppm for Mn, 10-17 ppm for Zn, and 1-2 ppm for Cu. Even though the content of Al may not present an imminent acute toxicological risk, for general population, heavy consumers, such as those participants in internet

challenges, as the Cinnamon Challenge, can be exposed much higher levels of those toxic elements. Periodic evaluation of such products is advisable since chronic exposure to these elements, at low levels, may induce neuro-, nephro- and/or hepatic toxicity.

1.3.3. Mercury in European eel *Anguilla anguilla* (Linnaeus, 1758) from a contaminated coastal lagoon

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Objective: Mercury is one of the most hazardous contaminants that may be present in aquatic environments, and the main pathway for metal exposure in humans is food consumption, particularly fish and fish derived products. The objective of this study was to evaluate the chemical and sanitary quality of the European eel Anguilla anguilla in a temperate coastal lagoon historically affected by mercury discharges (Ria de Aveiro, Portugal). The environmental quality of the ecosystem was also assessed.

Methodology: Water, sediment and biological samples were monthly collected from February 2012 to January 2013 at 9 sampling sites located inside the lagoon of Aveiro. A total of 44 eels were analyzed for total and organic mercury contents in 3 tissues (muscle, liver and gills). Water, suspended particulate matter and sediment chemical characterization complemented these analyses.

Results and conclusion: The mercury levels in eels tissues directly reflect environmental contamination, with higher tissue body burdens observed in the sampling site with the highest mercury levels in the dissolved, particulate and sedimentary fractions. Liver and muscle presented higher concentrations than gills for both total and organic forms. The concentration of mercury found in all tissues were low in all sampling sites except in the most contaminated area, where concentrations nevertheless did not exceed 0.50 mg/kg wet weight, considerably lower than those recommended by food safety legislation for the studied species. In conclusion, the consumption of eels from the Ria de Aveiro represents no risk for humans due to fish consumption.

1.3.4. Contribution of consumed vegetables to human dietary intake of As, Hg, Pb, Cd, Cu, and Zn in Estarreja urban area

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Taking in account that some agricultural soils in Estarreja urban area (NW Portugal) are contaminated it is advisable to analyse and monitoring the crops content of Potential Harmful Elements (PHEs) that grown there to ensure if it is safe or not to consume them. The soils are located around one of the most important Portuguese chemical industry and the inhabitants often use their own farm products in diary meals. The aim of the study was determine the content of PHEs (As, Hg, Pb, Cd, Cu and Zn) in cabbage (leaves) and tomato (fruit), frequently used in soups and salads, and estimate human daily intake through its consumption. Vegetables samples and respective topsoil were collected at two small farms (L1, L2) located 2-5 km far from Estarreja Chemical Complex and previously identified as ones of the most contaminated sites. Soil (dried and sieved to < 2mm) and vegetables (washed, weighted before and after dried at 40oC) were grinded for analysis that are performed at ACME laboratories (Canada) by ICP-MS, after digestion with aqua regia. The results show that total As, Hg, Pb, Cd, Cu and Zn at site L1 were 127, < 1, 40, < 0.5, 38 and 98 mg/kg, respectively, while in site L2 were 720, > 50, 422, 1.3, 178 and 546 mg/kg, respectively. At these sites the content of As (L1, L2), Hg, Pb, Cu and Zn (L2) on soils exceed the protective Health Canadian Soil Quality Guidelines for agricultural proposes (12, 6.6, 70, 63 and 200 mg/kg). The cabbages in site L2 show higher concentrations (1.4, 0.191, 0.9, 1.7, 19, 331 mg/kg dry weight (dw)) than the ones from site L1 (<0.1, 0.05, 0.42, 0.34, 9.35, 66.9 mg/kg dw). Tomato concentrates lower levels than cabbage. Considering local diet habits, daily intake of selected PHEs from consumption of both vegetables by an adult, were below the PTWI/PMTDI values set by WHO/FAO. At the studied sites highest contributions was from Cd and As (12.6, 4.5%). More research should be carried out to do a complete assessment of human health risks.

1.3.5. Inorganic contaminants and arsenic species contents in rice varieties consumed in Portugal

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Rice (*Oryza sativa L.*) is the second largest cereal crop in the world, being responsible for feeding about half the world's population. Recent data shows that Portugal has a rice consume of 15.8 kg/capita/year, the largest in Europe, producing 168 300 tons of rice per year being the fourth largest producer in Europe.

The aim of this work was to characterize whole grain rice, white rice and rice bran samples consumed in Portugal in terms of inorganic contaminants and arsenic species.

A total of 21 samples cultivated in seven different locations were analysed. For each location 3 types of samples (whole grain rice, white rice and rice bran) were collected. Contents in Cr, Ni, As, Cd and Pb were quantified using ICP-MS. Samples with the highest content of arsenic were analyzed for arsenic speciation with HPLC-ICP-MS. All laboratorial work was carried out in compliance with the internal quality criteria established by the laboratory.

Lead content was below the LOQ in all analysed samples. Cadmium was found above the LOQ only in one location; however it was present in all 3 sample types. Regarding the remaining elements bran was the type of sample with the highest levels. Arsenic content varied between 597-1527 µg/kg for bran, 266-593 µg/kg for whole grain, and 188-480 µg/kg for white rice.

Speciation data showed that the arsenic species present were As (III), As (V) and DMA. In rice bran the inorganic species were predominant while in whole grain and white rice DMA was the most abundant species.

This work demonstrates that, due to the toxicity of the species present, arsenic speciation studies are fundamental for characterize rice in terms of hazard identification. The content of all elements and species under study is in agreement with current legislation or available recommendations. This study also shows the need of powerful analytical techniques with very low detection limits to measure inorganic contaminants in foods.

1.3.6. Arsenic Bioaccumulation in Bivalve Samples

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The main objective of this study was determine the total Arsenic content and identify Arsenic species present in Oysters and Mussels common consumed in Portugal.

Arsenic (As) is a metalloid widely distributed in nature as a result of natural and anthropogenic contributions. Arsenic toxicity to human is largely dependent of chemical species presented whereas inorganic arsenic (iAs) exhibits high toxic levels, and organic forms (oAs) arsenobetaine (AsB), arsenocholine (AsC) and arsenosugars (AsS) are considered non-toxic. Food is the main route of exposure to arsenic and seafood is considered a major contributor to the intake of As. The increasing concerns about dietary intake of As, highlights the need for a robust method able to separate iAs from oAs.

Mussels and Oysters (20 kg) were acquired from local producers. Samples were cleared from water and sediments and lyophilized and then submitted to extraction process using ultrasonic bath. Analytical speciation of As has been achieved by use of coupled techniques which combine a separation process with a High Performance Liquid Chromatography (HPLC) with suitable detection as Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Separation of species was carried out using an anionic exchange column (Hamilton PRPX-100) which
allowed to separate the four species (AsB, DMA, AsIII and AsV). To guaranty the quality of results, samples were analyzed in triplicates and a reference material was used for speciation studies complying with metrological requirements.

In all samples speciation studies showed that AsB was the species founded at highest concentration ranging from 12.9 to 2.9 mg As/ kg bivalve whereas DMA was found at much lower concentration from 0.61 to 0.14 mg As/ kg bivalve. It can be concluded that there is no toxicological risk of arsenic associated with the consumption of oysters and mussels analyzed.

1.3.7. Trace elements profile in nectars and fruit juices consumed in Portugal

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Objective: The main objectives of the present work were 1) to study the inorganic contaminants present in nectars and fruit juices consumed in Portugal and 2) to evaluate the reliability of pooled versus single samples to derive consistent estimates of exposure assessment to inorganic contaminants.

Methodology: Twenty four samples of juices and nectars representative of the domestic market were acquired in May 2014 in the Lisbon region. Samples of representative brands were collected randomly in supermarkets of national implementation in accordance with consumer preference. Afterwards these were analyzed both as single units and as two pools, one of nectars and the other of juices, composed by 12 samples each.

The work focused on the determination of Copper, Manganese, Cobalt, Selenium, Zinc, Arsenic, Cadmium, Chromium and Lead using ICP-MS. Element determination was preceded by high pressure closed vessel microwave digestion. Speciation studies for the determination of inorganic arsenic were carried out through HPLC-ICP-MS.

Results: Cadmium was present in concentrations above the limit of quantification (LQ) only in one sample. Arsenic was found above the LQ (LQ=2 µg/L) in almost half the samples under study. The speciation study proved that most of this arsenic is present in the inorganic forms (As III and As V). However, there is no European legislation for arsenic in fruit juice. Inconsistent results were obtained for arsenic between pooled and single samples.

Conclusions: In the nectars and fruit juices studied metals and metalloids of known toxicity were found in levels below legislated limits for water intended for human consumption. In light of the obtained results arsenic speciation is crucial to clarify the toxicity of arsenic present in foodstuffs. Also, the present work provided a clear example of how, due to a dilution factor, pooling might mask the presence of a contaminant and therefore underestimate exposure assessments.

1.3.8. Heavy Metals Assessment of snail slime for cosmetic use

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Terrestrial gastropods constitute a niche food product, traditionally appreciated by Sicilian consumers. These products have a seasonally marketing, with a peak in the summer period (Tadde et al. , 2009). Genus Helix (*H. aspersa*, *H. pomatia*, *H. lucorum* or turkish snail) are the gastropods most commonly used for food production. Furthermore among the "active special", there is a particular interest in the use of snail slime as cosmetic. This gastropods produces a mucosal secretion composed of various substances, such as allantoin, glycolic acid, elastin, collagen, vitamins, proteins and peptides. Scholars have wondered if these and other protective substances, which are inside the body of these animals, could be exploited for the skin treatment. The aim of this study is to assess the presence of heavy metals (Cd, Pb, Hg) contaminations in slime of gastropods samples from Poland (*Helix aspersa maxima*) and Greece (*Helix aspersa muller*). 80 samples of each species of gastropods were examined by the Veterinary Institute of Sicily laboratories during February 2014. The determination of heavy metals was performed by an *Inductively Coupled Plasma Mass Spectrometry* (ICP-MS) (Agilent 7700 series), an ICP plasma torch analyzer that produce ionization and a mass spectrometer for the ion separation and detection. Results showed a mean concentration of Cadmium (0,35 ± 0,036 mg/Kg) and Lead (0,05 ± 0,013 mg/kg) that are over the limit of detection (LOD) of the method. Mercury levels in both species were not detected (< LOD of 0.06 mg/Kg).

1.3.9. Inorganic Arsenic Levels in Selected Canadian Retail Foods

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Arsenic is a naturally occurring element found in trace amounts throughout the environment. The primary routes of human exposure to arsenic are via drinking water and food.

Arsenic can exist in organic and inorganic forms in food with the inorganic forms being of greater toxicological significance to health. The ratios of inorganic/organic arsenic vary depending on the source of contamination and the commodities in which it is present. While inorganic arsenic is the major species in drinking water, organic arsenic species prevail in aquatic organisms.

This survey generated baseline data on the levels and proportions of the arsenic species in beverages, fruit products, grain products, rice and rice products, and seaweed products available on the Canadian retail market.

2015 samples were collected from retail stores between 2011 and 2013, and analyzed for two inorganic arsenic species (As(III) and As(V)), and up to four organic arsenic species(DMA, MMA, AsB and AsC). As anticipated, the majority of samples tested (87%) contained a detectable level of one or more arsenic species. As(III) and DMA were the most frequently detected species, being detected in more than 60% of samples analyzed. The species AsB and AsC were the least detected species, being detected in less than 10% of samples analyzed.

Beverages had the lowest prevalence of arsenic, with only 68% of samples containing a detectable level of one or more arsenic species, whereas 100% of seaweed products and rice/rice products tested contained a detectable level of one or more arsenic species. Beverages had the lowest average levels of inorganic arsenic (3.84 ppb), whereas rice and rice products had the highest average inorganic arsenic concentrations observed (94.19 ppb).

Potential long-term health risks from inorganic or total arsenic were assessed by Health Canada. It was found that the concentrations of inorganic and total arsenic in foods analyzed by the CFIA were not expected to pose a health risk.

1.3.10. Nutritional and safety assessment of a traditional Portuguese fermented sausage ('Alheira') from Baião

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Objective: "Alheira" is a traditional, smoked, fermented meat sausage, produced in the North of Portugal. Nitrates/nitrites can be found in low amounts due to its presence in the common salt or by production during the cure. In high levels, they can be deleterious to health (formation of methemoglobin or nitrosamines), thus, it is important to quantify their levels. The aim of this work was, then, to characterize the chemical profile of the "alheiras" produced in the county of Baião.

Methodology: Samples were collected from four different manufacturers. Moisture was evaluated using an infrared balance (50-3IR160N, Kern MLS). The ash and protein contents were analyzed according to NP 1615:2002 and NP 1612:2006, respectively, and total fat was determined using a Soxtest equipment (Raypa). Total carbohydrates were calculated by difference. Sodium chloride, nitrites and nitrates levels were determined as described in NP 1845:1982; NP1857:1987; NP 1846:1987, respectively.

Results: Protein, lipid and carbohydrate average levels were 23.8±3.2, 15.8±4.1, and 16.7±5.5 g/100 g, respectively, with a corresponding energetic value of 307 kcal/100 g. Mean moisture and ash contents were 38.1±7.2 and 5.2±0.9 g/100 g, correspondingly. Sodium chloride, nitrate and nitrite levels were 4.1±0.1 g NaCl/100 g, 14.5±3.4 mg $NaNO_3$/kg and 12.1±2.2 mg $NaNO_2$/kg.

Conclusions: As expected, nitrates and nitrites were below the maximum levels legally allowed (250 mg $NaNO_3$/kg and 50 mg $NaNO_2$/kg; Decreto-Lei nº 33/2008 de 25 de Fevereiro)

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1.3.11. Evaluation of pesticides and PBC's of *pterospartum tridentatum* extracts

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Pterospartum tridentatum (L.) Willk., a Fabacea known as ''prickled broom'', (previously named *Chamaespartum tridentatum*) is an autochthonous plant of the Northwest part of Iberian Peninsula and Morocco. This plant is commonly found in Portuguese mountains and is locally known as ''carqueja'' or ''carqueija''. *P. tridentatum* grows in acidic soils, in brushwoods and thickets. It is a shrub, with characteristic yellow flowers with a typical odor that are traditionally harvested during spring. Leaves and stems are normally used in cooking. The yellow flowers are also used in traditional medicine.

The aim of this work was the determination of Pesticides and PBC´s of *Pterospartum tridentatum* extracts. For that purpose, to evaluate the presence, or not, of Pesticides and PBC´s of *Pterospartum tridentatum* extracts was evaluate by gas chromatography GC-ECD-NPD, using the CEN 15662 extraction method QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) according Anastassiades, et al. (2003).

The results suggests the absence of Pesticides and PCB´s in all *Pterospartum tridentatum* extracts analysed, obtained from plants collected in February before the flowering period, in May during the flowering period and in June after flowering period .

This study is part of the overall project of the *Pterospartum tridentatum* biological characterization focus on its importance in the food industry, not only for its role flavor but also for its antioxidant and antimicrobial properties.

1.3.12. Risks of already banned pesticides in *Eruca sativa*

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Objective: *Eruca sativa* (rocket salad) is consumed all over the world. This vegetable is usually consumed fresh and it is described as containing several health promoting agents. Due to their importance in a healthy diet, the aim of this study was to assess if this vegetable are exposed to chemicals such as organochlorine pesticides (OCP).

Methodology: Rocket salad packed samples were bought from three supermarket brands and OCPs were evaluated, using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS). A methodology were optimized for the determination of 13 OCPs using gas chromatography coupled with electron-capture detector (GC-ECD) and the confirmations were made using GC tandem mass-spectrometry (GC-MS/MS).

Results: For the QuEChERS methodology optimization different amounts of sample and type QuEChERS were tested in order to improve the recoveries of the analytes. Due to the green vegetables such as rocket salad present pigments (chlorophyll) it is required the use of a clean-up containing graphitized carbon prior to GC analysis. Precision of the method was measured using rocket salad samples spiked at the levels of 40, 60 and 80 µg/kg. Satisfactory recoveries (from 55 to 149 %) were obtained with a relative standard deviation of ≤11%. The LOD values ranged from 0.9 to 3.5 µg/kg and LOQ values ranged from 3 to 11.6 µg/kg, the analysis obtained by GC-ECD shown 3 OCPs with concentrations above the LOQ values. After the confirmation by the GC-MS only β-HCH was confirmed to be present in levels above the EU maximum residue limited (MRL) in one of the samples of rocket salad with the concentration of 16.21 µg/kg.

Conclusion: The existence of OCPs in vegetables is an alert for a campaign of surveillance should be established. Although it has been banned the use of OCPs for some years ago, the presence of these pesticides remains a reality today. These results highlight the importance of monitoring the presence of OCPs in food.

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1.3.13. Dimethoate evaluation on Portuguese olives at the time of harvest and after brine

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Objective: Pesticides are among the most investigated priority pollutants in agricultural products due to their wide use and toxicity. Four olive groves were monitored and six samples were collected to assess the presence of dimethoate residues used for the combat of olive fruit fly in table olives at the time of harvest and after brine.

Methodology: Olive samples were collected in Porto Martins, Terceira Island (Azores, Portugal), in September 2011. QuEChERS extraction was optimized and dimethoate residues were assessed by liquid chromatography-photodiode array detection.

Results: The optimization of the QuEChERS procedure was achieved step by step through recovery studies. Thus, 3 g of olive sample were used and the extraction was performed using QuEChERS EN15662 with 10 mL of acetonitrile. Recoveries improved when extraction time increased from 1 to 2 minutes being constant subsequently and no clean-up step was needed.

Six olive samples were analyzed and positive results were obtained in two samples with concentrations of 3.58 and 4.34 mg/kg. Olive samples were kept in brine at two concentrations of sodium salt for six months. In both samples, dimethoate residues were not detected after brine.

Conclusions: For evaluation of residues of dimethoate used to combat olive fly in table olives, four olive groves were monitored. The obtained results are clearly influenced by the number of treatments performed, the concentration of the pesticide in the used formulation, and the proximity of the pesticide application with the harvest of the olives. It was also observed in our study that after brine, within the sodium chloride concentration range described above dimethoate residues are no longer detected.

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1.3.14. Polycyclic aromatic hydrocarbons in Mexican four-eyed octopus: levels and risks for human consumption

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Objective: The aim of this work was to characterize the Mexican four-eyed octopus species regarding its polycyclic aromatic hydrocarbon (PAHs) levels, in order to assess its status of contamination, and to evaluate the associated potential human health risks through its consumption.

Methodologies: Octopus samples were randomly purchased from the markets in NW region of Portugal and manually eviscerated. Edible tissues were microwave-assisted extracted with acetonitrile and analyzed by HPLC with photodiode array and fluorescence detectors on line. 18 PAHs (the 16 PAHs considered by U.S. EPA as priority pollutants, dibenzo(a,l)pyrene and benzo(j)fluoranthene) were analyzed.

Results: Total PAH concentrations ranged between 0.24 to 84.6 µg/kg ww. Recently the European Commission revised the established guidelines in order to set new maximum permitted levels for benzo(a)pyrene and also for the sum of benzo(a)pyrene, benz(a)anthracene,

benzo(b)fluoranthene and chrysene, being the recommended markers of the presence of carcinogenic and genotoxic PAHs in foodstuffs. The sampled octopus presented benzo(a)pyrene concentrations below 0.09 µg/kg ww; regarding the sum of the four recommended PAHs, concentrations ranged between 0.50-0.62 µg/kg ww. The mean levels reached were considerably lower than the established regulatory limits (12.0–35.0 µg/kg ww). The potential health risks through the non-carcinogenic (THQ) and carcinogenic risks (TR) risks were also estimated and ranged from 1.31×10-4 to 2.68×10-4 and 59×10-6 to 70×10-6, respectively.

Conclusions: Consumption of the characterized species is safe regarding non-carcinogenic and carcinogenic risks.

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1.3.15. Extraction and detection of mycotoxins in medicinal and aromatic plants: a case study with *Aloysia citrodora P.*

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Plants frequently suffer contaminations by toxigenic fungi, and their mycotoxins can be produced throughout growth, harvest, drying and storage periods. The objective of this work was to validate a method for detection of toxins in medicinal and aromatic plants, through a fast and highly sensitive method, optimizing the joint co-extraction of aflatoxins (AF: AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) by using *Aloysia citrodora* P. (lemon verbena) as a case study. For optimization purposes, samples were spiked (n=3) with standard solutions of a mix of the four AFs and OTA at 10 ng/g for AFB_1, AFG_1 and OTA, and at 6 ng/g of AFB_2 and AFG_2. Several extraction procedures were tested: i) ultrasound-assisted extraction in sodium chloride and methanol/water (80:20, v/v) [(OTA+AFs)1]; ii) maceration in methanol/1% NaHCO3 (70:30, v/v) [(OTA+AFs)2]; iii) maceration in methanol/1% NaHCO3 (70:30, v/v) (OTA1); and iv) maceration in sodium chloride and methanol/water (80:20, v/v)

(AF1). AF and OTA were purified using the mycotoxin-specific immunoaffinity columns AflaTest WB and OchraTest WB (VICAM), respectively. Separation was performed with a Merck Chromolith Performance C18 column (100 x 4.6 mm) by reverse-phase HPLC coupled to a fluorescence detector (FLD) and a photochemical derivatization system (for AF). The recoveries obtained from the spiked samples showed that the single-extraction methods (OTA1 and AF1) performed better than co-extraction methods. For in-house validation of the selected methods OTA1 and AF1, recovery and precision were determined (n=6). The recovery of OTA for method OTA1 was 81%, and intermediate precision (RSDint) was 1.1%. The recoveries of AFB_1, AFB_2, AFG_1 and AFG_2 ranged from 64% to 110% for method AF1, with RSDint lower than 5%. Methods OTA1 and AF1 showed precision and recoveries within the legislated values and were found to be suitable for the extraction of OTA and AF for the matrix under study.

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1.3.16. Assessment of mixtures of mycotoxins in breakfast cereals available in Portuguese market

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Objective: Mycotoxins are secondary metabolites of fungi that cause toxic and carcinogenic outcomes in humans exposed to them [1]. Mycotoxins affect several commodities including cereal grains and their finished products, infant formula and baby foods [2]. This study aimed to determine the incidence and levels of 20 mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, NIV, NEO, DAS, FUS-X, DON, 15-AC-DON, 3-AC-DON, HT-2, T-2, VER, T-2 TETROL, T-2

TRIOL), in breakfast cereals available in the Portuguese market, and compare the results with the maximum limits established by the EU.

Methodology: Twenty six breakfast cereal samples, including corn, wheat, oat, rice and multigrain, were collected from supermarkets in Lisbon region and analyzed by HPLC-FLD, LC-MS/MS and GC-MS.

Results: Results showed that 88 % breakfast cereals samples were contaminated with mycotoxins (with values above the detection limit), although all samples presented levels below the maximum limits established by the Commission Regulation 1881/2006 [3]. OTA and DON were the most commonly detected mycotoxins, with 88% and 73% of samples revealing values above the LOD, respectively. The co-occurrence of different mycotoxins in the same sample was observed in 92 % of the analyzed samples. From these, 46% include mixtures of 3 or 4 mycotoxins. These results are accordingly to those reported by Juan et al [4] and Iqbal et al [5].

Conclusions: These results contribute to the increased knowledge on mycotoxin contents in breakfast cereals marketed in Portugal, and they highlight the deep need of further studies to overcome the absence of legislated limits for mycotoxins in breakfast cereals other than DON and FB1 and the absence of legislated limits for mycotoxin mixtures in food. The last issue is particularly important considering the potential synergistic effects that could occur between mycotoxins and its potential impact on human and, mainly, children health.

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1.3.17. Trichothecenes type A and type B in cereal baby foods

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Processed cereal-based baby foods are particularly prone to be contaminated with trichothecenes (TRC), a group of mycotoxins produced by different species of *Fusarium,* commonly found in raw and processed cereals, such as wheat, maize, barley, oats, rice and rye.

The aim of this work was to optimize a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure followed by gas chromatography-mass spectrometry (GC-MS) analysis for the simultaneous quantification of 12 trichothecenes (type A and type B) in baby foods. Using this methodology, limits of detection and quantification ranging from 0.37-19.19µg/kg and 1.24-63.33µg/kg, respectively, were achieved. The screening of nine commercially available cereal-based baby foods revealed the presence of 4 out of 12 studied trichothecenes: DON (deoxynivalenol), 15AcDON (15-acetyl-deoxynivalenol), T2-Tetrol and NEO (Neosolaniol). DON was the most commonly found, being detected in 4 samples in significant levels (29-270 µg/kg), sometimes exceeding the maximum.

1.3.18. The occurrence of cereulide (emetic toxin of *B. cereus*) in the food chain

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Objective: Bacterial toxins are one of the important culprits of foodborne outbreaks throughout the world. One of the examples is cereulide (CER). This is a bacterial toxin produced by *B. cereus*, which induces emesis in acute intoxications. Besides acute toxicity the toxin has a potential chronic toxic effect. Concentrations from 0.2 ng/ml cereulide are cytotoxic for the beta cells and inhibit insulin secretion suggesting the possibility of adverse chronic effects. This study will focus on the occurrence of cereulide in the food chain in Belgium, in restaurant meals and in ready-to eat foods.

Methodology: In this study UPLC-MS/MS analysis of CER in fresh and/or frozen lasagne dishes, pizza from the supermarkets and ready-to-eat pasta dishes and other pasta products was performed. A complementary evaluation was done on the pasta and rice samples collected from the restaurants. In parallel the microbiological analyses (bacterial counts and PCR analysis) of the samples were performed to confirm presence/absence of the micro-organisms. The analyses of the samples were performed after different storage time (after collection, and at the end of expiry date) and conditions to mimic some household practices.

Results: Toxins were not detected in the samples coming directly from the market, microbiological analyses confirmed that the food was conform to food safety standards. On the contrary the toxin was detected in some food samples from restaurants. Experiments concerning the toxin occurrence when the meals were subjected to some household and kitchen practices are currently on-going.

Conclusions: Low toxin concentrations were detected in some restaurant meals. This warrants further investigation on the occurrence of this toxin, the exposure of certain population groups and on the chronic adverse health effects that might be related with the ingestion of this toxin.

1.3.19. Tetracycline antibiotic residues in small ruminant raw milk used in protected designation of origin Portuguese cheeses

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Tetracycline (TC) antibiotics are widely used to prevent and control diseases in small ruminants, mainly because of their broad spectrum activity and low cost. However antibiotic residues in milk represent a potential risk to the consumer, particularly with the development of allergic reactions and interference of intestinal micro-flora.

The objective of this study was to investigate the occurrence of TC residues in small ruminant raw milk used in the production of Protected Designation of Origin cheese [1]. Raw milk samples were collected from 35 dairies, along with a questionnaire regarding the management practices and TC use. Milk samples were analyzed through ELISA (B-ZERO, TECNA, Italy). The exposure of TC through cheese consumption was calculated by the Estimated Daily Intake (EDI). The consumption of cheese per capita in Portugal is 10 kg/year [2]; assuming that the proportion of national goat and sheep cheese production (17.82%) reflects the consumption of goat and sheep cheese, the latter corresponds to 1.782 kg/year.

About 86% (30) of the tested samples contained TC residues above the detection limit (7.5µg/kg), up to 34.8µg/kg. The concentrations detected were below the Maximum Residue Level (MRL) established by the EU (100µg/kg) [3]. Nevertheless, the positive results contradict the reported recognition of the security interval by the dairies' veterinaries, according to the questionnaire. Individual milk samples featured a higher mean value (23.7µg/kg) as compared to bulk tank milk (15.2µg/kg), which can be justified by a dilution effect. In the only similar study, in Nigeria, all the goat milk samples analysed contained TC residues, with a mean value of 4.0±1.1µg/kg [4]. The EDI of TC through consumption of goat and sheep cheese in Portugal was calculated as 0.00106 µg/kg.bw/day, and thus below the established ADI (3µg/kg.bw/day) [5].

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1.3.20. Coping with mercury in a warmer ocean: tissue partitioning and ecophysiological implications in seabass (*Dicentrarchus labrax*)

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Objectives: This study aimed to assess the effect of global warming on tissue partitioning of total mercury and methylmercury in seabass (*Dicentrarchus labrax*), as well as the ecophysiological consequences.

Methodology: Fish were maintained in tanks with water recirculation at different temperatures (T=18 ºC, i.e. average seawater temperature used in seabass rearing, or T=22 ºC, i.e. simulating

ocean warming, Δ=4ºC), while being fed with non contaminated (D1) or contaminated (8.0 mg MeHg / kg dry feed; D2) diets during 28 days. Four scenarios (S1, S2, S3, S4) were carried out: S1 (control): T=18 °C and fish fed D1; S2: T=22 °C and fish fed D1; S3: T=18 °C and fish fed D2; S4: T=22 °C and fish fed D2. Fish were sampled on days 0, 7, 14, 21 and 28, and different fish tissues were isolated). Total Hg and MeHg contents (muscle, liver and brain) and biochemical responses (brain, muscle, gills, liver and stomach/intestine) were investigated.

Results: MeHg was the main form of Hg in the analysed tissues, regardless of the treatment, and a significant increase in it's concentration was observed, throuhgout time, especially at higher temperatures. Our data clearly shows that temperature strongly influences Hg bioaccumulation and tissue partitioning. Hg exposure in combination with higher temperatures had a strong effect on fish metabolism and physiological status. Overall, data revealed widespread impairments in the enzymatic machinery, and that the rate of MeHg uptake was positively correlated with the metabolic rates.

Conclusions: The deleterious synergistic effects of ocean warming and Hg exposure observed in the this work suggest great biological challenges to marine vertebrate populations in the NE Atlantic coastal ecosystems in the future, as well as an increased risk of human exposure to MeHg through seafood consumption.

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1.3.21. Fungal contamination in feed production in Portugal: what to expect regarding mycotoxins contamination?

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Fungi on crops produce mycotoxins in the field, during handling, and in storage. Exposure of animals and humans are usually through consumption of contaminated feedstuffs or foods. Molds can grow and mycotoxins can be produced either pre-harvest or post-harvest, during storage, transport, processing, or feeding. Worldwide, approximately 25% of crops are affected by mycotoxins annually. Because of this is possible to concluded that mycotoxins occur frequently in a variety of feedstuffs that are routinely fed to animals causing effects on livestock productivity, such as subclinical losses in performance, increases the incidence of disease and reduced reproductive performance.

Taking this in consideration it was developed a study intending to know environmental contamination in a Portuguese feed production unit. Corn, wheat and soybeans were the most common cereals used in the feed production.

Air samples of 250L were collected through an impaction method with a flow rate of 140 L/min onto malt extract agar (MEA) supplemented with chloramphenicol (0.05%), using the Millipore air Tester (Millipore), during a work day. Surface samples, taken at the same time, were collected by the swabbing method. All the collected samples were incubated at 27ºC for 5 to 7 days. After laboratory processing and incubation of the collected samples, quantitative (colony-forming units - CFU/m^3) results were obtained.

Species from *Aspergillus fumigatus* complex were the most found (86.9%) in air but other species such as *Aspergillus ochraceus* complex and *Fusarium graminearum*, both with toxigenic potential, were also found. *Penicillium genus* was the most prevalent in surfaces (32.0%) but *A. flavus complex*, *A. ochraceus complex* and *Fusiarium verticilloides*, all with the ability to produce also mycotoxins, were also identified.

The results showed the presence of fungal species that are known as producers of several mycotoxins, such as aflatoxins, ochratoxins and fumunosins. The feed contamination can result from the cereal used as raw material but also can be occurring in the unit during the production, storage and later, during transport. More information is needed about why and when mycotoxins occur, how to prevent their occurrence and how to deal with their presence in the complete cycle of feed production.

1.3.22. Processed animal proteins in feeding stuff and human health

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Concerning the safety of both animal and human health, research and detection of animal sub products in feed of some species such as ruminants is required according to European Union legislation. The diseases generally known as Transmissible Spongiform Encephalopathy (TSE) are associated to the presence of prions. Quite a few of specific types of prions are found in different groups of animals, for e.g. Bovine Spongiform Encephalopathy (BSE), Creutzfeld Jacob in humans and scrapie in sheeps. The incidence of BSE in farmed animals indicated a serious risk in the animal food production chain. Banning processed animal proteins (PAPs) in feed for these animals led to an important reduction of the number of BSE cases. In order to analyze the presence of banned processed animal proteins (including meat and bone meal-MBM) the classical microscopy is the official method. The methodology is described in the Commission Directive EC/2003/126 and Regulation (EU) nº 51/2013, amending Regulation (EC) nº 152/2009, as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed. In combination with the classical microscopy, a new method of detection of animal constituents based on polymerase chain reaction (PCR) was validated by the EU reference laboratory for animal proteins in feeding stuffs (Regulation (EU) nº 51/2013). This new method is able to detect the presence of animal constituents in feeding stuff.

1.3.23. Feeding stuff contaminants and health

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Animal feed safety impacts in animal health, as well as in the safety of the human food supply. Safe feed helps to reduce production costs, maintains or increases food quality and reduces food losses and wastes. Is also an integral part of the food chain and its safety has been recognized as a shared value and a shared responsibility.

To assure a high level of protection feeding stuff should be consistently controlled.

Feed contamination may have its origin in drug residues (from bad manufacturing and management practices for example) or in environmental contamination.

There are incident reports of human illness due to contaminated raw materials and animal feed (human intoxications with clembuterol after the consumption of meat, for example). Feeding stuff has also showed the random presence of heavy metals in high concentration and other contaminants that can be toxic to animal and human beings.

Examples of environmental contamination are the presence of Salmonella and mycotoxins in feed ingredients. Additionally, *Salmonella* and moulds occasionally may multiply during storage. Concerning all that, evaluation of heavy metals (such as lead, cadmiun and mercury), drug residues, *Salmonella*, and mycotoxins are essential for monitoring and controlling feeding stuff.

EU regulations lay down specific rules for the assessment and quality control in the production and handling of these materials. This work presents some examples obtained in our laboratory concerning different types of contamination.

1.3.24. Zearalenone and Deoxynivalenol in gilts and sows

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Deoxynivalenol (DON, vomitoxin) and zearalenone (ZEA) are mycotoxins produced by several field fungi, including *Fusarium graminaerum* and *F. culmorum*, and thus co-occurrence is regularly observed. Pigs, and in case of ZEA female pigs, are considered to be the most sensitive animal species.

The follow up of the feed production chain was carried out from the cereal grains (unprocessed; n=12) up to the final compound feed (n=26). The cereal grains (unprocessed) were collected before incorporation in the factory mixing machine. The compound feed was sampled at the feed facility exit (in trucks), in the farm silo and at the feeder of the gilts and sows pavilions, in different steps (breeding, gestation and lactation). The mycotoxins were determined by competitive ELISA methods (Celer ZON v2, LOD 10 µg/kg and Celer DON v2, LOD 40 µg/kg; TECNA, Italy).

A histopathological study was performed in slaughtered gilts and sows fed, for at least two weeks, with the analysed compound feed, according to the production stage. Liver from 36 gilts and 54 sows were collected and stained with Hematoxylin and Eosin (HE) and Prussian blue.

The tested samples featured a widespread occurrence (71.1% for ZEA and 78.9% for DON), although more evident in compound feed, for both ZEA (84.6%) and DON (100%). Nevertheless, the average contamination levels were higher in cereal grains than compound feed for DON (644.9 vs. 286.4 µg/kg). Increasing levels were observed starting in the factory (14.1 µg/kg and 190 µg/kg for ZEA and DON, respectively) toward the farms silos (31.9 µg/kg and 350 µg/kg, for ZEA and DON, respectively) suggesting that for these field mycotoxins the storage step

could be a risk factor. In the preliminary histopathological results, both female groups presented structural changes (HE). Hemosiderin deposition, primarily at the liver perilobular area, was observed mainly in sows, which may be related with DON exposure and toxicity.

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2

Risk assessment of multiple contaminants in food

2.1. Multiple biomarker approach of mycotoxins and its contribution for the exposure assessment

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Abstract

One of the most important aspects in risk analysis of chemical substances is to determine the degree of human exposure, a particularly difficult task for contaminants present in foodstuffs, like mycotoxins. Exposure estimates can be based on data on consumption of contaminated foodstuffs, and on the average occurrence of the toxin. These classical approaches provide useful data, although not always ideal. Exposure biomarkers have been proposed for improving the direct exposure assessment to dietary mycotoxins. A biomarker of exposure refers to the quantification of the specific compound, its metabolite(s) or interaction products in a body compartment or fluid, which indicates the presence and magnitude of exposure to the agent. The available data on toxicokinetics of several mycotoxins in animal models and some human studies indicate that exposure to mycotoxins can be measured by biomarkers in several bio-specimens, especially in urine. In recent years, the liquid chromatography tandem mass spectrometry (LC-MS/MS) based on the multi-analyte approach has been successfully introduced into the field of mycotoxin analysis, including the evaluation of suitable biomarkers for assessment of human exposure to mycotoxins. However, this approach requires validation before using in human populations, to provide an accurate estimate of the ability of that measurement to predict the actual exposure to mycotoxins. Exposure assessment may be affected by a number of factors, such as level of the compound in food products, its bioavailability and the length of exposure. Thus implementation of single or multiple biomarker analyses in human populations should take into account the data from classical exposure estimates, to determine the correlation between the biomarker(s) data with the estimated intake of mycotoxins.

Introduction

Mycotoxins are toxic secondary fungal metabolites that are chemically diverse and occur in several agricultural commodities, especially in cereal products[1]. Human and animal diseases caused by mycotoxins include cancer, immune suppression and lesions in target organs such

as liver, kidneys, epithelial tissue (skin and mucous membranes) and central nervous system, depending on the type of the toxin. The main groups of toxin-producing fungi and their mycotoxins are distributed as follows [2]: i) *Aspergillus* species, mainly *A. flavus*, *A. parasiticus* and *A. nomius*, which produce the aflatoxins; ii) *Fusarium* species, which mainly produce fumonisins, trichothecenes, moniliformins and zearalenone; and iii) *Aspergillus alutaceus* (formerly *A. ochraceus*) and species of *Penicillium* which produce the ochratoxins.

Several mycotoxins, either from the same or different fungal species, co-occur in plant products and it is very likely that humans are exposed to mixtures rather than to individual compounds [3]. This is of increasing importance, due to the possibility of interactions of different mycotoxins and the resultant combined toxic that can be different from the individual effects.

The human exposure to dietary contaminants, including mycotoxins, has been traditionaly assessed on the basis of their intake from food (or feed), also known as “external exposure” or “oral dose”. The most common method of evaluating the dietary intake is based on the occurrence of the contaminant in food products and on consumption data, leading to a probable daily intake (PDI) value. Because of limitations inherent to that approach, especially for the individual measurement of those variables, biomarkers have been proposed as a suitable alternative whereby a more accurate assessment of exposure at the individual level can be performed.

Biomarkers of exposure for individual mycotoxins

A biomarker of exposure refers to the quantification of the specific compound, its metabolite(s) or interaction products in a body compartment or fluid, which indicates the presence and magnitude of exposure to the compound. Ideally, such a marker should reflect the toxicokinetics, transformation and fate of the assessed contaminant in the body. The first biomarkers of exposure for mycotoxins were validated in the 1970’s for aflatoxins, by comprehensive studies in animals and humans. Aflatoxin B_1 (AFB_1) is primary biotransformed in liver by enzymes of the cytochrome P450 family, which generate several metabolites that can be excreted in urine. These metabolites include the aflatoxins M_1 (AFM_1), Q_1 (AFQ_1) and P_1 (AFP_1), and AFB_1-8,9-epoxide, which bonds covalently to DNA at guanine residues and serum albumin at lysine residues, producing AFB_1-N^7-guanine and AFB_1-lysine adducts, respectively[4]. Serum AFB_1-lysine is a biomarker of long-term exposure to aflatoxins, since it indicates that an individual has been exposed to AFB_1 for a

2-3 month period [5]. Animal studies clearly indicated dose-response relationships between AFM_1 or AFB_1-N^7-guanine levels in urine and the incidences of liver tumors [6]. In human populations, several studies reported the incidence of aflatoxin metabolites in urine, especially in areas with high aflatoxin contamination of foods, such Gambia [7], Egypt and Guinea [8]. Percentages of aflatoxin metabolites excreted in urine were 4.4% and 7.6% of the AFB_1 ingested by women and men, respectively [7].

Biomarkers of exposure for other individual mycotoxins have also been investigated. For example, fumonisin B_1 (FB_1) levels in urine correlated with tortilla consumption in a Mexican population [9] and with fumonisin intake by farmers in the South African region of Centane [10]. Urinary excretion of ochratoxin A (OTA) has shown to correlate with OTA [11]. Deoxynivalenol (DON), zearalenone (ZEA) and their metabolites (e.g., e-epoxydeoxynivalenol (DOM-1) and deoxynivalenol-glucuronide (DON-GlcA), α- and β-zearalenol, respectively) can also be excreted in urine of intoxicated animals or humans exposed to contaminated foods [12]. However, validation of the urinary levels of those compounds as biomarkers of exposure to the respective mycotoxins in food products is still required.

Biomarkers of exposure for multiple mycotoxins

In recent years, the liquid chromatography tandem mass spectrometry (LC-MS/MS) based multi-analyte approach has been successfully introduced into the field of mycotoxin analysis, including the evaluation of suitable biomarkers for assessment of human exposure to mycotoxins. The development of new analytical techniques brought important contributions for the multi-mycotoxin biomarker approach, such as the measurement of a more realistic data on the mycotoxin exposure, since a mixture of mycotoxins is expected to occur under field conditions, and the potential application in risk assesments of combined mycotoxins and their possible interaction effects. However, sample preparation continues to be a chalenge for developing multi-mycotoxin methods of analyses, because of the wide range of chemical properties of the different mycotoxins and their metabolites [13].

One of the first studies describing a multi-mycotoxin method in urine was conducted by Solfrizzo et al. [14]. The authors analyzed FB_1, AFM_1, OTA, DON, DOM-1, and α- and β-zearalenol based on sample clean-up by multi-antibody immunoaffinity columns (IAC) and solid phase extraction (SPE) columns. Subsequently, Warth et al. [15] developed a multi-biomarker method based on a ‘dilute-and-shoot’ approach (no sample preparation other than centrifugation and dilution) for 15 mycotoxins, including FB_1, AFM_1, OTA, DON,

DON-3-GlcA, DOM-1, ZEA and α- and β-zearalenol. As a consequence, the rapid development of analytical methods for multiple mycotoxins in urine now requires additional studies for validation of those biomarkers, to provide an accurate estimate of the ability of that measurement to predict the actual exposure to mycotoxins.

Table 1 presents the main outcomes from some recent studies on biomarkers of exposure for multiple mycotoxins found in human urine. The data presented in those studies will allow for comparison of the exposure to multiple mycotoxins between different populations and/or places (e.g., higher and lower risk populations). However, the exposure assessment may be affected by the level of the compound in food products, its bioavailability and the length of exposure, among other factors. Therefore, additional studies are needed to determine the relationship between the mycotoxin concentrations found in urine and the actual levels of multiple ingested mycotoxins through the diet. This will be helpful in further assessments on the contribution of mycotoxin exposure for disease occurrence in human populations.

Table 1. Recent studies on urinary biomarkers for multiple mycotoxin exposure in human populations.

Country/region	Mycotoxins evaluated	Main outcomes	Reference
South Africa / Transkei	AFM_1, FB_1, FB_2, OTA, DON, DOM-1, DON-3-GlcA, DON-15-GlcA, NIV, T-2 toxin, HT-2 toxin, ZEA, ZEA-14-GlcA, α- and β-ZOL	Samples (*N=53*) were analyzed using clean-up methods and "dilute-and-shoot" approach. Urinary levels of FB1, DON, DON-3GlcA, DON-15-GlcA, NIV, ZEA, OTA, α- and β-ZOL were found in the Transkei population. FB1 in urine correlated with PDI values based on food analyses and corn consumption data.	12
Cameroon	AFM_1, FB_1, FB_2, OTA, DON, DOM-1, DON-3-GlcA, DON-15-GlcA, NIV, T-2 toxin, HT-2 toxin, ZEA, ZEA-14-GlcA, α- and β-ZOL	Samples (*N=175*) were analyzed by a "dilute-and-shoot" method. Multiple mycotoxin exposure (2 or more mycotoxins in one sigle urine sample) was found in 18% of urine samples analyzed.	16
Nigeria	AFM_1, FB_1, FB_2, OTA, DON, DOM-1, DON-3-GlcA, DON-15-GlcA, NIV, T-2 toxin, HT-2 toxin, ZEA, ZEA-14-GlcA, α- and β-ZOL	Samples (*N=120*) were analyzed by a "dilute-and-shoot" method. OTA, AFM_1 and FB_1 were the most frequently detected mycotoxins.	17
Italy (Southern)	AFM_1, FB_1, OTA, DON, DOM-1, ZEA, α- and β-ZOL	Samples (*N=52*) were analyzed using clean-up methods. ZEA, α- and β-ZOL, OTA, DON, FB_1 and AFM_1 were detected in 100%, 100%, 96%, 56% and 6%, of samples, respectively. All samples contained biomarkers of two or more mycotoxins.	18

Conclusions

Multiple biomarker approach for mycotoxins provides reliable information on individual exposure, which is especially important for the risk assessment, epidemiological studies or intervention trials.

Since dietary exposure may be affected by a number of factors, such as level of the compound in food products, its bioavailability and the length of exposure, biomarkers require validation to provide an accurate estimate of the ability of that measurement to predict the actual exposure to toxic compound.

Implementation of single or multiple biomarker analyses in human populations should take into account the data from classical exposure estimates to determine the correlation between the biomarker(s) data and the estimated intake of mycotoxins.

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2.2. OPEN platform for clinical nutrition - an online dietary assessment

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Abstract

This study presents the Open Platform for Clinical Nutrition (OPEN) as a tool for dietary exposure assessment. It highlights its potential application in clinical and research studies, the possibility to be translated to several languages, mobile applications and European users. The Portuguese projects MYCOMIX and MONITADITIVOS illustrate the successful application of OPEN to perform exposure assessment.

Introduction

OPEN is a web-based application that supports patients or study participants in food and physical activity recording, dieticians in diet planning, and researchers in food diary, 24-hour recall and food frequency questionnaires. The food recording includes also photos. OPEN enables online interaction between dietitians/nutritionists and their patients/clients.

To support its use in different countries and languages, OPEN allows translation of the user interface into any language as well as the use of any food composition dataset that complies with Food data structure and format standard (BS EN 16104, 2012). This standard defines harmonized procedures for description of food, food components, values, sampling, sample handling, analytical methods and data quality. OPEN has already been translated into English, Portuguese, Dutch, Italian and Greek. The Portuguese version of OPEN was translated in collaboration with the National Health Institute Doutor Ricardo Jorge (INSA) and uses the Portuguese Food Composition Database [1].

By default, OPEN refers to national and international dietary recommendations, which can be modified by the dietitian/nutritionist to suit the needs of individuals. The Slovenian version of OPEN considers D-A-CH recommendations for healthy people, ESPEN recommendations for adult patients (http://www.espen.org/education/espen-guidelines), ESPGHAN recommendations for pediatric patients (http://www.espghan.org/)

and EFSA (http://www.efsa.europa.eu/en/datex/datexfoodclass.htm) and WHO (http://www.who.int/nutrition/publications/nutrientrequirements/en/) recommendations for those nutrients and non-nutrients that have not been defined in other recommendations

OPEN has been extended with mobile applications for carbohydrates counting (Nutri), food barcode scanning (eDietetik) and dietary assessment of patients with Parkinson's disease (PARKDIET). A pocket-sized scale Libra was developed, which communicates wirelessly with Nutri to support easy counting of carbohydrates and other nutrients. As Nutri also wirelessly communicates with a blood glucose meter, bolus insulin doses can be easily calculated considering the amount of food eaten. Libra has also been connected with a mobile application for easy recording of food weights (FoodWiz) that is used by adolescents for weight control.

The non-profit European Association EuroFIR AISBL and the European Federation of the Association of Dietitians (EFAD) have supported further development of OPEN. It has been used in four Slovenian hospitals to support assessment of patient nutrient intake and support pregnant women in self-management of gestational diabetes, in the EU-funded projects QuaLiFY (http://qualify-fp7.eu) and PD-manager (http://parkinson-manager.eu), in the ERA Chair ISO-FOOD (http://isofood.eu), and last but not least in Portuguese projects for dietary exposure assessment to mycotoxins – MYCOMIX - and food additives - MONITADITIVES.

Application of OPEN in Portuguese projects

OPEN could be used as a tool for dietary exposure assessment. Within the safety evaluation procedure of any chemical substance, one crucial step is the dietary exposure assessment. Exposure assessment combines data on concentrations of a chemical substance present in food with the quantity of those foods consumed.

In a preliminary study to assess Portuguese children exposure to the mycotoxin patulin (PAT), the OPEN was used as a food diary to 20 children aged between 0 to 3 years old. Parents were asked to record detailed information about all food and beverages consumed during three days. The food consumption data obtained by OPEN was combined with patulin food contents to obtain the daily intake, expressed as µg PAT / kg of body weight / day. This value was compared with provisional tolerable weekly intake (PTWI) to assess the risk. The obtained results had shown that, even in the worst case, there is no risk of children exposure to patulin [2].

MYCOMIX project titled “Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact” aims to study the occurrence of multiple mycotoxins in infant foods consumed by the Portuguese children and its toxicity interactions. The infant foods consumption data were obtained within a pilot study that includes 103 children (0-3 years old) from the Primary Health Care Unit of Cidadela, Cascais. A three day food diary was applied by children parents and data was introduced in the web-based platform - OPEN. In this project, the presence of 12 mycotoxins (aflatoxins, ochratoxin A, fumonisins and trichothecenes) were quantified in several food items (identified on the consumption data), using HPLC-FLD, GC-MS and LC-MS/MS. This study revealed that Portuguese children are exposed to multiple mycotoxins throught food [3]. To complete information about children the platform provides anthropometric information and energy and nutrient intake.

MONITADITIVOS project aims to develop and implement a food additives monitoring system in Portugal. In this project, additives with established ADI are assessed for the general population in order to ensure the safety of consumers. However such a huge study is difficult, expensive and time consuming and can be performed using different methodologies. In this case, the European Commission [4] recommends to assess particular vulnerable population groups as children and adolescents and/or specific highly consumers of large amounts of foods containing high concentrations of additives (worst case study). Therefore, MONITADITIVOS study began performing the assessment of food consumption in schools using OPEN as a food diary for children and a 24-hours recall for adolescents. To calculate the additives daily intake, Maximum Permitted Levels (MPL) was first used. If daily intake values are higher than the corresponding Acceptable Daily Intake (ADI), the occurrence levels of these additives should be determined and the daily intake calculated again.

Conclusions and future perspectives

OPEN has proved to be an efficient tool for dietary assessment. It relies on evidence-based dietary recommendations and ensures repeatability and validity of measures including errors such as estimation of portion size, day-to-day variation in diet and physical activity as well as the frequency of consumption. OPEN is based on national food composition data, food-indexing systems (e.g. international LanguaL (http://www.langual.org/) and EFSA FoodEx2 (http://www.efsa.europa.eu/en/datex/datexfoodclass.htm) and translates results of the analysis in an illustrative and user-friendly fashion for the patients/clients to improve understanding.

OPEN revealed to be a very important tool in the exposure assessment of the national Portuguese projects related to mycotoxins and food additives. This platform will allow to perform, in future, a deterministic exposure assessment to food contaminants and additives. For this to become possible, data on food contaminants contents and maximum permitted levels of food additives should be introduced in OPEN as components of each food of the Portuguese Food Composition Database.

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2.3. Multifaceted approaches in risk assessment

2.3.1. Development of harmonised methodologies for risk assessment to multiple chemicals

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The development of harmonised methodologies for human and ecological risk assessment of combined exposure to multiple chemicals ("chemical mixtures") pose several challenges to scientists, risk assessors and risk managers. Key challenges to harmonisation include the large number of chemicals involved, their associated exposure patterns, toxicological profiles in humans and other species as well as the diversity of regulatory frameworks and legislations. In principle, once problem formulation has focused the purpose of the risk assessment, the use of tiered approaches for exposure assessment and hazard assessment combined for risk characterization, provide options for harmonisation. These tiered approaches, originally developed by the US-EPA and the WHO, range from qualitative/semi-quantitative to fully probabilistic tiers, the choice of which depending on the purpose of the risk assessment, data availability and, the time and resources available. In the human health area, these frameworks have been recently applied by EFSA to multiple pesticides with a similar and a dissimilar mode of action by the panel on plant protection products (PPR) and their residues and to multiple contaminants by the Panel on Contaminants in the food chain. In ecological risk assessment, recent examples include the methodology for assessing combined toxicity of pesticides in bees proposed by the PPR panel.

A number of recommendations for future work resulting from the consultation of EFSA panels and its scientific committee have been recently published and were recently discussed at the EFSA colloquium on "harmonisation of human and ecological risk assessment of multiple chemicals" [1,2]. For problem formulation, identification of priority chemicals using both exposure- and hazard-based criteria is recommended to provide guidance taking into account differences in legal frameworks (i.e. regulated substances versus contaminants). For exposure assessment, collection of occurrence data for multiple priority chemicals in individual food samples, development of case studies/training sets to compare deterministic versus probabilistic methods, and methodologies for aggregate exposure assessment for priority chemicals have been identified as priorities. For hazard assessment, further exploration of the scientific basis to deal with whole mixtures and to set assessment groups is recommended particularly

basis to deal with whole mixtures and to set assessment groups is recommended particularly using mode of action information. Further recommendations include the development of a guide for uncertainty analysis in hazard assessment and risk characterisation and methodologies for risk assessment of exposure to chemicals combined with other stressors (e.g. biological hazards, physical agents).

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2.3.2. Assessment of chemical mixtures within European regulations

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Objective: While humans and the environment are continuously exposed to a multitude of substances via different routes of exposure, the current regulatory risk assessment approach mainly focuses on the assessment of individual substances via a single exposure route.

Different types of mixtures are identified in current EU regulations, but currently there is no harmonised methodological approach to their assessment. This gap in the EU regulatory assessment framework has recently gained more attention, following a 2012 Commission Communication on the Combined Effects of Chemicals. The objective of our work was to review current regulatory requirements and available guidance, as well as to gather information on the application of different approaches in current risk assessments.

Methodology: More than 20 different pieces of EU legislation (food and non-food related such as REACH, plant protection products, biocides, medicines, cosmetics, food contaminants, food and feed additives etc.) were reviewed to analyse the regulatory requirements for the assessment of mixtures. Guidance documents from the EU and international bodies were scrutinized to summarise current guidelines on mixture assessments in different areas. Furthermore, a survey was performed to gather information on current practices and expert views.

Results and conclusions: An overview of the current EU regulatory requirements for the assessment of mixtures will be presented. Current assessment practices will be summarised, including an analysis of similarities and differences. Expert views on different methodologies will also be presented.

2.3.3. The EU project ACROPOLIS and exposure assessment to mixtures

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The overall objective of the ACROPOLIS project is to improve risk assessment strategies in Europe regarding cumulative and aggregate dietary exposure. In this project a framework for cumulative and aggregate risk assessment of pesticides was developed that is scientifically sound and accessible for all actors involved in the European risk assessment and risk management.

Within the project a model was developed to assess the dietary cumulative exposure to compounds belonging to a cumulative assessment group (CAG) according to the requirements as set out in the 2012 EFSA guidance probabilistic dietary exposure modelling. Assessments (both single and multiple compound). In this EFSA Guidance an optimistic and pessimistic model run are proposed aiming, respectively, at estimating the possible lower and upper range of exposures in a population. To test the implementation of the EFSA Guidance methods in the MCRA system, cumulative dietary exposure assessment to two CAGs of triazole pesticides was estimated using national food consumption and monitoring data of several European countries. These assessments were performed to test both the implementation as well as the practicality of the EFSA Guidance methods. It was concluded that some kind of intermediate scenario was needed, that can still be argued to be conservative (precautionary principle) but not over-conservative. The cumulative ACROPOLIS model was well-received throughout Europe and many member states and stakeholders are presently making use of the model.

Aggregate exposure combines dietary and non-dietary sources of exposure, which is relevant for pesticide residues. A conceptual framework of aggregated exposure was implemented in the MCRA system and tested addressing four different aggregated exposure scenarios in the form of case studies. Further testing and validation is recommended. Both the cumulative and the aggregate models were validated, and fully documented. Validation was performed against simulated data where the true outcome in known, and against the factor standard program used by the US-EPA, namely DEEM-FCID.

2.3.4. Intake assessment of associated neurotoxicants in seafood from the Mediterranean Sea from the coastal population

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Objective: To evaluate the associated intake of bio-accumulative neurotoxicants such as mercury/methylmercury (MeHg), no dioxin-like polychlorobiphenyls (NDL_6-PCBs) and polybromodiphenyls ethers (PBDEs) congeners nos. 47, 99 in the Italian population by consumption of Mediterranean seafood sold at local markets.

Methodology: Statistical descriptors of Hg/MeHg, NDL_6-PCBs, PBDEs 47, 99 occurrence in the most consumed seafood species; intake estimates accounting for food consumption database referred to seafood consumers; evaluation of the safety margin (MOS) as ratio between modelled intake and related guidance values (tolerable weekly intake for MeHg, tolerable daily intake for NDL6-PCBs and Bench Mark Dose Level10 for PBDEs nos. 47, 99) referred to the neurodevelopmental toxicity end-point; risk-oriented priority to reduce the associated exposure leading to a combined MOS ratio >1.

Results: Hg/MeHg represents the priority contaminant for Mediterranean seafood intake. A combined MOS <1 could be reached in vulnerable people accounting for: the selection of low trophic level seafood species and the farmed fish consumption.

Conclusions: A seafood species-specific risk assessment approach along with the geo-referenced traceability is seen as an effective management tool on which selective advisories on a responsible choice of seafood meals can rely on. This may allow also fulfilling recommended daily allowance for polyunsaturated fatty acids intake, without compromising the seafood meals number consumed per week. An open access to monitor data on considered contaminants could improve the empowerment in seafood consumers and make them resilient to the occurrence in seafood, without compromising the food security aspects related to people living on subsistence economy and/or low-mileage food chains.

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2.3.5. Risk assessment for lead in Cyprus & the use of improrisk model

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Objective: The objectives of the present work were to estimate the dietary lead intake of the adolescent population in Cyprus, to carry out the relevant risk assessment and to determine which food groups are the major contributors to the dietary lead exposure.

Methodology: Dietary lead exposure was calculated by a deterministic approach using the IMPRORISK model, an empirical distribution model. Specifically the dietary lead intake was determined by matching lower, middle and upper bound mean occurrence data of lead in Cyprus with mean daily consumption and body weight for each individual (Childhealth survey of Cyprus) at level 2 of the EFSA FoodEx food categories [1]. Middle bound mean exposure was used to establish a relative ranking for the contributions of the different broad food categories of FoodEx.

Results and Conclusions: Average lead dietary exposure ranged from 0,35 to 0,59 μg/kg b.w./day for mean consumers and 0,61 to 0,87 μg/kg b.w./day for high consumers. These exposure estimates are below or exceed (for high consumers) the BMDL10 intake value for nephrotoxicity (0,63 μg/kg b.w./day) and are below the BMDL10 intake value for cardiovascular effects (1,50 μg/kg b.w./day). The broad category “Grains and grain-based products” had the highest contribution to dietary lead intake. The above findings are consistent with the relevant EFSA estimations for Cyprus [2].

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2.3.6. Common cuttlefish: elemental characterization and risk assessment

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Objective: Cephalopods are part of the traditional diet of Mediterranean countries such as Portugal. The present work aimed to evaluate levels of Cd, Pb, As, Cu, Cr, Zn and Ni in the edible tissues of common sepia (*Sepia officinalis*). Another goal of this study was to assess the potential health risks for low and high cephalopod consumer populations. This evaluation was based on the daily minerals intake, the non-carcinogenic target hazard quotient (THQ) and target carcinogenic risk (TR) established by the U.S. Environmental Protection Agency.

Methodologies: Common sepia specimens from different origins were purchased from the markets in NW region of Portugal and biometrically characterized. Samples were digested with suprapur nitric acid and their elemental contents were quantified by high resolution continuum source graphite furnace atomic absorption spectrometry.

Results: Zn and Cu were the most abundant minerals, followed by As, Cd and Ni. Pb and Cr presented the lowest concentrations. In view of the long half-life of Cd, the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) decided recently to express its tolerable intake as a monthly value in the form of a PTMI. The reached estimated intakes were clearly below the respective PTMI established (25 µg/kg body weight). Regarding Pb, JECFA concluded that it is not possible to establish a new PTWI that would be considered to be health protective. The attained dietary Pb exposure are considerably below the exposure level of 1.2 µg/kg body weight per day calculated by the Committee to be associated with a population increase in systolic blood pressure of 1 mmHg. Consequently, it may be considered that any health risk that would be expected to occur at the estimated exposure level is negligible.

Conclusion: Still, due to the high Portuguese consumption of cephalopods, moderate consumption of this species is advised, principally by the most vulnerable population groups.

2.3.7. Evaluation of children exposure to Bisphenol A

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Objective: Bisphenol A (BPA) is the common name for 2,2-bis(4-hydroxyphenyl)propane.

The major human route of exposure to BPA has been shown to be the dietary pathway. Contamination of food with BPA is usually caused by contact with food packaging materials (or containers) containing epoxy resins and polycarbonate.

Nowadays it is known that this compound can stimulate several cellular responses at very low levels of concentrations. The effects of BPA are dependent on the dose and time window of exposure. Being the prenatal and neonatal period the most vulnerable and critical. The aim of this work was the evaluation of children exposure to BPA.

Methodology: Human plasma samples were collected from children, an SPE extraction procedure was developed and applied to samples' analysis. BPA was analyzed by GC-MS.

Results: The optimization of the SPE procedure was achieved step by step. Thus, 1 mL of sample was used and the extraction was performed using a SPE cartridge (Polymeric reversed phase from Phenomenex, 30mg/1ml). Good recoveries and linearity were obtained. Samples were analyzed and positive results were obtained.

Conclusions: There is extensive evidence that many consumer products contain and release BPA. There is also significant evidence that many of these products leach BPA under normal conditions of use.

Acknowledgements

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2.3.8. Cumulative health risk assessment of the co-occurring mycotoxins deoxynivalenol and its acetyl derivatives in cereal-based food for the Austrian population

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Objective: From 2007 to 2013 cereal-based food was collected from the Austrian market and analysed for deoxynivalenol (DON), 3-acetyl-DON (3ADON) and 15-acetyl-DON (15ADON). The purpose of this study was to estimate the dietary exposure and to perform a cumulative risk assessment of combined exposure to these mycotoxins for the Austrian population.

Methodology: 1890 samples of cereal-based food were collected and analysed for their levels of DON. 1746 of these samples were also examined for 3ADON and 15ADON.

Average and high exposures were estimated for DON, 3ADON and 15ADON by the deterministic approach. The Austrian consumption data of the four population groups preschoolers, school children, women and men were used. Dietary exposures to DON, 3ADON and 15ADON were added to calculate the total exposure for each population group.

Estimated total exposures were compared with the tolerable daily intake of 1 µg/kg bw/d for DON, 3ADON and 15ADON established by the World Health Organisation.

Results: In 39% of the samples DON was determined in quantifiable concentrations (≥ 50 µg/kg). The two derivatives occurred at a much lower frequency than DON. 3ADON was determined in only two samples and 15ADON in thirty one samples.

The estimated average combined intake of DON, 3ADON and 15ADON is 1 µg/kg bw/d for preschoolers, 0.5 µg/kg bw/d for school children and 0.4 µg/kg bw/d for women and men. DON contributed with more than 90 % to the total exposure. Bread and pastries followed by pasta were the main contributing food groups. The estimated high combined intake of DON and its derivatives is 0.9 µg/kg bw/d for school children, 1 µg/kg bw/d for adults and 2.3 µg/kg bw/d for preschoolers.

Conclusions: High consumption of cereal-based foods, such as bread and pastries, may lead to intakes above the maximum tolerable daily intake of 1 µg/kg bw/d. Risk for human health, especially for preschoolers, cannot be excluded at high consumption of contaminated cereal-based foods.

2.3.9. Risk assessment of Portuguese children exposed to single and multiple mycotoxins in breakfast cereals

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In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants, namely mycotoxins, a wide group of fungal secondary metabolites that cause toxic and carcinogenic outcomes in humans exposed to them [1].

Objectives: The present study aims to characterize, for the first time, the risk associated with the exposure of Portuguese children to single and multiple mycotoxins present in breakfast cereals (BC).

Methodologies: Portuguese children (0-3 years old) food consumption data (n=103) were performed using a 3 days food diary. Occurrence data concerned the quantification of 12 mycotoxins (aflatoxins, ochratoxin A, fumonisins and trichothecenes) were evaluated in 34 BC samples marketed in 2014 in Lisboa. Daily exposure of children to mycotoxins were performed using a deterministic (Microsoft Excel 2007) and probabilistic (@Risk 6 for Excel, Palisade) approaches. The output of exposure was compared to the dose reference values (TDI) in order to calculate the margin of safety (MOS). For the cumulative risk assessment of multiple mycotoxins, the concentration addition (CA) concept was used [2,3]. Different strategies had been considered to treat the left censored data [4].

Results: 88% of BC samples were contaminated with mycotoxins including 1 to 7 different toxins. Approximately 23 % of the studied children consumed BC at least one time in these 3 days. Preliminary results showed that children exposure to single mycotoxins present in BC were well below the TDI. MOS values for multiple mycotoxins were near 1.

Conclusions: This study concerns the first risk assessment of Portuguese children to single and multiple mycotoxins in BC. Children are a particularly vulnerable population group to food contaminants and the present results point out an urgent need to establish legal limits and control strategies regarding the presence of multiple mycotoxins in children foods in order to protect their health.

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2.3.10. Occupational co-exposure to several mycotoxins in the waste management setting

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Contrary to fungi, exposure to mycotoxins is not usually identified as a risk factor present in occupational settings. This is probably due to the inexistence of limits regarding concentration of airborne mycotoxins, and also due to the fact that these compounds are rarely monitored in occupational environments. In the waste management setting is important to consider that mycotoxins reside in the environment long after fungi elimination and this can implicate a serious problem due to multi and constant contamination of the waste during all the management process.

Recently, it was developed a study in Portugal aiming to assess occupational exposure to mycotoxins in the waste management setting.

Occupational exposure assessment started to measure Aflatoxin B_1 (AFB_1) in workers serum by enzyme-linked immunosorbent assay (ELISA). Forty-one workers from the waste company were enrolled in this study. A control group (n = 30) was also considered in order to know the AFB_1 background levels for the Portuguese population.

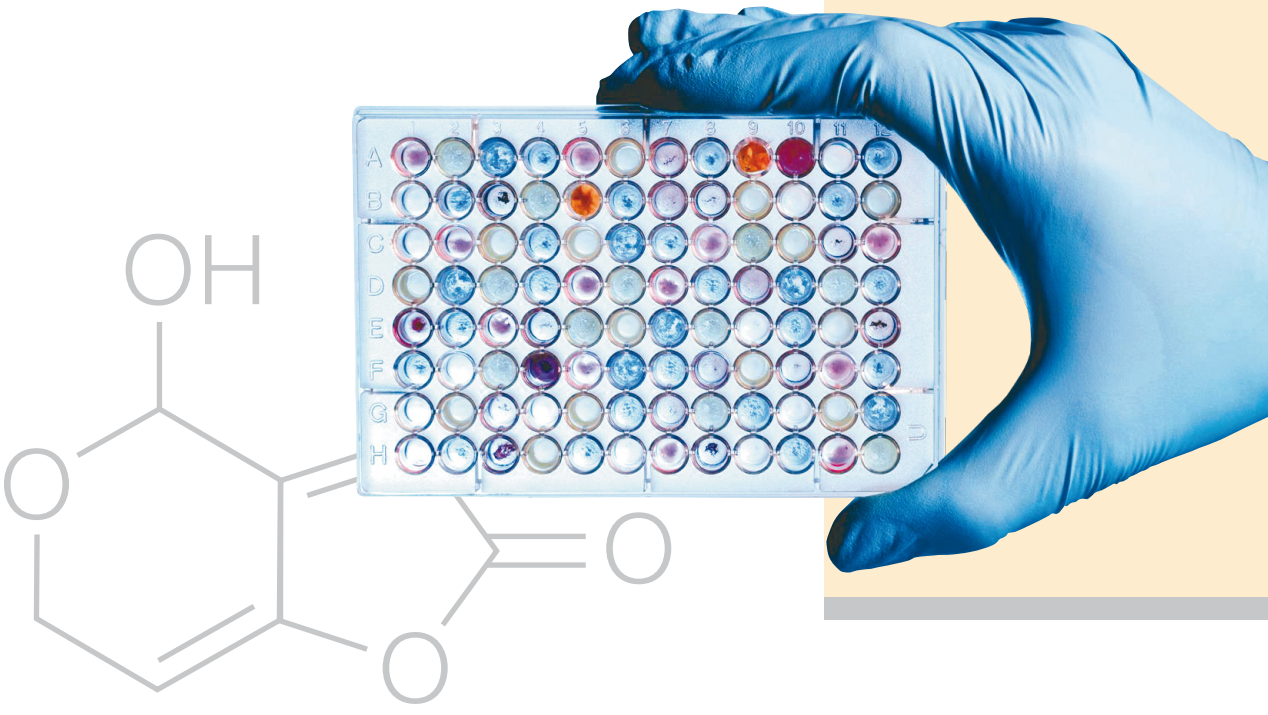
All the workers showed detectable levels of AFB_1 with values ranging from 2.5 ng/ml to 25.9 ng/ml with a median value of 9.9 ± 5.4 ng/ml. All of the controls showed values below the method's detection limit (LOD=1 ng/ml).

However, and taking in consideration that besides *A. flavus* complex were found other toxigenic fungi in the same workplaces, such as *A. niger* complex and *A. fumigatus* complex, we have to consider that probably there is a co-exposure to several mycotoxines.

In the near future it will be measured Ochratoxin A in the same serum samples. With this data it will be easier to understand what can be expected regarding health effects and to perform a more accurate risk assessment.

3

Advances in bioaccessibility and toxicology of multiple contaminants in food



3.1. An overview of the models for simulating food digestion

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During the last 30-40 years, there has been a dramatic increase in the diet-related pathologies such as type2-diabetes, hypertension, hart diseases and certain types of cancer. There is really an emergency to prevent the development of these pathologies rather than having to cure them. When we ingest food, the gut plays the first interface between the food and our body. Understanding the fate of food in the gastrointestinal (GI) tract has received a lot of attention recently. Up to now, the GI tract has mainly been considered as a black box and the mechanisms of food disintegration are still unknown. Similarly, little is known about the components released in the gut during digestion and their potential effects on human health.

Most of the protein and lipid digestion and absorption occur in the upper part of the GI tract and 3 major digestion phases can be distinguished. First, during the oral phase the food will be broken down by the mastication process and saliva will lubricate the food particles to allow the formation of the bolus. Digestion of starch will be initiated through the action of α-amylase. Then the bolus will arrive in the stomach where acidic gastric secretions and digestive enzymes like pepsin and the gastric lipase will start hydrolyzing proteins and lipids respectively. The pH of the empty stomach is between 1.5 and 2 for the adult but will increase within a few minutes to 5-6 depending on the pH and buffering capacity of the food. The food will be stored in the proximal stomach and then transferred to the distal stomach where contractions (3 to 6/min) will push the chyme (semifluid mass into which food is converted by gastric secretion) into the small intestine that is composed of 3 different parts: the duodenum (around 20 cm long in human), the jejunum (3 m) and the ileum (3 m). The small intestine and particularly the jejunum is considered as the major site of nutrient absorption. The pH will gradually slightly increase from 6.5 in the duodenum to 7.5 in the ileum and other enzymes like trypsin, chymotrypsin, pancreatic amylase and lipase will further disintegrate the food constituents.

Several models of different complexities have been proposed to simulate food digestion. *In vitro* static digestion models are the most widely used since they are cheap, easy-to-use, reproducible and do not require the use of animals or human volunteers. However, it is obvious

that it is impossible to perfectly mimic the complexity of the GI tract in a test tube. Therefore, these very simple models are particularly useful to compare foods of similar composition or can allow demonstrating mechanisms at the molecular level. For instance, Mandalari et al. [1] showed that native β-lactoglobulin interacts with gastric phosphatidylcholine in the stomach and is therefore protected from the hydrolytic action of pepsin. However, the experimental conditions can vary greatly from one study to another making comparison between studies difficult. Within the frame of the COST Action Infogest (www.cost-infogest.eu), a consensus *in vitro* digestion model based on physiologically-relevant parameters was set up by experts in the field [2]. Two inter-laboratory trials were performed to assess the reproducibility of the model. Forty scientists from all over Europe were trained on how to use the model that is also available through videos that can be watched on YouTube.

More sophisticated dynamic *in vitro* models are also available. They can be either mono-compartmental i.e. simulating only one part of the GI tract or multi-compartmental and have been reviewed by Guerra et al. [3]. Since they are dynamic, they take the transfer of the food in the different compartments into account as well as the regulation of the pH and the gastric and intestinal secretions. When relevant physiological parameters are available, they can represent alternatives to *in vivo* digestion especially on an ethical and economical point of view. Some have been validated towards *in vivo* data. For example, digestion of an infant formula has been performed using the DIDGI® system developed at INRA and, in parallel, studied using 18 piglets that were slaughtered after 30, 90 and 120 min [4]. *In vivo* effluents and *in vitro* digestates were analyzed for milk protein concentration and results showed that both casein and β-lactoglobulin concentrations were not statistically different demonstrating that the dynamic *in vitro* model used was physiologically-relevant.

Recently, some ingestible capsules initially developed to deliver drugs [5] have been used to monitor physiological parameters in humans like the pH, temperature and pressure in the different compartments. Some can even collect some samples at different locations of the GI tract.

Animal models are particularly adapted to investigate food digestion and the pig model is considered as the most relevant to mimic the upper part of the GI tract. Piglets have been used to simulate infant formula digestion [6] allowing to demonstrate that the composition of infant formula directly impacts the structure of the intestinal microbiota of the neonate [7]. Adult pigs can be canulated and catheterized to collect biological samples throughout digestion and are the most appropriate models to assess the bioavailability of a nutrient or

a bioactive compound. Using multi-canulated and catheterized mini-pigs, Barbe et al. [8] demonstrated that the food matrix structure drives the kinetics of protein digestion and of amino acid bioavailability.

Human studies remain the “gold standard” but are expensive and limited in terms of samples to collect. Digestion can be followed in humans by imaging techniques such as MRI [9]. However, in that case, effluents cannot be collected and characterized so the data collected will mainly provide information on the structure of the bolus in the stomach, the gastric emptying time etc. Patients with ileostomy can allow to access effluents. Finally, gastric and intestinal probes can also give access to effluents. Using this approach, Boutrou et al. [10] were able to identify and sequence more than 4700 peptides released in the jejunum after digestion of caseins or whey proteins.

Only few *in silico* models have been developed so far. Le Feunteun et al. [11] have published a mechanistic model of transit and absorption based on mini-pig *in vivo* data whereas Fouillet et al. [12] proposed a compartmental model of nitrogen metabolism. *In silico* models could constitute an interesting alternative in the future avoiding the use of animals or human volunteers.

In conclusion, several *in vitro*, *in vivo* and *in silico* digestion models are now available to investigate food digestion. They all have pros and cons. Besides ethical, financial and technical considerations that are, of course, to be taken into account, the choice of a model should mainly be based on the type of scientific question to answer.

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3.2. *In vitro* tools for bioaccessibility studies

3.2.1. Fate of chemical substances in food during human gastrointestinal digestion – Experiences on trace elements and nanomaterials

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Trace elements vary greatly in their ability to enter the human body and exert their biological effects. Characterizing element bioavailability is paramount in order to assess their beneficial or toxic effects in humans. For essential trace elements, bioavailability can be defined as the amount of the nutrient that is available for absorption in a form that is physiologically useful. Emphasis is on the utilization of the trace element for normal physiological functions, but it should not be forgotten that at high doses essential trace elements cause adverse effects as well. For non-essential, potentially toxic trace elements, bioavailability refers to their ability to be absorbed and reach the target organ, where they exert their adverse effects. Bioavailability varies considerably depending upon the element itself and many dietary and host-related factors. A key 'element-specific' property is the occurrence of the trace element in different chemical forms. Chemical speciation, along with aspects related to the interaction of the chemical species with the food matrix, critically affects the bioaccessibility of the element, i.e. the ability of the metals to be released from the food matrix, solubilized and become available for absorption through the gut wall. Selenium, an essential micronutrient for humans and animals that exists in a variety of organic and inorganic species in food, is a convenient example. Our studies on the speciation of the bioaccessible fraction of selenium in food, based on the combination of in vitro enzymolysis simulating human gastrointestinal digestion and hyphenated analytical techniques for selenium speciation (e.g. HPLC-ICP-MS), will be discussed.

In the case of nanomaterials, the fate during human GI digestion is the critical issue that influences the assessment of both the efficacy and safety of the chemical nano-sized agent. If the efficacy of an engineered nanomaterial added to food (e.g. increased bioavailability) is due to its nanoparticulate nature, degradation of nanoparticles (due to dissolution, aggregation, irreversible binding to food components, etc.) causes a loss of the specific (nano-related) activity. In terms of safety, if particles completely dissolve in the GI tract and are transformed

to the respective soluble (ionic or molecular) form, risk assessment will be based on the non-nanoform substance and not on the nanomaterial originally present.

3.2.2. A standardised static *in vitro* digestion method suitable for food – an international consensus

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Simulated gastro-intestinal digestion is widely employed in many fields of food and nutritional sciences, as conducting human trials are often costly, resource intensive, and ethically disputable. As a consequence, *in vitro* alternatives allowing for the determination of a variety of endpoints such as bioaccessibility of nutrients and non-nutrients, or digestibility of macronutrients such as lipids, proteins and carbohydrates, are used for screening and building new hypotheses. Various digestion models have been proposed, often impeding the possibility to compare results across research teams. For example, a large variety of enzymes from different sources such as of porcine, rabbit or human origin have been used, differing in their activity and characterization. Differences in pH, mineral type, ionic strength and digestion time, which alter enzyme activity and other phenomena, may also considerably alter results. Other parameters such as the presence of phospholipids, individual enzymes such as gastric lipase and digestive emulsifiers vs. their mixtures (e.g. pancreatin and bile salts), and the ratio of food bolus to digestive fluids, have also been discussed at length. In the present consensus paper, within the COST Infogest network [1], we propose a general standardised and practical static digestion method based on physiologically relevant conditions that can be applied for various endpoints, which may be amended to accommodate further specific requirements. A frameset of parameters including the oral, gastric and small intestinal digestion are outlined and their relevance discussed in relation to available *in vivo* data and enzymes. This consen-

sus paper [2] will give a detailed protocol and a line-by-line, guidance, recommendations and justifications but also limitation of the proposed model. This harmonised static, *in vitro* digestion method for food should aid the production of more comparable data in the future.

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3.2.3. Use of a validated, dynamic gastrointestinal model to determine the bioaccessibility of mycotoxins from multi-toxin contaminated diets

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A model of the stomach and small intestine (TIM-1) has been developed and validated as a reproducible system to simulate the gastrointestinal tract of monogastric animals (pigs, pre-ruminant calves and dogs) and humans (babies and adults). Simulating predetermined physiological parameters, such as meal size, peristaltic movements, pH, gastric and intestinal secretions, gastrointestinal transit, and absorption of digested products and water, the model is suitable for studies on digestion and bioaccessibility of food compounds and is a good alternative to *in vivo* experiments. The TIM-1 system, set-up to simulate the *in vivo* conditions of the porcine gastrointestinal tract, was used to determine the bioaccessibility of the ingested mycotoxins from two multi-toxin contaminated diets and the efficacy of a carbon/aluminosilicate-based product in reducing mycotoxin bioaccessibility. Mycotoxin levels in the diets were 19.9 and 5.9 mg/kg of fumonisins B_1 and B_2 (FB_1 and FB_2), 5.6 mg/kg of deoxynivalenol (DON), 1.3 mg/kg of zearalenone (ZEA), 0.187 mg/kg of ochratoxin A (OTA), and 0.193 mg/kg of aflatoxin B1 (AFB_1). Mycotoxins were absorbed from the small intestine at levels of 105% and 89% for FB_1 and FB_2, respectively, 87% for OTA, 74% for DON, 44% for AFB_1, and 25% for ZEA. The absorption of mycotoxins occurred mainly from the middle part

of the small intestine (jejunum) and less from the ileum. Samples collected at different time intervals (0-2, 2-4, and 4-6 h) showed that, with the exception of ZEA, maximum absorption of mycotoxins occurred in the first 2 h of digestion (0-2 h), was persistent for the following 2 h (2-4 h), and decreased during the later 2 h (4-6 h) of the experiment. ZEA was less and slowly absorbed in comparison to the other mycotoxins. These mycotoxin bioaccessibility data are similar to published *in vivo* data, showing the predictive quality of TIM-1. Supplementation of the diets with a carbon/aluminosilicate based product (up to 2%, w/w) significantly reduced the mycotoxin absorption in a dose-dependent manner, up to 88% for AFB_1, 44% for ZEA, and 29% for the fumonisins and OTA. The product was ineffective in reducing DON uptake. The findings of this study can help to interpret the *in vivo* studies on toxicology and carcinogenicity of mycotoxins and show that the TIM-1 system is a rapid and physiologically relevant method to test the efficacy of mycotoxin-binding agents.

3.2.4. Bioaccessibility of mycotoxins in artificially contaminated baby foods using the harmonized *in vitro* digestion model

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Objectives: Food products provide essential nutrients, but also contaminants that affect human health. Mycotoxins are fungal natural contaminants commonly found in a great variety of foods including baby foods. Patulin (PAT) is a mycotoxin found in fruits and fruit based products [1] and aflatoxin M_1 (AFM_1), the hydroxilated metabolite of AFB_1, is a potent carcinogen, mainly found in milk and milk based products [2]. Mycotoxins can form complexes with the food matrix that may cause a significant impact on their bioaccessibility - the proportion of the ingested contaminant in food that reaches the systemic circulation [3]. This study aimed to evaluate the bioaccessibility of the mycotoxins PAT and AFM_1 in powdered baby foods.

Methodology: A standardized static in vitro digestion method [4] was used to assess the bioaccessibility of PAT and AFM_1 in two different powdered baby foods: 3 cereal and fruit based baby food and 3 infant formulae artificially contaminated to 20 µg/kg of PAT and 500_µg/kg of AFM_1, respectively. Mycotoxins quantification was performed by HPLC-UV [1] for PAT and HPLC-FLD for AFM_1 [2].

Results: Patulin bioaccessibility in cereal and fruit based baby foods ranged between 49% to 61%. These results agree well with those reported by Brandon et al. [5] (84-100%) and are higher than those reported by Assunção et al [6] (28%), both in apple juices. AFM_1 bioaccessibility in infant formulae ranged between 86% and 104% which agree with results from Kabak et al [7]. Both methodologies had a RSD below 15%.

Conclusions: These are the first results on mycotoxins bioaccessibility using the standardised static in vitro digestion method developed by the COST action INFOGEST. Future work must be focused on analyzing a broader number of samples in order to assess the influence of different food matrix in mycotoxin bioaccessibility.

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3.2.5. Risk/benefit associated to the consumption of raw and cooked blue shark (prionace glauca) based on total mercury, methyl-mercury and selenium bioaccessibility

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The aim of this study was to identify the hazard associated with raw and cooked blue shark consumption taking into account the bioaccessibility of Se, Hg and MeHg, by using *in vitro* digestion method. Atomic absorption (graphite furnace and automatic mercury analyser) and ICP-MS techniques were used to determine the studied elements. Selenium, Hg and MeHg levels were higher in cooked samples, particularly in grilled blue shark. Selenium bioaccessibility was above 83% (grilled samples) whereas Hg and MeHg bioaccessibility was lower in grilled samples, with values reaching 50%. In addition, all Se-Health Beneficial Values were negative and the molar MeHg: Se ratios were higher than one. The risk-benefit assessment evidenced a maximum consumption of one yearly meal for raw or cooked blue shark, thus emphasising the need to recommend the diversification of seafood species consumption in a balanced and healthy diet.

3.2.6. Do cooking procedures influence mercury levels in commercial fish? Associated risk through human consumption

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Objective: The main goal of this study was to evaluate the influence of 3 cooking procedures (boiling, frying, grilling) and 3 seasonings (salt, lemon, combined) on the total and organic Hg levels found in 3 commercialized fish species (*Scomber scombrus*, *Aphanopus carbo*, *Dicentrarchus labrax*). The influence of boiling in releasing Hg to the cooking water was also evaluated.

Methodology: Fish samples were randomly purchased from 2 big supermarkets in Portugal. The edible part (dorsal muscle) was divided into portions that were weighed and randomly distributed to raw, fried, grilled and boiled procedure (n=10/ treatment). Cooking procedures were carried out in a domestic kitchen gas. Fish fillets were weighed before and after cooking and the results were expressed in wet weight. At the laboratory, all fish samples were freeze-dried and ground to a fine powder for further analyses of Hg concentrations. Total and organic Hg were analyzed by atomic absorption spectrometry with thermal decomposition and gold amalgamation, using an Advanced Mercury Analyser (AMA - LECO 254). The total Hg content of the water used in boiling procedure was also analyzed using the AMA – LECO 254. The estimation of the risk to human health by the intake of mercury due to fish consumption was characterized using the Hazard quotient (HQ).

Results and conclusion: The Hg levels found in muscle after cooking were species specific. While for *S. scombrus* and *A. carbo* the Hg levels decreased when cooked, for *D. labrax* concentrations remained similar in raw and cooked fish. Seasoning did not change Hg levels in fish. The Hg found in the boiling water reached the recommended threshold for drinking water in all species, and the maximum threshold for A. carbo, indicating significant Hg loss to the water during boiling. All cooking procedures effectively reduced organic Hg levels. *S. scombrus* and *D. labrax* emerge as healthy and safe choices, while moderate consumption of *A. carbo* is advised.

3.2.7. The impact of cooking process in bioacessibility of minerals from *Chenopodium quinoa*

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Quinoa seeds (*Chenopodium quinoa*) are usually reported as a good source of minerals, however, information about *in vitro* bioacessibility is scarce. The aim of the present study was to estimate the minerals bioaccessibility of quinoa seeds using different cooking processes: raw, steamed and boiled. The digestion of samples was performed using the harmonized static "*in vitro*" digestion protocol method (IVD) [1], with a minor modification concerning the oral phase enzymatic composition (bacterial α-amylase). The copper (Cu), manganese (Mn), iron (Fe), zinc (Zn), magnesium (Mg), calcium (Ca), phosphorous (P), sodium (Na) and potassium (K) contents were determined by inductively coupled plasma optical emission spectrometer (ICP-OES). Raw quinoa seeds presented the highest bioaccessibility values for Mn, K, P (100%) and the lowest for Ca (19%). After cooking process the highest values of bioaccessibility were observed for Ca (100% in boiled and 74% in steamed) and Cu (90% in boiled and 78% in steamed). Mn showed the highest decrease in bioacessibility when comparing raw and cooked samples (100% and 20% in raw and cooked samples, respectively). Zn presented bioaccessibility values near 40% for raw, boiled and steamed samples. The boiling process was the procedure that allowed to obtain the highest mineral bioaccessibility values for all the assayed minerals. These are the first results that describe the bioaccesisiblity of minerals of quinoa seeds using the harmonized IVD method.

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3.3. Toxicological interactions between mycotoxins

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Abstract

Humans and animals are exposed to several toxins at the same time. The toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic. Several approaches have been used to determine the interaction between mycotoxins. The theoretical biology-based models of additivity, especially the Chou-Talalay method, are the most advanced. Using this latter model in several cellular systems and in an ex vivo intestinal explants system, we have observed a synergistic toxicity for trichothecenes, especially at low concentrations. The synergistic effects observed after exposure to a mixture of low concentrations of mycotoxins could pose a significant threat to public health.

Introduction

Mycotoxins are toxic secondary fungal metabolites produced under specific environmental conditions by a variety of food commodity spoiling molds, mainly *Aspergillus*, *Penicillium* and Fusarium. Global surveys indicate that 72% of the samples of feed and feed raw materials are positive for at least one mycotoxin [1]. Human and animals are simultaneously exposed to several mycotoxins and there is a need for an update of the traditional single mycotoxin risk assessment approach [2]. Indeed, simultaneous exposure to different toxins could result in antagonistic, additive or synergistic effects. Therefore, an increasing number of mycotoxin studies are devoted to their combined toxicity, especially the exploration of the type of toxicological interactions.

The toxicity of a mixture is complex. Testing for a possible interaction in mixture toxicity requires a comparison of the actual experimentally determined effects of the mixture to theoretically expected no interaction effects, the so-called additive effects. This prediction of no interaction is made based on the toxicity of the individual compounds. Stronger-than-

expected effects indicate synergism whereas lower-than-expected effects indicate antagonism. Several methods have been proposed but a generally agreed definition of zero interaction does not yet exist [3].

As for other food contaminants, the gastro-intestinal tract can be considered the first target for mycotoxin toxicity, and gut damages caused by these contaminants may lead to poor intestinal health [4-5]. The possible overlapping intestinal residency times of the numerous contaminants that can be carried by food could also make the gastro-intestinal tract one of the most exposed organs to mixture toxicity. We present here the analytical approaches used in mycotoxin toxicological interaction studies and the preliminary lessons we learnt from the combined toxicity of *Fusarium* mycotoxins towards the intestine.

Experimental approaches to assess mycotoxin toxicological interactions

The arithmetic definition of additivity. Some mycotoxin combination studies considered the additive effect to equal the arithmetic sum of the sizes of the effects for individual mycotoxins when tested separately. So the expected (additive) size for the cytotoxic effect of a mixture was defined as the sum of the cytotoxic effects induced by each mycotoxin alone in mono-exposure experiments [6].

Cytotoxic effect (mycotoxin 1 + mycotoxin 2) = Cytotoxic effect (mycotoxin 1) + Cytotoxic effect (mycotoxin 2)

Although intuitively plausible and very easy to handle, most researchers in the biomedical area seem to agree that combined effects do not simply equal the sum of single effects [7].

Factorial design experiments. The general assumption for mycotoxin studies using factorial design experiments is that when testing the effects of mixtures by different patterns of combination on the one hand, and the effects of each individual compound on the other, the effect of any compound could be predicted by subtracting the mean of the groups not containing the compound from the mean of the other groups [3].

Despite the fact that interaction is definitely revealed by such statistical methods, the nature of interaction with regard to additivity, synergism or antagonism is not clearly explored and has to be inferred indirectly [8].

The theoretical biology models-based definitions of additivity. The most commonly used theoretical biology models-based definitions of zero interaction are the Bliss independence criterion also known as Response Addition, the Loewe additivity model also named Concentration or Dose Addition [9] and the Median Effect Principle of the Mass action law [10].

The Bliss independence criterion applies for combinations of mycotoxins exerting toxicity via different modes and possibly sites of action. Conversely, the Loewe additivity model studies lie on the assumption that the mycotoxins act on the same biological sites, by the same mechanisms of action and differ only in their potency. Relatively simple Loewe additivity model extensions are the isobolographic method and its algebraic variant, the Interaction index.

Another concept that is independent of the mode of action and just considers both the potency (EC50) and the shape of the dose-effect curve for each mycotoxin and their mixture has been proposed. In this new approach, a computerized simulation of the individual dose-effect curves and the additive response from the combined effect of several mycotoxins is obtained using the Median-Effect Equation of the Mass action Law [10].Then interactions can be analyzed by a Combination-index – isobologram method. Besides indicating the type of interaction (additivity, synergy or antagonism), this index allows a quantitative assessment of the magnitude of the interaction.

As of 2015, 82 publications described mycotoxin *in vitro* interactions. Most of these publications (54) described experiments lacking dose-response considerations and assuming arithmetic additivity; a few publications (7) were factorial design experiments; the theoretical biology model-based experiments are gaining increased attention (21 publications).

Analysis of mycotoxins' combined toxicity

The studies concerning the in vitro interactions between mycotoxins mainly concern the regulated mycotoxins, especially aflatoxins, ochratoxins, fumonisins, zearalenone and trichothecenes, a few studies also concern the "emerging" toxins such as beauvericin and enniatins.

Interaction between Ochratoxins and other toxins. Among the 82 publications, 21 concerned the nephrotoxic ochratoxins. Of course, most of these studies involved renal cell lines or renal primary cells cultures with cytotoxicity as the main endpoint. However, mycotoxins associations including ochratoxins have also been screened for genotoxicity via DNA damages, clastogenic effects and mutagenic activity.

Interaction between Aflatoxins and other toxins. 24 papers questioned the in vitro genotoxic and cell viability effects of the hepatocarcinogenic aflatoxins in association with other mycotoxins. Aflatoxins combinations have been assessed for their cytotoxic and genotoxic effects using mainly human or animal primary hepatocytes or transformed cell lines, while papers addressing specifically the mutagenic activity referred to the Ames test using *Salmonella* Typhimurium strains.

Interaction between Fusariotoxins. Papers analyzing the combined toxicity of Fusarium group mycotoxins were the most abundant (37/82). The *Fusarium* mycotoxin combinations studies can be grouped in 3 groups involved (i) the major *Fusarium* toxins (i.e. Deoxynivalenol, Zearalenone, Fumonisin B1) studies, (ii) the trichothecenes group mycotoxin association and (iii) other studies involving the emerging *Fusarium* toxins (beauvericin and enniatins group). Our team gathered evidences for low dose synergistic intestinal toxicity for type B trichothecenes and antagonistic interaction for the most prevalent emerging *Fusarium* toxin enniatin B1 and the type A trichothecene T-2 toxin using the combination index-isobologramm method [11-13]. Proliferating human colorectal adenocarcinoma Caco-2 cells exposed to binary or ternary mixtures of type B trichothecenes (Deoxynivalenol, Nivalenol, and their acetylated derivatives) demonstrated mainly synergistic cytotoxicity at low mycotoxin concentrations (cytotoxic effect between 10 and 30-40 %). At higher concentrations (cytotoxic effect around 50 %), the combinations had an additive or nearly additive effect. This synergistic intestinal cytotoxicity of type B trichothecenes was confirmed on non-transformed porcine intestinal epithelial IPEC cells, with magnitude of synergy for 10% cytotoxicity ranging evaluated to range from 2 to 7. Conversely, porcine jejunal explants culture and IPEC cell culture exposed to enniatin B_1 and T2-toxin mixture exhibited concordant antagonistic toxicity. Altogether, these results indicate that the simultaneous presence of mycotoxins in food commodities and diet may be more toxic than predicted from the mycotoxins alone. Moreover synergistic toxicity may result from co-exposure to trichothecenes. This synergy should be taken into account considering the frequent co-occurrence of trichothecenes in the diet and the concentrations of toxins to which consumers are exposed.

Conclusion

For the main mycotoxins, reference doses for regulatory purpose already exist. Exposure below these levels is usually considered safe. Whether the consumer is also protected against combined exposure to mycotoxins if each component is present below its individual threshold dose is gaining increasing interest. A crucial issue for toxicodynamic interaction analysis is

the statement of the non-interaction response. Factorial designs allow a reliable detection of departure from the additive response, while the Chou-Talalay method makes it possible to determine the type of the interaction and to optionally quantify its magnitude.

Many studies, using different methodological approaches have been used to explore the interactions in mycotoxin combined toxicity. The main conclusions from all these studies are that (i) very few studies used a robust methodological approach for the analysis of the combined effect of mycotoxins, and (ii) the type of interaction in terms of additivity, synergy or antagonism varies accordingly with the mycotoxin combinations, and even with the concentrations tested. More studies employing the isobologram approach are needed to feed a reliable database for the interactions between mycotoxins.

Experiencing the latest approach, our lab demonstrated that trichothecenes mycotoxins exert synergistic low dose intestinal toxicity. These in vitro synergistic interactions deserve to be confirmed *in vivo*.

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3.4. Predicting the toxicity of multiple chemicals by using conceptual mathematical models

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Abstract

Exposure is usually characterized by a multiple exposure rather than a single chemical exposure scenario. This assumption is both true for human health and the environment and several methodologies have been used to explore patterns of response upon multiple stressors exposure.

Conceptual models for additivity (Independent Action or Concentration Addition) are often used to predict toxicity of mixtures, leading often to conclusions on chemicals' interaction inside the organism(s) and therefore inducing synergistic or antagonistic patterns.

The approaches for mixture toxicity evaluation will be carried out exploring different methodologies.

Introduction

Exposure is usually characterized by multiple stress factors rather than single exposure scenario. This assumption is both true for human health and the environment and several methodologies have been used to explore patterns of response upon multiple stressors exposure [1].

In the beginning of the 20th century, aiming at improving pharmacological combinations to increase target effects, conceptual models were described and they were mainly based on the similarity of the chemicals' modes of action (MoA) and on the "no interaction" principal between these chemicals. In 1929, Loewe and Muischnek [2] discussed the dose or concentration addition (CA) model which assumes that chemicals have the same MoA and

it is characterized by a concentration-based summation of toxicity of chemicals, scaled to reflect their relative toxicities. In this approach chemicals can act as dilution of each other (equation 1).

$$\sum_{n}^{i=1} \left(\frac{Di}{EDxi} \right) = 1$$

Where Di denotes the doses of the individual chemicals in the mixture, and EDxi indicates the equivalent effect dose of the single chemicals, which alone would cause the same quantitative effect x as the mixture.

Alternatively, the model of independent action (IA) was described by Bliss [3] where the assumption that chemicals affect organisms through different MoA is presented, and their effects are therefore statistically independent of each other, calculating effects by multiplying the probabilities of responses (equation 2).

$$Y = u_{\max} \prod_{i=1}^{n} q_i \left(C_i \right)$$

where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, $q_i(C_i)$ the probability of non-response, umax the control response for the selected endpoint and Π the multiplication function.

This model is based on probabilities of effects in non-affected fractions but still is considered an additive model. Briefly, this concept assumes that when acting in different targets two chemicals will induce effects in non-affected fractions of the organism.

Therefore, the first insight during data modeling is usually to decide which conceptual model to use, based on the MoA. But often MoA are described for toxicity model organisms, or for certain target organs and ambiguity rules when transposing to the bio-testing model used. One clear example can be described when looking at studying effects of insecticides in plants. If one looks at an insecticide that was designed to interact in the central nervous system, by blocking acetylcholinesterase, this MoA is unmanageable to transpose to plants.

Therefore it has already been described by several authors (e.g. Altenburger et al. [4]) and recently pinpointed by EFSA that the conceptual model Concentration Addition can be used as default as the most conservative one. In addition, in the summary report of EFSA Scientific Colloquium 21 on "Harmonisation of human and ecological risk assessment of combined exposure to multiple chemicals" [5], the MoA concept and how to use it has been identified as one key issue for correctly approach combined stressors. In cumulative risk assessment, to support the use of the IA conceptual model the MoAs have to be clearly separated from each other, but also that this distinction can provide low to no advantage or relevance. To overcome this difficult issues, adverse outcome pathways (AOPs) may be of more interest has they can show a more integrated and overall picture of the effects of stressors. Therefore besides grouping chemicals by structural similarity in read across procedures, looking at the overall effects on a common target organ or organism may be also considered a foundation for grouping.

TU approach

In order to determine the potential additivity of multiple stressors, their concentration-effect relationship can be seen as their strength. The strength of a concentration (Ci) is usually converted to Toxic Units (TU) and it is related to an established endpoint like the EC50/ED50 (equation 3).

$$TU = \frac{C_i}{ED_{50}}$$

By establishing TUs for a multitude of chemicals, their cumulative strength can be calculated using both conceptual models and predicted effects. With the TU approach, strengths can be summed while for concentrations this is not advised as they are based on mass or molarity, being therefore impossible to specify which chemical will the mass refer to.

Experimental designs

To choose the most suitable experimental design for a certain study, several factors have to be taken into account. The first one regards the endpoint to be tested. Depending on this a full factorial design or a ray design may be an option to cover the larger range of exposure, as possible. When binary data is present (e.g. yes or no; dead or alive), full factorial designs

may be advised covering even high concentrations of both chemicals which will therefore derive a complete response (e.g. total cell unviability) (Fig. 1a).

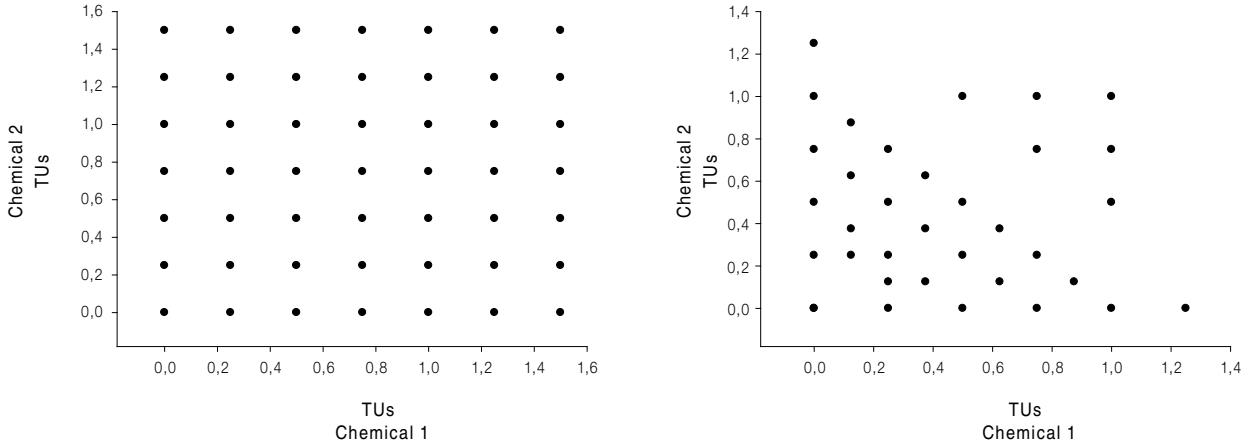

Fig.1 – A schematic design of toxic units combinations used for chemical binary mixtures – full factorial design (a) and fixed ray design (b).

In the case of continuous endpoints (e.g. growth, enzymatic activity), a fixed ray design is advised, choosing several dilution ratios (rays) and covering the lowest area of exposure. With this option, data will be not lost due to inviable points (Fig. 1b).

Binary experimental designs will combine single exposures of both chemicals and the trial for mixtures along with a control and positive and solvent controls whenever necessary. Considering the effort of covering the major area of exposure, decreasing the number of replicates may compensate the overload of experimental work. If an experimental setup includes a full factorial design with five concentrations for each chemical compound, the setup will be constituted of 35 treatments that will usually be replicated. Regarding the balance between what is labor feasible and the commitment of having a statistical viable experimental design, the statistical procedure must be adjusted.

Synergism and Antagonism

Often chemicals can interact after entering in the organism and depict synergistic or antagonistic patterns of toxicity. All these approaches rely on the previously studied single toxicity effects in order to estimate deviations from additivity (CA or IA). To evaluate if more or less toxicity was observed, a first approach on relying on the strength of the mixture has to be followed. By looking at the combined strength and on the isolates strength, one can calculate

what is expected to occur, based on the mathematical assumptions for additivity. If upon comparison of the observed (real) effects with the expected, predicted by the mathematical models, there are significant differences, then patterns for synergism and/or antagonism can be derived.

The Paracelsus paradigm that the dose makes the poison can be also pinpointed under the multiple chemicals umbrella, when looking at synergistic and antagonistic patterns. Looking at a binary mixture as the most basic example, besides the fact that one of these two patterns can be seen throughout a considerable concentration range of the mixture, both patterns can also be present. Patterns for synergism/antagonism can shift to antagonism/synergism depending on the concentrations present of both chemicals, having one pattern at low doses, shifting to the other one at higher doses. In addition, this shift can occur at high effect or low effect levels. Another deviation depending on the dominant chemical in the mixture can also occur, i.e. the chemical with highest strength or at concentrations inducing higher effects. Therefore the shift from synergism to antagonism is dependent on the chemical's ratio.

Data presentation

Several approaches can be used to present multiple chemical data effects. For binary mixtures, presenting or comparing EC50/ED50s from single with those from co-exposures can highlight changes in toxicity patterns. This approach is often defined as synergistic ratios and it is used especially when one of the chemicals does not induce toxicity or when a complete dose-response curve is not achieved within the concentration range tested. In this case a full factorial design may often be suggested while defining the experimental design [6].

Isobolograms are also often used to express patterns of response of binary combinations of chemicals. Although they can sometime be difficult to interpret, data in the isobols are

the responses obtained or modelled. The shapes/curves of isobols can be compared with the conceptual models for additivity and show the toxicity patterns observed [7,8] (Fig. 2).

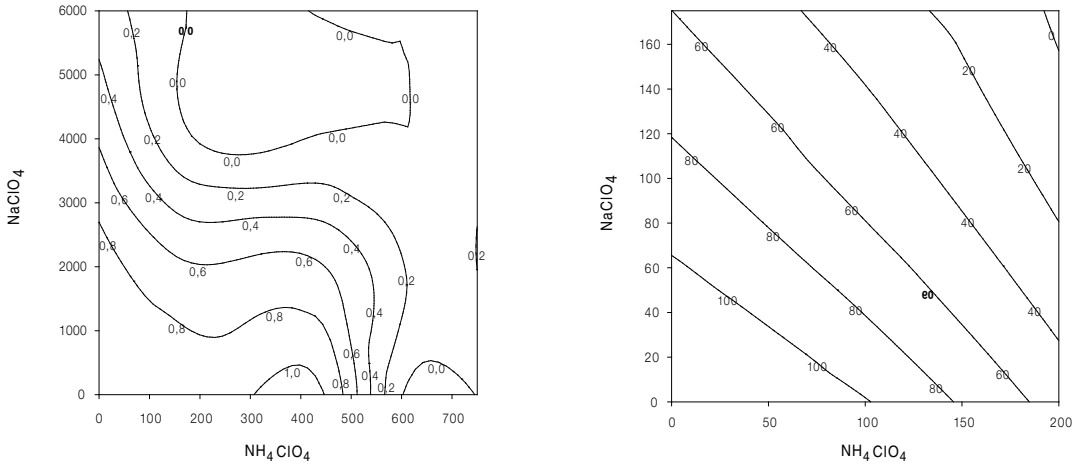

Fig. 2- Examples of isobolograms depicting response patterns for mixture toxicity of two perchlorates (sodium and ammonium perchlorates) to *Daphnia magna* (from Loureiro et al. [8]): left isobologram describes a dose-ratio deviation, for synergism when sodium perchlorate is dominant in the mixture and antagonism when ammonium perchlorate dominates (i.e. dominance is determined by having higher concentrations/strengths than the other chemical in the mixture); the right isobologram describes a additivity pattern (following the Concentration Adition conceptual model)

The curve-shift analysis is also another methodology that can be used to explore and present data for mixture toxicity. In this methodology, the single dose-response curves are calculated as well as the mixture dose-effect curves, using also the TU approach (Fig. 3). Then the shift for dose response curves to the left side will depict synergism, showing that that a smaller strength of the mixture is necessary to induce the same effect. If the shift is to the right side, the TUs inducing the same effect are higher and derive for antagonism.

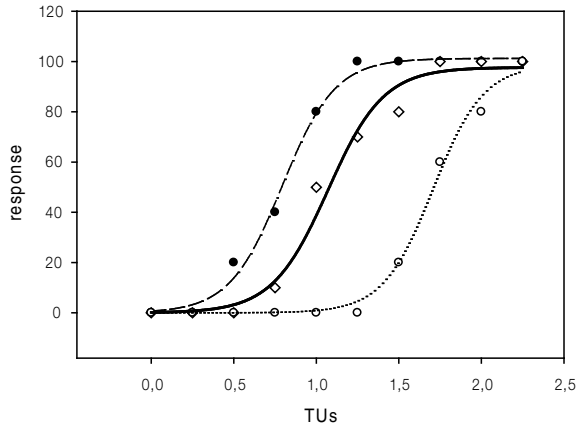

Fig. 3- Effect curves based on mixture TUs. The middle curve (solid) depicts the additivity effects with 1TU=50% effect; the left dashed line, 0.7TU=50%; the right point line, 1.7TU=50% effect.

Overall the experimental design will be crucial to derive accurate results on mixture toxicity. In addition, the procedure to analyse or model the data should be in accordance with the experimental design along with the results obtained. Data presentation is a way to clarify the results obtained showing them in a more efficient and clear manner. Therefore both tables and figures should be used to elucidate results and therefore the clearer approach should be chosen.

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3.5. Insights into individual and combined toxic effects

3.5.1. Combinatory effects of mycotoxins

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Despite tremendous efforts in prevention strategies and enhanced possibilities in preservation in the last decades, the occurrence of mycotoxins in food and feed still represents one of the most prevalent contaminations. Mycotoxins are secondary metabolites of fungi, whereby fungi species belonging to different genera are known to generate mycotoxins, thus resulting in toxins with high structural diversity.

The development of sophisticated MS-based “multi”-methods has opened the possibility to assess concomitantly the occurrence of a broad spectrum of mycotoxins in one sample in reasonable time. Respective data published so far demonstrate that often food and feed is not contaminated with only one single but a spectrum of different mycotoxins. This might result from co-contamination of the commodity with different toxin producing fungi genera, like e.g. *Alternaria*, *Fusarium*, *Aspergillus* or *Penicillium* and/or the activity of fungi generating different mycotoxins at the same time.

For example, *Alternaria* spp. are capable to produce more than 120 secondary metabolites of which about 25% are designated as mycotoxins. This highlights the importance of considering combined effects interactions between potentially co-occurring mycotoxins. Conceivable interactions might affect toxicokinetic as well as toxicodynamic parameters. Proceeding along the pathway of a compound through the body, absorption, distribution, phase I and phase II metabolism and excretion, but also very specific effects like genotoxicity or induction of oxidative stress may be modulated by other substances consumed at a time.

Recent studies demonstrated that several binary mixtures of mycotoxins exhibit significant differences in cytotoxicity compared to the expected values based on the effects of the single compounds. For example in human colon carcinoma cells, enniatin B1 has been found to modulate the cytotoxicity of several other *Fusarium* toxins. In this respect, studies on modulatory effects should not be restricted to genotoxic compounds. Recent *in vitro* studies on human colon carcinoma cells showed that tenuazonic acid, a mycotoxin produced by

Alternaria spp., has minor cytotoxicity in this model but is capable of modulating the toxicity of other mycotoxins produced by *Fusarium* spp..

These results underline the necessity of further studies on combinatory effects of mycotoxins and co-occurring secondary metabolites to elucidate potential relevance for risk assessment.

3.5.2. Combined toxicity of Aflatoxin B_1 and Ochratoxin A in *in vitro* and *in vivo* models

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Aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) are two mycotoxins that contaminate a great variety of foodstuffs. AFB_1 is a well-known liver genotoxic carcinogen for humans (IARC: group 1) while the mechanism of action of OTA, a potent renal carcinogen in rats, is still under debate (IARC: group 2B). Despite the well-known human co-exposure to mycotoxins, most toxicological studies have been carried out in conditions of single exposure to one mycotoxin. The aim of this project was to explore the combined toxicity of AFB_1 and OTA *in vitro* and *in vivo*. The ability of AFB_1, OTA and the combination of both to cause DNA strand breaks and oxidative damage was evaluated with the comet assay with and without S9 (3-24h) in Hep G2 cells. Cytotoxicity and radical oxygen species (ROS) induction capability was also evaluated. A single oral dose of AFB_1+OTA was administered to male F344 rats to explore the kinetics of the mixture *in vivo*. For *in vivo* genotoxicity evaluation, F344 rats were treated with a single dose of AFB_1 (0.25 mg/kg b.w.), OTA (0.5 mg/kg b.w.) or both mycotoxins. The micronucleus assay (MN) (bone marrow), comet assay (liver and kidney), biochemical/histopathological and transcriptomic analysis were performed. In vitro, a significant ROS formation was detected in single and combined treatments. AFB_1 was genotoxic after 3h (+S9) and after 24h (-S9). Co-exposure to OTA significantly decreased DNA damage induced by AFB_1. In the kinetic study, the effect of OTA on AFB_1 kinetics could not be assessed but AFB_1 seemed not to affect OTA kinetics. In the in vivo genotoxicity study, the combined treatment reduced the toxicity and number of MN produced by AFB_1. In the comet assay, positive results were obtained for AFB_1 in the liver and for OTA in the kidney. The combined treatment reduced DNA damage in the

liver and had no influence in the kidney. These results may be indicative of an antagonistic relationship regarding the genotoxicity of both mycotoxins in the liver.

Acknowledgments:

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3.5.3. Evaluation of combined cytotoxic effects of ochratoxin A and fumonisin B_1 in human liver and renal cells

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Mycotoxins are fungal food contaminants with potential to cause severe acute and chronic conditions [1]. Therefore, food contamination with mycotoxins such as ochratoxin A (OTA) and fumonisin B1 (FB_1) causes great concern. Previous studies addressed the co-occurrence of these toxins in foods [2], however there is little knowledge on their combined cytotoxic effects. In the present study we aimed to evaluate the cytotoxic effects of mixtures of OTA and FB_1 in two human-derived cell lines. For this purpose, neutral red and MTT assays were performed. In HepG2 cells, OTA caused a significant decrease in cell viability after 24h exposure (above 10 µM; $p<0.001$), with an IC_{50} of 27.5 µM. However, no significant cytotoxic effects were observed after 24h exposure with FB_1. When in mixture, both mycotoxins caused a non-significant decrease in the viability of HepG2 cells compared to the effects of the FB_1 individually.

In HK-2, OTA caused a significant decrease in cells viability after 24h exposure (above 5 µM; $p<0.001$), with an IC50 of 7.5 µM. Also, exposure to FB_1 during 24h caused significant cytotoxic effects (above 320 µM; $p<0.001$), with an IC_{50} of 1.1 mM. The mixture of both toxins was significantly different from all the respective individual treatments of OTA and FB_1 ($p< 0.006$).

After modelling these data with the Concentration Addition conceptual model, there was a significant deviation for the dose level pattern, depicting a synergism at low dose levels of both mycotoxins, but changing to antagonism at higher doses. Therefore, considering the lower doses as the more relevant and potential to occur, this results highlight the importance of studies like this, since an increase in toxicity was observed, being higher than expected. These results agree with those presented by Creppy *et al*. with synergistic effects between OTA and FB_1 in Vero cells [3]. Further work must be performed to disclose which genotoxic effects these toxins might cause to these cell lines.

Acknowledgments:

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3.5.4. Modulatory effects of enniatin B_1 on the cyotoxicity of selected *Fusarium* toxins

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Infestation of cereals and fruits by mycotoxin producing fungi and consequently the entry of mycotoxins into the food chain is a worldwide health issue. At present, risk assessment of mycotoxins is generally based on the evaluation of single compounds. Recent studies, analysing mycotoxin contaminations of feed and foodstuff by multi-methods, enabling the concomitant detection of a spectrum of contaminats, suggest, that defilement of a product by just one mycotoxin does hardly occur in contrast to co-contaminations with several compounds, which is found in most of the tested samples [1,2].

Therefore, exposure of humans and animals is not limited to one mycotoxin at a time. So far, the knowledge on combinatory effects of different mycotoxins is still scarce and, considering the diversity of compounds found in co-contaminations, the great need for further investigations of combinations becomes apparent.

Methods: In this study, special focus was layed on the fusarotoxin enniatin B_1 and its potential to modulate the toxicity of other selected mycotoxins produced by *Fusarium* spp., deoxynivalenol, nivalenol, zearalenone and aurofusarin. Preliminary tests on cytotoxicity of single compounds were conducted. Based on these data cytotoxic and non-cytotoxic concentrations were chosen for further tests on binary mixtures of mycotoxins in corresponding doses. Assessment of cytotoxicity was performed in the WST-1 in the colorectal adenocarcinoma cell line Caco-2.

Results: Evaluation of the cytotoxic effects of binary combinations in consideration of the cytotoxicity data of each single compound indicate that enniatin B_1 modulates the toxicity of several fusarotoxins in an extenuating manner, especially those of deoxynivalenol and nivlenol.

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3.5.5. Possible antagonistic effect of three *Fusarium* mycotoxins on genotoxicity of spermatozoa of breeding rabbit bucks

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Fusarium species can produce several mycotoxins e.g. FB_1, DON and ZEA which are frequent contaminants of cereals intended either for human or animal consumption. *F. verticillioides* and *F. proliferatum* produce FB_1 whereas *F. graminearum* is the main producer of DON and ZEA resulting in co-occurrence.

Although multimycotoxin contamination occurs very often, most studies focus on single effects. Moreover the mycotoxins' concentrations used in the experimental diets are usually high and unlikely to occur in nature. Data on the effect of combined mycotoxins consumption in low dosages (according to EU recommendations) are scarce. In addition there are no studies assessing the genotoxicity of these *Fusarium* toxins on reproductive system and especially on spermatozoa after *in vivo* exposure.

The aim of this study was to investigate the genotoxicity of FB_1, DON and ZEA alone as well as in combination in low dosages on spermatozoa derived from breeding rabbit bucks after *in vivo* treatment.

The rabbit bucks were fed for 65 days with the experimental diets; Control (C), FB_1 (F), ZEA+DON (ZD) and FB_1+ZEA+DON (FZD), the concentrations were 0 mg/kg, 5 mg/kg, 0.25+1 mg/kg and 5+0.25+1 mg/kg respectively. On day 65, semen was collected and Comet assay was performed to assess the genotoxicity (DNA damage).

According to the Comet assay results, F treatment resulted in significantly less 0 comets compared to other treatments. Regarding score 1 all toxin treatments had similar proportions. As for score 2, F had significantly increased number of cells compared to FZD. Few cells had score of 3 (maximum 0.625%) whereas no cells with score 4 were found. It can be concluded that the combined toxins act rather antagonistically than FB_1 alone since the combination led to lower DNA damage (higher score 0 and lower score 2).

This is the first time interactive genotoxic effects of FB_1, DON and ZEA on rabbit spermatozoa are reported.

3.5.6. Effect of gamma radiation on the citotoxicity and estrogenicity of Zearalenone

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Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by several species of *Fusarium* mostly on cereals and corn. ZEA has a relatively low acute toxicity but it interferes strongly with estrogen receptors and, consequently, with the reproductive tract of individuals. Many methods have been used to eliminate mycotoxins from foods and feeds. Gamma radiation has been also investigated for mycotoxins detoxification showing some promising results.

The purpose of present study was to evaluate the cytotoxicity of ZEA degradation products obtained after its irradiation and also to evaluate their estrogenicity. The effect of water during the irradiation process was also evaluated.

Vials with 60µM of ZEA at distinct moisture levels (dehydrated and in water) were irradiated with 0, 2.0 and 10.0 kGy doses. ZEA levels were determined by HPLC with fluorescence detection. Cytotoxicity of ZEA was assessed in Hep G2 cells using a battery of assays covering different modes of action including alterations of metabolic activity (AlamarBlue assay), plasma membrane integrity (CFDA-AM assay) and lysosomal function (NRU assay). The estrogenicity was assessed in HeLa 9903 cells, measuring luciferase activity.

It was observed that gamma radiation is effective in reducing ZEA concentration, and that the presence of water enhanced significantly its degradation. A reduction of irradiated samples cytotoxicity related to metabolic activity and lysosomal function was also observed. ZEA didn't show any toxicity in the plasma membrane integrity. Since ZEA reduction was more effective in water samples, the reduction of cytotoxicity was also higher in this case (up to 94% in NRU assay). ZEA estrogenicity was also reduced with the increase of radiation doses. This reduction was higher in aqueous solutions (less 80%) than in dried conditions. These results point out that irradiation may contribute to reduce levels of ZEA and its toxicity on food commodities.

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3.5.7. Insights of enteropathogenic effects of mycotoxins on the human intestinal gut mucosa

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Intestinal mucosa is the first biological barrier encountered by natural toxins and it could be exposed to high amounts of dietary mycotoxins[1,2]. Trichothecenes, ochratoxin A and patulin (PAT) are the best known enteropathogenic mycotoxins able to alter functions of the intestine[3].

Objectives: This study aimed to evaluate the effects of PAT, a mycotoxin produced by *Penicillium* spp. during fruit spoilage, on barrier properties and function of the gut mucosa.

Methodogies: Viability (MTT), proliferation (^{3}H-thymidine incorporation assay), transepithelial electrical resistance (TER), SDS-PAGE and immunoblotting and flow cytometry methodologies were applied in order to characterize the effects of PAT on intestinal cell model (Caco-2), human peripheral blood lymphocytes (PBL) and human blood monocyte-derived dendritic cells (DC).

Results: PAT exposure reduced Caco-2 cell viability at concentrations above 12µM. The integrity of the Caco-2 monolayer was affected by PAT exposure, as demonstrated by a decrease in TER values, becoming more pronounced at 50µM. No effects were detected

on the expression levels of the tight junction proteins occludin, claudin-1 and claudin-3 at 50μM. However, the expression of zonula occludens-1 (ZO-1) and myosin light chain (MLC) declined and levels of phospho-MLC increased, after 24h of exposure to 50μM of PAT. T cell proliferation was highly sensitive to PAT with the major effects for concentrations above 10nM of PAT. The same conditions did not affect the maturation of DC.

Conclusions: PAT causes a reduction in Caco-2 barrier function mainly by perturbation of ZO-1 levels and phosphorylation of MLC. Low doses of PAT strongly inhibited T cell proliferation induced by a polyclonal activator, but had no effect on the maturation of DC. These results provide new information that strengthens the concept that the epithelium and immune cells of the intestinal mucosa are important targets for the toxic effects of food contaminants like mycotoxins.

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3.5.8. Patulin induces primary keratinocytes proliferation involving EGFR-mediated Akt and MAPKs signaling pathways leading to Cyclin D1 and COX-2 expression

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Patulin (PAT), a present day major contaminant of commercial apple and apple products is reported to be carcinogenic, embryotoxic, and immunotoxic. While oral and inhalation are considered to be the most prevalent routes of exposure to this toxin, exposure through skin is now being extensively investigated. Our previous study showed that short-term dermal exposure to PAT resulted in toxicological injury to the skin, while long-term exposure induced skin tumorigenesis. In this study, we explore the mechanism involve in proliferation of mouse keratinocytes by PAT. Our study revealed that PAT rapidly induces phosphorylation of EGFR, activation of the Ras/MAPKs, and Akt pathways. This in-turn leads to the activation of NF-kB/AP-1 transcription factors which then binds to the promoter region of the cell growth regulatory genes Cyclin D1 and COX-2 inducing their expression leading ultimately to PMKs proliferation. Inhibition of EGFR or the Ras/MAPKs, PI3/Akt pathways with different pharmacological inhibitors or knockdown of NF-kB, c-jun, c-fos, Cyclin D1, and COX-2 with siRNA inhibited PAT-induced PMKs proliferation.

3.5.9. Cytotoxicity of mycotoxins after gamma irradiation

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Due to the high toxicity of mycotoxins, many methods have been used to reduce or eliminate them from food and feed. Gamma radiation is one technique that has been investigated with some promising results in the degradation of mycotoxins from food commodities. The aims of

this study were (i) to clarify the effect of gamma irradiation on aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin G_1, aflatoxin G_2 and ochratoxin A (OTA); (ii) to evaluate the effect of the presence of water during irradiation; and (iii) to evaluate the cytotoxicity of degradation products resulting from irradiation.

Solutions with the same initial mycotoxin concentration were submitted to gamma radiation doses ranging from 1 to 10.0kGy, at distinct moisture levels (dehydrated, in water and in methanol: water solution). After irradiation, mycotoxins levels were determined by HPLC with fluorescence detection and photochemical post-column derivatization (for aflatoxins). Mycotoxins cytotoxicity was assessed in Hep G2 cells using a battery of assays covering different modes of action including alterations of metabolic activity, plasma membrane integrity and lysosomal function.

Degradation of mycotoxins was observed at radiation doses above 3.0kGy, but only when irradiated in an aqueous environment. In dehydrated samples, no significant reduction of mycotoxins concentration and toxicity was observed comparing with controls. The production of hydroxyl radicals in presence of water could explain this difference. Cytotoxicity assays showed, for some mycotoxins (AFB_1, OTA and mix of aflatoxins) a significant reduction of cytotoxicity with increasing radiation doses. For aflatoxins, a 2kGy dose was sufficient to eliminate almost all toxicity. For OTA, a toxicity reduction of approx. 10% was only achieved. No increase of cytotoxicity was observed for any of the mycotoxins after irradiation. These results point out that irradiation may contribute for the reduction of some mycotoxins on food commodities.

3.5.10. Ameliorative effects of L-carnitine and vitamin E upon toxicological alterations induced by ochratoxin A (OTA) in white Leghorn cockerels

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L-carnitine is a quaternary ammonium compound biologically synthesized from amino acids methionine and lysine and vitamin E is an antioxidant. The present study was aimed to investigate the ameliorative effects produced by L-carnitine and vitamin E against the toxicopathological alterations induced by ochratoxin A in white Leghorn cockerels. A total of 240 cockerels were selected and divided to 12 groups containing 20 birds each. Each group was treated with different levels of OTA, L-carnitine or vitamin E or their combinations. Different parameters were studied like behavioral parameters, body weight gain, organ weights, hematological and serum biochemical parameters and histopathology of organs. Birds treated with OTA were depressed and less attractive to feed having ruffled feathers. Body weights and organ weights of the groups treated with OTA were also depressed. Hematology of OTA treated groups showed a decrease in PCV, Hb, TEC and TLC moving the birds in an anaemic state. Total proteins and albumen concentrations in the serum of OTA treated groups were significantly lower while serum urea and creatinine in OTA treated groups was significantly higher than control. Liver of control group showed normal hepatocytes and normal sinusoidal spaces. Nuclei were normal with exception of only few pyknotic nuclei. In kidneys, tubular epithelial cells had normal nuclei and urinary spaces were clear and dilated. In OTA treated groups, the sinusoidal spaces were congested and hepatocytes were pyknotic while in kidneys, there was pyknosis of nuclei of tubular epithelial cells and urinary spaces were also congested. All these alterations and lesions were more severe at higher doses (2.0 mg/kg OTA) while less severity was observed at low levels (1.0mg/kg OTA). Results confirmed that L-carnitine and vitamin E given alone or combination with 1.0 mg/kg OTA ameliorated OTA induced alterations in behavioural parameters, body weight gain, and organ weight, feed intake, haematological, serum biochemical and histopathological parameters. This amelioration, however, was not seen at 2.0 mg/kg OTA. The present study suggested that the products like L-carnitine, vitamin given alone or combination had the ameliorative effects against the toxic effects of OTA present in commercial feed in a dose dependent manner.

Note: This is the M. Phil research of Zain ul Abidin carried out at Department of Pathology, Faculty of Veterinary Sciences University of Agriculture Faisalabad.

3.5.11. Drinking water contaminants: toxicity of halogenated polycyclic aromatic hydrocarbons

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Food may be contaminated with polycyclic aromatic hydrocarbons (PAHs) in the process of smoking or heating. These contaminants or their derivatives can also be present in drinking water when raw water contacts with discharges of untreated industrial/waste water effluents, forest fires or by solubilisation of organic material from contaminated soils. A few studies have shown that water disinfection can lead to halogenated derivatives of PAHs (HPAHs) as chlorinated and brominated derivatives, and there are evidences that these compounds may have greater mutagenicity than the parent PAHs.

In this study the cytotoxic and genotoxic effects of chlorinated/brominated derivatives of pyrene (Pyr) and benzo[a]anthracene (BaA), 1-ClPyr, 1-BrPyr and 7-ClBaA, which can be formed as water disinfection by-products, were studied in HepG2 cells to assess their potential hazard to human health.

The formation of 1-ClPyr, 1-BrPyr and 7-ClBaA under aqueous disinfection conditions in waters contaminated with Pyr and BaA, was confirmed with an optimized gas chromatography method. Cells exposed (24h) to several concentrations of BaA and 7-ClBaA (1 to 50 M) displayed a dose-related and significant increase of cytotoxicity (neutral red assay) with IC50 values of 3.37 and 12.63μM respectively. For Pyr, 1-ClPyr and 1-BrPyr (10 to 50μM), a lower but significant dose-related cytotoxicity was observed. At non-cytotoxic concentrations (10 and 15μM), 7-ClBaA was able to induce a significantly higher level of oxidative DNA damage in HepG2 cells than its parent compound, as assessed by the FPG-modified comet assay. Under these conditions neither Pyr nor its derivatives were genotoxic.

In conclusion, the disinfection process may give rise to genotoxic HPAHs with potential impact on human health and it should be performed in raw waters with minimal content of total organic carbon. In real conditions, humans may be exposed to a mixture of these organic compounds and thus their combined toxic effects should be further evaluated.

3.5.12. Potential noxious effects of *Mentha aquatica* L. on mitochondrial bioenergetics

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Mentha aquatica (water mint) extracts are regularly used in food flavoring and pharmacology. In the present study, the possible effects of an ethanolic extract from leaves of *M. aquatica* L. on rat liver mitochondria bioenergetics were evaluated.

The plant extract (up to 25 µg.mg protein^{-1}) but not the vehicle, inhibited the mitochondrial oxidative system, as seen by a depression of respiration (state 3, respiratory control ratio (RCR), FCCP-stimulated respiration) and lower generation of the transmembrane electric potential using glutamate + malate or succinate as respiratory substrates. The depressing effects in oxidative phosphorylation can probably be related with the polyphenolic composition of the extract (mainly eriodictyol-7-O-rutinoside, luteolin-7-O-rutinoside, naringenin-7-O-rutinoside, hesperitin-7-O-rutinoside and rosmarinic acid), that can interact with membrane and change the inner mitochondrial membrane lipidic moiety. Despite decreasing the RCR, the presence of M. aquatica extract did not affect the mitochondrial phosphorylative capacity, as estimated by the ADP/O ratio. No significant increase in inner mitochondrial membrane permeability was observed and induction of mitochondrial permeability transition pore was not altered in the range of concentrations tested (up to 25 µg.mg protein^{-1}) either. For the highest concentrations tested (25 µg.mg protein^{-1} or higher) the inhibition observed on the mitochondrial respiratory chain, as reflected by FCCP-stimulated respiration, revealed that *M. aquatica* ethanolic extract is toxic for mitochondrial bioenergetics. In conclusion, the present study suggests that a highly daily consumption of an ethanolic extract of *M. aquatica* leaves should be regarded as hazardous.

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3.5.13. Toxicological evaluation and polyphenols characterization of *Pterospartum tridentatum* leaf extracts

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Pterospartum tridentatum Willk. (prickled broom) is a common autochthonous plant in Portugal. Leaves and stems are normally used in cooking, to flavour rice, roast meat or hunting animals. Leaves are also used as a condiment in fresh salads and, despite of its traditional use, no toxicological evaluation has been performed.

P. tridentatum leaves aqueous extract ESI-MS spectrum revealed the presence of several luteolin and isorhamnetin derived phenolic compounds, which can be associated to the health benefits claimed for this plant species. Still, *P. tridentatum* leaves extract (up to 100 µg plant extract.mg^{-1} protein) stimulated state 4 and FCCP-stimulated liver mitochondria respiratory rates and inhibited the state 3 respiratory rate. Respiratory control ratio was decreased, indicating a dysfunction in respiratory activity induced by *P. tridentatum* leaves extract and, in good agreement with the previous results cytotoxicity evaluation by MTT assay (50 and 125 µg plant extract.10^{-6} cells) showed a decrease on HepG2 cell viability. Overall, the present study suggests that the consumption of *P. tridentatum* leaves in high amounts or continuously should be regarded as potencially noxious.

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4

Screening and mitigation strategies for multiple contaminants in food

4.1. Screening for the major themes of scientific papers on “food contaminants” and “chemicals mixtures” using text mining tools

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Objective: Due to the high data repository of publications on food contaminants and complex mixtures, it is challenging for scientists to analyse all published data manually. The objective is to identify the major themes and working groups that have contributed to these topics. To aid in this effort, a text mining tool was used to screen a large number of publications.

Methodology: Publications on the topics of food contaminants and complex mixtures since 2000 to 7th of February 2015 were collected through a search of the Web of Knowledge. Analysis of titles, abstracts and authors from 146 publications was performed by KH Coder. The main relevant themes to this research area were identified and presented by hierarchical cluster analysis (HCA) and co-occurrence networks graphs.

Results: The most common nouns were exposure and mixture, both for abstracts and titles. In addition, environmental, human and organic were the most frequent adjectives. Titles analysis revealed biphenyl, pesticides, polychlorinated biphenyl and organochlorine as the most studied contaminants. By HCA, “organic contaminant in food” and “environmental potential of chemical mixture contamination by organic compound in food” were possible central themes for titles and abstracts, respectively. “Risk assessment and human health response to pesticide chemical mixture exposure” were noticeable subjects identified from co-occurrence network for titles. Abstracts delivered the combination “food contaminant and chemical mixture concentration level study”. In addition, eleven groups of different authors were identified.

Conclusions: Text mining is a useful tool to screen for the main fields of research on a large number of documents and the relations between them. Environmental, human, exposure and organic were the main terms related to food, contaminants, chemical and mixtures. To validate the effectiveness of this approach, the relevance of the articles selected to the topic should be individually confirmed.

4.2. Depuration of bivalve species as a mitigation strategy: effects on metal levels

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Depuration is only currently mandatory in the EU to diminish pathogenic microorganisms' levels in bivalves harvested for human consumption in polluted waters (B category) in order to ensure healthy and safe products for commercialization. However, the efficacy of depuration to eliminate chemical contaminants is still poorly understood. The main objective of this research was to evaluate the effectiveness of depuration on reduction of the levels of toxic metals of bivalve species from contaminated estuarine waters.

Bivalve species were collected in Tagus estuary (*Ruditapes philippinarum*, *Mytilus galloprovincialis* and *Scrobicularia plana*). Depuration was initiated 2 h after bivalves harvesting simulating the commercial practices commonly used. Thirty specimens from each species were randomly collected at 0, 2, 4, 6 and 8 days of depuration for Hg, Cd, Pb and As determination and mortality was recorded.

Mortality rate was very low after 8 days in *R. philippinarum* and *M. galloprovincialis* (below 1%), whereas *S. plana* had higher mortality rate particularly after 6 days (48%). Depuration was effective in reduction of levels of toxic elements (mainly Pb) in the three species, but particularly in *S. plana* after 2 and 8 days (39 and 60%, respectively). This species is currently declared unfit for human consumption due to the high levels of Pb, often found above the Maximum Permissible Limits (MPLs; 1.5 mg/kg). The levels of other toxic elements were always well below the MPLs (0.5 and 1.0 mg/kg for Hg and Cd, respectively) and the maximum allowable levels for total As (86 mg/kg) in all bivalve species, despite the depuration reduced Hg (32%; after 6 days), Cd (38%; after 8 days) and As (19%; after 4 days) levels in *R. philippinarum* as well as 10% of As (after 4 days) in *S. plana*. In conclusion, depuration may be employed as an excellent mitigation strategy to reduce toxic elements levels (e.g. lead) in contaminated bivalves to acceptable values for human consumption.

4.3. Influence of different activated carbons on Ochratoxin A decrease in wines

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The presence of mycotoxins in foodstuff is a matter of concern for food safety. Wines can also be contaminated with these toxicants. Several authors have demonstrated the presence of mycotoxins in wine, especially ochratoxin A (OTA) [1]. As these toxicants can never be completely removed from the food chain, many countries have defined levels in food in order to attend health concerns. The maximum acceptable level of OTA in wines is 2.0 µg/kg according to the Commission regulation No. 1881/2006 [2]. Although, higher levels of OTA have been detected in several wine samples.

In order to reduce OTA to safer levels, several oenological products can be used in wine; including activated carbons, as shown in previous experiments. Regarding this, the aim of present study was to evaluate the effectiveness of several activated carbons for reducing the amount of OTA present in white and red wines as well as to evaluate their effect on wines physicochemical characteristics.

Wine samples were artificially supplemented with OTA at a final concentration of 10.0 µg/L. The different activated carbons were applied at the concentration recommended by the manufacturer in order to evaluate their efficiency in reducing OTA levels. A mixture composed by gelatine, bentonite and activated carbon reduced 80% of OTA concentration in white wine. The same mixture was however less efficient in red wine, achieving only a reduction of 55%. Thereafter, the effect of activated carbon was evaluated in a red wine, achieving reductions of 66%. Considering these results more assays are being performed with other commercial activated carbons, in order to evaluate their efficiency. These results may provide valuable information for winemakers. Knowing the effect of commercial activated carbons they may choose most appropriate products to remove OTA, thus enhancing wine safety and quality.

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4.4. Removal of ochratoxin A from contaminated white and red wines using oenological fining agents

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Mycotoxins are toxic secondary metabolites produced by certain moulds, being ochratoxin A (OTA) one of the most relevant. Its chemical structure is a dihydro-isocoumarin connected at the 7-carboxy group to a molecule of L-β-phenylalanine via an amide bond. OTA contamination of wines might be a risk to consumer health, thus requiring treatments to achieve acceptable standards for human consumption [1]. According to the Regulation No. 1881/2006 of the European Commission, the maximum limit for OTA in wine is 2 µg/kg [2]. Therefore, the aim of this work was to know the effect of different fining agents on OTA removal, as well as their impact on white and red wine physicochemical characteristics. To evaluate their efficiency, 11 commercial fining agents (mineral, synthetic, animal and vegetable proteins) were used to get new approaches on OTA removal from white and red wines. Trials were performed

in wines artificially supplemented (at a final concentration of 10 µg/L) with OTA. The most effective fining agent in removing OTA (80%) from white wine was a commercial formulation that contains gelatine, bentonite and activated carbon. Removals between 10-30% were obtained with potassium caseinate, yeast cell walls and pea protein. With bentonites, carboxymethylcellulose, polyvinylpolypyrrolidone and chitosan no considerable OTA removal was verified. In red wine, removals between 6-19% were obtained with egg albumin, yeast cell walls, pea protein, isinglass, gelatine, polyvinylpolypyrrolidone and chitosan. The most effective fining agents in removing OTA from red wine were an activated carbon (66%) followed again by the commercial formulation (55%), being activated carbon a well-known adsorbent of mycotoxins. These results may provide useful information for winemakers, namely for the selection of the most appropriate oenological product for OTA removal, reducing wine toxicity and simultaneously enhancing food safety and wine quality.

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4.5. Effect of trazbon bread making on aflatoxins and *Fusarium* toxins

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The aim of the study was to investigate the fate of aflatoxins and *Fusarium* toxins during the Trabzon bread-making process. Trabzon bread, which is traditional sourdough bread, was prepared by baking of fermented dough at 175 - 220°C. During the process, wheat flour samples including aflatoxins ($AFLB_1$, $AFLB_2$, $AFLG_1$, and $AFLG_2$), deoxynivalenol (DON) and zearalenone (ZEA) were used. Fermentation process is based on two-stage fermentation; the long-term lactic acid fermentation and the short-term yeast fermentation. The mycotoxin levels were determined in dough samples before and after each fermentation step and in bread samples (in both crumb and crust).

Significant changes in $AFLB_1$, DON and ZEA levels were not observed while $AFLB_2$, $AFLG_1$ and $AFLG_2$ levels were reduced significantly after the lactic acid fermentation. After yeast (Saccharomyces cerevisiae) fermentation, it was observed that all mycotoxin levels significantly increased in dough samples, especially for aflatoxins (5.4 – 16.7%). With respect to the effect of Trabzon bread making process on mycotoxins, statistically significant reductions were observed for all mycotoxin levels in crust samples. The highest reduction in crust samples was found for $AFLG_2$ levels (29.5 – 30.4%). In crumb samples, significant changes were not observed in DON levels while aflatoxins and ZEA levels were significantly reduced. The highest reductions were observed in crumb samples for $AFLG_2$ (18.5 – 19.4%).

4.6. Control of Aflatoxigenic fungi and mycotoxins production by *Lactobacillus* species

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Molds play an important role in spoilage of food products. It is estimated that 5 to 10% of the world food's production is lost due to fungal contamination. Further, certain fungal species produce highly toxic metabolites designated of mycotoxins. Aflatoxins are the most toxics because they are proven carcinogenic. Biopreservation, defined as the control of one organism by another, has received much attention in recent years. In this field, lactic acid bacteria (LAB) are of great interest to be used as natural biopreservatives since they have broad probiotic properties and have been used traditionally in fermentation processes.

The aim of this work was to demonstrate the potential of *Lactobacillus* species to control the occurrence of aflatoxigenic fungi and their mycotoxins. For that, several aflatoxigenic species such as *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamari*, *A. arachidicola* and *A. minisclerotigenes* were cultured on MEA plates supplemented with 10% of sterile supernatant of different *Lactobacillus* species (obtained from liquid MRS cultures). Supernatants of most active strains inactivated with heat, proteases and NaOH (for pH neutralization) were also tested and compared with untreated ones. The fungal radial growth and the concentration of aflatoxins, cyclopiazonic acid and sterigmatocystin produced in each plate were determined and compared with controls.

L. casei LAB55 and *L. plantarum* LAB7 supernatants were the most active strains. Radial growth of *A. flavus* after 7 days of incubation at 25 ºC was reduced approx. by 31% and 25%, respectively. Aflatoxins production were inhibited approx. by 97 and 87%, respectively. Those reduction decreased slightly over 24 days of cultivation reaching at the end, about 13% and 70% for both strains and for growth and aflatoxins, respectively. The inhibitory properties of those strains was reverted when supernatants were treated with proteolytic enzymes or their pH adjusted to 7.

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4.7. Inhibitory effect of essential oils on *Aspergillus* growth and Aflatoxin accumulation

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Mycotoxins are produced as secondary products of filamentous fungi growth. Aflatoxins (AFs) are one of such toxins, being synthesized by various species of the genus *Aspergillus*. These mycotoxins have been widely studied, since they contaminate many foods used in human and animal diet, resulting in toxic effects in consumers. Thus, it is of extreme importance to find methodologies to reduce or inhibit the fungus and the production of toxins in food. Plants of some families are distinguished for their richness in essential oils and produce volatile fractions which have been used for various purposes. Research on essential oils has gained high attention in recent years due to their natural antimicrobiologic properties, which suppress the growth and the biosynthesis of mycotoxins. The aim of this study is to evaluate the effect of essential oils on fungal growth and on AF accumulation.

Essential oils from eight aromatic plants were tested for their inhibitory effect. The antifungal activity was carried out in in vitro conditions, on PDA, by assessing the volatile phase effect towards mycelial growth of *Aspergillus parasiticus* MUM 92.02 and aflatoxin production. Mycelial growth was monitored by measuring the diameter of growing colonies, while aflatoxin was quantified by HPLC. The Baranyi model was adjusted to the diameter values of colonies by nonlinear regression. In this model, the logarithmic term Dmax (maximum diameter) was deleted in order to omit the upper asymptote.

The results showed that the essential oil from the leaves of *Cinnamomum zeylanicum*, *Cymbopogon nardus* and *Melaleuca alternifolia* prevented or inhibit fungal growth and affected the production of aflatoxins. However, although inhibiting micelial growth, some essential oils, in lower concentration, enhance the production of aflatoxins. These results give important insights on the antimicrobial activity of essential oils in food commodities, preventing undesirable secondary effects on public health.

4.8. Enzymatic degradation of Ochratoxin A in wheat flour

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Ochratoxin A (OTA) is a mycotoxin that is presented in different food matrix with high thermal stability, allowing contamination persists during the processing steps. Based on this characteristic, biological methods of degradation become an interesting approach to avoid the drastic conditions required for the chemical and physical methods and to reduce the contamination to the legislated levels. Within this context, the objective of this work was to assess the enzymatic degradation of ochratoxin A in wheat flours with carboxypeptidase A from different sources (*R. oryzae*, pancreatin and soybean meal). *R. oryzae* was cultivated in PDA agar during 48h at 30ºC, soybean meal has its particle size standardized in 710µm. Carboxipeptidase A was extracted with water in an ultrasonic bath for 30 minutes followed by purification with acetone (1:3 v/v) overnight. The precipitated was dissolved in phosphate buffer pH 7.5 as well as pancreatin solution (2 $mg.mL^{-1}$). Wheat flour was contaminated at maximum legislated level (20$ng.g^{-1}$) and submitted to enzymatic hydrolysis during 30 minutes at optimum conditions for each extract: soybean (at 30ºC pH 7.5); *R. oryzae* and pancreatin (50ºC and pH 7.5), maintaining the proportion protein: OTA (1:2). After the hydrolysis the wheat flour was dried and OTA and OTα extraction were performed with chloroform and the extracts were analyzed by HPLC-FL on a previously validated methodology. All the enzymatic treatments were capable of reducing OTA levels in the flours (13.3-71.3%). The most promising enzymatic extract was from *R. oryzae*, showing a decrease of 71.3% in OTA concentration, with an increase (1.3 fold) on OTα concentration. The result is very promising to reduce the levels of contaminants in food since it is necessary only 30 minutes and soft conditions to reduce significantly OTA concentration in a matrix which is highly used in the food industry. Studies to confirm the lesser toxic effect of OTA metabolite (OTα) are being conducted.

4.9. Ochratoxin A biodegradation by *Pediococcus parvulus*

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Some lactic acid bacteria (LAB) have mycotoxin decontaminating properties due to the absorbing characteristics of their cells walls and because some can biotransform mycotoxins into less toxic compounds. One important mycotoxin found in agricultural commodities is ochratoxin A (OTA). OTA is known mainly for its nephrotoxicity and carcinogenicity being classified in Group 2B by IARC.

The present work reports on the ability of *Pediococcus parvulus* strains, which were isolated from Douro wines, to detoxify OTA. These strains were identified and characterised using a polyphasic approach that employed both phenotypic and genotypic methods. Strains were cultured in OTA-supplemented MRS media (1 µg/mL) at different conditions. The influence of bacteria inoculum size, OTA concentration in MRS medium and incubation temperature was evaluated.

OTA was biodegraded into OTα by *P. parvulus* strains in all conditions but not by reference strains. OTα was confirmed using LC-MS/MS. The conversion of OTA into OTα indicates that OTA amide bond was hydrolysed by a putative peptidase. The rate of OTA biodegradation was found to be dependent on the bacteria inoculum size and on the incubation temperature. Under optimum conditions (10^9 CFU/mL and 30 ºC), 50% and 90% of OTA was degraded in 6 and 19 h, respectively. Dead cells of *P. parvulus* adsorbed only 1.3% of OTA, excluding this mechanism in the elimination of OTA by strains. OTA biodegradation by P. parvulus UTAD 473 was also evaluated and observed in grape must. Vinification experiments were also conducted.

Because some *P. parvulus* strains have relevant probiotic properties, the strains that were identified could be particularly relevant to food and feed applications to counteract the toxic effects of OTA.

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4.10. Poisoning by *Amanita phalloides* mushrooms – regarding three cases

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Objective: Demonstrate that *Amanita phalloides* is the most toxic species of wild known mushrooms, presenting taste and morphology such as other species. Their toxins, resistant to the culinary operations, are capable of producing non-specific symptoms 6 hours after ingestion, with liver and renal failure before 3 days.

Methodology/Results: The author present three cases of death by unintentional poisoning by Amanita phalloides mushrooms ingestion, whose post mortem where performed in the services of Forensic Pathology of the National Institute of Legal Medicine, IP, with hospitable internment in the first 24 hours, being two of them subjected to liver transplantation.

Conclusions: When liver damage is reversible, the recovery is slow and late, being the rare total cure. In fatal cases, death is the outcome of the liver or kidney, and being the taxes of morbidity-mortality high, hence the need to alert the community to the risk of consumption of wild mushrooms.

Concluding remarks

The ICFC2015 conference was one of the first attempts to address the issues of multiple food contaminants and combined exposure in a scientific meeting. It gathered more than 130 scientists from 10 European countries and Brazil. It included 13 invited speakers, several dozens of posters, and 12 contributed talks. This book reflects the work and views presented at the ICFC2015 conference with complementary information gently provided by six invited lecturers. Different domains were involved to approach the emerging topic of challenges in chemical mixtures: analytical chemistry, exposure assessment, human digestion and its effects on food components and contaminants, toxicology and risk assessment.

The challenge of analyzing complex mixtures of food contaminants and the effort for proper method development and validation was evident. It was shown that LC-MS/MS for organic contaminants and MS coupled to ICP for inorganic contaminants in chemical species are extremely powerful analytical tools to address these analytical challenges. In addition, the possibility of simultaneous analytical determination of several hundreds of contaminants and/or metabolites is now a reality.

The importance of using multiple biomarkers of exposure to food contaminants (namely mycotoxins) was highlighted as a component of risk assessment, epidemiological studies and intervention protocols. Web-based applications for diet monitoring (e.g., the OPEN platform supported by the Slovenian Oncology Institute and Eurofir) are already available and are proving to be excellent tools to monitor dietary habits. Assessment of combined exposure to multiple contaminants is also a very challenging area and EFSA has already published and promoted scientific reports and meetings on this issue. Relevant insights on this topic have been additionally provided by projects ACROPOLIS on Aggregate and Cumulative Risk of Pesticides and its on-line integrated Strategy and by EuroMix, a project on new toxicological testing approaches, exploring physiologically-based pharmacokinetic (PBPK) models.

Bioaccessibility and combined toxicity of chemical contaminants were also considered as prominent topics in the food safety area. One harmonized *in vitro* digestion model developed by the INFOGEST Cost Action is now a reference and it has been used by several groups investigating nutrients, contaminants and their interactions. Co-occurrence of food contaminants has been clearly demonstrated and their combined biological effects are difficult to predict using exclusively *in silico* approaches. Hence, several *in vitro* and *in vivo* studies

have been addressing the potential interactions between food contaminants (particularly, mycotoxins) showing that the relative concentration of the individual chemicals affects the overall effect of the mixture and that the application of mathematical modeling is central to uncover the interactive effects.

Finally, some experimental approaches and strategies have been proposed to reduce the hazard of food contaminants, including the use of gamma irradiation, biodegradation, depuration, enzymatic processes and microorganisms.

Considering all contributions from ICFC 2015 it is possible to conclude that chemical mixtures is a hot topic in food safety domain considering the frequent co-occurrence and exposure to multiple contaminants through food, rather than to individual contaminants, and their potential synergistic effects that can pose a significant threat to public health. More studies are needed to develop multi analytical methods, cumulative risk assessment strategies and combined toxicity approaches allowing a holistic overview on food safety. Another key issue deserving future studies is the risk of vulnerable population groups (especially children) from exposure to food contaminants and the resulting adverse health effects. Novel risk assessment strategies should also take into account potential interactions of contaminants, allowing a more realistic and reliable regulation of food contaminants towards public health protection.

This book compiles the extended abstracts of some invited oral communications and the abstracts presented at the **"ICFC2015 – International Conference on Food Contaminants: challenges in chemical mixtures"** held in in Lisbon, on 13 and 14 April 2015

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