Mycotoxins: An Under-evaluated Risk for Human Health

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B P International

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India ▪ United Kingdom

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PREFACE

The growing interest in the consumers' Health Risks associated with mycotoxigenic fungi and their mycotoxins has led to the development of several research projects during the past decade in several European countries. All these efforts have generated a great deal of information on the natural occurrence of these unwanted contaminants in raw foods, feeds, vegetables, and transformed foods and, consequently, to evaluate human exposure to these natural toxins in human samples like biological fluids. Contaminants are chemical substances that pose a serious risk to animal and human health. These substances have not been intentionally added to food or feed and may be occurring as a result of the various stages of its field production, food processing, transport, or food storage and may result from environmental contamination or from the low quality of foods or the management of part or entire food chains.

The main emphasis of the present book is to collect effective, rapid, and reproducible methods, which can be easily applied to the identification of mycotoxins in routine food analysis.

Among the various available method approaches used in the pretreatment of samples, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach represents is largely employed to extract different groups of compounds and represents the most frequently used pretreatment technique in foods analysis.

The most frequent analytical methods adopted by research laboratories and surveillance government agencies are based on liquid chromatography coupled with MS. The use of ultra-high-performance liquid chromatography (UHPLC) provides higher sensitivity, a reduction in mobile phase consumption with consequent reduction of the environmental impact of analyses, and an increase in resolving power and peak shape. High-resolution mass spectrometry (HRMS) provides sensitive and specific measurements for the quantification of targeted compounds, with the additional features of making retrospective data analysis and the identification of untargeted compounds based on exact mass measurements.

This volume provides a detailed overview of the occurrence of mycotoxin and other contaminants in vegetables, foods, food supplements, and biological fluids that can serve as a basis for risk management-based regulatory decisions in charge of public institutions to shield consumers' health.

The main aim of this book is to highlight the practical aspects of mycotoxin contamination highlighting the importance of monitoring the quality of sources and moulds in order to protect consumers' health.

Careful attention is given to the understanding of toxicity and its impact on the performance of animals exposed to mycotoxin-contaminated feedstuffs.

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Mycotoxins: An Under-evaluated Risk for Human Health

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ABSTRACT

Mycotoxins are secondary toxic metabolites produced mainly by fungi belonging to the *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* genera. These moulds can colonize agricultural crops and produce mycotoxins during pre- and post-harvest practices, processing, and storage. Animals fed with feed contaminated with mycotoxins may be a natural and unwanted bioenhancer way to transfer mycotoxins, eventually metabolized, to animal-derived food addressed to humans. The natural occurrence of mycotoxins, also a low concentration, in food may cause adverse health effects in humans, rarely showing acute symptoms but the chronic exposure causes problems ranging from gastrointestinal and kidney disorders to immune deficiency and to develop some types of cancers. Human exposure to mycotoxins can happen by eating directly contaminated foods or through contaminated animal products. This alternative entry of mycotoxin into the human food chain is a signal of animals fed with contaminated feed. The exposure danger to mycotoxins can be monitored by following the biotransformation product occurrence in tissues and biological fluids, and these data are needed to evaluate their potential risk for humans, in particular for weak subpopulations like babies, children, old, or pressed by food security troubles.

In this regard, the main aim of this volume is to evaluate the occurrence of mycotoxins and other contaminants in food, nutraceuticals, and biological fluids in order to ensure human safety. To guarantee effective consumer safety, reliable methods have been validated for the analysis of contaminants in various matrices. In addition, the risk associated with the assumption of contaminated food was assessed. Risk characterization is an indispensable aspect to safeguard public health, which helps to identify risks threatening consumers.

Keywords: Mycotoxins; QuEChERS; UHPLC Q-Orbitrap HRMS; risk characterization.

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Chapter 1
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Preliminary Estimation of Deoxynivalenol Excretion through a 24 h Pilot Study

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ABSTRACT

A duplicate diet study was designed to explore the occurrence of 15 *Fusarium* mycotoxins in the 24 h-diet consumed by one volunteer as well as the levels of mycotoxins in his 24 h-collected urine. The employed methodology involved solvent extraction at high ionic strength followed by dispersive solid phase extraction and gas chromatography determination coupled to mass spectrometry in tandem. Satisfactory results in method performance were achieved. The method's accuracy was in a range of 68%–108%, with intra-day relative standard deviation and inter-day relative standard deviation lower than 12% and 15%, respectively. The limits of quantitation ranged from 0.1 to 8 µg/Kg. The matrix effect was evaluated and matrix-matched calibrations were used for quantitation. Only deoxynivalenol (DON) was quantified in both food and urine samples. A total DON daily intake amounted to 49.2 ± 5.6 µg whereas DON daily excretion of 35.2 ± 4.3 µg was determined. DON daily intake represented 68.3% of the established DON provisional maximum tolerable daily intake (PMTDI). Valuable preliminary information was obtained as regards DON excretion and needs to be confirmed in large-scale monitoring studies.

Keywords: Deoxynivalenol; duplicate diet study; GC-MS/MS; urine; risk characterization.

1.1 INTRODUCTION

Cereals are the most important source of food for both direct human consumption and livestock production. In fact, the latest published data by the Food and Agricultural Organization (FAO) reported an annually cereal global consumption (excluding beer) of 146.7 kg/capita [1]. Mycotoxins are secondary fungi metabolites produced in several commodities that could exert toxic effects on animals and humans [2] and mycotoxin contamination of cereals is also frequently reported as a public health threat [3,4]. Acute exposures to mycotoxins are related to gastrointestinal manifestations such as diarrhea, vomiting and melena, while chronic exposures and the most worrisome one are related to degenerative effects on the immune, neural and reproductive systems. Chronic exposure to some mycotoxins are also related to cancer induction [5]. Mycotoxins have also been classified as priority food contaminants by the Global Environment Monitoring System-Food Contamination Monitoring Assessment Programme (GEMS/Food) of the World Health Organization (WHO) [6]. Moreover, mycotoxins were the main hazards in the European Union with 425 border rejection notifications as highlighted its annual report for 2012, according to the Rapid Alert System for Food and Feed [7].

Among the wide number of mycotoxins, zearalonene and trichothecenes (type A mainly represented by T-2 and HT-2 toxins, and type B by deoxynivalenol (DON)) constitute one of the largest groups of mycotoxins produced by *Fusarium* in cereal grains [8,9]. Mycotoxin production in agricultural crops can occur at various stages in the food chain like pre-harvest, harvest, drying and storage. In addition, mycotoxins tend to persist during the transformation and processing of contaminated plants and are also still reported in cooked and sterilized food [10].

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The European Commission (EC) has set maximum limits for some mycotoxins in foodstuffs [11,12] and the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established maximum tolerable daily intakes to ensure food safety [13]. According to the guidelines published by the WHO, the basic approaches that can be used to determine the intake of a food contaminant include: total diet studies, duplicate diet studies, and selective studies of individual foods, which combine food consumption patterns and contamination level [14]. Duplicate diet studies may be a good alternative to total diet studies, especially when there are important economical limitations to perform a suitable total diet studies. In addition, duplicate diet studies are particularly interesting to consider not only basic cooking methods, but real cooking, something essential when evaluating the dietary intake in specific individuals, countries or regions [15]. A step forward in the individual exposure assessment could be provided by biomarkers measured in biological fluids. The identification of mycotoxins and their main metabolized products in urine could therefore serve as such biomarkers and could facilitate effective exposure assessment [16,17].

Knowledge of mycotoxins *in vivo* metabolism in humans has been rarely investigated and it is fundamental to carry out some studies which serve as an approach to assess the exposure. In this line, some experiments were designed to provide tentative information about the human *in vivo* metabolism of major *Fusarium* mycotoxins. For instance, Mirocha et al. [18] studied the zearalenone metabolite pattern in 24h urine after ingestion of 100 mg zearalenone at once by one volunteer. Similarly, Warth et al. [19] carried out a study to investigate the human deoxynivalenol and zearalenone *in vivo* metabolism through the analysis of urine samples obtained from one volunteer following a naturally contaminated diet containing 138 µg DON and 10 µg ZON over a period of four days. Thus, this preliminary work was designed as the previously ones to provide basic information about the human *in vivo* metabolism and serves for the purpose to develop a method which might principally suited as a screening tool. The dietary intake of 15 mycotoxins was studied in a single individual using the duplicate diet approach. All food products as prepared, served and consumed were analyzed. The occurrence of mycotoxins and metabolites was also evaluated in the 24-hour urine collection and expressed by µg/g of creatinine. The aims of the study were to investigate the daily mycotoxin intake through complete cooked meals, to estimate the mycotoxin urinary excretion, and to carry out a risk characterization approach for the participant.

1.2 RESULTS AND DISCUSSION

1.2.1 Method Performance

Regression equations were obtained using eight standard concentrations on the abscissa and the area of the chromatogram peaks as vertical coordinates. Linear range was tested at eight concentration levels in triplicate from LOQ to 100 µg/kg. Relative standard deviations among the triplicate were below 5% at all calibration curve points. The determination coefficients (R^2) of all analytes were >0.995. The matrix effect was observed (from 83% to 91%), and thus matrix-matched calibration curves were used for quantification purposes. Apparent recovery for each mycotoxin was determined in composite, beer and urine samples spiked at low and high level (Table 1.1).

Recoveries values obtained, from 68 to 108%, were in agreement with the range set in legislation [20]. Corrections based on recovery percentages were not performed. A precision study was performed by determining the repeatability (intra-day precision) (n = 6) and reproducibility (inter-day precision) (n = 4), and was fulfilled in conformity with the described criteria in current legislation. Intra-day and inter-day precision were lower than 12 and 15%, respectively in the assayed matrices. The sensitivity of the method was expressed in terms of limits of detection (LOD) and limits of quantitation (LOQ). LOD and LOQ values were calculated from spiked samples chromatograms based on a signal to noise ratio of 3:1 and 10:1, respectively (Table 1.1). LODs varied in the following ranges of 0.6–5 µg/kg, 0.05–8 µg/kg and 0.1–4 µg/kg for composite, beer and urine respectively. LOQs varied in the following ranges of 1.2–10 µg/kg, 0.1–16 µg/kg and 0.2–8 µg/kg, for composite, beer and urine respectively, which guaranteed quantitation at low ppb-level.

Table 1.1. Method performance for fifteen mycotoxins and metabolites in composite diet, beer and urine

Analyte	Composite diet				Beer				Urine			
	REC± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)	REC ± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)	REC ± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)
	Low level [a]	High level [c]			Low level [b]	High level [c]			Low level [a]	High level [c]		
DOM-1	87 ± 7	93 ± 5	0.6	1.2	73 ± 6	77 ± 8	0.1	0.2	84 ± 2	86 ± 4	0.2	0.5
DON	89 ± 5	91 ± 6	0.6	1.2	75 ± 9	83 ± 9	0.05	0.1	96 ± 4	94 ± 8	0.1	0.2
3-ADON	95 ± 4	90 ± 7	0.6	1.2	82 ± 6	80 ± 5	2	4	92 ± 5	94 ± 5	0.2	0.5
FUS-X	84 ± 5	89 ± 3	2.5	5	98 ± 8	93 ± 9	8	16	95 ± 3	90 ± 6	2	4
DAS	103 ± 3	99 ± 6	2.5	5	78 ± 6	82 ± 5	4	8	89 ± 4	84 ± 8	1	2
NIV	79 ± 6	82 ± 5	1.2	2.5	77 ± 12	81 ± 9	0.5	1	87 ± 3	93 ± 6	0.5	1
NEO	97 ± 8	92 ± 5	2.5	5	83 ± 8	88 ± 6	2	4	93 ± 5	94 ± 5	0.2	0.5
HT-2	93 ± 7	89 ± 8	1.2	2.5	97 ± 9	93 ± 4	2	4	96 ± 4	91 ± 8	1	2
T-2	84 ± 9	90 ± 6	2.5	5	108 ± 7	97 ± 8	4	8	102 ± 6	94 ± 9	0.5	1
ZAN	85 ± 5	90 ± 4	5	10	68 ± 9	73 ± 9	8	16	72 ± 7	74 ± 8	4	8
α-ZAL	72 ± 8	79 ± 8	5	10	70 ± 6	78 ± 7	4	8	79 ± 5	82 ± 5	4	8
β-ZAL	79 ± 6	77 ± 6	5	10	73 ± 8	79 ± 8	4	8	77 ± 8	74 ± 6	4	8
ZON	87 ± 8	84 ± 7	2.5	5	71 ± 5	78 ± 6	8	16	81 ± 5	84 ± 7	3	6
α-ZOL	83 ± 9	80 ± 7	2.5	5	78 ± 6	83 ± 4	2	4	88 ± 2	93 ± 5	1	2
β-ZOL	77 ± 6	78± 9	2.5	5	74 ± 8	73 ± 8	4	8	80 ± 6	84 ± 9	2	4

[a] Spiking level: 10 µg/kg; [b] Spiking level: 20 µg/kg; [c] Spiking level: 100 µg/kg.

1.2.2 Deoxynivalenol Reduction during Cooking

Mycotoxin analyses of pasta (spaghetti) and whole-wheat pasta (little stars) were carried out prior the cooking step and then cooked after the drying process. Analyses were performed in triplicate. The purpose was to evaluate the percentage of mycotoxin reduction during food preparation procedure. Most of the exposure assessment approach to contaminants has been carried out based on un-cooked food, and thus assuming some uncertainty in the reported data. In this work, not only cooked meals but also the regular cooking practices and the serving size were taking into account. The aim was not to serve as a representative data of percentage of reduction for wheat-based products, but to minimize the uncertainty of the obtained data in order to allow a closer exposure assessment approach. A reduction of 13% and 58% was obtained for whole-wheat pasta and pasta, respectively after culinary treatment (Fig. 1.1). That difference could be attributed to the distinct serving. While the cooking water is removed in spaghetti serving, little stars are consumed as a soup and thus, the amount of mycotoxin intake in little stars was higher than in spaghetti. The percentage of reduction obtained in this work were comparable than those reported Visconti et al. [10] in a larger study showing average DON reduction levels of 40%. Moreover, they indicated that the amount of DON retained by cooked pasta consistently decreased by increasing the pasta/water ratio during cooking.

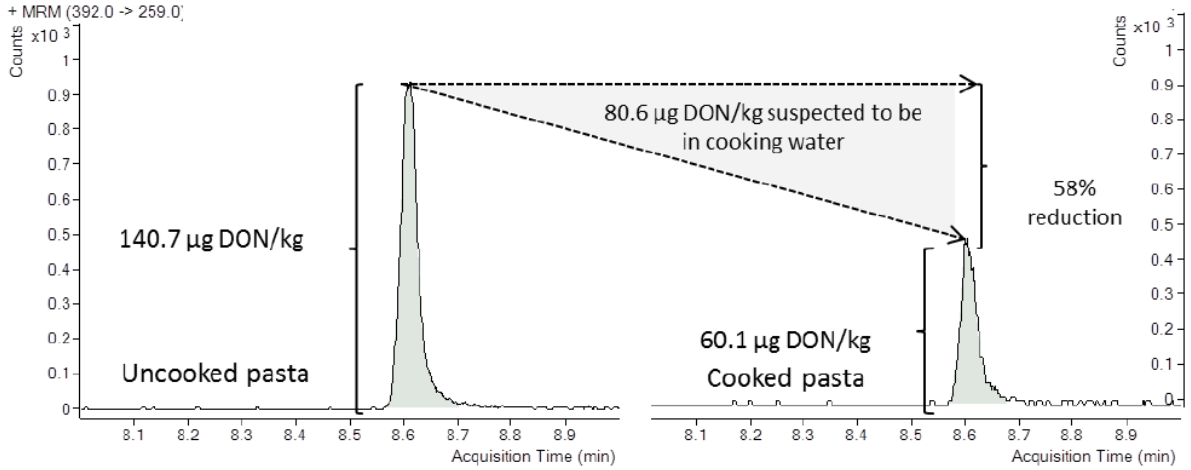

Fig. 1.1. Selected reaction monitoring (SRM) chromatograms of the naturally deoxynivalenol (DON) contaminated pasta before and after culinary treatment at 140.7 µg/kg and 60.1 µg/kg, respectively, on a dry weight basis, and percentage of mycotoxin reduction

1.2.3 Deoxynivalenol Content in Food

All analyzed meals were wheat-based. Within the 15 mycotoxins targeted in the proposed procedure only DON was detected in the analyzed food commodities. This high DON prevalence was highlighted before in cereal and their derivative products by several authors [5] as well as in urine samples [21]. Quantified DON (n = 3) in the different food items are presented in Table 1.2. The food items presenting the highest concentrations of DON were whole-wheat pasta and toasts with average values of 272.4 and 190.6 µg/kg, respectively. Despite beer was the matrix with the lowest DON content, it was the food item with the highest significant contribution to DON intake (28.6 µg) because of the consumption data (0.83L). A total DON daily intake throughout the consumption of the followed diet was estimated in 49.2 ± 5.6 µg. Mean DON contents of the studied food matrices were in line with results reported by other authors [22,23,24,25].

The estimated levels of mycotoxins in the composite are also presented in Table 1.2. DON was quantified at mean level of 120.5 ± 7.3 µg/Kg. A mean DON intake of 19.9 µg was obtained throughout the solid food items consumed. The composite's result was in line with the 20.5 µg of DON obtained from the sum of each food item. Thus, composite could be presented as an alternative tool

to reduce sampling and analysis cost. However, the resulting information concerning contaminants in each selected food item will be missed.

Table 1.2. Overview of contamination level, consumption data and mycotoxin intake contribution of the food items consumed

Time of consumption	Food	Consumption (g/day)	Mean DON ± SD (µg/kg)	Mean DON intake (µg)
8 am	Toast	45	190.6 ± 2.3	8.7
11 am	Breadsticks	30	49.7 ± 4.6	1.5
2 pm	Pasta	67 [a]	58.2 ± 2.7	3.9
7 pm	Wheat beer	500	36.4 ± 1.8	18.1
8 pm	Beer	330	32.1 ± 6.2	10.5
10 pm	Whole-wheat pasta	24 [a]	272.4 ± 5.9	6.5
				Σ DON Intake: 49.2 µg
	Composite [b]	166	120 ± 7.3	19.9 µg

[a] on a dry weight basis; [b] no beverages included.

1.2.4 DON Content in Urine

Quantifiable amounts of mycotoxins, and its toxin derivatives that result from its biotransformation, are expected to be found in urine. For instance, DON can be metabolized within the intestinal lumen by gut microbiota, generating the less toxic de-epoxy metabolite known as DOM-1. Further metabolism of DON and ZON to a less toxin metabolite involves the addition of glucuronic acid, catalyzed by UDP-glucuronyltransferase [26,27]. As regards mycotoxin conjugation, the uncertainness exists, since it has been related individual difference in the enzymatic system. On the other hand, Turner et al. [28,29] suggested that un-conjugated DON can also persist and it can be excreted in urine. In this line, this work was focused on the investigation of un-metabolized DON as a preliminar step since it needs to be extended in the future to understand the relation between the mycotoxin intake and mycotoxin levels in urine, both metabolized and un-metabolized fractions.

Analyses in urine carried out by other authors have revealed the occurrence of DON in a high incidence of samples. For instance, Gratz et al. [30] reported an incidence of DON in all analyzed samples (n = 54). Similarly, DON incidences in urine samples of 33.3% (n = 27) and 67.6% (n = 34) were reported by Rubert et al. [31] and Turner et al. [21], respectively. In this work, a total of 1.87 L urine was collected as 24-hour urine volume and was in the normal excretion range according to sex and age [32]. DON was quantified at 17.5 ± 2.7 µg/g creatinine (n = 3) (equivalent to 18.8 ± 3.5 µg/L). No other mycotoxin was found in the urine sample being according with the results reported by Rubert et al. [31] and Warth et al. [33] who did not find neither any other trichothecenes nor zearalenone in the analyzed urine samples. GC-MS/MS chromatograms of naturally DON contaminated composite, beer and urine are shown in Fig. 1.2. Urinary free DON levels of 18.8 ± 3.5 µg/L (equivalent to 35.2 ± 4.3 µg DON) was calculated in this study. Data found in literature were very similar. For instance, DON average contents of 20.4 µg /L were reported in an Austrian survey [27] (n = 27; incidence of 22%) and a range from 0.5 to 28.8 µg/L were reported in a French study (n = 76; incidence of 98.7%) [26].

1.2.5 Exposure Estimates

On the basis of the calculated data, DON daily intake was compared with the established PMTDI of 1 µg/kg bw [13]. The dietary exposure level to DON estimated in the present study was 0.683 µg/kg bw/day, lower than the value set by JECFA. Comparable values as regards dietary exposure to DON were reported in the second French total diet study [34] (mean: 0.379 µg/kg bw/day) as well as in a duplicate diet study carried out in Dutch young children (mean: 0.66 µg/kg bw/day) [35]. These results are also within the latest DON probable daily intake data (0.34 µg/kg bw/day) reported in the SCOOP task 3.2.10 derived from food analysis in Europe [36] and that reported by FAO/WHO (1.4 µg/kg bw/day) [37].

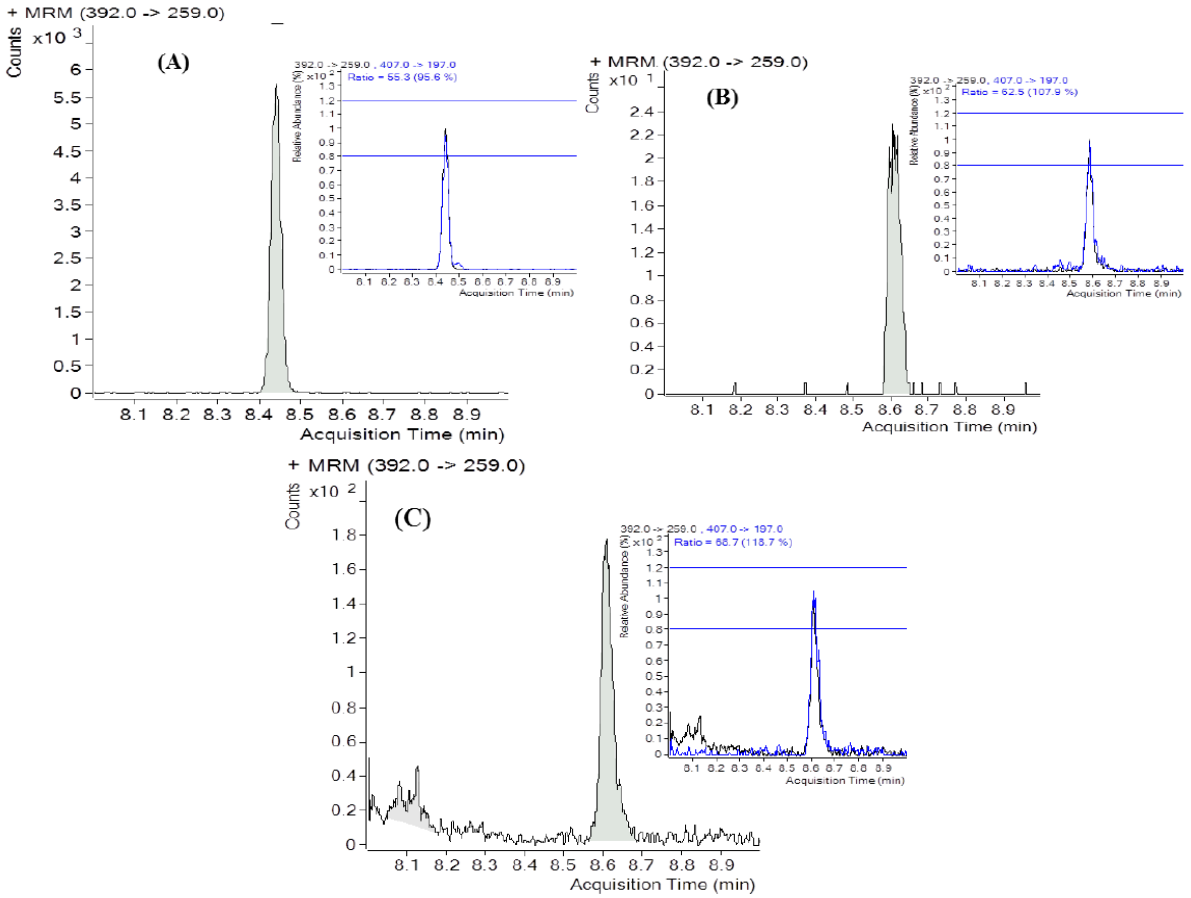

Fig. 1.2. (a) SRM chromatograms of the naturally contaminated composite; (b) wheat-beer and (c) urine by deoxynivalenol at 122.6 µg/kg, 37.2 µg/kg and 20.8 µg/L, respectively

1.3. EXPERIMENTAL SECTION

1.3.1 Materials

Mycotoxin standards and metabolites namely de-epoxydeoxynivalenol (DOM-1), DON, 3-acetyldeoxynivalenol (3-ADON), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), nivalenol (NIV), neosolaniol (NEO), HT-2, T-2, zearalanone (ZAN), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearelenone (ZON), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The derivatization reagent composed of BSA (*N*,*O*-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (*N*-trimethylsilyimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

Certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg/kg) was purchased from Biopure Referenzsubstanzen GmBH (Tulln, Austria).

All solvents, acetonitrile, hexane and methanol (HPLC grade), were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, CA, USA). Picric acid (moistened with water, ≥98%) and creatinine standard were purchased from Sigma-Aldrich (St. Louis, MO, USA) whereas sodium hydroxide was acquired from BDH Prolabo—VWR International (Barcelona, Spain).

1.3.2 Standard Preparation

Individual stock solutions of all analytes were prepared at the same concentration (1000 mg/L) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg/L). All standards were stored in darkness and kept at −20 °C until the GC-MS/MS analysis. Calibration function of both neat solvent standards and spiked samples were established by plotting peak areas *versus* analyte concentrations in the measured solutions and performing linear regression. Linear range was tested from 0.1 to 100 µg/kg by spiking at eight concentration levels. In order to reveal the presence of matrix effect, matrix-matched calibration prepared by spiking extracts of blank samples with mycotoxins at similar concentrations than the calibration built in neat solvent without any matrix were compared. The slopes of the resulting linear calibration functions were compared and the signal suppression/enhancement due to matrix effects (ME) was determined as follows:

$$ME\ (\%) = \frac{Slope_{matrix-matched\ calibration}}{Slope_{standard\ in\ solvent}}\ x\ 100$$

1.3.3 Study Design and Sampling

In this 24 h preliminary study, a farinaceous-based diet was designed and conducted by a 26 year old, healthy male volunteer. Duplicate meals as prepared, served and consumed, based on a “duplicate plate” method were provided for subsequent individual analysis in sterile plastic food containers and kept with ice packs in a cooler until they were returned to the laboratory. The selection of food items analyzed in the present study was based on two criteria: first, the food must be identified by GEMS/Food as potential sources of mycotoxins and second, their level of consumption must exceed 1g/person day. Four complete meals were consumed during the day of the study as well as two beers in the afternoon. The food groups items selected were bread, wholegrain cereals, pasta, and wheat or barley-based beer. Food items such as pasta (spaghetti) and whole-wheat pasta (little stars) were cooked for 7 min in boiling water. A pasta/water ratio of 1:5 (*w*/*v*) was respected as recommended the preparation mode established in the food packaging labels. Salt and spices were also added as in regular cooking practices.

Upon arrival at the laboratory all meals were weighed and dried in an oven at 100 ± 4 °C to reach constant weight for subsequent mycotoxin analysis. The dried products obtained were thoroughly grounded and homogenized using a laboratory mill and kept at 4 °C under dark and dry conditions into a specific plastic food containers. Note that once dried and milled, all dried products were wheat flour-related foods. For the cooked pasta-based products, both dry cooked form and the dry uncooked ones were subjected to mycotoxin analysis to evaluate the percentage of mycotoxin reduction during the cooking.

A urine sample was collected as 24 h urine throughout the day of the study, and due to this, it was demonstrated that the main part of absorbed *Fusarium* toxins showed a rapid elimination within 24 h after ingestion [38]. Urine collected was stored at −20 °C until analysis. The 24 h period lasted from 8 am to 8 am on the next day to include the first morning urine. A written and approved informed consent was obtained from the volunteer. This project was approved by the University of Valencia Institutional human research Committee and the study purposes and procedures were justified and accepted for this study.

1.3.4 Composite Diet Sample

The composite diet was intended to be representative of 24 h duplicate diet collected and included all food items consumed over the monitoring period, without beverages. The dried food items were briefly homogenized, carefully mixed, and finally combined keeping the diet proportions.

1.3.5 Sample Preparation

Composite and individual meals were analyzed as described in detail elsewhere [**39**] in order to know the contribution of each food item. In brief, 5 g of homogenized sample was added to 25 mL distilled water and 7.5 mL of acetonitrile followed by the addition of 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. After centrifugation the liquid extract was separated from the solid salts and finally the extract was evaporated to dryness under nitrogen flow.

Beer samples were first completely degased by sonication for 15 min prior the analysis. A 10 mL portion was then used for the analysis. 5 mL of acetonitrile were added to the sample followed by the addition of the mixture of salts ($MgSO_4$ and NaCl) and then submitted to a d-SPE as previously described.

The urine sample was first centrifuged at 4000 rpm for 5 min. A 10 mL portion of the centrifuged urine was then used for the subsequent analysis as indicated above.

All dry extracts were added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the samples were left for 30 min at room temperature. The derivatized samples were diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the diluted derivatized samples were added with 1 mL of phosphate buffer (60 mM, pH 7) shaken and the upper layers (hexane phases) were transferred to autosampler vials for the chromatographic analysis.

For quality control, certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg/kg) was used and included. Certified reference material was used as provided without further grinding. It was stored under the same conditions, extracted and determined with the same protocol as the analyzed samples. Each sample was analyzed in triplicate and measured in separate batches.

1.3.6 GC-MS/MS Method

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA) were used for MS/MS analysis. Chromatographic separation was achieved on a HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode (equivalent to 25 mg of dried food matrix) at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80ºC, and the temperature was increased to 245°C at 60°C/min. After a 3 min hold time, the temperature was increased to 260°C at 3°C/min and finally to 270°C at 10°C/min and then held for 10 min. Chromatographic analysis time was performed in 17 min, which reached the requirement for a high throughout determination [39].

The mass spectrometer operated in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230 and 280°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software [39].

1.3.7 Calculation of DON Daily Intake

A deterministic approach was applied for the calculation of DON dietary exposure. The volunteer filled a 1-day food consumption record and was asked to provide his body weight (bw: 72 kg). DON daily intake, expressed as µg DON/kg bw, was calculated by combining food consumption (g foodstuff/kg bw/day) with DON food contamination (µg DON/g foodstuff) data. The contribution of each food to the average dietary exposure was also calculated.

1.3.8 Creatinine Analysis

Creatinine urinary levels were determined based on a spectrophotometric method slightly modified [40]. In summary, 3.5 mM picric acid was reacted with 1000 mM NaOH to form alkaline picrate. This solution was stored in the dark in an amber glass recipient. Alkaline picrate (1 mL) was reacted with 1 mL of diluted urine (1/10, *v*/*v*, in ultrapure water). The optical density was measured at 500 nm after 30 min using a Shimadzu mini 1240 spectrophotometer. Mycotoxin urinary concentrations were correlated to the creatinine content of a sample expressed as µg/g creatinine.

1.4 CONCLUSIONS

A total DON daily intake derived from the 24 h duplicate diet study amounted to 49.2 ± 5.6 µg whereas 35.2 ± 4.3 µg of DON were quantified in the urine collected in the same. DON incidence in urine of the participant confirms its exposure to DON and evidences the usefulness of DON and its metabolites in urine as a biomarker of exposure to such contaminants. The values of DON PDI estimated herein with the urinary biomarker approach matched quite well the intake derived from food analysis. DON data was further correlated to the established DON PMTDI value in order to obtain a risk characterization approach. DON daily intake represented a 68.3% of the established PMTDI. The obtained data from this preliminary study is subjected to intra- and inter-day variations. Therefore, this experiment needs to be extended to a larger group of individuals to investigate these variations and to elucidate the relation between ingested mycotoxins and excreted ones and their corresponding metabolites in humans. In this sense, the *in vitro* digestibility/metabolic models are very useful to complete the full-scale metabolism studies.

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AUTHOR CONTRIBUTIONS

Guillermina Font and Jordi Mañes conceived and designed the experiments; Yelko Rodríguez-Carrasco, Houda Berrada performed the experiments, analyzed the data and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 2
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Development and Validation of a LC-ESI-MS/MS Method for the Determination of *Alternaria* Toxins Alternariol, Alternariol Methyl-Ether and Tentoxin in Tomato and Tomato-Based Products

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ABSTRACT

Alternaria species are capable of producing several secondary toxic metabolites in infected plants and in agricultural commodities, which play important roles in food safety. *Alternaria alternata* turn out to be the most frequent fungal species invading tomatoes. Alternariol (AOH), alternariol monomethyl ether (AME), and tentoxin (TEN) are some of the main *Alternaria* mycotoxins that can be found as contaminants in food. In this work, an analytical method based on liquid chromatography (LC) tandem mass spectrometry (MS/MS) detection for the simultaneous quantification of AOH, AME, and TEN in tomato and tomato-based products was developed. Mycotoxin analysis was performed by dispersive liquid-liquid microextraction (DLLME) combined with LC-ESI-MS/MS. Careful optimization of the MS/MS parameters was performed with an LC/MS system with the ESI interface in the positive ion mode. Mycotoxins were efficiently extracted from sample extract into a droplet of chloroform (100 µL) by DLLME technique using acetonitrile as a disperser solvent. Method validation following the Commission Decision No. 2002/657/EC was carried out by using tomato juice as a blank matrix. Limits of detection and quantitation were, respectively, in the range 0.7 and 3.5 ng/g. Recovery rates were above 80%. Relative standard deviations of repeatability (RSDr) and intermediate reproducibility (RSD_R) were ≤ 9% and ≤ 15%, respectively, at levels of 25 and 50 ng/g. Five out of 30 analyzed samples resulted positive to at least one *Alternaria* toxin investigated. AOH was the most common *Alternaria* toxin found, but at levels close to LOQ (average content: 3.75 ng/g).

Keywords: Alternaria; LC-MS/MS; dispersive liquid-liquid microextraction; tomato.

2.1 INTRODUCTION

Alternaria is a widely distributed fungal genus frequently isolated from different plant crops, and has been documented as a pre- and post-harvest pathogen causing decay [1]. The fungal species of *Alternaria* are considered relevant contaminants of refrigerated fruits, vegetables, and stored foodstuffs, mainly as a consequence of their occurrence and the ability to grow and produce toxins even at low temperatures and low water activity [2]. *Alternaria* species produce a liarge variety of secondary metabolites capable of causing several health problems in humans and animals. The most relevant mycotoxins produced by *Alternaria* spp. are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA), altenuene (ALT), and altertoxins (ATXs).

The toxic effects of *Alternaria* toxins are wide-ranging. To date some of these mycotoxins have shown to be teratogenic in vivo. Genotoxic effects of AOH and AME in vitro have also been described [3]. Recently, some authors reported that AOH and AME are able to induce cell cycle arrest, apoptosis of

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cells, and DNA damaging effects [4,5,6,7]. In spite of the before-mentioned, there are currently no guideline limits set for *Alternaria* mycotoxins by regulatory authorities yet. The European Food Safety Agency (EFSA) provided a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food [3]. EFSA evidenced a lack of robust occurrence data of *Alternaria* toxins in food and processed products, and recommended the collection of representative data across Europe to enable a proper risk assessment.

Tomatoes and many other soft-skinned vegetables and fruits can be easily infected by fungi and *Alternaria* is the main fungus responsible for spoilage. Tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) is considered to be one of the main important vegetable crops worldwide. Although tomatoes are commonly consumed fresh, over 80% of the tomato consumption comes from processed products, such as tomato juice, paste, puree, ketchup, and soup, such as gazpacho, a traditional Spanish, ready-to-serve cold vegetable soup, which contain fresh tomato (> 50%) and other ingredients, such as cucumber, pepper, olive oil, and other minor constituents, such as onion, garlic, wine vinegar, salt, and water.

Based on the increasing need for incidence data, a bunch of new analytical methods are demanded for detection and quantification of *Alternaria* toxins in foods. The coupling of both liquid (LC) and gas chromatography (GC) to tandem mass spectrometry (MS/MS) has enabled the development of highly selective, sensitive, and accurate methods for mycotoxin determination in both biological [8,9] and food samples [10,11]. For the analysis of mycotoxins in various food matrices, the traditional liquid-liquid extraction (LLE), solid phase extraction (SPE), and combinations of LLE and SPE have been commonly used as sample preparation procedures as recently reviewed by Turner et al. [12]. An ideal sample preparation procedure should be straightforward and rapid with low operational cost, as well as efficient in sample clean-up. Furthermore, to allow the trace-analysis of various compounds, a high enrichment factor could be of interest. To fulfill these ideal requirements, in 2006 it was proposed a novel very attractive dispersive liquid-liquid microextraction technique (DLLME) for the treatment of liquid samples, and has been recently used in the determination of some mycotoxins in several food samples [13,14,15,16,17]. Basically, DLLME consists of the formation of a cloudy solution promoted by the fast addition of a mixture of extraction and disperser solvents to an aqueous sample. The tiny droplets formed and dispersed among the aqueous sample solution are further joined by centrifugation. DLLME has been proved to be a powerful cleaning and preconcentration technique. Other benefits are its high speeds, the low solvent use, and final disposal.

The objective of the present work was to develop and validate a reliable DLLME-LC-ESI-MS/MS method for simultaneous determination of some *Alternaria* toxins in tomato and tomato-based products. Special attention was given on the optimization of the MS/MS parameters to attain the best response. Additionally, optimization of the DLLME procedure was also assessed, by careful evaluation of the nature and amount of extraction and disperser solvents as well as the amount of sample. The validated method was used to assess the occurrence of AOH, AME, and TEN in 30 tomato and tomato-based samples commercialized in Valencia, Spain.

2.2 RESULTS AND DISCUSSION

2.2.1 MS/MS Optimization

A preliminary study was conducted in order to obtain the best instrumental conditions affording high resolution and short analysis time with a suitable analyte separation. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS source using a syringe injection at a flow rate of 10 µL/min. Positive and negative ionization modes were tested, obtaining a better response in positive ionization mode for the studied *Alternaria* toxins. Compound-dependent parameters of quadrupole mode scans including declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP), and collision energies (CE) were also evaluated and optimized to provide the best combination of efficiency and finding the optimal response value for each analyte. The acquisition of two single reaction monitoring transitions per analyte allowed the confirmation of the identity of the positive results according to the criteria established in Commission Decision No.

2002/657/EC [18]. The product ion with the highest intensity was selected as a quantifier, whereas the other was used as a qualifier. Table 2.1 lists the characteristic ions and the optimized mass spectrometry parameters for each compound during multiple reaction monitoring (MRM) acquisitions.

Table 2.1. Retention times, main transitions, collision energies (CE), declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), and collision cell exit potential (CXP) for the *Alternaria* toxins analyzed in this study

Analyte	Rt (min)	Parent Ion Q1 (m/z)	Product Ion Q3 (m/z)	CE (V)	DP (V)	EP (V)	CEP (V)	CXP (V)
TEN	8.1	415	312 ^{Q}	29	55	8	21	2
		$[M + H]^+$	256 ^{q}	39				
AOH	8.3	259	128 ^{Q}	65	39	10	16	3
		$[M + H]^+$	184 ^{q}	42				
AME	9.1	273	128 ^{Q}	60	32	10	16	13
		$[M + H]^+$	228 ^{q}	40				

^{Q} *Quantification transition;* ^{q} *Qualification transition.*

2.2.2 DLLME Optimization

DLLME has been proved to be a powerful cleaning and preconcentration technique. Other benefits, such as its high speeds and the low solvent use (if compared with traditional sample preparation procedures) and final disposal, have caused it to fast become one of the most popular analytical sample preparations in developing reliable quantitative multi-analyte methods. Other promising techniques, such as dilute-and-shoot approaches, only requires the dilution of the sample, however, it does not remove any interference which can cause chromatographic troubles, such as carryover, loss of sensitivity, increase of background interferences, or co-eluting peaks. Therefore, its use as a routine procedure may cause a time consuming drawback coming from the troubleshooting and cleaning of the system.

Hence, the DLLME sample preparation procedure was selected here based on its benefit in routine on a daily basis. The effect of the following parameters affecting the extraction efficiency was evaluated: (i) the type of extraction and disperser solvents; (ii) the extraction-disperser solvent ratios; and (iii) the amount of sample. The method of optimization was performed by recovery experiments in three replicates using tomato extract blank samples (5 mL) spiked with 25 ng/g of each targeted mycotoxin.

2.2.2.1 Influence of the type of extraction and disperser solvents

Selection of both an appropriate extraction and disperser solvent is very crucial to achieve good performance. The extraction solvent must have properties, such as a greater density than water, high extraction capability of the analytes, as well as low solubility in water. The role of the disperser solvent is to increase the dispersion of the extraction solvent as tiny droplets in an aqueous medium solution resulting in a large contact area between the extraction solvent and aqueous solution, thus improving the extraction efficiency. In this study, three common halogenated solvents, including CCl_4, CH_2Cl_2, and $CHCl_3$ were tested for extraction, whereas acetonitrile, acetone, and methanol were selected as disperser solvents.

Mixtures of 1.0 mL of different disperser solvents and 100 µL of extraction solvent were injected to 5 mL of tomato sample extracts spiked with the standard solution at 50 ng/g. Furthermore, 1 g of NaCl was added to the sample extract to improve the extraction efficiency, as well as to facilitate the phase's separation [19]. Extraction efficiency was evaluated by comparing the recoveries of the analytes. Results indicated that the best conditions were accomplished with the AcN-$CHCl_3$ pair, with satisfactory recoveries between 81%–94% (Table 2.2). There were no differences in extraction for the studied analytes.

2.2.2.2 Influence of the extraction-disperser solvent ratio

To evaluate the influence of the extraction-disperser solvent ratios on the extraction efficiency different volumes of chloroform (60, 80, 100, and 120 µL) and acetonitrile (0.5, 1.0, and 1.5 mL) were used. The optimal volumes of AcN and $CHCl_3$ were evaluated with the MATrix LABoratory (MATLAB)-based surface response design (Fig. 2.1).

Table 2.2. Recovery range of *Alternaria* toxins obtained by using different combinations of extraction (Ac, AcN and MeOH) and disperser solvents (CCl_4, CH_2Cl_2, and $CHCl_3$)

Disperser Solvent	Recovery Range (%) [a]		
	Extraction Solvent		
	CCl_4	CH_2Cl_2	$CHCl_3$
Ac	45–67	35–56	69–78
AcN	71–86	47–66	81–94
MeOH	58–81	53–78	65–83

[a] *spiked level: 25 ng/g of each target mycotoxin.*

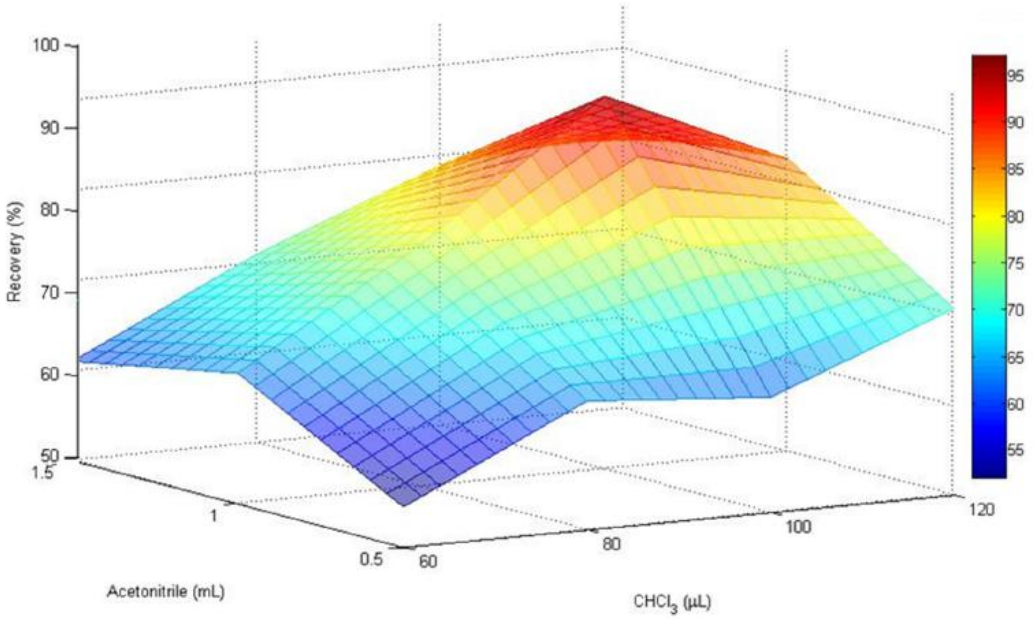

Fig. 2.1. MATLAB-based surface response design showing the influence of AcN and $CHCl_3$ ratio in the extraction efficiency of AOH. Recovery experiments were conducted by spiking tomato extract blank samples with 25 ng/g of each targeted mycotoxin

The enrichment factor improved with the lower volumes, but the lower the volumes the lesser the volume of the sedimented phase. Despite this, when the volume of extraction solvent was increased from 60 to 100 µL, the recoveries of the mycotoxins rose significantly. However, it should be noted that with the following combinations of AcN-$CHCl_3$: 1.50 mL–120 µL, 1.50 mL–100 µL, and 1 mL–120 µL, the matrix effect increased with respect to the other tested ratios. Hence, the combination of 1 mL of acetonitrile containing 100 µL of $CHCl_3$ was selected as a good compromise to reach the best DLLME conditions.

2.2.2.3 Influence of the amount of sample

The influence of the amount of sample was evaluated by testing different amounts of sample extract (5, 7.5 and 10 mL). Results showed that recoveries below 80% were obtained with 7.5 and 10 mL of

sample, whereas recoveries greater than 80% were achieved with 5 mL. Thus, 5 mL of tomato extract was selected as the optimum amount of sample for a reliable and efficient extraction (Fig. 2.2).

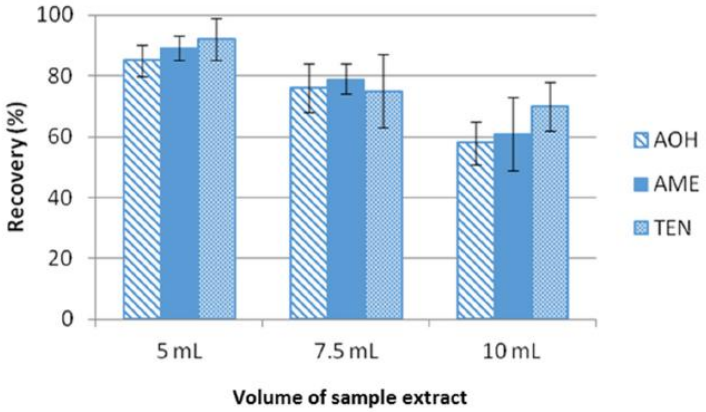

Fig. 2.2. Influence of the volume of sample extract in the extraction efficiency of the *Alternaria* toxins studied. Recovery experiments were conducted by spiking tomato extract blank samples with 25 ng/g of each target mycotoxin

2.2.3 Analytical Method Validation

Good linearity was achieved in all cases with regression coefficients higher than 0.990. Significant signal suppression was observed (from 65%–80%) between the slopes of the calibration lines meaning that the matrix effect is present (Table 2.3). Therefore, matrix-matched calibration curves were used for effective quantification in tomato samples.

Table 2.3. Overview of the correlation coefficient, extraction recovery, repeatability, and reproducibility (Rec (RSD), %), limits of detection (LODs) and quantitation (LOQ), and signal suppression/enhancement (SSE) for the studied analytes

Mycotoxin	Correlation Coefficient (r)	Repeatability (RSD_r, %) [a]		Reproducibility (RSD_R, %) [a]		LOD (ng/g)	LOQ (ng/g)	SSE (%)
		25 ng/g [b]	50 ng/g [b]	25 ng/g [b]	50 ng/g [b]			
AOH	0.998	81 (6)	82 (4)	84 (8)	89 (6)	1.40	3.50	65
AME	0.996	86 (4)	89 (7)	90 (7)	93 (10)	1.40	3.50	80
TEN	0.995	91 (9)	94 (6)	94 (15)	90 (12)	0.70	1.75	78

[a] n = 3; [b] spiked level.

Satisfactory results in terms of recoveries were found (recovery range from 81%–94% for both spiking levels). Precision studies showed that the method was repeatable (RSD_r < 9%) and reproducible (RSD_R < 15%) (Table 2.3).

LOQs were 3.5 ng/g for AOH and AME, while the LOQ for TEN was 1.75 ng/g. LODs were 1.40 ng/g for AOH and AME, while the LOD for TEN was 0.70 ng/g (Table 2.3). These results showed the suitability of the developed method for the determination of trace amounts of the selected mycotoxins in tomato samples. No obvious interfering peak from blank samples was detected. MRM chromatograms of tomato juice spiked at 10 µg/L of AOH, AME, and TEN are shown in Fig. 2.3.

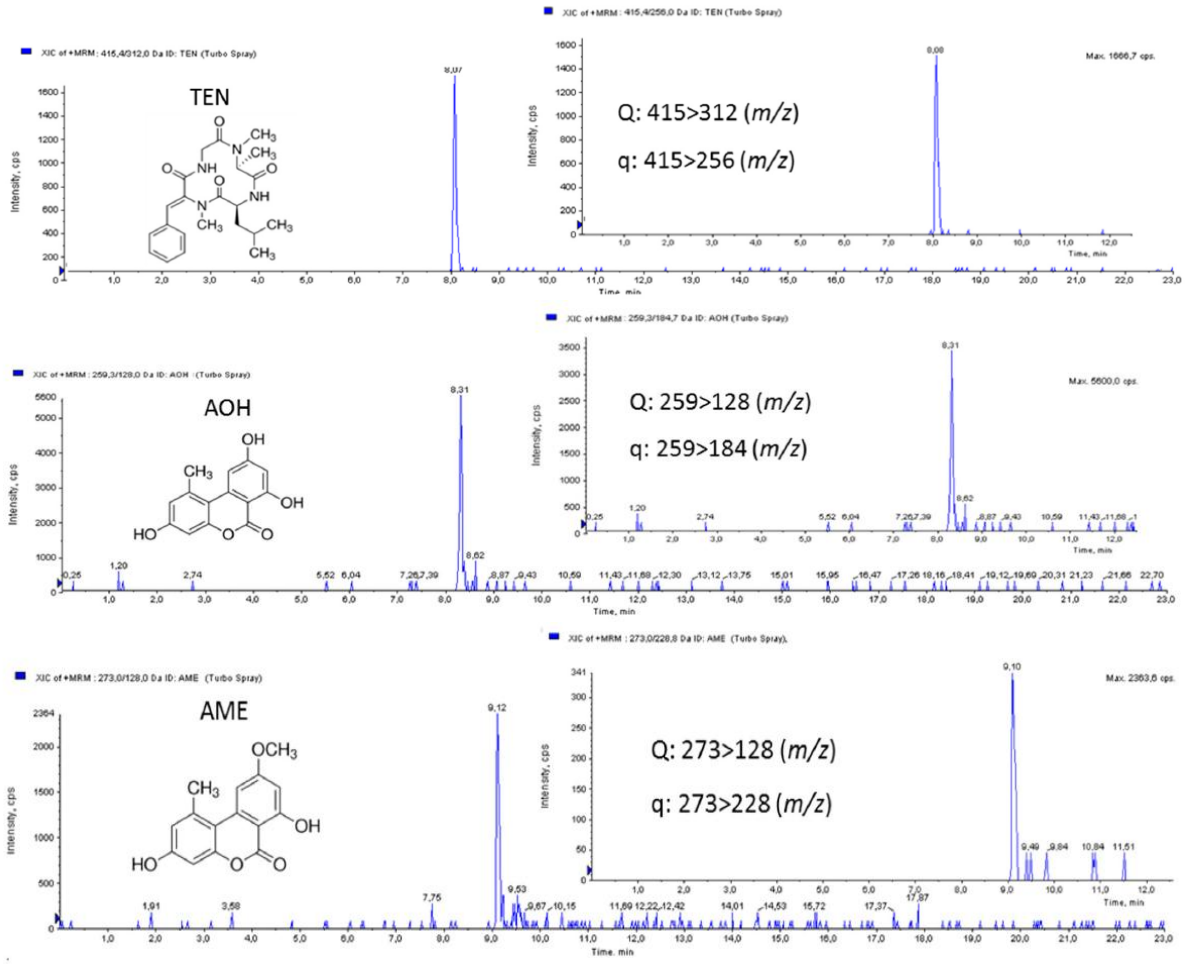

Fig. 2.3. MRM chromatograms of tomato juice spiked with 3.5 µg/L of AOH, AME, and TEN (corresponding to 1 ng of each injected toxin)

The results obtained in the present study were within the limits set by Commission Decision, No. 2002/657/EC. According to Commission Decision, No. 2002/657/EC, it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the analytes to a blank matrix and the guideline ranges for the deviation of the experimentally-determined recovery should be between 80% and 110% for a mass fraction ≥ 10 µg/kg. In the case of repeated analysis of a sample carried out under within-laboratory reproducibility conditions, the intra-laboratory relative standard deviation should not exceed 20% for a mass fraction of ≥ 10 µg/kg to 100 µg/kg. For analyses carried out under within-laboratory reproducibility conditions, the within-laboratory RSD shall not be greater than the reproducibility RSD.

2.2.4 Application to Samples

The developed method was evaluated carrying out a survey of AOH, AME, and TEN in 30 tomato and tomato-based products purchased in several Valencian supermarkets (Spain). Neither in tomato juice (n = 5) nor in gazpacho (n = 5) samples was the occurrence of target mycotoxins detected. However, five out of 20 fresh tomato samples (25%) resulted positive to at least one *Alternaria* toxin. AOH was detected in four out of the five contaminated samples but at levels close to LOQ (mean: 3.75 ng/g), whereas AME was identified in two fresh tomato samples but at levels between LOD and LOQ. TEN was not found in any analyzed sample. A MRM chromatogram of a naturally-contaminated tomato sample with AOH at 5.8 ng/g is shown in Fig. 2.4.

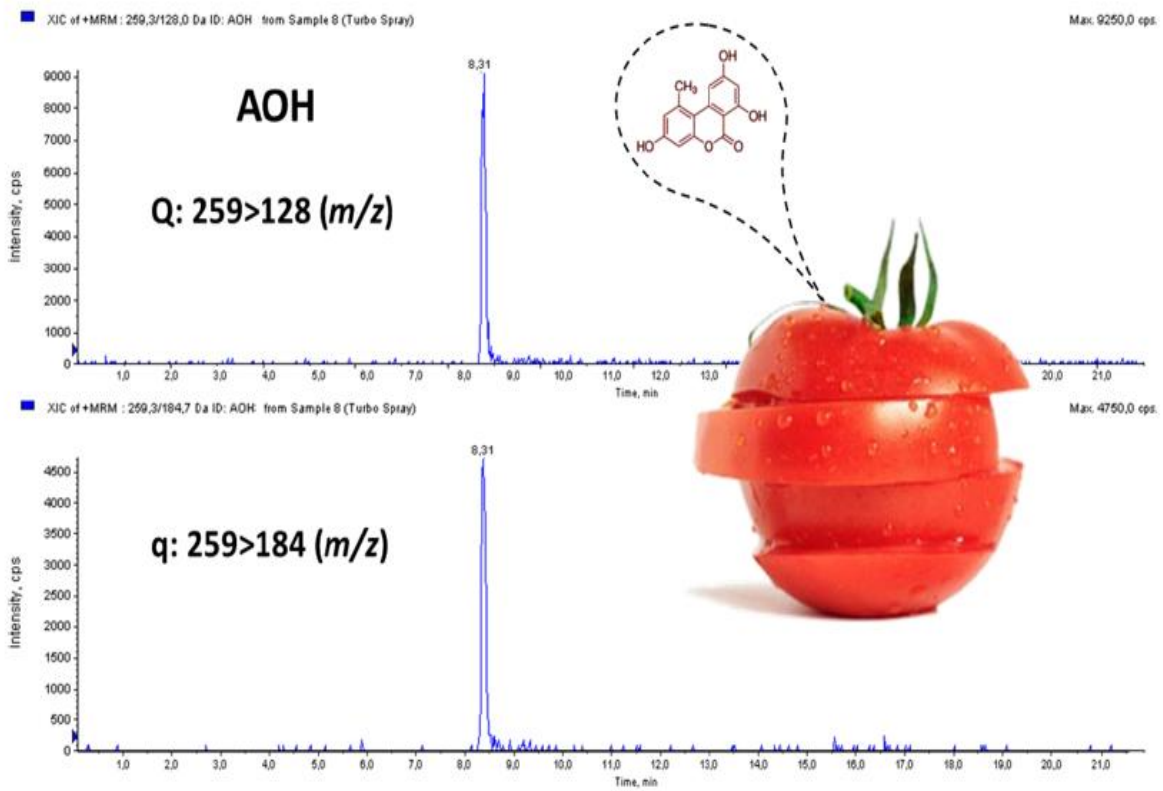

Fig. 2.4. MRM chromatogram of a naturally contaminated tomato sample with AOH at 5.8 ng/g

These findings are in agreement with the results reported in some European studies. In a study conducted in The Netherlands, AOH was detected in three out of 10 fresh tomato samples (levels ranging between 2–15 ng/g) but AME and TEN were not detected [20]. However, in the same study, AOH was quantified (at levels from 2–11 ng/g) in four out of 14 tomato juice samples. Similarly, in Switzerland, no *Alternaria* mycotoxins were found in fresh and whole tomato samples (*n* = 4) but AOH was detected in eight out of 24 tomato soup samples (levels from 4–10 ng/g). In the same products AME was detected in seven out of 24 samples but at lower concentration (from 1–4 ng/g). Only a few samples were positive for TEN (tomato puree, concentrated, and dried tomatoes) [21]. Those findings are also in line with the results reported in Italian tomato-based products (*n* = 10). Occurrence of AOH was detected in five out of 10 samples (levels from 4–6.8 ng/g) and TEN in one sample (4.4 ng/g) whereas AME was not found [22]. In Germany, a higher occurrence of *Alternaria* mycotoxins in tomato products (*n* = 34) was recently reported [23]. AME, AOH, and TEN were detected in 100, 70, and 26% of samples, respectively. AOH was found at levels from 6.1–25 ng/g and AME from 1.2–7.4 ng/g, whereas TEN contamination was set at levels from LOD and LOQ (<6.6 ng/g).

In China, no *Alternaria* mycotoxins were found in a study conducted in 70 fresh tomato samples [24]. In contrast, contamination of 26.2% of AME (up to 1734 µg/kg) and 6.2% of AOH (up to 8756 µg/kg) was reported in 80 tomato purees processed and sold in Argentina [25]. Similar results were also reported by Van de Perre et al. [26] who analyzed a total of 144 samples of derived tomato products such as ketchups, concentrates, pulp, dried tomatoes, and juices, which were collected from local markets in different countries (i.e., Belgium, Spain, Egypt, Brazil, and South Africa). Puree and concentrate tomato samples showed the highest occurrence of AOH and AME whereas, in tomato juice samples, none of the studied toxins were detected.

2.3 MATERIALS AND METHODS

2.3.1 Chemicals and Reagents

Acetonitrile (AcN), methanol (MeOH), acetone (Ac), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), and carbone tetrachloride (CCl_4) were supplied by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA).

Certified standards of AOH, AME and TEN were purchased from Sigma-Aldrich (Madrid, Spain). Standard solutions of AOH, AME, and TEN were prepared by dissolving 10 mg of each compound in 10 mL of MeOH. Stock solutions were diluted with pure MeOH afterwards order to get the appropriate working solutions. A multi-mycotoxin working standard solution was prepared by combining aliquots of each individual working solution and diluting with MeOH to obtain the final concentration of 0.02 mg/L for AOH, AME, and TEN. All solutions were stored at −20°C in amber glass vials and darkness before use.

2.3.2 LC-MS/MS Analysis

The determination was performed using a system LC-MS/MS triple quadrupole, consisted of a LC Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA)using a binary pump and automatic injector and coupled to a 3200 QTRAP® AB SCIEX (Applied Biosystems, Foster City, CA, USA). The chromatographic separation of the analyte was conducted at 25°C with a reverse phase analytical column Gemini® C18 (3 µM, 150 × 2 mm ID) and a guard-column C18 (4 × 2 mm, ID; 3 µM) from Phenomenex (Madrid, Spain).

Mobile phase was a time programmed gradient using as phase A methanol (1% formic acid and 5 mM ammonium formate), and as phase B water (1% formic acid and 5 mM ammonium formate). The following gradient was employed: equilibration during 2 min at 10% A at 0.25 mL/min, 10%–80% A in 3 min at 0.25 mL/min, 80% A for 1 min at 0.25 mL/min, 80%–90% A in 2 min, 90% A for 6 min at 0.25 mL/min, 90%–100% A in 3 min at 0.25 mL/min, 100% for 1 min at 0.35 mL/min, 100%–50% in 3 min at 0.4 mL/min at, return to initial conditions in 2 min and maintain during 2 min. Total run time was 21 min. The injection volume was 20 µL.

To analyze the mycotoxins, a triple quadrupole mass spectrometry detector (MS/MS) 3200 QTRAP® System AB SCIEX (Applied Biosystems, Concord, ON, Canada) was used. Electrospray ionization (ESI) interfaces were used to analyze these mycotoxins with the following settings for source/gas parameters: curtain gas (CUR) 20, ionspray voltage (IS) 5500 V, source temperature (TEM) 450°C, ion source gas 1 (GS1), and ion source gas 2 (GS2) 50. Therefore, in this study, the optimization of the MS/MS parameters was performed with an LC/MS system with the ESI interface in the positive ion mode using a mycotoxin standard mixture. The precursor ions (Q1) of each mycotoxin were confirmed in product ion (Q3) scan mode. As shown in Table 2.1, a protonated molecule was observed as the base peak ion in the mass spectra of AOH, AME, and TEN. Hence, these ions were selected as precursor ions (Q1) for each mycotoxin. The optimization of product ions (Q3) and their collision energy were performed in the product ion scan mode. The final selection of multiple reaction monitoring (MRM) transitions in positive ion mode for each compound, the optimal declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP), and collision energies (CE) are shown in Table 2.1. Data acquisition and processing were performed using Analyst® software version 1.5.2. (MDS Analytical Technologies, 2008 MDS Inc, ON, Canada).

2.3.3 Sampling and Sample Preparation

Thirty tomato and tomato-based samples were purchased from local supermarkets located in Valencia (Spain). Unwashed fresh tomato samples (n = 20) were chopped using a blender immediately after reception of samples in the laboratory. After blending, homogenization, and

centrifugation (3000 rpm, 4°C for 5 min), the tomato samples were placed in 100 mL closed polyethylene flasks before storing at 4°C until analysis. Analyses were carried out within the following two days after reception. Tomato-based samples consisted of gazpacho samples (*n* = 5) and tomato juice (*n* = 5).

A modified version of the DLLME method for sample preparation of fruit juices was used [**27**]. A mixture of 1 mL of AcN (as disperser solvent) and 100 µL of $CHCl_3$ (as extraction solvent), was rapidly injected into 5 mL of centrifuged tomato extract containing 1 g of NaCl. The mixture was vortexed for 1 min and centrifuged at 4000 rpm for 5 min, and the droplet formed was collected by a 100 µL syringe and transferred to a chromatography vial. Then, the droplet was evaporated to dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, *v/v*). The solution was filtered through 13 mm/0.20 µm nylon filter and injected into the LC–MS/MS system for mycotoxin analysis.

2.3.4 Method Performance

The method performance was performed under optimized conditions following the Commission Decision No. 2002/657/EC. The method validation included the evaluation of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision), and intermediate reproducibility (inter-day precision). All of the parameters were evaluated by spiking blank tomato juice samples at 25 and 50 ng/g. Samples were spiked and left to equilibrate over night before the analysis.

Linearity was assessed through six concentration levels in a linear range between LOQ and 100 × LOQ in triplicate. The correlation coefficient was obtained by plotting the signal intensity against analyte concentrations. A calibration curve was injected at the end of each batch to assess the response drift of the method. Components from matrix can negatively influence the method performance if they co-elute with the analyte of interest and can cause ion suppression or enhancement in the ion source. Therefore, matrix effect was also evaluated. The matrix effect (ME), is defined as the ratio between the slopes of the matrix-matched calibration and the solvent calibration one, and it was calculated as follows:

$$ME(\%) = \frac{Slope_{matrix-matched}}{Slope_{solvent}} \times 100$$

Matrix-matched calibration curves were built by spiking blank sample extracts with the studied analytes at the same concentration levels than those used in solvent standard calibration curves.

The accuracy was evaluated through recovery studies using spiked blank samples at 25 ng/g and 50 ng/g concentration levels. Recovery studies were performed in triplicate in the same day, as well as in three different days. Precision (expressed as %RSD) of the method was determined by repeatability (intraday precision, RSDr) and intermediate reproducibility (interday precision, RSDR). Intraday variation was evaluated in three determinations per concentration in a single day, whereas interday variation was tested on three different working days within 20 days. RSDr and RSDR were determined by spiking blank samples at the 25 ng/g and 50 ng/g concentration levels.

Limits of detection (LODs) and limits of quantitation (LOQs) were estimated from a blank juice tomato sample fortified with decreasing concentrations of the analytes. LODs were calculated using a signal-to-noise ratio of 3:1. LOQs Results were calculated using a signal-to-noise ratio of 10:1. The specificity of the method was evaluated with respect to interferences from endogenous compounds. Five samples of blank tomato juice samples were analyzed and compared with the corresponding spiked samples at the LOQ level to check for possible interference with the detection of the analytes.

2.3.5 Confirmation Criteria

Confirmation criteria were based on the following items; (i) chromatographic separation: the retention time of the analyte in the extract should correspond to that of the matrix-matched calibration within a ±

2.5% interval of the retention time; (ii) mass spectrometric detection: extracted ion chromatograms of sample extracts should have peak shapes and response ratios to those obtained from calibration standards analyzed at comparable concentrations in the same batch. The relative intensities or ratios of selective ions, expressed as a ratio relative to the most intense ion used for identification, should correspond to those of the calibration standard solutions. The ion ratio should not deviate more than 30% (relative).

2.4 CONCLUSIONS

A rapid, straight-forward, robust, sensitive and accurate analytical method based on DLLME-LC-ESI-MS/MS for determining various *Alternaria* toxins in tomato and tomato-based products was developed. Careful optimization of the MS/MS parameters was performed to reach the best analytical conditions. Additionally, parameters affecting the extraction efficiency of the DLLME were also evaluated and optimized. The method performance fulfilled the EU guideline standardized in the Commission Decision, No. 2002/657/EC. The recoveries were greater than 80% and relative standard deviations of repeatability and intermediate reproducibility were ≤9% and ≤15%, respectively, at levels of 25 and 50 ng/g. Under the optimized conditions LODs and LOQs were in the range 0.7–3.5 ng/g, respectively. Significant signal suppression was observed and matrix-matched calibrations were used for quantitation purpose. The developed method was successfully applied to 30 commercially available tomato and tomato-based products acquired in Valencia, showing the occurrence of various *Alternaria* toxins, at levels of few nanograms per gram in 20% of samples, being AOH the most commonly mycotoxin found. Due to its simplicity, and by allowing a faster extraction, the proposed methodology is proposed as a reliable analytical tool. Furthermore, this method could be applied to gather data on the presence of *Alternaria* toxins in foodstuffs, which are highly recommended by EFSA to enable a proper risk assessment of these toxins.

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AUTHOR CONTRIBUTIONS

Houda Berrada and Jordi Mañes conceived and designed the experiments; Yelko Rodríguez-Carrasco, Cristina Juan performed the experiments, analyzed the data and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 3
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The Natural Fungal Metabolite Beauvericin Exerts Anticancer Activity *in vivo*: A Pre-Clinical Pilot Study

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ABSTRACT

Recently, in vitro anti-cancer properties of beauvericin, a fungal metabolite were shown in various cancer cell lines. In this study, we assessed the specificity of this effect by comparing beauvericin cytotoxicity in malignant versus non-malignant cells. Moreover, we tested in vivo anticancer effects of beauvericin by treating BALB/c and CB-17/SCID mice bearing murine CT-26 or human KB-3-1-grafted tumors, respectively. Tumor size and weight were measured and histological sections were evaluated by Ki-67 and H/E staining as well as TdT-mediated-dUTP-nick-end (TUNEL) labeling. Beauvericin levels were determined in various tissues and body fluids by LC-MS/MS. In addition to a more pronounced activity against malignant cells, we detected decreased tumor volumes and weights in beauvericin-treated mice compared to controls in both the allo- and the xenograft model without any adverse effects. No significant differences were detected concerning percentages of proliferating and mitotic cells in tumor sections from treated and untreated mice. However, a significant increase of necrotic areas within whole tumor sections of beauvericin-treated mice was found in both models corresponding to an enhanced number of TUNEL-positive, i.e., apoptotic, cells. Furthermore, moderate beauvericin accumulation was detected in tumor tissues. In conclusion, we suggest beauvericin as a promising novel natural compound for anticancer therapy.

Keywords: Cyclohexadepsipeptide; beauvericin; cervix carcinoma; colorectal carcinoma; therapy.

3.1 INTRODUCTION

Malignant diseases are a major health concern worldwide [1] being the leading cause of death in most US states [2]. Although various treatment options including surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapeutics have been established [3], results in terms of progression-free and overall survival in several cancer types are still unsatisfying [4]. The most common limitations to currently-available approaches are severe adverse reactions and the development of multidrug resistance [5,6]. Therefore, there is a need for the development of new agents with novel mechanisms of action for cancer treatment [4]. Compounds isolated from different natural sources are promising candidates for the development of novel anticancer drugs, as shown previously [7]. Recently,

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naturally-occurring cyclic depsipeptides, which consist of hydroxyl- and amino acids linked by amide and ester bonds became of interest. They are secondary metabolites of bacteria, fungi and plants, or originate from algae, cyanobacteria, or sponges of the marine environment [8]. These compounds are known to exert a broad spectrum of biological effects, such as immunosuppressive, antibiotic, antifungal, as well as anti-inflammatory activities. Importantly, they were also shown to exhibit anticancer effects in different tumor models [9,10,11]. Recently, in vitro anticancer activity of the cyclodepsipeptides enniatins (ENNs) [12,13] and beauvericin (BEA) [14,15,16,17] were reported. In the case of ENNs, we showed high tumor cell specificity indicated by clearly-enhanced cytotoxicity against malignant, as compared to non-malignant, cells [12]. In addition, in vivo synergism of enniatin B with sorafenib, a clinically-approved tyrosine kinase inhibitor was observed in a cervical cancer model [18]. Similar anticancer effects were discussed for the structurally-related beauvericin. In the present pilot study we evaluated the cytotoxicity of beauvericin in normal versus malignant cell lines and assessed the in vivo anticancer activity of beauvericin to estimate its therapeutic potential.

3.2 RESULTS

3.2.1 Beauvericin Exerts Enhanced Cytotoxicity in Malignant as Compared to Non-Malignant Cells

Following previous studies in which beauvericin was suggested as potential anticancer drug [15,16,19] we compared the cytotoxic potency of beauvericin against malignant or non-malignant cells in vitro. At sparse conditions (5–10% cell confluency), beauvericin exerted cytotoxic effects on murine colon carcinoma cells (CT-26) and murine non-malignant fibroblasts (NIH/3T3) at micromolar concentrations (Table 3.1, Fig. S1). While IC_{25} values were similar, IC_{50} levels were 1.7-fold higher and IC_{75} values were even 2.4-fold higher for the non-malignant cell line as compared to the malignant CT-26 cells (Table 3.1). The difference between malignant and non-malignant cells became even more pronounced when treating cells at higher density (50–60%), representing better the in vivo situation. In the murine models, the IC_{50} value for beauvericin in CT-26 cells remained comparable (1.8 µM), in contrast to the IC_{50} value of the NIH-3T3 cells which rose to 9.4 µM. While at 1 µM no reduction of viability was observed in the non-malignant cells, tumor cell viability was already significantly reduced at 0.5 µM beauvericin at these conditions (Fig. S3).

Table 3.1. Cytotoxic activity of beauvericin (BEA) in murine cell lines

Cell Line	Tissue/	BEA (µM)	BEA (µM)	BEA (µM)
	Cell Type	Mean IC_{25} [1] ± SD	Mean IC_{50} [1] ± SD	Mean IC_{75} [1] ± SD
NIH/3T3	embryonic fibroblasts	1.2 ± 0.6	3.1 ± 0.2	6.5 ± 0.7
CT-26	colon carcinoma	1.4 ± 0.2	1.8 ± 0.2	2.7 ± 0.5

[1] *IC_{25}, IC_{50}, and IC_{75} values were calculated from dose-response curves and are given in means ± SD from at least three independent experiments performed in triplicate.*

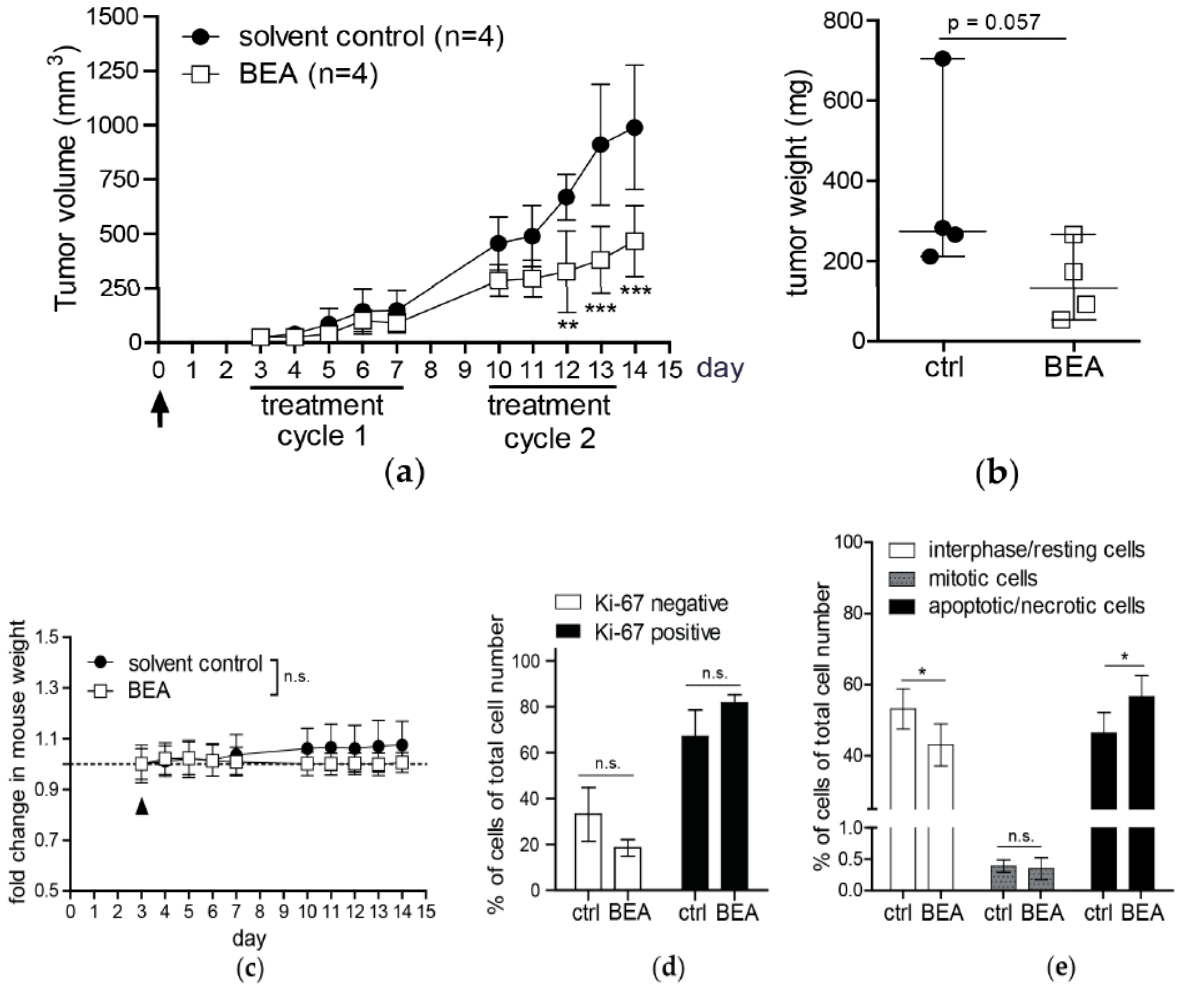
Regarding human cell lines, we treated non-malignant keratinocytes (HaCaT), three cervix cancer cell lines (KB-3-1, ME-180, GH354), and two cell lines originating from colon carcinomas (SW480, SW620), with increasing concentrations of beauvericin (Table 3.2, Fig. S2a,b). At sparse conditions, most malignant cell lines were more sensitive towards beauvericin treatment indicated by lower IC-values as compared to HaCaT cells (Table 3.2, Fig. S2a,b). Furthermore, SW620, the most dedifferentiated cell line used derived from a colon carcinoma metastasis [20], was most susceptible to beauvericin with an IC_{50} of 0.7 µM, i.e., 4.7-fold lower as compared to the non-metastatic colon carcinoma cell line SW480 and 5.6-fold lower than the normal HaCaT cells. When treating the cells at higher density (50–60% cell confluency) the difference between non-malignant and malignant cells was distinctly stronger. The IC_{50} values in KB-3-1 and SW480 cells increased only modestly (4.3 and 3.7 µM, respectively) while the one for non-malignant HaCaT cells rose distinctly to above 10 µM (Fig. S3).

To estimate the main mechanism underlying the reduced viability after beauvericin treatment, cell cycle and cell death analyses were performed in KB-3-1 cells. While at subtoxic concentrations a cell cycle arrest in the G0/G1-phase was prevalent (Fig. S4a), at concentrations >2 µM induction of apoptotic cell death was clearly detectable (Fig. S4b). In parallel, cleavage of poly-(ADP-ribose) polymerase (PARP) and caspase 9 was induced and upregulation of the proapoptotic Bcl-2 family members Bim and Bak was observed, while antiapoptotic Bcl-xL was reduced (Fig. S4c).

Table 3.2. Anticancer activity of BEA in human cell lines

Cell Line	Tissue/ Cell Type	BEA (µM) Mean IC_{25} [1] ± SD	BEA (µM) Mean IC_{50} [1] ± SD	BEA (µM) Mean IC_{75} [1] ± SD
HaCaT	Keratinocytes	2.7 ± 0.2	3.9 ± 0.4	4.8 ± 0.7
KB-3-1	Cervix carcinoma	2.6 ± 0.9	3.1 ± 0.7	3.6 ± 0.9
ME-180	Cervix metastasis	1.6 ± 0.8	2.2 ± 0.7	4.5 ± 1.2
GH354	Cervix adenocarcinoma	2.2 ± 0.6	3.6 ± 1.2	6.3 ± 2.1
SW480	Colorectal adenocarcinoma	2.1 ± 1.3	3.3 ± 0.3	4.2 ± 2.7
SW620	Colon metastasis (from SW480)	0.3 ± 0.02	0.7 ± 0.1	1.9 ± 0.2

[1] *IC_{25}, IC_{50}, and IC_{75} values were calculated from dose-response curves and are given in means ± SD from at least three independent experiments performed in triplicate.*



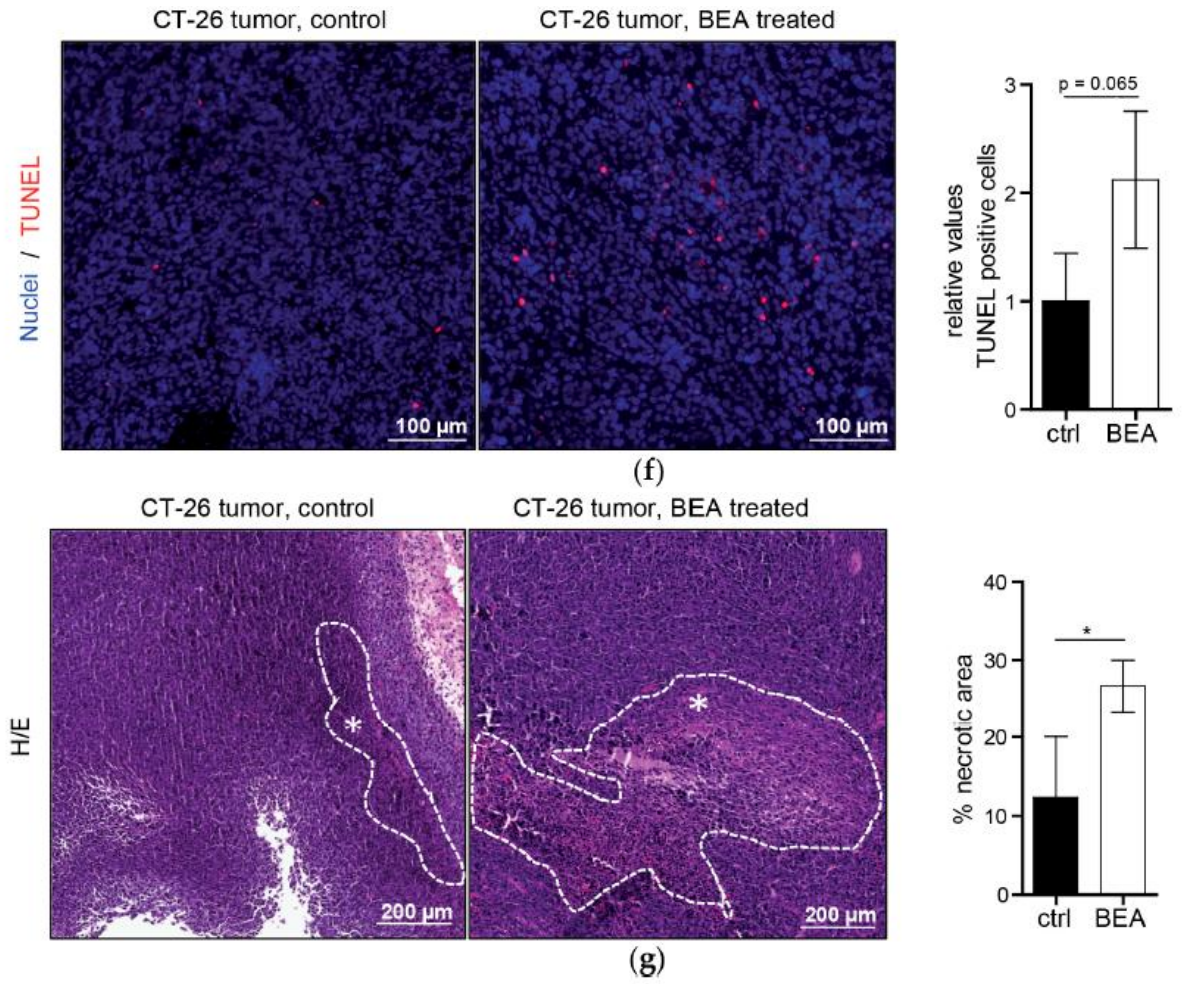


Fig. 3.1. In vivo anticancer activity of beauvericin (BEA) on CT-26-derived tumor allografts. (a) On day 0 tumor cells were injected (arrow) and beauvericin was administered in two cycles as indicated. Tumor volumes are given in mm^3 as mean values (±SD) for the solvent control (black circles) and beauvericin-treated group (open squares); (b) After sacrificing all mice on day 14, tumor weights were determined (median tumor weights in mg ± range); (c) Body weight of mice was measured during the study on the indicated 10 days and shown as mean fold change (±SD) relativized to baseline levels (dashed line) before treatment start (arrowhead); (d) Percentage of Ki-67-negative (open bars) and positive cells (black bars) in tumor sections from four treated and four control mice are shown; (e) Interphase and resting cells (open bars), mitotic (gray bars) and apoptotic/necrotic cells (black bars) counted in H/E-stained tumor sections are given in % of total cell number (±SD), counted in at least four optical fields of four tumors of both groups; (f) Representative images of tumor sections with TUNEL-positive cells (red) and DAPI-stained nuclei (blue) of a control (left) and of a treated mouse (middle) are shown. Results of TUNEL-positive cells counted in four tumor specimens of both groups respectively are given as relative values compared to the control (right); (g) Representative images of H/E-stained tumor sections of a control (left) and of a treated mouse (middle) are shown. Necrotic areas are encircled by white dashed lines and marked by asterisks. Areas of necrotic tissue were quantified by Definiens TissueStudio® 4.0 software from four tumors of both groups, respectively, and are depicted as the percent (±SD) of the total tumor area of the complete section (right). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.2.2 Beauvericin Treatment Induced Reduced Tumor Volumes and Increased Necrosis in an Allograft Mouse Model

Potential therapeutic effects of beauvericin were investigated in vivo in an allograft mouse model. Three days after subcutaneous injection of murine CT-26 colon-carcinoma cells into the right flank of BALB/c mice tumors were palpable and i.p. treatment with 5 mg beauvericin /kg body weight (bw)/day or with solvent alone was started (Fig. 3.1a). While during the first therapy cycle (days 3–7) only a minor effect on tumor volumes was observed; a marked reduction of tumor growth was detected in all four treated mice during the second treatment cycle (days 10–13). Differences in tumor volumes between the two groups were highly significant ($p < 0.01$) from day 12 onwards (Fig. 3.1a) and culminated in a 52.8% reduction of mean tumor volume in treated mice on day 14 at the end of the study. In addition to tumor volumes, tumor weights were measured showing that three of the four treated mice had lower tumor weights than the control group (Fig. 3.1b). Albeit not statistically significant ($p = 0.057$), an average 60% tumor weight reduction was observed in treated compared to untreated mice in accordance with the significant differences in tumor volumes (Fig. 3.1a).

Throughout the complete study period, mean body weight of mice remained virtually unaltered in both groups and no significant differences were observed between the treatment and the control group until the end of the second treatment cycle (Fig. 3.1c). Furthermore, behavior of the animals was monitored (see materials and methods) yielding no indication for beauvericin-attributed systemic toxicity (data not shown).

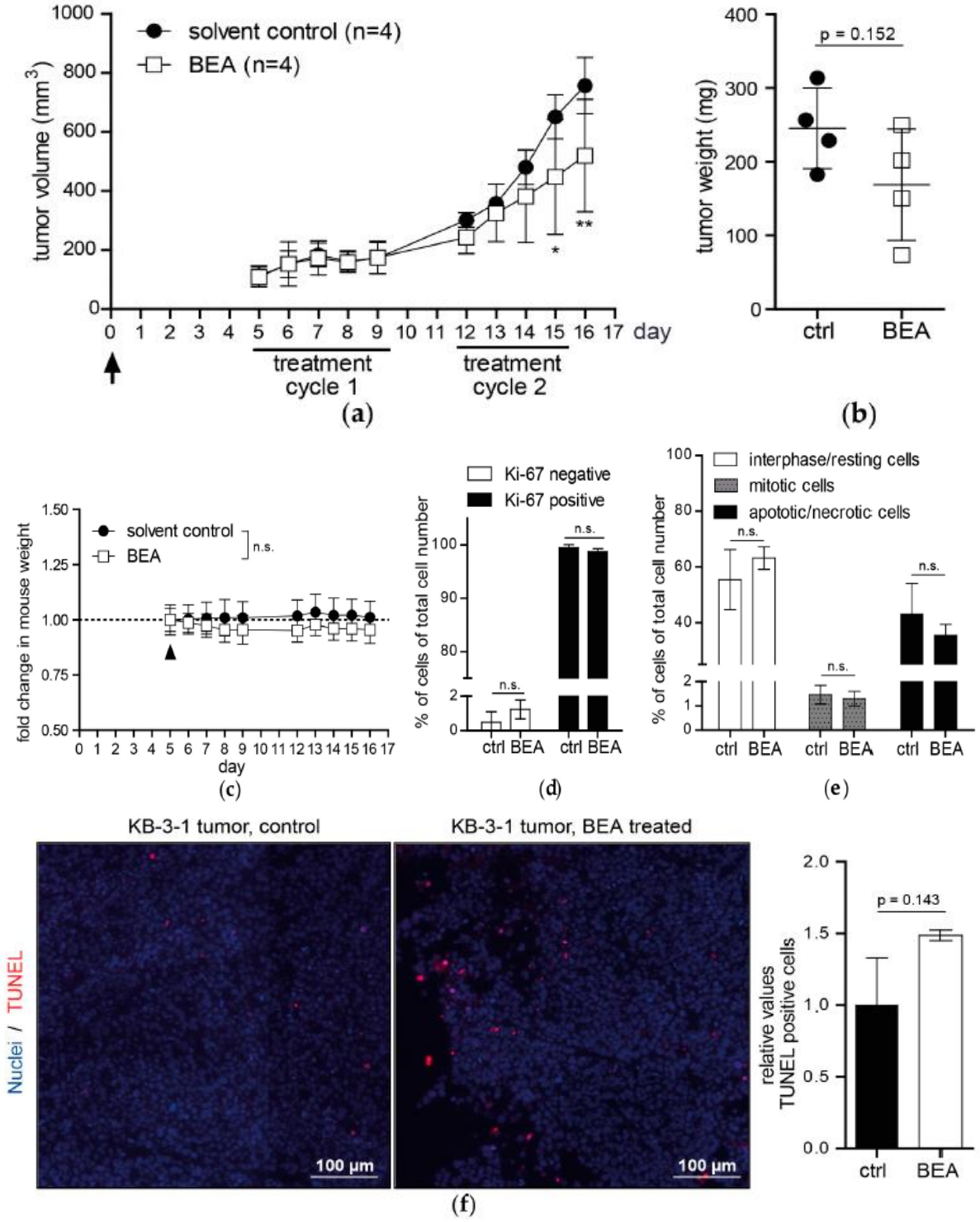
To investigate effects of beauvericin on tumor tissues in greater detail histological sections of tumor specimens obtained from treated and untreated mice were stained by different techniques. Staining for the proliferation marker Ki-67—expressed in cells during interphase or M-phase of the cell cycle [21]—revealed a slightly, but not significantly, higher percentage of proliferating tumor cells in the treated compared to the untreated group (81.6 ± 3.7% vs. 66.9 ± 11.7%. Fig. 3.1d). Assessment of fractions of mitotic cells in H/E-stained tumor sections yielded almost identical mean values for both groups (0.4 ± 0.1% control vs. 0.3 ± 0.2% treatment, Fig. 3.1e). However, in the group treated with beauvericin a 22% higher rate of scattered cells with signs of cell death (apoptosis or necrosis) became obvious within viable tumor regions ($p < 0.05$, Fig. 3.1e). In accordance, in TUNEL staining—a sensitive method for the detection of DNA strand breaks during apoptosis [22]—we observed a 2.1-fold increased average apoptosis rate in viable tumor areas of treated versus untreated mice, which did not reach statistical significance ($p = 0.065$, Fig. 3.1f). Additionally, a 2.2-fold increase of necrotic areas was detected in H/E-stained whole tumor sections of beauvericin-treated mice compared to the control group ($p < 0.05$, Fig. 3.1g).

2.3 Reduced Growth of Human Tumor Xenografts and Increased Necrosis in Tumor Tissue in Beauvericin-Treated Mice

To study therapy efficacy of beauvericin on human tumor growth, a cervix-carcinoma KB-3-1 xenograft mouse model was used. In accordance with the allograft experiments (Fig. 3.1a) tumor volumes of severe combined immunodeficiency (CB-17/SCID) mice treated with 5 mg/kg bw/day beauvericin were significantly reduced during the second treatment cycle (Fig. 3.2a) with a 31.3% reduction in tumor volume at the end of the experiment (day 16). Likewise, mean tumor weight was lowered by 31.2% in treated as compared to control mice ($p = 0.152$, Fig. 3.2b). In accordance with data of the allograft model (Fig. 3.1c) SCID mice did not show any indications for possible adverse treatment effects. Neither alterations of average body weight (Fig. 3.2c) nor abnormalities in behavior were observed (data not shown).

In Ki-67- and H/E-stained KB-3-1 tumor sections, similar results were obtained as compared to the allograft model in terms of rates of proliferating cells: in the control group 99.5 ± 0.6% and in the treatment group 98.8 ± 0.6% of Ki-67-positive cells were found (Fig. 3.2d) and 1.5 ± 0.4% mitotic cells were counted in the control versus 1.3 ± 0.3% in the treatment group (Fig. 3.2e). While almost no difference in percentages of apoptotic/necrotic cells within viable areas was observed between the two groups in H/E-stained tumor sections (Fig. 3.2e), a 1.5-fold increase of TUNEL-positive cells was

detected in tumor specimens of treated mice, indicating an not significant trend (p = 0.143) towards an increased rate of tumor cell death under beauvericin treatment (Fig. 3.2f). Similar to the allograft model, in H/E-stained whole tumor sections the proportion of necrotic areas was significantly increased by 34.2% in tumors of beauvericin-treated mice (Fig. 3.2g).



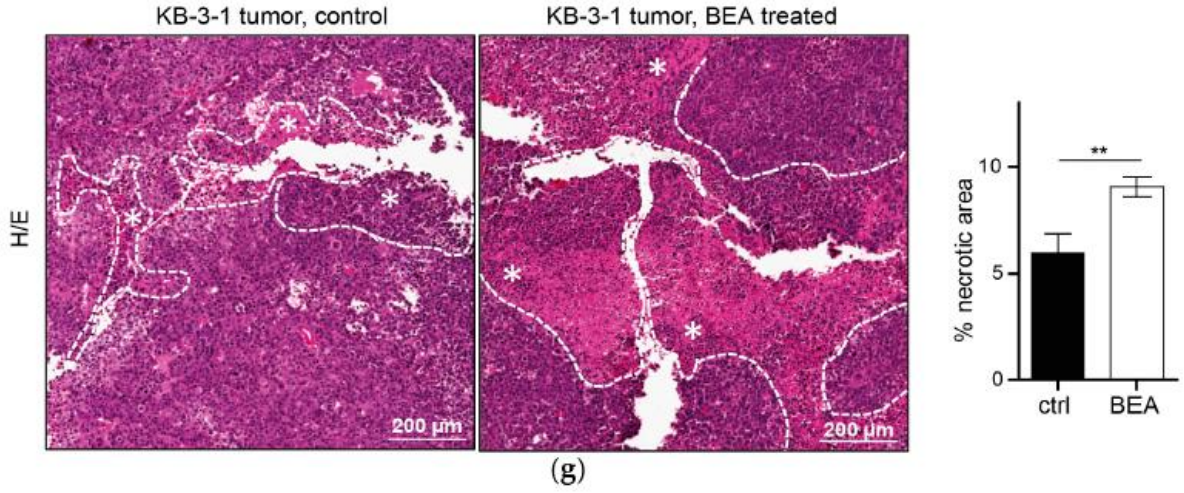


Fig. 3.2. In vivo anticancer activity of beauvericin on KB-3-1-derived tumor xenografts. (a) On day 0 tumor cells were injected (arrow) and beauvericin was administered in two cycles as indicated. Tumor volumes are given in mm^3 as mean values (±SD) for the solvent control (black circles) and beauvericin-treated group (open squares); (b) After sacrificing all mice on day 16, tumor weights were determined (mean tumor weights in mg ± SD); (c) Body weight of mice was measured during the study on the indicated 10 days and shown as mean fold change (±SD) relativized to baseline levels (dashed line) before treatment start (arrowhead); (d) Percentage of Ki-67-negative (open bars) and positive cells (black bars) in tumor sections from four treated and four control mice are shown; (e) Interphase and resting cells (open bars), mitotic (gray bars) and apoptotic/necrotic cells (black bars) counted in H/E-stained tumor sections are given in % of total cell number (±SD) and are counted in at least four optical fields of four tumors of both groups; (f) Representative images of tumor sections with TUNEL-positive cells (red) and DAPI-stained nuclei (blue) of a control (left) and of a treated mouse (middle) are shown. Results of TUNEL-positive cells counted in four tumor specimens of both groups, respectively, are given as relative values compared to the control (right); (g) Representative images of H/E-stained tumor sections of a control (left) and of a treated mouse (middle) are shown. Necrotic areas are encircled by white dashed lines and marked by asterisks. Areas of necrotic tissue were quantified by Definiens TissueStudio® 4.0 software (Definiens®, Munich, Germany) from four tumors of both groups, respectively, and are depicted as the percent (±SD) of the total tumor area of the complete section (right). * $p < 0.05$; ** $p < 0.01$

3.2.4 Distribution of Beauvericin in Tissue, Biological Fluids, and Tumor Specimens

Previously, a liquid chromatography mass spectrometry (LC-MS/MS) method was established to quantify beauvericin concentrations in various tissues and biological fluids after short-term treatment of healthy mice [19]. In the current study, we investigated tissue distribution of beauvericin after treatment of tumor-bearing mice as outlined above. In the allograft experiment with BALB/c mice beauvericin was measured in urine, serum, feces, and tissues, including CT-26 tumors, colon, liver, kidney, and adipose tissue (Fig. 3.3a), showing a significant 2.3-fold accumulation in tumors (81.0 ± 46.6 µg/kg) compared to serum (35.7 ± 13.5 µg/kg). However, the highest concentrations were found in adipose tissue (3701.3 ± 1091.5 µg/kg, 103.6-fold accumulation compared to serum) followed by feces (698.1 ± 257.2 µg/kg, 19.5-fold), excretory organs (kidney: 312.8 ± 104.6 µg/kg, 8.8-fold; liver: 266.5 ± 91.1 µg/kg, 7.5-fold), and colon (178.5 ± 170.1 µg/kg, 4.9-fold), while beauvericin concentrations in urine samples were very low (3.0 ± 3.3, 0.08-fold). In the xenograft experiment distribution of beauvericin was similar, but showed less pronounced accumulation in KB-3-1 tumor specimens (1.8-fold, 99.9 ± 35.7 µg/kg in tumor vs. 55.7 ± 23.4 µg/kg in serum) and in adipose tissue (2600.5 ± 513.9 µg/kg, 46.7-fold, Fig. 3.3b). Enrichment of beauvericin in colon (217.2 ± 104.0 µg/kg, 3.9-fold), liver (440.9 ± 165.9 µg/kg, 7.9-fold), kidney (305.7 ± 151.0 µg/kg, 5.5-fold), and feces (912.9

± 256.8 µg/kg, 16.4-fold) was comparable in both models, but in the xenograft experiment levels of beauvericin in urine were below the detection limit.

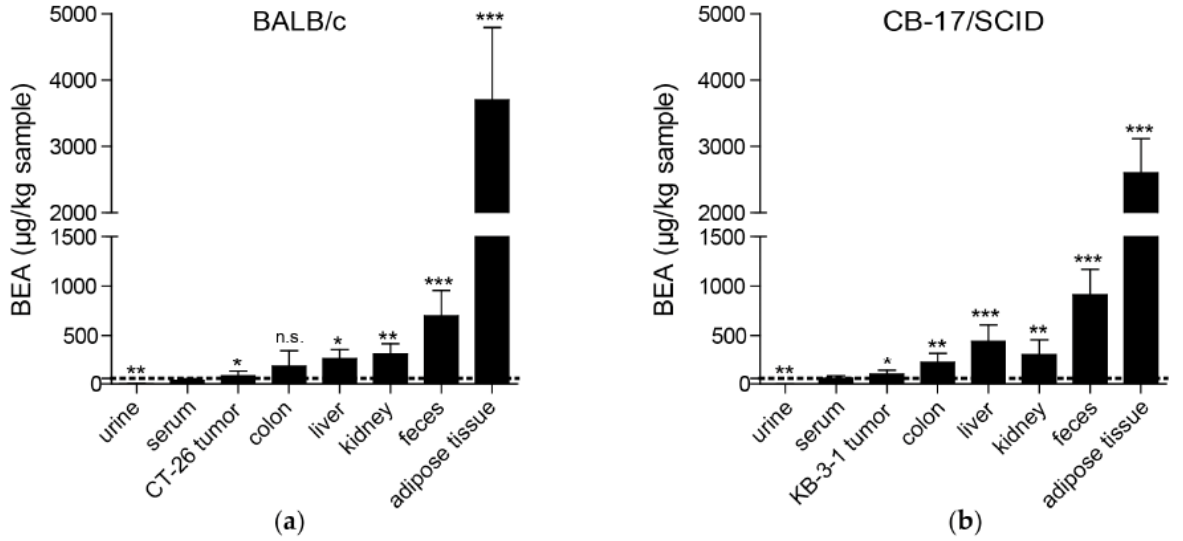


Fig. 3.3. Beauvericin distribution in mouse tissues and biological fluids after 9 days of treatment. Beauvericin levels (µg/kg sample) were determined in duplicates in all tissues indicated. The dashed line indicates the serum levels of beauvericin. Specimens were obtained from each mouse of both, the control (*n* = 4) and the treatment group (*n* = 4) of (a) the allograft model or (b) of the xenograft tumor model. * *p* < 0.05; ** *p* < 0.01; * *p* < 0.001**

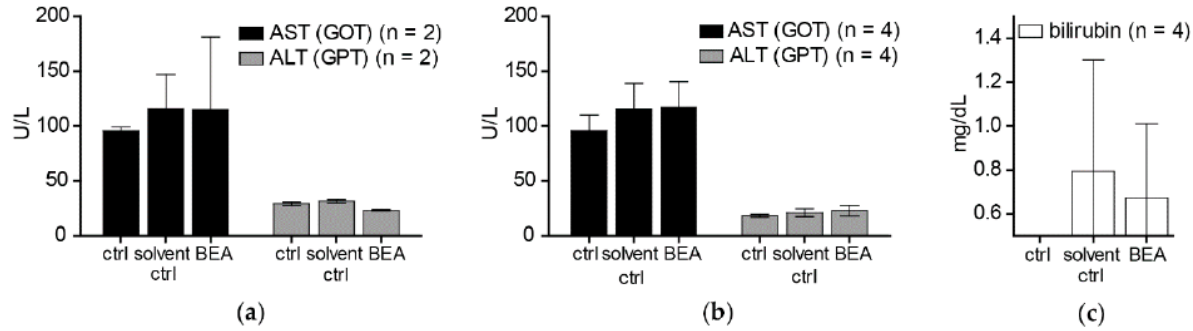


Fig. 3.4. Serum levels of aspartate aminotransferase (AST, black bars) and alanine aminotransferase (ALT, gray bars) were determined in CB17/SCID mice that were untreated (ctrl), treated with solvent (solvent ctrl) or with beauvericin (BEA) (a) one day after the last drug application and (b) two weeks after therapy finalization; and (c) bilirubin concentrations after two weeks of therapy in the sera of untreated (ctrl), solvent treated (solvent ctrl) and beauvericin-treated (BEA) mice are shown. Means (±SD) of replicates are shown for all measurements (a–c)

3.2.5 Concentrations of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Bilirubin, and Creatinine in Serum Indicate no Liver or Kidney Damage in Beauvericin-Treated CB-17/SCID Mice

Since considerable accumulation of beauvericin in liver and kidney was observed, possible hepato- and nephrotoxic effects of beauvericin were determined by monitoring concentrations of AST, ALT, bilirubin, and creatinine in the sera of beauvericin-treated mice in comparison to solvent-treated and untreated controls. One day after therapy, the serum level of AST in treated mice was 115.3 ± 66.0 U/L, which was similar to that of solvent treated (116.1 ± 30.9 U/L) and untreated (95.9 ± 3.1 U/L)

mice (Fig. 3.4a). Likewise, serum levels of ALT, a more specific indicator of liver damage, were 23.3 ± 0.6 U/L in treated mice, which was comparable to the levels determined in the sera of solvent-treated (31.7 ± 1.4 U/L) or untreated (29.4 ± 1.4 U/L) mice (Fig. 3.4a). The levels of total bilirubin, indicative for liver function, and of serum creatinine, reflecting glomerular filtration efficacy, were below the detection limit of 0.5 mg/dL in all mice at the end of the treatment period. As beauvericin was found to clearly accumulate in adipose tissue, a delayed release into the blood could be envisaged. Hence, in addition to measurements immediately after the last drug application, we further quantified these serum parameters two weeks after the last treatment at termination of the experiment. Results did not indicate any significant differences in serum levels of beauvericin-treated (AST: 117.3 ± 23.3 U/L, ALT: 23.1 ± 4.9 U/L, bilirubin: 0.6 ± 0.4 mg/dL), solvent treated (AST: 115.8 ± 23.3 U/L, ALT: 21.2 ± 3.6 U/L, bilirubin: 0.7 ± 0.5 mg/dL) and untreated (AST: 96.2 ± 13.9 U/L, ALT: 18.4 ± 1.4 U/L, bilirubin: <0.5 mg/dL) mice (Fig. 3.4b,c), while serum creatinine was below the detection limit (0.5 mg/dL) in all samples.

3.3 DISCUSSION

This study addressed in vivo anticancer efficacy and the therapeutic window of beauvericin. First, conforming to results from the structurally related enniatins [12] we found that beauvericin exerts modestly stronger cytotoxic effects in some malignant versus non-malignant cell types in vitro. The molecular mechanisms underlying these different sensitivities of cancer cell models are not fully understood but might include ABC-transporter-mediated drug efflux mechanisms or altered activation of cell survival pathways [23,24]. In addition, when seeding non-malignant fibroblasts or keratinocytes, but also cancer cells at higher density to mimic the tissue situation, beauvericin cytotoxicity markedly dropped in non-malignant cell types and the differences to cancer cells became more distinct. This selectivity is important for potential in vivo applications where cell death should be triggered in cancer cells while leaving non-malignant cells and tissues unaffected. In addition, our data revealed higher cytotoxic potency of beauvericin in colon carcinoma SW-620 cells from a metastatic lesion compared to the moderately dedifferentiated primary tumor cell line SW-480 of the same patient [20]. This is in agreement with migration inhibition by sublethal beauvericin concentrations in metastatic cancer cells (PC-3M, prostate cancer; MDA-MB-231, breast cancer) [15]. Together this data indicates that beauvericin might target especially dedifferentiated and invasive cancer types.

Investigating in vivo effects of beauvericin in mouse models of murine and human carcinomas, beauvericin treatment significantly reduced tumor volumes as compared to solvent-treated controls. Likewise, mean tumor weights were lower in the beauvericin-treated group of the xenograft and, even more pronounced, of the allograft model. Even though tumor growth was not completely inhibited, these effects suggest therapeutic potential of beauvericin, which may be further enhanced by optimization of the dose and the treatment schedule.

To examine mechanisms underlying the therapeutic effect of beauvericin histological stainings of tumor specimens were performed. In Ki-67-stained tissues the fractions of proliferating (Ki-67-positive) and resting (G0) cells (Ki-67-negative) did not show significant differences between treated and untreated groups in either model corroborated by similar rates of mitotic cells. Therefore, tumor growth reduction by beauvericin treatment was not caused by altered proliferation rates of malignant cells. Counting apoptotic/necrotic cells in H/E-stained tumor section, we observed a higher percentage of cells exhibiting apoptotic and necrotic features in beauvericin-treated mice, especially in the allograft model. Correspondingly, a distinct increase in TUNEL-positive cells, characteristic of apoptosis, was detected. Albeit, due to high variability between different regions of viable tumor parts, this alteration was not statistically significant. Whether uneven distribution of beauvericin in tumor nodules caused this variability needs to be determined. However, our results confirmed previous studies where beauvericin-induced apoptosis in diverse cancer cell types was shown in vitro [16,17,23,24].

The exact mechanisms of cell death induction by beauvericin is not yet established. However, in cervix carcinoma cells, we detected a G0/G1 phase arrest at subtoxic concentrations of beauvericin, followed by apoptosis induction at higher concentrations which was accompanied by the activation of the intrinsic mitochondrial cell death pathway. Additionally, the cytotoxic effects of beauvericin were

discussed to be based on its ionophoric characteristics increasing cytoplasmic calcium concentrations and stimulating calcium-dependent endonucleases finally resulting in DNA fragmentation and apoptosis [25]. Furthermore, others have also shown an influence on mitochondrial membrane potential [26,27], increased cytochrome C release followed by caspase 3 activation [17,27], a boost of reactive oxygen species (ROS) production [26], interaction with NF-KB [28] and/or MAPK pathways [16,28], as well as necrotic cell death [28] were suggested to underlie the multifaceted actions of beauvericin. Several of these suggested modes of action, like the boost of ROS production and the interaction with oncogenic NF-KB and/or MAPK pathways might have distinctly stronger impacts on malignant as compared to non-malignant cells and, hence, might contribute to the observed cancer selectivity. Cancer cells, for example, suffer from enhanced oxidative stress and are vulnerable to ROS-generating compounds [29]. For the MAP kinase pathway, inhibitory compounds are already in clinical use against cancer [30]. However, further investigations are necessary to estimate which cancer types might be primary targets for beauvericin treatment and which biomarkers might help to stratify respective patient subgroups.

Additionally to dispersed apoptotic/necrotic cells in the viable tumor, necrotic tissue areas—especially in the centers of the tumors—were significantly enhanced by beauvericin treatment in both tumor models. This is not necessarily a consequence of necrotic cell death but might be induced by focal, but massive, apoptosis of cancer cells or tissue breakdown due to starvation and lack of oxygen. This would suggest an interference of beauvericin with nutrient and oxygen delivery into malignant tissues probably based on inhibition of angiogenic processes. In accordance with this finding, anti-angiogenic activity of subtoxic beauvericin concentrations on human umbilical vein endothelial cells (HUVEC-2) has been described [15]. Likewise, in our hands both vessel forming and migratory ability of HUVEC cells were distinctly inhibited by subtoxic concentrations of beauvericin (unpublished data). Hence, reduction of blood supply in combination with the cytotoxic activity of beauvericin against tumor cells are likely to underlie the extensive necrosis detected in tumors of beauvericin-treated mice. Therefore, experiments investigating the impact of beauvericin on blood supply of tumors are currently initiated.

In our in vivo studies we observed a more pronounced anticancer activity of beauvericin on tumors in the allograft than in the xenograft model. This might result from the different cell-types used, i.e., cells derived from a colon carcinoma (CT-26) in the allograft and from a cervix carcinoma (KB-3-1) in the xenograft model. Accordingly, the former proved to be more susceptible to beauvericin also in vitro (IC_{50} for CT-26: 1.8 µM, for KB-3-1: 3.1 µM). Alternatively, the stronger activity in the allograft model might indicate a contribution of immune-related factors to the anticancer activity of beauvericin. Several chemotherapeutic agents support activation of tumor-targeting T-cell subclones partly based on enhanced tumor antigen presentation [31]. This immune-stimulating effect—synergizing with direct cytotoxic activity against malignant cells—is definitely lacking in SCID mice without functional B- and T-cells. However, immune-inhibitory effects of beauvericin have also been described in a Crohn's disease model [32]. Consequently, beauvericin might have an effect on invasion of immune cells e.g., T-cells, into the malignant tissue which is currently addressed in our allograft model. Generally, we did not see any signs of adverse effects or immune-related reactions in beauvericin-treated mice indicated by stable body weight, unaltered general physiological conditions (e.g., activity and coat appearance) or lack of abnormal behavior (e.g., grooming, fatigue). In addition, no signs of tissue alteration or inflammatory responses in H/E stained tissue sections of diverse organs were detected. Thus, we conclude that major adverse effects of beauvericin are unlikely.

As already suggested based on the lipophilicity of beauvericin [25], we found the highest beauvericin concentrations in adipose tissue followed by feces, kidney, and liver tissue. However, we also detected moderate beauvericin accumulation in tumor tissues in both mouse models in comparison to serum levels. In urine samples, we only detected minor beauvericin concentrations implying negligible renal clearance of the compound. Fecal enrichment, however, was also reported [25] suggesting elimination of beauvericin mainly through feces.

In contrast to the cytotoxic activity of beauvericin on non-malignant fibroblasts and keratinocytes shown in our in vitro experiments, no macro- and microstructural tissue changes were observed in liver and kidney [19], despite accumulation of beauvericin in these organs. In line with this observation, no alterations of serum markers indicating tissue damage (AST, ALT) or impairment of

kidney or liver function (bilirubin, creatinine) were detected both in treated and in control mice immediately and two weeks after the treatment period. This discrepancy between cytotoxic effects of beauvericin in vitro and lack of obvious adverse reactions in vivo might be explained at least in part by the loss of beauvericin cytotoxicity against non-malignant but not against malignant cell types at higher cell density. Additionally, pharmacokinetic parameters and blood vessel integrity effects might lead to tissue-specific drug exposure alterations in the in vivo situation.

Although in this study we observed a significant enrichment of beauvericin in tumors compared to serum levels, the accumulation was much more pronounced in adipose tissue, liver, kidney, or colon. In general, several approaches are possible to improve such sub-optimal tumor accumulation. Hence, drugs might be chemically modified to obtain derivatives with enhanced pharmacological characteristics. Alternatively, nanoformulations of several anticancer drugs were proven to be superior compared to the native substances also in clinical applications, such as the approved liposomal doxorubicin (Doxil®) [33]. Therefore, we plan to develop beauvericin derivatives and/or nanoformulations with improved therapeutic windows.

Beauvericin exerted a more pronounced anticancer activity in single drug regimens as compared to the closely related compound ENN B [18], maybe due to differences in metabolization. While no metabolites of beauvericin could be detected in mice after three days of treatment, ENN B was processed to three phase I metabolites [19]. Similar results were gained in vitro [34]. Due to this obviously higher metabolic stability sustained concentrations of beauvericin may be achieved in vivo. Additionally, rapid acquisition of beauvericin resistance seems unlikely as we did not induce beauvericin-unresponsiveness of KB-3-1 cells during a two-year in vitro selection process [24]. These favorable pharmacokinetic characteristics endorse the therapeutic potential of beauvericin.

3.4 MATERIALS AND METHODS

3.4.1 Chemicals

Beauvericin was purchased from BioAustralis (Smithfield, Australia) and, for animal experiments, purified from *Beauveria bassiana* (ATCC 7159). The culture conditions were adopted from Xu et al. [14] and the biomass harvested by suction filtration. The mycelium was lyophilized and extracted with ethyl acetate. The solvent was evaporated and the brownish residue resolved in methanol. Insoluble residues were removed by filtration and the solvent evaporated. The residues were dissolved in acetonitrile/water (80:20 *v/v*) and the solution was centrifuged to remove insoluble particles. The supernatant was then subjected to reversed phase chromatography using a GROM-Sil 120 ODS-5 HE, 10 µm, 250 × 20 mm column (Grace GmbH and Co KG, Worms, Germany) on an Agilent 1100 series preparative HPLC system (Agilent Technologies, Waldbronn, Germany) running isocratically on acetonitrile (+0.1% formic acid)/water (+0.1% formic acid) (70:30 *v/v*) with a flow rate of 15 mL/min. Beauvericin containing fractions were pooled, acetonitrile was evaporated and water was removed by lyophilization. Purity of the compound was verified by LC-MS on an Agilent ESI-Triple-Quadrupol-MS, 6460 Series (Agilent Technologies, Waldbronn, Germany) and by nuclear magnetic resonance (NMR) on a Bruker Avance III 700 MHz-NMR spectrometer (Bruker, Karlsruhe, Germany). Stock solutions of beauvericin were prepared in DMSO and stored at −20°C.

3.4.2 Cell Culture

All cancer cell lines used for this study are described in Table S1. Cultures were regularly screened for *Mycoplasma* contamination.

3.4.3 Cell Viability Assay

For the cell lines KB-3-1 and SW480 2×10^3 cells, for NIH/3T3, CT-26, HaCaT, and GH354 cells 3×10^3 cells and for ME-180 and SW620 cells 4×10^3 cells were seeded into 96-well pates and incubated at 37°C (5% CO_2) overnight. All cell numbers corresponded to a cell monolayer confluency of 5–10% 24 h after seeding and immediately before beauvericin treatment. For higher cell confluency, NIH/3T3,

CT-26, HaCaT, KB-3-1, and SW480 cells were grown to a cell density of approximately 50–60% before treatment. Cells were exposed to increasing concentrations of beauvericin for 72 h. The percentage of viable cells was detected after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 1–4 h, depending on the cell line, according to the user manual (EZ4U, Biomedica, Vienna, Austria). Cell viability after 72 h was determined and concentrations of beauvericin leading to a reduction of cell number by 25%, 50%, and 75% (IC_{25}, IC_{50}, IC_{75}), respectively, were calculated from whole dose-response curves. All experiments were conducted using full-growth media with 10% FBS in triplicate and repeated three times. The cell confluency was analyzed by Image J 1.50i (NIH, New York, NY, USA).

3.4.4 In Vivo Allo- and Xenograft Experiments

For the allograft experiment, 4×10^5 CT-26 cells were resuspended in 50 µL RPMI medium and injected subcutaneously into the right flank of eight, 6–8 weeks old, male BALB/c mice that were obtained from Harlan Laboratories (San Pietro al Natisone, Italy). Likewise, in the xenograft experiment, 1×10^6 KB-3-1 cells in 50 µL RPMI medium were injected subcutaneously into the right flank of eight, 6–8 weeks old, male CB-17/IcrHanHsd-Prkdc severe combined immunodeficiency (SCID) mice. Of each group, four animals were randomly assigned to the control or to the treatment group. After the tumor was palpable and reached an approximate size of 25 mm^3 the respective mouse was either treated intraperitoneally with 5 mg/kg bw/day beauvericin (dissolved in 10% DMSO) as described previously [19] or, for the control group, with solvent alone (10% DMSO). All animals were kept under pathogen-free conditions and all procedures were performed in a laminar flow hood. Effects of the treatment were assessed by daily recording of tumor size with a microcaliper and parameters indicating the animals' overall health condition (e.g., body weight, fatigue, grooming, ragged coat, food and fluid consumption). Tumor volumes (mm^3) were calculated using the formula: (length × $width^2$)/2. After two therapy cycles of four to five days, and 24 h after the last beauvericin injection, animals were sacrificed by cervical dislocation after anesthesia (Ketavet®/Rompun® mix) to collect blood by heart puncture. The tumor mass was weighed and organs and tissues for immunohistochemical experiments were fixed in 4% formalin/PBS (Roti®-Histofix 4%, Roth, Karlsruhe, Germany) or shock-frozen in liquid nitrogen and stored at −80 °C until the samples were prepared for LC-MS/MS analysis. The experiments were approved by the ethics committee of the Austrian Federal Ministry of Science, Research, and Economy (BMWF-66.009/0084-II/3b/2013, date of approval: 5 September 2013) and performed in line with the Arrive guidelines for animal care and protection and with guidelines from the Austrian Animal Science Association and from the Federation of European Laboratory Animal Science Associations (FELASA).

3.4.5 Immunohistochemistry

From each mouse from both the control (n = 4) and the treatment group (n = 4), tissue samples were formalin-fixed, paraffin-embedded and used to prepare serial 3 µm sections. Then, slices were deparaffinized and rehydrated. To evaluate the percentages of interphase/resting, mitotic and dead (apoptotic/necrotic) cell fractions in tumor specimens, sections were stained with hematoxylin and eosin (H/E) by means of standard protocols. Numbers of the three cell fractions were analyzed in a blinded setup in at least four images of H/E-stained tumor sections, taken under a 40 × objective microscope lens. In addition, for the quantification of areas of dead tumor tissue in whole tumor sections, the H/E-stained slides were scanned with a Pannoramic MIDI automated slide scanner (3DHISTECH, Budapest, Hungary) and evaluated using Definiens' TissueStudio® 4.0 (Definiens®, Munich, Germany) software and Pannoramic Viewer (3DHISTECH) software. To evaluate the fraction of apoptotic cells in tumor sections a terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was performed applying the In Situ Cell Death Kit TMR (red) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Afterwards, slides were covered with Vectashield anti-fade mounting medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA). Slides were protected from light until they were scanned with a Pannoramic MIDI automated slide scanner (3DHISTECH) and analyzed via digital image analysis software (Definiens' TissueStudio® 4.0, Definiens®). In order to visualize the proliferative cell fraction (cells in the interphase or M-phase) of tumors, KB-3-1 tumor sections were stained with the Ki-67 (clone MiB-1) antibody from DAKO (1:100; Glostrup, Denmark) and for CT-26 tumor sections

with Ki-67 (1:100; D3B5, rabbit, Cell Signaling, Danvers, USA). The primary antibody was incubated for 30 min at room temperature in a humid chamber and after rinsing the slides for 3 min in PBS + 0.1% Tween, the UltraVision LP detection system was applied according to the manufacturer's instructions (Thermo Fisher Scientific, Massachusetts, MA, USA). Sections were counterstained with hematoxylin. For analyzing the percentages of Ki-67-negative and -positive cells in the tumor sections, slides were scanned and analyzed via digital image analysis software (Definiens' TissueStudio® 4.0, Definiens®).

3.4.6 LC-MS/MS Analysis

Concentrations of beauvericin in tissue samples were analyzed according to Rodríguez-Carrasco et al. [19]. Briefly, tissues were collected from each mouse of both the control (n = 4) and the treatment group (n = 4). Samples of which were thawed on ice, divided and weighted. Afterwards, each slice was placed into a Precellys® hard tissue homogenizing CK28 tube (VWR, Radnor, PA, USA). After adding 2 mL of acetonitrile (LC-MS LiChrosolv®, Merck Millipore, Darmstadt, Germany) to each tube, samples were centrifuged for four cycles with 6000 rpm for 30 s with a 30 s break. Beauvericin from blood and urine samples was extracted by mixing 50 µL of sample with 1.5 mL acetonitrile and vortexing for 15 s. The supernatant was transferred into a fresh tube and stored at −80 °C until the LC-MS/MS analysis was performed. Therefore, a QTrap 5500MS/MS system (Applied Biosystems, Foster City, CA, USA) coupled to a TurboV electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) were used. Chromatographic separation was achieved at 25 °C on a Gemini® C18 column (150 × 4.6 mm i.d., 5 µm particle size) connected to a C18 security guard cartridge (4 × 3 mm i.d.; all from Phenomenex, Torrance, CA, USA) with a flow rate of 1 mL/min. Elution was performed in binary gradient mode and both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (*v*/*v*/*v*; eluent A) and 97:2:1 (*v*/*v*/*v*; eluent B), respectively. In the first 2 min of elution 100% eluent A was used, afterwards the proportion of eluent B was increased linearly to 50% within 3 min followed by a linear increase of B to 100% within 9 min. Finally, after a hold-time of 4 min with 100% of eluent B, the column was re-equilibrated with 100% eluent A for 2.5 min. ESI-MS/MS was performed in the scheduled selected reaction monitoring (sSRM) mode in positive mode and the target scan time was set to 1 s. The ESI source was set as follows: source temperature 550 °C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage + 5500, collision gas (nitrogen) medium.

3.4.7 Quantitative Determination of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Bilirubin, and Serum Creatinine

For the measurement of AST, ALT, bilirubin, and creatinine, ten male CB-17/IcrHanHsd-Prkdcscid mice (Harlan Laboratories, San Pietro al Natisone, Italy) that were 6–8 weeks old, were randomly assigned to three groups: two to the control (i.e., no treatment), four to the solvent control (10% DMSO), and four to the beauvericin treatment group (5 mg/kg bw/day beauvericin). Mice were treated in two cycles of five and four days, respectively. One day and two weeks after the last treatment, blood was collected from mice. Samples were allowed to coagulate at room temperature and serum was obtained by two centrifugation steps (1000 rpm, 10 min, 4 °C). Concentrations of AST, ALT, bilirubin, and serum creatinine were determined in serum samples by Reflotron® Plus System (Roche, Basel, Switzerland) according to the manufacturer's instructions.

3.4.8 Statistics

Data were analyzed using GraphPadPrism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Results are given as mean ± standard deviation (SD), if not indicated otherwise. Tumor volumes of the two groups were compared for each day by two-way ANOVA followed by Bonferroni post-test. For all other statistical analyses an unpaired two-tailed Student's *t*-test or, for non-parametric data-distribution, a Mann-Whitney test was performed.

3.5 CONCLUSIONS

In conclusion, we identified beauvericin as a modestly tumor-selective anticancer agent in vitro, exerting distinct activity and favorable tolerability in vivo in an allo- and a xenograft model of colon- and cervix cancer, respectively. Although tumor enrichment and the therapeutic margin of beauvericin still needs to be improved, our observations suggest further preclinical development of this natural compound as anticancer agent.

SUPPLEMENTARY MATERIALS

The following are available online at https://www.bookpi.org/wp-content/uploads/2022/05/Chapter-3_Supplementary-Materials.pdf, Fig. S1: Cell viability of murine fibroblasts NIH-3T3 and colon carcinoma CT-26 cells after treatment for 72 h with the indicated concentrations of beauvericin, Fig. S2: Cell viability of beauvericin-treated human malignant versus non-malignant cells, Fig. S3: Impact of beauvericin on cell viability of murine non-malignant fibroblasts NIH-3T3 and human non-malignant keratinocytes HaCaT, murine colon carcinoma CT-26, human cervix carcinoma KB-3-1 and human colon carcinoma SW480 cells at higher density, Fig. S4: Effects of beauvericin treatment on KB-3-1 cells, Table S1: Description of cell lines used in this study.

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AUTHOR CONTRIBUTIONS

D.H., R.D.-F. and W.B. conceived and designed the experiments; D.H. performed the experiments; S.v.S. performed the immunohistochemistry stainings; Y.R.-C. and M.S. performed the LC-MS/MS analysis; P.H. and W.B. contributed to and supervised the in vivo studies; D.H., G.T., B.E. analyzed the data, S.B., R.D.S. and R.L.-G. contributed reagents/materials/analysis tools; D.H. and W.B. wrote the paper. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 4
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Simultaneous Determination of AFB1 and AFM1 in Milk Samples by Ultra High Performance Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry

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ABSTRACT

Milk is the world's most consumed beverage, not counting water. Even though investigations on milk aflatoxin (AF) M1 contamination are regularly conducted, there is limited information on the contamination of milk with its parent compound, AFB1. Hence, the aim of this study was to develop a quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based method for the simultaneous analysis of AFB1 and AFM1 in milk, using ultrahigh performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS). The recoveries were in a range of 75–96% at 0.005, 0.01, and 0.05 µg/L spiking levels, with repeatability and reproducibility results expressed as relative standard deviations (RSDs) lower than 7% and 16%, respectively. The limits of detection (LODs) and quantification (LOQs) were 0.001 and 0.002 µg/L for AFM1 and AFB1, respectively. The LODs and LOQs that were obtained showed the suitability of the developed method for the determination of trace amounts of the selected mycotoxins in milk samples, and were up to ten times lower than those that had been reported in previous works using triple quadrupole mass analyzers. The matrix effect was evaluated and matrix-matched calibrations were used for quantification. The validated method was applied to 40 Italian milk samples. Neither AFB1 nor AFM1 were found above the LOD in any of the analyzed samples.

Keywords: Aflatoxin M1; aflatoxin B1; milk; mass spectrometry; QuEChERS; Orbitrap HRMS.

4.1 INTRODUCTION

Mycotoxins are secondary toxic metabolites that are produced by several species of fungi, mainly belonging to the *Aspergillus, Penicillium, Alternaria*, and *Fusarium* genera. These fungi are able to contaminate agricultural products and to produce mycotoxins under favorable conditions [1]. Contamination of food and feed with mycotoxins is a worldwide problem. In fact, the Food and Agriculture Organization (FAO) has estimated that a quarter of the world's crops are contaminated with mycotoxins, and it has a major economic impact [2]. Moreover, mycotoxins are of significant public health concern, based on their high toxic profile. Among these contaminants some metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, namely aflatoxins (AFs), can be found, which are classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) [3]. Hence, it is appropriate to keep the levels for AFs as low as reasonably achievable in both food and feed. In this sense, the Commission Directive 2003/100/EC set a maximum content of AFB1 of 20 µg/kg in all feed materials, with the exception of complete feeding stuffs for dairy animals, which has been reduced by up to 5 µg/kg [4]. AFM1 is the principal hydroxylated AFB1 metabolite that is

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present in the milk of cows that are fed with AFB1-contaminated feed, and it was classified as possibly carcinogenic to humans (Group 2B) by IARC. [5]. This metabolite is thermally resistant and is not completely inactivated after pasteurization, sterilization, or other milk treatment processes, and thus could represent a public health concern [6]. Consequently, the Commission Regulation (EC), No. 165/2010, and amending Regulation (EC), No. 1881/2006, with regards to aflatoxins, set the most restrictive limits for AFM1 content in milk at 0.05 µg/kg for raw milk, heat-treated milk, and milk for the manufacture of milk-based products, and of up to 0.025 µg/kg for infant formulae and follow-on formulae, including infant milk and follow-on milk [7].

Milk is the world's most consumed beverage, not counting water, and it is the primary source of nourishment for the normal growth of infants and children. According to the latest data that were reported by FAO, the European population has a significantly higher milk and dairy consumption than the global population, with an annual per capita data of 236.4 kg and 90 kg, respectively. Among the European countries, Italy is one of the highest milk and dairy consumers, with an annual per capita consumption of 246.9 kg [8]. On the other hand, the complexity of milk composition, containing fat, proteins, sugar, and other components, makes sample treatment difficult and, usually, different cleanup steps are necessary after extraction. Besides solid-phase extraction [9], liquid-phase extraction [10], and immunoaffinity column assay [11] for the purification and enrichment, QuEChERS (acronym for quick, easy, cheap, effective, rugged and safe) was a significant method for high–throughput determination and has been widely employed for the analysis of mycotoxins in different matrices [12,13,14,15], including milk [16,17]. Most of the studies regarding the occurrence of mycotoxins in milk are only focused on AFM1 analysis. Nevertheless, in recent years, the number of reports of milk being contaminated by multiple mycotoxins has increased, raising concerns about whether the synergistic effects of these coexisting mycotoxins could affect public health. In this sense, not much literature has considered evaluating the occurrence of AFB1 in milk, despite the fact that it is a carcinogenic compound to humans [18,19,20]. For instance, a recent study reported an incidence of AFB1 and AFM1 in 12.4% and 98.8%, respectively, of Chinese milk samples (n = 250) [21]. Therefore, humans are potentially exposed to these toxic metabolites and it becomes necessary to take a vigilant attitude in order to minimize the human intake of aflatoxins. To achieve this purpose, analytical methods must provide enough sensibility to reach the maximum limits that have been set by the Commission Regulation. In this sense, liquid chromatography mass spectrometry (LC-MS) methods, for multi-mycotoxin analysis in milk, have been reported in the literature [6,22,23]. In recent years, there has been increasing interest in evaluating the capability of the high-resolution mass spectrometry (HRMS) for multi-mycotoxin analysis because it provides not only a high resolution and accuracy mass results, but also a high sensibility and complementary structural information when compared with other MS detectors [21,24].

According to the aforementioned information, this method includes the analysis of AFM1 and its parent compound, AFB1, the latter having not been being frequently studied in milk. This method, with a simple extraction procedure based on the QuEChERS method, coupled with the high throughput determination that was provided by the ultrahigh performance liquid chromatography Q-Orbitrap mass spectrometry, has been successfully validated and applied to the analysis of these toxic compounds in 40 commercially available milk samples from Italy.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Materials

Acetonitrile, methanol, and water (LC-MS grade) were purchased from Merck (Darmstadt, Germany). Formic acid (mass spectrometry grade) and ammonium formate (analytical grade) were obtained from Fluka (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were supplied by Phenomenex (Castel Maggiore, Italy).

Sodium chloride and anhydrous sulphate sodium were acquired from Sigma Aldrich (Milan, Italy). Primary secondary amine (PSA) sorbent and C18 (analytical grade) were purchased from Supelco (Bellefonte, PA, USA). Standards of AFB1 and AFM1 (purity >98%) were acquired from Sigma Aldrich (Milan, Italy). Individual standard solutions of AFM1 and AFB1 were prepared at 1 µg/mL in methanol.

Working standard solutions at 5, 10, and 50 µg/L were prepared by adequate dilutions of the stock, for the spiking experiments. All solutions were stored at −20 °C in screw-capped glass vials.

4.2.2 Sampling

A total of 40 milk samples were randomly purchased between January and February 2018, from different supermarkets located in the Campania region, Southern Italy. The samples were shipped to the laboratory in their original packages and stored at 4°C until analysis. The milk analysis was carried out within two days after the arrival of the samples.

4.2.3 Sample Preparation

A QuEChERS-based procedure for the extraction of mycotoxins in milk was employed as a starting point, with minor modifications [16]. In short, 10 mL of sample was introduced into a 50 mL Falcon tube and 2.5 mL of distillate water, and 5 mL of acetonitrile, containing 3.35% of formic acid, (*v*/*v*) was added. The mixture was vortexed vigorously for 2 min and then subjected to ultrasonic extraction for 15 min (vortexed in every 5 min interval). After that, the tube involved the addition of 4.0 g of sulphate sodium anhydrous and 1.2 g of sodium chloride, which was shaken by hand for 2 min and then centrifuged for 3 min at 4000 rpm and 4°C. The upper organic layer was transferred to a 15 mL Falcon tube containing 300 mg of C18 sorbent, 140 mg of PSA, and 1.5 g of sulphate sodium. The mixture was vortexed for 1 min and was then centrifuged for 1 min at 1500 rpm and 4°C. The supernatant was transferred into a new glass tube and then evaporated under a gentle nitrogen flow at 45°C. Finally, the residue was reconstituted with 500 µL of MeOH:H_2O (70:30, *v*/*v*), filtered (0.22 µm membrane filter), and transferred into a vial for UHPLC-Q-Orbitrap HRMS analysis. Fig. 4.1 shows the schematic flow of the sample preparation procedure.

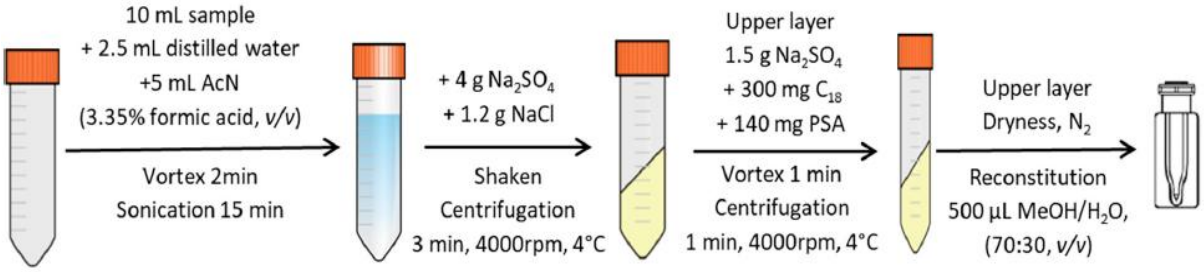

Fig. 4.1. Schematic flow of the sample preparation procedure

4.2.4 UHPLC-Q-Orbitrap HRMS Analysis

The analyses were performed using an UHPLC instrument (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Q Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, Waltham, MA, USA). The UHPLC system consisted of a degassing system, a Quaternary UHPLC pump working at 1250 bar, an auto sampler device, and a thermostated Luna Omega column (50 × 2.1 µm, 1.6 µm, Phenomenex) that was held at 30°C. The mobile phases were as follows: phase A, water with 0.1% formic acid and 5 mM ammonium formate; and phase B, methanol with 0.1% formic acid and 5 mM ammonium formate. A linear gradient elution program was applied as follows: initially 0% B was held for 1 min and then increased to 95% B in 1 min, and held for 0.5 min. Then, the gradient was linearly decreased to 75% in 2.5 min, and decreased again to 60% B in 1 min. After that, the gradient was reduced to 0% in 0.5 min and was held for 1.5 min for re-equilibration, giving a total run time of 8 min. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The detection was performed using a Q-Exactive mass spectrometer [25].

Table 4.1. Ultrahigh performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS) parameters of the studied mycotoxins

Mycotoxins	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (*m/z*)	Measured Mass (*m/z*)	Accuracy (Δ ppm)	Product Ion (*m/z*)	Collision Energy (eV)
AFM1	4.60	$C_{17}H_{12}O_7$	$[M - H]^+$	329.06558	329.06511	−1.43	273.07538 229.04909	40
AFB1	5.02	$C_{17}H_{12}O_6$	$[M - H]^+$	313.07066	313.06958	−3.45	285.07489 269.04373	36

The mass spectrometer was operated in positive ion mode by setting two scan events (full ion MS and all ion fragmentation [AIF]). Full scan data in positive mode were acquired at a resolving power of 70,000 FWHM at 200 *m*/*z*. The conditions in the positive ionization mode (ESI+) were as follows: spray voltage 4000 V; capillary temperature 290°C; S-lens RF level 50; sheath gas pressure (N_2 > 95%) 35; auxiliary gas (N_2 > 95%) 10; and auxiliary gas heater temperature 305 °C. The mass range in the full scan experiments was set at *m*/*z* 100–1000. The parameters in the positive ion mode for the scan event of AIF were as follows: mass resolving power = 10,000 FWHM; scan time = 0.10 s; HCD collision energy = 30 eV. UHPLC-HRMS parameters of the studied mycotoxins are shown in Table 4.1. The data analysis and processing were evaluated by the Quan/Qual Browser Xcalibur software, v. 3.1.66. (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA).

4.2.5 Validation of the Method

The validation of the method was carried out according to the EU Commission Decision 2002/657/EC [26]. The method performance was evaluated by the following parameters: linearity, matrix effect, trueness, precision, specificity, and sensitivity. All of the parameters were performed in triplicate.

Student *t*-test statistical analysis was performed for data evaluation: *p* values < 0.05 were considered significant.

4.2.6 Quality Assurance/Quality Control (QA/QC) in the Analysis of Real Samples

For the confirmation criteria, the retention times of the aflatoxins in the standards and samples were compared at a tolerance of ±2.5% and an accurate mass <5 ppm, thus providing a higher level of confidence in the analyses identification. In order to demonstrate the effectiveness of the validated method, a reagent blank, a procedural blank, a replicate sample, and a matrix-matched external calibration were included in each batch of samples for QA/QC. The fortified milk samples with AFB1 and AFM1 at three concentration levels (0.005, 0.01, and 0.05 µg/L) were used for analytical quality control (QC). The QC samples were stored and refrigerated at 4°C.

4.3 RESULTS AND DISCUSSION

4.3.1 Analytical Features of the Proposed Method

The method validation was performed in-house for the determination of AFM1 and AFB1 in milk samples. Table 4.2 shows the performance of the proposed method, namely, linearity, matrix effect, trueness, precision, specificity and sensitivity.

Table 4.2. Method performance

Parameters	R^2	SSE (%)	Recovery, % (*U'*, %; *k* = *2*)			LOD (µg/L)	LOQ (µg/L)
			0.005 µg/L	0.01 µg/L	0.05 µg/L		
AFM1	0.9994	65	75 (26)	84 (20)	91 (16)	0.001	0.002
AF B1	0.9996	72	81 (32)	87 (24)	96 (22)	0.001	0.002

R^2—correlation coefficients; SSE—signal suppression or enhancement; U'—expanded uncertainty (k = 2); LOD—limit of detection; LOQ—limit of quantification.

4.3.1.1 Linearity

Linearity was evaluated using both the standard solutions and matrix-matched calibrations at six concentrations levels, ranging from 0.002 to 20 µg/L. Calibration curves were performed in triplicate. The correlation between the response and the amount of analyte was verified by plotting signal intensity against analyte concentration. The correlation coefficients (R^2) of both analytes was >0.9990.

4.3.1.2 Matrix effect

The matrix effect (signal suppression or enhancement, SSE) was expressed as the ratio percentage between the slope of the matrix-matched calibration curve (A) and the curve in solvent (B). Thus, the

ratio (A/B × 100) was defined as the matrix effect (%). A value of 100% indicated that there was no matrix effect. There was signal suppression if the value was lower than 100% and a signal enhancement if the value was higher than 100%. The matrix effects for AFB1 and AFM1 were 72% and 65%, respectively. Based on the results that were obtained, the matrix-matched calibrations were used for quantification purpose.

4.3.1.3 Trueness and precision

The trueness was assessed throughout the addition of known amounts of the studied mycotoxins at 0.005, 0.01, and 0.05 µg/L to a blank milk sample, and were expressed as the percentage of recovery. The method provided satisfactory recoveries at each fortification level, ranging from 75% to 96% in all of the spiking levels, which were in agreement with the accepted values that were set at Commission Decision 2002/657/EC. The precision of the method was carried out by repeated measurements of the fortification levels that reported above, on the same day (repeatability, $n = 3$) and on three different days (reproducibility, $n = 3$), and were expressed as relative standard deviation (% RSD). The precision data showed that the method was repeatable (RSD < 7%) and reproducible (RSD < 16%). On the other hand, it was a requirement under ISO/IEC 17025 that laboratories determined and made available the expanded measurement uncertainty, which was associated with analytical results [27]. An expanded coverage factor of $k = 2$ was usually assumed to calculate the expanded measurement uncertainty that was represented by *u'*, from the relative standard uncertainty *u'* in Equation (1), as follows:

$$U' = k \times u' \quad (1)$$

The relative standard uncertainty *u'* was calculated using the laboratory reproducibility relative standard deviation combined with the estimated method bias, applying Equation (2), as follows:

$$u' = \sqrt{u'(RSD_R)^2 + u'(bias)^2} \quad (2)$$

where: u′ is the combine standard uncertainty; u′(RSDR) is the laboratory reproducibility; and u′(bias) is the uncertainty component arising from method bias.

4.3.1.4 Specificity

A blank milk extract, from a sample that was previously analyzed to confirm the absence of target aflatoxins, was injected 10 times to study the signals that were obtained from the matrix, and to evaluate the possible sample interferences. The good specificity of the HRMS made it possible to have no signal interferences in the blank matrix for any of the studied aflatoxins.

4.3.1.5 Limits of detection and limits of quantification

Sensitivity was evaluated by limit of detection (LOD) and limit of quantification (LOQ). The LOD was defined as the minimum concentration, where the molecular ion could be identified with a mass error below 5 ppm, and the LOQ were set as the lowest concentration of the analyte that produced a chromatographic peak with a precision and accuracy <20%. The method LODs and LOQs obtained were 0.001 and 0.002 µg/kg, respectively. The LODs and LOQs that were obtained showed the suitability of the developed methods for the determination of trace amounts of the selected mycotoxins in the milk samples. In addition, the LOQs that were obtained here were up to 10 times lower than those that were reported by other authors using triple quadrupole mass analyzer, and were similar than those that were reported in a recent studies employing HRMS [21] (Table 4.3). Nevertheless, this last study used immunoaffinity columns, which significantly increase thed sample preparation procedure in terms of time and cost, compared with the inexpensive QuEChERS.

4.3.1.6 QA/QC

The quality of results were controlled through all of the experiments. The spiked matrix was used in the QA/QC procedure to properly evaluate the accuracy and precision of the proposed methodology. In order for the data to be acceptable, the following QA/QC criteria had to be met. Extraction was

considered as conducted correctly when the recoveries were in the range from 70% and 120% (RSD < 20%). Out of this range, the extractions were repeated to meet these criterions.

Table 4.3. Simultaneous occurrence of AFB1 and AFM1 in milk samples, reported in literature

Milk Origin	Positive Samples		% Positive Samples	Range (µg/L)	LOQ (µg/L)	References
Mexico	AFM1	117/290	1.4	<LOQ-8.35	0.05	Carvajal et al. [33]
	AFB1	4/290	40.3	<LOQ-0.42	0.05	
Egypt	AFM1	11/80	13.8	0.144–0.378	*n.r.*	Nassib et al. [32]
	AFB1	1/80	1.2	<LOQ-3.54	*n.r.*	
Brazil	AFM1	21/40	52.5	0.7–1.5	0.50	Scaglioni et al. [18]
	AFB1	7/40	17.5	0.8–1.7	0.25	
Spain	AFM1	0/10	-	<LOD	0.025	Flores-Flores et al.
	AFB1	0/10	-	<LOD	0.020	[23]
Spain	AFM1	0/191	-	<LOD	0.025	Flores-Flores et al.
	AFB1	0/191	-	<LOD	0.020	[20]
China	AFM1	11/45	24.4	<LOQ-0.03	0.01	Zhou et al. [16]
	AFB1	0/45	-	<LOD	0.01	
China	AFM1	247/250	98.8	0.002–0.028	0.001	Mao et al. [21]
	AFB1	31/250	12.4	<LOQ-0.023	0.0033	
Italy	AFM1	0/40	-	<LOD	0.002	This study
	AFB1	0/40	-	<LOD	0.002	

n.r.: non reported.

4.3.2 Application to Samples

The validated UHPLC-Q-Orbitrap HRMS methodology was applied to 40 milk samples that were commercially available in Italy. The results showed that any analyzed sample was contaminated with AFM1 (LOD: 0.001 µg/L). The data that were reported here were in agreement with previous monitoring studies carried out in the milk from European countries, in which the absence or low AFM1 incidence was reported. For instance, Tsiplakou et al. [22] did not find AFM1 (LOQ: 0.002 µg/L) in any of the 21 milk analyzed samples from Greece. Similar results were recently reported by Flores-Flores et al. [20], who conducted a monitoring of AFM1 in tSpanish milk samples (*n* = 191), but none of them showed mycotoxin contamination (LOD: 0.025 µg/L). In France, Boudra et al. [28] reported that 3.4% of the analyzed milk samples (*n* = 264) were contaminated with AFM1, but at levels below the EU regulation. In contrast, 1.6% of Croatian milk samples (*n* = 61) [29] and 5% of Portuguese milk samples (*n* = 40) [30] presented AFM1 values that surpassed the legal maximum limit (0.05 µg/L). Despite that, Flores-Flores et al. [31] reviewed the occurrence of AFM1 in milk samples worldwide and reported that the European milk samples that exceeded the maximum EU limit (0.9%, *n* = 13566) were significantly lower than the milk samples that were commercialized in America (8.6%, *n* = 1267), Africa (25.8%, *n* = 980), and Asia (26.7%, *n* = 6376). Hence, Europeans seemed to be less exposed to AFM1 through milk consumption than other populations, and it could be probably related to the restrictive rules for AFM1 contamination in milk that were adopted by the EU (up to 10-fold lower than some countries).

Even though investigations on milk AFM1 contamination have been regularly conducted by European Economic Community (ECC) countries, there was limited information on the contamination of this food matrix by other major mycotoxins, such as AFB1. The existence of AFB1 in milk may have been as a result of contaminated feedstuffs that were not completely metabolized by the cow to AFM1, thus AFB1 was excreted in the milk. Table 4.3 summarizes the available studies focused on the occurrence of AFB1 and its metabolite AFM1 in milk samples. The occurrence of AFB1 was reported in 1.2% (*n* = 80) [32], 1.4% (*n* = 290) [33], 12.4% (*n* = 250) [21], and 17.5% (*n* = 40) [9] of Egyptian, Mexican, Chinese, and Brazilian milk samples, respectively, in which were found concentrations of up to 3.54 µg/L. These results were of particular concern because of the lack of regulation and considering that AFB1 was a recognized human carcinogen [3]. As far as the European milk samples were concerned, up to now, the occurrence of AFB1 had not been reported in the milk samples. The

absence of AFB1 that was found in these analyzed Italian milk samples were in agreement with previous studies that were conducted in samples from Spain [20,23]. These results seemed to be also related to the maximum AFB1 level that was allowed in the complete feeding stuffs for dairy animals, which was set at 5 µg/kg by Commission Directive 2003/100/EC.

4.4 CONCLUSIONS

Nowadays, a large number of studies regarding the occurrence of AFM1 in milk are available in the literature. However, there is limited information on the contamination of milk with AFB1, which could be found in this food matrix because of its incomplete metabolism. The present study was conducted to produce a sensitive, rapid, and accurate method in order to determine AFB1 and its metabolite AFM1 in milk. The determination of the studied analytes in a Q-Orbitrap system working in AIF mode, allowed an accurate determination of even ultratrace levels of mycotoxins because of the HRMS analyzer. It was, together with the use of an easy and cheap extraction procedure and the benefits from its application, the most important feature of this analytical procedure. Satisfactory results in terms of linearity, trueness, precision, specificity, and sensitivity were obtained, which fulfilled the requirements that were set by the Commission Decision 2002/657/EC. In addition, the validated method was applied to 40 milk samples. Despite the absence of AFs reported in the herein analyzed samples, the levels of these natural toxins should be continuously monitored to assess their exposure in the global population, as well as in susceptible groups such as children.

AUTHOR CONTRIBUTIONS

A.R., J.M., and Y.R.C. conceived and designed the experiments; L.I. performed the experiments; G.G. and A.G. analyzed the data; and Y.R.C. wrote the paper.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 5
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Identification and Quantification of Enniatins and Beauvericin in Animal Feeds and Their Ingredients by LC-QTRAP/MS/MS

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ABSTRACT

Emerging fusariotoxins, mainly enniatins (ENNs) and beauvericin (BEA), are secondary toxic metabolites produced by *Fusarium* spp. and are widely distributed contaminants of cereals and by-products. Mycotoxin contamination in these products supposes an important risk to feed supply security in the feed industry due to the common use of cereals in feed formulations. Hence, continuous monitoring of both raw materials and feed mixtures is highly recommended as stated by sanitary authorities. Therefore, an analytical procedure based on liquid chromatography tandem mass spectrometry and an acetonitrile-based extraction followed by a d-SPE (QuEChERS) step for the simultaneous determination of emerging *Fusarium* mycotoxins was in-house validated and successfully applied to raw materials (*n = 39*) and feed manufactured with them (*n = 48*). The analytical method was validated following the European guidelines and satisfactory results were obtained. Both raw materials and complete feedstuffs showed mycotoxin contamination at incidences of 18% and 92%, respectively. ENN B was the most commonly found mycotoxin in the analyzed samples at concentrations up to several tens of µg/kg. On the other hand, the co-occurrence of mycotoxins was observed in 47% of samples, ENN B and BEA being the most common combination. These results highlight the necessity to take a vigilant attitude to monitor the occurrence of contaminants in raw materials and feedstuffs throughout the manufacturing chain and storage.

Keywords: Mycotoxins; feed; raw materials; enniatins; beauvericin; co-occurrence.

5.1 INTRODUCTION

Mycotoxins are toxic secondary metabolites of fungi belonging, essentially, to the *Aspergillus*, *Penicillium* and *Fusarium* genera. While *Aspergillus* and *Penicillium* are the main mycotoxigenic postharvest mold genera reported, *Fusarium* seems to be the most important preharvest contaminant in crops [1]. Fungi from *Fusarium* genera frequently colonize small-grain cereals and are associated with grain diseases, such as *Fusarium* head blight, as well as the accumulation of potentially toxic metabolites in the kernels [2]. These toxic metabolites can contaminate a wide range of commodities such as food crops including grains, fibers and other agricultural feedstock and raw materials. The most widespread *Fusarium* mycotoxins occurring in cereals and derivates at high levels are fumonisins (FBs), trichothecenes (TCs), zearalenone (ZEN) and the so-called emerging fusariotoxins such as enniatins (ENNs) and beuvericin (BEA) [1].

Despite many years of research and the introduction of good practices in the human and animal meal manufacture, mycotoxins continue to be a significant health concern in feed manufacture because

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cereals and cereal by-products are the main ingredients included in feed formulation. Among crops, corn and wheat are the most commonly used for this purpose, but also for human consumption; however, they are also used in other industrial procedures, such as ethanol and flour production, and the by-products obtained in these processes, mainly dried distillers' grain with solubles (DDGS) are used to replace expensive grains in feed formulation [3].

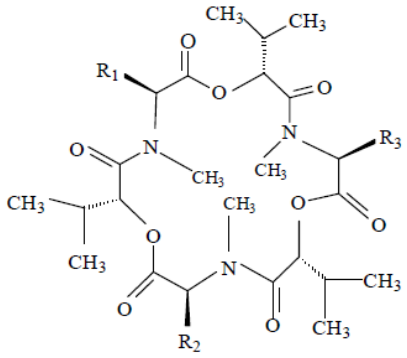
Physical processing such as cleaning, sorting and milling results in a reduction of the concentrations of mycotoxins in the refined product, but the application of these procedures results in an increase of the mycotoxin amount in the cereal by-products. For this reason, although published data confirm that milling can reduce the mycotoxin concentration in-fraction intended for human consumption, the co-products obtained, mainly DDGS, concentrate the initial mycotoxin levels up to three times compared to original grain into fractions which are commonly used as animal feed [3,4,5]. This effect is also favored by the presence in feed formulation of fractions that contain the outer part of the grain, which contain a higher mycotoxin content due to the external fungal contamination [4,6]. This high mycotoxin content has been reported in diverse studies, which indicated that over 70% of the BEA and ENNs in the original grains were found in by-products after food processing [7,8]. As a result, the use of by-products of cereal processing from contaminated grains represents a potential risk to livestock. This may explain, in part at least, the higher proportions of poultry feed samples reported as being contaminated with BEA and ENNs compared to whole maize grain [9]. In this sense, the inclusion of cereals and their by-products, mainly DDGS, in animals' diets must be carefully calculated since fungi can produce mycotoxins in all steps of both the food and feed chains [4,10,11]. Mycotoxin contamination in these by-products supposes an important risk to feed supply security in the feed industry due to the common use of cereals in feed formulations [1,3,5,12]. In fact, many studies have reported mycotoxin occurrence on different ingredients used in feed formulation and in finished feeds intended for terrestrial animals [13,14,15,16,17]. In summary, the inclusion of high amounts of vegetal origin sources, such as cereals and other raw materials, increases the risk of mycotoxin contamination [18] and, thus, the carry-over into edible tissues and/or animal byproducts, such as milk or eggs.

From a regulatory point of view, aflatoxin B_1 (AFB1) is the only mycotoxin under the European Union feed regulation up to now (20 µg/kg in raw materials), as it has been classified as carcinogenic according to the International Agency for Research on Cancer (IARC) [19,20]. For other mycotoxins, mainly *Fusarium* mycotoxins, guidance values have been set for feed ingredients and finished feed, including deoxynivalenol (DON), zearalenone (ZEN), and the sum of fumonisin B_1 and B_2 (FB1 + FB2), whereas for the T-2 and HT-2 toxins, only indicative levels for cereal products have been set [21,22,23].

However, the limits for some emerging *Fusarium* mycotoxins have not been set, although their presence has been assessed by the European Food Safety Authority (EFSA) in both food and feeds in high levels (up to mg/kg or ppm) [24]. This is the case of ENNs and BEA. ENNs are structurally related mycotoxins representing a large group of cyclic hexadepsipeptides while BEA is a cyclic hexadepsipeptide belonging to the enniatin antibiotic family (Fig. 5.1), which are primarily contaminants of cereals. BEA and ENNs are substantially stable during commercial cereal processing, including hot drying and ensiling procedures. Thus, they are widely present in livestock feeding as cereal grains or by-products resulting from cereal processing which are included in feedstuffs, and their presence in feed and raw materials has been reported in different studies [25,26,27]. In this sense, contents for enniatin B (ENN B) and BEA up to 2598 µg/kg and 988 µg/kg, respectively, have been reported [26]. Moreover, it should be taken into account that mycotoxin presence in livestock and poultry production may lead to economic losses and veterinary costs due to the negative effects on animal performance and the welfare of animals.

The European legislation on animal feed provides a legal framework for ensuring that feed does not suppose a risk to human or animal health. Nevertheless, the mycotoxin contents reported in the scientific literature indicate that sometimes the limits proposed for cereal-derived products by the European legislation may be not warranted. Therefore, the continuous monitoring and analysis of both raw materials and feed mixtures is highly recommended and, thus, included under specific projects [28], as, for example, in Spain, in the National Residue Monitoring Plan and the National Plan of Feed

Inspection and Monitoring, focusing on the analysis performed on mycotoxins to which production animals are vulnerable.



	R1	R2	R3
BEA	Phenyl-methyl	Phenyl-methyl	Phenyl-methyl
ENNA*	sec-butyl	sec-butyl	sec-butyl
ENNA1*	sec-butyl	sec-butyl	iso-propyl
ENNB*	iso-propyl	iso-propyl	iso-propyl
ENNB1*	iso-propyl	iso-propyl	sec-butyl

* ENNA, enniatin A; ENNA1, enniatin A1; ENNB, enniatin B; ENNB1, enniatin B1; BEA, beauvericin.

Fig. 5.1. The structure of Enniatins (ENNs) and Beauvericin (BEA)

From a toxicological point of view, it has been reported that BEA exerts cytotoxic activity against different animal and human cell lines, producing cellular damage by reactive oxygen species (ROS) generation and membrane lipid peroxidation (LPO), thus, producing oxidative stress. Moreover, the genotoxic activity of BEA has been also evidenced by different authors [29].

The ionophoric property seems to be the cause of BEA toxicity, as it also occurs in the case of ENNs. Due to the ionophoric properties, BEA is capable to promote the transport of mono- and divalent cations through membranes producing disturbances in the normal physiological cellular concentrations.

Regarding ENNs, it has been demonstrated that they are capable of inducing cytotoxicity under *in vitro* conditions. They can disturb the normal cell proliferation due to their hydrophobic nature, which affects the ionic homeostasis by the formation of dimeric structures that transport monovalent ions across the cellular membranes and can be easily incorporated in biological membranes, mainly the mitochondrial membrane. The cell damage is produced mainly due to the ROS generation and the induction of LPO [30].

Therefore, the accumulation of mycotoxins in foods and feeds represents a major threat to human and animal health as they are responsible for many different toxicities including the induction of cancer, mutagenicity and estrogenic, gastrointestinal, urogenital, vascular, kidney and nervous disorders.

Based on the information mentioned above, the aim of the present study was to carry out a survey on ENN and BEA occurrence in different raw materials (n = 39) commonly used in animal diets and feed intended for different animal species (n = 48). To achieve this objective, a confirmatory method based on liquid chromatography-tandem mass spectrometry was in-house validated and then applied to 87 samples to further broaden the knowledge on emerging fusariotoxins occurrence and co-occurrence in feedstuffs and by-products.

5.2 RESULTS AND DISCUSSION

5.2.1 Instrumental Optimization

The mycotoxin measurements were performed by an acetonitrile-based extraction followed by a d-SPE (QuEChERS) step followed by LC-MS/MS with a 3200 QTRAP® System AB Sciex (Applied Biosystems, Foster City, CA, USA) functioning as a triple quadrupole mass spectrometer detector (MS/MS). The mass spectrometric conditions were optimized by the direct infusion of individual working standard solutions, using an ESI source in both positive and negative modes. The results showed that the studied emerging fusariotoxins have higher peaks and response values in the positive ionization mode ($[M + NH_4]^+$). The most intense precursor ions were selected and the cone

voltage was optimized for each target mycotoxin, with the mass spectrometer operating in the product ion scan mode. Subsequently, collision energies were optimized for each transition and the product ions were selected for mycotoxin quantification and qualification. An entrance potential (EP) of 10 V and a collision cell exit potential (CXP) of 4 V were set for all the analytes. The final selection of the selected reaction monitoring (SRM) transitions in positive ion mode for each studied compound and the optimal MS parameters, namely, declustering potential (DP), collision energy (CE) and cell exit potential (CXP) are shown in Table 5.1.

Table 5.1. The optimized MS/MS Parameters

Mycotoxin	RT (min)	Precursor Ion	Product Ions	DP[c] (V)	CE[d] (V)	CXP[e] (V)
ENN A	15.8	699.400	228q[a]/210Q[b]	76/76	59/35	16/14
ENN A1	16.5	685.400	214q[a]/210Q[b]	66/66	59/37	10/8
ENN B	13.3	657.300	214q[a]/196Q[b]	51/51	59/39	10/8
ENN B1	14.7	671.200	228q[a]/214Q[b]	66/66	57/61	12/10
BEA	15.3	801.200	784Q[a] /244q[b]	116/116	39/27	6/10

[a] q, confirmation; [b] Q, quantitation; [c] DP, declustering potential; [d] CE, collision energy; [e] CXP, cell exit potential.

Previous studies have shown that adding a buffer and/or volatile acid into the mobile phase is beneficial to improve the efficiency of compound ionization. In this work, chromatographic behaviors of target fusariotoxins were comparatively investigated in the elution solution of methanol/water with and without ammonium formate (5 mM) and formic acid (0.1%). The results showed an improvement in both the peak shape and the analyte response by adding ammonium formate and formic acid. Therefore, methanol/water containing ammonium formate (5 mM) and formic acid (0.1%) were selected as the mobile phase in this study. Fig. 5.2 shows the LC-MS/MS chromatograms for target emerging fusariotoxins in the ESI positive mode.

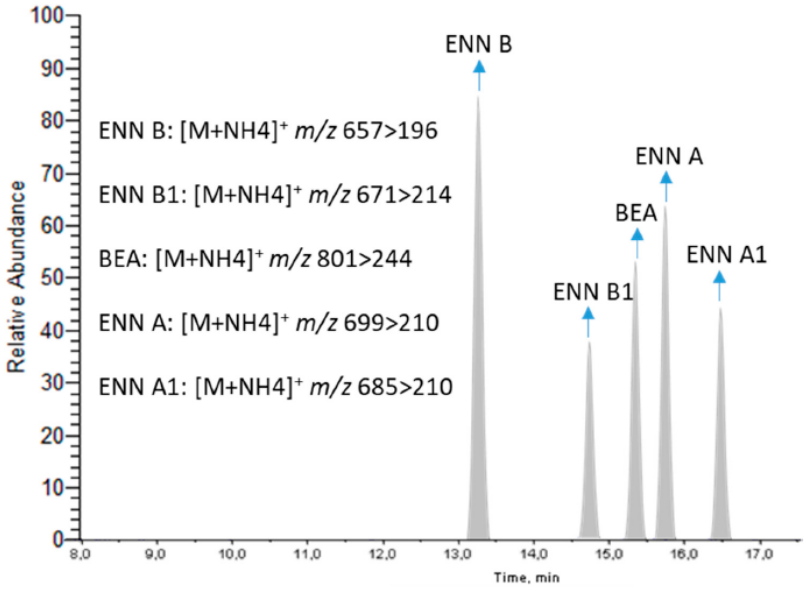


Fig. 5.2. The LC-MS/MS Chromatogram for Target Emerging Fusariotoxins at a Concentration of 10xLOQ

5.2.2 Method Validation

The specificity and selectivity of the method rely on the chromatographic retention time of each analyte and on the SRM transition used. The peaks for the studied analytes in the samples were confirmed by comparing the retention time of the peak with those of standards at the maximum tolerance of ±0.2 min or ±50% of the peak width at half height, recognizing both the quantitation (Q)

and confirmation (q) transitions, and matching the ion ratio. In addition, no interference peaks were observed at retention times of the target compounds in blank samples. Concerning linearity, all the studied mycotoxins showed correlation coefficients (R^2) greater than 0.990 over the working range (0.1–200 µg/kg). Co-eluting matrix components can negatively influence the accuracy of quantitative methods through signal ion suppression or enhancement (SSE) in the ion source; thus, the effects of the possible matrix mismatch were assessed. The results showed a significant signal suppression for the studied analytes (from 13 to 29%) and therefore matrix-matched calibration curves were used for a quantitative purpose. The sensitivity of the method was assessed by the limit of detection (LOD) and limit of quantitation (LOQ). LODs and LOQ_S were in a range from 0.2 to 1.0 µg/kg, and from 1.0 to 5.0 µg/kg, respectively. The trueness of the method, expressed as a recovery of analytes, was evaluated at three spiking levels (low level: LOQ; intermediate level: 10xLOQ; high level. 100xLOQ) and the results showed a recovery range from 86 to 98%, from 112 to 136%, and from 89 to 117%, at low, intermediate and high fortification levels, respectively (Table 5.2). As far as precision is concerned, the repeatability studies showed a relative standard deviation (RSD) lower than 5% at two spiking levels, whereas RSDs lower than 15% were obtained in the reproducibility studies.

Table 5.2. The method performance

Parameters	Recovery, % (RSD_R, %; *n* = 9)			LOD	LOQ
	LOQ (ng/g)	10xLOQ (ng/g)	100xLOQ (ng/g)	(ng/g)	(ng/g)
ENN A	95 (12)	112 (10)	93 (8)	1.0	5.0
ENN A1	91 (8)	95 (9)	89 (6)	0.2	1.0
ENN B	89 (9)	98 (8)	97 (5)	0.2	1.0
ENN B1	98 (10)	105 (9)	94 (7)	0.2	1.0
BEA	86 (7)	136 (15)	117 (12)	1.0	5.0

RSD_R: relative standard deviation (reproducibility).

Based on the before mentioned validation results, the proposed procedure is suitable for its purpose since it is a specific, sensitive, accurate, precise and robust method. The key performance characteristics fulfill the criteria set at the Commission Decision 2002/657/EC [31] and guidance document on the identification of mycotoxins in food and feed SANTE/12089 /2016 [32].

5.2.3 The Natural Occurrence of Mycotoxins in Raw Materials

The natural occurrence of ENNs and BEA was investigated in raw materials (*n = 39*) commonly used in feed manufacturing. The results revealed that the most prevalent mycotoxins were ENN B and BEA (18%), followed by ENN B1 (6%), while ENN A and ENN A1 were not detected in any of the analyzed samples. These results are in agreement with data reported in previous studies, ENNs type B being the major occurring mycotoxin in cereals and by-products in the following decreasing order: ENN B > ENN B1 > ENN A1 > ENN A. For instance, Mortensen et al. [33] reported ENN B contamination in all DDGS samples (*n = 7*) in by-products for animal feed collected from the official control in Denmark during 1998–2009. Similarly, Uhlig et al. [2], reported ENN B contamination in all analyzed samples of barley (*n = 75*), wheat (*n = 80*) and oat (*n = 73*) from Norway. This trend was also reported by Van Pamel et al. [1] who reported ENNs and BEA as the most frequently detected mycotoxins in all maize silage samples *(n = 10*) from Belgium, at trace levels (under the LOQ), with LOQ values of BEA 44 µg/kg, 48 µg/kg for ENN A, 43 µg/kg for ENN A1, 52 µg/kg for ENN B and 47 µg/kg for ENN B1. Those results were similar to those reported by Sørensen et al. [25] who found that up to 90% of the contaminated samples by ENN B in 2005 and in 100% in 2006 (*n = 30* and *n = 43* in 2005 and 2006, respectively), in maize silage from Denmark. On the other hand, ENNs type A (ENN A and ENN A1) were not detected in any sample of raw materials included in this study. In accordance with these results, Sørensen et al. [25], did not detect ENN A and ENN A1 in grain samples analyzed nor in 3-month-old silage stacks from the whole maize. Nonetheless, Streit et al. [17] reported ENN A1 contamination in 95% of raw materials analyzed (*n = 48*) and ENN A in 87% of samples. In those feed and raw materials, the occurrence of ENN type B (95%) and BEA (98%) was also reported.

Regarding the concentration of the emerging fusariotoxins found in raw materials here analyzed, ENN B was found in a range between 1.3 and 75.6 µg/kg, whereas ENN B1 contents ranged between 36.3

and 113.2 µg/kg, and BEA was found from 3.0 to 64.8 µg/kg. These results are in the same range than that reported by Shimshoni et al. [34] in corn and wheat silage from Israel, with mean values of 0.3 µg/kg, 0.9 µg/kg and 66 µg/kg, respectively, in corn and 0.3 µg/kg, 0.9 µg/kg and 5 µg/kg, respectively, in wheat samples. In addition, the results obtained in our study were in accordance with that reported by Warth et al. [26] in grain-based processed foods containing wheat. These authors found maximum contents of 16.4 µg/kg, 21.4 µg/kg and 47 µg/kg for ENN B, ENN B1 and BEA, respectively, in samples from Burkina Faso and 0.9 µg/kg, 4.1 µg/kg and 486 µg/kg, respectively, in samples collected in Mozambique. In spite of that, the above-reported concentrations significantly differ ($p < 0.05$) from those obtained in other studies performed in northern European regions. In these colder regions, higher contents have been reported compared to warmer zones. In this sense, Jestoi et al. [35] reported the presence of ENN B and ENN B1 in 100% of the analyzed raw material (mainly wheat and barley) from Finland (*n = 38*), reaching maximum levels up to 3980 µg/kg and 3240 µg/kg, respectively, in barley samples analyzed. These high levels have been also observed by Uhlig et al. [2], who found a maximum concentration of 5800 µg/kg for ENN B in wheat samples from Norway and by Sørensen et al. [25] who reported up to 2600 µg/kg of ENN B in whole fresh maize from Denmark. Similar results were also reported by Habler and Rychlik [36] in cereals from Germany; and Zachariasova et al. [27] reported average concentrations of ENN A and ENN B at 615 µg/kg in hay samples (*n = 4*) and 748 µg/kg in wheat-based DDGS samples (*n = 16*) from the Czech Republic, respectively. This fact could be explained due to the climatic conditions favorable to the proliferation of *Fusarium* spp. which produces ENNs. For BEA, the highest contents were found in rice bran and corn pulp samples, with an incidence of 25% and 50%, respectively (Table 5.3). According to Streit et al. [17], BEA was found in 98% of feed and raw material samples analyzed with a maximum content of 2326 µg/kg. In the survey reported by Lee et al. in Korea [37], 27% of feed ingredients were contaminated with BEA, at an average concentration of 480 µg/kg.

Table 5.3. The Enniatin and Beauvericin contents in the raw materials analyzed (*n* = 39). The results are expressed as an average (Concentration Range) in µg/kg

Raw Material (Number of Samples)	ENN A	ENN A1	ENN B	ENN B1	BEA
Wheat (*n* = 3)	*nd*	*nd*	50.2 (50.2)	36.3 (36.3)	*nd*
Maize (*n* = 2)	*nd*	*nd*	*nd*	*nd*	*nd*
Alfalfa (*n* = 3)	*nd*	*nd*	75.6 (75.6)	113.2 (113.2)	6.0 (6.0)
Sugar beet pulp (*n* = 1)	*nd*	*nd*	*nd*	*nd*	3.0 (3.0)
Barley (*n* = 10)	*nd*	*nd*	1.3 (1.3)	*nd*	*nd*
Rice bran (*n* = 4)	*nd*	*nd*	*nd*	*nd*	64.8 (64.8)
Corn pulp (*n* = 4)	*nd*	*nd*	1.8 (1.3–2.2)	*nd*	29 (20.4–37.8)
Meals (*n* = 7)	*nd*	*nd*	*nd*	*nd*	*nd*
Gluten feed (*n* = 5)	*nd*	*nd*	*nd*	*nd*	*nd*

nd: not detected.

5.2.4 Natural Occurrence of Mycotoxins in Feed

The results of acquired feedstuffs (*n = 48*) showed that 92% of the samples were contaminated with emerging fusariotoxins, ENN being B the most commonly found mycotoxin (89%), followed by ENN B1, BEA and ENN A1 (64%, 62% and 41.5%, respectively). ENN A was not detected in any of the analyzed samples. The results obtained are shown in Table 5.4. The highest average contents were found for ENNB in feed intended for rabbits, sheep, beef, dairy cattle and swine, which could be explained because of the contamination levels of the raw materials (pelleted diet and dried forages, mainly from cereals and vegetal protein). These results suggest that raw materials included in feed manufacture intended for those species showed higher contamination or were included in a higher proportion than feed intended for other species [38]. Nonetheless, there were no statistical differences ($p > 0.05$) among the contamination levels of the different feedstuffs.

Table 5.4. The Enniatin and Beauvericin Contents in the feed samples analyzed (*n* = 48). The results are expressed as an average (concentration range) in µg/kg

Animal Specie (Number of Samples)	ENN A	ENN A1	ENN B	ENN B1	BEA
Bovine (*n* = 8)	*nd*	9.7 (8.5–10.7)	24.1 (2.4–41.6)	15.2 (10.8–20.2)	27.4 (20.7–51.4)
Ovine (*n* = 13)	*nd*	10.2 (8.1–13.1)	32.4 (2.0–89.5)	16.7 (9.4–28.8)	32.6 (8.1–129.6)
Caprine (*n* = 1)	*nd*	8.4 (8.2–8.5)	16.8 (8.3–23.9)	12.7 (10.8–15.0)	13.9 (4.6–23.2)
Horses (*n* = 3)	*nd*	9.4 (8.7–10.1)	21.8 (6.0–43.8)	13.6 (10.0–15.5)	19.0 (8.2–29.8)
Porcine (*n* = 4)	*nd*	10.5 (9.1–11.9)	32.2 (22.1–55.1)	17.0 (14.1–24.0)	10.2 (5.7–14.6)
Poultry (*n* = 11)	*nd*	9.7 (8.1–11.9)	18.4 (3.0–51.1)	15.3 (7.4–23.1)	15.8 (8.1–23.8)
Rabbits (*n* = 2)	*nd*	11.8 (11.8)	47.4 (44.5–50.3)	23.5 (23.3–23.6)	13.5 (13.5)
Dogs (*n* = 3)	*nd*	*nd*	15.4 (7.5–24.8)	10.1 (10.1)	30.9 (21.3–40.5)
Cats (*n* = 3)	*nd*	*nd*	6.7 (6.7)	8.9 (8.9)	*nd*

nd: not detected.

The concentration range for the detected mycotoxins was the following: ENN A1 from 8.1 to 13.1 µg/kg; ENN B from 2.0 to 89.5 µg/kg; ENN B1 from 7.4 to 28.8 µg/kg; and for BEA, the concentration range was comprised between 4.6 and 129.6 µg/kg. The concentrations found in this study were in accordance with those reported by Warth et al. [26] in feed from Mozambique (*n = 10*), where ENN B and ENN B1 showed concentrations in a range between 2.2 and 114.0 µg/kg and 0.1 to 94.4 µg/kg, respectively; whereas BEA was detected in 100% of the analyzed samples at concentrations ranging from 3.3 to 418.4 µg/kg. In that study, the lowest contents corresponded to ENN A (from 0.6 to 7.9 µg/kg) and ENN A1 (from 3.4 to 43.9 µg/kg).

In the analyzed samples here, ENNs and BEA contamination were found in both raw materials and feedstuffs being higher in the latter. It could be justified taken into consideration that compound feed is particularly vulnerable to mycotoxin contamination as it typically contains a mixture of several raw materials, mainly cereals and seed proteins. Even raw materials are subjected to different processes, mainly pelletization and/or extrusion, which are supposed to reduce the initial mycotoxin concentration. Inappropriate conditions during feed storage or manufacture can result in fungal contamination and, consequently, mycotoxin production [9,11,26,38,39,40].

Regarding the different species that the feed is intended for, lower contents were reported for cat feedstuffs. This fact is probably due to the analysis of “grain free” feeds for cats. The main ingredients used in the elaboration are fish and fish by-products (herring, sole, hake), among other vegetal ingredients such as legumes or fruits.

Bovine feed samples (*n* = 8) were intended for different stages of production, mainly fattening calves (*n* = 4) and dairy cattle (*n* = 4). The results showed that two out four samples of feed intended to fattening calves presented higher contents for the analyzed mycotoxins compared with the other two samples. The highest incidence was for ENN B (88%), followed by ENN B1 and BEA (75%) and finally ENN A1 (63%). Regarding ENN and BEA contents, it was observed that the analyzed feedstuffs for dairy cattle showed higher BEA contents (51.4 µg/kg) compared to those obtained in fattening cattle feed. These results are in agreement with those presented by Lee and collaborators [37], in which a higher mean BEA content was observed in feed intended for dairy cattle (720 µg/kg) than in beef cattle (430 µg/kg), although the contents were much higher than those reported in the present study. In the study carried out by Zachariasova et al. in the Czech Republic [27], the maximum contents of 236 µg/kg of ENN B and 34 µg/kg of BEA were described in feed for dairy cows.

Regarding the contents obtained in the samples of feed destined to the ovine livestock (*n* = 13), samples included in the study were intended for lambs (*n* = 11) and dairy sheep (*n* = 2). The results showed that the highest incidence of contamination corresponded to ENN B (86%), followed by BEA (79%), ENN B1 (72%) and ENN A1 (57%). The contents obtained were similar for ENNs, however, lower BEA contents were reported in dairy feedstuffs (8.1 µg/kg).

In the porcine section, analyzed feedstuffs were intended for different stages of production, mainly breeding, farrowing and fattening. As it can be observed in Table 5.4, the highest incidence and the highest contents corresponded to the ENN B. In feed intended for adult pigs and breeder sows, no ENN A1 contents were detected, whereas, in feed intended for piglets and fattening pigs, BEA was not detected. However, according to Lee and collaborators [37], in a study carried out in Korea, samples of feed destined for pigs showed average levels of 740 µg/kg of BEA. However, the data obtained by these authors indicate higher levels in feed destined for piglets, in contrast to the present study, in which the feed contents destined for fattening pigs were higher. These results are in agreement with those described by Zachariasova et al. [27] in a study conducted in the Czech Republic, in which the ENN B reached maximum levels of up to 799 µg/kg in swine feed samples.

It has to be highlighted that a significant co-occurrence of BEA and ENNs (47% of samples) was found in the present survey. The presence of other *Fusarium* mycotoxins in raw materials has been reported by different authors, mainly FBs, TCs and ZEN [41,42,43], but the results obtained here also highlight that the emerging *Fusarium* mycotoxins can be found in feedstuff commodities simultaneously. Therefore, special attention should be paid to the co-occurrence of *Fusarium* mycotoxins since additive and/or synergistic effects could occur, as recently observed in in vitro studies by Prosperini et al. [44].

5.3 MATERIALS AND METHODS

5.3.1 Sampling

A total of 87 samples were analyzed for mycotoxin determination in this work. Samples were classified as follows: 48 feedstuffs and 39 raw materials commonly used as ingredients in feed manufacture. All of them were purchased from farms and feed factories located in the Valencia province, Spain.

Feed intended for different animal species was used as the criteria for samples sub-classification. Therefore, feedstuffs were grouped as follows: intended for ovine (*n = 13*); intended for poultry (*n = 11*); intended for bovine (*n = 8*); intended for domestic animals such as dogs and cats (*n = 6*); intended for swine (*n = 4*); intended for horses (*n = 3*); intended for rabbits (*n = 2*); and intended for caprine (*n = 1*). The raw materials analyzed were: barley (*n = 10*), meals (*n = 7*, mainly sunflower, rapeseed and soybean), gluten feed (*n = 5*, mainly wheat and corn gluten), rice bran (n = 4), corn pulp (*n = 4*), wheat (*n = 3*), maize *(n = 2*), alfalfa *(n = 3*) and sugar beet pulp (*n = 1*). These raw materials were selected by taking into account those with a high inclusion percentage in feed elaboration. The percentage of inclusion depended on the species the feed is intended for and the feed formulation.

All samples were homogenized in a food blender and then kept in dark and dry conditions at 4 °C until analysis.

Raw materials and feeds manufactured with them have been collected in factories from the region where the study has been performed. In this sense, feed production is intended for common livestock of the region. This is the reason why there are scarce samples intended for some animal species (caprine) compared to others, such as ovine and poultry.

5.3.2 Chemicals and Reagents

All solvents (acetonitrile (MeCN) and methanol (MeOH)) were acquired at Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Formic acid (HCOOH) and ammonium formate ($HCOONH_4$, 97%) were supplied by Sigma-Aldrich (Madrid, Spain). All solvents were filtered through a cellulose filter of 0.22 µm (Membrane Solutions, Plano, TX, USA) before use. The stock standards of ENNs and BEA were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Anhydrous magnesium sulfate was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18 was purchased from Phenomenex (Torrance, CA, USA).

5.3.3 Preparation of Standard Solution and Spiking of Blank Samples

Individual stock solutions of BEA and ENNs with a concentration of 1000 µg/mL were prepared in MeCN. They were stored in darkness conditions in glass-stoppered bottles at −20 °C. The working standard solutions consisting of individual compounds were prepared by the appropriate dilution of the stock solutions for spiking procedures and calibration curves. Samples of commercial feed and their ingredients containing none of the studied mycotoxins were used as a blank matrix for spiking experiments as well as for quality control. The spiked samples were left for overnight equilibration.

5.3.4 Sample Preparation

Two grams of the homogenized matrix were weighed into a 50 mL polypropylene (PP) tube and 10 mL of water containing 2% formic acid and 10 mL of MeCN were added and vigorously shaken for 30 min on a horizontal shaker (IKA, Staufen, Germany). Then, 1 g NaCl and 4 g of $MgSO_4$ were added and the tube was vortexed for 30 s and then centrifuged for 5 min at 2336 g and 4 ˚C (Eppendorf, Germany). Two mL of MeCN extract was collected and submitted to a dispersive solid phase extraction (dSPE, 15-mL PP tube), employing 0.1 g of C18 silica sorbent and 0.3 g of $MgSO_4$ and then centrifuged for 5 min at 1413 g and 4 ˚C. Finally, the purified extract was filtered through a 0.22 µm nylon filter and transferred into a vial for LC-MS/MS analysis.

5.3.5 LC-MS/MS Equipment and Conditions

The instrumental analysis was achieved on liquid chromatography coupled with a tandem mass spectrometry (LC-QTRAP/MS/MS) system. Chromatographic separation of the analytes was conducted at 25 ˚C using an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) with a binary pump and automatic injector. A reverse-phase Gemini-NX C18 (150 mm × 2 mm I.D., 3 µm particle size) analytical column (Phenomenex, Barcelona, Spain) was used. The analytical separation was performed using a gradient elution of 95% of phase A (water) and 5% of phase B (MeOH), both with 5 mM of ammonium formate and 0.1% formic acid, increasing linearly to 95% B for 10 min; then, decreasing linearly to 80% B for 5 min, and then gradually up to 70% B for 6 min. Finally, for the last 3 min, the initial conditions were maintained. The flow rate was maintained at 0.2 mL/min.

For the mass spectrometry analysis, a 3200 QTRAP® mass spectrometer operated in the Selected Reaction Monitoring (SRM) mode (AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionization (ESI) interface was used. The numerous heteroatoms in BEA and ENNs are the main reason why they ionize very well in the positive electrospray mode, and for this reason, the analysis was performed in positive ion mode (ESI+) and the ESI source values were as follows: capillary voltage: 3.50 kV; source temperature: 120 ˚C; desolvation temperature: 400 ˚C; cone gas: 50 L/h; desolvation gas (nitrogen 99.99% purity) flow: 800 L/h. The QTRAP® analyzer combines a fully functional triple quadrupole and a linear ion trap mass spectrometer within the same instrument. The resolution for the first and third quadrupoles was set to 12.0 (unit resolution); the ion energy to 0.5; the entrance and exit energies to 5 and 3, respectively; the multiplier to 650; the collision gas (argon 99.99% purity) pressure to 3.83×10^{-3} mbar; the interchanel delay to 0.02 s; the total scan time to 1.0 s; and the dwell time to 0.1 ms. SRM optimized parameters were calculated in triplicate (cone voltages, collision energies and precursor and product-ions selected) and are shown in Table 5.1.

5.3.6 Method Validation and Quality Assurance/Quality Control (QA/QC)

The method performance parameters were determined according to European guidelines [**28**]. The method was validated for mycotoxin standards with regards to its selectivity, specificity, linearity, matrix effect, sensitivity, trueness and precision.

Feed and raw material samples in which mycotoxins were not detected, were mixed to obtain an individual composite sample. This composite was used as the blank matrix to carry out both the matrix-matched calibration curves and the spiked samples used for recovery and quality control assays. The selectivity and specificity of the method were ascertained by analyzing the standard

solutions and the spiked samples. The peaks for the studied compounds in the samples were confirmed by comparing the retention time of the peak with those of the standard solution, as well as by recognizing both the precursor and product ions and their ratio. The linearity and matrix effects were studied using standard solutions in a neat solvent and matrix-matched calibrations. The calibration curves in both the pure solvent and matrix were constructed by plotting the signal intensity against analyte concentrations at eight levels (from 0.1 µg/kg to 200 µg/kg). The calibration curves were prepared in triplicate. To assess the matrix effects, the ratios between the slope of matrix-matched (A) and the slope of external calibration (B) were obtained. Thus, the ratio (A/B) × 100) is defined as the matrix effect and expressed as the signal suppression/enhancement (SSE, %). SSE values < 100% indicate signal suppression; >100% signal enhancement; whereas values equal to 100% indicate no matrix effect. Sensitivity was evaluated by the limits of detection (LOD) and the limits of quantitation (LOQ). LOD and LOQ were calculated as the lowest addition level constructed in the matrix-matched extract, corresponding to a signal to noise ratio of at least 3:1 and 10:1, respectively. The trueness and precision studies were evaluated by spiking the standard solution to blank samples at two concentration levels (10 x LOQ and 100 × LOQ). Trueness was expressed as a percentage of recovery. Precision was verified by three determinations on the same day (repeatability) and on three non-consecutive days (reproducibility).

Analytical quality control samples were evaluated during the method validation and analysis of samples according to the guidance document on the identification of mycotoxins in food and feed SANTE/12089 /2016 [32].

5.3.7 Statistics and Data Analysis

All experiments were performed in triplicate, and the results were expressed as the average values ± relative standard deviation (RSD, %). A Student's t-test statistical analysis was performed for data evaluation; *p* values < 0.05 were considered significant. Tests were carried out by using IBM SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

5.4 CONCLUSIONS

In this work, an LC-MS/MS method for the simultaneous determination of emerging *Fusarium* mycotoxins, namely, enniatins and beauvericin, in raw materials and complete feedstuffs was in-house validated. The results showed that the proposed analytical procedure was accurate (recovery range from 89 to 136% for the vast majority of analytes) and precise (RSDs < 15%, and sensitive (LODs from 0.2 to 1.0 µg/kg) to fulfill the criteria established in European guidelines. The validated method was successfully applied to 87 samples of raw materials and feedstuffs to monitor the occurrence levels of the studied mycotoxins. The results showed that ENNs and BEA were present in both raw materials and feeds, ENN B detected in up to 92% of samples. Mycotoxin concentrations found in analyzed samples varied depending on the type of sample, being those with a high level of cereal inclusion the most contaminated. In addition, the co-occurrence of mycotoxins was frequently detected in samples (47%). These data indicate that the contamination of feedstuffs with only one mycotoxin is rare and that mycotoxins occur more frequently together, representing a risk for animals. Thus, to monitor the occurrence of mycotoxins in raw materials and feedstuffs throughout the manufacturing chain and storage, we need to guarantee the safety of animals and trade requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, J.M. and A.R.; methodology, J.T., Y.R. and E.F.; investigation, J.T., Y.R. and E.F.; resources, J.M. and A.R.; writing-original draft preparation, J.T. and Y.R.; Writing—Review and Editing, J.T., Y.R. and E.F., supervision, J.M. and A.R.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Chapter 6
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Target Analysis and Retrospective Screening of Multiple Mycotoxins in Pet Food Using UHPLC-Q-Orbitrap HRMS

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ABSTRACT

A comprehensive strategy combining a quantitative method for 28 mycotoxins and a post-target screening for other 245 fungal and bacterial metabolites in dry pet food samples were developed using an acetonitrile-based extraction and an ultrahigh-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) method. The proposed method showed satisfactory validation results according to Commission Decision 2002/657/EC. Average recoveries from 72 to 108% were obtained for all studied mycotoxins, and the intra-/inter-day precision were below 9 and 14%, respectively. Results showed mycotoxin contamination in 99% of pet food samples (n = 89) at concentrations of up to hundreds µg/kg, with emerging *Fusarium* mycotoxins being the most commonly detected mycotoxins. All positive samples showed co-occurrence of mycotoxins with the simultaneous presence of up to 16 analytes per sample. In the retrospective screening, up to 54 fungal metabolites were tentatively identified being cyclopiazonic acid, paspalitrem A, fusaric acid, and macrosporin, the most commonly detected analytes.

Keywords: Mycotoxins; monitoring; pet food; HRMS-orbitrap; co-occurrence; retrospective screening.

6.1 INTRODUCTION

Mycotoxins are a group of toxic secondary metabolites produced by fungi mainly belonging to *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* genera [1]. Due to the great structural diversity of these toxic compounds, they display a wide range of deleterious effects, including carcinogenic, hepatotoxic, nephrotoxic, teratogenic, heamatotoxic, immunotoxic, and hormonal or reproductive effects [2,3]. Mycotoxins pose a challenge to food safety as they are unavoidable and unpredictable contaminants in crops. In fact, the Food and Agriculture Organization (FAO) estimated that over one-quarter of the world's food crop are contaminated with mycotoxins [4]. The mycotoxins with greatest agro-economic and health impact are aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), and trichothecenes [5]. In the last decade, attention to the risk posed to human and animal health has also been extended to the so-called emerging *Fusarium* mycotoxins (including enniatins (ENNs) and beauvericin (BEA)) as well as the *Alternaria* toxins [6]. The factors affecting molds growth and/or mycotoxin production, and thus contamination of raw materials and feed, are associated with yield conditions (i.e., temperature, humidity, insect damage). Moreover, in post harvesting, other factors, such as moisture and storage conditions, could contribute to increasing risk of mycotoxin production [7].

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Food crops susceptible to mycotoxin contamination include corn, wheat, barley, rye, rice, nuts, dried fruit, vegetables, and their derivatives [8]. It is remarkable that cereals and cereal by-products that are often unfit for human consumption are frequently used in feed formulations and act as excellent substrates for the fungal proliferation and production of mycotoxins. Recent surveys indicate that 70% of raw materials are contaminated with these toxins [9,10]. On the other hand, cereal processing, including dry milling, affects mycotoxin occurrence, especially for the fractions commonly designed for animal feeding [11,12]. Consequently, animal exposure to mycotoxins via plant-derived foods is of important consideration [13,14,15,16].

To limit the exposure to mycotoxins, the European Commission (EC) has set maximum limits of undesirable substances in both foodstuffs (EC/1881/2006 and amendments) and feedstuffs (2003/100/EC). As far as mycotoxins in feedstuffs are concerned, the Commission Directive 2003/100/EC has only established maximum admissible content of AFB1 in complete feedstuffs at 20 µg/kg. As regards the other mycotoxins, the European Union established in the Commission Decision 2006/576/EC guidance values regarding presence of deoxynivalenol (DON), ZEN, FBs, and OTA in products used as animal feeding (Table 6.1).

In the last decades, improvement of analytical methods for the detection of mycotoxins at low ng/g range in a wide variety of foodstuffs has been performed [17]. Mass spectrometry-based techniques, such as MS and MS/MS, in combination with gas chromatography (GC) or liquid chromatography (LC) allowed the development of multi-mycotoxins methodologies [18]. Over recent years, there have been improvements in the LC-technique with the development of ultra-high-performance liquid chromatography (UHPLC), leading to higher peak efficiency and shorter chromatography run time [19]. In addition, the use of high-resolution mass spectrometry (HRMS), such as Orbitrap mass analyzers, is growing up in the ambit of food toxicology. HRMS analyzers have good specificity and high resolution due to mass accuracy provided by the resolution of Q-Orbitrap detectors combined with structural information obtained in MS/MS mode [20]. This technique enable the identification of untarget compounds and retrospective data analysis without the need to re-run samples.

Even though investigations on mycotoxin distribution in feedstuffs are regularly conducted by competent authorities, the information on mycotoxin distribution of feedstuffs is limited [21]. Among the available studies focused on mycotoxins occurrence in feedstuffs, most of them have been performed in feed aimed to livestock production, whereas scarce literature have reported the occurrence of these toxic compounds in pet foods [22,23,24,25]. Therefore, the development and validation of analytical strategies to evaluate the occurrence of traditional and emerging mycotoxins in pet food to guarantee their quality, as well as to comply with trade requirements, are needed. Hence, the aim of this work was to develop an analytical tool based on a UHPLC-Q-Orbitrap HRMS method that combines quantitative target analysis for detection, quantification, and reliable identification of 28 mycotoxins from different fungi genera in pet food, with post-target screening (identification) of other 245 fungal and bacterial metabolites based on a comprehensive spectral library. In addition, the proposed methodology was applied to 89 dry commercially available pet food samples acquired from pet shops located in Campania region, Southern Italy.

6.2 RESULTS AND DISCUSSION

6.2.1 Optimization of the Ultrahigh-Performance Liquid Chromatography Coupled to High-Resolution Mass-Spectrometry (UHPLC-Q-Orbitrap HRMS) Analysis

The optimization of the Q-Orbitrap HRMS parameters was performed via direct infusion of each mycotoxin standard (n = 28) diluted at 1 µg/mL into the Q-Orbitrap system using a flow rate of 8 µL/min. According to the literature, the addition of formic acid-ammonium formate shows better ionization efficiency of the studied analytes than acetic acid-ammonium acetate, and thus these additives were added to the mobile phases [26]. The most intense and signal stable adducts were selected for each analyte. Precursor ions were subjected to different values of collision energies (between 10 and 60 eV) to perform their fragmentation. Table 6.2 shows the UHPLC-HRMS parameters for the determination of mycotoxins included in this study.

Table 6.1. Regulated and recommended maximum levels of mycotoxins in feed materials set by the European Commission

Mycotoxin	Products	[a] Regulated Maximum Level (mg/kg) Relative to a Feedingstuff	[b] Guidance Value (mg/kg) Relative to a Feedingstuff	[c] Guidance Value in mg/kg (ppm) Relative to a Feedingstuff with a Moisture Content of 12%
AFB1	All feed materials; complete feedingstuffs for pigs and poultry (except young animals); complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs); complementary feedingstuffs for pigs and poultry (except young animals); complete feedingstuffs for cattle, sheep and goats with the exception of:	0.02		
	complete feedingstuffs for dairy animals	0.005		
	complete feedingstuffs for calves and lambs	0.01		
DON	Maize by-products		12	
	Other cereals and cereal products		8	
	Complementary and complete feedingstuffs with the exception of:		5	
	complementary and complete feedingstuffs for pigs		0.9	
	complementary and complete feedingstuffs for calves (<4 months), lambs and kids		2	
ZEN	Maize by-products		2	
	Other cereals and cereal products		3	
	Complementary and complete feedingstuffs for piglets and young sows		0.1	
	Complementary and complete feedingstuffs for sows and fattening pigs		0.25	
	Complementary and complete feedingstuffs for calves, dairy cattle, sheep and goats		0.50	
OTA	Cereals and cereal products		0.25	
	Complementary and complete feedingstuffs for pigs		0.05	
	Complementary and complete feedingstuffs for poultry		0.1	
FBs	Maize and maize products		60	
	Complementary and complete feedingstuffs for pigs, horses, rabbits and pet animals		5	
	Complementary and complete feedingstuffs for fish		10	
	Complementary and complete feedingstuffs for poultry, calves (<4 months), lambs and kids		20	
	Complementary and complete feedingstuffs for adult ruminants (>4 months) and mink		50	
T-2 + HT-2 toxin	Compound feed for cats			0.05

[a] Directive Commission 2003/100/EC; [b] Recommendation Commission 2006/576/EC; [c] Commission Recommendation 2013/637/EU; Abbreviations: AFB1: aflatoxin B1; DON: deoxynivalenol; ZEN: zearalenone; OTA: ochratoxin A; FBs: fumonisins (FB1 and FB2).

Table 6.2. Ultrahigh-performance liquid chromatography coupled to high-resolution mass-spectrometry (UHPLC-HRMS) parameters for the determination of mycotoxins included in this study

Mycotoxins	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (*m/z*)	Product Ion	Collision Energy (eV)
AFB1	4.64	$C_{17}H_{12}O_6$	$[M + H]^+$	313.07066	285.07489; 269.04373	36
AFB2	4.98	$C_{17}H_{14}O_6$	$[M + H]^+$	315.08631	287.09064; 259.05945	36
AFG1	4.79	$C_{17}H_{12}O_7$	$[M + H]^+$	329.06558	243.06467; 200.04640	40
AFG2	4.61	$C_{17}H_{14}O_7$	$[M + H]^+$	331.08123	313.07010; 245.08032	37
OTA	6.50	$C_{20}H_{18}NO_6Cl$	$[M + H]^+$	404.08954	358.08304; 341.05658	16
FB1	6.03	$C_{34}H_{59}NO_{15}$	$[M + H]^+$	722.39575	352.32010, 334.30963	48
FB2	6.78	$C_{34}H_{59}NO_{14}$	$[M + H]^+$	706.40083	336.32547; 318.31488	58
DON	4.18	$C_{15}H_{20}O_6$	$[M + HCOOH]^-$	341.12451	295.1189; 265.10822	−12
3-ADON	3.83	$C_{17}H_{22}O_7$	$[M + H]^+$	339.14383	231.10118; 203.10638	20
15-ADON	4.02	$C_{17}H_{22}O_7$	$[M + H]^+$	339.14383	261.11154; 137.05957	20
HT-2	5.63	$C_{22}H_{32}O_8$	$[M + NH_4]^+$	442.24354	263.12744; 215.10641	27
T-2	6.13	$C_{24}H_{34}O_9$	$[M + NH_4]^+$	484.25411	215.10603; 185.09561	23
NEO	4.32	$C_{19}H_{26}O_8$	$[M + NH_4]^+$	400.19659	305.13803; 141.0053	10
DAS	5.11	$C_{19}H_{26}O_7$	$[M + NH_4]^+$	384.20168	307.15329; 105.06977	15
FUS-X	4.28	$C_{17}H_{22}O_8$	$[M + Na]^+$	377.12073	228.16002; 175.07550	20
ZEN	6.55	$C_{18}H_{22}O_5$	$[M - H]^-$	317.13945	175.03989; 131.05008	−32
α-ZEL	4.87	$C_{18}H_{24}O_5$	$[M - H]^-$	319.15510	174.95630; 129.01947	36
β-ZEL	4.98	$C_{18}H_{24}O_5$	$[M - H]^-$	319.15510	174.95604; 160.97665	36
α-ZAL	4.81	$C_{18}H_{26}O_5$	$[M - H]^-$	321.17044	259.09497; 91.00272	29
β-ZAL	4.94	$C_{18}H_{26}O_5$	$[M - H]^-$	321.17044	259.09497; 91.00272	40
ZAN	5.00	$C_{18}H_{24}O_5$	$[M - H]^-$	319.15510	273.01187; 131.05020	35
BEA	5.77	$C_{45}H_{57}N_3O_9$	$[M + NH_4]^+$	801.44331	262.76715; 244.18239	70
ENN A	8.17	$C_{36}H_{63}N_3O_9$	$[M + NH_4]^+$	699.49026	228.15900; 210.14847	43
ENN A1	8.16	$C_{35}H_{61}N_3O_9$	$[M + NH_4]^+$	685.47461	228.15900; 210.14847	48
ENN B	7.87	$C_{33}H_{57}N_3O_9$	$[M + NH_4]^+$	657.44331	214.14320; 196.13280	50
ENN B1	8.06	$C_{34}H_{59}N_3O_9$	$[M + NH_4]^+$	671.45986	214.14343; 196.13295	48
AOH	5.88	$C_{14}H_{10}O_5$	$[M - H]^-$	257.04555	215.03490; 213.05569	−32
AME	6.82	$C_{15}H_{12}O_5$	$[M - H]^-$	271.06120	256.03751; 228.04276	−36

Abbreviations: Aflatoxins (AFB1, AFB2, AFG1 and AFG2), ochratoxin A (OTA), fumonisins (FB1 and FB2), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-AcDON), 15-acetyl-deoxynivalenol (15-AcDON), HT-2 toxin, T-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS) fusarenon-X (FUS-X), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB and ENNB1), alternariol (AOH) and alternariol monomethyl ether (AME).

On the other hand, three gradient programs were tested to achieve a good separation of the 28 mycotoxins:

(i) Gradient 1: started with 20% B, kept up to 1 min, and then increased to 95% B in 1 min, followed by a hold-time of 0.5 min at 95% B. Afterward, the gradient switched back to 75% in 2.5 min, and decreased again reaching 60% B in 1 min. The gradient returned in 0.5 min at 20%, and 1.5 min column re-equilibration at 20%;

(ii) Gradient 2: started with 10% B, kept up to 1 min, and then increased to 95% B in 1 min, followed by a hold-time of 0.5 min at 95% B. Afterward, the gradient switched back to 75% in 2.5 min, and decreased again reaching 60% B in 1 min. The gradient returned in 0.5 min at 10%, and 1.5 min column re-equilibration at 10%;

(iii) Gradient 3: started with 0% B, kept up to 1 min, and then increased to 95% B in 1 min, followed by a hold-time of 0.5 min at 95% B. Afterward, the gradient switched back to 75% in 2.5 min, and decreased again reaching 60% B in 1 min. The gradient returned in 0.5 min at 0%, and 1.5 min column re-equilibration at 0%.

The results showed that several peaks eluted within the column dead time when starting the gradient program with high organic phase (20%, gradient 1) and the peak response was irregular. The second tested gradient (initial phase B set at 10%) decreased the number of analytes non-retained in the chromatographic column but still DON and its acetylated forms eluted within the first 1.0 min. The chromatographic separation of analytes was performed with a Luna Omega Polar C18 column. Optimal results in terms of retention time and good peak shape were achieved when the initial phase B was at 0%, obtaining good separation of the 28 mycotoxins in a total run time of 8 min (Table 6.2).

6.2.2 Optimization of Sample Preparation Procedure

Sample preparation has been recognized as a critical step in the chemical analysis workflow [27]. Few multi-mycotoxin methods have been reported in literature regarding pet food samples and most of them were performed with immunoaffinity column assays, increasing the cost of the method significantly [23,25,28]. Recently, a relatively cheap acetonitrile-based extraction was proposed in literature to determine seven *Fusarium* toxins in laboratory rat feed [11]. In this work, the sample preparation protocol reported by those authors was adopted as a starting point and slightly modified to extend it for the simultaneous determination of up to 28 target mycotoxins from different genera, including *Aspergillus, Penicillium, Fusarium,* and *Alternaria*. Critical extraction parameters were evaluated namely stirring time, sonication treatment, clean-up, and sample amount (Supplementary Table S1). All experiments were performed in triplicate using spiked samples at 20 µg/kg.

6.2.2.1 Stirring time

Three stirring times (15, 30, and 60 min) were tested to evaluate the effect of agitation in the extraction of mycotoxins. Results showed that 15 min of stirring time was not enough to reach acceptable recoveries (recovery range obtained for all mycotoxins: ≤40%) and RSD values (<23%). By increasing the stirring time up to 30 min, the recoveries for the wide majority of compounds increased (from 65 to 78%) except for AFs, for which recovery values lower than 55% were obtained. On the other hand, optimal results (recovery range: 72–105%, RSD < 16%) were achieved with 60 min of stirring for all studied compounds fulfilling the requirements set at Commission Decision EC 2002/657.

6.2.2.2 Sonication treatment

A sonication time of 15 min (with manual shaking every 5 min) was assayed and compared with samples in which the sonication step was not conducted. Results showed that when the sonication step was not performed, the accuracy and precision of the studied mycotoxins (recoveries ranging from 58 to 89%, RSD < 21%) were not as good as those obtained with sonicated samples (recoveries

ranging from 72 to 114%, RSD < 14%); and therefore sonication treatment was included in the sample preparation procedure.

6.2.2.3 Clean-Up Step

In the original method, a freeze-out step was carried out (minimum 2 h) to promote the precipitation of compounds that may interfere in the analysis based on the complexity of the samples [11]. Nonetheless, it significantly increases the time of the analysis. To overcome that, a clean-up step to reduce both matrix interferences and contamination of the instrument was evaluated. The efficiency of this strategy was evaluated by comparing the accuracy and precision data of the results obtained with samples stored in a freezer (2 h) and those submitted with a clean-up. According to literature, the mixture of 300 mg $MgSO_4$ and 100 mg C18 (ratio 3:1, *w/w*) was selected as appropriate dispersive clean-up [16]. The results showed an improvement in accuracy and precision data due to the efficacy of the clean-up in removing interferences. Furthermore, the matrix effect was significantly minimized (range from 71 to 86%) with the addition of the clean-up, leading to an improved selectivity and robustness. In the samples in which the freezing out was conducted, impurities appeared. The usage of the clean-up step instead of freezing out made the extraction procedure faster and the extract obtained was much cleaner, as evidenced by the chromatographic response.

6.2.2.4 Sample amount

Despite the significant reduction of interferences observed by the addition of a clean-up step, moderate signal suppression was obtained for most of the analyzed compounds, as specified in Section 2.2.3. To overcome that, the effect of reducing the sample amount was evaluated. Results showed that no matrix effect or slight signal suppression (≥85%) was obtained for all studied compounds when using 2 g of sample instead of 5 g, and therefore it allowed the quantification of the studied mycotoxins in pet food samples based on external calibration curves.

6.2.3 Method Validation

Calibration curves were prepared in triplicate at 8 concentration levels. Correlation coefficients (r^2) greater than 0.9990 were obtained for all studied analytes within the linear range from limits of quantification (LOQs) to 1000 µg/kg. No matrix effect or slight signal suppression was observed for all mycotoxins ranging from 75 to 98%. Limits of detection (LODs) obtained were between 0.06 and 0.62 µg/kg; LOQs were calculated from 0.013 and 1.25 µg/kg, being lower than those reported in recent literature (Table 6.3). Average recoveries were in the range 75–112% for all studied mycotoxins at the fortification levels assayed (10, 20, and 100 µg/kg). Those results highlighted that the proposed methodology is accurate enough for the quantitative determination of the target mycotoxins. Intra-day and inter-day relative standard deviations (RSDs) showed reliable repeatability (RSD < 12%) and within-laboratory repeatability (RSD < 17%) of the developed method (Supplementary Table S2). The carry-over was evaluated by injecting a blank sample after the highest calibration point. No carry-over was present since no peaks were detected in retention time zone of all studied mycotoxins. In the Quality Assurance/Quality Control (QA/QC) procedure, the spiked sample was used in each sample batch in order to assess the accuracy and precision of the proposed method. To guarantee the quality of the results, every one of QA/QC criteria had to be achieved. To provide method reliability, satisfactory recoveries (between 70% and 120%, RSD < 20%) for all samples were required. When the results did not fit the expected criteria, the extractions were repeated in order to achieve this range. After the optimization and validation procedure and during the sample analysis, none of the QA/QC samples were outside of the expected criteria in any batch of samples.

2.4 Occurrence of Mycotoxins in Pet Food Samples

The optimized and validated multi-mycotoxin method was applied to 89 dry pet food samples (55 for dogs and 34 for cats) acquired from different pet shops located in Campania region, Southern Italy. Table 6.3 shows the results here obtained, as well as reviewing the available studies published in the last decade regarding the occurrence of mycotoxins in pet food samples.

Table 6.3. Recent surveys reporting the occurrence of mycotoxins in pet foods samples

Analyzed Samples (n)	Analytes Investigated	Mycotoxins	Positive Samples (%)	[a] Range or Average (µg/kg)	Detection Methods	LOQ (µg/kg)	Reference
89	28	AFB1	25.8	3.3–7.9	UHPLC-Q-Orbitrap	0.013	This work
		AFB2	5.6	1.8–16.6		0.013	
		AFG1	1.1	11.1			
		AFG2	5.6	1.7–31.6		0.125	
		OTA	2.2	1.4–1.5		1.25	
		ZEN	91.0	0.9–60.6		0.013	
		Σ α + β-ZEL	87.6	0.9–58.9		α-ZEL = 1.25; β-ZEL = 0.125	
		Σ α + β -ZAL	79.8	<LOQ		α-ZAL = 1.25; β-ZAL = 0.125	
		ZAN	n.f.	n.f.		0.125	
		DON	30.3	7.6–297.3		1.25	
		Σ 3 + 15 AcDON	5.6	10.9–63.2		3-AcDON = 1.25; 15-AcDON = 1.25	
		NEO	n.f.	n.f.		0.188	
		HT2	32.6	3.3–110.1		1.25	
		T2	47.2	0.7–9.0		0.125	
		BEA	86.5	0.8–176.1		0.013	
		ENNA	10.1	0.3–9.6		0.125	
		ENNA1	22.5	0.4–28.1		0.125	
		ENNB	93.3	0.4–212.4		0.125	
		ENNB1	58.4	0.3–71.8		0.013	
		AOH	82.0	0.2–12.8		0.125	
		AME	84.3	0.1–15.6		0.125	
		FB1	66.3	11.8–990.1		0.125	
		FB2	52.8	10.5–556.3		0.250	
		DAS	n.f.	n.f.			
		FUS-X	n.f.	n.f.			
48	5	ENNA	n.a.	n.a.	LC-MS/MS	5	Tolosa et al., 2019 [**16**]
		ENNA1	41.5	8.1–11.9		1	
		ENNB	89	2.0–89.5		1	
		ENNB1	64	7.4–28.8		1	
		BEA	62	4.6–129.6		5	

Analyzed Samples (n)	Analytes Investigated	Mycotoxins	Positive Samples (%)	[a] Range or Average (µg/kg)	Detection Methods	LOQ (µg/kg)	Reference
32	8	AFB1	47.7	30.3–242.7	LC-MS/MS	1.7	Shao et al., 2018 [**29**]
		AFG1	13.9	13.9		0.7	
		OTA	16.2	15.1–17.3		10.7	
		ZEN	54.5	14.5–389.2		2.5	
		DON	66.3	22.8–421.3		16.5	
		T-2	15.4	15.4		3.3	
		BEA	19.1	0.2–153.4		2.5	
		FB1	87.2	6.6–191.9		10.0	
12	6	AFB1	n.a.	83.3	HPLC-FLD	41.57	Singh et al., 2017a [**24**]
		AFB2	n.a.	9.0		11.77	
		OTA	n.a.	1.0		-	
		ZEN	n.a.	5.7		-	
		FB1	n.a.	106.3		202.53	
		FB2	n.a.	61.9		118.37	
49	3	AFs	100	0.16–5.39	HPLC-FLD	AFB1 = 0.13; AFB2 = 0.59; AFG1 = 0.03; AFG2 = 0.22	Teixeira et al., 2017 [**25**]
		ZEN	95.9	4.07–98.3		3.95	
		FBs	77.6	37.4–1015		FB1 = 27.5; FB2 = 35.3	
100	4	AFLs	68	0.34–3.88	HPLC-FLD	B1=0.13; G1 = 0.03; B2 = 0.59; G2 = 0.22	Bissoqui et al., 2016 [**23**]
		ZEN	95	5.45–442.2		3.95	
		FB1	68	20.0–220		27.5	
		FB2	35	40.0–160		35.3	
20	3	AFs	n.a.	n.a.	ELISA-UV	5	Yasmina et al., 2016 [**21**]
		AFB1	15	2.6–18.4		1	
		OTA	70	2.62–6.65		2.5	
		ZEN	20	148–1170		1.75	
49	2	AFB1	8.2	<0.05–0.21	HPLC-FLD	0.15	Błajet-Kosicka et al., 2014 [**22**]
		OTA	46.9	<0.13–3		0.40	
	5	DON	100	22.7–436	LC-MS/MS	20.0	
		T-2	87.7	<0.5–13.3		1.50	
		HT-2	83.7	<1.60–19.6		5.00	
		ZEN	100	1.81–123		0.30	
7		FBs	28.6	<5–108		FB1 = 1.60; FB2 = 1.60; FB3 = 1.60	

Analyzed Samples (n)	Analytes Investigated	Mycotoxins	Positive Samples (%)	[a] Range or Average (µg/kg)	Detection Methods	LOQ (µg/kg)	Reference
76	4	DON	97	>250	ELISA-UV	-	Böhm et al., 2010 [**30**]
		OTA	5	3.5		-	
		ZEN	47	80		-	
		FBs	42	178		-	
29	3	DON	83	409	HPLC-FLD	25	
22		ZEN	68	185		20	
3		FBs	67	69		15	
180	1	AFB1	70.5	0.3–9.43	HPLC-FLD	0.1	Campos et al., 2008 [**31**]

Abbreviations: Aflatoxins (AFB1, AFB2, AFG1 and AFG2), ochratoxin A (OTA), fumonisins (FB1 and FB2), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-AcDON), 15-acetyl-deoxynivalenol (15-AcDON), HT-2 toxin, T-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS) fusarenon-X (FUS-X), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB and ENNB1), alternariol (AOH) and alternariol monomethyl ether (AME). [a] Range or arithmetic mean of all positive samples. n.f. (not found) is shown if there was no readable value below LOD. n.a. not available.

In these analyzed samples, 99% of pet foods showed mycotoxin contamination. Despite the significantly high incidence, the concentration levels found were below the maximum level and/or maximum permissible levels set for mycotoxins in feedstuffs (2003/100/EC; 2006/576/EC). Nonetheless, special attention must be considered for the aflatoxins group. 25.8% of analyzed samples showed AFB1 contamination in a concentration range from 3.3 to 7.9 µg/kg (average content: 4.3 µg/kg). Similar AFB1 findings were reported in petfoods from Poland (*n* = 49) in a concentration range from <LOQ to 0.2 µg/kg [22], and from Brazil (*n* = 180) with AFB1 contamination levels ranging from 0.3 to 9.4 µg/kg [31]. In another survey, 4 out of 70 Brazilian pet food samples showed AFB1 contamination in a range from 15 to 37 µg/kg. This high contamination level reported by these authors was related to the presence of contaminated peanuts present in all positive samples. Despite some samples exceeded the maximum limit set by the EU for complete feedstuffs (20 µg/kg), those levels were below the permitted limits adopted in Brazil (50 µg/kg).

On the other hand, OTA was quantified in 2.2% of the here analyzed pet food samples at average content of 1.5 µg/kg. These levels are in agreement with previous studies as reviewed in Table 6.3. However, a wide range of OTA incidence reported by the different surveys was observed. Concerning the occurrence of fumonisins in pet food samples, the available studies reported both high incidence (>50%) and concentrations up to hundreds/thousands µg/kg (Table 6.3). Recently, Teixeira et al. [25] reported FBs contamination in 70% (*n* = 87) of Brazilian pet food samples at a concentration range from 30 to 1015 µg/kg. These findings are also in line with the data here obtained in which 67% of analyzed samples showed FBs contamination ranging from 10.5 to 990.1 µg/kg, being FB1 the most commonly detected fumonisin. The particularly high levels and incidence of FBs in feed could be related to the quality of corn (grain) and corn-based ingredients used in the formulations of these feedstuffs.

As far as trichothecenes are concerned, DON (and its acetylated forms) were the most commonly reported type B trichothecene in pet food samples reported in literature at concentration levels of hundreds µg/kg (Table 6.3). Similar results were here found; in fact, 30% of samples were DON-contaminated at concentration range from 7.6 to 297.3 µg/kg. On the other hand, type A trichothecenes mainly represented by HT-2 and T-2 toxins, has been barely investigated in pet food samples despite the fact that these toxins have been proven to have a higher toxicity than DON. In these analyzed samples, HT-2 (32.6% positive samples) and T-2 (47.2% positive samples) were detected at levels from 3.3 to 110.1 µg/kg, and from 0.7 to 9.0 µg/kg, respectively. Higher HT-2 and T-2 incidences (of up to 87.7%) than those here obtained were reported by Błajet-Kosicka et al. [22], in the 49 Polish pet food samples, but the concentration levels in that study were below 20 µg/kg in all positive samples. In line with that, ZEN (and its derivative forms) were found in 91% of the here analyzed samples at levels ranging from < LOQ to 60.6 µg/kg. These results are in agreement with recent surveys carried out in Brazilian [23,25], Egyptian [21], Polish [22], and Austrian [30] pet food samples (Table 6.3).

Emerging *Fusarium* mycotoxins (ENs and BEA) and *Alternaria* mycotoxins (AOH and AME), have been barely investigated in feed samples. The results showed a high incidence (>80%) of enniatins with concentration up to hundreds µg/kg (Table 6.3). Among enniatins, ENNB was the most commonly detected mycotoxin in the assayed samples (83 out of 89). The results obtained in this work are according to contents reported in different feedstuffs samples. Tolosa et al. [32] reported a high incidence of ENs (100% positive samples) and BEA (95% positive samples) in 20 Spanish fish feed at levels ranging from 0.1 to 10.0 µg/kg and from 0.1 to 6.6 µg/kg respectively. These results are also according to those reported by Warth et al. [33] in which ENs and BEA were present in 70% and 100% (*n* = 10) of animal feed samples from Burkina Faso and Mozambique, with concentration levels ranging from 0.1 to 114.0 µg/kg and from 3.3 to 418 µg/kg, respectively. On the other hand, Warth et al. [33] reported also AOH and AME contamination in 75% and 25% in a low number of samples analyzed (*n* = 4) at average content of 15.1 and 11.1 µg/kg, respectively. Similar high incidence was reported by Streit et al. [34], in which AOH and AME were found in 80% and 82% of feed and feed raw materials (*n* = 83) from Europe at concentration levels of hundreds µg/kg.

6.2.5 Co-occurrence of Mycotoxins in Analyzed Samples

All contaminated pet food samples here analyzed showed co-occurrence from three to sixteen mycotoxins in a sum concentration range from 1.6 to 1700.0 µg/kg (Fig. 6.1). Three multicontaminated samples showed sum concentrations above 1000 µg/kg, with several *Fusarium, Aspergillus* and *Alternaria* toxins. A significant number of pet food samples (77.3%) were co-contaminated from 8 to 12 mycotoxins. Similarly, Böhm et al. [30] reported the co-occurrence of mycotoxins in 33% Austrian pet food samples (*n* = 76), and *Fusarium* toxins such as DON, ZEA, and FBs were the most predominant. The simultaneous occurrence complicates the evaluation of toxicological potential of feed. Additive and synergistic effects on overall toxicity are frequently observed when mycotoxin mixtures are evaluated [9,35,36].

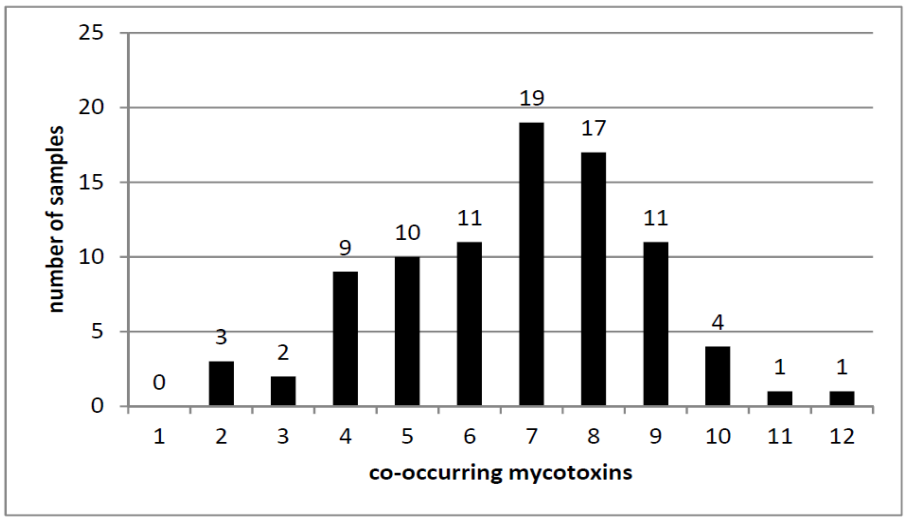

Fig. 6.1. Number of samples co-contaminated with a given number of mycotoxins (total samples analyzed; *n* = 89)

6.2.6 Identification of Non Target Coumpounds Based on A Retrospective Screening Analysis

The developed strategy based on Q-Orbitrap HRMS combines the quantitative target determination with the post-target screening approach. The possibilities of the Q-Orbitrap HRMS were further explored by subjecting the full scan data of the pet food samples to untargeted screening with the major data processing parameters set as follows: ionization patterns $[M + H]^+$ and $[M - H]^-$, a minimum peak area of 1×10^5 a.u., a maximum mass window of 5 ppm, and a retention time width of 1 min. The confirmation of the structural characterization of unknown compounds and untargeted analytes was based on the accurate mass measurement, elemental composition assignment, and MS/MS spectrum interpretation. Untargeted data processing was carried out using structural formula finder tool and the online high-quality mass spectral database. The advantage of using a full-scan acquisition mode is to allow the retrospective analysis of samples for the identification of up to 245 fungal and bacterial metabolites included in spectral library database by processing the raw data of the analyzed pet food samples. Fifty-four fungal metabolites were tentatively identified in the here analyzed samples (Fig. 6.2). Cyclopiazonic acid, paspalitrem A, fusaric acid, and macrosporin were the most commonly detected mycotoxins in the assayed samples (98.9%). Cyclopiazonic acid and paspalitrem A are produced by *Aspergillus* and *Penicillium* spp. Fusaric acid is produced by some *Fusarium* spp. Macrosporin is mainly produced by *Stemphylium* spp. These metabolites have been already found in contaminated cereal crops such as oats, barley, millet, corn, and rice [37,38,39]. In these analyzed samples, emodin was also identified in 97.8% of feed samples. This compound was already reported in Spanish feed and feed raw material but lower incidence (57.1%; *n* = 62) [40].

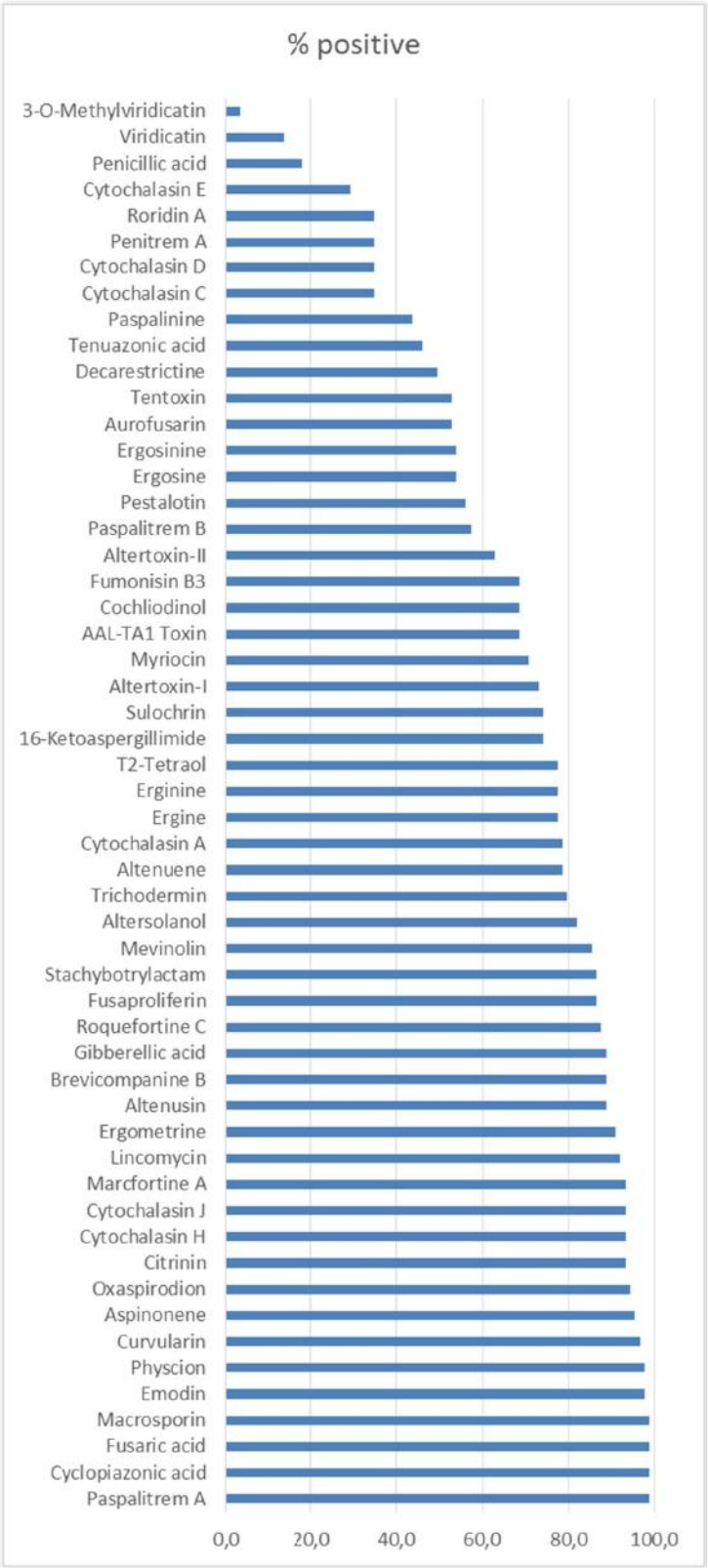

Fig. 6.2. Non-target mycotoxins identified in samples based on ultrahigh-performance liquid chromatography coupled to high-resolution mass-spectrometry (UHPLC-Q-Orbitrap HRMS) library

6.3 MATERIALS AND METHODS

6.3.1 Chemical and Reagents

Mycotoxin standards and metabolites namely aflatoxins (AFB1, AFB2, AFG1, and AFG2), ochratoxin A (OTA), fumonisins (FB1 and FB2), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-AcDON), 15-acetyl-deoxynivalenol (15-AcDON), HT-2 toxin, T-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB, and ENNB1), alternariol (AOH), and alternariol monomethyl ether (AME) were purchased from Sigma Aldrich (Milan, Italy). Individual stock solutions of all analytes were prepared by diluting 1 mg of each mycotoxin in 1 mL of methanol and further diluted for preparing working standard solutions. All these solutions were kept in safe conditions at −20°C.

All solvents, acetonitrile (AcN), methanol (MeOH) and water (LC-MS grade) were purchased from Merck (Darmstadt, Germany) whereas formic acid (mass spectrometry grade) and ammonium formate (analytical grade) were obtained from Fluka (Milan, Italy). Magnesium sulphate was obtained from VWR Chemicals BDH Prolabo, (Leuven, Belgium) and C18 (analytical grade) was purchased from Supelco (Bellafonte, Pennsylvania, PA, USA).

Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were provided by Phenomenex (Castel Maggiore, Italy); conical centrifuge polypropylene tubes of 50 mL and 15 mL were obtained from BD Falcon (Milan, Italy).

6.3.2 Sampling

A total of eighty-nine standard dry pet food samples was randomly purchased from different pet shops located in Campania region, Southern Italy. The acquired pet food samples were classified as follows: dogs (*n*= 55) and cats (*n* = 34). The nutritional composition of the analyzed samples is shown in Table 6.4. The main ingredients declared in labels from samples were rice, corn, corn flour, wheat, tapioca, wheat flour, oat, and barley. All samples were homogenized using a laboratory mill (particle size 200 µm) and then stored in a dark and dry place until analysis. The analysis was performed within 3 days after sample registration [40].

Table 6.4. Nutritional composition of standard dry pet food samples

Composition (%)	Dog (*n* = 55)	Cat (*n* = 34)
Proteins	25.8 ± 5.2	33.8 ± 3.8
Fats	14.9 ± 3.4	15.2 ± 4.4
Fibers	6.8 ± 1.2	7.5 ± 1.1
Total minerals	3.0 ± 2.1	3.9 ± 2.9

6.3.3 Sample Preparation

In this work, a sample preparation procedure for extraction of mycotoxins from laboratory rat feed reported in literature was selected as starting point and slightly modified [11]. In brief, homogenous representative samples (2 g) were weighted into 50 mL falcon tube and 10 mL of AcN:H_2O mixture (80:20, *v/v* with 0.1% of formic acid) were added. The mixture was placed in a horizontal shaker for 60 min at 245× *g* and then placed into an ultrasonic bath for 15 min. Samples were centrifuged for 3 min at 3435× *g* at 4°C, and 2 mL of the upper layer were submitted to a dispersive-SPE with a mixture of 300 mg of anhydrous $MgSO_4$ and 100 mg of C18, and vortexed for 1 min. The mixture was centrifuged for 1 min at 1472× *g* at 4°C. Finally, the extract was evaporated to dryness under gentle nitrogen flow at 45°C, reconstituted with 0.5 mL of MeOH/H_2O (70:30, *v/v*), and filtered (0.22 µm filter) prior to the UHPLC-Q Orbitrap HRMS analysis.

6.3.4 Method Validation

The method validation was performed in-house with respect to linearity, matrix effect, sensitivity, accuracy, and precision, as expected with compliance to Commission Decision 2002/657/EC. For the spiking and recovery studies, there were employed a pool of blank pet food samples (n = 10) (dog, n = 5; and cat, n = 5) of previous studies. Linearity was evaluated throughout standard solutions and matrix-matched calibrations. A graphic scatter plot test was used to assess the linearity, and lack-of-fit test was performed in linear regression model. Linear range of the method was assessed from limit of quantification to 1000 µg/kg for all mycotoxins. Matrix effect was evaluated by comparing the slopes of standard solutions built in neat solvent and the matrix-matched calibration curve. Values around 100% mean that there are no matrix effects, signal suppression, or enhancement if the value obtained was lower or higher than 100%, respectively. The sensitivity was evaluated by LODs and LOQs. LOD was defined as the minimum concentration where the molecular ion can be identified with a mass error below 5 ppm, and LOQ was set as the lowest concentration of the analyte that produce a chromatographic peak with precision and accuracy <20%. The accuracy of the method was evaluated with recovery studies. Blank samples were spiked and left to equilibrate overnight and then extracted as previously described. Method recovery was performed at three spiking levels (10, 20, and 100 µg/kg). Precision was expressed as relative standard deviation (% RSD) and calculated by triplicate measurements carried out on a single day (repeatability) and on three non-consecutive days (within-laboratory repeatability) [41].

6.3.5 Quality Assurance/Quality Control

For the confirmation criteria, the peaks for the studied compounds in the samples were confirmed by comparing the retention times of the peak with those of standard solutions at a tolerance of ± 2.5%. To ensure a higher level of confidence in the identification, the precursors and product ions were recognized with a mass error below 5 ppm. In the QA/QC procedure, a sample blank, a reagent blank, a replicate sample, and a matrix-matched external calibration were added at the beginning and end of each sample batch in order to assess the effectiveness of the developed method. Spiked pet food samples at three concentration levels (10, 20, and 100 µg/kg) were used for analytical quality control.

6.3.6 Ultrahigh-Performance Liquid Chromatography Coupled to High-Resolution Mass-Spectrometry (UHPLC-Q-Orbitrap HRMS) Analysis

Detection and quantitation were performed with a UHPLC instrument (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, Ma, USA) equipped with a degassing system, a Quaternary UHPLC pump working at 1250 bar, and an autosampler device. Chromatographic separation of analytes was performed with a thermostated Luna Omega Polar C18 column (50 × 2.1 mm, 1.6 µm, Phenomenex) kept at 30 °C. Both mobile phases contained 0.1% formic acid and 5 mM ammonium formate and were H_2O (phase A) and MeOH (phase B). The LC gradient started with 0% B, kept up to 1 min, and then increased to 95% B in 1 min, followed by a hold-time of 0.5 min at 95% B. Afterward, the gradient switched back to 75% in 2.5 min, and decreased again reaching 60% B in 1 min. The gradient returned in 0.5 min at 0%, and 1.5 min column re-equilibration at 0%. The injection volume was 5 µL with flow rate of 0.4 mL/min. The UHPLC system was coupled to a Q-Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, Waltham, Ma, USA). The mass spectrometer was operated in both positive and negative ion mode using fast polarity switching by setting two scan events (Full ion MS and All ion fragmentation, AIF). Full scan data were acquired at a resolving power of 35,000 FWHM at *m/z* 200. The conditions in positive ionization mode (ESI^+) were: spray voltage 4 kV; capillary temperature 290°C; S-lens RF level 50; sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, and auxiliary gas heater temperature 305°C. Ion source parameters in negative (ESI^-) mode were: spray voltage −4 kV; capillary temperature 290°C; S-lens RF level 50; sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, and auxiliary gas heater temperature 305°C. Value for automatic gain control (AGC) target was set at 1×10^6, a scan range of *m/z* 100–1000 was selected and the injection time was set to 200 ms. Scan-rate was set at 2 scans/s. For the scan event of AIF, the parameters in the positive and negative ion mode were: mass resolving power = 17,500 FWHM; maximum injection time = 200 ms; scan time = 0.10 s; ACG target = 1×10^5; scan range = 100–1000 *m/z*, isolation window to 5.0 *m/z*, and retention time window to 30 *s*. The collision energy

was optimized individually for each compound. Different collision energies were tested while the infusion of the compound was performed into the HRMS. The optimal energy was chosen when at least the parent compound remained at 10% intensity and it produced characteristic product ions from 80–100% intensity. Data processing were performed by the Quan/Qual Browser Xcalibur software, v. 3.1.66. (Xcalibur, Thermo Fisher Scientific). Retrospective screening was carried out on spectral data collected using a mycotoxin spectral library (Mycotoxin Spectral Library v1.1 for LibraryView™ Software, AB SCIEX, Framingham, USA). The identification was based on accurate mass measurement with a mass error below 5 ppm for the molecular ion; while regarding the fragments on the intensity threshold of 1000 and a mass tolerance of 5 ppm. Quantitative results were obtained working in scan mode with HRMS exploiting the high selectivity achieved in full-scan mode, whereas MS/HRMS information was used for confirmatory purposes.

6.3.7 Statistics and Data Analysis

All validation experiments were performed in triplicate, and the results were expressed as the average values ± relative standard deviation (RSD, %). Student's *t*-test statistical analysis was performed for data evaluation; p values < 0.05 were considered significant.

6.4 CONCLUSIONS

A UHPLC-Q-Orbitrap HRMS method for simultaneous determination of mycotoxins from different fungal species in pet food samples was in-house optimized and validated according to the criteria set by Commission Decision 2002/657/EC. In addition, mycotoxin spectral library of 245 analytes was used for post-run retrospective screening. The developed method was successfully applied to eighty-nine petfood samples and twenty-six different mycotoxins were found at high incidence (98.9%) but at concentrations below the maximum permissible limits. Co-occurrence of mycotoxins was found in all contaminated samples with up to sixteen analytes per sample. The established method was rapid and efficient, and capable of covering more analytes compared to the previous methods for the detection and quantitation of mycotoxins in pet food products. Moreover, this is the first work describing the simultaneous detection, quantification, and retrospective screening of a wide range of mycotoxins from different genera in pet food samples by using the capability provided by the UHPLC-Q-Orbitrap HRMS technology.

SUPPLEMENTARY MATERIALS

The following are available online at https://www.bookpi.org/wp-content/uploads/2022/05/Chapter-6_Supplementary-Materials.pdf, Table S1: Optimization of sample preparation procedure, Table S2: Accuracy and precision of the developed method.

AUTHOR CONTRIBUTIONS

A.R and Y.R.-C conceived and designed the experiments; L.I. and J.T. performed the experiments, G.G. and A.G. analyzed the data, L.C. and Y.R.-C. wrote the paper.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 7
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Ultra-High-Performance Liquid Chromatography Coupled with Quadrupole Orbitrap High-Resolution Mass Spectrometry for Multi-Residue Analysis of Mycotoxins and Pesticides in Botanical Nutraceuticals

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ABSTRACT

Cannabidiol (CBD) food supplements made of *Cannabis sativa* L. extracts have quickly become popular products due to their health-promoting effects. However, potential contaminants, such as mycotoxins and pesticides, can be coextracted during the manufacturing process and placed into the final product. Accordingly, a novel methodology using ultra-high-performance liquid chromatography coupled with quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) was developed to quantify 16 mycotoxins produced by major *C. sativa* fungi, followed by a post-target screening of 283 pesticides based on a comprehensive spectral library. The validated procedure was applied to ten CBD-based products. Up to six different *Fusarium* mycotoxins were found in seven samples, the most prevalent being zearalenone (60%) and enniatin B1 (30%), both found at a maximum level of 11.6 ng/g. Co-occurrence was observed in four samples, including one with enniatin B1, enniatin A and enniatin A1. On the other hand, 46 different pesticides were detected after retrospective analysis. Ethoxyquin (50%), piperonyl butoxide (40%), simazine (30%) and cyanazine (30%) were the major residues found. These results highlight the necessity of monitoring contaminants in food supplements in order to ensure a safe consumption, even more considering the increase trend in their use. Furthermore, the developed procedure is proposed as a powerful analytical tool to evaluate the potential mycotoxin profile of these particular products.

Keywords: Mycotoxins; pesticides; Q-Exactive Orbitrap; CBD capsule; nutraceutical.

7.1 INTRODUCTION

Nutrition is known to be an essential component of the health state, so having an unbalanced diet can lead to several disorders and diseases [1]. Due to current lifestyles, new and fast ways to maintain proper dietary habits are required. Nutraceuticals have emerged as an alternative to increase the input of nutrients, contributing to an improvement in health. These products are bioactive compounds naturally occurring in food or produced de novo in human metabolism, biologicals or botanicals, each intended to impart a physiological or medicinal effect after ingestion [2]. They can be delivered either in foods and beverages or in other non-conventional forms, such as capsules, tablets, powders or liquid extracts. In terms of marketing, nutraceuticals include a large number of different products packaged for specific groups by age, gender, physical conditions and activity level. The global market was valued at US$109 billion in 2015 and is projected to reach US$180 billion by 2020 [3].

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Inside the variety of products classified as nutraceuticals, food supplements based on botanical ingredients represent the second largest segment, behind vitamins and minerals. Most recently, cannabidiol (CBD) dietary supplements made of *Cannabis sativa* L. extracts have quickly become popular products. CBD is a phytocannabinoid present in the resin secreted from trichomes in female *C. sativa* plants, and is mainly found in inflorescences. The bioactivity of this compound has been related to an enhancement of its antioxidant and neurological activity, among others, by the promotion of several metabolic pathways [4,5,6]. However, the European Union (EU) does not consider CBD supplements as a novel food [7] and lets member states set their own rules over its marketing, leading to a convoluted situation in terms of regulation. Despite several ambiguities in its legislation, the European market for CBD-based supplements was valued at US$318 million in 2018 and with a strong growth projection [8].

Due to the complex nature of *C. sativa* and other botanicals, potential contaminants can be coextracted during the different stages of the manufacturing process and placed into the final product. Among all the potential non-desirable compounds in herbal-based supplements, mycotoxins and pesticides are the most commonly reported [9,10]. Mycotoxins are secondary metabolites mainly produced by the fungi genera *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps* and *Alternaria*. These compounds can be present in food and feed commodities and display immunosuppressive, nephrotoxic or carcinogenic effects, among others [11]. According to their carcinogenic potential, some mycotoxins, like aflatoxins, have been included in the classification list of human carcinogens provided by the International Agency for Research on Cancer (IARC) [12]. These mycotoxins are produced by the genera *Aspergillus*, which has been categorized as a major fungus occurring in *C. sativa* inflorescences alongside other mycotoxin producing fungi, like *Fusarium* spp., so different mycotoxins could be also expected [13,14]. On the other hand, pesticides include a broad range of compounds routinely applied to protect crops from different pests. However, residues coming from these products can accumulate in plants intended for human consumption, leading to several health issues related to neurotoxicity, carcinogenicity and pulmonotoxicity, as well as developmental and reproductive disorders [15,16,17,18].

In terms of regulation, maximum residue limits (MRL) for different types of contaminants have been set by the EU. Regulation (EC) No. 396/2005 [19] establishes limits for pesticides, whereas Regulation (EC) No. 1881/2006 [20] covers mycotoxins, attaching maximum limits in food and feeds. Nevertheless, nutraceutical products are not considered by the legislation yet but, due to a potential carryover during the manufacturing process, contamination could be expected not only in raw material, but also in other by-products. Moreover, several studies have reported the sole presence of pesticides [21,22], mycotoxins [23,24] and both types of contaminants [25,26,27] in diverse food supplements, remarking the necessity to evaluate the contamination profile of these products considering their rising consumption and popularity.

To overcome this point, the development of analytical procedures is needed. Concerning the extraction of contaminants, QuEChERS (quick, easy, cheap, effective, rugged and safe) [21,23,24] and “dilute and shoot” procedures have been recently applied to food supplements delivered as gelatin capsules, traditional capsules, tablets, powder extracts or liquid presentations [25,26,27]. Analytical methods used in the detection and quantification of contamination include ELISA detection [28], gas chromatography (GC) coupled with mass spectrometry (MS) [22] and ultra-high-performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry (MS/MS) [23,24] and high-resolution Orbitrap mass spectrometry (Q-Orbitrap HRMS) [25,26,27]. Due to its high resolving power, sensitivity and accurate mass measurement, high-resolution mass spectrometry stands as a suitable alternative for evaluating a large number of contaminants present in complex matrices at low concentrations. Therefore, the aim of the present study was to provide an analysis of pesticide residues and mycotoxins produced by major *C. sativa* fungi occurring in CBD-based food supplements, using ultra-high-performance liquid chromatography coupled with high-resolution Orbitrap mass spectrometry. To achieve this, a novel methodology was developed in order to identify and quantify 16 mycotoxins after evaluating different extraction procedures, followed by a post-target screening of 283 pesticides based on a comprehensive spectral library. To the best of the authors’ knowledge, this is the first multi-class analysis of CBD-based supplements through the use of high-resolution mass spectrometry techniques.

7.2 RESULTS

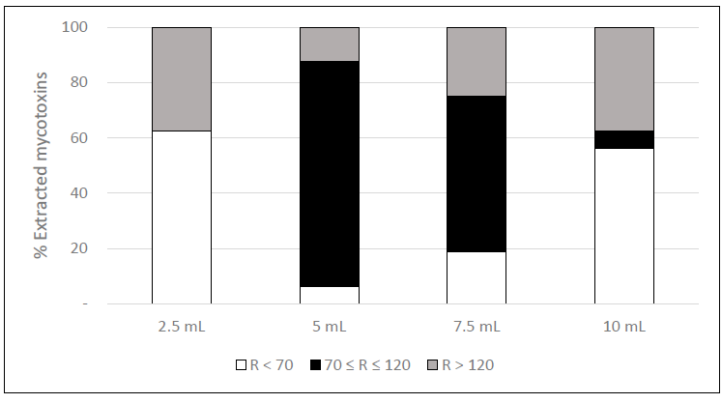
7.2.1 Optimization of Extraction Procedure

The molecular complexity of this matrix demands an effective extraction in order to detect and quantify several mycotoxins in a reliable way. A QuEChERS methodology previously developed on this typology of sample [24] was selected as the starting point, whereas different volumes of extraction solvent and the type of sorbent for clean-up was tested.

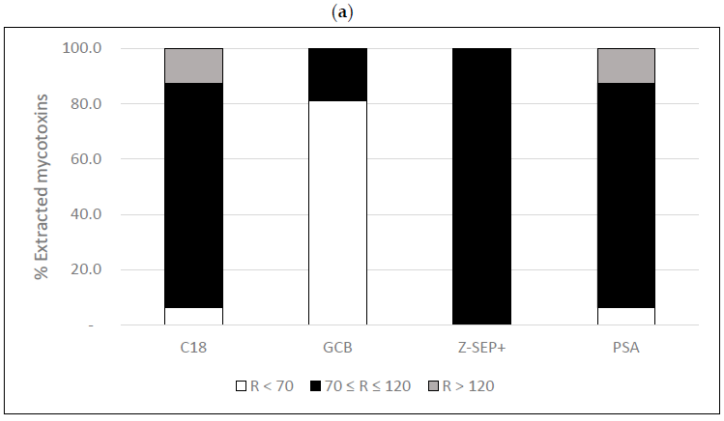
7.2.1.1 Evaluation of the volume of extraction solvent

The extraction procedure was first evaluated in triplicate by spiking the sample at 10 ng/g using the following volumes of extraction solvent per gram of sample: 2.5, 5, 7.5 and 10 mL.

The extraction performed with 2.5 mL showed recovery values below the minimum limit (70%) for the vast majority of the studied analytes as a consequence of solvent saturation (Fig. 7.1a). Satisfactory recoveries (70–120%) were obtained after performing the extraction with 5 mL of solvent for the majority of compounds, with the exception of β-ZEL (155%) and ZAN (150%), which were significantly more efficient than the other volumes tested ($p < 0.05$). On the other hand, the extractions performed with 7.5 and 10 mL showed a gradual decrease in recoveries due to the larger dilution of the analytes. Therefore, 5 mL of AcN was selected as the optimal volume of extraction solvent for this type of CBD capsule.



(a)



(b)

Fig. 7.1. Percentage of mycotoxins extracted with a recovery value (R) below 70% (white), between 70% and 120% (black) and above 120% (grey), corresponding to extractions performed with: (a) different volumes of solvent at a spiking level of 10 ng/g; (b) different sorbents for clean-up at a spiking level of 10 ng/g

7.2.1.2 Evaluation of the type of sorbent for clean-up

The molecular composition of the soft gel capsules mainly consists on fatty acids and proteins. Because of the complex nature of this matrix, an efficient clean-up is required in order to avoid interference with the analytes. To achieve this, clean-up with different sorbents (100 mg), including C18, as previously suggested [24], GCB, Z-Sep+ and PSA was performed.

PSA exhibited a good performance for the vast majority of analytes (Fig. 7.1b) but was unable to recover other important mycotoxins, such as AFB1 and AFG1. The moderate affinity of PSA with polar compounds may explain low recoveries for aflatoxins, being consistent with other works based on oily matrices [29,30]. Similarly, extraction with C18 was efficient for most compounds and only some low-polarity mycotoxins showed recoveries out of the range set, like ZAN (150%) and β-ZEL (155%). Clean-up using GCB showed poor results, allowing us to detect only NEO (85%), HT-2 (89%) and T-2 (89%). This sorbent is able to retain planar molecules and mycotoxin adsorption has been previously reported [31], which might be the reason for the low recoveries obtained here. Finally, extraction performed with Z-Sep+ showed satisfactory recoveries (70–120%) for all the mycotoxins studied.

On the other hand, the influence of the matrix was minimal (80% ≤ signal suppression/enhancement (SSE) ≤ 120%) for all targeted analytes when using Z-Sep+ and PSA. Clean-up based on Z-Sep+ has been successfully applied to the extraction of analytes from lipid matrices [32,33]. Furthermore, Z-Sep+ is also able to form irreversible links with carboxylic groups present in proteins [34], standing as the most suitable sorbent for the here-analyzed matrix. Similarly, the use of PSA has been suggested to remove coextracted fatty acids and other ionic lipids [35].

On the contrary, a strong matrix effect was evidenced for half the analytes when using C18 and GCB. Signal suppression was detected after using C18, obtaining SSE ranging from 40% to 69%, whereas signal enhancement occurred after GCB clean-up, with SSE increasing from 128% to 167%. Since both sorbents have a preferential affinity for non-polar compounds, matrix interferents were not fully removed but coextracted. The presence of these coextracted species can change the ionization efficiency, leading to improper SSE and preventing a reliable quantification. Although no significant differences were observed between the use of Z-Sep+ and PSA ($p > 0.05$), Z-Sep+ was chosen because of its better performance minimizing matrix interference.

7.2.2 Analytical Method Validation

The optimized method was validated for the simultaneous extraction of 16 mycotoxins in CBD-based products. Results are shown in Table 7.1. Good linearity was observed for all analytes in the range assessed (0.20–100 ng/g), with regression coefficients (r^2) above 0.990 and a deviation ≤20% for each level of the calibration curve. Comparison between calibration curves built in a blank matrix and in neat solvent showed a minimal interference in the matrix (±20%) for the studied analytes. Hence, external calibration curves were used for quantification purposes. Limits of quality (LOQs) obtained for all studied analytes were between 0.20 and 6.25 ng/g. Regarding trueness, recovery values corresponding to a fortification level of 20 ng/g ranged between 63 and 103% and between 63 and 113% for the lowest fortification level (10 ng/g). Referring to the additional spiking level (2 ng/g) for aflatoxins, recoveries ranged between 63% and 86%. Precision study revealed both RSD_r and RSD_R values below 20% for all the mycotoxins analyzed. These results confirmed that the optimized procedure is suitable for a reliable quantification of the mycotoxins analyzed, fulfilling the criteria set by Commission Decision 2002/657/EC [36]. Table 7.2 reviews the available literature regarding mycotoxins in herbal-based supplements. As shown, the here-obtained LOQs were lower than the ones reported in previous studies using UHPLC-Q-Orbitrap HRMS. As established by Regulation (EC) No. 1881/2006 [20], maximum limits for aflatoxins in many food matrices must not reach levels which are below those LOQs (5 ng/g), whereas LOQs obtained in this study were between 5 and 25 times lower. Other analytical methods based on low resolution mass spectrometry [37] required longer and more complicated extraction procedures than the QuEChERS developed here. Even ELISA detection has been used for quantification of mycotoxins in medicinal herbs [28], but a very specific extraction had to be performed for different groups of analytes using several multi-functional columns. The QuEChERS procedure developed in this study, in combination with UHPLC-Q-Orbitrap mass spectrometry, was extremely simple and reliable, allowing for the quantification of all mycotoxins with high sensitivity.

Table 7.1. Method performance: linearity, matrix effect (SSE %), recovery and LOQ

Analyte	Linearity (r^2)	SSE (%)	Recovery (%)				Precision (%) [RSD_r, (RSD_R)]				
			2 ng/g [1]	10 ng/g	20 ng/g	50 ng/g	2 ng/g [1]	10 ng/g	20 ng/g	50 ng/g	LOQ (ng/g)
AFG2	0.9975	111	78	77	81	98	16 (19)	5 (6)	6 (6)	5 (4)	0.78
AFG1	0.9982	106	81	86	86	105	12 (9)	16 (19)	7 (6)	11 (10)	1.56
AFB1	0.9984	115	71	91	98	107	14 (13)	10 (8)	4 (4)	4 (3)	0.20
AFB2	0.9998	111	86	88	91	103	18 (15)	10 (8)	7 (5)	5 (4)	0.20
NEO	0.9988	112		88	93	104		18 (14)	16 (18)	17 (18)	0.78
HT-2	0.9984	108		113	101	92		12 (14)	16 (11)	12 (15)	6.25
T-2	0.9990	83		89	98	110		19 (13)	9 (7)	7 (10)	0.78
α-ZEL	0.9943	81		81	94	100		11 (11)	10 (14)	5 (16)	6.25
β-ZEL	0.9985	84		106	103	89		8 (18)	15 (16)	9 (11)	3.13
ZAN	0.9992	108		111	100	105		15 (13)	18 (11)	5 (13)	1.56
ZEN	0.9991	109		104	103	93		5 (16)	15 (14)	10 (19)	3.13
ENN B	0.9998	102		63	63	65		18 (19)	18 (18)	6 (7)	6.25
ENN B1	0.9982	99		83	89	85		12 (11)	8 (6)	8 (8)	1.56
ENN A	0.9942	84		96	91	80		11 (9)	14 (17)	11 (12)	3.13
ENN A1	0.9972	87		92	101	90		12 (14)	9 (6)	7 (14)	1.56
BEA	0.9971	119		80	71	63		18 (17)	10 (18)	10 (19)	6.25

[1] *Additional fortification level only for AFs*

Table 7.2. Available methods for measurement of mycotoxins in herbal-based supplements [1]

Samples	Positives	Major Analytes	Concentration	Determination		
Procedence (no.)	Samples (%)	Detected	Reported (ng/g)	Sensitivity (LOQ, ng/g)	Detection Method	Reference
Medicinal or aromatic herbs (84)	99	ZEN	1.0–44.1	0.14	ELISA detection (EIA reader, SIRIO S)	[**28**]
		T-2	0.6–256.9	0.28		
		DON[3]	20.5–343.5	14.8		
		CIT[3]	14.9–354.8	16.5		
Traditional Chinese herbs (60)	83	ZEN	2.1–15.5	0.4	QQQ (Applied Biosystems) ESI+ MRM mode	[**37**]
		AFs[3]	0.2–19.5	0.1		
		MPA[3]	0.2–22.7	0.02		
Milk thistle (83)	19	AFB1	0.04–1.9	0.03	LC-FLD (Waters)	[**41**]

Samples	Positives	Major Analytes	Concentration		Determination	
Green coffee bean (50)	36	OTA[3]	1–136.9	2.5	QQQ (AB SCIEX) ESI^+ and ESI^- MRM mode	[**23**]
		OTB[3]	1–20.2	2.5		
		FB1[3]	50–415	100		
		MPA	5–395	10		
Milk thistle (7)	29	T-2	363–453.9	30.5	QQQ (AB SCIEX) ESI+ MRM mode	[**38**]
		HT-2	826.9–943.7	43.8		
Herbals (69)	96	ZEN	5–824	10	QQQ (AB SCIEX) ESI^+ and ESI^- MRM mode	[**24**]
		T-2	69–1870	10		
		HT-2	59–1530	50		
		ENNB	5–9260	5		
		ENNB1	5–10,900	5		
		ENNA	5–8340	5		
		ENNA1	5–2340	5		
Gingko biloba (8)	50	AFB1	5.0–54	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[**25**]
		AFB2	4–300	10		
		T-2	18–20	30.5		
Green tea (10)	10	AFB1	5.4	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[**26**]
Royal jelly (8)	0					
Soy (11)	27	AFB1	8.2–17.1	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[**27**]
		AFG2	6.4	5		
Cannabis sativa (10)	70	ZEN	4.2–11.6	3.13	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	Current study
		ENNB1	<LOQ–11.6	1.56		

[1] ESI+ = positive ion mode; ESI− = negative ion mode; HRMS = high-resolution MS; LOQ = limit of quantification; MRM = multiple reaction monitoring; QQQ = triple quadrupole. [2] Range of LOQs referring to the analyzed mycotoxins. [3] AFs = aflatoxins; DON = deoxynivalenol; CIT = citrinin; FB1 = fumonisin B1; MPA = mycophenolic acid; OTA = ochratoxin A; OTB = ochratoxin B.

7.2.3 Application to Commercial CBD-Based Products

The validated UHPLC-Q-Orbitrap HRMS procedure was applied to ten commercially available samples in order to evaluate the occurrence of mycotoxins. Results are shown in Table 7.3. A considerable occurrence of mycotoxins was observed, since contamination with at least one analyte was found in 70% of the samples. Up to six different mycotoxins (T-2, ZAN, ZEN, ENNB1, ENNA, ENNA1) were quantified at a range from below LOQ to 11.6 ng/g, all produced by *Fusarium* genera, reported as a major *C. sativa* pathogen fungus [14]. Previous studies regarding mycotoxins in different herbal-based extracts have revealed the occurrence of similar mycotoxins independently of the matrix and the dosage form (Table 7.2). Despite the fact that the percentage of positive samples varied among the different studies (19–99%), when the sensitivity of the analytical method increased, reaching lower LOQs, the number of positive samples dramatically increased. This indicated that mycotoxin contamination in herbal-based products at low levels is frequent.

Table 7.3. Occurrence of studied mycotoxins in the analyzed samples

Sample	Mycotoxin (ng/g)					
	T-2	**ZAN**	**ZEN**	**ENN B1**	**ENN A**	**ENN A1**
1			11.6	11.6	4.2	5.8
4			6.5			
5				<LOQ		
7			8.1			
8		1.9	4.7			
9			4.2	<LOQ		
10	2.0		6.3			

In the here-analyzed samples, ZEN appeared to be the most common mycotoxin, with an incidence of 60% and concentration levels ranging from 4.2 to 11.6 ng/g (mean level = 6.9 ng/g). A high incidence of ZEN has also been previously reported in supplements made of different herbals from Czech and US retail markets (84%, n = 69) at a wide range of concentrations (5–824 ng/g, mean value = 75.7 ng/g) [24]. Moreover, ZEN was previously found in 96% of medicinal herbals from Spain (n = 84) as well, but in a tighter range (1–44.1 ng/g, mean value = 8.9 ng/g) [28].

Referring to T-2, results reported contamination in one sample at 2.0 ng/g, in contrast with the prevalent presence of T-2 in 78% (n = 69) of the same Czech and US samples, at concentrations rising from 69 to 1,870 ng/g (mean value = 162 ng/g) [24]. High levels of T-2 were also observed in milk thistle samples from Spain (363–453.9, mean value = 408.9 ng/g) in only two out of seven samples [38]. In the other hand, T-2 was quantified in 98% (n = 84) of the Spanish medicinal herbals, but in much lower concentrations (0.6–256 ng/g, mean value = 22.645 ng/g) [28].

Similarly, ZAN was quantified in one sample at 1.9 ng/g. This mycotoxin has been scarcely targeted in dietary supplement studies, but has been previously quantified at similar concentrations as those here-reported in two samples of Chinese medicinal herbals (n = 33) [39].

Results also showed ENN contamination. ENNB1, ENNA and ENNA1 were found in the same sample at 11.6, 4.2 and 5.8 ng/g, respectively, whereas ENNB1 was detected in two other samples below the LOQ (1.56 ng/g). These emerging *Fusarium* mycotoxins have been previously found in herbal products (84–91%, n = 69) widely ranging from 5 ng/g up to 10,900 ng/g (mean value = 354 ng/g) [24]. Similarly, ENNB1 was the most common toxin out of these emerging *Fusarium* mycotoxins, being consistent with the results here obtained.

All the mycotoxins found in the present study correspond to low- to non-polar compounds, which should be prevalently expected due to the nature of the matrix.

Co-occurrence of at least two mycotoxins was also observed in four out of ten samples. Results showed the presence of ZEN in combination with ENNs B1, A and A1, ZAN or T-2, which are common associations found by previous studies in herbal-based supplements [24,28]. It must be

highlighted that synergic or additive effects have been observed as a consequence of these combinations in in vitro assays [40]. Based on what has been discussed and considering the uprising trend of *C. sativa*-based products, alongside the use of environment-friendly raw materials cultivated without pesticides, quality controls regarding mycotoxins should be set for these products in order to ensure safe consumption.

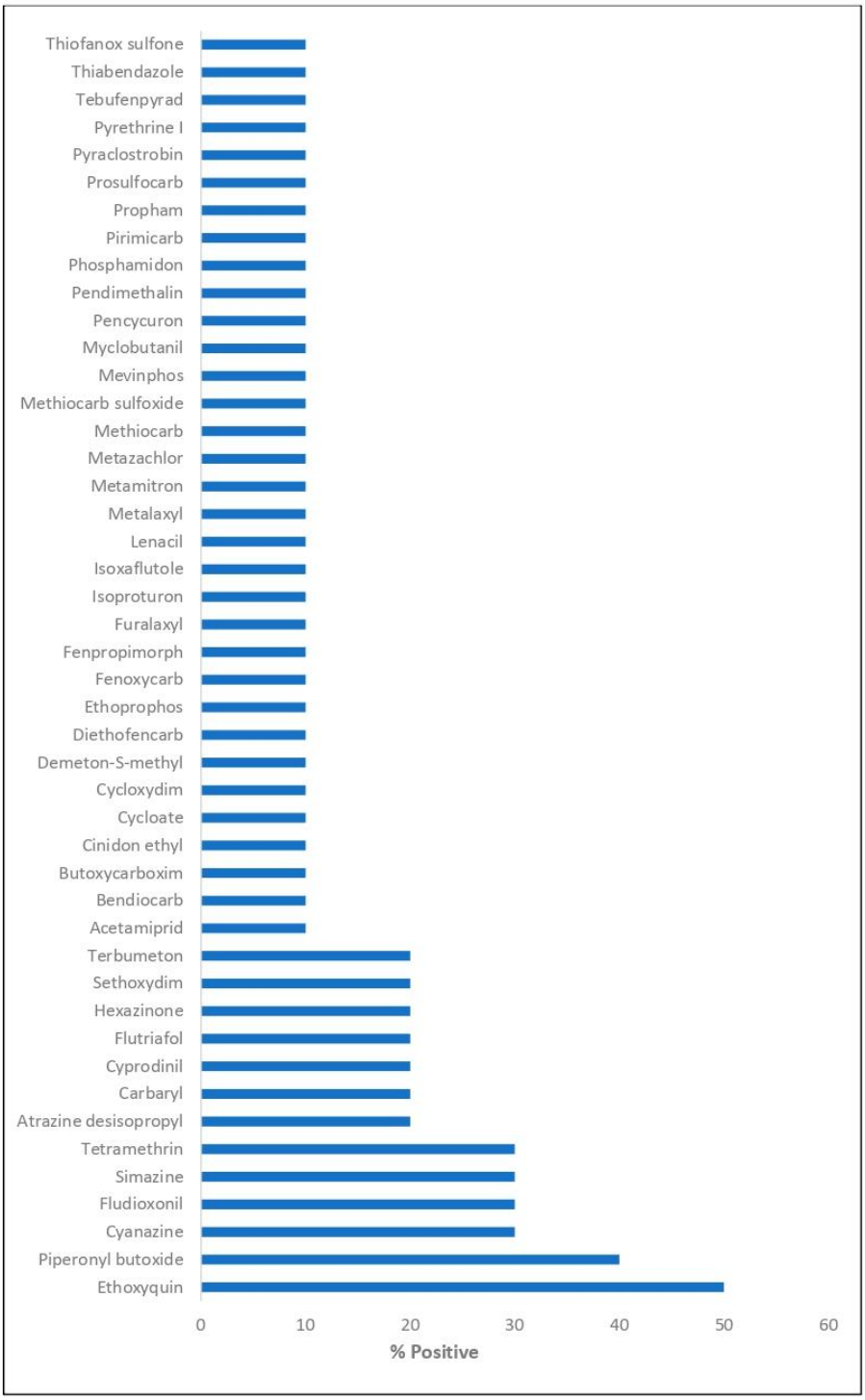


Fig. 7.2. Occurrence of non-target pesticides in analyzed samples after post-run retrospective screening

7.2.4 Identification of Non-Target Compounds through Retrospective Analysis in Studied Samples

The post-target screening approach allowed us to detect pesticide residues in the analyzed samples using a spectral library. Results are shown in Fig. 7.2. Up to 46 different pesticides were tentatively identified based on the pesticides mass spectral library. Ethoxyquin was putatively found in five samples, being the most prevalent pesticide. The main function of ethoxyquin is to avoid fungal contamination during the postharvest stage of the plant through its scald-preventive properties [42]. Surprisingly, the use of this pesticide is forbidden by the European Commission (EC) Decision 2011/143/EU. Piperonyl butoxide was found in four samples. This compound is not a pesticide by itself but can inhibit the resistance mechanisms of insects, being widely used in combination with other different pesticides [42]. The tentative presence of cyanazine and simazine, both found in three different samples, must also be noted. The use of these pesticides was prohibited by EC Regulation No. 1107/2009 [43] and Commission Decision 2004/247/EC [44], respectively. Therefore, the occurrence of forbidden pesticides found in the here-analyzed samples highlights the necessity of monitoring potential contaminants in *C. sativa*-derived products acquired from online shops.

7.3 MATERIALS AND METHODS

7.3.1 Chemicals and Reagents

Acetonitrile (AcN), methanol (MeOH), and water for LC mobile phase (HPLC grade) were acquired from Merck (Darmstadt, Germany). Formic acid and ammonium formate were obtained from Fluka (Milan, Italy). Sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), octadecyl carbon chain-bonded silica (C18), graphitized carbon black (GCB), primary-secondary amine (PSA) and zirconium oxide (Z-Sep$^+$) were obtained from Sigma Aldrich (Milan, Italy).

Mycotoxin standards and metabolites, namely aflatoxins (AFB1, AFB2, AFG1, and AFG2), HT-2 toxin (HT-2), T-2 toxin (T-2), neosolaniol (NEO), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), beauvericin (BEA) and enniatins (ENNA, ENNA1, ENNB, and ENNB1) were purchased from Sigma Aldrich (Milan, Italy). Individual stock solutions of all analytes were prepared by diluting 1 mg of each mycotoxin in 1 mL of methanol. The working standard solution including all the mycotoxins was made by adequate diluting in MeOH:H_2O (70:30 *v/v*) 0.1% formic acid to reach the required concentrations for performing the spike experiments: 20, 10 and 2 µg/mL. All solutions were kept in safe conditions at −20°C.

7.3.2 Sampling

For the analysis of real samples, ten different CBD gelatin capsules were obtained from online shops based in different European countries. The capsules are made of gel mass, which contains gelatin, water, glycerin and other minor additives whereas the fill formulation consists of olive oil mixed with hemp oil containing CBD at certain concentrations. The weight of each capsule depended on the manufacturer; there were 0.25, 0.5 and 1 g capsules. Only soft gel capsules were studied since it was the prevalent presentation available for CBD supplements. On the other hand, one sample of CBD supplements delivered as soft gel capsules was acquired from a local store (Naples, Italy). After confirming the absence of contaminants, they were used for preparing fortified samples for recovery assays and matrix-matched standards for calibration purposes. All the samples were conserved in dark and cool conditions, as recommended by the manufacturer, until further analysis.

7.3.3 Sample Preparation

The sample preparation procedure developed by Veprikova et al. [**24**] was selected as a starting point and then slightly modified, as follows: 1 g of sample was weighed into a 50 mL polytetrafluorethylene (PTFE) tube and mixed with 5 mL of 1% aqueous formic acid. The mixture was placed in an SKO-D XL orbital shaker (Argo Lab, Italy) for 30 min at 294 × *g*. Then, 5 mL of AcN were added and the mixture was shaken for an additional 30 min at 294 × *g*. After that, 0.5 g of sodium chloride and 2 g of

magnesium sulfate were added and the tube was shaken for 1 min by hand, followed by centrifugation at 4907 × *g* for 15 min in an SL 16R centrifuge (Thermo Fisher Scientific LED GmbH, Germany). A 2 mL aliquot of the upper acetonitrile layer was taken for dispersive solid phase extraction (d-SPE) cleanup in a 15 mL PTFE tube containing 100 mg of Z-Sep+ sorbent and 300 mg of magnesium sulfate. The tube was vortexed for 1 min and then centrifuged at 4907 × *g* for 15 min. An aliquot of the supernatant (1 mL) was collected and filtered through a 0.2 µm PTFE filter (Phenomenex, Italy) into a vial prior to UHPLC-Q-Orbitrap HRMS analysis.

7.3.4 UHPLC-Q-Orbitrap HRMS Analysis

The qualitative and quantitative profiles of the mycotoxins were obtained using an ultra-high-pressure liquid chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000, a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T = 30 °C) Luna Omega 1.6 µm (50 × 2.1 µm) column.

The eluent consisted of two different phases: A (H_2O containing 0.1% formic acid and 5 mM ammonium formate) and B (MeOH containing 0.1% formic acid and 5 mM ammonium formate). The gradient elution for LC-Orbitrap HRMS analyses was applied as follows: an initial 0% of phase B was held for 1 min, which linearly went up to 95% B over 1 min and held for 0.5 min. Next, the gradient decreased to 75% B over 2.5 min and then decreased again to 60% B over 1 min. Finally, the gradient turned to 0% B over 0.5 min and then the column was equilibrated for 1.5 min at 0% B. The total run time was 8 min, at a flow rate of 0.4 mL/min. A total of 5 µL of the sample was injected. Detection was performed using a Q-Exactive mass spectrometer. The mass spectrometer was operated in both positive and negative ion mode using fast polarity switching by setting two scan events (full ion MS and all ion fragmentation (AIF)). Full scan data were acquired at a resolving power of 35,000 FWHM at *m/z* 200.

The ion source parameters were: spray voltage 4 kV (-4 kV in ESI− mode); capillary temperature 290°C; S-lens RF level 50; sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, and auxiliary gas heater temperature 305 °C. The value for automatic gain control (AGC) target was set at 1×10^6, a scan range of *m/z* 100 to 1000 was selected and the injection time was set to 200 ms. The scan rate was set at 2 scans/s. For the scan event of AIF, the parameters in the positive and negative ion mode were: mass resolving power = 17,500 FWHM; maximum injection time = 200 ms; scan time = 0.10 s; ACG target = 1×10^5; scan range = 100–1000 *m/z*, isolation window to 5.0 *m/z*, and retention time window to 30 s. The Orbitrap-MS parameters were optimized in a previous work [45]. The exact mass for the studied compounds, including elemental composition, retention time (RT), theoretical masses and accurate mass errors for the detected ions are shown in Table 7.4. A mass error below 5 ppm, referring to the molecular ions, was set for identification. Retrospective screening was carried out on spectral data collected using a pesticide spectral library (Pesticide Spectral Library Version 1.1 for LibraryView™ Software, AB SCIEX, Framingham, USA). For accurate mass measurement, identification and confirmation were performed at a mass tolerance of 5 ppm for the molecular ion and for both fragments at the intensity threshold of 1000. Data analysis and processing were performed using the Xcalibur software, v. 3.1.66.10.

7.3.5 Validation Parameters

An in-house validation study was conducted following the EU Commission Decision 2002/657/EC [36]. The parameters evaluated were selectivity, specificity, linearity, trueness, repeatability (intra-day precision), within-reproducibility (inter-day precision), limit of quantification (LOQ) and limit of detection (LOD). The selectivity and specificity of the method were evaluated by analyzing both standard solutions and samples, comparing the retention time of the peaks corresponding to the analytes of interest alongside the determination of its precursor and product ion, with a mass error below 5 ppm. For linearity, standard solutions built in neat solvent and matrix-matched calibration were analyzed by spiking blank samples at eight concentration levels from 0.2 to 100 ng/g. The slopes of each linear calibration function were compared in order to detect a signal suppression/ enhancement (SSE) effect due to the matrix interference. This effect was quantified following the

equation: SSE (%) = matrix-matched calibration slope/solvent calibration slope x 100. An SSE value of 100% was interpreted as no matrix interference in the concentration range evaluated. An SSE value above 100% revealed signal enhancement whereas a value below 100% indicated signal suppression. For trueness, recovery studies were evaluated by spiking three blank samples at three different levels. Additionally, a lower spike level was used only for aflatoxins. Intra-day precision (RSD_r) was expressed as the relative standard deviation after three determinations in a single day (n = 3). Inter-day precision was calculated by repeating the measurements in triplicate on three non-consecutive days (n = 9) and expressed as relative standard deviation (RSD_R). The LOD was defined as the minimum concentration where the molecular ion can be identified by the instrument (mass error value below 5 ppm) and the LOQ as the minimum concentration where a linear response (mass error value below 5 ppm) can be observed with an accuracy and precision of ≤ 20%.

Table 7.4. Retention times, accurate mass and mass accuracy of mycotoxins evaluated

Analyte	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (m/z)	Measured Mass (m/z)	Accuracy (Δ ppm)
NEO	4.25	$C_{19}H_{26}O_8$	$[M+NH_4]^+$	400.1966	400.1963	−0.67
AFG2	4.50	$C_{17}H_{14}O_7$	$[M+H]^+$	331.0812	331.0808	−1.36
AFG1	4.52	$C_{17}H_{12}O_7$	$[M+H]^+$	329.0656	329.0655	−0.27
AFB2	4.58	$C_{17}H_{14}O_6$	$[M+H]^+$	315.0863	315.0862	−0.51
AFB1	4.62	$C_{17}H_{12}O_6$	$[M+H]^+$	313.0707	313.0705	−0.42
HT-2	4.74	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2435	442.2432	−0.7
α-ZEL	4.83	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1550	−0.31
T-2	4.85	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2541	484.2543	0.39
β-ZEL	4.97	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1550	−0.31
ZAN	4.98	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1549	−0.6
ZEN	5.01	$C_{18}H_{22}O_5$	$[M+H]^+$	317.1395	317.1393	−0.54
ENN B	5.56	$C_{33}H_{57}N_3O_9$	$[M+NH_4]^+$	657.4433	657.4435	0.26
ENN B1	5.68	$C_{34}H_{59}N_3O_9$	$[M+NH_4]^+$	671.4599	671.4594	−0.76
BEA	5.73	$C_{45}H_{57}N_3O_9$	$[M+NH_4]^+$	801.4433	801.4432	−0.16
ENN A1	5.82	$C_{35}H_{61}N_3O_9$	$[M+NH_4]^+$	685.4746	685.4745	−0.18
ENN A	5.99	$C_{36}H_{63}N_3O_9$	$[M+NH_4]^+$	699.4903	699.4899	−0.56

7.3.6 Statistical Analysis

Validation experiments were performed in triplicate and the results expressed as the average values alongside relative standard deviation (RSD, %). The Saphiro–Wilk test was applied to evaluate normality and multivariant analysis was performed using a non-parametric Kruskal–Wallis test, considering p values < 0.05 as significant. Analysis of data was carried out using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA).

7.4 CONCLUSIONS

A sample preparation procedure based on a QuEChERS followed by UHPLC coupled with high-resolution Q-Orbitrap mass spectrometry was optimized in order to determine and quantify 16 mycotoxins in *C. sativa*-based supplements. The proposed methodology was validated following the EU criteria, ensuring a proper specificity, selectivity, linearity, trueness and precision with a fast chromatography run performance (8 min). The validated procedure was applied to ten CBD-based supplements that are commercially available online, allowing us to quantify up to six different *Fusarium* mycotoxins in 70% of samples. ZEN was the most prevalent mycotoxin (60%) found at a maximum level of 11.6 ng/g (mean value = 6.9 ng/g). Co-occurrence was observed in four out of ten samples, including one sample with ENNB1, ENNA and ENNA1. Additionally, a retrospective analysis of pesticide residues was performed. Up to 46 different pesticides were tentatively detected, including some forbidden in *C. sativa* cultivation. Considering the uprising trend of CBD-based products, quality controls regarding contaminants should be set for these products in

order to ensure a safe consumption. Furthermore, the developed procedure is proposed as a powerful analytical tool to evaluate the potential mycotoxin profile of these particular products.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.R.-C. and A.N.; methodology, L.I. and A.N.; validation, L.C. and A.N.; formal analysis, A.N.; investigation, L.C., L.I. and A.N.; resources, A.R.; writing—original draft preparation, A.N.; writing—review and editing, Y.R.-C.; supervision, Y.R.-C. and A.R.; project administration, Y.R.-C. and A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 8
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Biomonitoring of Enniatin B1 and Its Phase I Metabolites in Human Urine: First Large-Scale Study

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ABSTRACT

Enniatins (Enns) are mycotoxins produced by *Fusarium* spp. which are a fungus widely spread throughout cereals and cereal-based products. Among all the identified enniatins, Enn B1 stands as one of the most prevalent analogues in cereals in Europe. Hence, the aim of this study was to evaluate for the first time the presence of Enn B1 and its phase I metabolites in 300 human urine samples using an ultrahigh-performance liquid chromatography high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) methodology. Enn B1 was detected in 94.3% of samples ranging from 0.007 to 0.429 ng/mL (mean value: 0.065 ng/mL). In accordance with previous in vitro and in vivo analysis, hydroxylated metabolites (78.0% samples) and carbonylated metabolites (66.0% samples) were tentatively identified as the major products. Results from this biomonitoring study point to a frequent intake of Enn B1 in the studied population, suggesting that in-depth toxicological studies are needed in order to understand the potential effects in humans.

Keywords: Enniatin B1; biomonitoring; in vivo; metabolomics; high resolution mass spectrometry (HRMS).

8.1 INTRODUCTION

Mycotoxins are toxic secondary metabolites mainly produced by the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps,* and *Alternaria*. These compounds can be found in food and feed commodities, and consuming contaminated products can lead to adverse health effects in humans and animals. *Fusarium* fungi are frequent pathogens of cereal grains and maize, with a large impact in temperate regions of America and Europe [1]. Considering occurrence, toxicity, and consumption data, maximum limits have been set in foodstuffs for several mycotoxins alongside tolerable daily intakes (TDI) or provisional TDIs by a Joint Food and Agriculture Organization and World Health Organization (FAO/WHO) Expert Committee on Food Additives [2]. During the last few years attention has been put into emerging *Fusarium* mycotoxins, such as enniatins (Enns). Its core structure consists of a cyclohexadepsipeptide with alternating residues of three *N*-methyl amino acids and three hydroxyisovaleric acid [3]. To date, 29 different enniatins have been isolated as single compounds or as mixtures of homologues, being Enn B, Enn B1, Enn A1, and Enn A, being the most relevant in that order [4]. These Enns have been reported in cereal samples from Mediterranean and Scandinavian countries in concentration levels ranging from µg/kg to mg/kg [5,6] and also Enns carry-over potential from feedstuffs to animals has been suggested [7,8]. However, no maximum limits have been set for Enns in foodstuffs yet. In 2014, the European Food Safety Authority (EFSA) released a scientific opinion on the risk related to the occurrence of Enns in food and feed, concluding that there might be a concern about chronic exposure but the lack of toxicological studies hampers the risk assessment of dietary exposure [9]. To date, data is still being collected [10].

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The toxicity of Enns is based on its ionophoric properties, being able to integrate themselves in biological membranes forming cation selective pores. Transport of mono and divalent cations through these pores disrupts normal physiological concentrations, leading to a wide range of toxicological effects. In vitro studies have reported phytotoxic, insecticidal, antibacterial, enzyme inhibition, antifungal, and immuno-modulatory activities. Besides, cytotoxic effects have been observed in several animal and cell lines at a low micromolar range [4,11]. Nevertheless, Enns have shown low toxicity in vivo, and rapid metabolization and elimination of Enns might be the main reason [12].

Regarding bioavailability, the Enn analogue with the highest oral absorption is Enn B [13,14]. There might also exist a specie-dependent relation, since absolute Enn B1 bioavailability in pigs (91%) is in strong contrast with the one observed in broiler chicken (5%) after single oral application [12,14]. Referring to metabolism, transformation of Enn B1 predominantly occurs via cytochrome P-450 3A-dependent oxidation reaction. Therefore, only phase I products seem relevant, since no phase II metabolites have been found yet. After incubation of Enn B1 with human and pig liver microsomes, 11 different metabolites were structurally characterized using liquid chromatography coupled to: iontrap mass spectrometry (ITMS), multiple-stage mass fragmentation (MS^n), and high resolution mass spectrometry (HRMS) coupled to an Orbitrap mass spectrometer [15,16]. Biotransformation processes of Enn B1 consisted in carbonylation, carboxylation, hydroxylation, and *N*-demethylation, following the same pattern observed for Enn B except for carbonylation [15,17].

There is scarce literature referring to the detection of enniatins and its resultant metabolites in biological samples, making it harder to assess the risk related to enniatins. Recently, the occurrence of Enn B and its tentative phase I metabolites in human urine was evaluated, finding the parental toxin and some of its tentative metabolites at a concentration of a few ng/mL [18]. Nevertheless, to date, there is no data available concerning the occurrence of Enn B1 and its tentative phase I metabolites in human biological fluids. In order to obtain a risk evaluation precisely, mycotoxins potentially present in foods and its metabolites have to be included in biological studies since the overall toxic profile could be influenced. Therefore, the aim of the present study was to evaluate for the first time the presence of Enn B1 and Enn B1 phase I metabolites in 300 urine samples from volunteers residing in southern Italy using ultrahigh-performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC-Q-Orbitrap HRMS) and to tentatively identify the main Enn B1 metabolic pathway for producing scientific evidence on the pharmacokinetics process of this toxin.

8.2 RESULTS AND DISCUSSION

8.2.1 Evaluation of UHPLC-Q-Orbitrap HRMS Conditions

The Enn B1-dependent tandem mass spectrometry (MS/MS) parameters were optimized by injection of 1 µg/mL of Enn B1-analytical standard into the UHPLC-Q-Orbitrap instrument. Results revealed a peak at 8.06 min in full scan mass spectrometry (MS), which showed a stable and abundant ammonium adduct $(M + NH_4)^+$ at *m/z* 671.45986 under positive electrospray ionization (ESI) mode corresponding to Enn B1. The mass error of the observed *m*/*z* was less than 2.5 ppm indicating exceptional agreement with the calculated *m/z.* In addition, product ions (*m/z* 558, 549, 458, 214 and 196) were generated by collision-induced dissociation of the Enn B1 ammonium adduct. These ions correspond to the loss of one or various *N*-methyl-valine, *N*-methyl-isoleucine, and hydroxyisovaleryl residues leading to the formation of main Enn B1 fragments.

Apart from the parent compound, in literature, up to 11 metabolic products were detected and their structures tentatively characterized through high-performance liquid chromatographic/mass spectrometric analyses. In this work, the ammonium adducts of the reported molecular masses were targeted in the Q-Orbitrap HRMS to evaluate the occurrence of the enniatin B1 metabolites in human urine samples. Therefore, a qualitative procedure was developed for detecting theoretical masses of the metabolites previously reported, whereas the same chromatographic gradient was used since all the investigated compounds were able to elute in that short run time.

Data for retention times, observed ion mass, and mass accuracy for Enn B1 and its metabolites are shown in Table 8.1.

Table 8.1. Retention times, observed mass, and mass accuracy of Enn B1 and its phase I metabolites

Compound	Retention Time (min)	Molecular Formula	Observed Mass (M + NH_4)$^+$	Accuracy (Δppm)
Enn B1	5.75	$C_{34}H_{59}N_3O_9$	671.4591	−0.60
M1	4.76	$C_{33}H_{55}N_3O_{11}$	687.4175	−0.78
M2	5.44	$C_{34}H_{59}N_3O_{10}$	687.4532	−1.78
M3	5.5		687.4535	−1.34
M4	5.55		687.4536	−1.19
M5	5.6		687.4540	−0.61
M6	5.61	$C_{34}H_{57}N_3O_{10}$	685.4380	−1.12
M7	5.64		685.4384	−0.54
M8	5.67		685.4378	−1.42
M9	4.1	$C_{34}H_{57}N_3O_{11}$	701.4328	−1.26
M10	4.77		701.4335	−0.26
M11	4.79		701.4333	−0.55

8.2.2 Method Performance

Method results referring to matrix effects, linearity, trueness, repeatability, within-lab reproducibility, limit of quantification (LOQ), and limit of detection (LOD) were obtained following the guidelines set at Commission Decision 2002/ 657/EC [19] are shown in Table 8.2. A matrix influence was observed, leading to a signal suppression effect (63%) for Enn B1 in the urine samples. Hence, matrix-matched calibration was chosen for quantitative purposes. A coefficient of linearity (R^2) of 0.9997 was obtained within the range from 0.001 to 5 ng/mL. In order to evaluate the carry-over, a blank sample (n = 10) was analyzed just after the highest calibration sample. Since no peaks eluted in the same Enn B1 retention time area, no carry-over was assumed. Acceptable recoveries, ranging from 78% at 0.5 ng/mL to 95% at 5 ng/mL of spiking levels, were obtained. The RSD_r and RSD_R were ≤12% for all the concentrations studied, remarking the satisfactory precision of the developed method. LOQ and LOD values were extracted from matrix-matched solutions, being 0.0005 and 0.001 ng/mL, respectively. The developed method allowed a reliable detection and quantification of Enn B1 at low ppt range (Table 8.3).

8.2.3 Occurrence of Enniatin B1 in Human Urines

Enn B1 has been detected in 283 out of 300 urine (94.3%) ranging from <LOQ to 0.429 ng/mL (mean value: 0.065 ng/mL; Table 8.4). In a previous study, Escrivá et al. [21] evaluated the presence of Enn B1 in urine samples from the Spanish population, reporting occurrence in six samples (60%, n = 10), but only two samples could be quantified (0.1–0.34 ng/mL) due to analytical limitations (LOQ = 0.1 ng/mL). Serrano et al. [20] detected Enn B1 in one urine sample (10%, n = 10) collected from Italian volunteers, despite having high instrumental sensitivity (LOQ = 0.005 ng/mL). Differences showed among the mentioned studies could be due to the limited sampling. A more recent work, conducted by Liu et al. [22], analyzed 60 urine sample from the Chinese population, reporting the absence of Enn B1 (LOQ = 0.0002 ng/mL). The variation between the Chinese population urinary patterns of Enn B1 and the obtained results could be explained either by dietary habits or the quality of foodstuffs. Wheat has been reported as one of the most susceptible cereals for *Fusarium* spp. contamination [1] and, according to the data reported by FAO [23], the per capita annual consumption of wheat and wheat-based products during 2017 in Italy (146.22 kg) was more than double the consumption in China (62.75 kg). Following the same line, the urinary excretion pattern of Enn B, a structurally similar mycotoxin, was also reported to vary depending on geographical areas with different dietary habits [18]. Recently, strong associations to some cereals and to dietary fiber was reported for Enn B urinary concentration in Sweden [24]. These authors also reported associations to other *Fusarium* mycotoxins, such as deoxynivalenol, in these food categories; nonetheless, Enn B association to rice was negative but strong, which indicates that rice was not as contaminated as the other cereals (e.g., wheat). Hence, if rice consumption was increased in the diet, as in the case of the Asiatic people, the

occurrence of Enns in biological fluids would consequently be reduced, which is in line with previous studies [22]. Therefore, the occurrence of Enn B1 in the majority of Italian cereals and cereal-based products analyzed by previous studies [25,26,27] alongside the high consumption of cereals (45–60% kcal per day for an Italian adult) [23] may account for the high incidence reported in the obtained results.

Table 8.2. Method performance

Parameters	R^2	SSE (%)	Recovery, % (RSD_R, %; n = 9)				LOD (ng/mL)	LOQ (ng/mL)
			5 ng/mL	1 ng/mL	0.5 ng/mL	0.1 ng/mL		
Enn B1	0.9997	63	95 (7)	88 (6)	78 (7)	84 (12)	0.0005	0.001

R^2, coefficient of correlation; RSD_R, inter-day relative standard deviation; LOD, limit of detection; LOQ, limit of quantification

A statistical study for the evaluation of the occurrence of Enn B1 in urines from the three different population groups was conducted. The highest Enn B1 concentration was observed within the low-age group (age ≤ 30 years, mean = 0.071 ng/mL); however, no significant differences were found between occurrence of Enn B1 and age groups (p = 0.93). Similarly, gender did not show any correlation referring to Enn B1 concentrations (p = 0.85) in accordance with previous mycotoxins monitoring studies in urine [28,29,30].

8.2.4 Urinary Excretion Pattern of Enn B1 Phase I Metabolites

To date, there is scarce literature regarding Enn B1 metabolism. In vivo studies have been carried out in pigs and broiler chickens fed with Enn B1 contaminated feed, reporting the presence up to 11 tentative major Enn B1 metabolites in plasma and showing a good correlation with previous in vitro studies [14,15]. In a recent investigation conducted by Ivanova et al. [16], the same tentative metabolites were detected in vitro after the incubation of human liver microsomes with Enn B1. These metabolites are products of oxidative demethylation (M1), hydroxylation (M2–M5), carbonylation (M6–M8), and carboxylation (M9–M11) reactions. From a chromatographic point of view, all the identified metabolites eluted before the parental compound in reversed-phase chromatography since they became more hydrophilic after going through phase I metabolism pathways (Table 8.1). Nevertheless, the lack of standards of Enn B1 metabolites avoid an accurate quantification. To overcome that, the matrix-matched calibration curve from the parent compound was used as an approach in order to quantify its metabolites. The results obtained are shown in Table 8.4. The most prevalent groups were the hydroxylated and carbonylated Enn B1 metabolites, ranging from 0.006 to 0.233 ng/mL (78% samples) and from 0.012 to 1.763 ng/mL (66% samples), respectively. The carboxylated metabolites were found between 0.008 and 0.656 ng/mL (26.3% samples) and the demethylated products ranged from 0.007 to 0.177 ng/mL (5.3% samples). Within the hydroxylated group, the main metabolites were M5 > M3 > M4 > M2, in this order, with M5 representing 68% of all the hydroxylated metabolites quantified. Significant differences were found among concentration values of each metabolite (p ≤ 0.05). Similarly, the main carbonylated metabolites were M8 > M6 > M7, and statistical analysis revealed significant differences among them (p ≤ 0.05), with M8 being 48% of the total carbonylated metabolites. Whereas results reported by Ivanova et al. [16] showed a similar trend for hydroxylated compounds with M5 as the major one, the most relevant carbonylated product was M6, differing from the here-analyzed samples in which M6 only represented a 32% of the carbonylated products. The most important Enn B1 carboxylated metabolites were M10 and M11, whereas M9 showed very low incidence (0.7%). Ivanova et al. [15] detected M9 and M11 only in in vitro samples of pig liver microsomes, whereas the occurrence of M10 was restricted to in vivo samples. However, the results highlight M11 as another important product of carboxylation pathways in human. Differences observed between this work and in vitro Enn B1 biotransformation assays stand as additional evidence of potential pre-systemic metabolism. The demethylated metabolite M1 appeared to be irrelevant and the multiple reactions needed to generate this product may account for the low incidence showed (5.3%). Fig. 8.1 shows the chromatograms of a sample contaminated with Enn B1 at 0.036 ng/mL; hydroxylated Enn B1 metabolites (M3: 0.053 ng/mL; M5: 0.174 ng/mL); carbonylated Enn B1 metabolites (M6: 0.034 ng/mL; M8: 0.045 ng/mL); and carboxylated Enn B1 metabolites (M10: 0.028 ng/mL; M11: 0.017 ng/mL).

Table 8.3. Available methods for measurement of Enn B1 in human urine

				Determination			
Urine Samples (*n*)	**Origin**	**Positives Pamples (*n*, (%))**	**Sample Preparation**	**Range (ng/mL)**	**Sensitivity (LOQ, ng/mL)**	**Detection Method**	**Reference**
10	Italy	1 (10)	SPE	<LOQ	0.005	QQQ (Thermo Fisher Scientific) ESI+ SRM mode	Serrano et al. (2015) [**20**]
10	Spain	6 (60)	DLLME	<LOQ–0.34	0.1	QQQ (Applied Biosystems) ESI+ SRM mode	Escrivá et al. (2017) [**21**]
60	China	0 (0)	SPE	-	0.0002	QQQ (AB SCIEX) ESI+ MRM mode	Liu et al. (2020) [**22**]
300	Italy	283 (94)	SALLE	<LOQ–0.429	0.001	Q-Orbitrap (Exactive, Thermo Fisher Scientific) ESI+ HRMS	This work

ESI+, positive ion mode; HRMS, high-resolution MS; SRM, selected reaction monitoring transition; MRM, multiple reaction monitoring transition; LOQ, limit of quantification; QQQ, triple quadrupole; SPE, solid phase extraction; DLLME, dispersive liquid-liquid microextraction; SALLE, salting-out liquid-liquid extraction.

Table 8.4. Occurrence of Enn B1 and Enn B1 metabolites in the analyzed human urine samples (*n* = 300)

Compound/Group	Incidence (%)	Range (ng/mL)	Mean [a] (ng/mL)
Parent Compound			
Enn B1	94.3	0.007–0.429	0.069
Enn B1 Biotransformation Products			
M1	5.3	0.007–0.177	0.035
Demethylated and hydroxylated (M1)	5.3	0.007–0.177	0.035
M2	11.0	0.006–0.019	0.010
M3	50.0	0.005–0.076	0.023
M4	*18.0*	0.002–0.143	0.025
M5	77.3	0.006–0.186	0.047
Hydroxylated group (M2–M5)	78.0	0.006–0.233	0.069
M6	40.0	0.012–1.511	0.105
M7	30.7	0.008–0.510	0.085
M8	48.0	0.042–1.310	0.128
Carbonylated group (M6–M8)	66.0	0.012–1.763	0.196
M9	*0.7*	0.019–0.045	0.032
M10	21.0	0.008–0.241	0.047
M11	14.0	0.002–0.451	0.053
Carboxylated group (M9–M11)	26.3	0.008–0.656	0.066

[a] *Mean values are based in positive samples only.*

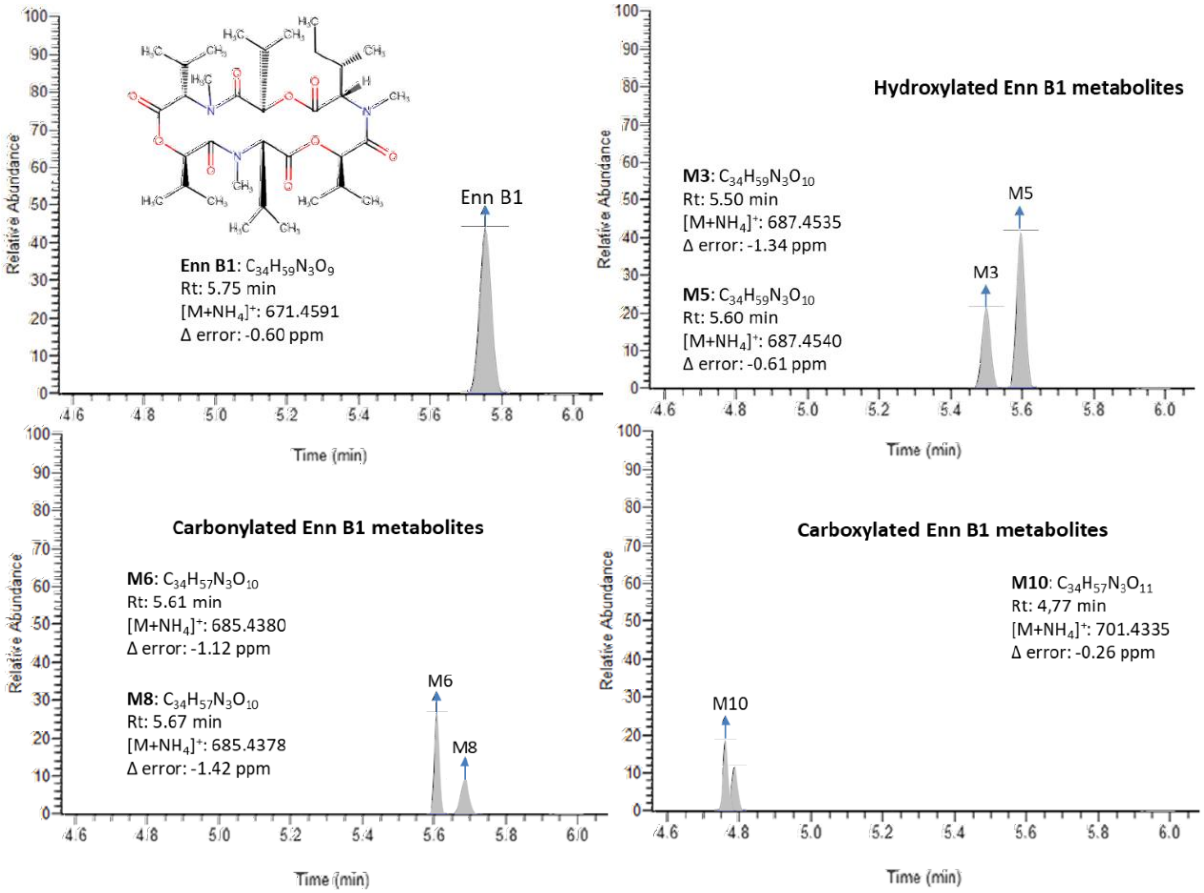

Fig. 8.1. Chromatograms of a human urine sample containing Enn B1 at 0.036 ng/mL; hydroxylated Enn B1 metabolites (M3: 0.053 ng/mL; M5: 0.174 ng/mL); carbonylated Enn B1 metabolites (M6: 0.034 ng/mL; M8: 0.045 ng/mL); and carboxylated Enn B1 metabolites (M10: 0.028 ng/mL; M11: 0.017 ng/mL)

Referring to the samples with no Enn B1 contamination (5.7%, *n* = 17), only 1.3% (*n* = 4) did not show any Enn B1 metabolite, whereas at least two different metabolites were found in the 4.3% (*n* = 13) of the remaining samples, pointing to a complete biotransformation. The results reported above showed that the metabolization of Enn B1 in humans mainly occurs via hydroxylation and carbonylation reactions. Although cytochrome P450 3A4 (CYP3A4) is the main enzyme in the metabolism of Enn B and Enn B1, additional enzymes are also involved; CYP1A2 and CYP2C19 are present in Enn B pathway, whereas CYP3A4/5 is responsible for carbonylated products of Enn B1 as evidenced by Fæste et al. [31] and Ivanova et al. [16]. In fact, a previous work conducted by Rodríguez-Carrasco et al. [18] remarked the hydroxylated and demethylated products as the most relevant Enn B metabolites, whereas demethylated Enn B1 metabolite (M1) showed very low incidence in the present study. Despite a similar pattern of metabolic pathways for Enn B1 and Enn B has been reported in in vitro assays, major metabolites found in human urine are different for each mycotoxin. In the here analyzed samples, major metabolites frequently co-occurred with minor metabolites from the same group; M5 was found alongside any other hydroxylated metabolite in 99% (*n* = 232) of the positive samples (*n* = 234). Referring to carbonylated products, the presence of M8 combined with at least one different metabolite was detected in 52% (*n* = 103) of the positive samples (*n* = 198). Finally, co-occurrence was detected in 34% (*n* = 28) of samples containing carboxylated metabolites (*n* = 79).

8.3 MATERIALS

8.3.1 Chemicals, Reagents and Materials

Methanol (MeOH), acetonitrile (AcN), and water for liquid chromatography (LC) mobile phase (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were acquired from Fluka (Milan, Italy). Sodium chloride and C18 were provided from Sigma–Aldrich (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE; 15 mm, diameter 0.2 µm) were purchased from Phenomenex (Castel Maggiore, Italy). Analytical standard of Enn B1 (>95% HPLC purity) was obtained from Sigma–Aldrich (Milan, Italy). A stock solution (1 mg/mL in MeOH) was prepared and working standard solutions were built by serial dilution of the stock and stored at −20°C.

8.3.2 Sampling

During January and February 2018, 300 Italian volunteers residing in Campania region (southern Italy) provided first-spot morning urine samples. The following exclusion criteria were considered for the study: (i) only one member per family allowed; (ii) people with severe issues in liver, kidney, or bile were not allowed due to potential interferences in the metabolic processes related to mycotoxins; (iii) people exposed to high amounts of mycotoxins in a different way from food, such as farmers and veterinarians, were not allowed. The use of medication was not an exclusion criterion due to the lack of information available about interferences with mycotoxins. Urine was stored in plastic containers at −20°C within 2 h after collection. All volunteers signed informed consent following the Helsinki Declaration on ethical principles for medical research when humans are involved. The present study was accepted by the University of Valencia Institutional human research Committee and the procedures and purposes were properly justified and approved. The numerosity of the sampling (*n* = 300) is in accordance with the International Federation of Clinical Chemists (IFCC) recommendations [32].

Volunteers were asked to specify their age and gender on their own container, in order to classify the sample. The sampling tried to keep the gender parity (male: 45.7%, female: 54.3%). According to the age of participants, three different groups were considered for statistical analysis: ≤30 years old (*n* = 94), from 31 to 59 years old (*n* = 72), and ≥60 years old (*n* = 134). Samples with undetected levels of the analytes of interest were chosen as “blank” and used in spiking and recovery studies. The consumption data were set according to age and gender, following the Guidelines for Healthy Italian Food Supply reported by the National Institute for Food Research and Nutrition [33].

8.3.3 Sample Preparation

The sample was processed following a previous procedure slightly modified [34]. Briefly, 1.5 mL of sample was placed into a 2 mL Eppendorf Safe-Lock Microcentrifuge tube and centrifuged for 3 min at 4000 rpm. After that, 1 mL of the supernatant was transferred to a 15 mL screw cap test tube with conical bottom and 1 mL of acetonitrile was added. The mixture was vortexed for 30 s and subsequently 30 mg of C18 sorbent and 0.3 g sodium chloride were incorporated to minimize interference from matrix. The solution was vortexed again for 30 s and centrifuged for 3 min at 4000 rpm and 4°C. Then, the upper layer was collected, evaporated under gentle nitrogen stream in a water bath at 45°C, reconstituted with 0.5 mL of MeOH/H_2O (70:30 *v*/*v*) and filtered through a 0.2 µm filter before to UHPLC-Q-Orbitrap HRMS analysis.

8.3.4 UHPLC-Q-Orbitrap HRMS Analysis

Quantitative and qualitative profiles of Enn B1 and Enn B1 phase I metabolites were acquired through Ultra High Pressure Liquid Chromatograph (UHPLC; Thermo Fisher Scientific, Waltham, MA, USA) equipped with an auto sampler device, a degassing system, a thermostated (T = 30°C) Luna Omega 1.6 µm (50 × 2.1 µm) column, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar.

The eluent consisted of two different phases, both H_2O (phase A) and MeOH (phase B) containing 0.1% formic acid and 5 mM ammonium formate. The gradient elution program for LC prior to Orbitrap HRMS analysis was developed as follows: 0–1 min–0% of phase B, 2 min–95% of phase B, 2.5 min–95% of phase B, 5 min–75% of phase B, 6 min–60% of phase B, 6.5 min–0% of phase B, and 1.5 min–0% phase B for equilibrating the column. The flow rate was set at 0.4 mL/min. A total of 5 µL of the sample was injected. Detection was performed using a Q-Exactive mass spectrometer. Data were acquired through full scan in positive mode at a resolving power of 70,000 FWHM at *m/z* 200. Ion source parameters in positive (ESI+) mode were: sheath gas (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, spray voltage 4 kV, capillary temperature 290 °C, auxiliary gas heater temperature 305°C, S-lens RF level 50. Data analysis and processing were carried-out using the Xcalibur software, v. 3.1.66.10 (Thermo Fisher Scientific, Waltham, MA, USA). A scan range of *m*/*z* 100–800 was set for the compounds of interest; the injection time was set to 200 ms and the automatic gain control (AGC) was selected at 1×10^6. Scan-rate was set at 2 scans/s.

8.3.5 Metabolomic Data Processing

Data processing and data pretreatment were performed to allow the putative identification of significant metabolites. Screening was done by investigating spectral data collected using a mycotoxin spectral library (version 1.1 for Library View Software, AB Sciex, Framingham, MA, USA) containing spectral data for 245 mycotoxins and other fungal/bacterial metabolites and 236 full MS/MS spectral library entries. The features, defined by their m/z and retention time, and their intensities in different samples were used to carry out the statistical analysis. Then, samples were grouped to perform the statistical analysis.

8.3.6 Method Validation

An in-house validation study was conducted according to the Commission Decision 2002/657/EC [19]. The parameters measured included linearity, matrix effect, trueness, precision, LOQ, and LOD. Linearity was evaluated using solvent and matrix-matched calibration curves, analyzing in triplicate six concentration levels ranging between 0.001–5 ng/mL. The matrix-matched calibration curves were prepared spiking aliquots of the corresponding matrices with Enn B1 at similar concentrations than the calibration curve made in solvent. Signal suppression or enhancement effect due to matrix co-elution interferences, was evaluated through a comparison between the slope of pure standard curve with the slope of matrix-matched standard curve following the next equation: SSE (%) = Slope matrix-matched calibration/Slope standard in solvent × 100. Trueness and precision were assessed using recovery studies since no suitable reference material was available. Recovery measurements were performed

by spiking blank urines with the standard working solution of Enn B1 at the levels of 0.1, 0.5, 1, and 5 ng/mL. Intra-day (RSD_r, %) and inter-day precision (RSD_R, %) were expressed as the relative standard deviation after repeating three measurements per concentration level on the same day and in three non-consecutive days, respectively. LOD was established as the minimum concentration at which the molecular ion can be identified (mass error < 5 ppm) and the LOQ as the lowest concentration of the analyte at which the concentration can be determined with accuracy and precision ≤20%.

8.3.7 Statistical Analysis

Statistical analysis of data was carried out using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA). For comparison of categorical data, the Pearson chi-square and Fisher exact tests were evaluated in order to assess whether Enn B1 occurrence in several subgroups (age, gender, cereal consumption) were significantly different. A non-parametric Kruskal–Wallis test was used to evaluate significant differences in Enn B1 metabolites concentrations. A confidence level of 95 % was assumed for examining data, whereas a *p*-value below 0.05 was considered as significant.

8.4 CONCLUSIONS

In this study, the occurrence of the *Fusarium* mycotoxin Enn B1 and its phase I metabolites was evaluated in 300 human urine samples throughout ultrahigh-performance liquid chromatography high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS). Results confirmed the presence of Enn B1 in 94.3% of samples ranging from 0.007 to 0.429 ng/mL (mean value: 0.065 ng/mL). Furthermore, the occurrence of Enn B1 metabolites previously found in in vitro and in vivo analysis was evaluated for the first time in human urine samples. In accordance with literature, demethylated and oxidated metabolite (5.3% samples, mean content = 0.035 ng/mL), hydroxylated metabolites (78.0% samples, mean content = 0.069 ng/mL), carbonylated metabolites (66.0% samples, mean content = 0.196 ng/mL), and carboxylated metabolites (26.3%, mean content = 0.066 ng/mL) were tentatively identified. Statistical analysis confirmed hydroxylated and carbonylated products as the most prevalent metabolites of Enn B1 in human urine. Differences observed between this work and in vitro Enn B1 biotransformation assays stand as additional evidence of potential pre-systemic metabolism. The characterization of metabolites derived from food contaminants is an important issue when performing safety and risk evaluations, and according to the here-obtained results a frequent exposure to Enn B1 is highlighted in Italian population.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.R.-C. and A.R.; methodology, L.I. and A.N.; software, A.G. and G.G.; validation, L.I. and Y.R.-C.; formal analysis, L.I., A.N. and Y.R.-C.; data curation, A.G. and G.G.; writing—original draft preparation, A.N.; writing-review and editing, Y.R.-C.; supervision, Y.R.-C and A.R. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 9
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Occurrence and Exposure Assessment of Mycotoxins in Ready-to-Eat Tree Nut Products through Ultra-High Performance Liquid Chromatography Coupled with High Resolution Q-Orbitrap Mass Spectrometry

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ABSTRACT

Tree nuts have become popular snacks due to their attributed benefits in the health state. Nevertheless, their susceptibility to fungal contamination lead to the occurrence of potentially dangerous mycotoxins. Hence, the aim of this work was to evaluate the presence of mycotoxins in ready-to-eat almonds, walnuts, and pistachios from Italian markets. The most relevant mycotoxin found in almonds was α-zearalanol in 18% of samples (*n* = 17) ranging from 3.70 to 4.54 µg/kg. Walnut samples showed frequent contamination with alternariol, present in 53% of samples (*n* = 22) at levels from 0.29 to 1.65 µg/kg. Pistachios (*n* = 15) were the most contaminated commodity, with β-zearalenol as the most prevalent toxin present in 59% of samples ranging from 0.96 to 8.60 µg/kg. In the worst-case scenario, the exposure to zearalenone-derived forms accounted for 15.6% of the tolerable daily intake, whereas it meant 12.4% and 21.2% of the threshold of toxicological concern for alternariol and alternariol monomethyl-ether, respectively. The results highlighted the extensive presence of *Alternaria* toxins and zearalenone-derived forms, scarcely studied in ready-to-eat tree nut products, highlighting the necessity to include these mycotoxins in analytical methods to perform more realistic risk assessments.

Keywords: Almonds; pistachios; walnuts; mycotoxins; Q-Exactive Orbitrap; risk characterization.

9.1 INTRODUCTION

Tree nuts have become a popular alternative to unhealthy snacks due to their attributed benefits. The intake of tree nuts has been related to a lower risk of suffering from cardiovascular diseases through several mechanisms, and they can also act as antioxidant suppliers [1,2,3,4]. According to the International Nut and Dried Fruit Council (INC), the annual production of tree nut products has increased over the last ten years, especially forn almonds, walnuts and pistachios, reaching a maximum of 4.6 million metric tons in 2019 and highlighting a global trend in tree nut consumption [5].

Nevertheless, tree nuts are susceptible to fungal growth that can occur for several reasons related to environmental factors, such as moisture and temperature. In addition, improper post-harvest practices and storage conditions can also promote fungal contamination [6]. As a consequence of these mentioned factors, mycotoxins could also be expected in crops. These are secondary metabolites produced by several filamentous fungi, mainly *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium*, and *Penicillium* spp., which can exert severe adverse effects including neurotoxicity, nephrotoxicity,

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immunosuppression, or carcinogenesis [7]. According to the carcinogenic potential, some mycotoxins have been included in the list of human carcinogens released by the International Agency for Research on Cancer (IARC).

In order to control the content of potentially dangerous mycotoxins in ready-to-eat tree nut products, the European Commission released the Regulation (EC) 1881/2006 [8] amended by Regulation (EU) 165/2010 [9] setting maximum limits (MLs) for certain mycotoxins. Almonds and pistachios cannot exceed 8 µg/kg for aflatoxin B1 and 10 µg/kg for the sum of aflatoxin B1, B2, G1, and G2, whereas MLs in walnuts were set at 2 and 4 µg/kg, respectively. However, different mycotoxins could be expected due to the susceptibility of tree nuts to fungal contamination. In this line, *Aspergillus* and *Fusarium* genera have been characterized as other major pathogens in tree nuts, so their secondary toxic metabolites could also be expected [6,10,11]. Alongside aflatoxins, relevant toxins from the *Fusarium* genus included in the IARC classification, such as T-2 or zearalenone, have been evaluated in tree nut products [12,13,14,15,16,17]. However, zearalenone-derived forms have been scarcely studied and, recently, the European Food Safety Authority (EFSA) highlighted the necessity to include these metabolites in risk assessment studies due to their estrogenic activity [18]. Furthermore, the presence of other mycotoxins-producing fungi such as *Alternaria*, able to produce the genotoxic compounds alternariol monomethyl-ether (AME) and alternariol (AOH), has been detected in tree nuts [11]. According to the EFSA CONTAM Panel, these mycotoxins have been scarcely studied, so the toxicological potential is still unknown, and seems to be major contributors to mycotoxin exposure in several commodities [19]. Therefore, there is a necessity to develop analytical methods able to detect and quantify these less studied mycotoxins, since they could also contribute towards overall exposure when consuming ready-to-eat nut products.

To overcome this, sensitive methods are required in order to detect low levels of mycotoxins occurring in tree nut products. In addition, environmental conditions, geographical area, or harvest practices can lead to a strong variety among products regarding mycotoxin occurrence, so constant monitoring based on sensitive and multi-analyte methods are required for having proper mycotoxin profiles. The most recent methods for detecting mycotoxins in tree nuts are based on liquid chromatography coupled with tandem mass spectrometry [12,13,14,15,16,17]. However, other alternatives are able to provide a more precise detection and quantification. The use of high-resolution mass spectrometry stands as the best alternative when performing simultaneous determinations of analytes. Identification based on full scan-all ion fragmentation analysis represents an improvement in mass accuracy when compared to traditional multiple reaction monitoring analysis based on triple quadrupole [20]. Furthermore, Q-Orbitrap offers a better performance than other high-resolution mass spectrometers for low *m*/*z* compounds, as most mycotoxins. Therefore, the aim of this work was to evaluate the presence of eighteen mycotoxins from different genera in ready-to-eat tree nut products (n = 54), including almonds, walnuts, and pistachios from Italian markets through ultra-high performance liquid chromatography coupled with high-resolution Q-Orbitrap mass spectrometry. To achieve this, a single QuEChERS-based extraction was validated in the three commodities. In addition, the risk characterization resulting from mycotoxin contamination in ready-to-eat tree nut products was performed in the Italian population for the first time.

9.2 RESULTS

9.2.1 Analytical Method Validation

The proposed methodology was validated for the simultaneous detection and quantification of 18 mycotoxins in almonds, walnuts, and pistachios. Results are shown in Table 9.1 Linearity, expressed through correlation coefficient (R^2), ranged from 0.9908 to 0.9998 for all compounds analyzed. The matrix effect was quantified through the percentage of signal suppression/enhancement (%SSE) ranging from 77 to 120%, from 74 to 148%, and from 82 to 149% in almond, walnut, and pistachio, respectively. There were only two analytes out of the range 80–120%: AME showed a %SEE of 148% and 149% in pistachio and almond, respectively, whereas %SEE for ENNB were 77% and 74% in almond and pistachio. In order to avoid miscalculation, matrix-matched calibration curves were used for quantification purposes. Despite the complexity of those matrices consisting mainly of proteins and fatty acids, a simple acetonitrile-based extraction including a clean-up step with C18 was enough to

almost completely remove the matrix interference. The method also displayed a high sensitivity, with limits of quantification (LOQs) ranging from 0.20 to 0.78 µg/kg for all the analytes in each matrix. The recovery studies showed satisfactory results. For almonds, values ranged from 81 to 106% at the highest fortification level (20 µg/kg), from 73 to 95% at a medium level (5 µg/kg), and from 71 to 95% at the lowest level (1 µg/kg). Similarly, walnuts showed values from 79 to 105%, 70 to 99%, and 71 to 100% for the highest, medium, and lowest levels, respectively. Finally, recovery values for mycotoxins in pistachios ranged from 80 to 113%, from 70 to 105%, and 72 to 107% for the highest, medium, and lowest fortification levels. The precision evaluated through RSD_r and RSD_R was below 20% for all the analytes at three spiking levels.

Table 9.1. Method performance in almonds, walnuts, pistachios

		Recovery (%) (RSD_R (%))			
Analyte	**SSE (%)**	**20 ng/g**	**5 ng/g**	**1 ng/g**	**LOQ (ng/g)**
almonds					
NEO	106	83 (12)	77 (16)	88 (7)	0.78
AFG2	101	88 (16)	82 (19)	78 (14)	0.20
AFG1	111	83 (20)	78 (20)	82 (13)	0.39
AFB2	106	93 (15)	94 (16)	83 (12)	0.20
AFB1	117	100 (10)	95 (15)	95 (5)	0.39
HT-2	115	105 (8)	89 (17)	79 (8)	0.78
A-ZAL	102	88 (11)	92 (12)	83 (10)	0.39
A-ZOL	109	92 (7)	88 (9)	87 (6)	0.78
AOH	105	81 (14)	73 (14)	71 (14)	0.20
T-2	114	104 (16)	87 (11)	89 (13)	0.78
B-ZAL	118	96 (10)	95 (13)	89 (10)	0.78
B-ZOL	98	95 (9)	90 (15)	76 (9)	0.20
ZON	120	94 (18)	81 (17)	86 (18)	0.20
AME	149	94 (15)	85 (16)	81 (15)	0.78
ENN B	77	94 (6)	90 (9)	98 (10)	0.78
ENN B1	106	106 (14)	84 (13)	74 (10)	0.78
ENN A1	111	87 (3)	86 (5)	95 (18)	0.39
ENN A	102	86 (12)	83 (13)	89 (12)	0.78
walnuts					
NEO	91	85 (9)	81 (14)	92 (10)	0.78
AFG2	93	85 (5)	78 (9)	82 (10)	0.20
AFG1	91	79 (5)	70 (8)	76 (11)	0.78
AFB2	103	91 (4)	84 (8)	79 (12)	0.39
AFB1	97	102 (6)	83 (12)	78 (10)	0.39
HT-2	107	97 (9)	88 (11)	76 (9)	0.78
A-ZAL	103	99 (10)	81 (12)	100 (9)	0.78
A-ZOL	110	88 (7)	78 (15)	75 (9)	0.78
AOH	105	83 (4)	71 (6)	74 (8)	0.20
T-2	96	86 (10)	84 (13)	79 (8)	0.78
B-ZAL	105	95 (5)	91 (11)	99 (12)	0.78
B-ZOL	97	94 (7)	85 (7)	95 (12)	0.20
ZON	100	81 (7)	78 (7)	73 (5)	0.20
AME	114	80 (7)	74 (9)	81 (15)	0.78
ENN B	74	101 (5)	90 (9)	84 (16)	0.78
ENN B1	99	88 (8)	89 (10)	76 (8)	0.78
ENN A1	96	105 (8)	99 (9)	76 (13)	0.78
ENN A	107	99 (8)	87 (15)	86 (8)	0.78
pistachios					
NEO	103	86 (10)	75 (14)	72 (8)	0.78
AFG2	82	106 (10)	82 (14)	82 (9)	0.39
AFG1	91	80 (11)	70 (11)	73 (6)	0.78
AFB2	87	113 (10)	103 (11)	97 (7)	0.39

		Recovery (%) (RSD_R (%))			
Analyte	**SSE (%)**	**20 ng/g**	**5 ng/g**	**1 ng/g**	**LOQ (ng/g)**
AFB1	94	87 (18)	82 (18)	79 (13)	0.39
HT-2	105	91 (14)	85 (17)	88 (14)	0.78
A-ZAL	86	99 (13)	99 (16)	88 (17)	0.78
A-ZOL	97	83 (15)	85 (15)	89 (7)	0.78
AOH	83	84 (16)	77 (17)	83 (13)	0.39
T-2	85	92 (11)	98 (14)	85 (18)	0.78
B-ZAL	96	87 (10)	89 (15)	81 (9)	0.78
B-ZOL	112	92 (9)	92 (13)	89 (12)	0.78
ZON	118	104 (9)	105 (9)	107 (13)	0.20
AME	148	87 (10)	77 (16)	75 (14)	0.78
ENN B	115	99 (14)	92 (16)	83 (15)	0.78
ENN B1	116	102 (6)	94 (8)	96 (16)	0.78
ENN A1	118	105 (13)	99 (16)	96 (13)	0.78
ENN A	114	100 (14)	92 (17)	96 (15)	0.78

Table 9.2. Quantitative methods for multi-mycotoxin detection in several tree nut products

Analytes (*n*)	**Method**	**Sample Treatment**	**Sensitivity (µg/kg)**	**References**
Aflatoxins (B1, B2, G1, G2), CIT, DON, FB1, FB2, FUS-X, HT-2, OTA, T-2, STE, ZEN (14)	UHPLC-MS/MS	QuEChERS-DLLME	0.61–150	Arroyo-Manzanares et al., 2013 [12]
Aflatoxins (B1, B2, G1, G2), BEA, DAS, enniatins (A, A1, B, B1), FB1, FB2, FB3, HT-2, OTA, T-2 (16)	LC-MS/MS	QuEChERS-SPE cartridge	0.2–45	Azaiez et al., 2014 [13]
Aflatoxins (B1, B2, G1, G2), DAS, 3AC-DON, 15AC-DON, DON, FB1, FB2, FUS-X, HT-2, NEO, OTA, T-2, ZEN (16)	LC-MS/MS	QuEChERS-Z-Sep$^+$ + C18	1.25–5	Cunha et al., 2018 [14]
Aflatoxins (B1, B2, G1, G2), AME, AOH, BEA, enniatins (A, A1, B, B1), OTA, OTB, T-2, TEN, ZEN (16)	UPLC-MS/MS	QuEChERS-C18	0.1–5	Wang et al., 2018 [15]
3-ADON, aflatoxins (B1, B2, G1, G2, M1), DAS, ERGC1, ERGC2, FB1, FB2, GLI, HT-2, OTA, T-2, α-ZEL, ZEN (17)	Nano flow LC-HRMS	QuEChERS EMR-Lipid	0.05–5	Alcantara et al., 2019 [16]
Aflatoxins (B1, B2, G1, G2), α-ZEL, ZEN (6)	UHPLC-MS/MS	QuEChERS-C18	0.5–1	Hidalgo et al., 2019 [17]
Aflatoxins (B1, B2, G1, G2), AME, AOH, enniatins (A, A1, B, B2), HT-2, NEO, T-2, α-ZAL, α-ZEL, β-ZAL, β-ZEL, ZEN (18)	UHPLC-HRMS	QuEChERS-C18	0.2–0.78	Present work

AME: Alternariol monomethyl-ether; AOH: Alternariol; BEA: Beauvericin; CIT: Citrinin; DAS: Diacetoxyscirpenol; DLLME: Dispersive liquid–liquid microextraction; 3AC-DON: 3-acetyl-deoxynivalenol; 15AC-DON: 15-acetyl-deoxynivalenol; DON: Deoxynivalenol; EMR: Enhanced matrix removal; ERGC1: Ergocornine 1; ERGC2: Ergocornine 2; FB1: Fumonisin B1; FB2: Fumonisin B2; FB3: Fumonisin B3; FUS-X: Fusarenon-X; GLI: Gliotoxin; HRMS: High-resolution mass spectrometry; LC: Liquid chromatography; MS/MS: Tandem mass spectrometry; NEO: Neosolaniol; OTA: Ochratoxin A; OTB: Ochratoxin B; STE: Sterigmatocystin; TEN: Tentoxin; UHPLC: Ultra-high performance liquid chromatography; UPLC: Ultra performance liquid chromatography; α-ZAL: Alpha-zearalanol; α-ZEL: Alpha-zearalenol; β-ZAL: Beta-zearalanol; β-ZEL: Beta-zearalenol; ZEN: Zearalenone.

Several multi-mycotoxin methods for tree nut products based on QuEChERS methodology have been recently published, as shown in Table 9.2. Although methodologies are focused on detecting aflatoxins since they are the only regulated mycotoxins in tree nuts, less attention has been put into *Fusarium* and *Alternaria* mycotoxins, which are a common genera causing fungal contamination in almonds, pistachios, and walnuts, as stated by Marín and Ramos [10] and Escrivá, Oueslati, Font, and Manyes [11]. In addition, ZEN-derived forms have also been here validated for their simultaneous detection, as recommended by the EFSA [18]. The main feature of the present methodology lies in its

high sensitivity when compared to previous ones, with LOQs ≤ 0.78 µg/kg. Sensitivity also plays a key role when performing exposure assessment studies, allowing a more realistic analysis and avoiding underestimation of mycotoxins as highlighted by the EFSA.

9.2.2 Analysis of Real Samples

Up to nine different mycotoxins were detected and quantified in the here-analyzed samples. Results are shown in Table 9.3. At least one mycotoxin occurred in 33 out of 54 nut products. The most commonly found mycotoxins belong to *Fusarium* species, whereas *Alternaria* metabolites were also found in all the studied commodities.

Table 9.3. Occurrence of mycotoxins in the analyzed tree nuts

			Range (µg/kg)	
Analyte	**Incidence (*n*, (%))**	**Mean (µg/kg)**	**Min**	**Max**
Almonds (n = 17)				
AFB1	1 (6)	0.45	-	-
α-ZAL	3 (18)	3.99	3.70	4.54
α-ZEL	1 (6)	1.40	-	-
β-ZEL	2 (12)	0.54	0.46	0.62
AOH	2 (12)	0.35	0.34	0.37
Walnuts (n = 22)				
α-ZAL	2 (12)	2.18	2.13	2.24
β-ZAL	3 (18)	3.13	1.67	5.24
β-ZEL	4 (24)	0.39	0.3	0.55
ZEN	3 (18)	0.44	<LOQ	0.93
AOH	9 (53)	0.67	0.29	1.65
AME	3 (18)	1.63	1.13	1.95
ENN B1	1 (6)	1.30	-	-
Pistachios (n = 15)				
α-ZAL	2 (12)	25.75	2.16	49.35
β-ZAL	1 (6)	11.86	-	-
α-ZEL	2 (12)	1.50	1.26	1.74
β-ZEL	10 (59)	3.42	0.96	8.60
AOH	1 (6)	7.75	-	-

Referring to almonds (*n* = 17), five mycotoxins were identified in 41% of samples. The most relevant compound was α-ZAL, a *Fusarium* toxin that results from the metabolism of its parental mycotoxin, ZEN. This toxin was present in 18% of the samples, ranging from 3.70 to 4.54 µg/kg. β-ZEL, another product from the metabolization of ZEN, was found in 12% of samples at low levels going from 0.46 up to 0.62 µg/kg. ZEN has also been studied in almonds, and contamination at 1.2 and 3.48 µg/kg was reported by Škrbić, et al. [21] in the two analyzed samples. According to the present results, ZEN metabolites resulted as the major contaminants in almonds and scarce literature regarding them is available. Since ZEN appears to be a common toxin in tree nuts, its metabolites could also be expected, as observed in this study. Despite not being a major fungus in almonds, *Alternaria* toxins have been usually reported. AOH was also quantified in 12% of samples ranging from 0.34 to 0.37 µg/kg. In a previous work conducted by Varga et al. [22], AOH was found in one sample (*n* = 8) at 1.5 µg/kg, more than three times the contamination here reported. Wang, Nie, Yan, Li, Cheng, and Chang [15] reported AOH contamination in four samples (*n* = 25) with a maximum level of 54.24 µg/kg. On the contrary, aflatoxins are one the most studied toxins in every nut typology. AFB1 was found in one sample at 0.45 µg/kg, complying with the regulation regarding maximum limits. Liao, et al. [23] reported contamination with AFB1 at 0.3 µg/kg in one almond sample (*n* = 9), similar to the concentration found in this study. Due to the toxicological potential of aflatoxins, special treatments are used in order to eliminate fungal contamination or to inactivate the toxins, such as roasting, sorting, and physical segregation [10], so it is not usual finding aflatoxins at concentrations above the maximum limits (8 for AFB1 and 10 µg/kg for the sum of aflatoxins).

Walnuts (*n* = 22) showed contamination with seven mycotoxins, finding at least one in 59% of samples. ZEN was quantified in 18% of samples ranging from <LOQ to 0.93 µg/kg. Several metabolites were also detected: β-ZEL was the most common one, present in 24% of samples varying from 0.3 to 0.55 µg/kg; β-ZAL was found in 18% of samples and, quantitatively, meant the most relevant one at levels going from 1.67 to 5.24 µg/kg; lastly, α-ZAL was present in 12% of samples at 2.13 and 2.24 µg/kg. ENNB1, an emerging *Fusarium* mycotoxin, was quantified at 1.3 µg/kg in one sample (6%). As previously observed in almonds, walnut samples also contained ZEN and ZEN metabolites. In the same study conducted by Wang, Nie, Yan, Li, Cheng, and Chang [15], a high contamination with ZEN was observed in one sample (*n* = 35) at 49.35 µg/kg, whereas Arroyo–Manzanares, Huertas–Pérez, Gámiz–Gracia, and García–Campaña [12] reported the presence of ZEN in one sample (*n* = 6) at 221.8 µg/kg. These levels of contamination strongly vary from those here obtained, with concentrations below 1 µg/kg. Furthermore, ZEN metabolites were quantitatively more relevant than ZEN, remarking the necessity of taken into consideration these mycotoxins when performing contaminant analysis in walnuts.

Alternaria toxins are not common mycotoxins included in tree nut studies. Nevertheless, AOH was extensively found at low concentrations ranging from 0.29 to 1.65 µg/kg in 53% of samples. AME was also present in 18% of samples at slightly higher levels, going from 1.13 up to 1.95 µg/kg. Therefore, sensitive analytical methods are required in order to understand the incidence of these toxins. In this line, Wang, Nie, Yan, Li, Cheng, and Chang [15] quantified AOH and AME in 23% and 31% of the walnut samples analyzed (*n* = 35), respectively, with a LOQ of 2 µg/kg. AOH was quantified in a range of 5.78–142.9 µg/kg and AME ranged from 1.53 to 110.5 µg/kg. Similar to the here-presented data, *Alternaria* toxins resulted as the most common toxins occurring in walnuts.

Lastly, five mycotoxins were quantified in pistachios samples (*n* = 15), with at least one occurring in 80% of them. Among the matrices analyzed, pistachios resulted as the most contaminated. β-ZEL was the most prevalent mycotoxin, being detected in 59% of samples ranging from 0.96 to 8.6 µg/kg. α-ZEL and α-ZAL were both quantified in 12% of samples, ranging from 1.26 to 1.74 µg/kg and from 2.16 to 49.35 µg/kg, respectively. β-ZAL was only found in one sample at 11.86 µg/kg and, similarly, AOH was found at 7.75 µg/kg.

Quantitatively, pistachios showed a significantly heavier contamination ($p < 0.05$) when compared to almond and walnuts. In addition, the highest levels of AOH and ZEN metabolites were detected in pistachios. Although aflatoxins or ZEN have been analyzed in this matrix [22,24,25], there is scarce literature regarding the here-found toxins. Furthermore, the available validated procedures for ZEN metabolites cannot reach a high sensitivity as the here obtained (LOQ = 0.78 µg/kg), with Spanjer et al. [26] establishing the LOQs at 40 µg/kg and Hidalgo–Ruiz, Romero–González, Martínez Vidal, and Garrido–Frenich [17] at 1 µg/kg for α-ZEL. Sensitivity becomes a crucial feature in analytical procedures, even more when the contamination reported only reach a few µg/kg. In this line, Alcántara–Durán, Moreno–González, García–Reyes, and Molina–Díaz [16] did not find α-ZEL occurring in pistachios despite having a low LOQ (0.05 µg/kg). *Alternaria* toxins have not been deeply studied in pistachio neither. Varga, Glauner, Berthiller, Krska, Schuhmacher, and Sulyok [22] developed a procedure for AOH with the LOQ at 9.6 µg/kg, which would not have been sensitive enough for detecting the contamination here reported (7.75 µg/kg).

Co-occurrence of mycotoxins was observed in the three different commodities, as shown in Table 9.4. Walnuts presented a wide variety of combinations, including AOH in most of them. In total, co-occurrence happened in a 45% of walnuts samples. On the contrary, pistachios and almonds showed a lower incidence of co-occurrence, accounting for 27% and 1% of the total samples, respectively.

There is a strong variability referring to the content of mycotoxins depending on several parameters including temperature, moisture, or pre- and post-harvest practices, among others. This variability observed highlights the necessity of constantly monitoring these kinds of products using highly sensitive analytical procedures in order to ensure a safe consumption. Furthermore, an investigation carried out by the EFSA [18] considered the application of potency factors for ZEN metabolites ranging from 0.2 to 60 times the toxicity associated with ZEN. Considering the high uncertainty related

to these metabolites, the analytical procedures should include these mycotoxins that could account for future exposure assessment studies.

Table 9.4. Co-occurrence of mycotoxins in the analyzed tree nuts

Combinations of Mycotoxins	Incidence (n, (%))
Almonds (n = 17)	
α-ZAL + α-ZEL	1 (6)
Walnuts (n = 22)	
AOH + α-ZAL	1 (6)
AOH + β-ZEL	1 (6)
AOH + ZEN	2 (12)
α-ZAL + AME	1 (6)
α-ZAL + β-ZAL	1 (6)
α-ZEL + β-ZEL	1 (6)
AOH + α-ZAL + ZEN	1 (6)
AOH + β-ZEL + AME	1 (6)
AOH + β-ZEL + β-ZAL + AME	1 (6)
Pistachios (n = 15)	
α-ZAL+ β-ZAL	1 (6)
β-ZAL + β-ZEL	1 (6)
α-ZEL + β-ZEL	1 (6)
AOH + β-ZEL	1 (6)

9.2.3 Exposure Assessment

An exposure assessment and risk characterization were performed taking into consideration the left-censored data for the more prevalent mycotoxins detected in the samples, including ZEN-derived forms, AOH and AME. Results are showed in Table 9.5.

The tree nut consumption did not vary much throughout the age groups, so children with the lowest body weight showed the heaviest exposure. The percentages of relevant TDI or TTC calculated for children were two-fold higher than those for teenagers and adults, but below the maximum tolerable values established by the Scientific Committee on Food of the European Commission at 0.25 µg/kg bw/day for the sum of ZEN and its metabolites and 0.0025 µg/kg bw/day for both AOH and AME.

Under the worst-case scenario, the percentages of relevant TDI or TTC calculated for children meant between a tenth and a fifth of the established safety levels. Nevertheless, it might be a concern since ZEN, its derived forms, AOH and AME, can be found in different commodities such as cereals and other vegetal products, which are more commonly consumed by children.

The results evidence a negligible exposure of these mycotoxins due to ready-to-eat nut products, but these scarcely studied mycotoxins might be of importance when performing risk assessment through total diet studies.

9.3 MATERIALS AND METHODS

9.3.1 Chemicals and Reagents

Methanol (MeOH), acetonitrile water, and formic acid (FA) for LC mobile phase (HPLC grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl), ammonium formate (NH_4HCO_2), magnesium sulfate ($MgSO_4$), and octadecyl carbon chain-bonded silica (C18) were acquired from Sigma Aldrich (Milan, Italy). Eighteen mycotoxin standards (purity >98%) including aflatoxins (AFB1, AFB2, AFG1, and AFG2), zearalenone (ZEN), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), β-zearalanol (β-ZAL), neosolaniol (NEO), T-2 toxin, HT-2 toxin, enniatins (A, A1, B, and B1), alternariol monomethyl ether (AME), and alternariol (AOH) were obtained from Sigma-Aldrich (Milan, Italy).

Table 9.5. Risk characterization of detected mycotoxins in different population groups based on the percentage of tolerable daily intake considering four different scenarios per group: Mean and 95th percentile consumption value combined with lower and upper bound of contamination

		Risk Characterization (%TDI or %TTC)																	
				Child				Teenager				Adult				Elderly			
		C (µg/kg)		Mean		P95th		Mean		P95th		Mean		P95th		Mean		P95th	
Mycotoxins	**TDI or TTC (µg/kg bw/day)**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**
∑ ZEN_d	0.25	7.25	59.70	0.7	5.9	1.9	15.6	0.4	3.2	1.2	10.1	0.4	3.3	1.0	8.5	0.5	3.8	1.3	10.5
AOH	0.0025	0.10	0.48	0.8	4.8	2.8	12.4	0.4	2.4	1.6	8.0	0.4	2.8	1.6	6.8	0.8	3.2	1.6	8.4
AME	0.0025	0.25	0.82	2.4	8.0	6.4	21.2	1.2	4.4	4.4	14.0	1.2	4.4	3.6	11.6	1.6	5.2	4.4	14.4

∑ ZEN_d: Sum of ZEN and its derived forms α-ZAL, α-ZEL, β-ZAL, β-ZEL; TDI: Tolerable daily intake; TTC: Threshold of toxicological concern; C: Contamination; LB: Lower bound; UP: Upper bound; P95th: 95th percentile.

Stock solutions of each mycotoxin were built by dissolving 1 mg of solid reference standard in 1 mL of methanol. An intermediate mixed solution containing all the mycotoxins at a concentration of 30 µg/mL was obtained after mixing individual stock solutions and diluting in MeOH:H_2O (70:30 *v*/*v*) 0.1% formic acid. Working standard solutions at 1.6, 0.4, 0.08 µg/mL were used for spiking experiments (fortification levels at 20, 5, and 1 µg/kg). All solutions were stored in safe conditions at −20 °C in screw-capped glass vials.

9.3.2 Sampling

Fifty-four commercially available nut products were randomly purchased from supermarkets located in the Campania Region, Southern Italy. Products were classified as walnuts (*n* = 22), pistachios (*n* = 15), and almonds (*n* = 17) and sent to the laboratory in their original packages. Samples were kept in dark and cool conditions as recommended by the manufacturer, and analyses were carried out within five days after receiving them.

9.3.3 Sample Preparation

A procedure previously developed by Cunha, Sá, and Fernandes [14] was used as the starting point, with some modifications. Briefly, 10 g of homogenized sample was introduced into a 50 mL Falcon tube (Conical Polypropylene Centrifuge Tube; Thermo Fisher Scientific, Milan, Italy) and 5 mL of distilled water and 5 mL of acetonitrile containing 1% formic acid (*v*/*v*) were added. The sample was vortexed (ZX3; VEPL Scientific, Usmate, Italy) for 2 min. Then, 0.5 g of sodium chloride and 2.0 g of anhydrous sulfate sodium were added. The tube was manually shaken for 1 min and then centrifuged (X3R Heraeus Multifuge; Thermo Fisher Scientific, Kalkberg, Germany) for 10 min at 4907× *g* at room temperature. The supernatant (1.5 mL) was transferred to a 15 mL Falcon tube containing 50 mg of C18 sorbent, then vortexed for 1 min and centrifuged for 3 min at 4907× *g* at room temperature. Lastly, 0.4 mL were collected, filtered through a 0.22 µm filter and injected into the UHPLC-Q-Orbitrap HRMS instrument.

9.3.4 UHPLC-Q-Orbitrap HRMS Analysis

Detection and quantification analysis were performed using a Dionex UltiMate® 3000 system consisting of a quaternary UHPLC pump working at 1250 bar (125 MPa), a degassing system, an autosampler device, and a thermostatically controlled column coupled with a Q-Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). Chromatographic separation of analytes was carried out with a thermostated Luna Omega Polar C18 column (50 × 2.1 mm, 1.6 µm; Phenomenex, Torrance, CA, USA) kept at 30 °C. Water (A) and methanol (B), both containing 0.1% formic acid and 5 mM ammonium acetate were used as mobiles phases. The gradient profile started with 0% B for 1 min, increased to 95% B over 1 min and kept for 0.5 min. Next, the gradient linearly decreased to 75% B over 2.5 min and decreased again until 60% in 1 min. Lastly, the gradient went back to 0% in 0.5 min and held for 1.5 min for column re-equilibration. Total run time was 8 min with an injection volume of 5 µL and a flow rate of 0.4 mL/min.

The mass spectrometer operated in positive and negative ion modes by setting 2 scan events: Full ion MS and all ion fragmentation (AIF). The following settings were used in full MS mode: Resolution power of 35,000 FWHM (defined for *m*/*z* 200), automatic gain control (AGC) target 1×10^6, scan range 100–1000 *m*/*z*, injection time set to 200 ms, and scan rate set at 2 scans/s. The ion source parameters were: Capillary temperature 290°C, S-lens RF level 50, spray voltage 4 kV (-kV en ESI- mode), sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, and auxiliary gas heater temperature 305 °C. The AIF mode used the following settings in both modes: Scan time = 0.10 s, maximum injection time = 200 ms; mass resolving power = 17,500 FWHM, ACG target = 1×10^5, scan range = 100–1000 *m*/*z*, retention time window 30s, and isolation window 5.0 *m*/*z*. The collision energy and Orbitrap-MS parameters corresponding for each analyte were individually optimized in a previous work of our group [27]. The mass spectrometer was regularly calibrated using calibration solutions provided by ThermoFisher during three-day intervals and before each sequence. Retention time, elemental composition, theoretical and measured mass, accurate mass error, collision energy,

and product ions for the analyzed compounds are shown in Table 9.6. A mass tolerance of 5 ppm was set for identification and confirmation of the molecular ion and both products. For accurate mass measurement, identification, and confirmation were performed at a mass tolerance of 5 ppm for the molecular ion and for both fragments. Xcalibur software, v.3.1.66.10. was used in order to analyze and process the data.

9.3.5 Validation Parameters

An in-house validation study was carried out for the three different matrices here analyzed following the EU Commission Decision 2002/657/EC guidelines referring to linearity, selectivity, trueness, intra-day precision (repeatability), inter-day precision (reproducibility), and sensitivity expressed as LOQs [28]. Linearity was determined by injecting a series of neat solvent and matrix-matched calibration curves at eight concentrations levels ranging from 0.2 to 200 ng/mL with a deviation of ≤20% for each calibration level. The coefficient of determination was calculated using the means of the least square approach. For evaluating a potential interference of the matrix, the slopes corresponding to each linear function were compared. The %SSE occurred when a deviation ≥20% was observed after comparing both slopes. The selectivity of the method was assessed by injecting ten blank samples, observing no peaks that could interfere in the same retention time area as the analytes, considering a mass error of 5 ppm. Trueness was evaluated through recovery studies, spiking three blank samples at three different fortification levels: 1, 5, and 20 µg/kg. The measurements were made during three non-consecutive days. Values ranging from 70 to 120% of recovery were considered as optimal. Precision was assessed in terms of repeatability (relative standard deviation after three determinations in a single day, RSD_r) and reproducibility (relative standard deviation after determinations in triplicate on three non-consecutive days, RSD_R). Sensitivity was determined through the LOQ for each analyte, which was established as the minimum concentration with a linear response that can be observed with a deviation ≤20% considering a mass error of 5 ppm.

9.3.6 Exposure Assessment

A deterministic approach was followed for performing the exposure assessment. The latest data consumption published by the Italian National Food Consumption Survey INRAN-SCAI 2005-06 were considered [29]. The commodities here analyzed were all included in the “nuts” category according to the survey, so exposure assessment was performed considering both the mean and the 95th percentile values. Population was divided in four age groups: Children (3–9.9 years) average consumption 6.4 g/day, P95 17.0 g/day; teenagers (10–17.9 years) average consumption 7 g/day, P95 22.3 g/day; adults (18–65 years) average consumption 9.5 g/day, P95 24.7 g/day; and elderly (65 < years) average consumption 11.1 g/day, P95 30.7 g/day. The mean body weights attached to each group were 26.1, 52.6, 69.7, and 70.1 kg, respectively, as detailed in the INRAN-SCAI 2005-06 survey. The probable daily intake (PDI) values were calculated using the next equation:

$$PDI = C \times I/bw, \quad (1)$$

where *C* represents the contamination (for the lower or upper bound) of each mycotoxin (µg/kg); *I* accounts for the mean or 95th percentile consumption established for each age group (g/day); and *bw* means the body weight assigned to its corresponding age group (kg). Once the PDIs were calculated, the tolerable daily intakes (TDIs) established for the detected mycotoxins were considered for performing the risk characterization. In case of finding a mycotoxin without any TDI assigned by the EFSA yet, a threshold of toxicological concern (TTC) was used. The risk characterization was calculated following the next equation:

$$\%TDI = PDI/TDI \times 100, \quad (2)$$

Because of the high proportion of left-censored data, two scenarios of exposure were defined considering negative samples as zero or LOQ for the lower-bound and upper-bound, respectively.

Table 9.6. UHPLC-HRMS parameters corresponding to the here-analyzed mycotoxins

Analyte	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (*m/z*)	Measured Mass (*m/z*)	Accuracy (Δ ppm)	Collision Energy (eV)	Product Ions (*m/z*)
NEO	4.25	$C_{19}H_{26}O_8$	$(M+NH_4)^+$	400.19659	400.19632	−0.67	10	305.13803 141.00530
AFG2	4.52	$C_{17}H_{14}O_7$	$(M+H)^+$	331.08123	331.08078	−1.36	37	313.07010 245.08032
AFG1	4.55	$C_{17}H_{12}O_7$	$(M+H)^+$	329.06558	329.06549	−0.27	40	243.06467 200.04640
AFB2	4.60	$C_{17}H_{14}O_6$	$(M+H)^+$	315.08631	315.08615	−0.51	36	287.09064 259.05945
AFB1	4.64	$C_{17}H_{12}O_6$	$(M+H)^+$	313.07066	313.07053	−0.42	36	285.07489 269.04373
HT−2	4.74	$C_{22}H_{32}O_8$	$(M+NH_4)^+$	442.24354	442.24323	−0.70	27	263.12744 215.10641
α-ZAL	4.81	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17065	0.65	29	259.09497 91.00272
α-ZEL	4.83	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	−0.31	36	174.95604 129.01947
T-2	4.84	$C_{24}H_{34}O_9$	$(M+NH_4)^+$	484.25411	484.25430	0.39	23	215.10603 185.09561
AOH	4.85	$C_{14}H_{10}O_5$	$(M-H)^-$	257.04555	257.04581	1.01	−32	215.03490 213.05569
β-ZAL	4.94	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17059	0.47	40	259.09497 91.00272
β-ZEL	4.97	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	−0.31	36	174.95604 160.97665
ZEN	5.01	$C_{18}H_{22}O_5$	$(M+H)^+$	317.13945	317.13928	−0.54	−32	175.03989 131.05008
AME	5.13	$C_{15}H_{12}O_5$	$(M-H)^-$	271.06120	271.06140	0.74	−36	256.03751 228.04276
ENN B	5.56	$C_{33}H_{57}N_3O_9$	$(M+NH_4)^+$	657.44331	657.44348	0.26	50	214.14320 196.13280

Analyte	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (*m/z*)	Measured Mass (*m/z*)	Accuracy (Δ ppm)	Collision Energy (eV)	Product Ions (*m/z*)
ENN B1	5.68	$C_{34}H_{59}N_3O_9$	$(M+NH_4)^+$	671.45986	671.45935	−0.76	48	214.14343 196.13295
ENN A1	5.82	$C_{35}H_{61}N_3O_9$	$(M+NH_4)^+$	685.47461	685.47449	−0.18	48	228.15900 210.14847
ENN A	5.99	$C_{36}H_{63}N_3O_9$	$(M+NH_4)^+$	699.49026	699.48987	−0.56	43	228.15900 210.14847

9.7 Statistical Analysis

Validation experiments were conducted in triplicate and expressed as mean values alongside the corresponding relative standard deviation (RSD, %). The normality was evaluated through Saphiro–Wilk test and multivariant analysis was carried out using the Kruskal–Wallis test in order to compare the contamination levels among different matrices. A p-value < 0.05 was considered as significant. The statistical software package IBM SPSS version 25 was used for data analysis.

9.4 CONCLUSIONS

An analytical method based on QuEChERS extraction and ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry was validated for the simultaneous detection of eighteen mycotoxins in ready-to-eat almonds, walnuts, and pistachios. The features fulfilled the requirements set by the European Union regarding selectivity, linearity, trueness, precision, with high sensitivity based on limits of quantification. The procedure was then applied to almonds (n = 17), walnuts (n = 22), and pistachios (n = 15) acquired from Italian markets. The most relevant mycotoxin in almonds was α-zearalanol, found in 18% of samples (n = 17) ranging from 3.70 to 4.54 µg/kg. Walnut samples showed frequent contamination with alternariol, present in 53% of samples (n = 22) at levels from 0.29 up to 1.65 µg/kg. Pistachios were the most contaminated commodity, with β-zearalenol as the most prevalent toxin present in 59% of samples ranging from 0.96 to 8.60 µg/kg. In the worst-case scenario, the exposure to zearalenone-derived forms accounted for 15.6% of the tolerable daily intake, whereas it meant 12.4% and 21.2% of the threshold of toxicological concern for alternariol and alternariol monomethyl-ether, respectively. The results highlighted the extensive presence of zearalenone-derived forms and *Alternaria* toxins in ready-to-eat nut products. The relevance showed in this study suggests the inclusion of these mycotoxins in analytical methods to perform more realistic risk assessments, even more when only little toxicological data are available for setting a proper legislation.

AUTHOR CONTRIBUTIONS

Conceptualization, A.R. and Y.R.-C.; methodology, G.G. and Y.R.-C.; validation, L.C. and A.N.; formal analysis, L.I. and A.N.; investigation, L.C. and L.I.; data curation, A.N.; writing—original draft preparation, A.N.; writing—review and editing, Y.R.-C.; supervision, A.R. and Y.R.-C. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 10
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Target Quantification and Semi-Target Screening of Undesirable Substances in Pear Juices Using Ultra-High-Performance Liquid Chromatography-Quadrupole Orbitrap Mass Spectrometry

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ABSTRACT

Fruit juices are common products in modern diets due to the supply of vegetal nutrients combined with its tastiness. Nevertheless, potential contaminants, such as mycotoxins and pesticides, can be present in commercial products due to a potential carry-over. Therefore, the aim of this study was to investigate for the first time the presence of 14 *Fusarium* mycotoxins using a quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based extraction followed by an ultra-high-performance liquid chromatography-quadrupole Orbitrap high-resolution mass spectrometry in 21 pear juice samples from Italian markets. Up to nine different mycotoxins were detected, particularly an extensive presence of zearalenone (67%, *n* = 21, mean value = 0.88 ng/mL). Emerging *Fusarium* mycotoxins enniatins B, B1, A, and A1 were also detected. Additionally, 77 pesticide residues were tentatively identified through a retrospective analysis based on a mass spectral library. The prevalent presence of some non-approved pesticides, such as ethoxyquin (64%, *n* = 21) and triazophos (55%, *n* = 21), must be highlighted. The results obtained indicate an extensive contamination of marketed pear juice with undesirable compounds, and they should be taken into consideration when performing risk assessment studies.

Keywords: Pear juice; mycotoxins; pesticides; Fusarium; Q-Exactive Orbitrap.

10.1 INTRODUCTION

During the last years, diets have gravitated to higher intakes of fruits and vegetables, mainly due to its beneficial effects on health status and its protective role against chronic diseases [1]. In this line, fruit juices have become an appealing alternative, recommended as a good vitamin C source for children, and have been introduced as part of breakfast in conventional diets [2]. According to the European Fruit Juice Association (AIJN), juice consumption was 9.2 billion liters in 2017, with pear juice being one of the most consumed flavors in several countries, such as Italy [3]. The frequent intake of pear juice demands strict quality controls, especially when children become an important target group, in order to ensure safe consumption.

Several harmful compounds, originally present in pears, can also be present in marketed pear juice due to a potential carry-over during the manufacturing process. Among all the different contaminants in pears, mycotoxins and pesticide residues are some of the most impactful [4]. Mycotoxins are fungal secondary metabolites that can display several adverse effects, such as immunosuppression, carcinogenicity, or nephrotoxicity, among others [5]. In pears, the most relevant mycotoxin-producing

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genera are *Alternaria, Aspergillus*, and *Penicillium* [4], so analytical methods have been focused on the detection of their respective mycotoxins. Patulin (PAT), produced by *Aspergillus* and *Penicillium* spp., is the most studied mycotoxin in pear juice. Diverse effects have been attributed to PAT, including hepatotoxicity or neurotoxicity, among others [6]. Furthermore, citrinin (CIT) and ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* spp. too, and even *Alternaria* mycotoxins, such as alternariol (AOH) and alternariol monomethyl ether (AME), have been studied in this matrix [7,8]. Apart from these genera, *Fusarium* has also been classified as another pathogenic fungus in pear, so its presence was recently reported for the first time, causing postharvest decay [9].

On the other hand, pesticide residues include a broad range of toxic compounds widely used to prevent crops from pests. Nevertheless, a routine application can lead to the accumulation of residues in plants meant for human consumption, causing severe adverse effects, like neurotoxicity, carcinogenicity, and reproductive and developmental disorders [10]. In pear, these products are mainly used to avoid postharvest diseases caused by fungi, so pesticides from the benzimidazole group are commonly used. Similarly, pyrethroids represent another group of pesticides routinely applied due to its insecticidal capacity [11]. Despite the accumulation of residues being due to the intended use of pesticides, there are other factors to take into consideration, such as a potential run-off from contaminated soils and waters or even cross-contamination between different crops. Consequently, the overall pesticide profile of crops can vary from what it is expected to be. In terms of regulation, maximum residue limits (MRLs) have been established by the European Commission in pear or pear juice. Pesticide residues are brought under Regulation (EC) No. 396/2005 [12], but no specific MRLs have been set for pear juice so those corresponding to pears are applied instead. Referring to mycotoxins, Regulation (EC) No. 1881/2006 [13] covers contamination in pear juice, setting an MRL at 50 ng/mL for PAT. Several studies have reported the presence of PAT [14,15,16] and pesticide residues in pear juice [11,17,18], but no literature regarding *Fusarium* mycotoxins is available. Considering that *Fusarium* spp. has previously been identified as another pathogen in pears, its own mycotoxins could be expected in pear-derived products, even coexisting with other contaminants, like pesticides. Consequently, it is necessary to evaluate the contamination profile of pear juice even more when children represent one of the largest targets.

To overcome this, powerful analytical tools are needed. Concerning the extractive procedures, the most recent studies have used dispersive liquid-liquid microextraction (DLLME) [7], liquid-liquid extraction (LLE) [8,14], and QuEChERS (quick, easy, cheap, effective, rugged, and safe) combined with (DLLME) [11] for the extraction of contaminants from pear juice. Analytical methods include high-performance liquid chromatography-fluorescence detection (HPLC-FD) [7], HPLC-ultraviolet-visible detection (HPLC-UV-VIS) [8], HPLC-UV detection [14], and gas chromatography-electron capture detection (GC–ECD) [11]. Based on its high-resolution power, sensitivity, and accurate mass measurement, high-resolution mass spectrometry represents an optimal choice for evaluating trace contaminants occurring in complex matrices. Hence, the aim of the present study was to evaluate the presence of pesticide residues and mycotoxins produced by *Fusarium* spp. in 21 pear juice samples available in Italian markets, using ultra-high-performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry. To achieve this, an extractive methodology was validated for identifying and quantifying 14 *Fusarium* mycotoxins, followed by a screening of 283 pesticides. To the best of the authors' knowledge, this is the first multi-class analysis including *Fusarium* toxins in marketed pear juice.

10.2 MATERIALS AND METHODS

10.2.1 Chemicals and Reagents

All solvents, acetonitrile (AcN), methanol (MeOH), and water (LC-MS grade), were purchased from Merck (Darmstadt, Germany). Formic acid (MS grade) was acquired from Carlo Erba reagents (Cornaredo, Italy), whereas ammonium formate (analytical grade) was provided by Fluka (Milan, Italy). Magnesium sulfate ($MgSO_4$) (anhydrous), sodium chloride (NaCl), primary-secondary amine (PSA) (analytical grade), and octadecyl carbon chain-bonded silica (C18) (analytical grade) were obtained from Sigma Aldrich (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane

(PTFE, 15 mm, diameter 0.2 µm) were purchased from Phenomenex (Castel Maggiore, Italy). Conical centrifuge polypropylene tubes of 50 and 15 mL were acquired from BD Falcon (Milan, Italy).

Mycotoxin standards and metabolites (purity ≥98%), namely neosolaniol (NEO), HT-2 toxin, α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), T-2 toxin, β-zearalanol (β-ZAL), β-zearalenol (β-ZEL), zearalanone (ZAN), zearalenone (ZEN), enniatins (ENNA, ENNA1, ENNB, and ENNB1), and beauvericin (BEA), were provided by Sigma Aldrich (Milan, Italy). For the preparation of individual stock solutions, 1 mg of each mycotoxin was diluted in 1 mL of methanol. A working standard solution including all the analytes was built by diluting in MeOH:H_2O (70:30 v/v, 0.1% formic acid) until the concentrations needed for the spiking experiments were reached: 100, 20, and 10 ng/mL. The analytical standards were kept in a tightly closed container under cool dry conditions at −20°C in a well-ventilated place as stated in the safety data sheets provided by the manufacturer.

10.2.2 Sampling

A total of 21 pear juices samples from different European brands were randomly purchased between January and February 2020 from different supermarkets located in Campania region, southern Italy. The sampling was limited to one product per brand. The samples were divided into organic (n = 7) and conventional (n = 14) samples as indicated on the label by the manufacturer. In all cases, the percentage of fruit in the analyzed samples was above 50%; other ingredients declared in the labels from the samples were water, sugar, glucose-fructose syrup, lemon juice, and citric and ascorbic acid. All samples were stored in a refrigerator at 4°C into their original packages and analyzed within 3 days after sample registration.

10.2.3 Sample Preparation

In this work, the sample preparation procedure reported by Desmarchelier et al. [19] was selected as the starting point and slightly modified. Briefly, 10 mL of sample were placed into a 50-mL Falcon tube and mixed with 5 mL of water prior to be shaken for 1 min in a vortex. Then, 10 mL of AcN were added and the mixture was horizontally shaken for 30 min at 294× g. After that, 4 g of magnesium sulfate and 1 g of sodium chloride were added. The mixture was shaken by hand for 1 min and centrifuged at 4907× g for 10 min at 15°C in an SL 16R centrifuge (Thermo Fisher Scientific LED GmbH, Langenselbold, Germany). Then, 3 mL of the upper acetonitrile layer were placed into a 15-mL Falcon tube containing 900 mg of magnesium sulfate, 150 mg of C18 sorbent, and 150 mg of PSA, and vortexed for 1 min. The mixture was centrifuged for 10 min at 4907× g at 15°C, and 0.5 mL of the upper layer was added. Finally, the extract was evaporated to dryness under gentle nitrogen flow, reconstituted with 0.5 mL of MeOH/H_2O (70:30 v/v; 0.1% formic acid), and filtered through a 0.2-µm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

10.2.4 UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic analysis was performed using an ultra-high pressure liquid chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Dionex Ultimate 3000 (Waltham, MA, USA), a degassing system, an auto sampler device, a Quaternary UHPLC pump working at 1250 bar, and a thermostated (30 °C) Luna Omega (50 × 2.1 mm, 1.6 µm, Phenomenex) column. The mobile phases were water (phase A) and methanol (phase B), with both phases containing 0.1% formic acid and 5 mM ammonium formate. The separation gradient for the UHPLC-Orbitrap HRMS analyses consisted of an initial 0% of phase B kept for 1 min, and then increased up to 95% B in 1 min, followed by a hold-time of 0.5 min. Then, the gradient switched back to 75% B in 2.5 min and decreased again up to 60% B for 1 min. Finally, the gradient returned in 0.5 min at 0% B and then the column was re-equilibrated during 1.5 min at 0% B, giving a total run time of 8 min. The flow rate was 0.4 mL/min and a total of 5 µL of the samples were injected.

The UHPLC system was coupled to a Q-Exactive Orbitrap mass spectrometer. The spectrometer worked in positive and negative mode through fast polarity switching, setting two scan events (full ion MS and All ion fragmentation, AIF). Full scan data were obtained at a resolving power of 35,000 full

width at half maximum (FWHM) at 200 *m/z*. The ionization parameters were capillary temperature, 290°C; spray voltage, 4 kV (−4 kV in ESI− mode); sheath gas pressure (N_2 > 95%), 35; S-lens radio frequency (RF) level, 50; auxiliary gas heater temperature, 305 °C; and auxiliary gas (N_2 > 95%), 10. The selected value for the automatic gain control (AGC) target was 1e6, the injection time was set to 200 ms, and a scan range of *m/z* 100 to 1000 was selected. The scan rate was set at 2 scans/s. The parameters for the scan event of AIF were: maximum injection time, 200 ms; mass resolving power, 17,500 FWHM; ACG target, 1e5; scan time, 0.10 s; *m/z* scan range, 100–1000; retention time window, 30 s; and *m/z* isolation window, 5.0. The Orbitrap-MS parameters were optimized in a previously published article [20]. Table 10.1 shows the analytical parameters of the studied mycotoxins, including the elemental composition, retention time (RT), adduct ion, theoretical and measured mass, accurate mass error, collision energy, and product ion as well as tolerable daily intake (TDI). The energy was chosen when at least the parent ion was held at 10% and the generating product ions at a 90% intensity. A mass tolerance below 5 ppm was set for identification of the ions. Retrospective semi-target screening was performed using spectral data provided by a pesticide spectral library (Pesticide Spectral Library Version 1.1 for LibraryView™ Software, AB SCIEX, Framingham, MA, USA). The identification and confirmation were carried out according to the accurate mass measurement with a mass tolerance below 5 ppm for the molecular ion and for both fragments at the intensity threshold of 1000. Precursor and product ions were required to identify both targeted and nontargeted compounds. Data analysis and processing were conducted using Quan/Qual Browser Xcalibur software, version 3.1.66 (Thermo Fisher Scientific, Waltham, MA, USA).

10.2.5 Validation Parameters

In-house validation was carried out according to the guidelines established by the EU Commission Decision 2002/657/EC [21] and the SANTE criteria (SANTE/12682/2019) [22]. The method validation was based on the following parameters: Selectivity, trueness, repeatability (intra-day precision), reproducibility (inter-day precision), linearity, limit of detection (LOD), and limit of quantification (LOQ). The selectivity of the method was evaluated by analyzing blank samples (n = 10) to detect signals that could interfere with the analytes. The peaks for the analytes of interest in the samples were confirmed by comparing the retention times of the peak with those of standard solutions and also identifying both the precursor and product ions, with a mass tolerance below 5 ppm. To determine the linearity (R^2), standard solutions built in neat solvent and matrix-matched calibration were compared by spiking blank samples with selected mycotoxins at eight concentration levels over a range of 0.4–100 ng/mL. Calibration curves were prepared in triplicate. In order to reveal the presence of matrix effects, the slopes of each linear calibration function were evaluated. The signal suppression/enhancement (%SSE) due to matrix effects was determined according to the following equation:

$$\%SSE = S_m/S_s \times 100, \quad (1)$$

where S_m is the matrix-matched calibration slope and S_s is the solvent calibration slope. An SSE of 100% indicates that no matrix effect occurred in the concentration range evaluated. An SSE value higher than 100% revealed signal enhancement, whereas there was signal suppression if the SSE value was below 100%. Trueness was determined by spiking three blank samples at three different levels (100, 20, and 10 ng/mL) during three non-consecutive days and the results were expressed as percentage of recovery. Values in the range 70–120% in relation to the theoretical concentrations were considered as satisfactory Intraday precision (repeatability) was expressed in terms of the relative standard deviation (RSD_r) after comparing the recoveries from three determinations in a single day (n = 3) for each fortification level. Inter-day precision (reproducibility) was expressed as the relative standard deviation (RSD_R) of a triplicated determination on three non-consecutive days (n = 9) for each fortification level. LODs were set considering the lowest concentration where the molecular ion could be identified (mass error value below 5 ppm) and LOQs were defined as the minimum concentration inside the linear range (mass error value below 5 ppm) with deviation below 20%.

Table 10.1. Ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) parameters and tolerable daily intakes (TDIs) corresponding to the evaluated mycotoxins

Analyte	Retention time (min)	Elemental Composition	Adduction	Theoretical Mass (*m/z*)	Measured Mass (*m/z*)	Accuracy (Δppm)	Collision Energy (eV)	Product Ions (*m/z*)	TDI (ng/kg bw)
NEO	4.25	$C_{19}H_{26}O_8$	$(M + NH_4)^+$	400.19659	400.19632	−0.67	10	305.13803 141.00530	n.d. [a]
HT-2	4.74	$C_{22}H_{32}O_8$	$(M + NH_4)^+$	442.24354	442.24323	−0.7	27	263.12744 215.10641	20 [b]
α-ZAL	4.81	$C_{18}H_{26}O_5$	$(M - H)^-$	321.17044	321.17065	0.65	29	259.09497 91.00272	250 [c]
α-ZEL	4.83	$C_{18}H_{24}O_5$	$(M - H)^-$	319.15510	319.15500	−0.31	36	174.95604 129.01947	250 [c]
T-2	4.85	$C_{24}H_{34}O_9$	$(M + NH_4)^+$	484.25411	484.25430	0.39	23	215.10603 185.09561	20 [b]
β-ZAL	4.94	$C_{18}H_{26}O_5$	$(M - H)^-$	321.17044	321.17059	0.47	40	259.09497 91.00272	250 [c]
β-ZEL	4.97	$C_{18}H_{24}O_5$	$(M - H)^-$	319.15510	319.15500	−0.31	36	174.95604 160.97665	250 [c]
ZAN	4.98	$C_{18}H_{24}O_5$	$(M - H)^-$	319.15510	319.15491	−0.6	35	273.01187 131.05020	250 [c]
ZEN	5.01	$C_{18}H_{22}O_5$	$(M + H)^+$	317.13945	317.13928	−0.54	−32	175.03989 131.05008	250 [c]
ENN B	5.56	$C_{33}H_{57}N_3O_9$	$(M + NH_4)^+$	657.44331	657.44348	0.26	50	214.14320 196.13280	n.d. [a]
ENN B1	5.68	$C_{34}H_{59}N_3O_9$	$(M + NH_4)^+$	671.45986	671.45935	−0.76	48	214.14343 196.13295	n.d. [a]
BEA	5.73	$C_{45}H_{57}N_3O_9$	$(M + NH_4)^+$	801.44331	801.44318	−0.16	70	262.76715 244.18239	n.d. [a]
ENN A1	5.82	$C_{35}H_{61}N_3O_9$	$(M + NH_4)^+$	685.47461	685.47449	−0.18	48	228.15900 210.14847	n.d. [a]
ENN A	5.99	$C_{36}H_{63}N_3O_9$	$(M + NH_4)^+$	699.49026	699.48987	−0.56	43	228.15900 210.14847	n.d. [a]

[a] not determined; [b] sum of T-2 and HT-2; [c] sum of ZEN and its forms α-ZEL, β-ZEL, α-ZAL, β-ZAL, and ZAN in terms of ZEN equivalents being 60, 0.2, 4, 2, and 1.5, respectively, its molar potency factors. Neosolaniol (NEO), HT-2 toxin, α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), T-2 toxin, β-zearalanol (β-ZAL), β-zearalenol (β-ZEL), zearalanone (ZAN), zearalenone (ZEN), enniatins (ENNA, ENNA1, ENNB, and ENNB1), and beauvericin (BEA).

10.2.6 Quality Assurance/Quality Control

For a proper confirmation of the peaks, the retention times corresponding to each analyte in the samples were compared to those in standard solutions at a tolerance of ± 2.5%. A mass error of 5 ppm was set for identification of both the precursor and product ions. Referring to the quality assurance/quality control (QA/QC) procedure, a reagent blank, a sample blank, and a replicate sample were put at the beginning and end of each sample batch in order to evaluate the efficacy and stability of the system throughout the whole batch. A potential carry-over was also evaluated through blank samples (n = 10) injected right after the highest calibration value, with 100, 20, and 10 ng/mL being the concentrations chosen for the analytical quality control.

10.2.7 Exposure Assessment

The exposure assessment was performed following a deterministic approach. Data reported by the Italian National Food Consumption Survey INRAN-SCAI 2005-06 was considered so five different age groups were made: Infants (0.1–2.9 years), mean consumption of juice of 150 mL/day; children (3–9.9 years), mean consumption of 127 mL/day; teenagers (10–17.9 years), mean consumption of 122 mL/day; adults (18–65 years), mean consumption 58 mL/day; and elderly (>65 years), mean consumption 50 mL/day. The body weight assigned to each group was 11.3, 26.1, 52.6, 69.7, and 70.1 kg, respectively. For the calculation of the probable daily intake (PDI) corresponding to each mycotoxin, the consumption data provided by the Survey INRAN-SCAI 2005–06 was combined with the contamination data here obtained, following the next equation:

$$PDI_m = (C_m \times I)/bw, \quad (2)$$

where PDI_m is the probable daily intake (ng/kg bw/d) corresponding to each mycotoxin, m; C_m is the average content of a certain mycotoxin in pear juice (ng/mL); I represents the intake of juice (mL); and bw is the body weight attached to each age group (kg). After PDI_m was calculated, the risk characterization, considered as the percentage of relevant TDI_m, was evaluated by dividing the resultant PDI_m by its TDI_m value (Table 10.1). Since ENNB, ENNB1, ENNA, ENNA1, and BEA do not have an assigned TDI, a theoretical 20 ng/kg bw value was used, corresponding to the lowest one for a *Fusarium* toxin:

$$\%TDI_m = PDI_m/TDI_m \times 100. \quad (3)$$

10.2.8 Statistical Analysis

The Mann–Whitney U test was used to evaluate differences between juice typology considering p values < 0.05 as significant. Statistical analysis of the results was carried out using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA).

10.3 RESULTS

10.3.1 Analytical Method Validation

The method was validated in order to extract and quantify 14 different *Fusarium* mycotoxins in pear juice. The results are shown in Table 10.2 All the analytes showed good linearity, with the regression coefficients (R^2) above 0.990 in the range evaluated (0.4–100 ng/mL), and a deviation ≤20% for each level of the calibration curves. Signal suppression/enhancement (%SSE) as a consequence of matrix interference was evaluated by comparing the curves built in neat solvent and blank matrix, with a minimal deviation (≤17%) being observed. Therefore, the external calibration curves were considered for quantification purposes. Sensitivity was assessed through the limits of quantifications (LOQs), that ranged from 0.4 to 3.1 ng/mL. To evaluate trueness, recovery studies were carried out in triplicate at three different spiking levels. Values corresponding to fortification at 100 ng/mL ranged between 70% and 106%, from 72–106% at 20 ng/mL, and from 70–103% at 10 ng/mL, meaning an efficient extraction procedure even at low concentrations levels. Precision was evaluated through both RSD_r

and RSD_R, showing values below 19% for all the mycotoxins analyzed. These results fulfill the criteria set by the European EU Commission Decision 2002/657/EC [21] established for a reliable quantification. This methodology, based on a simple QuEChERS extraction, stands as a powerful tool for detecting *Fusarium* mycotoxins in pear juice at low levels, reaching a higher sensitivity for several of the analytes than previous methods developed for detecting a single analyte in pear juice and carried out by Spadaro, Garibaldi, and Gullino [16] (PAT, LOQ = 1.7 ng/mL); Bonerba, Ceci, Conte, and Tantillo [15] (PAT, LOQ = 1 ng/mL); and Pan et al. [23] (AOH = 1.3 ng/mL).

Table 10.2. Method performance: linearity, matrix effect (SSE %), recovery, and limit of quantification (LOQ)

Analyte	Linearity (R^2)	SSE (%)	Recovery (%)			Precision (%) (RSD_r, (RSD_R)			LOQ (ng/mL)
			100 ng/mL	20 ng/mL	10 ng/mL	100 ng/mL	20 ng/mL	10 ng/mL	
NEO	0.9971	98	99	99	91	5 (13)	8 (18)	16 (12)	1.6
HT-2	0.9967	104	88	89	72	13 (13)	15 (13)	14 (17)	1.6
A-ZAL	0.9944	83	84	77	72	10 (8)	10 (8)	7 (7)	3.1
A-ZOL	0.9967	90	97	93	102	15 (19)	8 (18)	10 (19)	3.1
T-2	0.9998	105	106	106	103	11 (14)	16 (18)	7 (17)	1.6
B-ZAL	0.9941	113	93	85	89	9 (12)	13 (16)	12 (16)	1.6
B-ZOL	0.9997	112	81	87	77	10 (8)	8 (7)	8 (6)	0.8
ZAN	0.9993	118	83	85	77	7 (5)	13 (9)	6 (5)	0.4
ZON	0.9994	117	84	91	87	5 (5)	4 (4)	6 (8)	0.4
ENN B	0.9995	103	73	76	71	5 (8)	7 (5)	10 (7)	0.8
ENN B1	0.9980	94	76	81	75	10 (9)	8 (9)	8 (8)	0.4
BEA	0.9977	96	78	84	78	5 (5)	10 (6)	15 (10)	1.6
ENN A1	0.9994	101	70	74	71	3 (6)	7 (5)	13 (9)	0.8
ENN A	0.9994	103	70	72	70	3 (2)	7 (5)	6 (4)	0.8

RSD_r: repeatability relative standard deviation; RSD_R: reproducibility relative standard deviation

10.3.2 Analysis of Real Samples

Up to nine different *Fusarium* mycotoxins were detected in the analyzed pear juice samples. In total, 20 out of 21 samples showed contamination with at least one mycotoxin, generally at low levels or even below the LOQ, as shown in Table 10.3. ZEN was the most frequently detected compound, present in 67% of the samples, with concentrations ranging from below the LOQ to 1.5 ng/mL. T2 was also a relevant mycotoxin in the analyzed samples, showing an incidence of 33% at concentrations from <LOQ up to 2.0 ng/mL. Similarly, HT-2 was detected in 33% of the samples, ranging from below <LOQ to 7.0 ng/mL. The main enniatins were also detected: ENNB and ENNA1 were present in 19% of the samples, ranging from <LOQ to 0.8 ng/mL and 1.2 ng/mL, respectively; ENNB1 was found in 14% of the samples at concentrations going from <LOQ up to 0.5 ng/mL; and ENNA was only found in one sample (5%) at 1.0 ng/mL. Lastly, ZEN metabolites were also observed. ZAN was present in 10% of the samples at concentrations below the LOQ, whereas α-ZAL was quantified in 14% of the samples, ranging from <LOQ to 10.5 ng/mL. To date, several studies have only evaluated the presence of PAT in Italian pear juice as a consequence of *Penicillium expsansum* contamination, which is the main fungus causing postharvest diseases. Spadaro, Garibaldi, and Gullino [16] reported an incidence of 64% (n = 39), with 17 samples showing a contamination below 10 ng/mL and 8 samples above 10 ng/mL. Similarly, Bonerba, Ceci, Conte, and Tantillo [15] found patulin in 40% of the pear juice samples (n = 35) at concentrations ranging from 5 to 92 ng/mL. Recently, *Alternaria* mycotoxins have been studied in pear and pear-derived foodstuffs. Pan, Sun, Pu, and Wei [23] investigated *Alternaria* toxin AOH in fresh pears (n = 5), observing an absence of contamination despite having good sensitivity (LOQ = 1.3 ng/mL). A specific methodology for detecting AOH and AME in pear juice has been developed by Ruan, Diao, Zhang, Zhang, and Liu [7]. The results obtained here show that the *Fusarium* toxin ZEN extensively occurred in pear juice samples, having a larger incidence than the one reported for PAT or AOH in the mentioned studies, whereas other less detected mycotoxins, such as T2 or HT-2, also had a considerable impact.

Table 10.3. Incidence and range of concentrations of the mycotoxins detected in conventional and organic pear juice samples

Juice Typology (n)	ZEN		ZAN		A-ZAL		T-2		HT-2		ENNB		ENNB1		ENNA		ENNA1	
	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)	Incidence (n (%))	Range (ng/mL)
Conventional juice (14)	7 (50)	<L [2] - 1.5	0 (0)	nd [1]	1 (7)	10.5	2 (14)	<L	4 (29)	<L - 7.0	0 (0)	nd	1 (7)	<L	1 (7)	1.0	3 (21)	<L - 1.2
Organic juice (7)	7 (100)	<L - 0.6	2 (29)	<L	2 (29)	<L - 3.5	5 (71)	<L - 2.0	3 (43)	<L - 1.6	4 (57)	<L - 0.8	2 (29)	<L - 0.5	0 (0)	nd	1 (14)	0.8
Total	14 (67)	<L - 1.5	2 (10)	<L	3 (14)	<L - 10.5	7 (33)	<L - 2.0	7 (33)	<L - 7.0	4 (19)	<L - 0.8	3 (14)	<L - 0.5	1 (5)	1.0	4 (19)	<L - 1.2

[1] Not determined; [2] Limit of quantification

Table 10.4. Risk characterization of mycotoxins found in pear juice samples according to the tolerable daily intake values

Mycotoxins	C_m (ng/mL)	Probable Daily Intake (PDI) (ng/kg bw/d)					Risk Characterization (%TDI)				
		Infants	Children	Teenager	Adult	Elderly	Infants	Children	Teenager	Adult	Elderly
ZEN + α-ZAL	2.88	38.24	14.01	6.69	2.3	2.04	15.3	5.6	2.68	0.92	0.82
T-2 + HT-2	0.88	11.68	4.28	2.04	0.7	0.62	58.40	21.40	10.20	3.50	3.10
ENNB + ENNB1 + ENNA + ENNA1	0.25	3.32	1.22	0.58	0.2	0.18	16.60	6.10	2.90	1.00	0.90

Bearing in mind the type of sample, significant differences ($p < 0.05$) were found when comparing the occurrence of mycotoxins in both organic and conventional juice, being more frequent in organic samples, as expected. Additionally, co-occurrence of mycotoxins was observed in high frequency in organic juice samples. Up to 65% of the conventional juice samples ($n = 14$) showed contamination with only one mycotoxin, whereas four or more mycotoxins co-occurred in the majority of organic samples (71%, $n = 7$). The most common associations were ZEN alongside its two metabolites, and ZEN, T-2, and ENNB, which seems to be a frequent mixture in several plant-based foodstuffs [24,25]. Furthermore, ENNB1 and ENNA1 co-occurred with ENNB in organic samples, and the combination ENNA and ENNA1 was also observed in one conventional juice sample. The presence of multiple mycotoxins could affect its toxicological potential, deriving into synergic or additive effects as observed in in vitro assays [26]. Based on the above-discussed points and considering the popular trend of organic and environment-friendly products, *Fusarium* mycotoxins should also be taken into consideration in exposure assessment studies involving pear and pear juices. Moreover, further toxicological knowledge in terms of the combination of food contaminants is needed in order to ensure safe consumption.

10.3.3 Exposure Assessment

As reflected by the Italian National Food Consumption Survey INRAN-SCAI 2005-06, juices are mostly consumed by the young population, so a bigger intake accounts for a higher risk. Considering that *Fusarium* mycotoxins have not been studied in pear juice, the exposure to these mycotoxins might be underestimated, so an exposure assessment and risk characterization were performed.

Table 10.4 summarizes the risk characterization of the mycotoxins found in the juice samples. The mean content of mycotoxins was 2.88, 0.88, and 0.25 ng/mL for ZEN + α-ZAL, T-2 + HT-2, and enniatins, respectively. Among the different age groups, the probable daily intake strongly varied. Infants were identified as the group with the highest PDIs due to a heavier consumption of juice and a lower body weight. Values corresponding to the rest of the groups ranged from 2 to 20 times lower in comparison with the infants' results. These PDIs values are below the TDIs established by the Scientific Committee on Food of the European Commission, set as 250 ng/kg bw/day for the sum of ZEN and its derived products, 20 ng/kg bw/day for the sum of T-2 HT-2, and a theoretical value of 20 ng/kg bw/day for the sum of enniatins. Considering the results, the pear juices analyzed here account for 0.78% to 14.65% of the TDI for ZEN + α-ZAL, from 3% to 55.95% of the TDI set for T-2 and HT-2, and from 0.85% to 15.90% for enniatins. This suggests that the exposure to *Fusarium* mycotoxins as a consequence of juice consumption might not represent a health concern, but the intake of mycotoxins by infants due to regular consumption could be of importance. Therefore, the results suggest having a watchful attitude in order to ensure safe consumption.

10.3.4 Identification of Non-Target Compounds through Retrospective Analysis in Studied Samples

The semi-target screening was performed using a mass spectral library, allowing the detection of 283 different pesticide residues in the analyzed samples. The pesticides present in more than 25% of the samples are shown in Fig. 10.1. Up to 77 pesticide residues were tentatively identified, but the presence of several compounds that have not yet been approved by the EU should be noted. Ethoxyquin was detected in 64% of the samples ($n = 21$), being the third most frequently found residue. This pesticide acts as a fungicide during the postharvest stage of the crops through its scald-preventive properties [27]. Currently, the use of ethoxyquin is suspended by European Commission Decision 2011/143/EU [28]. Triazophos is an insecticide, which was found in 55% of the samples ($n = 21$). The use of products containing this compound is not allowed under Regulation No. 1107/2009 [29] due to its toxicity, as reported by the European Food Safety Authority (EFSA) [30]. Similarly, the insecticide bifenthrin was also detected in 50% of the samples ($n = 21$). Although bifenthrin would fulfil the safety requirements according to the last update of report SANCO/12946/2011 released in 2018, it has not received any authorization yet. Oxadixyl was another relevant compound in the analyzed samples. This fungicide was found in 46% of the samples ($n = 21$) despite its use not allowed being in pears, as brought under Regulation (EC) No. 2076/2002 [31]. According to the mentioned legislation,

butoxycarboxim was established as another forbidden insecticide, but it was present in 46% of the samples (*n* = 21).

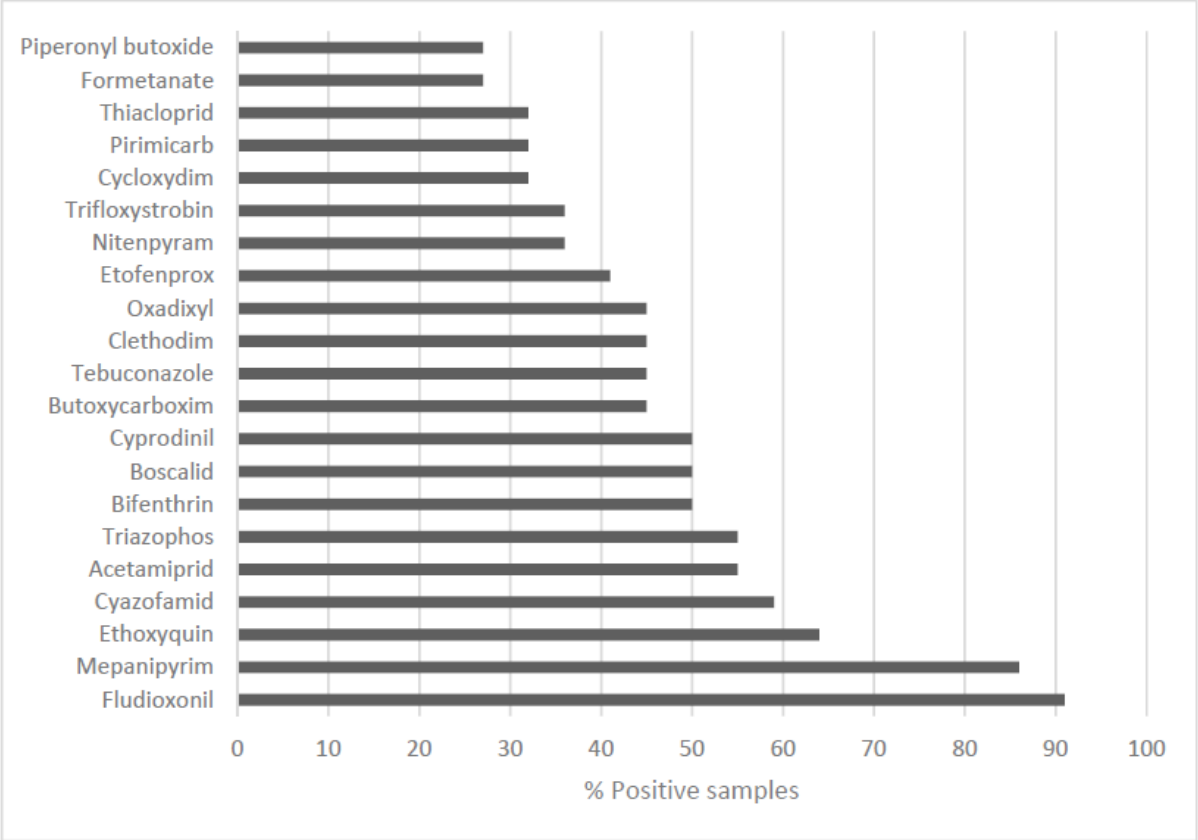

Fig. 10.1. Occurrence of non-target pesticides in pear juice samples after retrospective screening

Considering that pesticide residues occurred in all samples, organic juice samples showed significantly less ($p < 0.05$) residues than conventional samples, as expected. This fact could also explain the extensive contamination of organic samples in comparison with conventional samples. Therefore, the presence of non-approved pesticides in the analyzed samples indicates the necessity of monitoring potential contaminants of pears and pear-derived products.

10.4 CONCLUSIONS

A QuEChERS-based extraction was validated in order to detect and quantify 14 *Fusarium* mycotoxins for the first time in pear juice, using ultra-high-performance liquid chromatography coupled to high-resolution Q-Orbitrap mass spectrometry. The extraction fulfilled all the criteria set by the EU in terms of linearity, trueness, specificity, selectivity, and precision. This methodology was then applied to 21 pear juice samples purchased from Italian markets. Up to 95% of the samples showed mycotoxin contamination, indicating the extensive presence of ZEN and its metabolites and T-2 and its metabolites. The emerging *Fusarium* mycotoxins enniatins B, B1, A, and A1 were also detected in the samples. Organic juice samples showed a significantly higher contamination ($p < 0.05$), with at least four mycotoxins co-occurring in 71% of the samples ($n = 7$), when compared to conventional ones, with only one mycotoxin per sample in 65% of the cases ($n = 14$). Additionally, 77 pesticide residues were tentatively identified through a retrospective analysis based on semi-target screening. The prevalent presence of some non-approved pesticides, such as ethoxyquin (64%, $n = 21$) and triazophos (55%, $n = 21$), must be highlighted. The results obtained highlight an extensive contamination of marketed pear juice with undesirable compounds, including mycotoxins and

pesticide residues. Hence, cumulative risk characterization studies of undesirable substances with chronic effects need to be performed, as well as more realistic risk assessment studies. Considering that children represent one of the largest targets for juices alongside the uprising trend of environmental-friendly products, there is a necessity of evaluating the contamination profile of these products to ensure safe consumption.

AUTHOR CONTRIBUTIONS

Conceptualization, A.N. and Y.R.-C.; methodology, L.I. and L.C.; validation, L.I., and L.C.; data curation, A.N.; writing—original draft preparation, A.N.; writing—review and editing, Y.R.-C. and A.R.; supervision, Y.R.-C. and A.R. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 11
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Mycotoxin Occurrence and Risk Assessment in Gluten-Free Pasta through UHPLC-Q-Exactive Orbitrap MS

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ABSTRACT

Celiac disease (CD) is a genetic-based autoimmune disorder which is characterized by inflammation in the small intestinal mucosa due to the intolerance to gluten. Celiac people should consume products without gluten, which are elaborated mainly with maize or other cereals. Contamination of cereals with mycotoxins, such as fumonisins (FBs) and aflatoxins (AFs) is frequently reported worldwide. Therefore, food ingestion is the main source of mycotoxin exposure. A new analytical method was developed and validated for simultaneous analysis of 21 mycotoxins in gluten-free pasta, commonly consumed by celiac population as an alternative to conventional pasta. Ultrahigh-performance liquid chromatography coupled to quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Exactive Orbitrap MS) was used for analyte separation and detection. The mycotoxins included in this work were those widely reported to occur in cereal samples, namely, ochratoxin-A (OTA), aflatoxins (AFB1, AFB2, AFG1 and AFG2), zearalenone (ZON), deoxynivalenol (DON), 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol (3-AcDON and 15-AcDON, respectively), nivalenol (NIV), neosolaniol (NEO), fusarenone-X, (FUS-X), T-2 toxin (T-2) and HT-2 toxin (HT-2), fumonisin B1 and B2 (FB1 and FB2, respectively), enniatins (ENN A, ENN A1, ENN B and ENN B1) and beauvericin (BEA). The validated method was successfully applied to 84 gluten-free pasta samples collected from several local markets of Campania region (Italy) during September to November 2020 to monitor the occurrence of mycotoxins and to assess the exposure to these food contaminants. A significant number of samples (95%) showed mycotoxin contamination, being *Fusarium* mycotoxins (FB1, ZON and DON) the most commonly detected ones. Regarding the risk assessment, the higher exposures were obtained for NIV, DON and FB1 for children and teenagers age group which can be explained due to their lower body weight.

Keywords: Mycotoxins; gluten-free pasta; liquid-chromatography; HRMS-Orbitrap; multiresidue method; exposure.

11.1 INTRODUCTION

Celiac disease (CD), is a serious autoimmune disease occurring in genetically-predisposed people caused by gluten ingestion, a complex of high molecular-weight proteins found in the endosperm of grass-borne grains including wheat, barley and rye [1]. Currently, CD is one of the most common food-induced diseases, with an estimated prevalence ranging from 0.5 to 1% of the population [2]. The treatment of CD is the removal of gluten from the diet (i.e., consumption of a gluten-free (GF) diet) [3]. This is achieved by the consumption of GF foodstuffs, mainly maize, rice, potatoes and other few cereals and pseudo-cereals, which can be safely employed as carbohydrate source since these products do not contain gluten. Apart from celiac population, in recent years, many people prefer to

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avoid gluten in their diets for fashionable or non-scientific beliefs or because consumers perceive that GF products are healthier. This trend is supported by the increase market of GF products which was valued at 5.6 billion dollars in 2020 and is estimated to reach 8.3 billion dollars in 2025 [4]. Cereals, including those highly consumed in GF diets, are one of the main dietary sources of mycotoxins, and therefore one of the leading foods which increase the exposure to mycotoxins worldwide [5].

Mycotoxins are common contaminants found in cereals and by-products. They can be produced under favorable conditions of temperature and humidity by different filamentous fungi, such as *Aspergillus*, *Penicillium* and *Fusarium*, which can affect crops both in the field and during storage [6]. The most frequently contaminated cereals are wheat, maize, rice, among others. Maize and rice are recognized as good substrates for fungal growth and mycotoxin production, especially aflatoxins (AFs) [7,8], fumonisins (FBs) [9,10], trichothecenes (TCs) [11] and zearalenone (ZON) [12]. Mycotoxins are of significant public health concern, due to their high occurrence and toxic properties [13]. The toxic properties of mycotoxins associated with animals and human beings include carcinogenicity, genotoxicity, teratogenicity, mutagenicity, nephrotoxicity and immunotoxicity. Because of their recognized harmful effects, many countries have adopted regulations to control the mycotoxin exposure [6]. In Europe, maximum residue levels have been set for FBs, AFs, deoxynivalenol (DON), ochratoxin A (OTA), patulin (PAT) and ZON by Commission Regulation 1881/2006/EC [14] and its amendments, setting maximum levels (MLs) for these mycotoxins in maize and maize-based products, which has been modified by Regulation EC 1126/2007 [15], as regards the limits for *Fusarium* mycotoxins in corn and derivatives, based on successive risk assessments and EFSA scientific opinions.

Based on the abovementioned data, the population most exposed to the mycotoxins present in maize and rice foodstuffs (GF products) are celiac people and also some ethnic groups, and within these groups, the highest exposure level corresponds to children. In this sense, the aims of this research were (i) to validate in-house a multi-mycotoxin method for the analysis of 21 different mycotoxins in GF pasta (maize and rice) by ultra-high-performance liquid chromatography coupled to quadrupole Orbitrap mass spectrometry (UHPLC-Q-Orbitrap HRMS) and (ii) to assess the exposure to mycotoxins in celiac population groups through the mycotoxin occurrence data obtained from a surveillance on GF pasta here performed.

11.2 RESULTS AND DISCUSSION

11.2.1 Chromatographic and Mass Spectrometric Optimization

The front-end parameters of the Q-Orbitrap, including electrospray ionization (ESI) parameters, the heated capillary and the S-Lens radio frequency (RF) level, were optimized. ESI parameters are responsible for the spraying of the sample carried in the mobile phases followed by either positive or negative ionization modes of the analytes. Those parameters are influenced by both flow rate and mobile phase composition, and hence the mycotoxin standard solutions diluted at 1 µg/mL with mobile phases were individually infused directly into the mass spectrometer through syringe infusion (flow rate: 0.4 mL/min) to mimic working conditions. The flow of sheath gas and auxiliary gas were optimized along with the heater temperature to ensure optimum desolvation of the sample being adjusted to 35 and 10 arbitrary units, respectively. Heater and capillary temperatures were kept at 290 and 305 °C, respectively. The ionization was achieved by applying a spray voltage of 4kV in both ESI modes. Ions are then focused by the S-lens through the application of RF voltage. Different HRMS scan modes were also used (e.g., data-dependent acquisitions) to permit a retrospectively analyze the data if needed for further research purposes [16].

On the other hand, several chromatographic conditions were tested to achieve the best compound separation. Methanol and acetonitrile were tested as organic mobile phase. Both organic solvents showed efficient separation of the studied mycotoxins but methanol was selected due to the superior signal intensity observed. Similarly, ammonium formate (5mM) and formic acid (0.1%) were added as additives to both mobile phases due to enhanced signal intensity. Retention time of the 21 target mycotoxins ranged from 3.83 to 8.24 min with a total run time of 10.0 min. Optimal parameters of studied mycotoxins are presented in Table 11.1.

Table 11.1. UHPLC/ESI Q-Orbitrap optimized parameters of analyzed mycotoxins

Mycotoxin	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (m/z)	Measured Mass (m/z)	Accuracy (Δ ppm)
3-AcDON	3.83	$C_{17}H_{22}O_7$	$[M + H]^+$	339.14383	339.14331	−1.53
15-AcDON	4.02	$C_{17}H_{22}O_7$	$[M + H]^+$	339.14383	339.14331	−1.53
DON	4.18	$C_{15}H_{20}O_6$	$[M + HCOOH]^-$	341.12451	341.12454	0.09
NIV	4.35	$C_{15}H_{20}O_7$	$[M + H]^+$	313.12810	313.12785	−0.74
FUS-X	4.47	$C_{17}H_{22}O_8$	$[M + H]^+$	355.13874	355.13866	−0.92
NEO	4.58	$C_{19}H_{26}O_8$	$[M + NH_4]^+$	400.19659	400.19653	−0.15
AFG2	4.61	$C_{17}H_{14}O_7$	$[M + H]^+$	331.08123	331.08032	−2.75
AFG1	4.79	$C_{17}H_{12}O_7$	$[M + H]^+$	329.06553	329.06553	−0.05
AFB2	4.98	$C_{17}H_{14}O_6$	$[M + H]^+$	315.08631	315.08521	−3.49
AFB1	5.02	$C_{17}H_{12}O_6$	$[M + H]^+$	313.07066	313.06958	−3.45
HT-2	5.63	$C_{22}H_{32}O_8$	$[M + NH_4]^+$	442.24354	442.24323	−0.70
FB1	6.03	$C_{34}H_{59}NO_{15}$	$[M + H]^+$	722.39575	722.39539	−0.50
T-2	6.13	$C_{24}H_{34}O_9$	$[M + NH_4]^+$	484.25411	484.25418	0.14
OTA	6.50	$C_{20}H_{18}NO_6Cl$	$[M + H]^+$	404.08954	404.08801	−3.79
ZON	6.53	$C_{18}H_{22}O_5$	$[M + H]^+$	317.13945	317.13910	−1.10
FB2	6.78	$C_{34}H_{59}NO_{14}$	$[M + H]^+$	706.40083	706.40192	1.54
ENN B	7.81	$C_{33}H_{57}N_3O_9$	$[M + NH_4]^+$	657.44331	657.44299	−0.49
BEA	7.96	$C_{45}H_{57}N_3O_9$	$[M + NH_4]^+$	801.44330	801.44323	−0.09
ENN B1	8.06	$C_{34}H_{59}N_3O_9$	$[M + NH_4]^+$	671.45986	671.45923	−0.94
ENN A1	8.11	$C_{35}H_{61}N_3O_9$	$[M + NH_4]^+$	685.47461	685.47351	−1.60
ENN A	8.24	$C_{36}H_{63}N_3O_9$	$[M + NH_4]^+$	699.49026	699.48926	−1.43

11.2.2 Method Validation

The optimized multi-mycotoxin methodology was in-house validated to ensure the reliability of the results. Results are shown in Table 11.2. The method was found to be selective and specific based on the absence of interference peaks at the retention times of the studied mycotoxins in a blank GF sample (QA/QC sample) injected ten times. Linearity was established in a concentration range from 0.25 to 1000 µg/kg at eight concentration levels in both neat solvent and matrix extract. Calibration standards accuracy of all analytes were within ±15% of the nominal concentrations. Correlation coefficients were greater than 0.990 for all target mycotoxins. Strong to moderate matrix effects were observed (from 38 to 99%) probably due to the simple sample preparation procedure and therefore matrix-matched calibrations were used for quantitation purposes. Spiked samples were used to evaluate trueness and precision of the method. Recoveries ranged between 71 and 125% at all fortification levels (125, 62.5 and 12.5 µg/kg and at 500 µg/kg only for DON and FBs). Repeatability and reproducibility were determined at same spiking levels than trueness and relative standard deviation lower than 10.5 and 12.1% were obtained, respectively. The comprehensive results of accuracy are shown in Table 11.2. Quality control samples were also included in each batch to guarantee the accuracy of the results. For regulated mycotoxins, all LOD and LOQ were much lower than the maximum level (ML) set in current Regulation [14] and for non-regulated mycotoxins, results were acceptable and comparable to those obtained for regulated mycotoxins. In detail, LODs ranged between 0.05 to 7.8 and LOQs from 0.14 and 23.5 µg/kg.

11.2.3 Mycotoxin Occurrence in Gluten-Free Pasta

Although multi-mycotoxin methods in GF products have been commonly based on the detection of AFs, FBs, and ZON, in the present study, the determination of up to 21 mycotoxins in GF pasta samples has been carried out by in house method validation, thus supposing an improvement for the field of mycotoxin determination on GF products. Moreover, as our knowledge, multi-mycotoxin determination in GF pasta by Orbitrap mass spectrometry have not been reported to date.

Table 11.2. Analytical parameters (recovery at spiking level of 12.5, 62.5 and 125 µg/kg and 500 µg/kg for deoxynivalenol and fumonisins, intraday and interday precision, signal suppression enhancement (SSE), limits of detection and quantitation (LOD and LOQ, respectively), calibration curves and correlation coefficient (r^2) for each mycotoxin in analyzed samples)

Mycotoxin	Recovery (%)			Repeatability RSD %	Reproducibility RSD %	SSE (%)	LOD (µg/kg)	LOQ (µg/kg)	Calibration Curves	r^2
	12.5 µg/kg	62.5 µg/kg	125 (500 *) µg/kg							
3-AcDON	71	92	83	10.2	10.5	73	1.1	3.3	$y = 282{,}673x$	0.992
15-AcDON	70	94	82	9.9	10.1	75	1.1	3.3	$y = -382{,}359 + 25{,}694.5x$	0.992
DON	89	85	98 (95 *)	5.8	6.7	94	2.1	6.4	$y = -451{,}745 + 27{,}781.9x$	0.997
NIV	68	79	75	6.2	5.6	75	5.4	16.1	$y = -558{,}243 + 37{,}636.8x$	0.996
FUS-X	97	110	90	10.5	10.1	48	7.8	23.5	$y = -593{,}326 + 10{,}261x$	0.997
NEO	114	118	122	5.2	4.0	39	2.3	6.9	$y = -1.20903 \times 10^6 + 275{,}818x$	0.997
AFG2	102	112	98	6.5	7.2	78	0.10	0.23	$y = 71{,}914 + 3.10136 \times 10^6 x$	0.996
AFG1	95	110	102	6.3	7.5	84	0.10	0.23	$y = 68{,}876 + 3.09432 \times 10^6 x$	0.997
AFB2	105	125	120	8.4	10.4	80	0.06	0.17	$y = 71{,}914.2 + 3.13427 \times 10^6 x$	0.992
AFB1	121	120	124	1.8	2.1	83	0.06	0.17	$y = 72{,}965.1 + 3.08148 \times 10^6 x$	0.994
HT-2 toxin	112	95	98	8.7	9.1	87	1.2	3.6	$y = -146{,}011 + 186{,}971x$	0.997
FB1	104	115	99 (104 *)	8.3	8.2	72	1.9	5.6	$y = -285{,}447 + 74{,}040.9x$	0.998
T-2 toxin	121	107	98	9.4	11.6	66	0.8	2.5	$y = -1.7152 \times 10^6 + 1.58156 \times 10^6 x$	0.999
OTA	95	100	118	10.3	12.1	91	0.08	0.25	$y = -40{,}0195 + 21{,}4596x$	0.998
ZON	90	93	105	8.7	7.9	87	0.13	0.38	$y = 1.37666 \times 10^6 + 937{,}950x$	0.997
FB2	99	110	115 (108 *)	7.1	8.2	85	0.26	0.76	$y = -414{,}600 + 89{,}251.2x$	0.998
ENN B	90	108	117	7.4	10.7	41	0.05	0.16	$y = -497{,}411 + 82{,}621.5x$	0.990
BEA	99	96	112	6.2	8.5	88	3.6	10.9	$y = -272{,}121 + 58{,}199.7x$	0.995
ENN B1	102	123	120	7.8	11.4	38	0.05	0.76	$y = -435{,}829 + 78{,}581x$	0.992
ENN A1	113	110	109	3.5	2.1	86	0.10	0.30	$y = -476{,}813 + 79{,}612.5x$	0.996
ENN A	123	118	115	3.7	4.0	99	0.05	0.14	$y = -387{,}123 + 68{,}591.5x$	0.998

** Additional fortification level (500 µg/kg) for deoxynivalenol (DON) and fumonisins (FB1 and FB2).*

The validated method was successfully applied for the screening of mycotoxins in eighty-four commercially available GF pasta commodities. The mycotoxin occurrence and their levels obtained in the present study are reported in Table 11.3. As it can be observed, ninety-five percent of GF pasta samples included in the survey shown to be contaminated by one or more than one mycotoxin, being common the mycotoxin co-occurrence of more than one mycotoxin simultaneously. Only one sample showed no mycotoxin contamination (a parboiled Italian rice sample).

Table 11.3. Incidence and mycotoxin contents in samples analyzed, IARC classification and MLs established for cereal foodstuffs

Mycotoxin	Incidence (%)	Range (Mean) (µg/kg)	IARC Classification	MLs (EC) No. 1881/2006 (µg/kg)
NIV	33.3	209.2–367.6 (241.3)	3	No limits established
DON	66.7	182.2–377.4 (239.8)	3	750
HT-2 toxin	9.5	18.2–26.3 (22.2)	NC	No limits established
ZON	71.4	9.2–26.9 (13.5)	3	20–200 ***
FB1	90.5	39.9–246.9 (116.2)	2B	200 **–400
FB2	33.3	44.0–53.4 (48.0)	2B	
ENN A1	4.8	1.7 *	NC	No limits established
BEA	9.5	17.3–21.9 (19.6)	NC	No limits established

: only one positive sample; **: products intended for children; *: maize-based products; NC: not classified*

Regarding mycotoxin occurrence, the most prevalent mycotoxins found in analyzed samples were those belonging from *Fusarium* genera. The incidence of contamination was FB1 > ZON > DON > NIV = FB2. In detail, the highest percentages of occurrence corresponded to FB1, ZON and DON, with an overall incidence of 90.5%, 71.4% and 66.7%, respectively. On the other hand, regarding mycotoxin contents, the order of mycotoxin levels was DON > NIV > FB1 > FB2 > ZON > HT-2. The highest contents corresponded to DON (377.4 µg/kg), followed by NIV (367.6 µg/kg). The highest DON content (377.4 µg/kg) corresponded to a sample of "pasta dietetica" (fusilli) consisting in rice flour (67%) and corn flour (33%). In this sample, DON was found simultaneously with NIV (211.7 µg/kg), ZON (10.4 µg/kg) and FB1 (39.9 µg/kg). A 100% buckwheat pasta sample showed the highest NIV content (367.6 µg/kg) in co-occurrence with DON (240 µg/kg), HT-2 toxin (11.3 µg/kg), and ZON (26.3 µg/kg). Although mycotoxin contents detected were high, especially for DON, NIV and FBs, none of the samples exceed the MLs established by the European Regulation [14].

The co-occurrence of two or more mycotoxins has been commonly reported in analyzed samples. In this sense, only the 9% of commodities were contaminated by one mycotoxin. The 11% of samples were contaminated simultaneously by two mycotoxins, being the most common combination DON with FB1 and ZON with FB1. The combination of three mycotoxins with (NIV, DON and FB1 and DON, ZON and FB1) were the most commonly detected (44% of samples). The 26% of samples showed the presence of four mycotoxins simultaneously, being DON, ZON, NIV and FBs in different combinations the most frequently detected. Finally, 4% of samples were contaminated by five mycotoxins and 5% of samples presented co-occurrence of up to six mycotoxins. These results suggest that special attention should be paid to samples showing the presence of more than one mycotoxin simultaneously, as their toxic effects produced by their interaction could be synergistic.

Emerging *Fusarium* mycotoxins have been extensively reported to occur in cereal samples even at high contents [17], however, ENN A and ENN B were not detected in GF pasta samples analyzed in the present study, and lower incidence and contents were detected for ENN A1 and BEA (4.8% and 9.5% at 1.7 µg/kg and 19.6 µg/kg, respectively). Regarding mycotoxins *from Aspergillus* and *Penicillium* genera included in the study (AFs and OTA, respectively), no mycotoxin contents were detected in GF samples analyzed.

With regard to mycotoxin occurrence in pasta samples, differences have been observed between GF pasta and durum wheat pasta in relation to mycotoxin contamination, as different ingredients are used for the elaboration and some matrices are more prone to contamination by certain mycotoxins than others. Thus, whereas FBs, mainly FB1 and FB2, were the most detected mycotoxins in the present

study in GF pasta, no FB contents were detected in a previous survey on wheat-based pasta commodities [17]. DON and ZON were the predominant mycotoxins in durum wheat pasta (100% and 93%, respectively); however, although higher incidence was reported in these samples, higher contents were reported for DON in GF pasta (239.8 µg/kg in GF pasta vs. 96.9 µg/kg in durum wheat pasta). On the other hand, ENNs showed higher incidence and contents in durum wheat pasta in contrast to GF pasta. ENN B was detected in 90% of durum wheat pasta with contents up to 710.9 µg/kg; however, ENN B was not detected in GF pasta samples [17].

Mycotoxin contents detected in GF pasta samples surveyed in this study was in accordance with data reported by other authors. The detected mycotoxins have been previously reported in different GF samples [18,19], especially FBs, which were the most analyzed and reported mycotoxins in GF foodstuffs. In the study conducted by Esposito et al. (2016) [18], 154 Italian GF products (breakfast cereals, biscuits, bread, canned corn, cornmeal, rice, pasta, cookies, sweet and savory snacks) were analyzed, showing an incidence of 85% for FBs (FB1, FB2 and FB3), with levels up to 272 µg/kg. Furthermore, high FBs levels were found in maize, corn-meal, and maize-flour samples in the survey conducted by Magro et al. (2011) [19], where 7% of analyzed samples showed contents above the ML fixed by the EU Regulation 1126/2007/EC [15]. FBs were also reported by Dall'Asta et al. (2009) [20] in 90% of the GF food samples reaching a maximum concentration level of 3310 µg/kg. Moreover, in many cases the sum of free and bound FBs exceeded the EU legal limit set for total FBs. Dall'Asta et al. [21] reported an incidence of 89% of FBs in GF products intended for celiac population and 7% of the samples exceed the EU legal limits [14]. Regarding pasta samples, these authors found a 93% of incidence and contents ranged from 27 µg/kg to 335 µg/kg. Results reported by Cano-Sancho et al. [22] showed the co-occurrence of FB1 and FB2 in most of the analyzed GF commodities together with AFs, DON and ZON. Our results were in accordance with those FBs contents reported by the above-mentioned studies, where FB1 showed higher incidence than FB2. Other studies have also reported high FBs (FB1 and FB2) contents [6,23], with maximum levels of 421 and 759 µg/kg, respectively. Furthermore, Huong et al. [24] found that 24% of maize samples and 8% of rice analyzed in their study were contaminated by FBs with contents ranging from 5.6 to 89.8 µg/kg and from 2.3 to 624 µg/kg, respectively.

Regarding DON occurrence, Cano Sancho et al. [22] reported DON levels only in two samples out of 18 GF pasta analyzed, with respective amounts of 163 and 270 µg/kg. In the survey performed by Herrera et al. [25], DON was investigated in 27 samples of GF samples intended for children age between 4 to 6 months, showing an incidence of 22% of positive samples with contents ranging from 33 µg/kg to 194 µg/kg. As regards the presence of ZON in GF samples, Cano Sancho et al. [22] reported ZON contamination in 6 ethnic food samples but ZON was not detected in any GF sample, which was in accordance with results reported by Brera et al. [6] where no ZON contamination was found in GF pasta samples. Contrary to these surveys, ZON showed a high incidence in GF pasta analyzed in our study.

Regarding emerging *Fusarium* mycotoxin levels, different pattern of contamination was observed. In the present study, only ENN A1 and BEA were detected in one and two samples, respectively. These findings agree with those reported by Decleer et al. [26], where trace levels of ENN A1 were reported. However, these authors reported ENN B and ENN B1 as the most prevalent mycotoxins, while ENNs type B were not detected in our study. In addition, BEA levels found by these authors were higher (up to 209.0 ± 39.7 µg/kg) than those obtained in our study.

Regarding AFs contamination, although AFs have been widely reported especially in maize-based foodstuffs, AFB1 was not detected in the present survey, according to studies carried out by Brera et al. [6] and Cano Sancho et al. [22], where AFB1 was not detected in any sample. On the other hand, other mycotoxins such as OTA and T-2 toxins were not detected in our study, whereas Brera et al. [6] found contents of these two mycotoxins in analyzed samples with some samples exceeding the maximum level set for OTA by the European legislation (Commission Regulation 1881/2006) [14].

Table 11.4. Risk characterization based on Exposure assessment of mycotoxins studied in different population groups for pasta. The results are reported per age/sex category; overall exposures are reported

Mycotoxin	Age/Sex (Years)	Bodyweight (kg) *	Mean Consumption (g) *	Consumption P95 (g) *	Contamination Range (µg/kg)	Exposure (ng/kg bw/day)	Exposure P95 (ng/kg bw/day)	TDI (ng/kg bw/day)
NIV	3–9.9 M/F	26.1	58.2	104.9	209.2–367.6	466.5–819.7	840.8–1477.4	1200
	10–17.9 M	57.1	63.6	128		233.0–409.4	468.9–824.0	(SCF, 2013)
	10–17.9 F	49.1	56.6	105.3		241.1–423.7	448.6–788.3	
	18–64.9 M	78.4	60.3	118.4		160.9–282.7	315.9–555.1	
	18–64.9 F	62.2	47.4	100		159.4–280.1	336.3–590.9	
	≥65 M	78.1	61.1	109.6		163.6–287.6	293.6–515.9	
	≥65 F	65	50.7	100.6		163.2–286.7	323.8–569.0	
DON	3–9.9 M/F	26.1	58.2	104.9	182.2–377.4	406.3–841.5	732.3–1516.8	1000
	10–17.9 M	57.1	63.6	128		202.9–420.4	408.4–846.0	(SCF, 2004)
	10–17.9 F	49.1	56.6	105.3		210.0–435.0	390.7–809.4	
	18–64.9 M	78.4	60.3	118.4		140.1–290.3	275.1–570.0	
	18–64.9 F	62.2	47.4	100		138.8–287.6	292.9–606.7	
	≥65 M	78.1	61.1	109.6		142.5–295.2	255.7–529.6	
	≥65 F	65	50.7	100.6		142.1–294.4	282.0–584.1	
HT-2 toxin	3–9.9 M/F	26.1	58.2	104.9	18.2–26.3	40.6–58.6	73.1–105.7	Σ T-2 + HT-2
	10–17.9 M	57.1	63.6	128		20.3–29.3	40.8–59.0	100
	10–17.9 F	49.1	56.6	105.3		21.0–30.3	39.0–56.4	(SCF, 2011a)
	18–64.9 M	78.4	60.3	118.4		14.0–20.2	27.5–39.7	
	18–64.9 F	62.2	47.4	100		13.9-20.0	29.3–42.3	
	≥65 M	78.1	61.1	109.6		14.2–20.6	25.5–37.0	
	≥65 F	65	50.7	100.6		14.2–20.5	28.2–40.7	
ZON	3–9.9 M/F	26.1	58.2	104.9	9.2–26.9	20.5–60.0	37.0–108.1	250
	10–17.9 M	57.1	63.6	128		10.2–30.0	20.6–60.3	(SCF, 2011b)
	10–17.9 F	49.1	56.6	105.3		10.6–31.0	19.7–57.7	
	18–64.9 M	78.4	60.3	118.4		7.1–20.7	13.9–40.6	
	18–64.9 F	62.2	47.4	100		7.0–20.5	14.8–43.2	
	≥65 M	78.1	61.1	109.6		7.2–21.0	12.9–37.7	
	≥65 F	65	50.7	100.6		7.2–21.0	14.2–41.6	

Mycotoxin	Age/Sex (Years)	Bodyweight (kg) *	Mean Consumption (g) *	Consumption P95 (g) *	Contamination Range (µg/kg)	Exposure (ng/kg bw/day)	Exposure P95 (ng/kg bw/day)	TDI (ng/kg bw/day)
FB1	3–9.9 M/F	26.1	58.2	104.9	39.9–246.9	89.0–550.6	160.4–992.3	Σ FB1 + FB2
	10–17.9 M	57.1	63.6	128		44.4–275.0	89.4–553.5	2000
	10–17.9 F	49.1	56.6	105.3		46.0–284.6	85.6–529.5	(SCF, 2003)
	18–64.9 M	78.4	60.3	118.4		30.7–189.9	60.3–372.9	
	18–64.9 F	62.2	47.4	100		30.4–188.2	64.1–397.0	
	≥65 M	78.1	61.1	109.6		31.2–193.2	56.0–346.5	
	≥65 F	65	50.7	100.6		31.1–192.6	61.8–382.1	
FB2	3–9.9 M/F	26.1	58.2	104.9	44.0–53.4	98.1–119.1	176.8–214.6	Σ FB1 + FB2
	10–17.9 M	57.1	63.6	128		49.0–59.5	98.6–119.7	2000
	10–17.9 F	49.1	56.6	105.3		50.7–61.6	94.4–-114.5	
	18–64.9 M	78.4	60.3	118.4		33.8–41.1	66.4–80.6	
	18–64.9 F	62.2	47.4	100		33.5–40.7	70.7–85.9	
	≥65 M	78.1	61.1	109.6		34.4–41.8	61.7–74.9	
	≥65 F	65	50.7	100.6		34.3–41.7	68.1–82.6	
ENN A1	3–9.9 M/F	26.1	58.2	104.9	1.7	3.8	6.8	No TDI
	10–17.9 M	57.1	63.6	128		1.9	3.8	established
	10–17.9 F	49.1	56.6	105.3		1.9	3.6	
	18–64.9 M	78.4	60.3	118.4		1.3	2.6	
	18–64.9 F	62.2	47.4	100		1.3	2.7	
	≥65 M	78.1	61.1	109.6		1.3	2.4	
	≥65 F	65	50.7	100.6		1.3	2.6	
BEA	3–9.9 M/F	26.1	58.2	104.9	17.3–21.9	38.6–48.8	69.5–88.0	No TDI
	10–17.9 M	57.1	63.6	128		19.3–24.4	38.8–49.1	established
	10–17.9 F	49.1	56.6	105.3		19.9–25.2	37.1–47.0	
	18–64.9 M	78.4	60.3	118.4		13.3–16.8	26.1–33.1	
	18–64.9 F	62.2	47.4	100		13.2–16.7	27.8–35.2	
	≥65 M	78.1	61.1	109.6		13.5–17.1	24.3–30.7	
	≥65 F	65	50.7	100.6		13.5–17.1	26.8–33.9	

**: Data reported by Leclerq et al. [27]. M: male; F: female.*

11.2.4 Exposure Assessment

To evaluate the mycotoxin exposure through the diet for CD patients, contents detected in the present survey were combined with data consumption of Italian population. Table 11.4 summarizes the exposure assessment of mycotoxins evaluated in different population groups based on pasta consumption of Italian population. The results were calculated following the equation described in Section 4.7. The Probable Daily Intake (PDI) was calculated only for detected mycotoxins, including NIV, DON, HT-2, ZON, FB1, FB2, ENN A1 and BEA. The PDI have been calculated by using the mean consumption value for the total population consulted for each age group and, as a worst-case scenario, exposure was calculated at 95th percentile of consumption. The PDI for age group from 0 to 2.9 years was not calculated since this age group are not regular consumers of the products included in the study. As reported in Table 11.4, the PDI for all detected mycotoxins were below the corresponding TDI established; however, the TDI levels for children (3–9.9 years) were surpassed for NIV, DON and the sum of T-2 and HT-2 toxins when assumed the 95th percentile of consumption. It must be highlighted that for all mycotoxins children age group (3–9.9 years) showed the highest PDI among all the age groups although the mean consumption and the P95 consumption were lower than that reported for other age groups. This can be explained by their lower body weight and thus, the unfavorable body weight/intake ratio, which result in a higher exposure [6].

The results reported in Table 11.4 suggest that mycotoxin exposure level might not pose a health risk for the average consumers; however, for certain populations, especially infants or children age group, and for heavy consumers, mycotoxin intake with GF pasta samples could exceed the safety limits. In this sense, although the common GF foodstuffs available for consumers are mainly intended for adults, children can be common consumers of this kind of products because CD is often diagnosed in early ages. For this reason, mycotoxin limits established for maize-based products intended for children should be considered, as they are lower than those established for adult population.

In addition, some studies revealed that celiac population may have important health implications consuming GF products due to the high occurrence of FBs in these products which implies a high FBs intake for celiac patients [21]. Nevertheless, in this study, PDI calculated for FBs (FB1 and FB2) did not exceed the TDI established for the sum of FBs (Table 11.4). On the other hand, PDI calculated for emerging *Fusarium* mycotoxins could not be compared to the limits established as no TDI has been established nor for ENNs neither for BEA.

11.3 MATERIALS AND METHODS

11.3.1 Chemicals and Reagents

Mycotoxin standards, namely aflatoxins (AFB1, AFB2, AFG1 and AFG2) fumonisins (FB1 and FB2), ochratoxin A (OTA), zearalenone (ZON), enniatins (ENN A, ENN A1, ENN B and ENN B1), beauvericin (BEA), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2), neosolaniol (NEO), and fusarenon-X (FUS-X), with purity ≥98%, were obtained from Sigma Aldrich (Milan, Italy) and they were stored at -20°C. Acetonitrile (MeCN), methanol (MeOH), and water were LC-MS grade and acquired from Merck (Darmstadt, Germany). Formic acid and ammonium formate were supplied by Fluka (Milan, Italy), whereas magnesium sulphate and sodium chloride were purchased from Sigma Aldrich (Milan, Italy).

Syringe filters with politetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 mm) were purchased from Phenomenex (Castel Maggiore, Italy). Centrifuge polypropylene tubes Corning PQ of 50 and 15 mL were provided by Corning Cable Systems (SRL, Turin, Italy).

Standard stock solutions of individual mycotoxins were prepared dissolving them in MeCN to reach a final concentration of 1 mg/mL, except for FB1 and FB2, which were prepared in MeCN/H_2O 50:50 *v/v* solution. Subsequently, working standard solutions containing all the investigated analytes were prepared at appropriate concentration levels to conduct spiking experiments. All stock and working standards solutions were stored in safety conditions at −20°C.

11.3.2 Sampling

Eighty-four GF pasta samples were evaluated to perform a surveillance of mycotoxins. Commercially available samples were acquired from several local markets of Campania region (Italy) during September to November 2020. This region was chosen due to the incidence of CD per 100,000 person-years amounted to 7.3 and 27.4 for adults and children, respectively, representing the third region for CD frequency in Italy [28].

As far as sample composition is concerned, all of collected samples contained maize and rice flour in different proportions (generally 30:70 *w/w*, respectively), with the exception of one 100% rice sample, one 100% buckwheat sample and two 100% maize flour samples. According to Commission Regulation EC/401/2006 [**29**], samples were milled throughout a high-speed food blender (Ika, mod. A11 basic, Staufen, Germany) and stored in a dark and dry place at 4°C until analysis.

11.3.3 Sample Preparation

Sample preparation procedure reported by Rodríguez-Carrasco et al. (2014) [11] was followed and slightly modified. Briefly, 4 g of ground sample were weighted in a 50 mL centrifuge tube and 7.5 mL of water with 0.1% (*v/v*) formic acid and 10 mL of MeCN were added. Then, the mixture was vortexed for 3 min and then 1 g of NaCl and 4 g of $MgSO_4$ were added. After that, the mixture was vortexed for 2 min and centrifuged at 5000 rpm (1960× *g*) for 5 min and a 0.5 mL of supernatant was diluted with deionized water in 1:1 (*v/v*) ratio. Finally, the extract of sample was filtered through the 0.2 µm filter and transferred to an autosampler vial for the UHPLC-Q-Orbritrap HRMS analysis.

11.3.4 Ultra-High Performance Liquid Chromatography Couple to Q Exactive Orbitrap Mass Spectrometry (UHPLC-Q-Orbritrap HRMS) Analysis

An Ultra-High-Performance Liquid Chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 Quaternary UHPLC pump working at 1250 bar, an autosampler device and a thermostated (40°C) Luna Omega C18 (50 × 2.1 mm, 1.6 µm particle size) column (Phenomenex, Castel Maggiore, Italy) were used to obtain the qualitative and quantitative profile of mycotoxins. The mobile phase composition consisted of (A) H_2O in 0.1% formic acid and 5 mM ammonium formate, and (B) MeOH in 0.1% formic acid and 5 mM ammonium formate. Mycotoxins were eluted using a 0.4 mL/min flow rate with the following gradient elution program: 0–0.5 min 20% of phase B, 1 min 40% of phase B, 6 min 100% of phase B, 8 min 20% of phase B, 10 min 20% of phase B. Injection volume was of 1 µL.

Detection was performed using a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Full scan data in both positive and negative mode were acquired at resolving power of 70,000 FWHM at m/z 200. Ion source parameters in negative mode (ESI-) were: spray voltage −4kV, sheath gas (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, capillary temperature 290°C, S-lens RF level 50, auxiliary gas heater temperature 305°C. Ion source parameters in positive mode (ESI+) were: spray voltage 4kV, sheath gas (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, capillary temperature 290°C, S-lens RF level 50, auxiliary gas heater temperature 305°C. A scan range of m/z 100–1000 was selected. The automatic gain control (AGC) was set at 1×10^6 and the injection time was set at 200 ms. Scan rate was set at 2 scans/s [30]. The accuracy and calibration of the HRMS instrument was tested daily using the reference standard mixture obtained from Thermo Fisher Scientific. Data processing was performed using the Xcalibur software, v. 3.0.63 used in 2020 (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA).

11.3.5 Method Performance

The proposed UHPLC-Q-Orbitrap HRMS method was in-house validated in terms of linearity, trueness, repeatability, within-laboratory reproducibility, limit of detection (LOD) and limit of quantitation (LOQ), according to the EU Commission Decision 2002/657/EC [**31**]. Linearity, expressed as correlation coefficient, was assessed by constructing calibration curves for all mycotoxins ranging

from 0.25 to 1000 µg/kg. The data were fit to a linear least-squares regression curve with a 1/x weighting, and they were not forced through the origin. Linearity was assumed when regression coefficients were greater than 0.990 with residuals lower than 30%. To assess the matrix effect on the chromatographic response, the slopes of the calibration in standard solution with those obtained in matrix-matched standards were compared and expressed as percentage of signal suppression/ enhancement (% SSE), as follows:

$$\%\mathrm{SSE} = \left(1 - \frac{Sm}{Ss}\right) \times 100 \quad (1)$$

where *Sm* is the slope of calibration curve in matrix-matched standard and *Ss* is the slope of calibration curve in standard solution.

The trueness was evaluated throughout recovery studies. Recovery assays were performed in triplicate at three fortification levels (125, 62.5 and 12.5 µg/kg). An additional spiking level of 500 µg/kg was included for DON and FBs due to the higher permitted maximum limits of these mycotoxins in cereal-based foodstuffs. Spiked samples were placed overnight, and then the samples were extracted as previously mentioned. The precision of the method was calculated by the repeated analysis of spiked samples at all tested fortification levels and expressed as the relative standard deviation (%RSD) of measurements (n = 3) carried out in the same day (repeatability) and in three different days (within-laboratory reproducibility). LOD was established as the minimum concentration where the molecular ion can be identified (mass error value below 5 ppm) and LOQ was set as the lowest concentration that allowed the concentration of the analyte to be determined with accuracy and precision ≤20%.

11.3.6 Quality Assurance/Quality Control (QA/QC)

Chromatographic and mass spectrometry data were used for confirmation. In detail, mycotoxin identification was carried out by two identification points: the peaks for the mycotoxins included in the study were confirmed by comparing the retention time from extracted ion chromatogram (XIC) of the peak in samples with those of standard solutions at a tolerance of ± 2.5%; and by the exact mass set to five decimal places. Mycotoxin standards chromatograms are provided in Supplementary material (Fig. S1). The mass accuracy (Δ) for a measured ion was calculated according to the following formula and expressed as part-per-million (ppm) [17]:

$$\Delta(\mathrm{ppm}) = 1 \times 10^6 \frac{(\mathrm{m/z_{measured}} - \mathrm{m/z_{theoretical}})}{\mathrm{m/z_{theoretical}}} \quad (2)$$

In order to demonstrate the effectiveness of the validated method, a reagent blank, a blank sample, a replicate sample, and a matrix-matched calibration were included at the beginning and at the end of each batch of samples for QA/QC analysis. Samples were analyzed in duplicate; measurable concentrations of mycotoxins were detected in both replicates.

11.4.7 Mycotoxin Probable Daily Intakes Calculation

The exposure assessment was carried out based on a deterministic approach by combining the mean content of a mycotoxin *Cm* (µg/kg) obtained from the samples here analyzed and the food consumption data *K* (g/day) consulted in the survey published by the Italian Institute of Nutrition (INRAN) [28] of different population groups: children (3–9.9 years), teenagers (10–17.9 years), adults (18–64.9 years), and elderly (65 years and above). Starting from the teenagers age group, food consumption was also differentiated per gender. And body weight data were assumed those reported in the INRAN survey for the defined population groups. Thus, the probable daily intake (PDI, µg/kg bw/day) of each mycotoxin *m*, was assessed as follows [32]:

$$\mathrm{PDI}_m = \frac{C_m \times K}{\mathrm{bw}} \quad (3)$$

Additionally, a worst-case scenario was also considered by taking into account the 95th percentile of food consumption data to assess the exposure for those large scale consumers.

11.4 CONCLUSIONS

An UHPLC-Q Exactive Orbitrap MS method has been in-house validated for the simultaneous determination of 21 mycotoxins. Results showed that the proposed analytical procedure was accurate (recovery range from 71 to 125% for vast majority of analytes), precise (RSDs < 12.1%), and sensitive (LODs from 0.05 to 7.8 µg/kg) to fulfil the criteria established in European guidelines. FBs, ZON and DON were found as common contaminants in maize-based foodstuffs, underlining that these foodstuffs can be contaminated with *Fusarium* mycotoxins at levels that could represent a risk for the higher consumers of these products, especially celiac people, due to the continuous ingestion of these foodstuffs. Regarding exposure assessment, it has been determined that GF consumers are exposed to mycotoxin ingestion, and exposure to mycotoxins could suppose a risk especially in the worst-case scenario for higher consumers and concretely for children age group. Thus, with the aim of reducing the risk associated to the ingestion of mycotoxins commonly present in these foodstuffs, research on mycotoxin contamination in these products and their mitigation should be carried out.

SUPPLEMENTARY MATERIALS

The following are available online at https://www.bookpi.org/wp-content/uploads/2022/05/Chapter-11_Supplementary-Materials.pdf, Fig. S1: UPLC-Q-Orbitrap HRMS extracted ion chromatogram of a blank GF sample spiked at 12.5 µg/kg of each mycotoxin.

AUTHOR CONTRIBUTIONS

Conceptualization, E.F., J.M. and A.R.; methodology, J.T.; software and validation, G.G., A.G., Y.R.-C. and J.T.; formal analysis, J.T.; investigation, J.T. and E.F.; resources, A.R. and J.M.; data curation, G.G. and A.G.; writing—original draft preparation, J.T., G.G. and A.G.; writing—review and editing, J.T. and Y.R.-C.; visualization, Y.R.-C.; supervision, A.R. and J.M.; project administration, A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 12
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Cytoprotective Effects of Fish Protein Hydrolysates against H_2O_2-Induced Oxidative Stress and Mycotoxins in Caco-2/TC7 Cells

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ABSTRACT

Many studies report the potent antioxidant capacity for fish protein hydrolysates, including radical scavenging activity and inhibition ability on lipid peroxidation (LPO). In this study, the in vitro cytotoxicity of protein hydrolysates from different salmon, mackerel, and herring side streams fractions was evaluated in the concentration range from 1 to 1:32 dilution, using cloned human colon adenocarcinoma cells TC7 (Caco-2/TC7) by MTT and PT assays. The protein hydrolysates' antioxidant capacity and oxidative stress effects were evaluated by LPO and reactive oxygen species (ROS) generation, respectively. The antioxidant capacity for pure and bioavailable hydrolysate fraction was also evaluated and compared. Additionally, mycotoxin levels were determined in the fish protein hydrolysates, and their cytoprotective effect against T-2 toxin was evaluated. Both hydrolysates and their bioavailable fraction induced similar cell viability rates. The highest cytoprotective effect was obtained for the salmon viscera protein hydrolysate (HSV), which increased the cell viability by 51.2%. ROS accumulation induced by H_2O_2 and LPO was suppressed by all pure hydrolysates. The cytoprotective effect of hydrolysates was observed against T-2. Moreover, the different fish fraction protein hydrolysates contain variable nutrients and unique bioactive peptide composition showing variable bioactivity, which could be a useful tool in developing dietary supplements with different target functional properties.

Keywords: Fish hydrolysates; cytotoxicity; oxidative stress; cytoprotective effect; bioavailability.

12.1 INTRODUCTION

Synthetic antioxidants are commonly used to preserve food products, including butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ) and butylated hydroxyanisole (BHA), but their use is limited due to the potential toxic effects in humans [1,2,3]. Therefore, there is an increasing consumer demand for natural antioxidants in order to delay food deterioration caused by oxidation, as well as to alleviate the oxidative stress caused by mitochondrial generation of reactive oxygen species (ROS) [4,5,6,7], proteins and peptides being of great interest [8,9,10].

Protein hydrolysates and peptides exhibit multiple physiological functionalities such as antimicrobial, antioxidant, antihypertensive, and cholesterol-lowering effects and immunomodulatory activities [11,12,13]. Moreover, in some recent studies, the ability of fish protein hydrolysates to suppress DSS-Induced colitis by modulating intestinal inflammation in mice, as well as to suppresses diabetes and modulates intestinal microbiome in a murine model of diet-induced obesity, has been observed [14,15].

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Filleting fish generates large amounts of protein-rich side stream materials, such as heads, backbones, viscera, and trimmings, with potential use for human consumption when handled properly and according to food-grade regulations. In order to use side stream fish proteins directly, the production of fish protein hydrolysates is a promising option, where the proteins are cleaved into smaller and more water-soluble peptides compared with the native protein [16].

Fish protein hydrolysates can have great application potential in the food, medical, and cosmetic industries [17]. An example is collagen, abundant in connective tissue rich fish fractions, such as skin and bones [18]. Marine collagen has shown promising activities such as antioxidant, wound healing, anti-aging, the adipogenic differentiation inhibition, and anti-freezing, among others [13,19,20,21,22]. Numerous studies have reported that peptides derived from fish collagen and protein hydrolysates, of a variety of fish species such as tuna, salmon, cuttlefish, sardinella, tilapia, and shrimp, showed potent antioxidant activities including radical scavenging activity, reducing power, and inhibition ability on lipid and protein oxidation [23,24]. Moreover, Zhao et al. [25] and Wong et al. [26] reported that protein hydrolysates of miiuy croaker swim bladders and hydrolysate of blue-spotted stingrays could scavenge radicals and inhibit lipid peroxidation. Yarnpakdee et al. [27] studied lipid oxidation in protein hydrolysates from the muscles of Indian mackerel. Sheriff et al. [28] reported powerful antioxidant, lipid peroxidation inhibition and free radical scavenging activity of hydrolysate from the backbones of Indian mackerel. Kumar et al. [29] found a good ability to scavenge hydroxyl radicals from antioxidant peptides obtained from horse mackerel viscera protein.

The bioavailability of fish protein hydrolysates has been evaluated in several studies [30,31,32,33,34] using the in vitro digestion technique. The human colorectal adenocarcinoma (Caco-2) cell line is the international validated model used for evaluating the intestinal absorption of food nutrients, also recognized as a reliable cell model for cell-based bioassays of food antioxidant activity [35]. These cells are able to differentiate in long-term culture and to polarize, when seeded on semi-permeable membranes, on which they form a continuous monolayer with tight junctions mimicking the intestinal barrier [36]. Nevertheless, Caco-2 cells are heterogeneous, and their physiological performance is highly dependent on culture conditions, often leading to variable transport properties and permeability. In order to reduce the heterogeneity of the cells, several clones have been isolated from Caco-2 cells. The TC7 clone was obtained from a late passage of the parental Caco-2 cell line and characterized for its ability to transport [36]. Zucco et al. [37] compared the characteristics of four Caco-2 cell lines and concluded that the TC7 clone consisted of a more homogeneous cell population, with more representative functions of the small intestinal enterocytes. Thus, in our study we evaluated the suitability of Caco-2/TC7 as a predictive in vitro model for the intestinal transport of fish protein hydrolysate compounds.

Mycotoxins are secondary toxic metabolites produced by fungi found in cereals and their side streams, monitored in both food and feed by national and international public health programs to evaluate compliance with the current regulations [38,39]. Aquaculture fish are commonly exposed to feed borne mycotoxins, especially from wheat and maize derived raw materials largely used in aquafeed formulations due to their favorable availability, price, and protein content [40]. There are severable studies evaluating the presence of mycotoxins in terrestrial animals, whereas the data on the occurrence of mycotoxins in farmed fish is limited [41].

The aims of this study were: (i) to evaluate the viability of Caco-2/TC7 cells after 24 h exposure of protein hydrolysates based on heads, backbones, and viscera from salmon, mackerel, and herring (the latter providing only viscera), and fish collagen based on flounder skin before and after an in vitro digestion process; (ii) to determine the cytoprotective/cytotoxic effects of these protein hydrolysates against oxidative stress-induction in Caco-2/TC7 cells; (iii) to identify possible fungal metabolites in these hydrolysates using a liquid chromatography high resolution mass spectrometry technique, and to evaluate the cytoprotective effect of hydrolysates in co-exposure with T-2 toxin.

12.2 MATERIALS AND METHODS

12.2.1 Reagents

The reagent grade chemicals and cell culture compounds used, namely Dulbecco's Modified Eagle's Medium (DMEM + GlutaMAX™), antibiotic solution (penicillin-streptomycin), non-essential amino

acids (NEAA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fungizone, trypsin/EDTA solutions, Phosphate Buffer Saline (PBS), Fetal Bovine Serum (FBS), methylthiazoltetrazolium salt (MTT) dye, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), deferoxamine mesylate salt (DFA), di-ter-butylmethylphenol (BHT), 1,3,3,3 tetramethoxipropan (TMP), 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA), t-octylphenoxypolyethoxyethanol, glacial acetic acid, H_2O_2, NaOH, NaCl, glycine, Coomassie Brilliant Blue, ethyl acetate, and $CaCl_2$, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) was purchased from VWR International (LLC, Monroeville, PA, USA). Deionized water (resistivity < 18 MΩ cm) was obtained by filtering tap water through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standards of mycotoxins were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Enzymatic protein hydrolysates based on mackerel heads (HMH), mackerel backbones (HMB), mackerel viscera (HMV), salmon heads (HSH), salmon backbones (HSB), salmon viscera (HSV), and herring viscera (HHV) were produced according to Aspevik et al. [42]. In brief, the raw materials were mixed with tap water (1:1) and subjected to enzymatic hydrolysis for 50 min at 55 °C using the protease Food Pro PNL (DuPont Wilmington, DE). After thermal inactivation of the enzyme (>90 °C, 10 min), the water phase (hydrolysate) was separated from the insoluble fraction and lipids and further purified using a 0.1 µm ceramic membrane filter before spray drying to a light-yellow powder. The chemical properties of protein hydrolysates of mackerel and salmon are shown in Aspevik et al. [42]. Flounder skin collagen was kindly provided by Seagarden (Karmøy, Norway).

12.2.2 Cell Culture and Treatment

The Caco-2/TC7 cells were cultured in DMEM medium, supplemented with 10% FBS, 1% HEPES, 1% NEAA, 0.2% fungizone, 100 U/mL penicillin, and 100 mg/mL streptomycin. The incubation conditions were pH 7.4, 5% CO_2 at 37°C and 95% air atmosphere at constant humidity. The medium was changed every 2–3 days. All experiments were carried out 7 days post-seeding, when the integrity of the Caco-2/TC7 cell monolayers had a measure of transepithelial electrical resistance (TEER) above 300 Ω/cm^2. The clonal line Caco-2/TC7 cells were kindly provided by Central Service for Experimental Research (SCSIE) of the University of Valencia (Valencia, Spain).

12.2.3 In Vitro Cytotoxicity

Cytotoxic effects were determined in Caco-2/TC7 cells by the MTT and total protein content (PC) assays. The two assays have been extensively used for in vitro toxicological studies used to measure cell proliferation and survival. The MTT assay determines the viability of cells by the reduction in yellow soluble MTT, only in the metabolically active cells, via a mitochondrial-dependent reaction to an insoluble purple formazan crystal. The MTT viability assay was performed according to Ruiz et al. [43].

Briefly, the Caco-2/TC7 cells were plated in 96-well tissue culture plates at a density of 2×10^4 cells/well. In Caco-2/TC7, the culture medium was replaced with fresh medium containing serial dilutions of each hydrolysate. The amount of 1 mg of the powdered hydrolysates was resuspended in 1 mL of fresh medium. Following this, 1:2 dilutions were carried out using fresh medium. The range of concentrations of the hydrolysates were: 1 (mg/mL), 1:2 (0.5 mg/mL), 1:4 (0.25 mg/mL), 1:8 (0.125 mg/mL), 1:16 (0.0625 mg/mL), and 1:32 (0.03125 mg/mL). The hydrolysates were exposed over 24 h. During the exposure time, neither the medium nor the hydrolysate was replenished. After 24 h of exposure, the medium was removed and 200 µL of fresh medium was added to each well. Then, 50 µL/well of MTT was added, and the plates were returned to the incubator in the dark. After 3 h of incubation, the MTT solution was removed and 200 µL of DMSO was added, followed by 25 µL of Sorensen's glycine buffer. Plates were gently shaken for 5 min to achieve the complete dissolution. The absorbance was measured at 540 nm using an automatic ELISA plate reader (MultiSkanEX, Thermo Scientific, Walthman, MA, USA).

The PC method is based on the increase in absorbance of Coomassie Brilliant Blue dye when binding to proteins. The assay was performed in the same 96-well plates where the MTT test was carry out. First, the plates were washed with PBS and each well received 200 µL of NaOH 0.1 N to dissolve the

proteins. After 2 h of incubation, 170 µL of NaOH was removed from each well and 180 µL of diluted 22% Coomassie Brilliant Blue was added. The plates remained for 30 min at room temperature and the absorbance was measured at 620 nm in an automatic ELISA plate reader (MultiSkanEX, Thermo Scientific, Walthman, MA, USA).

For MTT and PC assays, cell viability was expressed as a percentage relative to the control solvent (medium DMEM + GlutaMAX™). Determinations were performed in three independent experiments with 4 replicates for each one. The mean inhibition concentration (IC_{50}) values were calculated using SigmaPlot version 11 (Systat Software Inc., GmbH, Düsseldorf, Germany).

12.2.4 Intracellular ROS Generation

Early intracellular ROS production was monitored in Caco-2/TC7 cells by adding H_2-DCFDA. The H_2-DCFDA is taken up by cells and then deacetylated by intracellular esterase's; the resulting non-fluorescent 2′,7′-dichlorodihydrofluorescein (H_2-DCF) is switched to highly fluorescent dichlorofluorescein (DCF) when oxidized by ROS. The generation of ROS was monitored according to Ruiz-Leal and George [44]. Briefly, 2×10^4 cells/well were seeded in a 96-well black culture microplate. In Caco-2/TC7 cells the culture medium was replaced, and cells were loaded with 20 µM H_2-DCFDA in a fresh medium for 20 min. Then two assays (A and B) were simultaneously carried out. Assay A: H_2-DCFDA was removed and 200 µL/well of fresh medium or medium with hydrolysates 1 mg/mL was added. Assay B: H_2-DCFDA was removed and 200 µL/well of fresh medium or medium with hydrolysates (1 mg/mL) and 5 mM H_2O_2 was added. In both assays, the ROS level was monitored by measuring the increase in fluorescence on a Wallace Victor^2, model 1420 multilabel counter (PerkinElmer, Turku, Finland), at intervals up to 2 h at excitation/emission wavelengths of 485/535 nm, respectively. Results were expressed as fluorescence intensity. Determinations were performed in three independent experiments with 16 replicates each.

12.2.5 Lipid Peroxidation Assay

Lipid peroxidation (LPO) assay was carried out by determining the formation of reactive thiobarbituric acid reactive substances (TBARS), according to Ferrer et al. [45]. The TBARS allow us to determine the production of a red adduct between TBA and malondialdehyde (MDA), which is a biomarker used to prove that the LPO process has occurred. Briefly, 2.25×10^5 cells/well were seeded in six-well plates. In Caco-2/TC7 cells two assays (A and B) were simultaneously carried out after confluence. Assay A: the culture medium was replaced with fresh medium with hydrolysates 1 mg/mL. Assay B: the culture medium was replaced with fresh medium with 5 mM H_2O_2 for 5 min, except the control. After that, the culture medium with H_2O_2 was replaced with fresh medium with hydrolysates (1 mg/mL). In both assays, the Caco-2/TC7 cells were exposed to 1 mg/mL of hydrolysates for 24 h. Then, the medium was removed, and cells were washed with PBS, homogenized in 150 mM sodium phosphate buffer (NaH_2PO_4) pH 7.4 and lysate with the Ultra-Turrax T8 IKA®-WERKE. Immediately, cells were boiled at 100 °C in a water bath for 20 min under acidic conditions in the presence of 0.5% TBA, 1.5 mM DFA, and 3.75% BHT. After that, the samples were placed on ice for 5 min and centrifuged at 2880× *g* for 15 min. The absorbance was measured at 532 nm. Three independent experiments were conducted with three replicates each. Results were expressed as ng of MDA/mg of protein measured by the Lowry method.

12.2.6 Comparison of Cytotoxicity between Pure Hydrolysates and Their Bioavailable Fraction

The Caco-2/TC7 were exposed to 0.0625 mg/mL of bioaccessible fraction of each hydrolysate. To obtain the bioaccessible fraction, the standardized method INFOGEST was applied. Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), Simulated Intestinal Fluid (SIF), and enzymatic activity assays were prepared according to Minekus et al. [46]. Due to the high percentage of proteins in the hydrolysate samples, the salivary step was carried out without amylase enzyme. Briefly, 2.5 mL of hydrolysate solution, 2 mL of SSF, 12.5 µL of 0.3 M $CaCl_2$, and deionized water to a final volume of 5 mL were mixed for 2 min. Afterwards, to simulate the gastric phase, 3.75 mL of SGF, 0.8 mL of

pepsin solution (25.000 U/mL), and 2.5 µL of 0.3 M $CaCl_2$ were added. Then, the pH was adjusted to 3.0, and deionized water was added up to a final volume of 10 mL. This gastric mixture was incubated for 2 h. Subsequently, 5.5 mL of SIF containing pancreatin (100 U trypsin activity/mL) and bile salts (10 mmol/L), and 20 µL of 0.3 M $CaCl_2$ were added. The pH was adjusted to 7.0, deionized water was added up to a final volume of 20 mL, and the intestinal mixture was incubated for 2 h. The oral, gastric, and intestinal steps were performed by mechanical shaking at 95 *x*rpm and 37 °C. At the end of the in vitro digestive process, samples were cooled in an ice bath and centrifuged at 3100× *g* and 4 °C for 60 min to obtain the bioaccessible fraction.

The Caco-2/TC7 cells were seeded at a density of 2.25×10^5 cells in inserts 6-well Transwell Permeable Supports, 12 mm diameter (Corning Life sciences, Corning, NY, USA) and 0.4 mm of pore size, and grown for 7 days. The culture medium in the apical and basolateral sides was replaced every 2–3 days. The integrity of the Caco-2/TC7 cell monolayers was confirmed by measuring the transepithelial electrical resistance (TEER) using an electric resistance device (Millicell-ERS, Millipore Corp), and monolayers with TEER above 300 Ω/cm^2 were used for the bioavailability assay. Medium of apical (upper compartment) and basolateral side (lower compartment) was removed, and transport was assessed by paracellular passage of bioaccessible fraction of hydrolysates (0.0625 mg/mL) in the apical side to the basolateral side. Therefore, 1.5 mL of bioaccessible fraction of digested hydrolysates was added to the apical side and 2 mL of medium without serum was added to the basolateral side. Control samples composed by transport medium without serum were also evaluated. The medium of the basolateral side, which is the bioavailable fraction, is collected. Then, with the bioavailable fraction, the MTT assay was carried out to determine the cytotoxicity of bioavailable fraction of hydrolysates at 0.0625 mg/mL. The results obtained were compared with the pure hydrolysates at 0.0625 mg/mL. The concentration 0.0625 mg/mL was chosen as it is amongst those which exerted the highest cytoprotective effect in Caco-2/TC7 cells during the MTT assay (Fig. 12.1). Determinations were performed in three independent experiments with 4 replicates each.

12.2.7 Cytoprotective Effect of Hydrolysates Exposed Together with T-2 Toxin

The T-2 toxin (T-2) is a *Fusarium* mycotoxin frequently occurring in cereals worldwide and the most toxic fusariotoxin. The recent animal dietary exposure to T-2 conducted by EFSA, reported the occurrence of T-2 in feed in concentrations of up to 405 µg/kg [47]. Hence, the cytoprotective effect of hydrolysates in Caco-2/TC7 cells was evaluated by MTT assay, exposing 60 nM T-2 to each hydrolysate at 0.0625 mg/mL. To develop the MTT assay, 2×10^4 cells/well were plated in 96-well tissue culture plate and the assay was performed as described in Section 2.3 In vitro cytotoxicity. The results obtained were compared with cells exposed to 60 nM T-2 and pure hydrolysates at 0.0625 mg/mL individually. The concentration of 60 nM was chosen as it showed a cytotoxic effect in previous studies in our laboratory [48]. Determinations were performed in three independent experiments with 4 replicates each.

12.2.8 Determination of Fungal Metabolites in Fish Hydrolysates through Liquid Chromatography Quadrupole Time of Fliht Mass Spectrometry

The extraction of mycotoxins from hydrolysates of fish samples was performed according to the sample preparation procedure described by Taroncher et al. [48] slightly modified. In brief, 0.5 mL of hydrolysate fish sample was collected and transferred to a 4 mL Eppendorf Safe-Lock microcentrifuge tube. Then, 3 mL of ethyl acetate was added, and the mixture was shaken for 2 min with an Ultra-Turrax Ika T18 basic (Staufen, Germany) and centrifuged at 5600× *g* for 3 min (Centrifuge 5910R, Eppendorf, Germany). The supernatant phase was collected and evaporated under a gentle N_2 stream at 45°C to dryness with a TurboVap-LV (Zymark, Allschwil, Switzerland), re-dissolved in 0.14 mL of a mixture methanol:water (70:30, *v*/*v*), and then filtered through a 0.2 µm filter prior to the analysis.

The analysis was performed using a liquid chromatography quadrupole time of flight mass spectrometry (LC-Q-TOF MS) system consisting of an LC Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, an autosampler and a binary pump. The column was a Gemini NX-C_{18} (150 × 2 mm, i.d. 3 µm, Phenomenex, Torrance, CA, USA)

with a guard column C_{18} (4 × 2 mm, i.d. 3 µm). Mobile phases consisted of Milli-Q water with 0.1% formic acid and 5 nM ammonium formate as solvent system A, and methanol with 0.1% formic acid and 5 nM ammonium formate as solvent system B, with the following elution gradient: 0.2 min, 20% B; 0.5 min, 20–40% B; 5 min, 100% B; and 2 min, decrease to 20% B, maintained for 6 min. The flow rate used was 0.200 mL/min, and the total run time was 14 min. The sample volume injected was 20 µL.

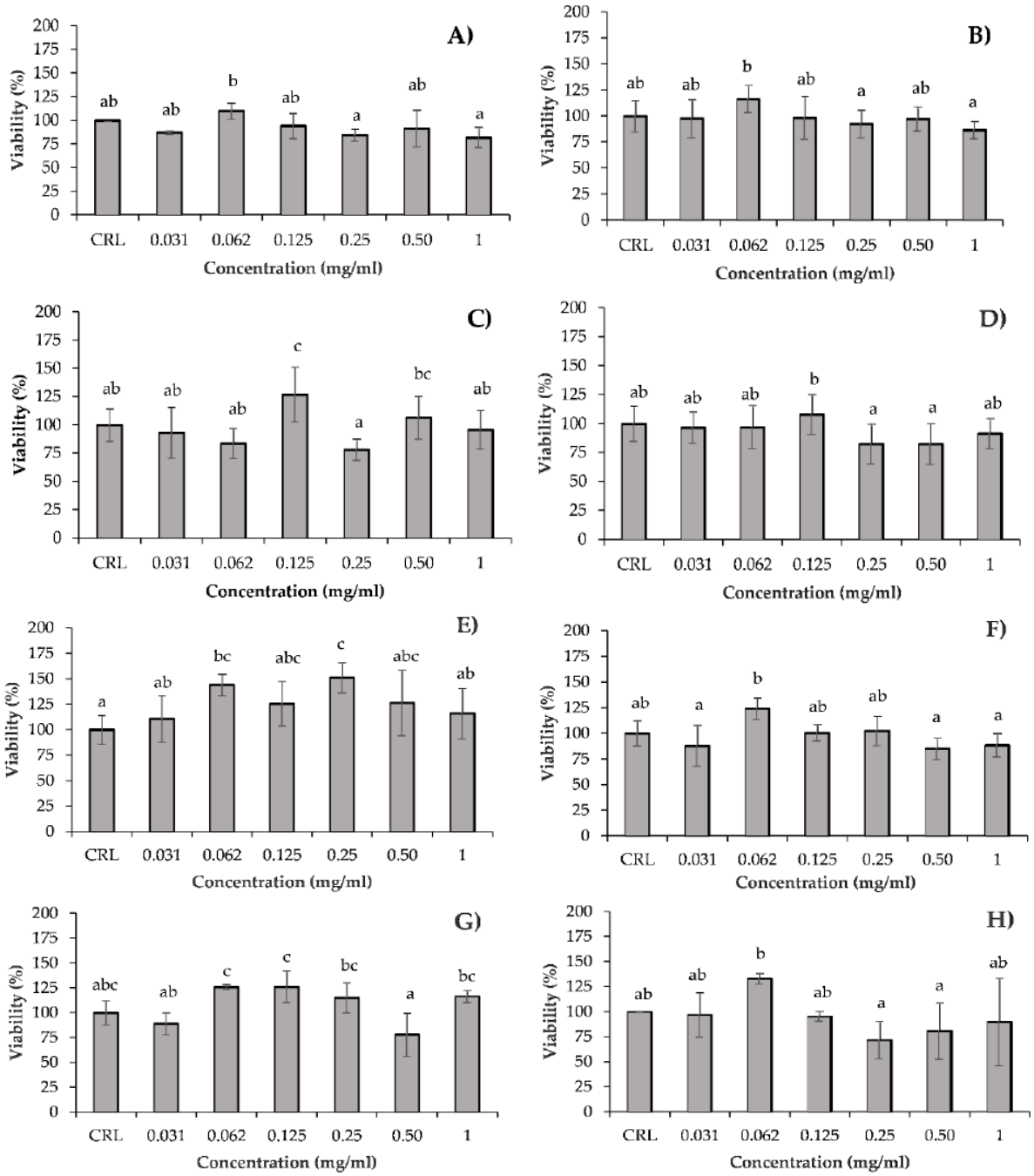

Fig. 12.1. Effect of HSH (A); HMH (B); HSB (C); HMB (D); HSV (E); HMV (F); HHV (G); and collagen (H) on cell viability (MTT assay) in Caco-2/TC7 cells after 24 h of exposure at increasing concentrations from 0 to 1 mg/mL. Values are expressed as mean ± SEM (*n* = 3). Values in the same figure with different superscript letter are significantly different (*p* < 0.05). CRL: control

The mass spectrometry (MS) analysis was carried out using a 6540 Agilent Ultra-High Definition Accurate-Mass Q-TOF MS, equipped with an Agilent Dual jet Stream electrospray ionization (Dual AJS ESI) interface in both positive and negative ionization modes. The conditions were as follows: sheath gas temperature 350 °C at a flow rate of 8 L/min, capillary voltage 3500 V, nebulizer pressure 45 psig, drying gas 10 L/min, gas temperature 300 °C, skimmer voltage 65 V, octopole RF peak 750 V, and fragmentor voltage 130 V. Analyses were performed using AutoMS/MS mode in a mass range of 50–1200 *m*/*z*. The acquisition rate was 3 scans/s for three different collision energies (10, 20, and 40 eV). Internal mass correction was enabled using two reference masses in positive mode (121.050873 and 922.009798 *m*/*z*) and two reference masses in negative mode (112.985587 and 1033.988109 *m*/*z*). Instrument control and data acquisition were performed using Agilent MassHunter Workstation software B.08.00. Potential analytes were identified by the MassHunter METLIN Metabolite PCD (Personal Compound Database) and PCDL (Personal Compound Database and Library) from Agilent Technologies.

12.2.9 Statistical Analysis

The statistical analysis of the data was carried out using the Statgraphics version 16.01.03 statistical package (IBM Corp., Armonk, NY, USA). Data were expressed as the mean ± standard error of the mean (SEM) of different independent experiments. The statistical analysis of the results was performed using the Student t-test for paired samples. Differences between groups were analyzed using the one-way analysis of variance (ANOVA), followed by a Tukey HDS post-hoc test for multiple comparisons. Statistical significance was considered as $p \leq 0.05$ and tendencies as $0.1 < p < 0.05$.

12.3 RESULTS

12.3.1 In Vitro Cytotoxicity

The safe supplementation level of hydrolysates HSV, HSB, HSH, HMH, HMB, HMV, HHV, and collagen based on the evaluation of cytotoxic effect in Caco-2/TC7 cells was evaluated by MTT and PC assays over 24 h. The concentration range of the hydrolysates selected to study the potential cytotoxic/cytoprotective effects on these cells was: 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL.

After 24 h of treatment, most hydrolysates did not show significant effects on the mitochondrial function of Caco-2/TC7 cells at any of the concentrations tested, except HSB at 0.125 mg/mL, and HSV at 0.0625 mg/mL and 0.25 mg/mL (Fig. 12.1). The maximum measured MTT increase was 27% for HSB, and 51.2% for HSV. The increase in cell viability can be due to the hormetic effect of the test hydrolysates in Caco-2/TC7 cells.

In the PC assay (Fig. 12.2) all hydrolysates, except HSB and HMV, showed a hormetic effect when the cells were exposed to specific dilutions, including 0.0625 mg/mL. The maximum measured PC increases were 18% for HMB, 19% for HSV, 69% for collagen, 139% for HHV, 140% for HSH, and 214% for HMH. At 0.0625 mg/mL used in the subsequent tests, the PC increasing effect of the different hydrolysates was as follows: HSV = HMB < collagen < HSH < HHV < HMH ($p = 0.000$).

12.3.2 Intracellular ROS Generation

The intracellular accumulation of ROS in Caco-2/TC7 cells exposed to hydrolysates (HSV, HSB, HSH, HMH, HMB, HMV, HHV, and collagen) (1 mg/mL) without (Fig. 12.3) and with (Fig. 12.4) H_2O_2 was analyzed using H_2-DCFDA. Fig. 12.3 shows ROS generation grouped by type of fish hydrolysate. Fig. 12.3 shows differences between ROS generation of hydrolysates with respect to the control. In general, ROS production increased slightly at 5 min, and then decreased and stabilized 60 min after exposure, at lower levels than at trial start, but without significant differences with respect to the control. Only significant differences of HMH and collagen, at 90 and 15 min, respectively, were determined with respect to the control (Fig. 12.3; Table 12.1). The production of ROS in Caco-2/TC7 cells treated with salmon heads hydrolysate (HSH) and collagen was lower, whereas was higher in those treated with HSB, HSV, HMH, and HMB as compared to the control (Table 12.1). No

differences were found between HMV, HHV, and control (Table 12.1). Among the different hydrolysates, the lowest ROS accumulation in Caco-2/TC7 cells was observed by exposure with HSH, followed and at increasing ROS accumulation levels by collagen, then HMV, HHV, HMB, HSB, HMH, and lastly HSV at 120 min, but not all differences were statistically significant (Table 12.1). Significant differences appeared at 15 min exposure, when higher ROS accumulation was found in the HMH and HSV as compared to the HSH treatment (p = 0.003; Tukey HSD), and again at 90 min exposure, when ROS accumulation was significantly higher in HSB as compared to the collagen treatment (p = 0.001; Tukey HSD).

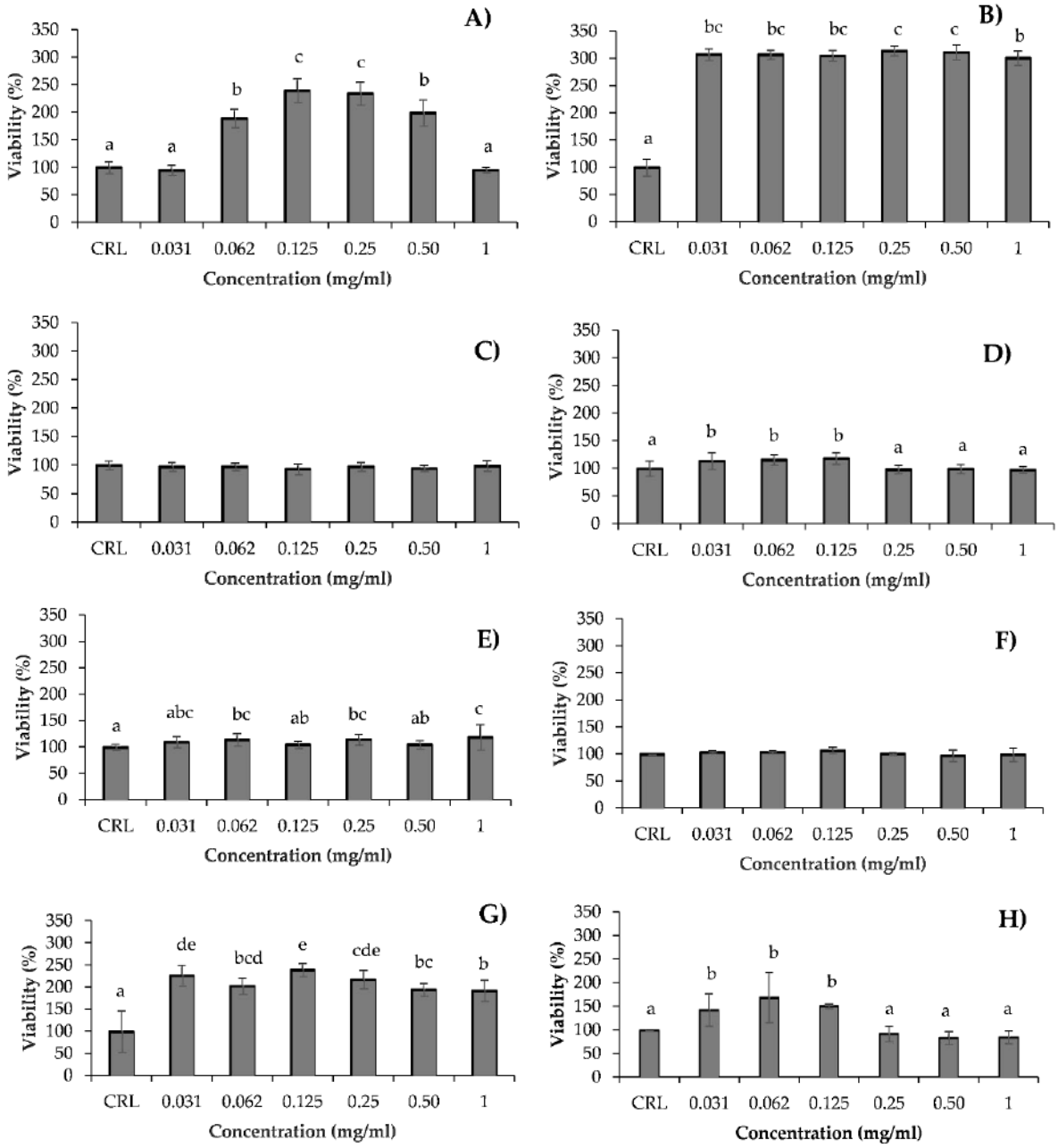

Fig. 12.2. Effect of HSH (A); HMH (B); HSB (C); HMB (D); HSV (E); HMV (F); HHV (G); and collagen (H) on cell viability (PC assay) in Caco-2/TC7 cells after 24 h of exposure at increasing concentrations from 0 to 1 mg/mL. Values are expressed as the mean ± SEM (n = 3). Values in the same figure with different superscript letter are significantly different (p < 0.05). CRL: control

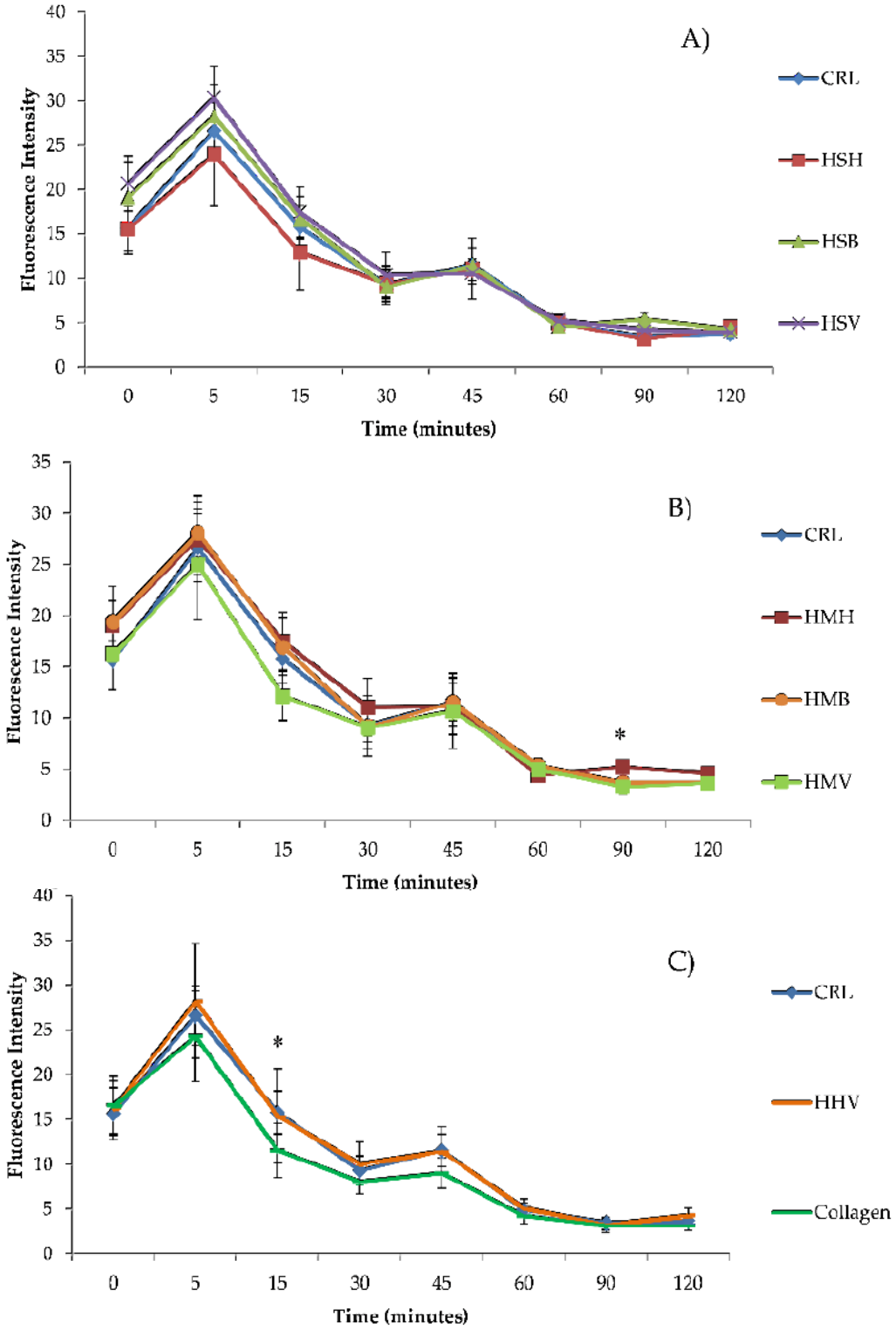

Fig. 12.3. Time dependence of ROS-induced fluorescence in Caco-2/TC7 cells exposed to (A) salmon hydrolysates, (B) mackerel hydrolysates, and (C) herring hydrolysates and fish collagen at 1 mg/mL without oxidative stress induced by H_2O_2. Results are expressed as the mean ± SEM (*n* = 3). (*) $p \leq 0.05$ indicates significant differences compared to the control (CRL)

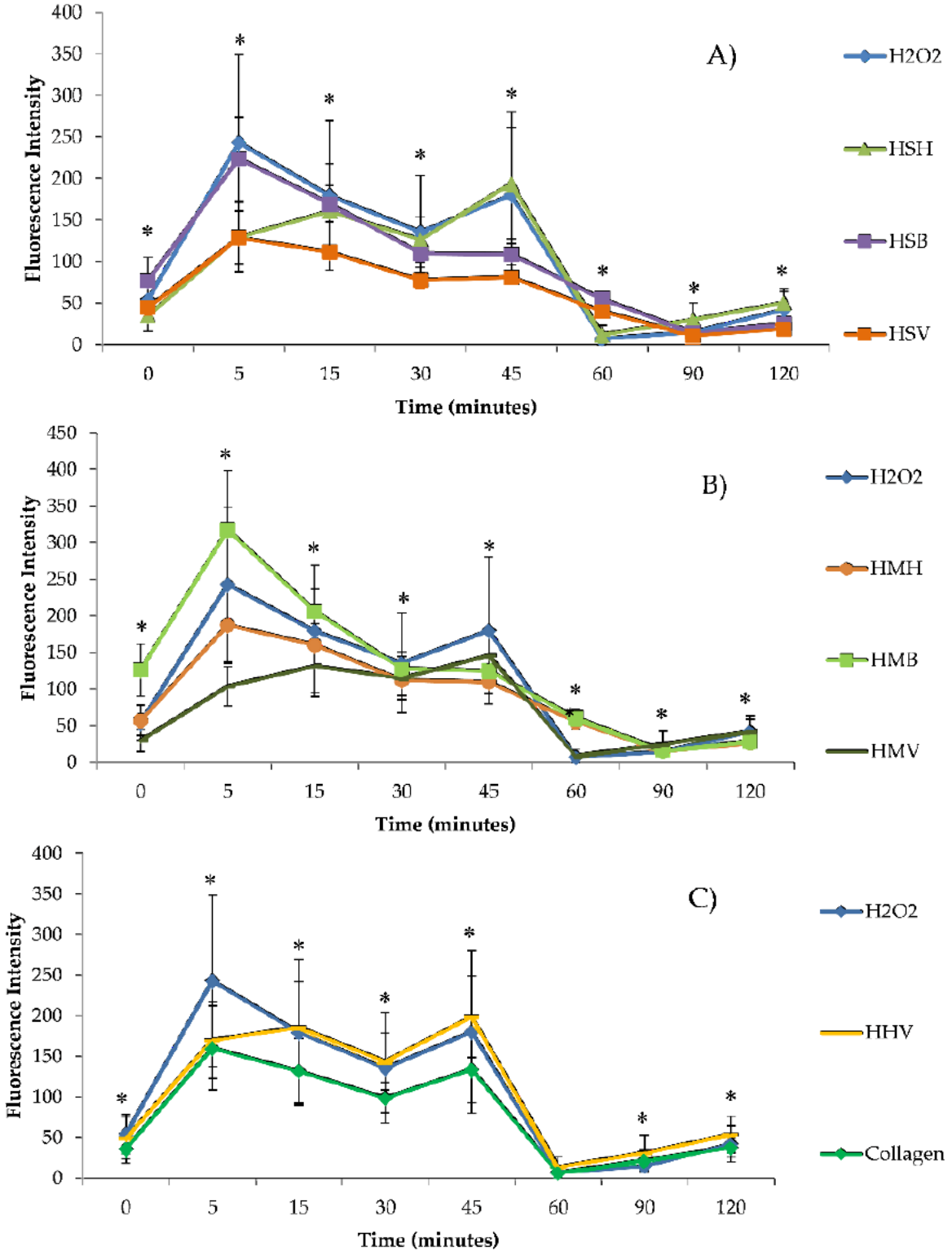

Fig. 12.4. Time dependence of ROS-induced fluorescence in Caco-2/TC7 cells exposed to (A) salmon hydrolysates, (B) mackerel hydrolysates, and (C) herring hydrolysates and fish collagen at 1 mg/mL with oxidative stress induced by H_2O_2. Results are expressed as the mean ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant differences compared to the H_2O_2

Table 12.1. Outcomes of the differences between hydrolysates and collagen without H_2O_2 and control from 0 to 120 min of exposure using ANOVA followed by a Tukey HDS post-hoc test for multiple comparisons

			Salmon			**Mackerel**			**Herring**
		CTR	**HSH**	**HSB**	**HSV**	**HMH**	**HMB**	**HMV**	**HHV**
Salmon	HSH	HSH < CTR (0.1 > *p* > 0.05)							
	HSB	HSB > CTR (0.1 > *p* > 0.05)	HSB > HSH (*p* ≤ 0.05)						
	HSV	HSV > CTR (0.1 > *p* > 0.05)	HSV > HSH (*p* ≤ 0.05)	NS					
Mackerel	HMH	HMH > CTR (*p* ≤ 0.05)	HMH > HSH (*p* ≤ 0.05)	NS	NS				
	HMB	HMB > CTR (0.1 > *p* > 0.05)	HMB > HSH (0.1> *p* > 0.05)	NS	NS	NS			
	HMV	NS	NS	HMV < HSB (*p* ≤ 0.05)	HMV < HSV (*p* ≤ 0.05)	HMV < HMH (*p* ≤ 0.05)	HMV < HMB (*p* ≤ 0.05)		
Herring	HHV	NS	HHV > HSH (0.1 > *p* > 0.05)	HHV < HSB (0.1 > *p* > 0.05)	HHV < HSV (0.1 > *p* > 0.05)	HHV < HMH (*p* ≤ 0.05)	HHV < HMB (0.1 > *p* > 0.05)	HMV < HHV (0.1 > *p* > 0.05)	
Flownder	Collagen	Collagen < CTR (*p* ≤ 0.05)	NS	Collagen < HSB (*p* ≤ 0.05)	Collagen < HSV (*p* ≤ 0.05)	Collagen < HMH (*p* ≤ 0.05)	Collagen < HMB (*p* ≤ 0.05)	Collagen < HMV (*p* ≤ 0.05)	Collagen < HHV (*p* ≤ 0.05)

NS: non-significant

Table 12.2. Outcomes of the differences between hydrolysates and collagen with H_2O_2 and control from 0 to 120 min of exposure using ANOVA followed by a Tukey HDS post-hoc test for multiple comparisons

					Salmon			Mackerel		Herring
		CTR	**H_2O_2**	**HSH_{H2O2}**	**HSB_{H2O2}**	**HSV_{H2O2}**	**HMH_{H2O2}**	**HMB_{H2O2}**	**HMV_{H2O2}**	**HHV_{H2O2}**
	H_2O_2	H_2O_2 > CTR ($p \leq 0.05$)								
Salmon	HSH_{H2O2}	HSH > CTR ($p \leq 0.05$)	NS							
	HSB_{H2O2}	HSB > CTR ($p \leq 0.05$)	NS	NS						
	HSV_{H2O2}	HSV > CTR ($p \leq 0.05$)	HSV < H_2O_2 ($0.1 > p > 0.05$)	NS	HSV < HSB ($p \leq 0.05$)					
Mackerel	HMH_{H2O2}	HMH > CTR ($p \leq 0.05$)	NS	NS	NS	HMH > HSV ($p \leq 0.05$)				
	HMB_{H2O2}	HMB > CTR ($p \leq 0.05$)	NS	NS	HMB > HSB ($p \leq 0.05$)	HMB > HSV ($p \leq 0.05$)	HMB > HMH ($0.1 > p > 0.05$)			
	HMV_{H2O2}	HMV > CTR ($p \leq 0.05$)	NS	HMV < HSH ($p \leq 0.05$)	NS	NS	NS	NS		
Herring	HHV_{H2O2}	HHV > CTR ($p \leq 0.05$)	NS	HHV > HSH ($p \leq 0.05$)	NS	HHV > HSV ($p \leq 0.05$)	NS	NS	HHV > HMV ($p \leq 0.05$)	
Flownder	$Collagen_{H2O2}$	Collaben > CTR ($p \leq 0.05$)	Collagen < H_2O_2 ($p \leq 0.05$)	NS	Collagen < HSB ($p \leq 0.05$)	NS	Collagen > HMH ($0.1 > p > 0.05$)	Collagen < HMB ($p \leq 0.05$)	NS	Collagen < HHV ($p \leq 0.05$)

NS: non-significant.

Fig. 12.4 shows ROS generation of hydrolysates exposed simultaneously to H_2O_2 grouped by type of fish hydrolysate. The ROS generation of hydrolysates with H_2O_2 was compared with H_2O_2 tested alone. When Caco-2/TC7 cells were exposed to oxidative stress induced by H_2O_2, they showed an increase in ROS levels which ranged from 1.9-fold to 17.2-fold at 0 and 45 min, respectively, compared to the control. Nevertheless, at 5 min collagen and most fish hydrolysates except HMB induced lower ROS accumulation as compared to H_2O_2 treatment alone, statistically significant only for HSH, HSV, and HMV (p = 0.000; Tukey HSD). After 5 min, the strongest antioxidant effect was induced by HMV. The antioxidant effect of fish hydrolysates was maintained until 45 min following exposure, and gradually disappeared as compared to the H_2O_2 treatment at 60 min following exposure, when there were higher ROS levels analyzed in HSV, HSB, HMH, and HMB treatments as compared to both the control and the H_2O_2 treatment (Fig. 12.4). Considering the whole trial period, it was collagen which induced significant antioxidant effects against H_2O_2 exposure, and HHV which induced the highest ROS accumulation, though not significantly as compared to all treatments (Table 12.2).

12.3.3 Lipid Peroxidation Assay

The LPO on Caco-2/TC7 cells was determined by the TBARS method in the presence of hydrolysates HSV, HSB, HSH, HMH, HMB, HMV, HHV, and collagen (1 mg/mL) with and without H_2O_2. As shown in Fig. 12.5, the incubation for 24 h in the absence of oxidative stress significantly decreased the LPO production in Caco-2/TC7 cells ($p \leq 0.05$). Cells treated with H_2O_2 alone showed significant LPO increase compared to the control, whereas all the hydrolysate treated cells showed similar LPO levels to that of the control, though there was an overall increase in LPO in the combined hydrolysate/H_2O_2 treatments, compared to the non-pretreated cells, of a magnitude between 40.2 and 146.3%. Respect to hydrolysates simultaneously exposed to H_2O_2, HSB, HMH, and HMV showed cytotoxic protection respect to H_2O_2 exposure. The highest cytoprotective effect was obtained for HMV, significant only in comparison to HSV, collagen, and H_2O_2 treatments. In cells without H_2O_2 pretreatment, HSV offered highest antioxidant effect, and HSH the lowest.

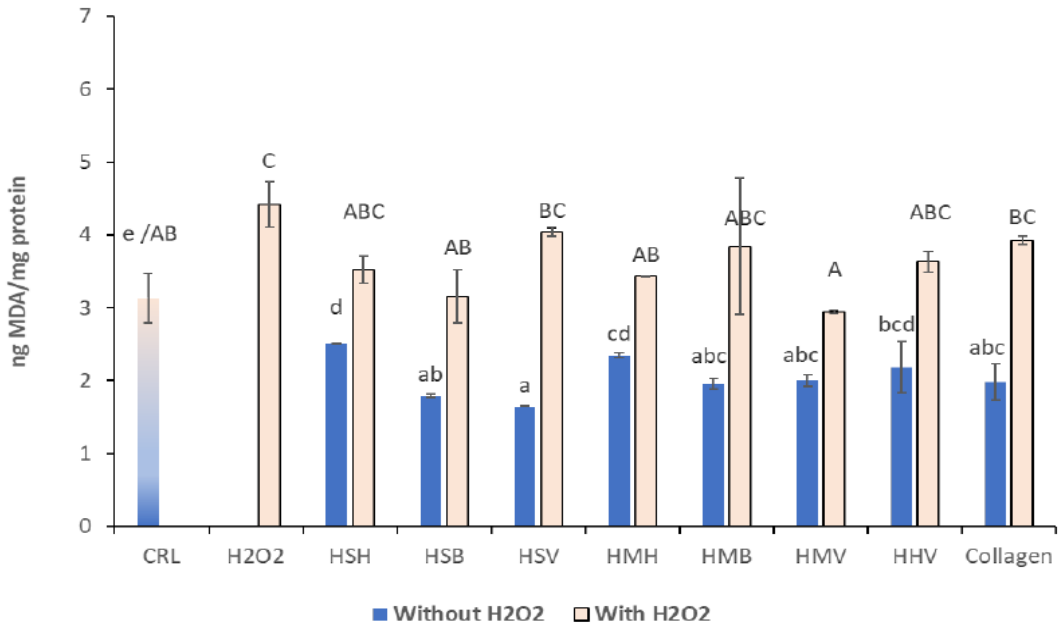

Fig. 12.5. The LPO measured by MDA production in Caco-2/TC7 cells incubated with hydrolysates HMH, HSV, HSB, HMB, HHV, HSH, HMV, and collagen (1 mg/mL) with and without oxidative stress (H_2O_2). Results are expressed as the mean ± SEM (n = 3) in ng of MDA/mg of protein measured by the Lowry method. Values with common small or capital letters, for the treatment without or with H_2O_2 induced stress, respectively, are not significantly different (p < 0.05; Tukey HSD). CRL: control

12.3.4 Comparison of Cytotoxicity between pure Hydrolysates and Their Bioavailable Fraction

In general terms, the bioavailable fraction of the tested fish hydrolysates showed similar effects as the respective pure hydrolysates. There were no significant differences in the cytoprotective effect of pure fish hydrolysates as compared to their bioavailable extracts ($p > 0.1$), with the exception of the bioavailable fraction of herring viscera hydrolysate (HHV) which increased cell viability significantly as compared to the respective pure hydrolysate (Fig. 12.6) ($p < 0.001$; Tukey HSD).

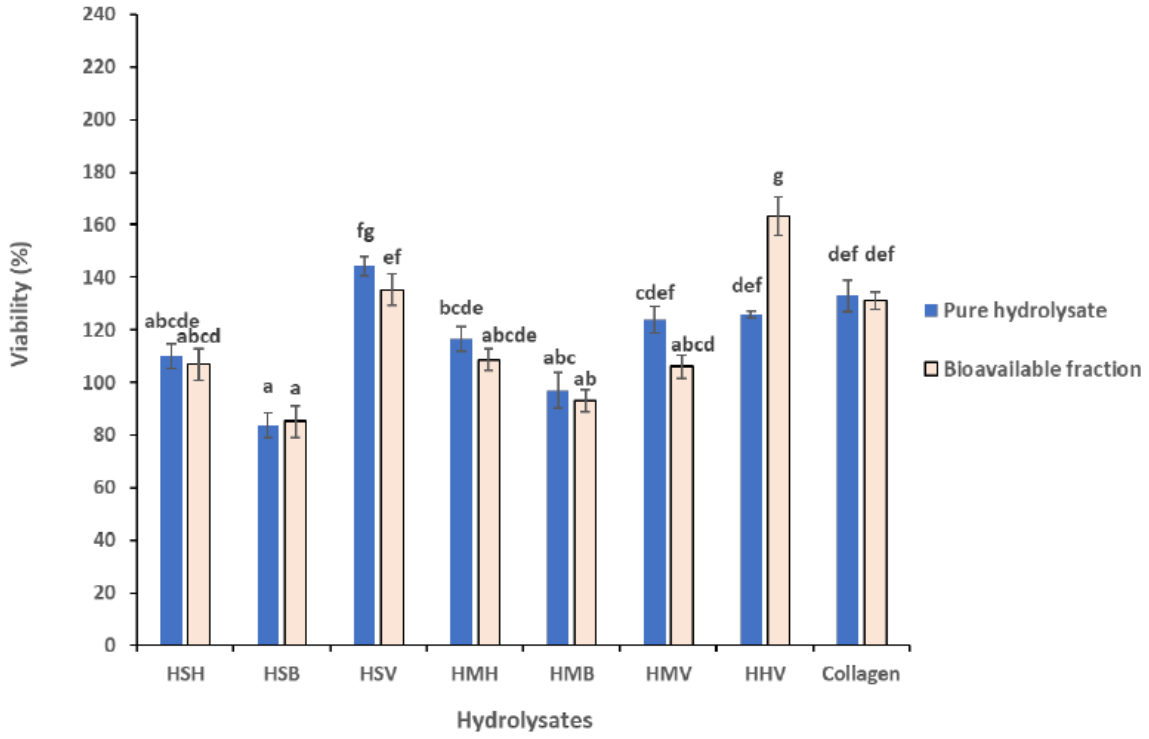

Fig. 12.6. Viability (%) of the fish-based protein hydrolysates compared with the bioavailable fraction of the same protein hydrolysate at 1:16 dilution in Caco-2/TC7 cells after 24 h of exposure by MTT. Values are expressed as the mean ± SEM ($n = 3$). Values with common letters are not significantly different ($p < 0.001$; Tukey HSD)

Comparing the three salmon and three mackerel fraction hydrolysates together, the ANOVA analysis did not show significant species effects. On the other hand, significant differences were observed between the different side stream fractions. The backbone hydrolysate fractions did not induce a cytoprotective effect (−10.47% viability as compared to the pure hydrolysates). Head hydrolysates induced a 10.98% increase in cell viability, and the viscera induced significantly higher cell viability (28.78%) as compared to the other two fractions ($HB^a < HH^b < HV^c$; $p < 0.001$; Tukey HSD). Comparing the visceral hydrolysate fraction effects, HMV (mackerel) induced significantly lower viability improvement (12.8% increased viability as compared to the pure hydrolysates) as compared to HSV (salmon) (39.72%), and HHV (herring) (50.70%) ($p < 0.001$; Tukey HSD).

12.3.5 Cytoprotective Effect of Hydrolysates against T-2 Mycotoxin

Exposing Caco-2/TC7 cells to T-2 mycotoxin significantly reduced their viability. All tested fish side stream hydrolysates exerted cytoprotective effects against the T-2 mycotoxin, though not statistically significantly in the backbone hydrolysates HSB, HMB, and collagen as observed in Fig. 12.7.

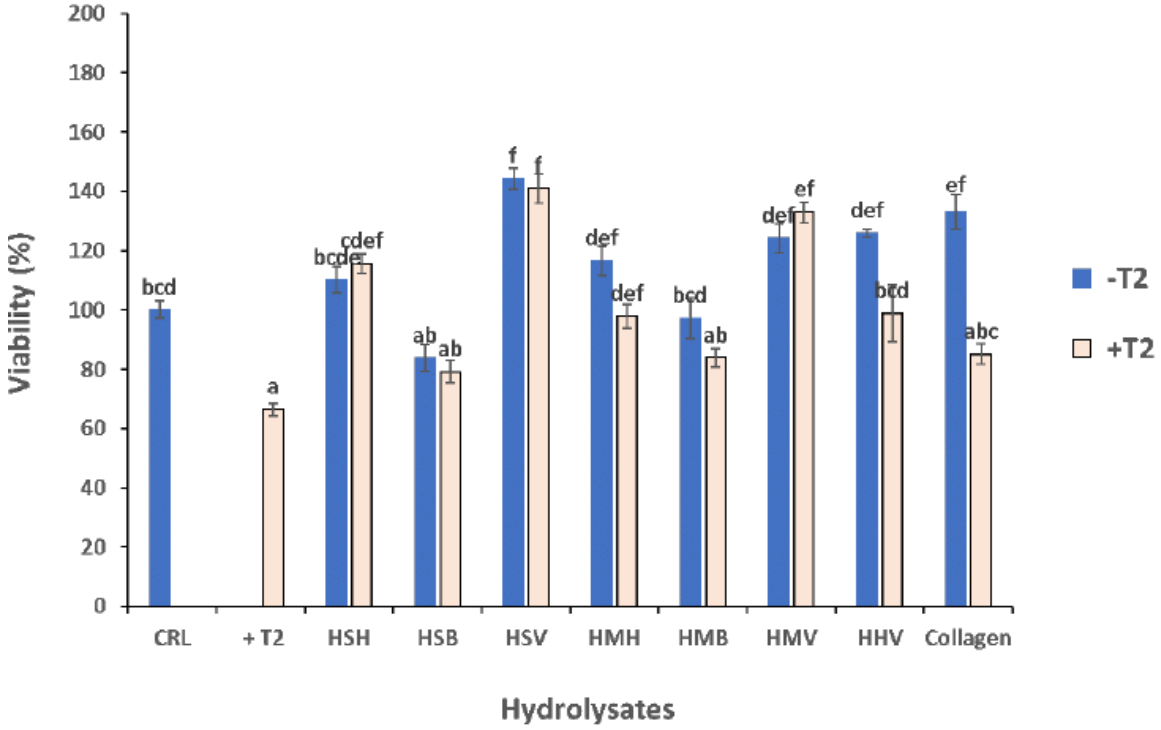

Fig. 12.7. Viability (%) of T-2 toxin, pure hydrolysates, and hydrolysates combined with T-2 in Caco-2/TC7 cells after 24 h of exposure by MTT. All hydrolysates were added at 1:16 dilution and T-2 at 60 nM. Values are expressed as the mean ± SEM (*n* = 2). Values with common letters are not significantly different (*p* < 0.001; Tukey HSD). CRL: control

As seen in the viability trial without exposure to T-2 mycotoxin, the cytoprotective effect of salmon hydrolysates was higher than that of mackerel in cells exposed to T-2; this time the species factor showed a tendency for effect (p = 0.1). Likewise, the side stream fraction induced statistically significant effects, with the backbone fractions inducing the lowest cytoprotective effect (19.16% increased viability as compared to the T-2 treated cells), followed by the heads (43.46% increase in cell viability), and the viscera, which induced significantly higher cell viability (69.92%) as compared to the other two fractions ($HB^a < HH^b < HV^c$; $p < 0.001$; Tukey HSD). Comparing the visceral hydrolysate fraction effects, it was HSV (salmon) which induced the highest cytoprotective effect against T-2 mycotoxin exposure (76.18% increased viability as compared to the T-2 treated cells), significantly higher as compared to HHV (herring) (62.47%), and HMV (mackerel) (60.80%) ($p < 0.01$; Tukey HSD).

12.3.6 Identification of Fungal Metabolites in Fish Hydrolysates

The retrospective screening was applied to high resolution mass spectrometry data acquired in AutoMS/MS mode using a Q-TOF MS system. The screening of suspected fungal metabolites was performed using the MassHunter METLIN Metabolite PCD and PCDL. No fungal metabolites were identified in analyzed fish hydrolysates. To guarantee the quality control and quality assurance of the results, a pool sample of fish hydrolysates spiked with a mixture of mycotoxins at instrumental detection limit was performed and included in the same batch (Fig. 12.8).

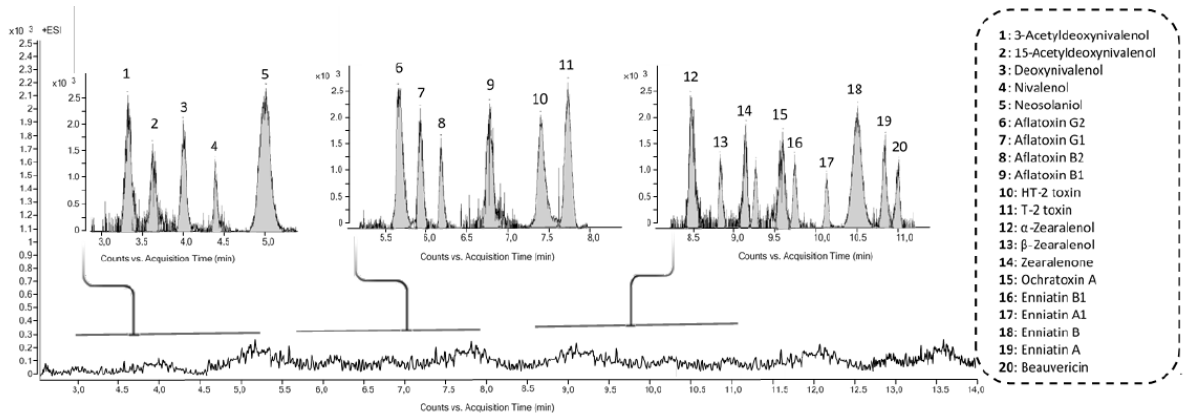

Fig. 12.8. LC-Q-TOF MS extracted ion chromatogram of a pool sample of fish protein hydrolysates spiked with a mixture of mycotoxins

12.4 DISCUSSION

Fish filleting side stream materials contain high levels of structural and bioactive proteins and smaller peptides, and lower levels of lipids containing long chain ω-3 polyunsaturated fatty acids (ω-3-PUFAs), phospholipids, bioactive nitrogenous compounds, organic minerals, and vitamins, among others. Most fish side streams are considered waste, though they are highly nutritious, containing, among other things, significant amounts of protein [49]. Effective use of fish side stream raw materials reduces the environmental impact of aquaculture and fisheries, and could provide high-added-value products to increase economical income for the marine product processing industries. In recent years, the use of extracts and compounds obtained from marine side stream biomass to improve the functionality of food products, in different processes such as organoleptic, health, and technological, has attracted much interest [50]. Among different applications, extracts and compounds obtained from fish side streams can be used as colorants, antimicrobials, antioxidant compounds, PUFAS, essential amino acids, and emulsifiers. These properties allow them to act as preservatives, to improve the nutritional and health profile of foods, as well as to improve the technological characteristics of the products. On the other hand, the use of these products may also have a great interest from the pharmaceutical point of view such as nutraceuticals, biorefinery, or development of new packaging systems [51].

In the present study, protein hydrolysates based on different side stream fractions from salmon, mackerel and herring, and flounder skin collagen have been selected to evaluate their potential use as functional food ingredients. Serial dilutions of each hydrolysate were chosen to study the potential cytotoxic/cytoprotective effects of the hydrolysates in Caco-2/TC7 cells. The hydrolysates did not show cytotoxic effects, however a cytoprotective effect was observed when Caco-2/TC7 cells where exposed to HSB at 0.125 mg/mL, and HSV at 0.0625 mg/mL and 0.25 mg/mL by MTT assay (Fig. 12.1). The increase in cell viability at lower concentrations can be due to the hormetic effect. It can be observed as either a xenobiotic or an antioxidant substance, a hormone or a metabolite in several studies [52,53,54].

By the PC assay, all hydrolysates except HSB and HMV showed an increase in cell viability when Caco-2/TC7 cells were exposed to specific dilutions, including 0.0625 mg/mL (Fig. 12.2). Wiriyaphan et al. [55] reported that hydrolysate of side streams of *Nemipterus* spp. did not have cytotoxic effects on Caco-2 cells. Furthermore, several studies on fish-based protein hydrolysates on multiple human cell lines have shown low toxicity and high cell viability [51,52]. Gómez et al. [56] showed a cytoprotective effect after 24 h of treatment with red tilapia side streams in Caco-2 cells, demonstrating an increase in cell viability in a dose-dependent manner. Similar to our results, in which the viscera hydrolysate showed highest cytoprotective effect, these authors demonstrated that the highest protection was achieved at 0.1 and 0.25 mg/mL for RTVH-A (red tilapia viscera hydrolysate with higher antioxidant activity) and FRTVH-V (red tilapia viscera hydrolysate with molecular weight

cut-offs of <1 kDa fraction), respectively. In this line, Zhong et al. [57], found that protein hydrolysates isolated from silver carp by-products showed a cytoprotective effect on Caco-2 cells exposed to low concentrations of H_2O_2 and the highest capacity to neutralize radicals. Hu et al. [58] did not observe any significant cytotoxic effects on HepG2 cells after exposure of hydrolysate of monkfish (*Lophius litulon*) muscle during 24 h, by MTT assay. However, they reported an increase in a concentration-dependent manner of cell viability, compared with control, when the cells were exposed to peptides with antioxidant activity with H_2O_2-induced oxidative damage. For instance, the peptide MMP-12 increased the HepG2 cell viability from 48.85% ± 1.68% to 63.28% ± 2.06%, to 79.35% ± 2.85%, and to 88.65% ± 3.42% at the concentrations of 10, 50, and 100 µM, respectively. These findings suggest that the peptides from hydrolysate of monkfish muscle could strongly protect H_2O_2-induced oxidative damage HepG2 cells, especially at high concentrations. These results were consistent with the data presented by Zheng et al. [59], who showed that hydrolysate of swim bladders of *Nibea japonica* exposed to human umbilical vein endothelial (HUVECs) cells during 24 h did not decrease cell viability. Indeed, a collagen peptide of the swim bladders, named SNNH-1, promoted the growth of these cells. Moreover, Yang et al. [60] evidenced that marine collagen peptides from *Nibea japonica* skin have the potential to promote NIH-3T3 fibroblasts cells.

Reactive oxygen species (ROS) are generated in aerobic organisms during mitochondrial respiration to detoxify substances, chemical defense, cell signaling, and biosynthetic reactions [35,61]. When the elimination of ROS is inadequate, or their generation exceeds their elimination, ROS are accumulated in cells and oxidative stress occurs. Excess of ROS alters the normal cell metabolism oxidizing cell membrane phospholipids, proteins, lipids, DNA, and enzymes, among others [62]. Oxidative stress contributes to many noncommunicable disease pathologies such as neurodegenerative conditions, cardiovascular and inflammatory diseases, emphysema, and certain types of cancer [63]. Therefore, there is a significant interest in the development and application of functional food supplements with antioxidant properties to promote disease prevention and protect human health. The maintenance and proliferation of cell viability by a treatment with salmon, mackerel and herring hydrolysates could be related to the fact that these hydrolysates do not produce more ROS at a cellular level than cells not exposed to these hydrolysates. The ROS are generated by different physiological oxidative processes in the organisms, and associated with pathogenesis of various human diseases [64]. The overproduction of ROS culminates in oxidative damage to some key biological macromolecules which leads to significantly reduced cell viability [58]. In our study, none of the tested hydrolysates at 1 mg/mL concentration leads to a significant increase in the intracellular ROS levels in Caco-2 cells (p > 0.05) compared to the control, except HMH and collagen at 90 and 15 min, respectively (Fig. 12.3; Table 12.1). However, cells pre-treated with H_2O_2 showed an increase in ROS generation at all times of exposure compared to the control (Fig. 12.4). The decrease in intracellular ROS levels using a pretreatment with these hydrolysates suggested that sequestration or neutralization of free radicals is a possible mechanism of action in the antioxidant peptides found in hydrolysates. Similar results were obtained by Zhen et al. [59] who studied the effect of SNNH-1 on ROS levels in HUVECs cells. After H_2O_2 treatment, the fluorescence intensity in HUVECs cells was significantly higher compared to the control. However, SNNH-1 pretreatment reduced ROS levels in a concentration-dependent manner on HUVECs cells. These results were in agreement with the data presented by Li et al. [65], who found that collagen peptides from sea cucumbers (*Acaudina molpadioides*) could protect RAW264.7 cells from H_2O_2-induced damage. Hu et al. [58] observed the effects of MMP-4, MMP-7, and MMP-12 (three peptides of hydrolysate of monkfish, *Lophius litulon,* muscle), on the level of ROS in oxidative damage HepG2 cells. The level of ROS observed in HepG2 cells exposed to only H_2O_2 was 231.7% ± 13.5%, which was significantly higher than the control (100%). The intracellular ROS levels were significantly attenuated by MMP-4, MMP-7, and MMP-12 pretreatment in a dose-dependent manner. Among them, MMP-7 showed the strongest scavenging effect of ROS, and decreased the ROS level from 229.5% ± 16.8% to 165.2% ± 11.9%, to 137.3% ± 14.3%, and to 129.1% ± 8.6% at the concentrations of 10, 50, and 100 µM, respectively. Chen et al. [12] reported that the exposure of HepG2 cells to tilapia fish skin gelatin hydrolysates for 24 h decreased the level of ROS in a dose-dependent manner. Similarly, according to Wang et al. [32], the HepG2 cells caused significant inhibition of oxidative damage compared with cells treated only with H_2O_2, after pretreatment with mackerel proteins hydrolysate (5 mg/mL). Gómez et al. [56] determined that Caco-2 cells treated with H_2O_2 showed a 5 times increase in intracellular ROS levels compared to untreated cells, but these levels decreased by approximately 40% with pretreatment with red tilapia viscera hydrolysates RTVH-

A and FRTVH-V. Other antioxidant peptides with high cellular antioxidant activity via neutralization of intracellular ROS have been identified in different sources of proteins, such as tilapia muscle [35] and tilapia scale gelatin [66].

Antioxidant collagen peptides are also known to provide cell protection from oxidative stress damage, through scavenging ROS and increasing the levels of intracellular antioxidant enzymes [67]. This work shows that flounder skin collagen protects cells from oxidative stress, decreasing ROS levels and MDA. Moreover, comparing with hydrolysates, it is amongst those which exert the most protective effect. These finding are corroborated with the results obtained by other authors. Collagen peptides from crimson snapper (*Lutianus erythroptrus*) scales [12] and royal jelly [64] could prolong the average life of Drosophila treated with H_2O_2 by decreasing the contents of peroxide products, such as MDA and protein carbonylation. Moreover, Wang et al. [68] determined that collagen of the red lip croaker could protect H_2O_2-damaged HepG2 cells from oxidative stress by decreasing ROS and MDA levels and enhancing endogenous antioxidant enzyme defense system, so it could serve as antioxidant used in food and health products. Moreover, collagen peptides are popular in cosmetics as, for example, they can protect the skin from ultraviolet (UV) radiation injuries [68].

The LPO occurs as a non-specific process secondary to initial cell damage, which can be blocked by antioxidants. The MDA is the oxidative metabolite of cell lipid oxidation and can attack unsaturated fatty acids in the cell membrane, which causes cell damage. Therefore, the degree of LPO and cell damage can be evaluated according to the MDA content in cells [30]. Our results showed that salmon, mackerel, and herring hydrolysates prevented the propagation of LPO, decreasing the MDA levels in Caco-2/TC7 cells. These results demonstrated that salmon (HSB) and mackerel (HMH and HMV) hydrolysates may protect Caco-2/TC7 cells from oxidative damage. Similar results were obtained by Zheng et al. [59], who reported that MDA content in HUVEC cells was significantly higher after H_2O_2 treatment compared to the control; however, pretreatment of HUVECs with SNNH-1 decreased the amount of MDA in a dose-dependent manner. In addition, Cai et al. [69] reported that FPYLRH (S8) from the swim bladders of miiuy croaker (*Miichthys miiuy*) could down-regulate the contents of MDA, suggesting that it plays a protective role in the antioxidant effects on HUVECs cells against H_2O_2-induced damage. Following this, Zhen et al. [59] determined the effect of SNNH-1 pretreatment on the levels of MDA after oxidative damage of HUVECs induced by H_2O_2. The results concluded that SNNH-1 inhibited intracellular LPO and enhanced the cell´s antioxidant defense system. Hu et al. [58] studied the effects of protein hydrolysate of monkfish on MDA levels in H_2O_2-induced HepG2 cells. The MDA content (21.63 ± 0.81 nM/mg protein) in HepG2 cells exposed to H_2O_2 was significantly increased compared with the control group (9.32 ± 0.35 nM/mg protein). At the 10, 50, and 100 µM concentration, the MDA content of MMP-12 peptide was 18.8 ± 0.56, 16.46 ± 0.74, and 12.43 ± 0.62 nM/mg protein, respectively, which was lower than those of MMP-4 and MMP-7 peptides, and the H_2O_2 treated group. Therefore, MMP-4, MMP-7, and MMP-12 peptides could reduce the oxidative stress injury by the decrease in LPO.

The results obtained in this study indicate that fish protein hydrolysates represent great candidate molecules for the development of antioxidant dietary supplements. However, peptides are often chemically and physically unstable, display rapid clearance, and are quickly degraded by enzymes [70]. Therefore, ensuring the bioaccessibility of peptides should be evaluated. Thus, the bioavailable fraction of the different protein hydrolysates was achieved and a comparison between the cell viability of pure hydrolysates and their bioavailable fraction was carried out. These results revealed the same trend as the as the pure hydrolysates. This indicates that there were no statistically significant differences, in terms of toxicity or antioxidant capability, of the protein hydrolysates once the digestion process had been carried out.

Additionally, the viability of cells exposed to pure hydrolysates and to hydrolysates combined with T-2 was compared to corroborate the cytoprotective effect of fish hydrolysates. The results evidenced that almost all the hydrolysates combined with T-2 showed an increase in viability compared to cell exposure to T-2 alone. This may be due to the fact that these hydrolysates counteract the cytotoxic effect of T-2, and they act as if the cell were only exposed to the hydrolysate. Hydrolysates based on viscera, regardless of the species, combined with T-2, exercised the same effect on cell viability than if they were not exposed to T-2, therefore viscera hydrolysates exert the most protective effect. As

can be observed in Fig. 12.7 and according to MTT assay (Fig. 12.1), 1:16 dilution of HSV exercised the most cytoprotective effect compared with the rest of hydrolysates. Moreover, when hydrolysates were combined with T-2, HSV also exercised the most cytoprotective effect. However, when collagen was combined with T-2, we saw significantly decreased cell viability with respect to its corresponding pure hydrolysates. This could be as collagen evidenced the highest bacterial content of all hydrolysates analyzed (data not shown), which may be related with conditions of the degradation, or breakdown, of the fish. Furthermore, collagen evidenced higher levels of trimethylamine-N (TMA; 66 mg N/100 g) [42], which is an indicator of poor product quality. This could explain the fact that a co-exposure of collagen with T-2 decreased the viability, as the collagen did not have the necessary quality parameters to protect the cell against this damage.

Finally, it is important to know whether the feedstuffs used for farmed fish are contaminated. Fish from aquaculture are fed using different feeds and raw materials from vegetal origin and plant products, which could be contaminated by mycotoxins [71]. Different studies have shown that some mycotoxins carry-over from feed into edible tissues or fluids from animals [72,73,74]. As far as the foodstuffs from aquaculture industries are concerned, the occurrence of aflatoxins, ochratoxin A, and *Fusarium* mycotoxins, such as thrichotecenes and the emerging fusariotoxins, have been reported in the literature [75,76,77,78,79]. Tolosa et al. [76] determined the occurrence of enniatins (ENs) in feed for farmed fish and in fish samples of sea bass and sea bream. They reported an occurrence of ENs in up to 25% of analyzed samples (n = 20) at levels from 1.01 to 119.0 µg/Kg, and revealed higher average concentrations of EN A1 (11.1 µg/Kg), EN B (13.0 µg/Kg), and EN B1 (19.0 µg/Kg) in head than in liver (5.1, 12.6, and 5.3 µg/Kg for EN A1, EN B, and EN B1, respectively). Those findings corroborate the ability of ENs to distribute and persist into tissues, and even penetrate different barriers, including the blood-brain barrier, in higher organisms as previously evidenced in the literature [80,81]. However, mycotoxins were not detected in the protein hydrolysates, suggesting that the fish had not been exposed to mycotoxins.

To summarize, HSV showed the highest cytoprotective effect in Caco-2/TC7 as evidenced by MTT, ROS, and LPO assays. HMV treatment also showed a reduction in ROS values when compared to H_2O_2. On the other hand, the HHV bioavailable fraction showed the highest percentage of cell viability in Caco-2/TC7 cells compared to the rest of hydrolysates. Moreover, HSV presented the highest cytoprotective effect when hydrolysates are combined with T-2 toxin.

12.5 CONCLUSIONS

Taken together, the findings from this study suggest that heads, backbones, and viscera from salmon, mackerel, and herring could be utilized as valuable raw materials in the development of health-promoting functional foods with potential antioxidant capacities. Both hydrolysates and their bioavailable fraction induced similar cell viability rates. Moreover, from the results obtained, it can be concluded that the highest cytoprotective effect was obtained for the salmon viscera protein hydrolysate. In addition, ROS accumulation induced by H_2O_2 and LPO was suppressed by all pure hydrolysates, and the cytoprotective effect of all hydrolysates was observed against T-2. This scientific basis may provide a solution for dealing with the high cost and environmental problems associated with the disposal of such waste material. However, more studies are needed to investigate the biological effects of fish hydrolysates in order to transform protein rich side stream products into valuable products, as well as to ensure food safety.

AUTHOR CONTRIBUTIONS

Conceptualization, M.T. and M.-J.R.; methodology, M.T. and T.A.; software, M.T.; validation, M.T. and M.-J.R.; formal analysis, M.T.; investigation, M.T.; resources, M.T.; data curation, M.T.; K.K.; writing—original draft preparation, M.T.; writing—review and editing, M.-J.R.; Y.R.-C.; F.J.B.; K.K. and T.A.; visualization, M.-J.R.; Y.R.-C.; F.J.B. and K.K.; supervision, M.-J.R.; Y.R.-C.; F.J.B. and K.K.; project administration, F.J.B.; and funding acquisition, F.J.B. and Y.R.-C. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data is contained within the article.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 13
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Human Biomonitoring of T-2 Toxin, T-2 Toxin-3-Glucoside and Their Metabolites in Urine through High-Resolution Mass Spectrometry

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ABSTRACT

The metabolic profile of T-2 toxin (T-2) and its modified form T-2-3-glucoside (T-2-3-Glc) remain unexplored in human samples. Therefore, the present study aimed to investigate the presence of T-2, T-2-3-Glc and their respective major metabolites in human urine samples (*n* = 300) collected in South Italy through an ultra-high performance liquid chromatography (UHPLC) coupled to Q-Orbitrap-HRMS methodology. T-2 was quantified in 21% of samples at a mean concentration of 1.34 ng/mg Crea (range: 0.22–6.54 ng/mg Crea). Almost all the major T-2 metabolites previously characterized in vitro were tentatively found, remarking the occurrence of 3′-OH-T-2 (99.7%), T-2 triol (56%) and HT-2 (30%). Regarding T-2-3-Glc, a low prevalence of the parent mycotoxin (1%) and its metabolites were observed, with HT-2-3-Glc (17%) being the most prevalent compound, although hydroxylated products were also detected. Attending to the large number of testing positive for T-2 or its metabolites, this study found a frequent exposure in Italian population.

Keywords: Human biomonitoring; biomarkers; metabolites; high-resolution mass spectrometry; urine; T-2 toxin; exposure.

13.1 INTRODUCTION

Mycotoxins are toxic metabolites resultant from the secondary metabolism of several species belonging to the *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps* and *Alternaria* genera. Under certain conditions, these fungi can colonize a broad variety of crops, eventually leading to the accumulation of mycotoxins. Among the mentioned fungal genera, the *Fusarium* species represents the major mycotoxin-producing pathogens of warm areas from America, Europe and Asia, especially affecting cereal grains and their derived products [1]. Throughout the consumption of contaminated materials, mycotoxins can cause severe adverse health effects in humans, such as immunotoxic, neurotoxic or even carcinogenic effects [2]. Therefore, regulatory authorities have established maximum limits (MLs) in susceptible foods and foodstuffs alongside tolerable daily intake (TDI) values for certain mycotoxins, setting a maximum level of dietary exposure to avoid the appearance of toxic effects.

The traditional methods for estimating dietary exposure are mainly based on the combination of consumption surveys and occurrence data throughout total diet studies or meta-analysis approaches [3,4]. Nevertheless, the outcomes can be considerably biased due to interindividual differences in consumption patterns or to assuming an homogeneous contamination within the same food category. In order to obtain a more reliable estimation of the exposure, human biomonitoring (HBM) studies represent an ideal alternative [5]. These studies involve the measurement of parent mycotoxins and/or

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their respective metabolites in biological samples, preferably urine, considering its easy and non-invasive collection. In this line, a considerable amount of literature about HBM studies of *Fusarium* toxins and their metabolites occurring in human urine is available, including studies on deoxynivalenol (DON), nivalenol (NIV), fumonisins B1 and B2 (FB1 and FB2), zearalenone (ZEN), enniatin B (ENB) and B1 (ENB1) [6,7,8,9,10]. Nevertheless, the extensive metabolism of T-2 toxin (T-2), a major *Fusarium* toxin included in group 3 of the International Agency for Cancer Research (IARC), has been scarcely studied in biological samples.

T-2 is a type-A trichothecene containing an epoxy group between C12 and C13, a double bond between C9 and C10, and variable acetoxy groups. This toxin mainly occurs in wheat, maize and oat, although it can be found in other cereal grains and derived commodities [11]. Once ingested, T-2 can represent a health concern. T-2 is a major cause of alimentary toxic aleukia, affecting the mucosa and immune system [12]. At the cellular level, T-2 binds to the 60S ribosomal subunit, inhibiting protein synthesis. In addition, T-2 causes oxidative stress, impairs the mitochondrial function by altering the electron transport chain and stimulates apoptosis after activating several MAPK and caspases [13,14,15]. Nevertheless, the toxicity of T-2 highly depends on its metabolites, considering the intensive and rapid metabolization reported after in vitro assays with human liver microsomes. In this line, numerous metabolic pathways were described by Yang et al. [16] for the biotransformation of T-2 into its major metabolic products: hydrolysis for the production of HT-2 toxin (HT-2) and neosolaniol (NEO); hydroxylation, generating 3′-hydroxy-T-2 (3′-OH-T-2) and 3-hydroxy-HT-2 (3′-OH-HT-2, also known as T-2 triol); and glucuronidation, for the production of T-2-3-glucuronic acid (T-2-3-GlcA), HT-2-3-glucuronic acid (HT-2-3-GlcA) and HT-2-4-glucuronic acid (HT-2-4-GlcA).

In addition, T-2 can also be modified in plants by the addition of polar molecules, such as glucose, producing T-2-3-glucoside (T-2-3-Glc) as a result [17]. This modified form has also been repeatedly reported in cereal grains [18]. Therefore, humans could also be exposed to this T-2 modified form through dietary intake. Exposure to T-2-3-Glc represents a health concern, as it can be deconjugated into its free parent toxin within the intestinal tract. However, other metabolites were recently characterized by Yang et al. after in vitro assays with human liver microsomes [19]. Among them, the major metabolites were the hydrolyzed forms HT-2-3-glucoside (HT-2-3-Glc) and NEO-3-glucoside (NEO-3-Glc), and the hydroxylated 3′-hydroxy-T-2-3-Glc (3′-OH-T-2-3-Glc) and 4′-hydroxy-T-2-3-Glc (4′-OH-T-2-3-Glc).

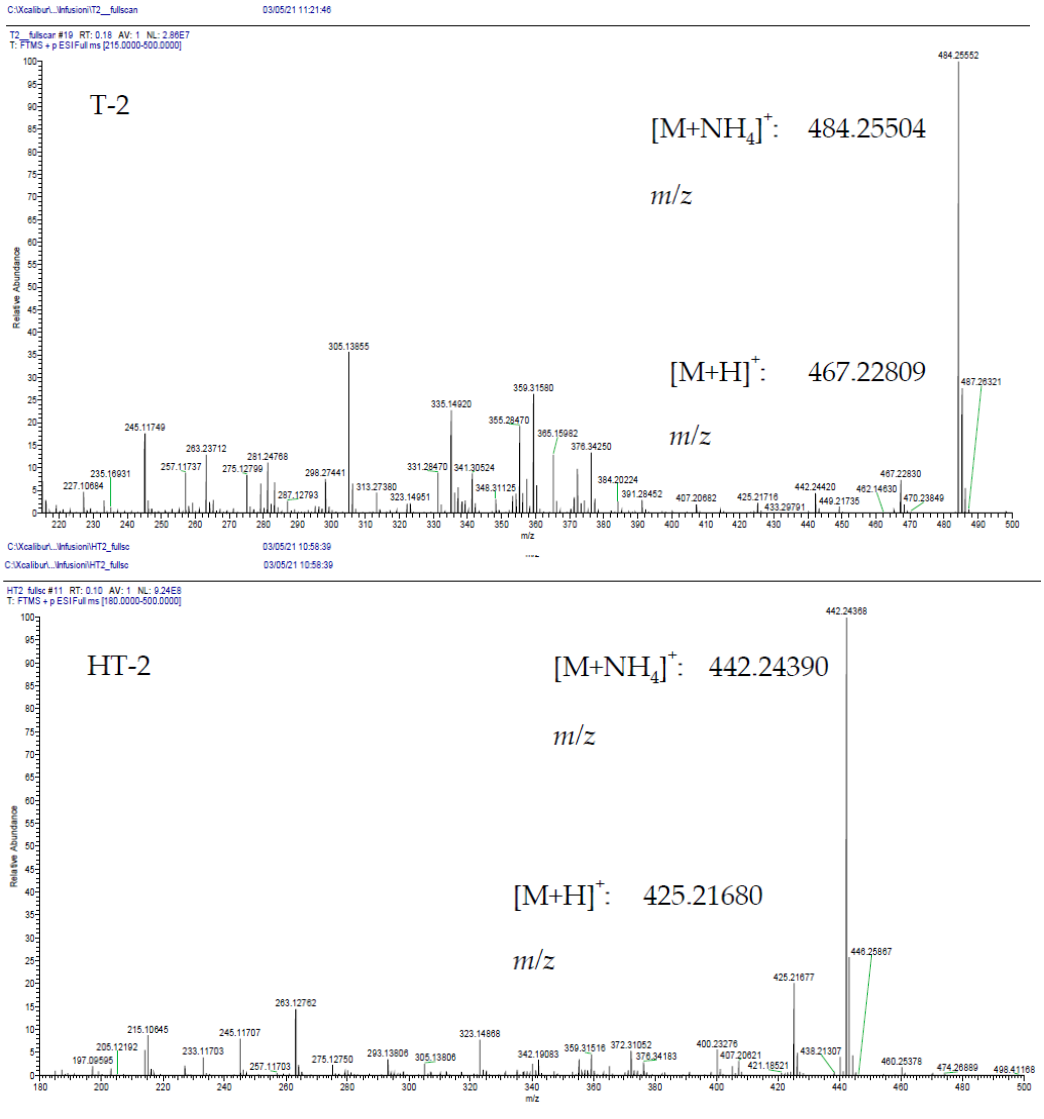
Although Gerding et al. [20] and, recently, De Ruyck et al. [21] have investigated the presence of HT-2-4-GlcA and T-2 triol, respectively, in human urine samples, the complete metabolic profile of major biotransformation products remains unexplored. To overcome the lack of analytical standards, high-resolution mass spectrometry (HRMS) represents an optimal tool for characterizing the metabolic profiles of these parent toxins that require consideration when conducting exposure assessment studies. Therefore, the present study aimed to investigate the presence of T-2, T-2-3-Glc and their respective major metabolites in human urine samples (n = 300) collected in South Italy through an ultra-high performance liquid chromatography (UHPLC) coupled to Q-Orbitrap-HRMS methodology. A combined strategy consisting of targeted quantification of T-2 and HT-2, and suspect screening based on exact mass measurement was applied.

13.2 RESULTS AND DISCUSSION

13.2.1 Optimization of Q-Orbitrap HRMS Parameters

Analytical standards of T-2 and HT-2 diluted at 1 µg/mL in methanol were directly infused into the Q-Orbitrap HRMS system at a constant flow rate of 8 µL/min in order to evaluate the MS parameters of both analytes. The system operated in both positive and negative ESI mode to determine the best ionization pattern, and exact mass measurements were compared to the theoretical masses to assess the accuracy, as shown in Fig. 13.1. Both mycotoxins formed stable ammonium adducts in the positive ESI mode with high accuracy (mass error < 2 ppm) that were further used for targeted quantification in the urine samples. Protonated adducts were also observed for both mycotoxins but at a lower relative intensity, whereas negative ionization showed not only lower relative intensity but also lower exact mass accuracy.

Previous in vitro studies with human liver microsomes have tentatively identified the major biotransformation products of T-2 and T-2-3-Glc, and elucidated their structure through mass spectrometry analysis [16,19]. Therefore, the following T-2 metabolites were targeted for retrospective analysis: hydrolysis products (NEO and 4-deAc-NEO), hydroxylation products (3′-OH-T-2 and T-2 triol) and phase II metabolites (T-2-3-GlcA, HT-2-3-GlcA and HT-2-4-GlcA). In addition, the modified form T-2-3-Glc was also included alongside its hydrolyzed (NEO-3-Glc and HT-2-3-Glc) and hydroxylated (3′-OH-T-2-3-Glc and 4′-OH-T-2-3-Glc) metabolic products. Although the biotransformation of T-2-3-Glc could also originate T-2 and its corresponding products, only the glucoside forms were included as T-2-3-Glc metabolites for classification purposes. Tentative identification was performed following a suspect screening approach based on the exact mass measurements. Both the protonated and ammonium adducts of each analyte were initially targeted to select the fragment with the higher intensity for further analysis. In addition, the order of elution was compared to previous methodologies that used reverse-phase chromatography, considering that a more polar character implies a sooner elution, while also allowing a proper differentiation of the isomeric forms. A stringent mass error of < 2 ppm was also set, aiming for a more accurate identification in urine samples. Data for the retention times, theoretical mass, observed mass and mass error for all the assayed compounds are shown in Table 13.1.



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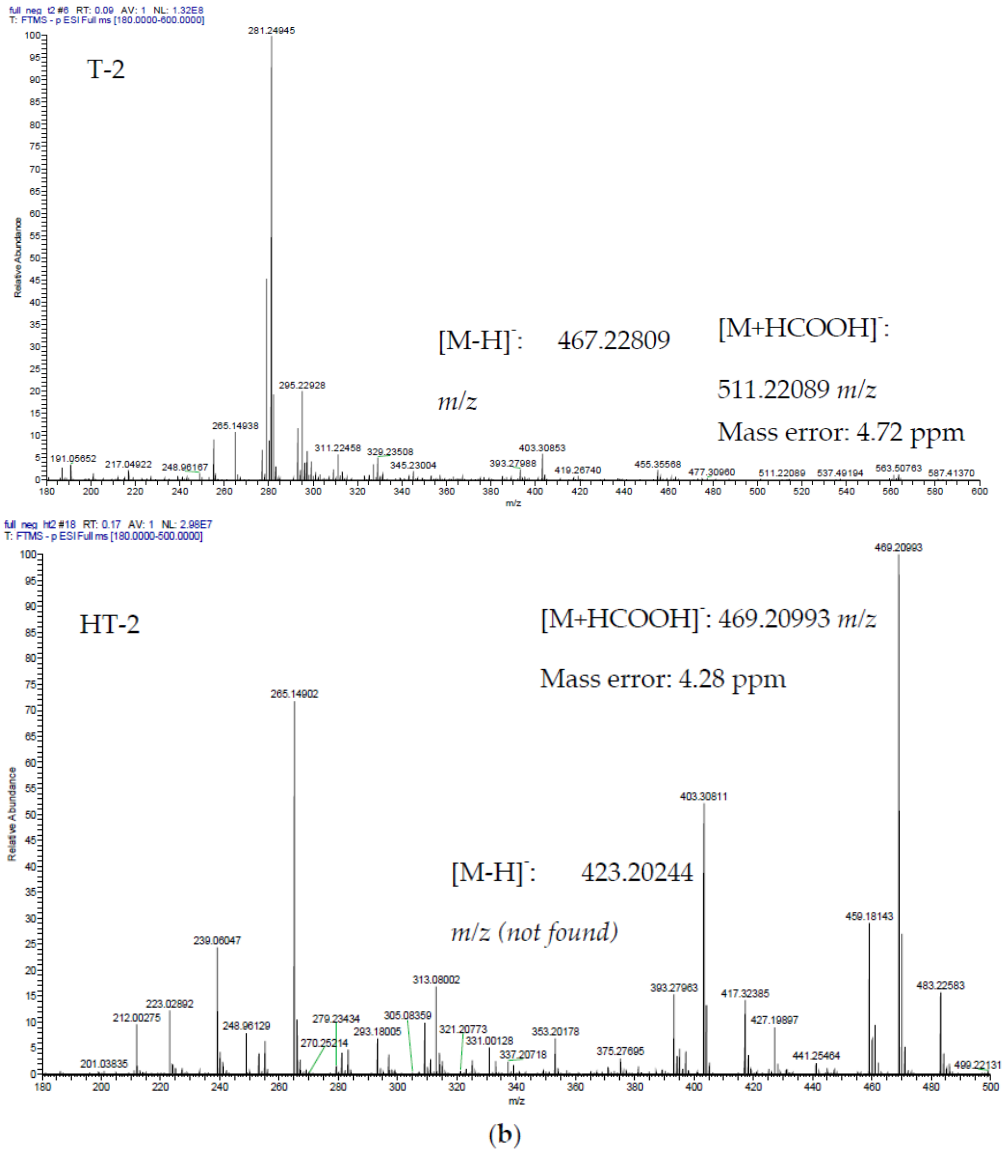

Fig. 13.1. Mass spectra of T-2 and HT-2 in (a) positive ionization mode and (b) negative ionization mode after a full scan

13.2.2 Method Validation

The proposed methodology for the analysis of urine samples was validated in-house according to the in-force legislation [22]. The results from validation experiments are shown in Table 13.2. The calibration curves built in neat solvent and blank matrix showed good linearity ($r^2 > 0.990$) throughout the assayed range of concentrations (20–0.1 ng/mL). After comparing both slopes of the calibration curves, a negligible matrix interference was detected (98–102%). Therefore, the external calibration based on the neat solvent curve was used for quantification purposes. The spiking experiments determined a proper recovery of analytes that ranged from 79% to 116% for the selected fortification levels (5, 1 and 0.5 ng/mL). The maximum relative standard deviations of 19% and 17% after intraday (RSD_r) and interday (RSD_R) precision studies were respectively obtained. The absence of coelutant peaks in the matrix after the analysis of blank samples (n = 10) confirmed the selectivity of the proposed methodology. LOQs corresponding to T-2 and HT-2 were set at 0.2 ng/mL and 0.4 ng/mL, respectively. Therefore, the proposed methodology fulfilled the validation criteria and was further applied for quantification of T-2 and HT-2 in human urine samples.

Table 13.1. UHPLC-Q-Orbitrap-HRMS parameters for the targeted and non-targeted analytes

Analyte	Retention Time (min)	Molecular Formula	Adduct Ion	Exact Mass (*m*/*z*)	Observed Mass (*m*/*z*)	Mass Error (ppm)
Parent mycotoxin						
T-2	4.82	$C_{24}H_{34}O_9$	$[M + NH_4]^+$	484.25411	484.25504	1.92
Phase I metabolites—Hydrolyzed group						
4-deAc-NEO [a]	-	$C_{17}H_{24}O_7$	$[M + NH_4]^+$	358.18602	-	-
			$[M + H]^+$	341.15950	-	-
HT-2	4.79	$C_{22}H_{32}O_8$	$[M + NH_4]^+$	442.24354	442.24390	0.81
NEO	4.25	$C_{19}H_{26}O_8$	$[M + NH_4]^+$	400.19669	400.19659	−0.25
Phase I metabolites—Hydroxylated group						
T-2 triol	4.7	$C_{20}H_{30}O_7$	$[M + H]^+$	383.20642	383.20662	0.52
3′-OH-T-2	4.78	$C_{24}H_{34}O_{10}$	$[M + NH_4]^+$	500.24902	500.24926	0.48
Phase II metabolites—Conjugated group						
T-2-3-GlcA	4.69	$C_{30}H_{42}O_{15}$	$[M + H]^+$	643.25964	643.25974	0.16
HT-2-3-GlcA	4.67	$C_{28}H_{40}O_{14}$	$[M + H]^+$	601.24908	601.24998	1.5
HT-2-4-GlcA	4.39	$C_{28}H_{40}O_{14}$	$[M + H]^+$	601.24908	601.24998	1.5
Parent mycotoxin						
T-2-3-Glc	4.38	$C_{30}H_{44}O_{14}$	$[M + NH_4]^+$	646.30693	646.30793	1.55
Phase I metabolites—Hydrolyzed group						
HT-2-3-Glc	4.59	$C_{28}H_{42}O_{13}$	$[M + NH_4]^+$	604.29636	604.29616	−0.33
NEO-3-Glc	3.85	$C_{25}H_{36}O_{13}$	$[M + NH_4]^+$	562.24941	562.24897	−0.78
Phase I metabolites—Hydroxylated group						
3′-OH-T-2-3-Glc	3.91	$C_{30}H_{44}O_{15}$	$[M + NH_4]^+$	662.30184	662.30121	−0.95
4′-OH-T-2-3-Glc	4.04	$C_{30}H_{44}O_{15}$	$[M + NH_4]^+$	662.30184	662.30121	−0.95

[a] *Neither the protonated nor the ammonium adducts were observed.*

Table 13.2. Method performance for T-2 and HT-2

Analyte	Linearity (r^2)	SSE (%)	Recovery (%)			Precision (%) [RSD_r, (RSD_R)]			
			5 ng/mL	1 ng/mL	0.5 ng/mL	5 ng/mL	1 ng/mL	0.5 ng/mL	LOQ (ng/mL)
HT-2	0.9901	102	116	86	85	11 (9)	5 (6)	12 (17)	0.4
T-2	0.9944	98	116	87	79	8 (7)	11 (17)	19 (14)	0.2

Several methodologies have been validated for the quantification of T-2 and HT-2 in urine samples, as shown in Table 13.3. Liquid chromatography coupled to tandem low-resolution mass spectrometry appears to be the gold standard, although gas chromatography has also been used. Similar to the present study, the use a Q-Orbitrap mass spectrometer in the recent study conducted by Ndaw et al. [23], which remarks the rising use of HRMS techniques in HBM studies, must be highlighted. Apart from quantification, HRMS allows retrospective data analysis for untargeted compounds and suspect screenings. Therefore, HRMS could represent the main tool for future HBM studies considering the complex metabolome occurring in biological samples [24]. In terms of sensitivity, most of the available analytical techniques have reported LOQs ≤ 1 ng/mL for T-2, whereas sensitivity to HT-2 seems to be critically impacted not by the analytical technique but the sample treatment. In this line, direct approaches, such as dilute and shoot or filter and shoot, have shown a strong variability in sensitivity between both analytes. Although these procedures represent a good alternative in HBM studies, based on an easy and quick workflow, these are unable to selectively remove any interference present in the matrix, thus reducing the performance of the methodology. Other sample treatments including a clean-up step, such as QuEChERS or SALLE procedures, obtained better sensitivities for HT-2 when compared to direct approaches. The here-presented methodology, based on a simple SALLE + clean-up procedure and later UHPLC-HR-Q-Orbitrap-MS/MS, showed suitable sensitivity for detecting both analytes at low ng/mL levels.

Table 13.3. Previous methodologies for the quantification of T-2 and HT-2 in human urine samples

Sample Treatment	Analytical Method	LOQ [a] (ng/mL)		Reference
		T-2	HT-2	
Dilute and shoot	UHPLC-Q-TRAP-MS/MS	0.5	4	Gerding et al. [20]
SALLE	UPLC-QqQ-MS/MS	0.013	0.036	De Ruyck et al. [21]
Clean-up	UHPLC-HR-Q-Orbitrap-MS/MS	1	0.5	Ndaw et al. [23]
LLE	HPLC-Q-TRAP-MS/MS	6.7	67	Abia et al. [25]
QuEChERS	GC-QqQ-MS/MS	1	2	Rodríguez-Carrasco et al. [26]
Dilute and shoot	UHPLC-Q-TRAP-MS/MS	1	40	Ezekiel et al. [27]
LLE	HPLC-Q-TRAP-MS/MS	1	40	Warth et al. [28]
Filter and shoot	UHPLC-QqQ-MS/MS	0.03	0.5	Heyndrickx et al. [29]
LLE	UHPLC-QqQ-MS/MS	0.1	0.84	
LLE	HPLC-Q-TRAP-MS/MS	0.2	9	Gerding et al. [30]
LLE	UHPLC-Q-TRAP-MS/MS	0.1	0.5	Fan et al. [31]
IA-SPE	UHPLC-QqQ-MS/MS	0.013	0.031	Gratz et al. [32]
DLLME	GC-QqQ-MS/MS	1	2	Niknejad et al. [33]
Dilute and shoot	HPLC-Q-TRAP-MS/MS	10	5	Duringer et al. [34]
SALLE + clean-up	UHPLC-HR-Q-Orbitrap-MS/MS	0.2	0.4	Present study

[a] *Values considering the dilution/concentration factor of each methodology; LLE = liquid-liquid extraction; QuEChERS = quick, easy, cheap, effective, rugged and safe; IA = immunoaffinity; SPE = solid-phase extraction; DLLME = dispersive liquid-liquid microextraction; SALLE = salt-assisted liquid-liquid extraction; HPLC = high-performance liquid chromatography; Q = quadrupole; MS/MS = tandem mass spectrometry; GC = gas chromatography; QqQ = triple quadrupole; UHPLC = ultra-high performance liquid chromatography; HR = high resolution*

13.2.3 Urinary Levels of T-2 and HT-2

The validated methodology was applied to 300 human urine samples to assess the urinary levels of T-2 and HT-2. Table 13.4 reviews previous studies with positive urine samples for any of both mycotoxins alongside the here-obtained results. In the present study, T-2 was quantified in 21% of samples at a mean concentration of 1.34 ng/mg Crea (range: 0.22–6.54 ng/mg Crea), whereas HT-2 showed a slightly higher prevalence, occurring in 30% of samples at a similar mean concentration of 1.23 ng/mg Crea. No significant differences were observed when comparing the concentration levels of both analytes throughout the whole dataset. Nevertheless, a statistically significant positive association between the occurrence of T-2 and HT-2 within the same sample was revealed (p-value < 0.01, phi coefficient = 0.479). This co-exposure might be explained by the correlated occurrence of

both toxins in foodstuffs, which has been extensively reported in the literature [11,35,36]. Across age and gender groups, both mycotoxins reflected similar distributions in terms of prevalence and concentration, supported by statistical analysis which determined non-significant differences.

Scarce literature about T-2 or HT-2 occurring in urine samples is available. In addition, most of the published studies have focused on specific cohorts, compiling several criteria in terms of age range, occupational exposure or diseases potentially related to T-2 exposure, as shown in Table 13.4. In general, a low rate of positive samples has been reported when considering healthy cohorts, with T-2 occurring in a higher number of samples than HT-2 (T-2 < 21.8%; HT-2 < 13.6%). Quantitatively, HT-2 seemed to occur at higher concentrations when compared to T-2 (T-2 < 1.75 ng/mg Crea; HT-2 < 16.81 ng/mg Crea). In contrast with these previous studies, the here-presented results demonstrated a higher prevalence rate for both mycotoxins but at lower concentration values. These inconsistencies in urinary levels could be due to several factors. First, the analytical performance of the abovementioned studies displayed differences in terms of sensitivity for each mycotoxin, with LOQs being 3–8-times higher for HT-2. This could directly translate into lower rates of positive samples and higher mean concentration values. Second, both mycotoxins can be found in foods and foodstuffs, so variable levels can be expected according to dietary habits. In this line, the latest data on food supply quantity by the Food and Agricultural Organization of the United Nations (FAO) presented a higher consumption of cereals and cereal-based products in Italy (160.97 kg/capita/year) when compared to Germany (115.03 kg/capita/year), the United Kingdom (126.75 kg/capita/year) Belgium, the Czech Republic, the Netherlands and Norway (<102.12 kg/capita/year) [37], so higher prevalence could also be expected in the present study. In addition, the results obtained in HBM studies that have focused on a specific age segment (see Table 13.4, Gratz et al. [32], 2–6 years old; Gerding et al. [20], 20–30 years old) may not be comparable to studies with larger sample sizes due to the potential bias generated by age-related consumption patterns. Last, although little is known about toxicokinetics of T-2 and HT-2 in human, in vivo studies with rats and pigs have reported low half-life values for both toxins in urine, as reviewed by Schelstraete et al. [38], so this might hamper the understanding of urinary levels in human samples too. Therefore, these results provide even more evidence on the occurrence of T-2 in human urine and support the development of more sensitive analytical techniques for its application in HBM studies to clarify the impact of T-2 in humans.

13.2.4 Retrospective Analysis of Urine Samples

Data collected after UHPLC-Q-Orbitrap-HRMS analysis were manually examined in Xcalibur Qual Browser v.3.1.66 to evaluate the tentative presence of NEO, 4-deAc-NEO, 3′-OH-T-2, T-2, T-2-3-GlcA, HT-2-3-GlcA, HT-2-4-GlcA, T-2-3-Glc, NEO-3-Glc, HT-2-3-Glc, 3′-OH-T-2-3-Glc and 4′-OH-T-2-3-Glc in 300 human urine samples. Identification was based on exact mass identification in at least two of the three replicates, with a stringent mass error of 2 ppm using the molecular formulas previously reported in the literature [16,19], thus corresponding to a level 5 of certainty as established by Schymanski et al. [39]. The results are shown in Fig. 13.2.

Almost all the major T-2 metabolites previously characterized in vitro were tentatively found in human urine within the following order of prevalence: 3′-OH-T-2 (99.7%) > T-2 triol (56%) > HT-2 (30%) > NEO (21%) > HT-2-3-GlcA (6%) > T-2-3-GlcA (1.3%) > HT-2-4-GlcA (0.3%) > 4-deAc-NEO (0%). These results could indicate phase I metabolism as the preferential biotransformation pathway, as evidenced by the high proportion of 3′-OH-T-2 and T-2 triol and the low relevance of conjugated metabolites, although dietary exposure could also take part on these outcomes. Furthermore, among phase I metabolites, statistical analysis revealed a significantly higher frequency of hydroxylated metabolites when compared to the hydrolyzed products (p-value < 0.01). Therefore, hydroxylation seemed to be the main biotransformation pathway of T-2. Although the presence of HT-2 and NEO could indicate hydrolysis as an alternative reaction, their prevalence could be partly due to dietary exposure considering that they extensively occur in foodstuffs [40]. The low relevance of conjugated metabolites in human urine has been previously reported by Gerding et al. [20], who did not find any positive samples for HT-2-4-GlcA.

Table 13.4. Previous human biomonitoring studies with positive urine samples for T-2 and/or HT-2

Provenance	Cohort (age)	Samples (n)	LOQ (ng/mL)[a]		Prevalence (%)		Range of Concentration (ng/mg Crea)		Mean (ng/mg Crea)		Reference
			T-2	HT-2	T-2	HT-2	T-2	HT-2	T-2	HT-2	
Germany	Adults (20–30)	101	0.5	4	1	nd	<LOQ	nd	na	nd	Gerding et al. [20]
Belgium, Czech Republic, Netherlands, and Norway	Adults (45–65)	188	0.013	0.036	21.8	6.4	<LOQ—0.77	<LOQ—4.60	0.05 [c,d]	0.48 [c,d]	De Ruyck et al. [21]
France	Adults, grain elevator workers (19–56)	18	1	0.5	4	4	<LOQ—2.73	<LOQ—3.29	na	na	Ndaw et al. [23]
Spain	Children (8–14)	16			nd	6.2	nd	12.6 [b]	nd	12.6	Rodríguez-Carrasco et al. [26]
	Young adults (18–28)	16			nd	nd	nd	nd	nd	nd	
	Adults (>28)	22			nd	13.6	nd	15.8 [b]	nd	14.3	
	Total	54	1	2	nd	7.4	nd	15.8 [b]	nd	na	
China	Adults (18–66)	260	0.1	0.5	2.3	nd	0.392–4.23	nd	1.75	nd	Fan et al. [31]
United Kingdom	Children (2–6)	21	0.013	0.031	5	5	0.03	6.13	0.03	6.13	Gratz et al. [32]
Iran	Adults, esophageal cancer patients (50–92)	17			6	18	na	na	44.7	29.09	Niknejad et al. [33]
	Adults, control group (20–46)	10	1	2	nd	10	nd	na	nd	16.81	
Uganda	Children, nodding syndrome patients (5–18)	50			74	nd	0–288 [c]	nd	29 [c]	nd	Duringer et al. [34]
	Children, control group (5–18)	50			70	nd	0–425 [c]	nd	49 [c]	nd	
	Total	100	10	5	72	nd	0–425 [c]	nd	39 [c]	nd	
Italy	Children, teenagers and adults (≤30)	94			20	32	0.42–2.37	0.44–2.32	1.26	1.19	Present study
	Adults (31–59)	72			19	28	0.33–6.54	0.46–2.75	1.48	1.48	
	Elderly (≥60)	134			22	30	0.22–2.51	0.44–2.39	1.4	1.13	
	Total	300	0.2	0.4	21	30	0.22–6.54	0.44–2.75	1.34	1.23	

[a] Values considering dilution/concentration factor of each methodology; [b] Only maximum value available; [c] Values expressed as ng/mL without applying; creatinine correction; [d] Referring to median values instead of mean; nd = not detected; na = not available.

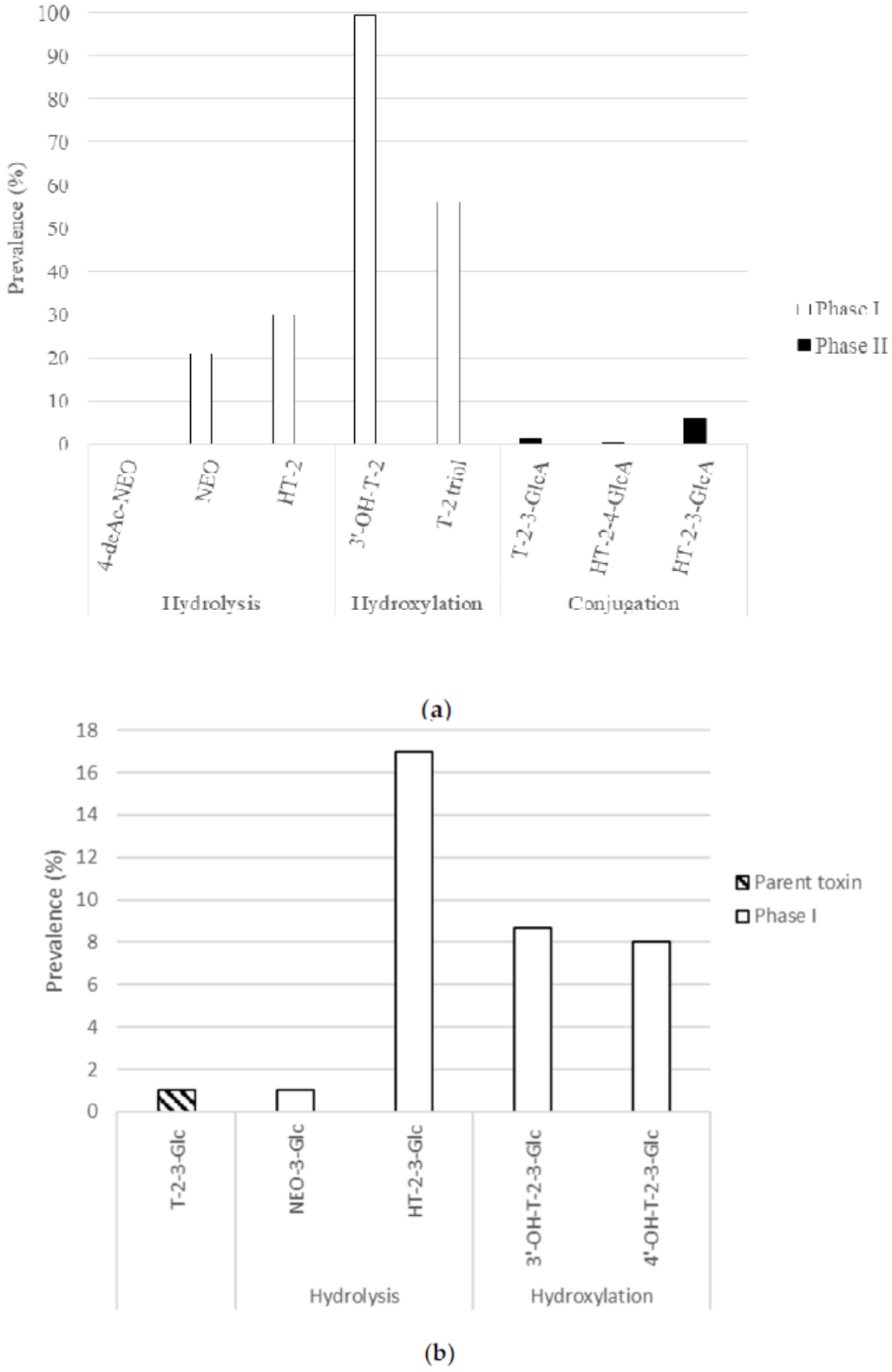

Fig. 13.2. Prevalence of (a) T-2 metabolites and (b) T-2-3-Glc and its phase I metabolites in 300 human urine samples. Although HT-2 was assessed through a targeted methodology, it was introduced in this section as a major T-2 metabolite

These results are in contrast with the human in vitro assays conducted by Yang et al. [16], which found HT-2 as the most predominant metabolites, whereas T-2 triol was the preferred hydroxylation product. Nevertheless, different metabolic profiles have been observed after comparing the in vivo and in vitro data from other species. Similar to human in vitro data, other previous assays with liver microsomes of chickens and rats have revealed a predominance of hydrolyzed metabolites, whereas in vivo experiments in both species have remarked hydroxylation as the main biotransformation pathway and did not observe conjugation [16,41], in line with the here-observed findings. The high relevance of hydroxylated compounds, especially 3′-OH-T-2, could represent a concern considering that it does not exert a significantly lower toxicity. Moreover, 3′-OH-T-2 might even display a faintly higher toxicity when compared to its parent mycotoxin [42,43].

Regarding the T-2-3-Glc metabolic profile, a low prevalence of the parent mycotoxin (1%) and its metabolites was observed: HT-2-3-Glc (17%) > 3′-OH-T-2-3-Glc (8.7%) > 4′-OH-T-2-3-Glc (8%) > NEO-3-Glc (1%). The observed low urinary prevalence is in agreement with findings reported by Yang et al. [19], who observed that T-2-3-Glc and its metabolites were mainly excreted in feces, whereas only traces were observed in urine after the oral administration of T-2-3-Glc to rats. Nevertheless, only T-2, HT-2 and 3′-OH-T-2-3-Glc were reported in urine, whereas the here-obtained results highlighted HT-2-3-Glc as the major T-2-3-Glc metabolite, being tentatively detected at a significantly higher frequency when compared to the rest of products (p-value < 0.01) This discrepancy could be addressed by considering not only in vitro/in vivo and interspecies differences but also potential dietary exposure. Although the presence of HT-2-3-Glc has been scarcely studied in foodstuffs, the available studies have reported a high prevalence in oats, wheat and barley samples [44].

The vast occurrence of metabolites in human urine samples indicated an extensive biotransformation of T-2 and T-2-3-Glc. Although scarce information is available on in vivo metabolism, a similar pattern across species has been observed with a preferential metabolism through phase I reactions for T-2 and low-to-no relevance of conjugation reactions.

13.3 MATERIALS AND METHODS

13.3.1 Chemicals, Reagents and Materials

Water for the LC mobile phase (LC-MS grade), acetonitrile (AcN) and methanol (MeOH) were acquired from Merck (Darmstadt, Germany). Formic acid (MS grade) was supplied by Carlo Erba reagents (Cornaredo, Italy). Ammonium formate (analytical grade) was purchased from Fluka (Milan, Italy). Octadecyl carbon chain-bonded silica (C18) (analytical grade) and sodium chloride (NaCl) were obtained from Sigma Aldrich (Milan, Italy). Conical centrifuge polypropylene tubes of 15 mL were provided by BD Falcon (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were acquired from Phenomenex (Castel Maggiore, Italy).

Analytical standards of T-2 and HT-2 (HPLC purity > 98%) were supplied by from Sigma-Aldrich (Milan, Italy). Stock solutions were built by diluting 1 mg of each standard mycotoxin in 1 mL of MeOH. Then, working solutions were prepared by properly diluting with MeOH/H_2O (70:30 *v*/*v*) 0.1% formic acid to reach the concentrations needed for spiking experiments (5, 1 and 0.5 ng/g). The solutions were kept in securely closed vials at −20°C.

13.3.2 Sampling

First-spot morning urine (50 mL) samples from 300 volunteers were collected during January and February 2018 in the Campania region (Southern Italy) and stored in sterile plastic vessels. Samples were aliquoted and storage at −20 °C until further analysis to avoid stability issues. Volunteers were selected among students, academic and non-academic staff of the Faculty of Pharmacy of University of Naples “Federico II” considering the following exclusion criteria: (i) susceptible people to occupational exposure, such as veterinarians and farmers, were excluded; (ii) only one member per family was allowed; (iii) people with serious bile, kidney or liver problems were not eligible due to potential interferences with the metabolism of mycotoxins. No diet limitations were established during

the sampling. All participants provided written consent in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects. This project was approved by the University of Valencia Ethics Committee. The sample size (n = 300) selected is consistent with previous HBM studies of food contaminants [5].

Participants were asked to specify their age and gender in the vessel for further data treatment. The sampling attempted to maintain the gender parity (male: 45.7%, female: 54.3%). Three age groups were considered: ≥60 years old (n = 134), from 31 to 59 years old (n = 72) and ≤31 years old (n = 94). Samples with undetectable levels of mycotoxins were used for recovery studies.

13.3.3 Extraction Proczedure

Sample preparation procedure was conducted following a methodology previously developed by Rodríguez-Carrasco et al. [8]. Briefly, 1.5 mL of urine sample was transferred into a 2 mL Eppendorf Safe-Lock Microcentrifuge tube and centrifuged at 3926× *g* for 3 min. Afterward, 1 mL of the supernatant was collected and placed into a 15 mL screw cap test tube with conical bottom alongside 1 mL of acetonitrile. The mixture was vortexed for 30 s. Then, a blend of 30 mg of C18 sorbent and 0.3 g of NaCl were added and vortexed for 30 s and centrifuged at 3926× *g* at 4°C for 3 min. Finally, the upper layer was transferred into another 15 mL screw cap test tube with conical bottom and evaporated to dryness under nitrogen flow at 45°C, reconstituted with 0.5 mL of MeOH/H_2O (70:30 *v/v*) 0.1% formic acid and filtered through a 0.2 µm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

13.3.4 UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic separation through UHPLC was conducted using a Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) instrument equipped with a degassing system, an auto sampler device, a quaternary UHPLC pump working at 1250 bar and a thermostated (30°C) Luna Omega column (50 × 2.1 mm, 1.6 µm, Phenomenex). As mobile phases, water (A) and methanol (B), both containing 0.1% formic acid and 5 mM ammonium formate, were used. The chromatographic gradient followed the next configuration: initial 0% of phase B was kept for 1 min, then increased to 95% in 1 min and held for 0.5 min. Next, the gradient went back to 75% of B in 2.5 min and then decreased again until 60% in 1 min. Finally, the gradient switched back to 0% of B in 0.5 min and was held during 1.5 min for column re-equilibration, accounting for an entire run time of 8 min. The flow rate was set at 0.4 mL/min, and an aliquot of 5 µL of sample was injected.

After chromatographic separation, samples were assessed through HRMS using a Q-Exactive Orbitrap system. The analysis was conducted in positive electrospray ionization (ESI) and full scan mode. The ionization parameters were: spray voltage, 4 kV; capillary temperature, 290°C; auxiliary gas (N2 > 95%), 10; auxiliary gas heater temperature, 305°C; sheath gas pressure (N2 > 95%), 35; S-lens radio frequency (RF) level, 50. Full scan data collection was performed with the following settings: resolving power 70,000 full width at half maximum (FWHM) at 200 *m*/*z*, automatic gain control (AGC) target 1 × 106, injection time 200 ms, scan range from 100 to 800 *m*/*z*, scan rate 2 scans/s. A stringent mass tolerance of 2 ppm was set for identification. Data analysis was performed using Quan/Qual Browser Xcalibur v.3.1.66 (Thermo Fisher Scientific, Waltham, MA, USA).

13.3.5 Method Validation

In-house validation was conducted following the in-force legislation [22] in terms of linearity, matrix-induced deviations, selectivity, trueness, within-laboratory reproducibility, repeatability and limits of quantification (LOQs). Linearity (r2) was obtained after building the neat solvent and matrix-matched calibration curves using T-2 and HT-2 analytical standards. Concentrations ranged from 20 to 0.1 ng/mL, with each level of the calibration curves showing a relative standard deviation (RSD) < 20% compared to the theoretical concentration. The comparison of both calibration curves throughout their corresponding slopes allowed the assessment of the signal suppression/enhancement effect (%SSE), following the next equation:

$$\%SSE = Sm/Ss \times 100 \quad (1)$$

where Sm is the matrix-matched calibration slope and Ss is the solvent calibration slope. An %SSE below 100% was translated into signal suppression, whereas values above 100% represented signal enhancement in the range of concentrations assayed. Trueness was evaluated through recovery experiments, spiking known blank samples at three different levels (5, 1 and 0.5 ng/mL). Experiments were conducted in triplicate on three nonconsecutive days and reflected as interday (within-laboratory reproducibility, RSDR) or intraday (repeatability, RSDr) relative standard deviation. Selectivity was assessed to determine the potential presence of coelutants in the matrix. Thus, blank samples (n = 10) were injected immediately after the highest calibration sample. For confirmation criteria, the retention times of the analytes in standards and samples were compared. LOQs were considered as the lowest concentration where the molecular ion could be identified inside the linear range, considering a mass error below 5 ppm.

13.3.6 Quality Control/Quality Assurance

Spectral and chromatographic data were combined for the correct identification of the analytes. Retention times attached to the assayed analytes were compared in both positive samples and standards in neat solvent at a tolerance of ±2.5% of the total run time (8 min). Data quality was verified through the inclusion of a comprehensive range of quality assurance and quality control procedures. Each batch of samples contained a reagent blank, a procedural blank and a matrix-matched calibration in order to evaluate the robustness and stability of the system throughout the whole analysis.

13.3.7 Creatinine Analysis

Urinary levels of creatinine were calculated throughout a spectrophotometric assay previously performed by Rodríguez-Carrasco et al. [23]. In brief, 1000 mM NaOH was mixed with 3.5 mM picric acid to obtain alkaline picrate. The solution was stored in dark conditions in an amber glass container. Urine samples were then diluted using ultrapure water (1:10 *v*/*v*) and 1 mL was reacted with 1 mL of alkaline picrate solution. The optical density was measured after 30 min at 500 nm using a Shimadzu mini 1240 spectrophotometer. Finally, concentrations of mycotoxins were related to the creatinine content of the corresponding sample and expressed as ng/mg Crea.

13.3.8 Statistical Analysis

Statistical data treatment was carried out in software package IBM SPSS v.25. The Mann–Whitney U test was used to detect quantitative differences between T-2 and HT-2 in the assayed samples according to gender and age groups. Categorical data, as the prevalence of T-2, T-2-3-Glc and its corresponding metabolites across gender and age groups, were compared throughout the Pearson chi-square tests. A confidence level of 95% was selected for data treatment, and a p-value < 0.01 was considered as significant.

13.4 CONCLUSIONS

A simple SALLE procedure followed by a UHPLC-Q-Orbitrap HRMS methodology was applied to monitor T-2, T-2-3-Glc and their corresponding metabolites in 300 urine samples collected from volunteers in South Italy. After validation of the analytical procedure according to the current legislation, a combined approach was successfully used for the quantification of T-2 and HT-2, and a suspect screening for the tentative identification of the major T-2 and T-2-3-Glc metabolites.

The metabolic profile of T-2 in urine samples indicated hydroxylation as the main biotransformation pathway, with T-2 triol and 3′-OH-T-2 occurring at significantly higher prevalence when compared to the rest of metabolites, whereas conjugation reactions appeared to be residual in line with in vivo findings from other species. The hydroxylated metabolite 3′-OH-T-2 was tentatively found in almost all samples, which could represent a concern considering that it does not show significantly lower toxicity

compared to its parent mycotoxin. The modified form T-2-3-Glc and its corresponding metabolites were less frequent in urine samples, in accordance with other previous in vivo studies that observed an almost complete excretion in feces and only determined traces of metabolites in urine. Similarly, hydroxylated products 3′-OH-T-2-3-Glc and 4′-OH-T-2-3-Glc were tentatively identified. Therefore, the observed urinary metabolic profile of T-2 and T-2-3-Glc reveal similarities with other species.

Attending to the large number of samples testing positive for T-2 or its metabolites, this study also found a frequent exposure to T-2, although there is a considerable variability in the available HMB studies that have used urine samples. Thus, more sensitive analytical techniques should be validated for their application in biological matrices in order to clarify the impact of T-2 in humans. In addition, considering the frequent exposure to several metabolites that can also occur in foodstuffs, such as T-2 triol or HT-2-3-Glc, analytical methodologies in food analyses should incorporate them. This could help to elucidate whether the presence of these metabolites is due to dietary exposure or to T-2/T-2-3-Glc metabolism.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.R.-C. and A.R.; methodology, L.I. and L.C.; software, A.N. and N.P.; validation, L.I. and L.C.; formal analysis, A.N. and N.P.; investigation, A.N. and Y.R.-C.; data curation, A.N. and N.P.; writing—original draft preparation, A.N.; writing—review and editing, Y.R.-C.; supervision, Y.R.-C. and A.R.; funding acquisition, Y.R.-C. and A.R. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Comité Ético de Investigación en Humanos, Universitat de València (Ref: 1564214).

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author. The data are not publicly available for preserving the privacy of the volunteers that participated in the present study.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 14
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High-Throughput Determination of Major Mycotoxins with Human Health Concerns in Urine by LC-Q TOF MS and Its Application to an Exposure Study

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ABSTRACT

Human biomonitoring constitutes a suitable tool to assess exposure to toxins overcoming the disadvantages of traditional methods. Urine constitutes an accessible biological matrix in biomonitoring studies. Mycotoxins are secondary metabolites produced naturally by filamentous fungi that produce a wide range of adverse health effects. Thus, the determination of urinary mycotoxin levels is a useful tool for assessing the individual exposure to these food contaminants. In this study, a suitable methodology has been developed to evaluate the presence of aflatoxin B2 (AFB2), aflatoxin (AFG2), ochratoxin A (OTA), ochratoxin B (OTB), zearalenone (ZEA), and α-zearalenol (α-ZOL) in urine samples as exposure biomarkers. For this purpose, different extraction procedures, namely, the Solid Phase Extraction (SPE); Dispersive Liquid–Liquid Microextraction (DLLME); and Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) methods were assessed, followed by Liquid Chromatography coupled to Quadrupole Time of Flight Mass Spectrometry with Electrospray Ionization (LC-ESI-QTOF-MS) determination. Then, the proposed methodology was applied to determine mycotoxin concentrations in 56 human urine samples from volunteers and to estimate the potential risk of exposure. The results obtained revealed that 55% of human urine samples analyzed resulted positive for at least one mycotoxin. Among all studied mycotoxins, only AFB2, AFG2, and OTB were detected with incidences of 32, 41, and 9%, respectively, and levels in the range from <LOQ to 69.42 µg/L. Risk assessment revealed a potential health risk, obtaining MoE values < 10,000. However, it should be highlighted that few samples were contaminated, and that more data about mycotoxin excretion rates and their BMDL10 values are needed for a more accurate risk assessment.

Keywords: Biomarkers; mycotoxins; QuEChERS; LC-ESI-QTOF; urine; risk assessment.

14.1 INTRODUCTION

Traditional evaluation exposure to mycotoxins is often carried out combining the analysis of chemicals in foodstuffs with food consumption data. However, this indirect approach presents some disadvantages such as the lack of information related to the individual exposure situation, toxicokinetics, and bioavailability of the selected food contaminants [1]. Furthermore, this approach presents the difficulty of obtaining accurate data on food consumption and the bioavailability of toxins. The distribution of mycotoxin levels in food is not homogeneous and some mycotoxins may be linked to food matrix components and may, therefore, be underestimated [2].

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Human biomonitoring constitutes a suitable alternative in order to assess toxin exposure at individual level and has already been applied to study exposure to mycotoxins in different countries and cohorts. The typical biomarkers used for exposure assessment are the parent toxins and the major phase I and II metabolites. Blood, urine, or breast milk samples are the biological fluids most often used in biomonitoring. Among them, the use of non-invasive urine sampling is the most frequent [3,4,5].

Mycotoxins are low-molecular weight secondary metabolites produced by fungi during preharvest, harvest, or storage steps. These toxic compounds are produced to a response of oxidative stress during fungi colonization and infestation. *Aspergillus*, *Fusarium,* and *Penicillium*, are the major mycotoxin producers [6]. Although more than 300 mycotoxins have been identified, only some of them have been regulated in food by the European Commission (EC 1881/2006) [7]. Aflatoxins (AFs) are produced by species of *Aspergillus* genera; ochratoxin A (OTA) is produced by both *Aspergillus* and *Penicillium*; trichothecenes (HT-2, T-2, deoxynivalenol (DON) as well as nivalenol (NIV)), zearalenone (ZEA), fumonisins (FB1 and FB2) and emerging mycotoxins are produced by *Fusarium* species. Aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), trichothecenes, fumonisins (FBs), and patulin (PAT) constitute the mycotoxins most likely to occur in foodstuffs [8,9].

Human exposure to mycotoxins occurs through the consumption of contaminated crops or derivatives or through the ingestion of animal origin products from animals fed with contaminated feed. Humans can also be exposed to mycotoxins by inhalation and dermal contact. The impact of mycotoxins on human health depends on different factors such as the type of toxin, its metabolism, the pharmacokinetics, the exposure conditions, and the health status of the individual [2]. Long-term exposure to high doses can produce health problems such as mutagenicity, carcinogenicity, teratogenicity, hepatotoxicity, nephrotoxicity, gastrointestinal toxicity, immunotoxicity, and neurotoxicity [10].

Mycotoxins can occur in three possible forms: unmodified as they are biosynthesized by fungal metabolisms (basic or free forms of mycotoxin structures); matrix-associated as complexes with matrix compounds; modified mycotoxins that have undergone chemical or biological modifications to their structure. These modifications of mycotoxin structures can be produced by fungi, plants, or animals that are able to modify toxins because of their metabolic processes. Modified mycotoxins can be converted to free toxin forms during digestion, thus increasing exposure to these toxins [11]. All mycotoxin forms (free, metabolites, and conjugates) should be included in biomonitoring studies for a more realistic approach of risk assessment [12].

This study focuses on the presence of the major mycotoxins with human health concerns: AFs, OTA, and ZEA.

AFs are very toxic compounds classified as carcinogenic to humans by the International Agency for Research in Cancer (IARC) [13]. O-dealkylation, ketoreduction, epoxidation, and hydroxylation constitute the AFB1 major metabolic pathways. These reactions lead to the production of highly toxic forms, such as AFB1-8,9-epoxide (AFBO) and aflatoxin M1 (AFM1), as well as relatively nontoxic forms: aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1), or aflatoxin 2a (AFB2a) [14].

OTA constitutes a toxic compound with relatively rapid absorption and slow elimination. The liver and kidneys are the main organs involved in OTA biotransformation. OTA has affinity to plasma proteins, approximately 99% of the circulating OTA is bound to plasma proteins. OTA is eliminated both via urine and feces after biliary excretion [15]. The major OTA metabolic pathways are hydrolysis, hydroxylation, lactone-opening, and conjugation. OTalpha (OTα) constitutes the major OTA metabolite and is formed by the cleavage of the peptidic bond. OTA can also be metabolized into its hydroxylated derivatives: 4-R-hydroxyochratoxine A (4-R-OH OTA), 4-S-hydroxyochratoxine A (4-S-OH OTA), 10-hydroxyochratoxin A (10 OH-OTA), ochratoxin B (OTB), open lactone of ochratoxin (OP-OTA) and ochratoxin hydroquinone (OTHQ), which can be found in blood or urine in the cited forms or conjugated to glutathione. Most of the OTA metabolites such as OTα and OTB are considered less toxic than the parent compound [16].

ZEA presents a hormonal action higher than others naturally occurring non-steroidal estrogens due to its structural similarity to 17β-estradiol. ZEA is mainly metabolized in the liver and intestine in different ways: reduction reactions, resulting in α-zearalenol (α-ZOL), β-zearalenol (β–ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAL) metabolites, monohydroxylation producing ZEA catechols, and a conjugation reaction that implies the conjugation of ZEA and its reduced metabolites with sulfate and glucuronic acid. α-ZEA presents more estrogenic potential than the ZEA parent compound [17,18].

Urine samples constitute a complex matrix, in which mycotoxins presented in trace amounts may be masked by some interfering compounds, making a clean-up step necessary. Different techniques such as liquid–liquid extraction (LLE), solid phase extraction (SPE), dispersive liquid–liquid micro extraction (DLLME), QuEChERS, and immunoaffinity columns (IAC) have been reported in the literature for mycotoxin determination in urine [19,20,21,22].

Previous enzymatic treatment of urine samples with β-glucuronidase is required to release the parent mycotoxin from the mycotoxins–glucuronides. After enzymatic deconjugation, the total amount of (free +conjugated) mycotoxins can be measured.

The aim of the present study was to develop a suitable methodology to assess human exposure to mycotoxins (AFB2, AFG2, OTA, OTB, ZEA, and α-ZOL) through biomonitoring analysis. For this purpose, different extraction procedures (SPE, DLLME, QuEChERS) were evaluated with determination by Liquid Chromatography coupled to Quadrupole Time of Flight Mass Spectrometry with an Electrospray Ionization (LC-ESI-QTOF-MS) system. Then, the proposed procedure was validated and applied to determine mycotoxin concentrations in 56 human urine samples from volunteers.

14.2 RESULTS

14.2.1 Evaluation of SPE, DLLME, and QuEChERS Extraction Methods

QuEChERS extraction was selected as the most appropriate methodology, as it provided better recovery values for the mycotoxins studied at both recovery levels, from 55 to 90% at 50 µg/L and from 75 to 93% at 100 µg/L (Fig. 14.1). Lower recovery values ranging from 7 to 30% were obtained with the SPE method, being OTA the mycotoxin with lowest recovery percentage achieved (7%). The same trend was observed also for OTA employing DLLME and QuEChERS methods. Finally, DLLME extraction provided at 100 µg/L, recovery values under 30% for OTA and ZEA, 51% for AFG2, and 68% for AFB2, and near to 100% for OTB and α-ZOL. As QuEChERS extraction revealed adequate recovery percentages for all studied mycotoxins at the two levels of concentration assayed, it was proposed as the most appropriate for further validation.

In a previous mycotoxin extraction comparative study, where was optimized the AFB1, AFB2, AFG1, AFG2, OTA, ZEA, beauvericin (BEA), enniatins (ENNs) extraction from urine samples, QuEChERS extraction showed similar recovery percentages (71–109%); however, DLLME was selected by these authors as it provided slightly better values (79–113%) [20]. Contrary to the present study, better extraction efficiency values (70–98%) were obtained employing SPE cartridges for NIV, DON, Deepoxy-deoxynivalenol, Aflatoxin M1, FB1, Dihydrocitrinone, Alternariol, Citrinine, α-ZOL, β-ZOL, OTA, and ZEA determination in urine. The different results obtained in the present study may be due the different type of cartridge used, and the different alternatives of solvent employed for the elution and reconstitution steps [23].

14.2.2 Validation of the QuEChERS Method

All analytical parameters obtained (recoveries, matrix effects, limits of detection, and quantification and linearity) were in accordance with the limits established by European Commission Decision 2002/657/EC [24] (Table 14.1).

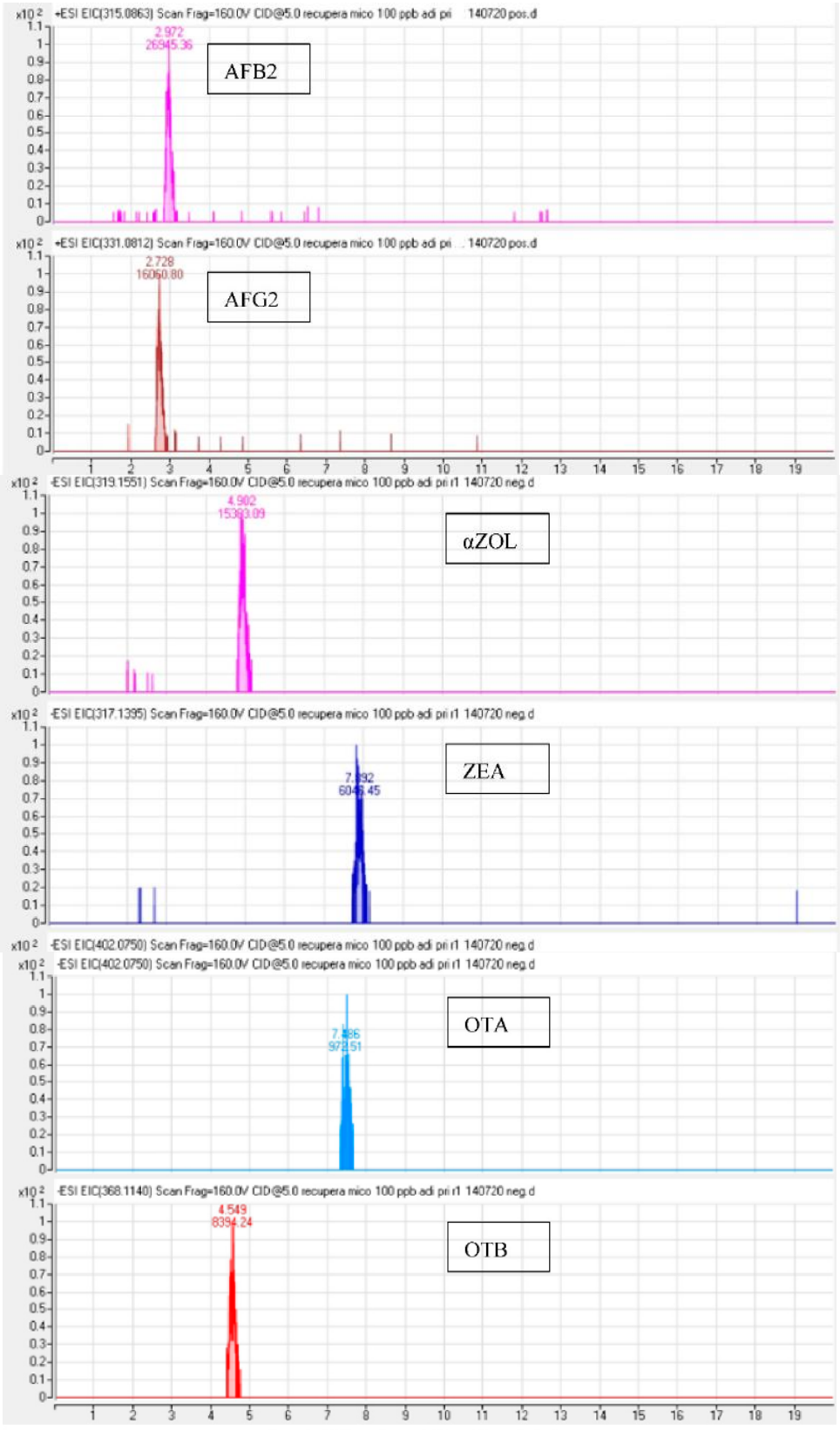

Fig. 14.1. LC-ESI-QTOF chromatogram of a spiked urine sample at 100 µg/L after QuEChERS extraction

Table 14.1. Analytical parameters for QuEChERS extraction in urine samples: recoveries, matrix effects, limits of detection, and quantification and linearity

Mycotoxin	Recoveries 50 µg/L± RSD (%)		Recoveries 100 µg/L ± RSD (%)		Matrix Effects (SSE %)	Limits of Detection (LODs) (µg/L)	Limits of Quantification (LOQs) (µg/L)	Linearity R^2
	Intra-Day Analysis	Inter-Day Analysis	Intra-Day Analysis	Inter-Day Analysis				
AFB2	90 ± 20	115 ± 12	80 ± 2	98 ± 20	66	1.5	5	0.997
AFG2	72 ± 19	77 ± 20	75 ± 16	105 ± 20	86	1.5	5	0.990
OTA	55 ± 8	52 ± 13	75 ± 19	67 ± 19	21	3	10	0.994
OTB	56 ± 18	72 ± 3	93 ± 20	99 ± 17	23	3	10	0.992
ZEA	85 ± 17	108 ± 10	80 ± 4	96 ± 20	22	1.5	5	0.994
αZOL	76 ± 5	84 ± 6	84 ± 1	68 ± 20	49	1.5	5	0.994

Recoveries obtained ranged from 52 to 115% at 50 µg/L and from 68 to 105% at 100 µg/L, respectively.

SSE (%) obtained evidenced a signal suppression (less than 50%) for all mycotoxins except for AFB2 and AFG2. Therefore, matrix-matched calibration curves were used to compensate the signal suppression effects and for effective quantification of the samples. Matrix-matched calibration curves were constructed by spiking blank urine extract samples at levels between <LOQ and 1000 µg/L.

Calibrations curves revealed good linearity, with correlation coefficients (R^2) between 0.990 and 0.999.

Finally, LOD values ranged from 1.5 to 5 µg/L, while LOQ ranged from 3 to 10 µg/L.

14.2.3 Mycotoxin Biomarker Occurrence in Urine Samples

About 55% of the 56 human urine samples resulted positive for at least one of the studied mycotoxins. Comparing genders, 15 of (*n* = 24) male urine samples were contaminated, while only 16 of (*n* = 32) female urine samples were positive. Regarding the analyzed mycotoxins, only AFB2, AFG2, and OTB were detected in hydrolyzed human urine samples with incidences of 32, 41, and 9%, respectively, and concentrations ranging from <LOQ to 69.42 µg/L (Table 14.2).

Table 14.2. Incidences (%) and contents (µg/L) of mycotoxins detected in urine samples

Mycotoxin	AFB2	AFG2	OTB
Incidence (%)	32	41	9
Minimum concentration (µg/L)	<LOQ	<LOQ	<LOQ
Maximum concentration (µg/L)	60.98	69.42	38.88
Mean of total samples (µg/L)	5.30	9.26	1.62
Mean of positive samples (µg/L)	16.48	23.81	18.17
Mean in male urine samples (µg/L)	19.16	24.97	38.88
Mean in female urine samples (µg/L)	14.78	22.28	12.99

AFG2 turned out to be the most prevalent mycotoxin, being reported in 41% of total samples with contents, ranging from <LOQ to 69.42 µg/L. The mean of positive samples was 23.81 µg/L. Comparing genders, 54% of male urine samples resulted contaminated with AFG2, against 31% of female samples. No significant differences were found between the mean of positive samples in both genders, with 24.97 µg/L for males and 22.28 µg/L for females, respectively. AFB2 was reported in 32% of total urine samples at levels comprised between <LOQ and 60.98 µg/L, and a mean of positive samples of 16.48 µg/L. A similar mean concentration was observed when male and female urine samples were compared (19.16 µg/L and 14.78 µg/L, respectively). Similar results were obtained by Jonsyn-Ellis [25], who analyzed mycotoxin levels in urine samples from 97 boys and 93 girls from Sierra Leone during the rainy season. These authors reported AFB1 in 33% and 41% of the boys' and girls' samples, respectively, at concentrations ranging from 0.08 to 127 µg/L. AFB2 was detected in 9% and 20% of the boys' and girls' samples, respectively, with levels of up to 48 µg/L, while AFG1 was detected in 28 and 19% of samples, respectively, at concentrations of up to 57.4 µg/L in boys and 150 µg/L in girls. Finally, AFG2 was only detected in 2 and 3% of samples, respectively, at low concentrations (≤2 µg/L). AFB2 was reported by these authors at similar concentrations to those observed in the present study: although, AFG2 was detected at lower concentrations than in the present study.

Slightly lower concentrations were reported by Ritieni et al. [26] in Italy after analyzing 18 urine samples from pregnant women. AFG1 was reported by these authors in four samples at concentrations ranging from 14.0 to 18.8 µg/L, while AFB1 and AFB2 were presented in two samples at concentrations of 0.4–2 µg/L and 0.3–3 µg/L, respectively. Contrary to these results, in a study performed in Brazil, Jager et al. [27] did not detect AFs in any of the 16 analyzed urine samples, while AFM1 was reported in 61% of the samples. Similar to these authors, Rubert et al. [28] did not report the presence of AFB1, AFB2, and AFG1 in any urine samples acquired from 27 volunteers, while

AFG2 was detected only in one sample at trace levels, at a concentration value comprised between LOD (0.8 µg/L) and LOQ (2 µg/L).

Regarding ochratoxins, OTA was not detected in any of the analyzed samples. Concerning the information available in the literature about the presence of OTA in urine samples, similarly to the present study, Ritieni et al. [26] did not report OTA in any of the 18 urine samples from pregnant Italian women. Rubert et al. [28] only reported the presence of OTA at trace level in 3 of 27 urine samples in a study performed in Spain. Contrary to these results, also in a Spanish population, Coronel et al. [29] reported OTA and ochratoxin α (OTα) presence in 72 human urines samples enzymatically treated, with an occurrence of 12.5% and 61.1%, respectively, and concentrations of up to 0.562 ng/mL and 2.894 ng/mL, respectively. In Portugal, Martins et al. [21] revealed the exposure of the Portuguese population to OTA, among other mycotoxins. However, OTA was observed in first-morning urine samples only at levels of up to 0.610 µg/L. Higher contents and incidences were also reported by Jonsyn-Ellis [25], who studied the presence of aflatoxins and ochratoxins in children's urine samples from Sierra Leone. In the urine samples collected during the dry season, OTA was detected in 21% of the boys' samples at contents ranging between 0.07 and 59 ng/mL, while in girls an occurrence of 31% was detected and contents in the range of 0.08–148 ng/mL. The contents detected in the urine collected in the rainy season ranged from 0.6 to 72.2 ng/mL for boys and from 0.7 to 4.9 ng/mL for girls, respectively.

In contrast to OTA, the other ochratoxin studied in the present work, OTB, was observed in 9% of the urine samples with concentrations ranging from <LOQ and 38.88 µg/L, and a mean of positives of 18.17 µg/L. The mean amount detected in male samples (38.88 µg/L) was higher than that detected in females (12.99 µg/L). Similar to the present study, Jonsyn-Ellis [25] reported some of the urine samples from boys and girls from Sierra Leone positive for OTB. During the rainy season, ranges between 0.05 and 45 ng/mL and between 0.06 and 81 ng/mL were observed for boys and girls, respectively. Similar concentrations were reported in the present study, although a higher OTB occurrence (up to 44%) was reported by Jonsyn-Ellis [25]. In contrast, Liu et al. [30] did not detect OTB in any of the 60 human urine samples collected in Beijing (China) after enzymatic digestion with β-glucuronidase. Contrary to OTB, OTA, and OT-alpha were detected by these authors in one-third of samples at concentrations of up to 0.14 and 2.38 ng/mL, respectively.

ZEA and its metabolite α-ZOL, were not detected in any of the analyzed urine samples. Similar to the present study, Solfrizzo et al. [31] did not report α-ZOL presence in 10 human urine samples after enzymatic pre-treatment of urine with β-glucuronidase/sulfatase. Contrary to the present work, in China, Li et al. [32] analyzed 301 urine samples collected from volunteers aged 0–84 with and without enzyme hydrolysis to determine total and free ZEA biomarkers (α-ZOL, β-ZOL, α-ZAL, β-ZAL, and ZAN). ZEA and α-ZOL were reported by these authors at incidences of 71 and 4%, respectively, after enzyme hydrolysis and concentrations ranging from <LOQ and 3.7 µg/L. Further, in a Chinese population, Zhang et al. [33] studied human biomonitoring of ZEA and its metabolites (α-ZOL, β-ZOL, ZAN, α-ZAL, and β-ZAL) in 199 urine samples, both free and total after β-glucuronidase digestion. After enzymatic hydrolysis, total ZEN, α-ZEL, and β-ZEL were detected in 87.8, 25.6, and 24.1% of samples, respectively, with average amounts of 0.383 ng/mL, 0.089 ng/mL, and 0.142 ng/mL, respectively. These authors also observed that positive rates and amounts increased after enzymatic hydrolysis.

14.2.4 Mycotoxin Biomarker Risk Assessment

For AFB2, a PDI of up to 28.3 µg/kg bw/day was calculated considering the mean of positives samples, decreasing to values of 9.1 µg/kg bw/day and 10.85 µg/kg bw/day under the LB and UB scenarios, respectively (Table 14.3). Regarding AFG2, a PDI of up to 40.9 µg/kg bw/day was calculated, decreasing to 15.9 µg/kg bw/day (LB scenario) and 17.2 µg/kg bw/day (UB scenario). Similar to the present study, Martins et al. [34] assessed the Portuguese exposure to aflatoxins employing a biomonitoring approach in urine and obtained AFs PDIs of 13.4 and 16.7 µg/kg bw/day considering the probabilistic and deterministic approaches. However, all PDI obtained in the present study estimated a MoE < 10,000, thus revealing a potential health risk.

Table 14.3. PDIs calculated based on the mycotoxin biomarker urinary levels among the participants

Mycotoxin	Mean Positive Samples		LB Scenario		UB Scenario	
	Mean PDI (µg/kg bw/day)		Mean PDI (µg/kg bw/day)		Mean PDI (µg/kg bw/day)	
	Males	Females	Males	Females	Males	Females
AFB2	23.19	28.3	7.46	9.1	8.89	10.85
AFG2	33.52	40.9	13.03	15.9	14.1	17.2
OTB	0.66	0.81	0.06	0.07	0.16	0.19

Concerning OTB, PDI values of up to 0.81, 0.07, and 0.19 µg/kg bw/day, were calculated within mean of positive samples estimation, LB and UB scenarios, respectively (Table 14.3). These PDIs exceeded the TWI established for OTA in all scenarios. In Catalonia (Spain), Vidal et al. [35] reported a median OTA PDI of 0.031 µg/kg bw/day after the analysis of urine samples, with most samples exceeding the safety TWI value fixed for OTA as in the present study. These authors also concluded that the dietary exposure approaches may result in an underestimation of mycotoxin exposure. Moreover, the margin of exposure (MoE) obtained using the BMDL10 established for OTA also revealed a potential health risk with values close to 100 and 200 in LB and UB scenarios. However, several studies revealed that OTB is metabolized and eliminated quickly and completely, unlike OTA, presenting a lower nephrotoxicity. Furthermore, in vitro and in vivo studies suggested that OTB could be approximately an order of magnitude less toxic than OTA [36]. Thus, much more accurate risk assessment could be performed with the OTB excretion rate and BMDL10 specific values.

14.3 MATERIALS AND METHODS

14.3.1 Reagents and Chemicals

Solvents, acetonitrile (ACN) and methanol (MeOH) TOF grade and ethyl acetate (EtOAc) were supplied by Merck (Darmstadt, Germany). Deionized water (<18.2 MΩcm resistivity) was prepared in the laboratory using a Milli-QSP® Reactive Water System (Millipore, Beadford, MA, USA). Formic acid (CH_2O_2) (grade ≥ 95%) was supplied by Sigma-Aldrich (St. Louis, MO, USA), and acetic acid ($C_2H_4O_2$) (grade ≥ 99%) was acquired from Fisher Scientific (UK). All solvents were filtered through a 0.45 µm cellulose filter supplied by Scharlau (Barcelona, Spain).

Salts, ammonium formate (99%) was supplied by Panreac Quimica S.A.U (Barcelona, Spain), sodium chloride (NaCl) was obtained from VWR Chemicals (Leuven, Belgium), magnesium sulfate (MgSO4) anhydrous powder (99.5%) was supplied by Alfa Aesar (Karlsruhe, Germany) and C18-E (50 µm, 65 A) was purchased from Phenomenex (Madrid, Spain).

Cartridges used for SPE extraction consisted in Strata 33 µm polymeric reversed phase supplied by Phenomenex (USA).

Ammonium acetate was obtained from Merck. Helix pomatia type H-1 β-glucuronidase (glucuronidase activity: ≥300,000 units/g solid and sulfatase activity: ≥10,000 units/g solid) was purchased from Sigma-Aldrich.

Mycotoxin standards (OTA, OTB, AFB2, AFG2, ZEA, and α-ZOL) were obtained from Sigma Aldrich. Individual stocks solutions of all analytes were prepared to obtain 100 mg/L in methanol, and 1 mg/L multi-analyte working solutions were prepared by diluting the individual solutions. All standards were stored in the dark at −20°C.

Before the injection, samples were filtered through a nylon syringe filter (13 mm diameter, 0.22 µm pore size) obtained from Membrane Solutions (Texas TX, USA).

14.3.2 Sample Collection

Fifty-six urine samples from adult participants were collected during the December 2019–January 2020 period. First-morning urine samples collected were obtained from 24 males and 32 females in a wide age group. To collect the samples, sterile vessels were used and then stored at −20°C until analysis. No exclusion criteria were set, and volunteers provided a signed informed consent following the Helsinki Declaration on ethical principles for medical research. This research was approved by the University of Valencia Institutional Human Research Committee (reference number: 1564214).

14.3.3 Urine Sample Preparation

All urine samples were centrifuged at 5000 rpm for 5 min at 4°C prior to extraction.

The urine samples were hydrolyzed according to a previous study [5]: 1 mL of the previously centrifuged urine was collected in a 2 mL Eppendorf tube and 250 µL of ammonium acetate buffer (1 M, pH5) containing 20,000 U of β-glucuronidase/mL was added. Hydrolysis was performed with continuous stirring at 550 rpm for 18 h at 37°C.

14.3.4 Mycotoxin Extraction Procedures

14.3.4.1 Solid Phase Extraction (SPE)

Firstly, 1 mL of previously centrifuged urine was introduced into the cartridges pre-conditioned with 1 mL of MeOH and 1 mL of H_2O. Samples were eluted from the cartridges using 600 µL of 2% formic acid MeOH/ACN (50:50). Then, the samples were dried under a nitrogen stream and reconstituted with 1 mL of 0.1% formic acid ACN/MeOH.

14.3.4.2 Dispersive Liquid–Liquid Microextraction (DLLME)

For DLLME extraction, 1 mL of previously centrifuged urine was placed in a tube with 0.3 g of NaCl and mixed with the vortex, then 1 mL of ACN and 100 µL of EtOAc were added and vortexed for 1 additional minute. Thereafter, the samples were centrifuged at 5000 rpm at 4 °C for 3 min, the organic phase, separated and placed at the top of the tube, was collected. Then, it was evaporated under a N_2 stream and reconstituted with 1 mL MeOH /H_2O (70:30, *v*/*v*) prior to being filtered through a 13 mm/0.22 µm nylon filter.

14.3.4.3 QuEChERS

Firstly, 1 mL of the previously centrifuged urine was placed into a 15 mL tube with 0.3 g of MgSO4, 0.030 g of C18 and 1 mL of ACN. After vortex for 1 min and centrifugation at 4500 rpm for 3 min, the supernatant was then filtered through a 13 mm/0.22 µm nylon filter inside 1.5 mL glass vials prior to injection.

14.3.4.4 Optimization of Extraction Procedures

To optimize the extraction of AFB2, AFG2, OTA, OTB, ZEA, and α-ZOL mycotoxins from urine samples different extraction protocols were tested: SPE, DLLME, and QuEChERS.

Optimization was carried out through recovery experiments. For this, the absolute peak areas of each analyte in a blank urine sample spiked before extraction was compared with the absolute peak areas of the analyte spiked after extraction. Recovery experiments were performed in triplicate at two levels of contamination (50 µg/L and 100 µg/L).

14.3.4.5 Validation of the QuEChERS Method

QuEChERS method was characterized in terms of recoveries, linearity, limits of detection (LODs), and limits of quantification (LOQs) and matrix effects according to European directive 2002/657/EC [24].

Recovery experiments were performed at two levels (50 and 100 µg/L). Intra-day analysis was obtained by three determinations on the same day, and inter-day was assessed based on three determinations on nonconsecutive days.

To evaluate possible matrix effects, which evidence a possible suppression or enhancement of mycotoxin signals, the slope of mycotoxin calibration curves prepared in blank urine extract samples were compared with the slope of calibration curves performed in solvent. SSE (%) were calculated as follows: SSE (%) = slope of curve prepared in extracted matrix/slope of curve in methanol × 100.

Calibrations curves of mycotoxins dissolved in blank urine extract samples and in methanol were constructed at concentration levels ranging from the LOQ of each mycotoxin to 1000 µg/L.

Finally, LODs and LOQs were obtained using the criterion of a signal-to-noise ratio (S/N) of ≥3 for LOD and S/N ≥ 10 for LOQ.

14.3.5 LC-ESI-qTOF-MS Determination

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, autosampler, and binary pump was used for the chromatographic determination. The column used consisted of a Gemini® NX-C18 (3 µM, 150 × 2 mm ID) (Phenomenex). The mobile phases were made of water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient program was as follows: 0–6 min, 50% B; 7–12 min, 100% B; 13–20 min, 50% B. The injection volume was set at 5 µL and the flow rate at 0.2 mL/min. Mass spectrometry (MS) analysis was performed employing a 6540 Agilent Ultra-High-Definition Accurate-Mass q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive and negative ionization modes under the following conditions: interface in positive and negative ionization modes; drying gas flow (N2) 12.0 L min^{-1}; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage, 160 V. Analysis was carried out in MS mode and MS spectra were collected within the scan range of 50–1500 *m*/*z*. Integration and data acquisition were performed employing the Mass Hunter Workstation software.

14.3.6 Risk Assessment

Risk assessment provides an overview of the potentially hazardous exposure to mycotoxins. Risk assessment employing biomarker quantification uses the excreted levels of the contaminant in urine and aims to estimate the intake level. Molecular biomarkers of mycotoxins (mycotoxin metabolites or mycotoxin bioconjugated forms) can be used to measure human exposure.

According to Solfrizzo et al. [37], the Probable Daily Intake (PDI) can be calculated based on the results of mycotoxin biomarkers detected in urine, using the following expression:

$$\text{PDI (µg kg bw/day)} = C \times V \times 100/W \times E$$

where C refers to the concentration of mycotoxins biomarker in urine (µg/L), V to the volume of urine excreted in 24 h, established in a mean of 1.5 L [38]. W refers to body weight (kg), established at 82 kg for males and 67.2 kg for females according to EFSA guidance [39]. Finally, E constitutes the mycotoxin excretion rate (%), calculated as approximately 50% for OTA [40], 10% for ZEA [41], and 1.3% for AFs [42].

In order to calculate mycotoxin PDIs, different exposure scenarios were considered: the mean of positive urine samples, where only positive samples were considered; and the lower bound (LB) and upper bound (UB) scenarios, where data below LOD were processed according to EFSA recommendations [43]. Thus, zero was assigned when mycotoxins were not detected or were detected below the limit of quantification in the LB scenario, while the limit of detection was assigned in the UB scenario.

Then, PDIs were compared with the established TDIs to estimate the potential risk of exposure to the mycotoxins. A tolerable weekly intake (TWI) of 0.12 µg/kg bw/week has been fixed for OTA by the EFSA CONTAM Panel [44] and a TDI of 0.25 µg/kg bw/day has been established for ZEA [45]. However, no TDI has been established for AFs, as they are considered genotoxic and carcinogenic compounds, causing hepatocellular carcinoma [46,47]. In this case, risk assessment is typically based on the margin of exposure (MoE) [48]. Moreover, for OTA, EFSA [49] concluded that the use of a health-based guidance value for OTA is no longer appropriate, but instead a margin of exposure (MoE) should be calculated for both neoplastic and non-neoplastic effects.

The MoE tool is used in risk assessment to evaluate substances that are both genotoxic and carcinogenic. MoE can be calculated using the benchmark dose lower confidence limit (BMDL), obtained from animal studies, divided by estimated PDI. Subscript 10 indicates the percentage of the confidence level of the dose response curve. Thus, MoE can be calculated following this equation:
MoE = (BMDL 10)/(intake values)

An MoE ≥ 10,000 indicates low public health risk associated with exposure to a genotoxic carcinogen [48].

An AFs BMD lower confidence limit (BMDL10) for a 10% increase in cancer incidence obtained from animal study data modeling of 170 ng/kg bw/day was proposed by EFSA [50], while a BMDL10 (14,500 ng/kg bw/day for neoplastic effects) was considered according to EFSA guidelines concerning OTA risk assessment [49]. To the best of our knowledge no BMDL10 has so far not been established for OTB.

14.4 CONCLUSIONS

Among all extraction procedures evaluated (SPE, DLLME and QuEChERS), QuEChERS extraction followed by LC-ESI-qTOF determination was selected as the most appropriate methodology to determine AFB2, AFG2, OTA, OTB, ZEA, and α-ZOL biomarkers in urine samples, as it produces better recovery results (55–93%). The remainder of the analytical parameters obtained was also in accordance with the limits established by European Commission Decision 2002/657/EC. The application to actual urine samples from volunteers revealed the presence of AFB2, AFG2, and OTB in 32, 41, and 9% of the analyzed samples at levels ranging from <LOQ to 69,42 µg/L. Risk assessment revealed a potential health risk to AFB2, AFG2, and OTB exposure. However, it is important to highlight that only some samples were contaminated and that more data about mycotoxin excretion rates and BMDL10 values are needed to obtain a more accurate risk assessment.

AUTHOR CONTRIBUTIONS

Formal analysis, investigation, validation, writing—original draft preparation: N.P. and D.C. Conceptualization, supervision, writing—review and editing: H.B., E.F. and Y.R.-C. All authors have read and agreed to the published version of the manuscript.

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INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chapter 15
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Multiclass and Multi-residue Screening of Mycotoxins, Pharmacologically Active Substances, and Pesticides in Infant Milk Formulas through ultra-High-performance Liquid Chromatography Coupled with High-resolution Mass Spectrometry Analysis

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ABSTRACT

Infant milk formulas are designed to substitute human milk when breastfeeding is unavailable. In addition to human milk and milk-derived products, these formulas can be a vehicle of contaminants. In this work, a multiclass method based on the QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach was developed for the simultaneous determination of contaminants (n = 45), including mycotoxins and veterinary drug residues, occurring in infant milk formulas. By using an ultra-high-performance liquid chromatography quadrupole-Orbitrap coupled with high-resolution mass spectrometry analysis (UHPLC-Q-Orbitrap HRMS; Thermo Fisher Scientific), further retrospective analysis of 337 contaminants, including pesticides, was achieved. The method was validated in accordance with European regulations and applied for the analysis of 54 infant milk samples. Risk assessment was also performed. Dexamethasone was detected in 16.6% of samples (range: 0.905–1.131 ng/mL), and procaine benzyl penicillin in 1 sample at a concentration of 0.295 ng/mL. Zearalenone was found in 55.5% of samples (range: 0.133–0.638 ng/mL) and α-zearalenol in 16.6% of samples (range: 1.534–10.408 ng/mL). Up to 49 pesticides, 11 veterinary drug residues, and 5 mycotoxins were tentatively identified via retrospective analysis based on the mass spectral library. These findings highlight the necessity of careful evaluation of contaminants in infant formulas, considering that they are intended for a vulnerable part of the population.

Keywords: Infant milk formula; mycotoxins; veterinary drug residues; pesticide; UHPLC-Q-Orbitrap HRMS.

1. INTRODUCTION

Infant milk formulas (**IMF**) are a complementary food that occupies a special place in childhood feeding and that become an essential product when breastfeeding is unavailable. Infant milk formulas are the only processed foodstuff that totally fulfills the nutritional requirements of infants through the first months of life [1,2]. Worldwide recommendations for infant feeding are sole breastfeeding up to 6 mo old and nutritionally complementary feeding from 6 mo to 2 yr of age or more [3]. To safeguard infant health, it is essential to ensure that the products marketed are suitable. An adequate selection of raw materials, which includes strict limitations on contaminant residues, should be demanded in

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their manufacture. Infant milk formulas are principally produced based on cow milk subsequently diluted, skimmed, and enriched with minerals and vitamins [4]. However, IMF can contain toxic substances due to potential contamination from the chain production of the raw material and may serve as a vehicle for the transmission of toxic substances such as mycotoxins [5].

Mycotoxins are common secondary metabolites produced by commensal genera of filamentous fungi such as *Penicillium*, *Aspergillus*, *Fusarium*, *Claviceps*, and *Alternaria*. Mycotoxin contamination represents a great worldwide concern due to their serious health hazards, including mutagenicity, teratogenicity, and carcinogenicity. Even though several hundred mycotoxins have been identified to date, those responsible for common mycotoxicological risk concerns are represented by aflatoxins (**AF**), trichothecenes, ochratoxins, fumonisins, patulin, citrinin, and ergot alkaloids [6]. Among these, AF are classified as carcinogenic to humans and included in group 1 by the International Agency for Research on Cancer; fumonisins and ochratoxins are included in group 2B and classified as possibly carcinogenic to humans [7]. Generally, mycotoxin contamination occurs directly in the field, or during the post-harvest period, which includes processing, storage, and distribution of harvested products. In addition, when animals ingest contaminated foodstuffs, mycotoxins are metabolized, biotransformed, and secreted into animal products, such as milk or meat [8,9].

Among mycotoxins, aflatoxin M_1 (**AFM_1**), the hydroxylated metabolite of AFB_1, represents the mycotoxin of greatest incidence in milk and dairy products [10]. Moreover, when animal feed was supplemented with silage, ochratoxin, zearalenone, T2 toxin, fumonisins, and deoxynivalenol were also identified in milk and milk derivatives, although these have not been as extensively studied as AFM_1 [11,12]. To protect consumers' health, the regulatory authorities set a maximum limit for AFM_1 of 0.05 µg/kg in raw milk, heat-treated milk, and milk for the manufacture of milk-based products, restricted to 0.025 µg/kg for infant formulas and follow-on formulas and dietary foods for special medical purposes intended specifically for infants, under regulation no. 1881/2006 [13].

Another group of contaminants of relevant concern in IMF are represented by pesticides, synthetic compounds used for the management of parasites in agricultural production. These organisms can reach the food chain through consumption of contaminated fodder and pastures [14,15]. Several classes of pesticides, such as organo-phosphorus, pyrethroids and carbamate, and organo-chlorines, have been reported in milk and milk derivatives [16]. Milk consumption is not free from risk of exposure to pesticides, and this deserves more attention from researchers. Maximum residue levels (**MRL**) for pesticides have been established by the European Union in regulation no. 839/2008 [17].

In addition, IMF may also contain residues of pharmacologically active substances. Veterinary drugs are frequently used to prevent and treat diseases in food-producing animals. However, incorrect use or noncompliance with the withdrawal period after treatment may allow release of residues from active substances that, after accumulation in the body, lead to injurious effects in humans [5]. The normal use of veterinary drugs to control animal diseases or improve production efficiency is acceptable when meeting the MRL tolerated in food products set in regulation EC 37/2010 by the Codex Alimentarius Commission [18]. However, the withdrawal period that must elapse between the administration of the veterinary drug and the recovery of raw material for food production intended for human consumption is set by directive 2004/28/CE [19].

To guarantee effective consumer safety, reliable methods have been validated for the analysis of contaminants in food matrices [20,21]. Until now, few scientific works have reported methods for the simultaneous analysis of pharmacologically active substances, pesticides, and mycotoxins in milk [22,23,24,25]. The development of an optimal method should allow good recovery of the analytes and good reproducibility of the data, and, at the same time, it should be fast, easy to reproduce, and guarantee lower toxic organic solvent consumption.

Among the various available method approaches used in the pretreatment of samples, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach represents the one that comes closest to achieving the characteristics mentioned above. It is largely employed to extract different groups of compounds and represents the most frequently used pretreatment technique in foods analysis [26,27,28,29]. The most frequent analytical methods are based on liquid chromatography

coupled to MS. The use of ultra-high-performance liquid chromatography (**UHPLC**) provides higher sensitivity, a reduction in mobile phase consumption, and an increase in resolving power and peak shape. High-resolution mass spectrometry (**HRMS**) provides sensitive and specific measurements for the quantification of targeted compounds, with the additional features of retrospective data analysis and identification of untargeted compounds based on exact mass measurements [30,31,32].

Bearing in mind the lack of studies reporting multiclass analysis, the current scientific study aimed (1) to develop a multi-residue method for the identification of target mycotoxins and pharmacologically active substances (n = 45) in IMF through a QuEChERS-based extraction coupled to UHPLC quadrupole (**Q**)-Orbitrap HRMS (Thermo Fisher Scientific); (2) to apply the developed method for evaluating the occurrence in 54 samples collected from the Italian market; and (3) to detect possible untargeted compounds (n = 337), including pesticides, through retrospective analysis.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Methanol, acetonitrile, and water for chromatography (LC-MS grade) were purchased from Merck. Ammonium formate (NH_4HCO_2, analytical grade) and formic acid (HCOOH, MS grade) were acquired from Fluka. Sodium chloride (NaCl), anhydrous sulfate sodium (Na_2SO_4), anhydrous sodium acetate ($C_2H_3NaO_2$), primary secondary amine sorbent (**PSA**), and discovery octadecyl silica (C_{18}, analytical grade) were supplied by Sigma-Aldrich.

Conical polypropylene centrifuge and microcentrifuge tubes and polypropylene syringes without needles were acquired from Microtech Srl. Syringe filters with a nylon membrane (Phenex-NY 15-mm syringe filters, 0.2-µm diameter) and glass amber vials with septum screw caps were supplied by Phenomenex.

Standards of mycotoxins and pharmacologically active substances (purity >98%) were obtained from Sigma-Aldrich. European Commission regulation no. 37/2010 [18] was used as a starting point to select pharmacologically active substances with lower MRL or those that are prohibited in milk.

The acquired pharmacologically active substances (n = 23) standards included abamectin, amoxicillin, ampicillin, ceftiofur, chloramphenicol, clenbuterol hydrochloride, cyhalothrin, cypermethrin, colchicine, danofloxacin, dapsone, dexamethasone, deltamethrin, doramectin, eprinomectin, imidocarb, ivermectin, metronidazole, meloxicam, monensin sodium, procaine benzylpenicillin, sulfadimidine, and trichlorfon.

The acquired mycotoxin (n = 22) standards included aflatoxins (AFM_1, AFB_1, AFB_2, AFG_1, and AFG_2), alternariol, alternariolmonomethyl ether, beauvericin, deoxynivalenol, enniatins (ENNA, ENNA1, ENNB, and ENNB1), fusarenon-X, neosolaniol (**NEO**), HT2 toxin, T2 toxin, zearalanol (**ZEN**), α- and β-zearalenol (α- and β-**ZEL**), α-zearalanol, and β-zearalanol.

The individual standard solution of each analyte was prepared in the optimal solvent recommended by the supplier at a concentration of 1 mg/mL. After that, 2 stock solutions were prepared: stock solution 1, which included all mycotoxins, and stock solution 2, which included the remaining pharmacologically active compounds. These stock solutions were optimally diluted in methanol to obtain appropriate working standard solutions to use in spiking tests. The working standard solutions were prepared at 0.5, 5, and 25 ng/mL. Stock solutions were kept at −20°C in screw-capped glass vials.

2.2 Sampling

Three lots of 18 different brands of IMF were purchased from supermarkets in the Campania region, in southern Italy. The analysis was performed on a total of 54 samples. Samples included infant milk powder (n = 18) and liquid infant milk (n = 36) commonly consumed in childhood. Samples were kept

in their original packages and stored at 4°C until analysis, which wasperformed within 48 h after their arrival in the laboratory.

2.3 Sample Preparation

Extraction was performed in accordance with the procedure described by [25], with slight modifications. Aliquots of 1.4 g of infant milk powder were weighed into 50-mL propylene tubes (Conical Polypropylene Centrifuge Tube, Thermo Fisher Scientific) and dissolved with 10 mL of pure water as recommended by the manufacturer. In short, to 10 mL of the liquid sample, 2.5 mL of distilled water and 5 mL of acetonitrile containing 3.35% formic acid (vol/vol) were added. The 50-mL Falcon tube was vortexed (ZX3, VEPL Scientific) for 1 min and sonicated (LBS 1, Zetalab SRL) for 15 min. A mixture of salts, which included 4.0 g of anhydrous sulfate sodium, 1.2 g of sodium chloride, and 0.5 g of anhydrous sodium acetate, was added. This step was followed by manual shaking for 1 min and centrifugation (X3R Heraeus Multifuge, Thermo Fisher Scientific) for 3 min at 1,792 × *g* and 4°C. After centrifugation, all the supernatant was recovered and transferred into a 15-mL Falcon tube in which 300 mg of C18 sorbent, 140 mg of PSA, and 1.5 g of anhydrous sulfate sodium were weighed. The 15-mL falcon tube was vortexed for 1 min and centrifuged at 4°C and 252 × *g* for 1 min. The upper layer was collected into a clear 15-mL Falcon tube and dried under gentle nitrogen flow at 45°C. The residue was reconstituted with 500 µL of methanol:water (70:30, vol/vol), filtered through a 0.22-µm filter, and transferred into an amber vial for the UHPLC-Q-Orbitrap HRMS analysis.

2.4 UHPLC-Q-Orbitrap HRMS Analysis

Analysis was performed as previously described by [25], with some modifications. For the qualitative and quantitative analyses of compounds, a UHPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) equipped with a degassing system, a quaternary UHPLC pump working at 125 MPa, and a refrigerated autosampler device were used. Chromatographic separation was carried out with a thermostated (T = 25°C) Kinetex Biphenyl 2.6-µm column (100 × 2.1 mm, Phenomenex) coupled to a guard column (5 × 2 mm, 1.8-µm particle size) and an inline filter. The mobile phase consisted of water containing 5 m*M* ammonium acetate (A) and methanol containing 5 m*M* ammonium acetate (B). The injection volume was 5 µL, and the flow rate was 0.5 mL/min. Separation was achieved under the following gradient conditions: 0 to 0.5 min, 10% B; 0.5 to 2.5 min, 10 to 80% B; 2.5 to 5.5 min, 100% B; 5.5 to 7.5 min, 100 to 10% B; and then the column was re-equilibrated at 10% B for 1.5 min before the next injection.

The Q-Orbitrap HRMS system was operated in both positive and negative ionization modes. Full scan and data-independent all-ion fragmentation spectra were collected.

The following scan parameters were used in full MS mode: mass resolution power of 35,000 full width at half maximum, scan range 90 to 1,000 *m*/*z*, automatic gain control target 1×10^6, maximum inject time set to 200 ms, and scan rate 3 scan/s. The ion source parameters were as follows: sheath gas (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, spray voltage 2.8 kV; capillary temperature 310°C; S-lens RF level 50; and auxiliary gas heater temperature 305°C.

The following scan parameters were used in all-ion fragmentation mode: mass resolution power of 17,500 full width at half maximum, scan range 80–1,000 *m*/*z*, automatic gain control target 1×10^5, maximum inject time set to 200 ms, and scan rate 3 scan/s; scan time = 0.10 s; isolation window 5.0 *m*/*z*; and retention time 30 s. The collision energy was varied in the range of 10 to 45 eV to obtain representative product ion spectra. A mass tolerance below 2 ppm was set for the identification of the molecular ion and fragments. Data processing was performed by using Xcalibur software, version 3.1.66.10 (Thermo Fisher Scientific; Rodríguez-Carrasco et al., [33]).

2.5 Retrospective Screening

Retrospective analysis was carried out as previously described by Izzo et al. [25], with slight modifications. Qualitative analysis of compounds for which a reference standard was not available

was carried out by retrospective analysis of the data. The retrospective analysis of data was performed on spectral data collected using 3 spectral libraries (Mycotoxin Spectral Library version 1.1 for LibraryView Software, AB Sciex; Antibiotics Spectral Library version 1.0 for LibraryView Software, AB Sciex; and Pesticide Spectral Library version 1.1 for LibraryView Software, AB Sciex). Identification was performed by searching in the extract for the exact mass to the fifth decimal place. The sample was considered suspect positive for the occurrence of a specific analyte if a peak was identified by setting a mass tolerance of 1 ppm for the molecular ion.

2.6 Validation of the Method

Validation of the method was performed as previously described by Narváez et al. [9] with modifications. An analytical method for the determination of both mycotoxins and veterinary drug residues in IMF was validated in-house in accordance with European regulations [34,35,36]. Data quality was verified using a comprehensive range of quality guarantee and quality control procedures. To achieve a rigorous control, the in-house validation method included a reagent blank, a procedural blank, a triplicate sample, a matrix-matched calibration in each batch of samples, and 3 spiking levels. The following analytical performance parameters were evaluated: linearity, matrix effect, specificity, trueness, precision (repeatability and intermediate precision), and sensitivity expressed in terms of limit of detection, limit of quantification (**LOQ**), decision limits (**CCα**), and detection capability (**CCβ**).

Linearity was evaluated using solvent and matrix-matched calibration curves, injecting in triplicate concentration levels ranging from 0.0049 to 25 ng/mL.

Signal suppression or enhancement effect was evaluated through a comparison between the slope of matrix-matched standard curve (A) and the slope of pure standard curve (B), calculated as the ratio (A/B × 100). Signal suppression occurred if the value was lower than 100% and a signal enhancement if the value was higher than 100%. A value of 100% indicated no matrix effect.

Specificity was evaluated by injecting a blank milk extract 10 times to confirm the absence of target analytes and to evaluate possible sample interference.

Trueness was assayed by the addition of known quantities of the studied analytes at 0.5, 1, and 5 ng/mL to a blank milk sample, and was expressed as the percentage of recovery.

Precision of the validated method was determined by repeated measurements of the 3 fortification levels, on the same day (repeatability, n = 9) and on 3 nonconsecutive days (reproducibility, n = 27), and was expressed as relative standard deviation [% **RSD**; intra-day (RSDr, %) and inter-day precision (RSDR, %)].

Sensitivity was evaluated by limit of detection and LOQ. The limit of detection was established as the minimum concentration that allows molecular ion identification with a mass error below 2 ppm. The LOQ referred to the lowest concentration of the analyte that generates a chromatographic peak with a precision and accuracy <20%. For confirmation criteria, the retention times of analytes in standards and samples were compared (tolerance of ± 2.5%).

In the case of substances for which MRL was fixed, the CCα values were determined by analyzing 20 blank samples fortified at the corresponding permitted limit (CMRL). Detection capability was the value of the CMRL plus 1.64 times the corresponding standard deviation (SD) of the within-laboratory reproducibility (α = 5%):$CC\alpha = CMRL + 1.64 \times SD_{20\ \text{representative MRL spike}}$. In the case of substances for which MRL was not established, CCα was calculated as 3 times the signal-to-noise ratio (S/N) of the baseline of 20 representative blank samples:$CC\alpha = 3\ S/N_{20\ \text{representative blank samples}}$. The CCβ was estimated by analyzing 20 blank samples fortified at the previously estimated CCα decision limit (β = 5%):$CC\beta = CC\alpha + 1.64 \times SD_{20\ \text{representative spike at CC}\alpha\ \text{level}}$.

2.7 Statistical Analysis

Sample analysis was performed in triplicate and results expressed as mean ± RSD. Statistical analysis of data was performed using the software Info-Stat version 2008

(https://www.infostat.com.ar/index.php?mod = page&id = 15). The level of $P \leq 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSIONS

3.1 Optimization UHPLC-Q-Orbitrap HRMS Conditions

During the method development stage, each of 45 standards were infused at a concentration of 1 µg/mL into the Q-Orbitrap system using syringe injection at a flow rate of 10 µL/min. The obtained MS parameters ensured assignment of the correct identities to the respective compounds in a mixture of standards. The instrument was operated in the positive and negative electrospray modes. For the studied analytes, the best fragmentation patterns were obtained in positive electrospray mode, producing the quasi-molecular ion $[M+H]^+$. Due to the presence of ammonium formate in the mobile phases, the compounds ivermectin, cyhalothrin, cypermethrin, deltamethrin, abamectin, doramectin, NEO, T2 and HT2, enniatins, and beauvericin exhibited ammonium adduct species as the most predominant ions in the mass spectrum [37,38,39].

The collision energy of each studied compound was carefully set to obtain the best fragmentation patterns. The MS parameters for all compounds are shown in Table 1. For accurate mass measurement, identification and confirmation were performed at a mass tolerance of 2 ppm for the precursor molecular ion and for relative fragments.

In this experiment, the column chosen for chromatography separation reached superior resolution, speed, and sensitivity compared with another column tested in a previous study [25]. A first comparison was made by comparing a Luna Omega column (50 × 2.1 mm, 1.6-µm; Phenomenex) used in the chromatographic separation of our previously published study with that of the present study's Kinetex Biphenyl 2.6-µm (100 × 2.1 mm, Phenomenex). The characteristics of the Kinetex Biphenyl column allowed a suitable separation of hydrophobic and hydrophilic compounds simultaneously. The ability to reach 100% aqueous mobile phase in the initial run time was useful for competent retention of hydrophilic analytes. Optimal results in terms of retention time, good peak shape, and chromatographic separation of the analytes were obtained with this chromatographic column. Moreover, ammonium formate was added to both mobile phases to help reduce the formation of unwanted adducts and stabilize the pH [40,41].

Having made the choice of the column, different gradients were tested to obtain the best chromatographic separation of all analytes. Chromatographic separation was evaluated under the following gradient conditions:

Gradient 1: 0 to 1.5 min, 0% B; 0.5 to 3.5 min, 10 to 50% B; 3.5 to 6.5 min, 100% B; 6.5 to 9.5 min, 100 to 0% B; column re-equilibrated at 10% B for 2.5 min before the next injection; total run time 12 min;

Gradient 2: 0 to 1.5 min, 10% B; 0.5 to 2.5 min, 10 to 80% B; 2.5 to 5.5 min, 100% B; 5.5 to 9.5 min, 100 to 0% B; column re-equilibrated at 10% B for 1.5 min before the next injection; total run time 12 min;

Gradient 3: 0 to 0.5 min, 10% B; 0.5 to 2.5 min, 10 to 80% B; 2.5 to 5.5 min, 100% B; 5.57.5 min, 100 to 10% B; column re-equilibrated at 10% B for 1.5 min before the next injection; total run time 9 min.

Using gradient 1, some compounds were not retained by the stationary phase and were eluted within the dead time of the chromatographic separation. The total run time was 12 min, which led to greater organic solvent consumption. With gradient 2, compounds were eluted between 3.5 and 7 min, although peak response for some analytes was not regular. For gradient 3, good separation and peak shape were obtained for all studied analytes (Table 1). The retention times were reproducible under ± 0.2 min for most of the target analytes.

Table 1. Chromatographic retention time and optimized MS/MS parameters for 45 target analytes in commercial infant milk formula samples

Compound	Retention time (min)	Chemical formula	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy[1] (Δ ppm)	CE[2] (V)
Veterinary drugs (n = 23)							
Amoxicillin	1.84	$C_{16}H_{19}N_3O_5S$	$[M+H]^+$	366.11182	366.11163	−0.52	20
Metronidazole	2.10	$C_6H_9N_3O_3$	$[M+H]^+$	172.07167	172.0715	−0.99	19
Trichlorfon	3.35	$C_4H_8Cl_3O_4P$	$[M+H]^+$	256.92985	256.92965	−0.78	20
Procaine benzyl penicillin	3.38	$C_{13}H_20N_2O_2$	$[M+H]^+$	237.15975	237.15928	−1.98	20
Ampicillin	3.39	$C_{16}H_{19}N_3O_4S$	$[M+H]^+$	350.11693	350.11629	−1.83	20
Imidocarb	3.40	$C_{19}H_20N_6O$	$[M+H]^+$	349.17714	349.17672	−1.20	25
Dapsone	3.75	$C_{12}H_{12}N_2SO_2$	$[M+H]^+$	249.06922	249.06885	−1.49	18
Clenbuterol	3.81	$C_{12}H_{18}Cl_2N_2O$	$[M+H]^+$	277.08690	277.08657	−1.19	21
Chloramphenicol	3.81	$C_{11}H_{12}Cl_2N_2O_5$	$[M-H]^-$	321.00505	321.00526	0.65	20
Danofloxacin	3.84	$C_{19}H_20FN_3O_3$	$[M+H]^+$	358.15615	358.15548	−1.87	20
Sulfadimidine	3.91	$C_{12}H_{14}N_4O_2S$	$[M+H]^+$	279.09102	279.09062	−1.43	25
Ceftiofur	4.21	$C_{19}H_{17}N_5O_7S_3$	$[M+H]^+$	524.03629	524.03536	−1.77	28
Dexamethasone	4.40	$C_{22}H_{29}FO_5$	$[M+H]^+$	393.20718	393.20645	−1.86	26
Colchicine	4.43	$C_{22}H_{25}NO_6$	$[M+H]^+$	400.17546	400.17469	−1.92	25
Meloxicam	4.56	$C_{14}H_{13}N_3O_4S_2$	$[M-H]^-$	350.02747	350.02725	−0.63	25
Cyhalothrin	5.09	$C_{23}H_{19}ClF_3NO_3$	$[M+NH_4]^+$	467.13438	467.13436	−0.04	21
Abamectin	5.17	$C_{48}H_{72}O_{14}$	$[M+NH_4]^+$	890.52603	890.52463	−1.57	20
Eprinomectin	5.18	$C_{50}H_{75}NO_{14}$	$[M+H]^+$	914.52603	914.52406	−2.15	20
Cypermethrin	5.21	$C_{22}H_{19}Cl_2NO_3$	$[M+NH_4]^+$	433.10802	433.10769	−0.76	23
Monensin	5.23	$C_{36}H_{61}NaO_{11}$	$[M+H]^+$	693.41843	693.41645	−2.86	70
Deltamethrin	5.29	$C_{22}H_{19}Br_2NO_3$	$[M+NH_4]^+$	521.00699	521.00619	−1.54	25
Ivermectin	5.32	$C_{48}H_{74}O_{14}$	$[M+NH_4]^+$	892.54168	892.54123	−0.50	30
Doramectin	5.33	$C_{50}H_{74}O_{14}$	$[M+NH_4]^+$	916.54168	916.53905	−2.87	20
Amitraz	5.55	$C_{19}H_{23}N_3$	$[M+H]^+$	294.19647	294.19635	−0.41	25
Mycotoxins[3] (n = 22)							
DON	2.70	$C_{15}H_{20}O_6$	$[M+H]^+$	297.13326	297.13345	0.64	13
FUS-X	3.58	$C_{17}H_{22}O_8$	$[M+Na]^+$	377.12073	377.12063	−0.27	20
NEO	3.74	$C_{19}H_{26}O_8$	$[M+NH_4]^+$	400.19659	400.19662	0.07	31
AOH	4.23	$C_{14}H_{10}O_5$	$[M-H]^-$	257.04555	257.04622	2.61	32
HT2	4.30	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.24354	442.2432	−0.77	27
α-ZAL	4.31	$C_{18}H_{26}O_5$	$[M-H]^-$	321.17044	321.17136	2.86	26
β-ZAL	4.40	$C_{18}H_{26}O_5$	$[M-H]^-$	321.17044	321.17136	2.86	26
α-ZEL	4.44	$C_{18}H_{24}O_5$	$[M-H]^-$	319.15510	319.15563	1.66	26
T2	4.49	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.25411	484.2542	0.19	23
β-ZEL	4.69	$C_{18}H_{24}O_5$	$[M-H]^-$	319.15510	319.15549	1.22	26
ZEN	4.70	$C_{18}H_{22}O_5$	$[M-H]^-$	317.13945	317.13913	−1.01	32
AFG_1	4.73	$C_{17}H_{12}O_7$	$[M+H]^+$	329.06558	329.06553	−0.15	40
AME	4.75	$C_{15}H_{12}O_5$	$[M-H]^-$	271.06120	271.06128	0.30	36
AFM_1	4.88	$C_{17}H_{14}O_7$	$[M+H]^+$	331.08123	331.08033	−2.72	37
AFG_2	5.03	$C_{17}H_{12}O_7$	$[M+H]^+$	329.06558	329.0651	−1.46	40
ENNB	5.15	$C_{33}H_{57}N_3O_9$	$[M+NH_4]^+$	657.44331	657.44292	−0.59	50
AFB_2	5.16	$C_{17}H_{14}O_6$	$[M+H]^+$	315.08631	315.0852	−3.52	36
ENNB1	5.18	$C_{34}H_{59}N_3O_9$	$[M+NH_4]^+$	671.45986	671.45926	−0.89	48
ENNA1	5.24	$C_{35}H_{61}N_3O_9$	$[M+NH_4]^+$	685.47461	685.47355	−1.55	48
AFB_1	5.26	$C_{17}H_{12}O_6$	$[M+H]^+$	313.07066	313.06958	−3.45	36
ENNA	5.28	$C_{36}H_{63}N_3O_9$	$[M+NH_4]^+$	699.49026	699.48928	−1.40	43
BEA	5.40	$C_{45}H_{57}N_3O_9$	$[M+NH_4]^+$	801.44331	801.44339	0.10	35

[1] Accuracy = [(measured mass m/z *– theoretical mass* m/z*)/theoretical mass* m/z*] × 10^6 = ppm.*
[2] CE = collision energy.
[3] Mycotoxin standards were as follows: aflatoxins (AFM_1, AFB_1, AFB_2, AFG_1, and AFG_2), deoxynivalenol (DON), HT2 toxin, T2 toxin, neosolaniol (NEO), fusarenon-X (FUS-X), zearalanol (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB, and ENNB1), alternariol (AOH), and alternariolmonomethyl ether (AME).

3.2 Optimization of Sample Preparation

In this work, critical extraction parameters such as percentage of acidification and clean-up stage were evaluated. In total, 3 different sample preparation procedures were tested for the determination of different compounds in IMF. Although the extraction process has as its starting point our previously optimized scientific on milk, because IMF represents a different matrix, a new validation is required to ensure that the developed methodology is suitable and meets the requirements of the European regulation in force. Using the protocol reported in the Sample Preparation of the Materials and Methods section as a starting point, slight changes in mixture acidification were performed. The percentage of acetonitrile acidification was 3.35% formic acid (vol/vol) in the first procedure, 4% in the second and 1% in the third protocol. Optimal conditions in terms of peak shape for all the target analytes were reached by using acetonitrile containing 3.35% formic acid (vol/vol). Data regarding recovery and matrix effect were comparable between the first 2, whereas the third protocol showed unsatisfactory results. In addition, 2 different clean-up stages were tested: the first foresaw 300 mg of C18 sorbent, 140 mg of PSA, and 1.5 g of anhydrous sulfate sodium, and the second 100 mg of C18 sorbent, 500 mg of PSA, and 1.5 g of anhydrous sulfate sodium. The conditions for the second clean-up were not suitable to reach appropriate recovery values (<50%). Moreover, interference from the matrix was observed for all studied compounds (percentage of signal suppression or enhancement effect outside the range 80–120%). Special attention is given to the various adsorbent phases used that strictly depend on the matrix. Primary secondary amine (PSA) is a common sorbent used to remove fatty acids, organic acids, lipids, and sugars from the preliminary extract. Sometimes the clean-up step with PSA sorbent may retain analytes and lead to recoveries below 50% [42]. Octadecyl silica (C18) provides optimal results in the purification of samples with significant fat content; at the same time, recoveries of the more lipophilic pesticides may suffer [43]. The clean-up of the first protocol represents a fair compromise between matrix effect and recovery values.

3.3 Analytical Features of the Proposed Method

Results of the in-house validation method for the determination of 45 analytes in milk samples are reported in Table 2. Correlation coefficients (r) > 0.990 were obtained within the range from 0.0049 to 25 ng/mL. The matrix effects were in the range from 78% to 116%, resulting in accordance with the limits reported by EC regulations [34,35,36]. The pure standard calibration curves were used for quantification purposes. As established by Commission decision 2002/657/EC, the values of recoveries at each tested spiked level ranged between 83% and 118%, and the method was repeatable (RSD <19%) and reproducible (RSD <16%). As regards specificity, the instrumentation used showed no signal interferences in the blank matrix for any target studied analytes. As regards sensitivity, the LOQ obtained ranged from 0.049 to 0.390 ng/mL. Both CCβ and CCα were also determined, and results are shown in Table 3. The developed method met the requirements set in European regulations and proved to be suitable for the determination of studied analytes in IMF.

3.4 Application to Samples: Target Mycotoxins and Pharmacologically Active Substances

3.4.1 Substances in milk samples

To demonstrate the suitability of the validated method, it was applied to the analysis of 3 lots of 18 IMF brands acquired from different supermarkets in the Campania region of southern Italy. The results reported in this study referred to the average obtained from analysis of 3 different lots for each brand available in Campania supermarkets (n = 54 samples). Although most studies on milk have focused on evaluating occurrence of AFM_1, in the last few years researchers have extended the analysis to other mycotoxins [8,12].

Table 2. Method performance: linearity, matrix effect (signal suppression or enhancement, SSE %), recovery, and limit of quantification (LOQ, ng/mL) for 45 targeted analytes in commercial infant milk formula samples[1]

Analyte	Linearity (r)	SSE (%)	Recovery (%)			Precision (%) [RSD_r (RSD_R)]		
			25 ng/mL	5 ng/mL	0.5 ng/mL	25 ng/mL	5 ng/mL	0.5 ng/mL
Veterinary drug residues (n = 23)								
Abamectin	0.9969	103	107	91	90	14 (10)	19 (9)	17 (15)
Amoxicillin	0.9975	84	99	91	97	11 (16)	18 (15)	19 (9)
Ampicillin	0.9998	83	104	98	83	18 (12)	17 (12)	18 (11)
Ceftiofur	0.9996	102	106	90	84	15 (10)	17 (15)	18 (5)
Chloramphenicol	0.9983	87	107	100	103	14 (10)	12 (9)	13 (10)
Clenbuterol hydrochloride	0.9981	116	110	95	92	12 (6)	16 (4)	18 (6)
Colchicine	0.9988	102	92	95	100	17 (7)	17 (8)	19 (14)
Cyhalothrin	0.9951	91	116	95	82	10 (9)	14 (9)	12 (7)
Cypermethrin	0.9961	94	91	102	96	12 (16)	16 (8)	18 (8)
Danofloxacin	0.9986	88	92	89	93	13 (16)	12 (7)	19 (7)
Dapsone	0.9956	116	92	94	94	10 (16)	18 (11)	19 (15)
Deltamethrin	0.9940	84	99	99	97	7 (14)	12 (7)	10 (12)
Dexamethasone	0.9993	110	111	113	102	15 (16)	15 (14)	13 (16)
Doramectin	0.9982	81	85	116	97	18 (9)	19 (8)	9 (13)
Eprinomectin	0.9990	83	101	102	105	17 (13)	17 (11)	14 (8)
Imidocarb	0.9917	105	103	100	95	15 (12)	19 (12)	15 (7)
Ivermectin	0.9916	107	93	97	93	11 (5)	15 (6)	19(11)
Meloxicam	0.9932	104	116	107	108	14 (13)	8 (12)	12 (7)
Metronidazole	0.9936	89	90	83	100	8 (3)	14 (8)	11 (9)
Monensin sodium	0.9987	99	90	91	93	14 (5)	14 (3)	13 (7)
Procaine benzyl penicillin	0.9956	83	102	93	85	10 (13)	10 (5)	18 (8)
Sulfadimidine	0.9976	86	93	95	85	12 (8)	10 (15)	17 (8)
Trichlorfon	0.9986	90	104	109	90	12 (10)	12 (10)	13 (3)
Mycotoxins[2] (n = 22)								
DON	0.9973	93	96	97	92	15 (18)	14 (6)	15 (6)
FUS-X	0.9922	85	109	98	94	7 (9)	11 (5)	15 (11)
NEO	0.9987	89	118	97	100	14 (8)	15 (6)	12 (11)
AFG_2	0.9975	78	102	105	90	11 (7)	14 (13)	12 (8)
AFG_1	0.9963	85	101	106	92	16 (12)	8 (9)	7 (7)
AFM_1	0.9991	81	96	86	93	18 (5)	10 (16)	9 (6)
AFB_2	0.9943	82	114	117	93	10 (14)	5 (10)	16 (3)
AFB_1	0.9948	89	104	114	113	14 (13)	5 (6)	15 (8)
HT2	0.9953	88	116	81	88	16 (9)	16 (15)	8 (16)
α-ZAL	0.9966	86	83	84	91	15 (5)	13 (6)	15 (10)
α-ZEL	0.9967	80	95	87	88	13 (6)	11 (15)	9 (7)
AOH	0.9956	98	104	98	91	18 (11)	17 (10)	10 (6)
T2	0.9995	96	103	113	91	18 (4)	12 (14)	18 (12)
β-ZAL	0.9930	105	106	107	92	12 (5)	14 (12)	12 (11)
β-ZEL	0.9962	94	97	92	89	19 (5)	17 (12)	10 (7)
ZEN	0.9968	95	103	99	89	14 (11)	8 (14)	7 (6)
AME	0.9992	96	117	113	109	11 (8)	15 (5)	14 (16)
ENNB	0.9990	91	102	104	92	9 (11)	15 (13)	18 (10)
ENNB1	0.9992	89	110	115	77	9 (11)	12 (7)	15 (8)
BEA	0.9935	92	113	107	87	14 (12)	19 (11)	7 (3)
ENNA1	0.9996	96	115	93	105	12 (10)	15 (6)	14 (5)
ENNA	0.9969	93	106	113	90	11 (9)	10 (8)	14 (2)

[1] RSD_r = intra-day relative standard deviation; RSD_R = inter-day standard deviation.

[2] Mycotoxin standards were as follows: aflatoxins (AFM_1, AFB_1, AFB_2, AFG_1, and AFG_2), deoxynivalenol (DON), HT2 toxin, T2 toxin, neosolaniol (NEO), fusarenon-X (FUS-X), zearalanol (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB, and ENNB1), alternariol (AOH), and alternariolmonomethyl ether (AME).

Table 3. Limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ) obtained for mycotoxins and veterinary drug residues in infant milk formulas, using ultra-high-performance liquid chromatography quadrupole-Orbitrap high-resolution MS (Thermo Fisher Scientific)

Analyte	LOQ (ng/mL)	CCα (ng/mL)	CCβ (ng/mL)
Veterinary drug residues (n = 23)			
Abamectin	0.390	0.224	0.257
Amoxicillin	0.390	4.114	4.238
Ampicillin	0.390	4.082	4.137
Ceftiofur	0.390	101.047	101.380
Chloramphenicol	0.049	0.072	0.395
Clenbuterol hydrochloride	0.049	0.091	0.097
Colchicine	0.049	0.049	0.221
Cyhalothrin	0.390	32.585	32.678
Cypermethrin	0.390	101.691	102.766
Danofloxacin	0.390	30.963	31.130
Dapsone	0.049	0.071	0.371
Deltamethrin	0.195	31.889	32.245
Dexamethasone	0.049	0.383	0.417
Doramectin	0.390	16.318	16.348
Eprinomectin	0.390	20.963	21.364
Imidocarb	0.390	50.858	51.062
Ivermectin	0.390	10.665	10.949
Meloxicam	0.195	16.055	16.382
Metronidazole	0.049	0.072	0.087
Monensin sodium	0.390	2.180	2.322
Procaine benzyl penicillin	0.097	4.145	4.252
Sulfadimidine	0.195	25.963	26.472
Trichlorfon	0.390	51.141	51.434
Mycotoxins[1] (n = 22)			0.000
DON	0.390	0.439	0.501
FUS-X	0.390	0.447	0.467
NEO	0.390	0.397	0.419
AFG_2	0.195	0.209	0.227
AFG_1	0.195	0.202	0.215
AFM_1	0.049	0.058	0.266
AFB_2	0.097	0.105	0.111
AFB_1	0.097	0.099	0.113
HT2	0.390	0.424	0.450
α-ZAL	0.390	0.438	0.447
α-ZEL	0.390	0.455	0.478
AOH	0.390	0.428	0.451
T2	0.390	0.466	0.478
β-ZAL	0.390	0.435	0.457
β-ZEL	0.390	0.390	0.390
ZEN	0.049	0.073	0.080
AME	0.049	0.049	0.064
ENNB	0.390	0.480	0.496
ENNB1	0.390	0.445	0.445
BEA	0.390	0.488	0.520
ENNA1	0.390	0.458	0.524
ENNA	0.390	0.431	0.500

[1] Mycotoxin standards were as follows: aflatoxins (AFM_1, AFB_1, AFB_2, AFG_1, and AFG_2), deoxynivalenol (DON), HT2 toxin, T2 toxin, neosolaniol (NEO), fusarenon-X (FUS-X), zearalanol (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB, and ENNB1), alternariol (AOH), and alternariolmonomethyl ether (AME).

In this work, IMF tested positive for 2 veterinary drug residues, dexamethasone (16.6%, n = 9) and procaine benzyl penicillin (5.5%, n = 3), and tested positive for up to 6 mycotoxins produced by *Fusarium* species, including emerging mycotoxins (ENN; Table 4).

Table 4. Occurrence of target compounds in 3 lots of 18 different brands of commercial infant milk formulas from southern Italy (n = 54)

Analyte	Range (ng/mL)	Mean (ng/mL)	Positive samples (%)	MRL[1] (ng/mL)
Mycotoxins[2]				
FUS-X	0.735	0.736	3/54 (5.5)	—
NEO	1.735	1.736	3/54 (5.5)	—
α-ZEL	1.534–10.408	6.948	9/54 (16.6)	—
ZEN	0.133–0.638	0.295	30/54 (55.5)	—
ENNB	0.552–0.626	0.576	12/54 (22.2)	—
ENNA1	0.903	0.903	3/54 (5.5)	—
Veterinary drug residues				
Dexamethasone	0.905–1.131	1.149	9/54 (16.6)	0.3
Procaine benzyl penicillin	0.295	0.295	3/54 (5.5)	4

[1] *MRL = maximum residue level.*
[2] *Mycotoxin standards were as follows: neosolaniol (NEO), fusarenon-X (FUS-X), zearalenone (ZEN), α-zearalenol (α-ZEL), and enniatins (ENNA1 and ENNB).*

Similarly, the presence of up to 7 pharmacologically active substances, including benzylpenicillin procaine and dexamethasone, in Italian milk samples (n = 56) has been previously reported [25]. Particularly, procaine benzyl penicillin was found in 8.9% of samples at a concentration range between 1 and 4.530 ng/mL and dexamethasone in 1.7% of samples at a concentration of 0.140 ng/mL. In that case, the concentration of procaine benzyl penicillin found in one sample was slightly higher than the MRL (4 µg/kg). In our study, the concentration of 0.295 ng/mL did not exceed the EU limit.

Antibiotics are drugs widely used for therapeutic purposes and promotion of growth. Around 80% of food animals receive antibiotics for part or most of their lifetime. Veterinary drug residues remain one of the major concerning issues that affect the safety of the dairy industry [44]. Dexamethasone is a potent corticosteroid indicated for the treatment of ketosis in postpartum dairy cows and as an anti-inflammatory drug in bovines but also used as an illegal growth promoter in livestock production [45].

Among mycotoxins, the occurrence of ZEN and its metabolite, α-ZEL, have frequently been reported in feed components in dairy cattle diets. Mycotoxins are natural contaminants known to adulterate a wide heterogeneity of feed ingredients and final products [46]. In this study, ZEN was the most common detected mycotoxin (55.5%, n = 30), at concentrations ranging between 0.133 and 0.638 ng/mL. Although ZEN is not one of the main mycotoxins occurring in dairy products, several scientific studies have reported ZEN contamination [47,48,49]. Pleadin et al. [50] analyzed concentrated dairy cattle feeds (n = 56), cow milk samples (n = 105), and maize silage (n = 21) from Croatia and detected the occurrence of ZEN in 9.5% of maize silage samples and in 94.3% of milk samples, ranging from 0.3 to 88.6 ng/mL. As regards ZEN, considered as not carcinogenic to humans and classified in the third group by IARC, no maximum limits are available in milk.

We detected ENN in up to 4 analyzed milk samples: ENNB (22.2%, n = 12) at concentration ranges of 0.552 to 0.626 ng/mL, and ENNA1 (5.5%, n = 3) at concentrations of 0.903 ng/mL. The ENN are emerging mycotoxins derived from *Fusarium* species, commonly found in cereal grains, animal feeds, and food commodities worldwide [33,51]. Presently, ENNB is the most studied, because it has been the most often found in grains from European countries. The European Food Safety Authority has established that acute exposure to ENN does not indicate concern for human health. It has not been possible to evaluate chronic exposure, due to overall lack of toxicity data [52].

3.4.2 Retrospective Screening Analysis of Real Samples

The developed Q-Orbitrap HRMS strategy combines quantitative target analysis with identification of untargeted compounds. A retrospective approach was taken, without the need to re-run samples. Retrospective analysis of data enabled detection of untargeted contaminants in analyzed milk samples, although it was not possible to carry out a quantitative analysis based on a reference standard. The findings of retrospective data analysis are based only on a match of one precursor ion and do not include any product ion data. Results of untargeted mycotoxins (n = 14) and veterinary drug residues (n = 40) are reported in Figure 1, whereas data on pesticides (n = 283) are shown in Fig. 2.

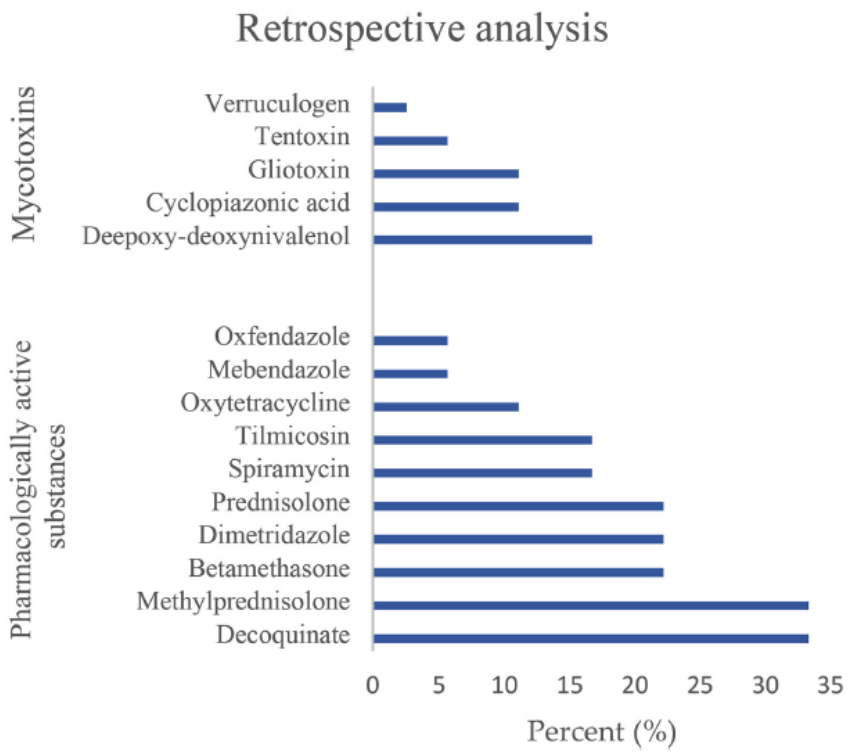

Fig. 1. Percentages of samples and types of mycotoxins and pharmacologically active substances tentatively identified in commercial infant milk formulas, using retrospective analysis of data by matching the exact mass of theoretical precursor ions within 1 ppm

Data tentatively identified the occurrence of up to 5 mycotoxins and fungal metabolites, 11 pharmacologically active substances, and up to 49 different pesticides in the analyzed IMF. Regarding veterinary drugs, oxfendazole, mebendazone, and oxytetracycline were putatively identified in 5.5% samples. Similarly, oxfendazole was found in 2.7% Greek milk samples at an average concentration of 1 µg/kg [53]. Aguilera-Luiz et al. [54], reported oxfendazole, tilmicosin, trimethoprim, thiabendazole, and albendazole in 55.5% of commercial Spanish IMF (n = 9). In our study, betamethasone and methylprednisolone were detected in 22.2% and 33.3% of samples, respectively. Corticosteroids are widely used veterinary drugs, often in combination with antimicrobial drugs. Although licensed for treatment of diseases in breeding animals, MRL for betamethasone and dexamethasone, prednisolone, and methylprednisolone have been set in the European Union at very low levels [18].

Of special concern was the putative identification of decoquinate in 33.3% samples, although it is recommended not to use it in animals from which milk is produced for human consumption. Nebot et al. [55] reported the occurrence of decoquinate in 0.8% analyzed Spanish milk samples, at a concentration of 5 µg/kg.

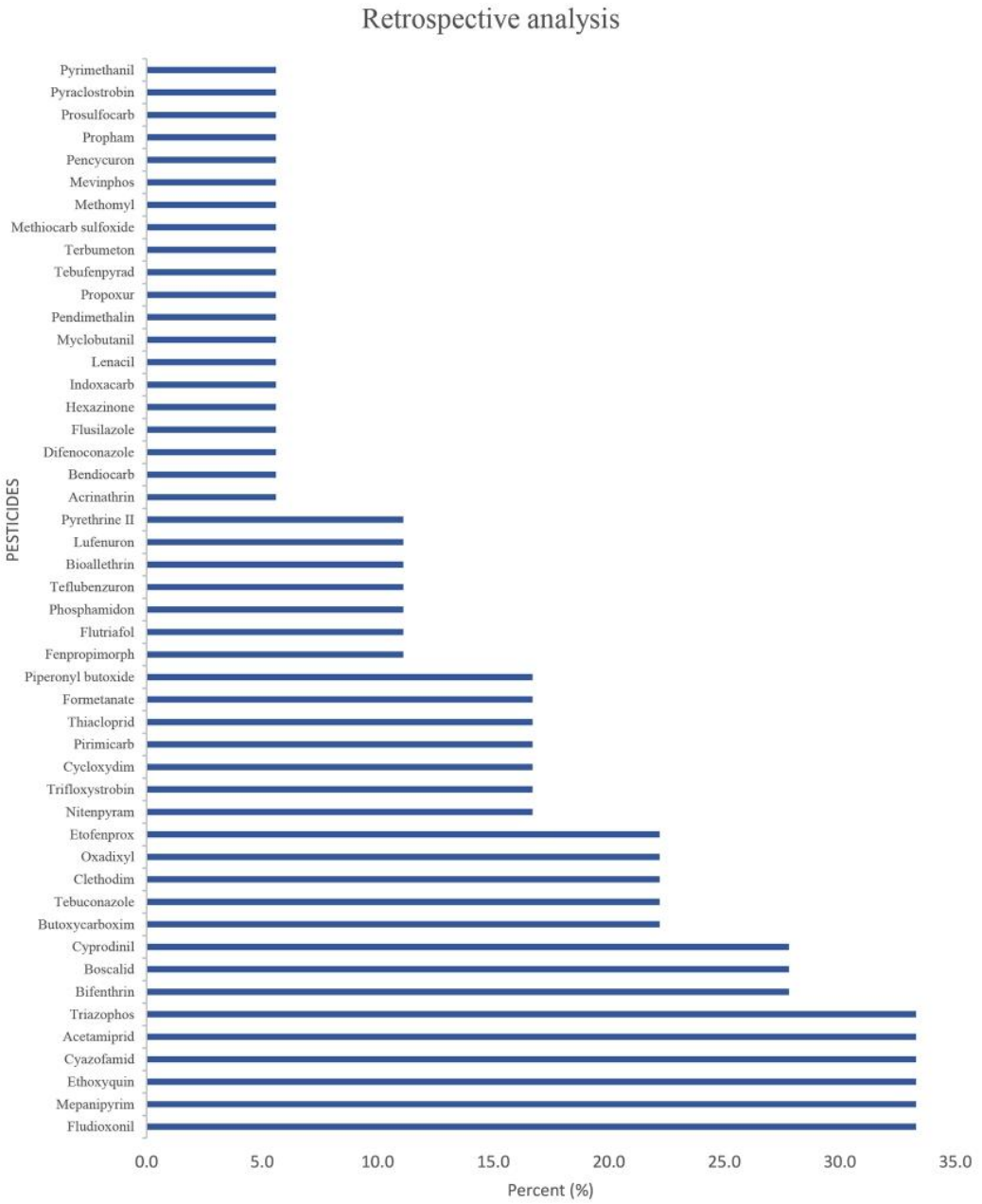

Fig. 2. Percentages of samples and types of pesticides tentatively identified in commercial infant milk formulas, using retrospective analysis of data by matching the exact mass of theoretical precursor ions within 1 ppm

Ethoxyquin, fludioxonil, mepanipyrim, ethoxyquin, cyazofamid, acetamiprid, and triazophos were detected in 33.3% of the samples (n = 6). Aguilera-Luiz et al. [15] reported detecting pesticides, including thiabendazole, acetamiprid, thiacloprid, carbendazim, thiophanate methyl, and bendiocarb, in milk. In particular, imidacloprid and atrazine desisopropyl were found in 4 milk samples at concentration levels higher than 10 µg/kg in Aguilera-Luiz et al. [15], although until now no MRL have been set for them in milk. Pesticides listed in Table 1 of Annex VIII of commission directive 2006/141/EC [56], not recommended in agricultural products intended for the production of IMF, were not tentatively found in analyzed samples.

Co-occurrence of pharmacologically active residues was found in a significant number of infant milk samples (83.3%). Up to 4 pharmacologically active substances, decoquinate, dimetridazole, betamethasone, and oxfendazole, were detected simultaneously in one analyzed sample. Additive or synergistic effects of more contaminants coexisting in the same sample should be considered in risk assessment studies [57,58,59]. Given the large consumption of milk and milk derivates, a thorough investigation of the occurrence of contaminants, as well the adoption of measures to reduce their contamination of milk, is essential.

3.5 Risk Characterization

Risk characterization is an indispensable aspect to safeguard public health, which helps to identify risks threatening consumers. Therefore, it is extremely important to determine the percentage of tolerable daily intake (**TDI**) to avoid adverse effects due to dietary exposure. For calculation of TDI percentage, consumption data, body weight, and maximum concentration of each contaminant occurring in IMF were taken into consideration. As reported by the latest global individual food consumption data [60], Italian infant formula milk-based consumption is 22 and 6.91 g/d for infants 1 to 3 yr old and neonates 0 to 12 mo old, respectively, and body weight of 11.9 and 6.7 kg for infants 1 to 3 yr old and neonates 0 to 12 mo old, respectively, were evaluated [61]. Hence, to calculate TDI percentage, the following equation was used:%TDI=DI×CTDI×BW×1,000,where DI = daily substance intake (ng/mL); C = food consumption (kg per capita per year); TDI = tolerable daily intake (µg/kg of BW); and BW = body weight (kg).

To achieve a more accurate estimation of exposure, 2 scenarios were established: the upper bound and the lower bound. For the upper bound, samples reported as negative or <LOQ were substituted by the LOQ, whereas the lower bound considered these samples as strictly negative. To estimate exposure, the mean concentration value was considered.

Table 5. Exposure assessment for mycotoxins and pharmacologically active substances exposure in infants calculated on the consumption of infant food according to the latest FAO data in Italy [65]

Analyte	Concentration[1] (µg/kg)		TDI[2] (µg/kg of BW)	% TDI, infants aged 0–12 mo[3]		% TDI, infants aged 1–3 yr[3]	
	Upper bound	Lower bound		Upper bound	Lower bound	Upper bound	Lower bound
Mycotoxins[4]							
FUS-X	0.409	0.041	None established	—	—		—
NEO+T2+HT2	0.465	0.096	0.02	76.69	1.58	1.35	0.28
Σ ZEN + α-ZEL	217.360	69.64	0.25	286.77	91.88	50.49	16.18
ENNB	0.431	0.128	None established	—	—	—	—
ENNA1	0.422	0.050	None established	—	—	—	—
Veterinary drug residues							
Dexamethasone	0.350	0.192	0–0.015[5]	7.70	4.22	1.36	0.74
Procaine benzyl penicillin	0.108	0.016	None established		—	—	—

[1] *Highest residue concentration detected in the tested samples.*
[2] *TDI = tolerable daily intake, expressed as µg/kg of BW.*
[3] *1- to 3-yr-old child with 11.9 kg of BW; 0- to 12-mo-old child with 6.7 kg of BW (EFSA, 2012). Dashes indicate that no TDI was established for a given analyte.*
[4] *Mycotoxin standards were as follows: fusarenon-X (FUS-X), neosolaniol (NEO), zearalenone (ZEN), α-zearalenol (α-ZEL), and enniatins (ENNA1 and ENNB).*
[5] *Percent TDI is shown for a TDI of 0.015.*

For risk characterization, TDI stated by the European Food Safety Authority was used. In the case of ZEN and its derived forms, TDI have been established for the sum of ZEN and its derived forms through a relative potency factors system according to their reported toxicity. The relative potency

factors of 1, 60, 0.2, and 1.5 are respectively assigned to ZEN, α-ZEL, β-ZEL, and ZAN. Similarly, NEO is included in the TDI value established for T2 in the same relative potency factors system: T2, HT2, and NEO are given values of 1, 1, and 0.3, respectively [62,63]. Results are shown in Table 5. Taking into consideration the lower bound, for a child 1 to 3 yr old, the calculated TDI percentages are in a range of 0.28% to 16.18%, whereas for infants 0 to 12 mo old, the calculated TDI percentages are in a range of 1.58% to 91.88%. In the worst-case scenario represented by the upper bound, for a child 1 to 3 yr old, the calculated TDI percentages are in a range of 1.35% to 50.49%, whereas for infants 0 to 12 mo old, the calculated TDI percentages are in a range of 7.70% to 286.77% Therefore, the European Food Safety Authority Scientific Committee [64] does not recommend grouping infants from 0 to 3 yr, because their intake and body weights are variable, and instead suggested the evaluation of risk on a case-by-case basis. In the worst-case scenario, the percentage found does not represent a health hazard but a significant contribution to TDI. For some contaminants, TDI is not established, making it impossible to assess the risk of exposure.

As expected, infants are the most vulnerable category of the population, considering the high infant milk formula intake compared with older children and adolescents in relation to their low body weight. Based on risk assessment, a smaller quantity of contaminant residues could be enough to provoke adverse effects on infants. Hence, it is highly recommended to have a watchful attitude regarding food intended for susceptible groups. The total process of infant milk formula manufacturing should be controlled to ensure the use of raw materials with acceptable MRL.

4. CONCLUSIONS

This work proposed a multi-class analysis of mycotoxins (n = 22) and veterinary drug residues (n = 23), in addition to retrospective analysis of other contaminants for which analytical standards (n = 54) are not available and pesticides (n = 283) in IMF. Advantages of the validated method include a single extraction for all the studied analytes, rapid determination, simple sample pretreatment, and high sensitivity. Results of evaluated parameters are in accordance with the limits reported by European Commission regulations. The validated method was applied to the analysis of 3 lots of 18 different brands of IMF. Results showed occurrence of contaminant residues in analyzed IMF: dexamethasone, procaine benzyl penicillin, zearalenone, and α-zearalenol. Up to 65 contaminants were tentatively identified via retrospective analysis based on the mass spectral library. Orbitrap technology represents a practical tool for sure and precise identification of a wide number of contaminants in a 9-min analytical runtime. Moreover, the in-house validated method could be proposed for simultaneous identification of contaminants in routine analysis of IMF, reassuring consumers on the safety of purchased foods.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 16
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Analysis of Mycotoxin and Secondary Metabolites in Commercial and Traditional Slovak Cheese Samples

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ABSTRACT

Cheese represents a dairy product extremely inclined to fungal growth and mycotoxin production. The growth of fungi belonging to *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, *Alternaria*, and *Trichoderma* genera in or on cheese leads to undesirable changes able to affect the quality of the final products. In the present investigation, a total of 68 types of commercial and traditional Slovak cheeses were analyzed to investigate the occurrence of fungal metabolites. Altogether, 13 fungal metabolites were identified and quantified. Aflatoxin M1, the only mycotoxin regulated in milk and dairy products, was not detected in any case. However, the presence of metabolites that have never been reported in cheeses, such as tryptophol at a maximum concentration level from 13.4 to 7930 µg/kg (average: 490 µg/kg), was recorded. Out of all detected metabolites, enniatin B represents the most frequently detected mycotoxin (0.06–0.71 µg/kg) in the analyzed samples. Attention is drawn to the lack of data on mycotoxins' origin from Slovak cheeses; in fact, this is the first reported investigation. Our results indicate the presence of fungal mycotoxin contamination for which maximum permissible levels are not established, highlighting the importance of monitoring the source and producers of contamination in order to protect consumers' health.

Keywords: Mycotoxins; Slovak cheeses; fungi growth; enniatin B; tryptophol.

16.1 INTRODUCTION

Fungi are the major producers of secondary toxic metabolites, which represent the main cause of food spoilage. Mycotoxins are toxic compounds produced by several molds belonging to the main genera of fungi—*Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, *Alternaria*, *Stachybotrys* and many others—which have a wide variety of properties that influence our common life [1]. The occurrence of toxic metabolites in food, as well as in feed, represents a serious global problem for human health due to their toxic effects, e.g., neurotoxic, nephrotoxic, carcinogenic and mutagenic effects [2]. Concerning food safety, European institutions established guidelines to minimize human exposure to different mycotoxins, e.g., to aflatoxins, ochratoxins, patulin, fumonisins and others, where their occurrence and maximum levels have been established [3,4].

Milk can contain various mycotoxins when lactating animals ingest contaminated feeds. Mycotoxins are metabolized, biotransformed, and transferred to animal products (milk, meat). There are mycotoxins of primary concern detected in dairy products and also mycotoxins that can be found as a

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result of in situ production by potentially toxigenic fungi frequently associated with cheese [5]. In fact, rumen flora can change a number of mycotoxins into metabolites that are less biologically inactive at common exposure levels. However, this does not apply to all mycotoxins that contaminate feed materials [6]. Milk and dairy product contamination may arise from several causes, such as animal feed contamination, starter strains, environment, processing equipment or incorrect manipulation [7]. The preparation and storage conditions of animal feedstuffs (grain, silage) can reconcile mycotoxin contamination. Toxic fungal metabolites can be produced and excreted by toxigenic species growing on cheese and penetrating the product [8]. It is concluded that the penetration depends on the type of cheese and the type of mycotoxin. However, it has also been reported by several authors that toxin concentrations and visible mold colonies may not always correlate [9,10,11,12].

The most common molds that contaminate cheese are *Penicillium* species [13], which can grow even at refrigerator temperature and produce the toxins ochratoxin A, citrinin, penicillic acid, patulin, mycophenolic acid, penitrem A, and cyclopiazonic acid [1]. The most frequently and commonly dominant *Penicillium* species on spoiled cheeses from different countries were identified. *P. commune* was the most widespread and most frequently occurring species. Most of the isolates belonged to the following species: *P. commune*, *P. nalgiovense*, *P. verrucosum*, *P. solitum*, *P. roqueforti*, *A. versicolor*, *P. crustosum*, *P. atramentosum*, *P. chrysogenum* and *P. echinulatum* [14,15]. Some of them, especially *P. commune*, *P. verrucosum*, *A. versicolor* or *P. Roqueforti,* are species reported as players in cheese ripening microbiota [16]. Moreover, a new *Penicillium* species, *P. gravini caseii,* was isolated and described from cave cheese [17]. As reported by Kandasamy et al. [18], that investigated ten dairy farms from six different provinces in the Republic of Korea, mycological analysis in cheese factories was important in an attempt to prevent mold growth on cheese. From this study emerged fungal contamination in 8 out of 10 dairy farms which were out of the acceptable range, as per hazard analysis critical control point regulation. The environment of cheese ripening rooms persuades a favorable niche for mold growth. The proper management of hygienic and production practices, and air filtration systems, would be effective to eradicate contamination in cheese processing industries [18].

To date, EU General Food Law, and other worldwide organizations, do not regulate the mycotoxins in cheese. In the European Union (EU), AFM1 is the only regulated mycotoxin in raw milk, heat-treated milk and milk for the manufacture of dairy products, at a concentration level not exceeding 0.05 μg/kg for adult consumption, and 0.025 μg/kg for food products meant for infants and young children [4]. Although AFM1 is the only mycotoxin regulated by the EU, the list of detected mycotoxins in cheeses is more extensive. The occurrence of ochratoxin A, patulin and citrinin [15,17,19,20], cyclopiazonic acid [21,22], roquefortine C and mycophenolic acid (ROQC) [23,24], isofumigaclavines [25], penitrems [26] or andrastins [27] were reported in the literature.

Thanks to the development of specific and sensitive tools for quantitative assessment of contaminants in foods, we are witnessing increasing work on this topic. Recent advances in mycotoxin quantifications have been focused on analytical methods for multi-mycotoxin analysis with minimal sample clean-up adapted to different types of food [28,29,30,31,32]. Until now, few scientific works have focused on the investigation of mycotoxins on Slovak cheeses. The occurrence and diversity of yeasts and filamentous fungi in Brydza cheeses were investigated by [33,34]. Cisárová et al. [35] studied the ability to produce cyclopiazonic acid by strains of fungi from Camembert-type cheese from different European countries, including Slovakia.

Based on the above, the present scientific work is focused on the analysis, for the first time, of mycotoxins and secondary metabolites in 68 types of commercial and traditional Slovak cheeses through LC-ESI-MS/MS analysis.

16.2 RESULTS AND DISCUSSIONS

16.2.1 Mycotoxins and Metabolites Detected in Slovak Cheeses

From altogether 68 analyzed Slovak cheese samples, 13 different fungal metabolites in different concentrations were detected. Of all the tested samples, four samples (samples: 4, 6, 30 and 37)

reported contamination from nearly all the tested mycotoxins, including samples 30 and 37, derived from commercial sheep cheeses, not smoked and smoked, respectively. Results obtained from original traditional Slovak cheeses came from markets and local farmers using a traditional biotechnical process at manufacture. Instead, the occurrence of target compounds investigated in common commercial cheeses that came from markets are reported in Table S1. A summary of the analyzed Slovak cheeses divided from milk origin and their concentrations range are shown in Table 16.1.

Among 28 target mycotoxins and metabolites investigated in analyzed samples, AFM1, the only mycotoxin regulated in milk and dairy products, was not detected in any case, which highlighted the overall milk quality (goat's, cow's, sheep's) used for the cheese manufacturing.

The complex diet of ruminants, consisting of forages, concentrates, and preserved feeds, can be a source of very diverse mycotoxins that contaminate individual feed components. In a brief review [36] was reported the assessment of mycotoxins in the diet of dairy cows in terms of exposure assessment. A direct consequence of the complex and variable composition of ruminant diets is the risk of exposure to more than one mycotoxin or a set of mycotoxin clusters produced by an individual fungal species. This includes, for example, aflatoxins, fumonisins, zearalenone, trichothecenes and ergot alkaloids; from pasture grasses it can be a source of lolitrems, paspalitrems, penitrem A, ergovaline and associated ergot alkaloids; and finally, contamination from preserved feeds (silage) where we talk about patulin, mycophenolic acid, roquefortines, fumitremorgens, verruculogen, monacolines, and others.

16.2.1.1 Enniatin B

Out of all detected metabolites, enniatin B (ENN B) represents the only one detected in all analyzed samples, as well as in commercial and traditional cheeses. Although present in all samples (100%, *n* = 68), its concentration was low compared with other metabolites. ENN B concentrations ranged from under 0.01 to 0.71 µg/kg. Particularly, traditional cheeses were contaminated with slightly higher average levels compared with commercial cheeses: 0.21 and 0.15 µg/kg, respectively (Table S1, Table 16.1).

ENNs belong to the so-called "emerging mycotoxins" family because of their increase in food and feed, and also the great concern about their worldwide presence, originally reported as being produced by *Fusarium* spp. [37,38]. ENNs occur as contaminants mainly in cereals, although their presence has been also reported in other matrices, including products of animal origin, as a consequence of the carry-over of these compounds into animal tissues after the feeding of contaminated feed [39,40,41,42,43,44,45]. As reported, high contamination levels of ENNs in food commodities have been reported. ENNs B, B1, B4, A, and A1 were quantified in baby food, including infant formula kinds of milk, dairy products such as cheese and yogurt, cereal-based baby food, fruit and vegetable compotes, fruits, and vegetable puree from the Italian market. The detected concentrations ranged from 11.8 to 832 µg/kg and the highest reported contamination levels were represented by ENN B [46]. In particular, ENN B is the most prevalent compound belonging to this group of mycotoxins and its presence represents relatively high concentrations in *Fusarium*-contaminated food and feed. In a multi-mycotoxin validation of an efficient multi-analyte method for the detection and quantification of mycotoxins of maize silage from Spain dairy farms, Dagnac et al. [47] found that ENN B was the most frequent mycotoxin; it achieved the highest average detection frequency and was detected in 51% of the samples (*n* = 148; average concentration: 157 µg/kg). In addition, ENN B was confirmed by a pilot study for the presence of fungal metabolites in sheep milk from first spring milking. Out of 700 bacterial, fungal and plant metabolites tested for, only one mycotoxin—ENN B—was detected in sheep milk samples (0.0055–0.0121 µg/kg; average concentration: 0.0078 µg/kg) [48]. Unlike other *Fusarium* mycotoxins, such as deoxynivalenol, T-2, HT-2, fumonisins, and zearalenone, whose presence in food and feed has been regulated by authorities, no limits have been set for ENN B, up to now.

Table 16.1. Summary of the analyzed cheeses (*n* = 68) and their concentration ranges expressed as µg/kg

Type of Mycotoxin	Cow's Cheeses/45 Samples			Sheep's Cheeses/19 Samples			Goat's Cheeses/4 Samples			Numbers of Total Positive Samples
	Commercial	Traditional	Concentration Range µg/kg	Commercial	Traditional	Concentration Range µg/kg	Commercial	Traditional	Concentration Range µg/kg	
3-NPA *	0/25	2/20	10.6–66.7	3/10	0/9	3.8–27.2	0/4	-	-	5/68
Andrastin A, B, C, D	0/25	8/20	89.7–8890	8/10	0/9	96.7–9140	0/4	-	-	16/68
Chanoclavine	0/25	2/20	3.1–7.5	2/10	0/9	4.8–6.9	0/4	-	-	4/68
Enniatin B	25/25	20/20	0.02–0.71	10/10	9/9	0.04–0.57	4/4	-	0.09–0.17	4/68
Festuclavine	0/25	2/20	1.2–2	2/10	0/9	0.21–1.3	0/4	-	-	4/68
iso-Fumigaclavine	0/25	2/20	178–294	2/10	0/9	90–135	0/4	-	-	4/68
Mycophenolic acid	0/25	2/20	20.7–29.4	2/10	0/9	16.1–28.7	0/4	-	-	4/68
Roquefortine C, D	0/25	4/20	591.8–17900	4/10	0/9	679–13700	0/4	-	-	8/68
Tryptophol	8/25	8/20	15–7930	4/10	2/9	13.4–171	0/4	-	-	22/68

***** *3-Nitropropionic acid.*

16.2.1.2 Tryptophol

From all analyzed cheeses, the second most frequently detected metabolite was tryptophol. Tryptophol was detected in 30.8% of commercial cheeses (*n* = 12) and 34.5% of traditional cheeses (*n* = 10). Concentrations exhibited a broad range; in traditional cheese samples, the range varied from 13.4 to 7930 µg/kg (average concentration: 916 µg/kg), while the range in commercial cheese samples was between 15.5 and 354 µg/kg (average concentration: 136 µg/kg).

Tryptophol (indole-3-ethanol) is a metabolite produced by plants, bacteria, fungi and sponges. The central role of indolic compounds as plant growth regulators is well established [49]. The dairy yeast *Debaryomyces hansenii* was investigated for its production of alcohol-based quorum sensing (QS) molecules [50]. The addition of tryptophol was found to influence both the adhesion and sliding motility of this yeast. This fungus has great importance for the food industry; thus, it is used as a starter culture for the production of cheese products [51]. More recently, tryptophol was identified as a QS molecule released by cells and capable of inducing the morphogenetic switch in response to nitrogen starvation, which stimulates morphogenesis of pseudohyphal growth in *Sacchromyces cerevisiae* [52]. Tyrosol and tryptophol have been reported to impart slightly bitter flavors to beer. However, they have not been identified in cheese. The production of indolic compounds was screened, also, by rumen bacteria isolated from grazing ruminants. Fresh isolates from sheep and dairy cows produced indole, indole propionic acid, tryptophol and skatole from the fermentation of tryptophan and indoleacetic acid [53].

16.2.1.3 3-Nitropropionic Acid

Moreover, the metabolite 3-nitropropionic acid (3NPA) was found in one sample of Camembert cheese, at a concentration level of 66.7 µg/kg, and in one sample of traditional blue cheese Niva, at a concentration level of 10.6 µg/kg. In addition to 3NPA, all metabolites are confirmed, such as typical metabolites of *P. Roquefortii,* and their evidence in cheeses is not so strange and surprising. Our Camembert cheese sample (number 4) represented the white ripening Camembert cheese where *P. candidum* was used as a ripening culture. *P. Candidum* (synonym of *P. camemberti*) is a species domesticated from *P. commune*. This species has never been found outside the white mold cheese environment [54]. There is no evidence of the production of 3-NPA by this species. The neurotoxin 3-nitropropionic acid (3-NPA) is a mitochondrial toxin produced by several plants and fungi. The first production of 3-NPA (ß-nitropropionic acid) was derived from a strain of *A. flavus*. Since then, more fungi species have been added to producers of this mycotoxin: *A. wentii*, *A. oryzae*, *P. atrovenetum*, *Arthrinium* spp., *Mucor circinelloides* [55,56,57]. The consumption of 3-NPA by humans is likely through the consumption of both commercially and domestically prepared foodstuffs using fungi (*Penicillium* spp., *Aspergillus* spp.), or through forage consumption that includes contaminated plants [58]. Unfortunately, the survey of the natural contamination of 3-NPA is limited in the literature, although the presence of this metabolite was reported in cheese [10,59].

16.2.1.4 Clavine Alkaloids, Isofumigaclavine, Festuclavine, and Chanoclavine

Among clavine alkaloids, isofumigaclavine, festuclavine, and chanoclavine were detected in our samples, especially in Niva cheeses. Chanoclavine and festuclavine varied relatively in low concentrations (3.1–7.5 µg/kg and 0.21–2 µg/kg, respectively; 5.9%, *n* = 4). Iso-fumigaclavine concentrations were higher (90–294 µg/kg). Ergot alkaloids are fungal metabolites with high biological activity, distinct in two subgroups. The first represents simple clavine-type alkaloids (for example, fumigaclavines) produced by fungi of the Aspergillaceae (*A. fumigatus*) [60], and the second includes lysergic acid-derived ergot alkaloids produced by parasitic or endophytic Clavicipitaceae fungi [61]. All three alkaloids were isolated from the collection and mutant strains of *P. roquefortii* [25]. *P. roqueforti* is able to accumulate the intermediates festuclavine and agroclavine [62]. *P. roqueforti* is used industrially as inoculum for ripening cheeses, and this saprophytic fungus produces a variety of enzymes and secondary metabolites (including mycotoxins) based on various types of substrates including, for example, PR-toxin, roquefortine C, isofumigaclavines (A, B), mycophenolic acid, andrastins (A, D), eremofortines (A, B, D) and others [27,63]. Fabian et al. [64] reported that the *P. camemberti* genome contains a cluster of five genes required for the synthesis of the ergot alkaloids

intermediate chanoclavine-I aldehyde. They analyzed samples of Brie and Camembert cheeses, as well as cultures of *P. camemberti*, and did not detect chanoclavine-I aldehyde or its derivatives. The production of festuclavine and iso-fumigaclavine was confirmed by *P. carneum* [62,65], which is included in the *P. roqueforti* complex.

16.2.1.5 Andrastins

Andrastins are interesting anticancer drug candidates which could represent an idea for the future production of "functionalized cheeses" with higher quantities of andrastin A [66]. Even though they are considered to be beneficial for human health, there are no studies that support its lack of toxicity when accumulated in high levels in cheeses. The first finding of andrastins in blue cheese was described by Nielsen et al. [27], where it was produced by the secondary starter culture *P. roqueforti*. In 23 representative samples of European blue cheeses, andrastin A was consistently found in quantities between 0.1 and 3.7 µg/g of mold-ripened cheese. Andrastin A was accumulated inside blue cheeses inoculated with this secondary starter. Not so far away, there was sequenced and annotated a genomic region that is involved in the biosynthesis of andrastin A in *P. roqueforti* [67]. Secondary metabolites produced by species of *P. roqueforti* complex (*P. roqueforti* and *P. paneum*) were established and two metabolites (roquefortine C and andrastin A) were consistently produced by both species [65]. A total of four white-mold-ripened cheeses were also purchased and used as controls; andrastins A-D were not detected. No correlation was observed between the level of sporulating mycelium (assessed visually) in the cheeses and the quantity of the andrastins [27]. This was expected, because they have never detected the production of andrastins by *P. camemberti* cultures [54]. Our investigation discovered the presence of andrastins (andrastin A, B, C and D) in 16 Slovak cheeses, traditional and commercial samples. Andrastin concentration levels ranged from 89.7 to 9140 µg/kg, with an average range of 3310 µg/kg (positive samples, n = 16). In our samples, andrastin A levels were the highest compared with others.

16.2.1.6. Roquefortine C (ROQC), Roquefortine D (ROQD), Mycophenolic Acid (MPA)

P. roqueforti is highly appreciated in biotechnological applications and is a very important filamentous fungus, used during the process of making, and in the maturation of blue Roquefort-type cheeses. This fungus oxidizes fatty acids into methyl ketones, 2-haptanone and 2-honanone, which are considered to be responsible for the specific and unique sensorial flavors and odors of the blue cheese. On the other hand, there is increasing evidence that has been reported on the ability of this species to produce secondary metabolites in different culture media, or in the blue cheeses. This species is known as a producer of roquefortines, as well as of mycophenolic acid, amongst other mycotoxins [54,68]. Although ROQC and MPA present low acute cytotoxicity on the human intestinal cell, they have been shown to possess neurotoxic and immunosuppressive effects, respectively, and may thus cause secondary (indirect) mycotoxicosis [54]. Moreover, a high variability of these metabolites was observed in an unprecedented worldwide blue-veined cheese collection. Overall, 75% of samples contained less than 792 µg/kg ROQC and 705 µg/kg MPA [69]. Zambonin et al., [70] found MPA in five samples of Gongonzola and Danablu cheeses, ranging from 100 to 500 µg/kg. Another investigation [71] conducted on 53 blue cheeses reported that the levels of MPA achieved a concentration level from <10 to 1200 µg/kg. Our results show lower values of MPA range from 0.129 to 0.235 mg. ROQC and ROQD detected in the concentration levels varied from 592 to 17,900 µg/kg (positive sample, n = 8). Another study was conducted on industrial batches of nine *P. roqueforti* strains used in the production of the Gorgonzola cheese to verify the production of secondary metabolites. In vitro, only one *Penicillium spp* out of nine produced ROQC and four strains produced MPA. ROQC concentrations ranging from 50 to 1470 µg/kg were quantified after the analysis of 30 blue cheeses [72]. Moreover, higher values were obtained by Kokkonen et al. [24], from 600 to 12,000 µg/kg, respectively. There is a consensus that roquefortines in cheeses do not pose human health risks [62,68,69,73].

16.2.2 Method Performance Data

Method validation was performed in accordance with Sulyok et al. [31]. Table 16.2 reports the apparent recovery expressed as a percentage (%) and standard deviation (RSD %) for the 28

positively identified analytes in the investigated cheese samples. Apparent recovery was determined by adding a known quantity of a multi-analyte stock solution at a high concentration level to five individual blank samples.

Table 16.2. Apparent recovery expressed as percentage (%) and standard deviation (RSD) for the 28 positively identified analytes in Slovak cheese samples

Analyte	Apparent Recovery (%)	RSD (%)
3-Nitropropionic acid	53.2	14.9
Aflatoxin B1	28.3	10.4
Aflatoxin M1	50.7	13.9
Alternariolmethylether	75.1	13.9
Andrastin A	54.9	18.2
Andrastin B	59.5	12.7
Andrastin C	60.8	7.4
Chanoclavine	39.8	19.2
Citreoviridin	84.1	16.2
Citrinin	107	13.5
Cyclopiazonic acid	102.9	13.9
Deoxynivalenol	77.8	9
Enniatin B	93	7.2
Fumigaclavine A	48	15.9
Fumonisin B1	61.2	17.5
Fumonisin B2	72.3	15.5
Griseofulvin	49.1	10.4
Mycophenolic acid	71.6	14.2
Ochratoxin A	68.7	9.3
Patulin	47.8	18.9
Penitrem A	116.8	12.8
Quinolactacin A	43.5	17.2
Roquefortine C	71.7	12.2
Roquefortine D	36.3	13.3
Sterigmatocystin	61	18.1
T-2 Toxin	56.3	5.6
Tryptophol	31.8	9.6
Zearalenone	71.2	9.1

16.3 MATERIALS AND METHODS

16.3.1 Chemicals

Acetonitrile, methanol and glacial acetic were purchased from VWR Chemicals (Vienna, Austria). Ammonium acetate was acquired from Sigma-Aldrich (Vienna, Austria). A Purelab Ultra system (ELGA LabWater, Celle, Germany) was used for further purification of reverse osmosis water. Standards of fungal metabolites were purchased from several sources as described in [31].

16.3.2 Sampling

Altogether, 68 samples of cheese were randomly purchased from food markets, as well as directly from local cheesemakers located in the south-western part of Slovakia. All cheese samples originated from 3 types of Slovak milk: cow, sheep, goat. Our sampling can be divided into 2 types of cheeses: common commercial Slovak cheeses came from markets (*n* = 39, Table S2), and original traditional Slovak cheeses came from markets and local farmers using a traditional biotechnical process at manufacture (*n* = 29, Table S2). Traditional cheeses represent unique and specific types of cheese not found anywhere else in the world. We talk about traditional sheep cheese, Bryndza, Camembert cheese, Encián, Plesnivec, and blue cheese, Niva, from cow's milk. Camembert white brine cheese is

made by a culture of *P.candidum,* while Niva, as an internally mold-ripening cheese, is formed by *P. roquefortii*. Other origin types of cheeses were korbáčik-traditional Slovak, Nite traditional cheese threads, Parenica and Oštiepok, all derived from cow's milk.

16.3.3 Mycotoxin Extraction

Mycotoxin extraction involved the addition of 20 mL of acetonitrile/water/acetic acid (79:20:1, *v*/*v*/*v*) to 5 g of homogenized cheese samples according to the procedure described by [31]. The mixture was placed on a rotary shaker (GFL 3017, Burgwedel, Germany) for 90 min. After centrifugation for 5 min at 5000 rpm the supernatant was diluted two times with acetonitrile/water/acetic acid (20:79:1, *v*/*v*/*v*) and analyzed through mass spectrometry.

16.3.4 LC-ESI-MS/MS Analysis

The analytical conditions used for the analysis of mycotoxins are reported by [31]. Analyses were performed using a QTrap 5500 MS/ MS (Sciex, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was carried out by using a Gemini C18 column, 150 × 4.6 mm, 5 µm particle size, equipped with a security guard cartridge (Phenomenex, Torrance, CA, USA) held at 25 °C. The mobile phase consisted of (A) methanol:water (10:90 *v*/*v*) and (B) methanol:water (98:2 *v*/*v*), both containing 5 mM ammonium acetate and 1% acetic acid. Elution was carried out as follows: an initial 100% A was held for 2 min and decreased to 50% A over 3 min. Then, the gradient was linearly decreased to 0% A over 9 min, followed by a hold time of 4 min at 0% A and held for 2.5 min for re-equilibration. The flow rate was 1 mL/min. The injection volume was set to 5 µL. ESI MS/ MS was performed both in positive and negative polarity. The target cycle time was set to 1000 ms, the MS pause time at 3 ms, and the detection window width was 52 and 40 s in the negative and positive ESI mode, respectively. For confirmation criteria, the ion ratio was compared with the related value of the standard at a tolerance within 30%, and the retention time at a tolerance of ±0.03 min.

16.3.5 Validation Method

External quantification was performed using a serial dilution of a multi-analyte stock solution. Results were corrected for apparent recoveries that were determined through spiking experiments. The limits of detection and quantification were determined following the EURACHEM guide [31]. The accuracy of the method is verified on a routine basis by participation in a profinite testing scheme organized by BIPEA (Geneviliers, France).

16.3.6 Statistical Analysis

Statistical analysis was performed by using the software Info-Stat (https://www.infostat.com.ar/index.php?mod=page&id=15 accessed on 27 January 2022; Córdoba, Spain), version 2008. Tukey's test was used to assess differences between the various typologies of the studied samples. Tukey's test was considered significant at a $p < 0.05$ level. Three independent replications were used to assess the results, expressed as mean ± standard deviation (SD).

16.4 CONCLUSIONS

The results of this current study indicate the presence of fungal mycotoxin contamination: ENN B was found in all samples (100%, $n = 68$), although its concentration was low compared with other metabolites (0.01 to 0.71 µg/kg), and tryptophol, the second most frequent detected metabolite, present in 30.8% of commercial cheeses ($n = 12$) and 34.5% of traditional cheeses ($n = 10$). Contamination with AFM1, the only metabolite regulated in EU milk and milk products, has not been confirmed. This is evidence of good milk quality and safety for human health.

Continuing to monitor and describe the source and producers of contamination is essential. To protect consumers´ health, it is very important to verify and monitor the presence of secondary metabolites,

including mycotoxins, in food that is widely consumed, to avoid mycotoxin risk. To our knowledge, this is the first preliminary investigation focused on Slovak cheeses and the contamination of these dairy products with mycotoxins.

SUPPLEMENTARY MATERIALS

The following are available online at https://www.bookpi.org/wp-content/uploads/2022/05/Chapter-16_Supplementary-Materials.pdf, Table S1: Occurrence of 13 target compounds in Slovak cheese samples (*n* = 68). Results are expressed as µg/kg samples, Table S2: Characteristics of the 68 analyzed samples, original traditional Slovak cheeses came from markets and local farmers using a traditional biotechnical process at manufacture (*n* = 29) and common commercial Slovak cheeses came from markets (*n* = 39).

AUTHOR CONTRIBUTIONS

Conceptualization, P.M. and A.R.; methodology, P.M.; formal analysis, P.M.; investigation, L.I.; resources, A.R.; data curation, L.I. and S.L.; writing, original draft preparation, L.I. and P.M.; writing, review and editing, M.S. and A.R.; supervision, A.R. and M.S.; project administration, A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

There are no conflict of interest declared by the authors.

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