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“Wheat integrity”: new insight into safety, quality and authenticity, through high throughput analytical techniques.

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“Difficulties mastered are opportunities won”

Winston Churchill

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Preface

Wheat represents one of the most important cereal crops, with over 700 million tonnes being harvested worldwide annually. Its success depends on its adaptability and high yield potential, as well as its role in providing basic nutrients to human diet. Wheat, indeed, is relatively rich in micronutrients, including minerals and B vitamins, and supplies up to 20% of the energy intake of the global population. Wheat is a valuable food due to its chemical composition related to carbohydrates, proteins, minerals, and lipids composition. On this regard, wheat is a susceptible matrix, since transport, storage, industrial and cooking processes, may have a critical influence on its quality. In fact, another drawback is related to wheat safety, considering its widespread contamination by fungal pathogens, especially those of the *Fusarium* genera, and subsequent accumulation of mycotoxins. Besides possible toxicological implications, presence of *Fusarium* micromycetes influences both quantitative and qualitative aspects of wheat production, potentially leading to enormous economic losses. In an effort to assess wheat safety and quality, the concept of authenticity have to be also taken into account, since the substitution of one ingredient by a similar or cheaper one may occur and have a direct implication for both quality and safety, demonstrating that this three aspects are strongly linked one to each other, contributing together to the assessment of the wheat integrity. Note that hundreds of different varieties, having different characteristics and thus prices, are available, and therefore, wheat adulteration represents a critical issue to be aware of.

As a result, ensuring the safety, quality, and authenticity of wheat is a concrete and challenging issue. Nowadays, there are many targeted techniques for the evaluation of food compliance in terms of safety, quality, and authenticity. These analytical strategies involve the analysis of one or a few compounds (pesticides, mycotoxins, proteins, DNA, etc.) from a complex mixture. Nevertheless, a major requirement to effectively apply the targeted approach is to know the identity of the compound before it can be detected, excluding its application to the analysis of unknown compounds. Considering that limitation and the increasing requirements from regulatory authorities for control of food safety and quality with scientifically based guarantees, the development of novel and advanced untargeted analytical methodologies is highly advisable. Novel research in food science is, indeed, moving from classical methodologies to advanced analytical strategies in which high throughput MS-based techniques play a crucial role. In this context, foodomics has been recently defined as a new discipline that studies food domains through the application of advanced omics technologies in which MS techniques are considered indispensable.

The main of this doctoral project was to provide concrete outputs about the potential of these new high throughput MS-based techniques for assuring wheat safety, quality and authenticity.

This Ph.D. thesis was organized in three main sections. The first section was devoted to study wheat safety, by applying two analytical approaches that have become increasingly prevalent. First, the applicability of rapid multi-analyte screening methods, such as ELISA, was investigated. Secondly, the development of highly sophisticated multi-analyte methods based on liquid chromatography coupled to high resolution mass spectrometry was explored to gain insights into three different safety-related issues. The second section was focused on the investigation of wheat species and their quality, focusing on the lipid profile of modern and ancient wheat varieties. Throughout the last section untargeted metabolomics approaches were also used to differentiate between common and durum wheat with an authenticity purpose.

For each section a brief introduction is provided to allow an immediate contextualization of the treated subject. The results obtained highlighted the power of emerging analytical techniques as a valuable approach to gain new insight and successfully assess wheat safety, quality and traceability.

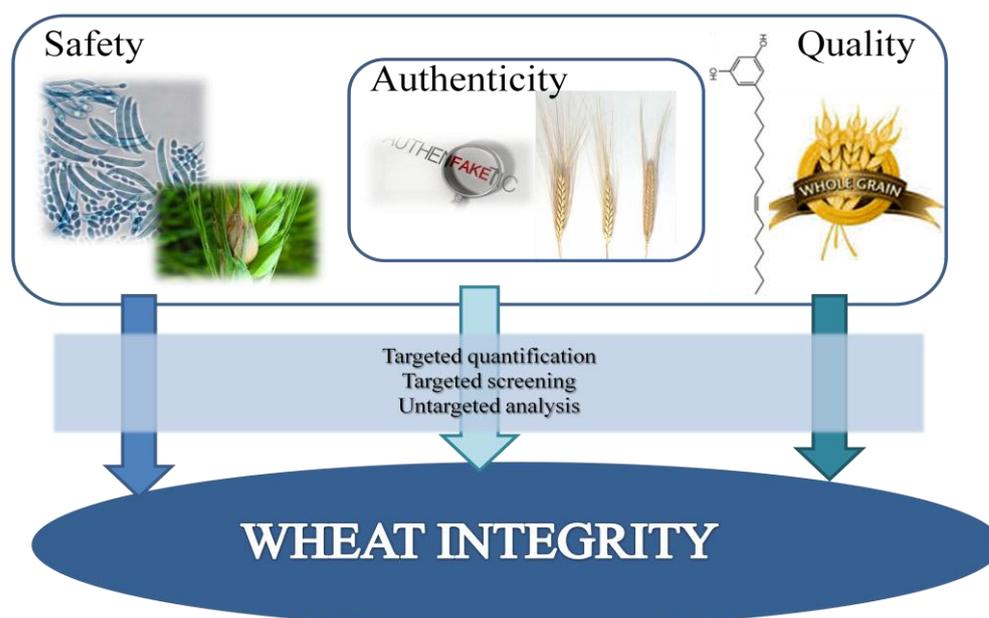


Figure 1. Wheat integrity scheme, including safety, authenticity and quality assessment through both targeted and untargeted approaches.

1.INTRODUCTION

Literature Review

Two reviews were written during this PhD Thesis covering different aspects studied: advance mass spectrometry approaches and new metabolomics strategies.

Among different approaches, mass spectrometry plays a key role providing new methods that are fundamental tools in all branches of food science. New developments in high resolution mass spectrometry, ion mobility, targeted and untargeted analysis yielded more and more sensitive, specific, fast, reliable and validated approaches in food analysis.

The first review presents advances in liquid chromatography-mass spectrometry methods, with a particular focus on the improvement on detection and structural characterization of new modified forms of mycotoxins. The analysis of these modified forms remains still challenging due to their chemical diversity, large number of isomeric forms and due to the lack of reference material and analytical standards. However, monitoring their occurrence and assess whether they can represent an additional threat, is one of the main tasks for ensuring food safety.

Moreover, technical improvements in ultrahigh performance liquid chromatography, high-resolution mass spectrometry and software for processing large analytical data sets have been responsible for the rapid and recent development of metabolomics. Because metabolomics allows the simultaneous characterization of hundreds of small metabolites in food matrices, it may offer a more detailed and comprehensive molecular picture of cereal composition or plant response to fungal infection. The book chapter summarizes the recent metabolomics approaches applied to decipher the pathways involved and the changes occurring in the plant and pathogen metabolites upon interaction. These findings will help in elucidating the *Fusarium* resistance mechanisms activated by the host, essential to select resistant cereals varieties and to prevent and control mycotoxins contamination at a *pre-harvest* stage.

The first review was published on Toxins and the book chapter on “Metabolomics” Book (InTech). For additional details see section “Author”.

Review I

Recent advance and future challenging in modified mycotoxins analysis: why HRMS has become a key instrument in risk assessment.

Recent Advances and Future Challenges in Modified Mycotoxin Analysis: Why HRMS Has Become a Key Instrument in Food Contaminant Research

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Abstract: Mycotoxins are secondary metabolites produced by pathogenic fungi in crops worldwide. These compounds can undergo modification in plants, leading to the formation of a large number of possible modified forms, whose toxicological relevance and occurrence in food and feed is still largely unexplored. The analysis of modified mycotoxins by liquid chromatography–mass spectrometry remains a challenge because of their chemical diversity, the large number of isomeric forms, and the lack of analytical standards. Here, the potential benefits of high-resolution and ion mobility mass spectrometry as a tool for separation and structure confirmation of modified mycotoxins have been investigated/reviewed.

Keywords: modified mycotoxins; high resolution mass spectrometry; ion mobility spectrometry

1. Introduction

The presence of food and feed contaminants, in particular secondary fungal metabolites, has become of increasing concern for consumers and producers. Indeed, more than 400 mycotoxins with widely different chemical structures have been identified so far, and their number is expected to increase further due to climate changes [1]. Extreme weather conditions are increasingly affecting the mycotoxin map in Europe and also world-wide, leading to an unpredictability of the range of mycotoxins occurring in food crops. In addition, it is already well known [2] that plants and other living organisms (i.e., fungi, bacteria, mammals) can alter the chemical structure of mycotoxins as part of their defense against xenobiotics, and thus contribute to further increase the wide spectrum of possible occurring contaminants. Mycotoxins, indeed, may undergo [2,3] phase-I, and phase II metabolism, involving in the former, chemical reactions such as oxidation, reduction and hydrolysis, and in the latter conjugation with amino acids, glucoses, sulfate groups and glutathione. All these modifications significantly change the chemical structure of the parent compounds. According to the recent EFSA opinion [3], which aimed to harmonize the terminology across the scientific community, all these metabolites are referred to as “modified mycotoxins” [4], being structurally altered forms of the parent mycotoxins. The definition also covers the metabolites originating after thermal/ process degradation. Modified mycotoxins may co-occur as contaminants in addition to parent compounds in food and feed; so far modified forms for trichothecenes, zearalenone (ZEN), fumonisins, *Alternaria* toxins and ochratoxin A (OTA) have been identified. However, up to now their occurrence in naturally infected cereals has been exclusively confirmed for deoxynivalenol (DON), ZEN and fumonisins [5–7]. In fact, the lack of analytical standards and reference materials, has substantially complicated their identification, partially restraining research progress in the field as well.

The metabolic conjugation with polar molecules is commonly considered as an inactivation reaction, because the aglycone usually loses its biological activity. However, the possible hydrolysis of modified mycotoxins back to their toxic parents during mammalian digestion raises toxicological concerns [8] and thus the requirement for their detection. Modified mycotoxins were originally considered “masked” [9] since they may elude conventional analysis because of impaired extraction efficiency caused by increased polarity when a less polar solvent is used for the extraction of non-modified mycotoxins. Moreover, the changed physicochemical properties of their molecules lead to modified chromatographic behavior and due to the lack of analytical standards they are currently not routinely screened.

All of these effects may lead to a potential underestimation of the total mycotoxin content of the sample. Therefore, monitoring the presence of these potentially hazardous metabolites remains one

of the main tasks for ensuring food safety and human/animal health. In this frame, liquid chromatography coupled with mass spectrometry has represented the golden standard for at least a decade. This review aims, therefore, at pointing out the possible advantage of innovative MS techniques in mycotoxin analysis, and to highlight the improvements still needed to meet the future challenges in the field.

2. From Targeted LC-MS/MS Determination to Untargeted HR-MS Analysis

In the field of residues and contaminants, analytical methods used for surveillance purposes must ensure optimal sensitivity and accuracy. For this reason, chromatographic analysis coupled to fluorescence or UV detection was the reference techniques for many decades. Over the last two decades, the LC-MS/MS platform became the method of choice, in particular for its ability to allow the development of multi-residue and multi-class methods [10–14]. First attempts were aimed at quantifying a single mycotoxin, but later on the research moved to the simultaneous determination of multiple mycotoxins, leading to the development of the so-called multi-toxin methods for quantitative as well as screening purposes [13–15].

The current “golden standard” in routine food safety control is represented by unit resolution tandem mass spectrometric analyzers such as triple quadrupole (QqQ) [16,17], mainly because this technique ensures analytical parameters that easily meet quality criteria required by law [18–20]. Multiple reaction monitoring (MRM) has been traditionally selected for mycotoxin analysis, monitoring in parallel quantitative and qualitative ion transitions, providing both sensitivity and selectivity. Achieved limits of quantification (LOQs) and detection (LODs) usually match regulatory requirements for the official control method for contaminants. The first low-resolution multi-toxins method was developed in 2006 [13] for the quantitative determination of 39 parent and modified mycotoxins. Among the modified mycotoxins, the methods included 3Ac-DON, 15Ac-DON, DON3Glc, ZEN14Glc, ZEN14Sulf, and hydrolysed FB1. More recently [21] a method for the simultaneous quantification of both parent and modified *Alternaria* mycotoxins in cereals based food, including alternariol (AOH), AOH3Sulf, AOH3Glc, alternariol-methyl ether (AME), AME3Sulf, and AME3Glc was developed. *Alternaria* modified mycotoxins were in-house synthesized, since they are not commercially available yet. In addition, the applicability of the developed methods has been demonstrated by analysis of a variety of naturally contaminated cereals and real-life samples purchased on the market [7,13,21–23].

Despite having become a well-established technique, the QqQ method set-up is time-consuming when aimed at determining a large number of substances. Likewise, this technique presents limitation on the number of compounds that can be analyzed in one run. In addition, only targeted

analytes can be detected, making necessary the use of an analytical standard, which is a critical issue in the modified mycotoxins field.

Thus, with the introduction of benchtop high resolution mass spectrometers (HRMS), such as Time-of-Flight (ToF) and Orbitrap, full-scan techniques started to be investigated as a complementary approach for the triple-quadrupole-based methods on the basis of increased resolution power and detectability. In addition, the use of LC coupled to HRMS offers some advantages over QqQ, since the acquisition of high resolution full scanned data permits the combination of target analysis with screening of non-target compounds, novel compound identification, and retrospective data analysis. The increasing popularity of HRMS is mainly due to the advantages of using the full-scan acquisition mode [10,24,25] with high sensitivity, combined with high resolving power (up to 100,000 FWHM) and accurate mass measurement (<5 ppm). Moving from low resolution MS to high resolution should improve in principle specificity, although this is difficult to be transferred to a superior performance of the target analysis when moving from LC-MS/MS to HRMS methods, since ion suppression/enhancement phenomena due to the matrix may occur in both approaches.

A possible approach to cope with the matrix effect is represented by stable isotope dilution assay (SIDA) [26]. Multi-mycotoxins methods applying this technique have been developed [27,28], also thanks to in-house synthesis of labeled isotopologue mycotoxin standards, including isotopologues of modified forms (i.e., DON3Glc, 3Ac-DON, 15Ac-DON) [29]. Authenticity and method performance have been demonstrated by analyzing naturally contaminated samples, such as malt, beer and maize [30]. Despite ion suppression phenomena, the enhanced selectivity and sensitivity provided by HRMS allow the development of methods that cover a wide range of compounds with different physicochemical properties, as demonstrated by Dzuman and coworkers [31] who developed an LC-HRMS method for the detection of 323 pesticides, 55 mycotoxins, and 11 plant toxins. The major advantage of using HRMS over MS/MS techniques is actually due to the possibility to perform retrospective data analysis [32], thus enabling the possibility to reconsider analytical results for stored data. The measurement of accurate MS and MS/MS spectra (resolution <5 ppm) allows the determination of compounds without previous compound-specific tuning, to carry out retrospective analysis of data, and to perform structural elucidation of unknown or suspected compounds. This is particularly worth noting when modified mycotoxins are considered, especially if combined toxicological effects are in the pipeline.

3. Use of HRMS Methods for Targeted Quantification of Natural Toxins

From a quantitative point of view, as main advantage, a full-scan technique allows the extraction from the HRMS full scan data of a theoretically unlimited number of analytes without any

compromise regarding the resulting detectability. Generic tuning setting can be used, without the need for optimizing parameters for each analyte.

Although the use of HRMS in food is very recent, over the last few years there was a significant increase in the number of studies reporting LC-HRMS-based approaches for targeted quantitative analysis of residues and contaminants in complex food matrices [10,24,33,34], most of them using a multi-contaminant approach [11,12,31,32]. Among the classes of food contaminants, the possible use of HRMS-based methods for natural toxins was successfully applied to the analysis of a large variety of samples from the market, as recently reviewed by Senyuva et al. [35]. In general, the collection of exact m/z values, HRMS/MS spectra and retention time allow the build up of (myco)toxins spectral libraries potentially sharable between Q-Orbitrap instruments. As an example, Ates et al. [36] created a database containing empirical formulae, polarity, fragment ions (up to five), and retention times for 670 plant and fungal metabolites.

The library was validated by correct identification of known mycotoxins in proficiency test materials, and then applied to the screening of emerging mycotoxins in cereal samples from the market. While the use of HRMS for the quantification of one or few analytes does not pose any significant advantage compared to MRM-based methods, multi-contaminant methods seem to be the most promising approach for food and feed surveillance in the coming years. It must be underlined, however, that the validation steps required for HRMS-based methods does not differ from those applied for QqQ methods. Moreover, sample preparation (i.e., extraction, enrichment, clean up, chromatographic separation) still remains a crucial step to reduce ion suppression phenomena, and ensure the required specificity and detectability.

Concerning mycotoxins, the possible set up of HRMS multi-toxin methods has been increasingly exploited in recent years, especially for monitoring the co-occurrence of regulated and emerging mycotoxins. In this frame, the potential capability of HRMS to return a full picture of the pool of modified mycotoxins in a selected food may represent the basis for future studies of combined toxicity.

However, it should be mentioned that only five modified mycotoxins have been included so far in multi-toxin HRMS quantitative methods, since analytical standards are available on the market only for DON3Glc, 3Ac-DON, 15Ac-DON, as well as α and β zearalenol (ZEL) [31]. Other modified forms have been included in semi-quantitative or screening methods, based on in-house prepared reference compounds, usually obtained by chemical/enzymatic synthesis or isolation from natural sources. In other studies, conjugates are semi-quantified on the base of the parent compounds. Although inaccurate, in case of novel compounds, when neither commercial calibrants nor in-house

synthesized standards are available, this assumption allows for a rough semi-quantitation of the novel conjugate compared to the parent form. For instance, zearalenone biotransformation to zearalenone malonyl-glucoside in wheat was estimated by assuming that both the parent and the modified form had the same response during MS ionization [37]. As an alternative, the formation of DON-oligoglucosides during malting and brewing processes was expressed as their molar ratio to DON [38].

In consideration of the lack of commercial standards, HRMS actually provides more qualitative than quantitative benefits to modified mycotoxin analysis.

4. Use of HRMS Non-Targeted Screening Methods for Natural Toxins

In consideration of the possible collection of full-scan spectra, a theoretically unlimited number of compounds from different classes may be detected simultaneously by HRMS at low concentration level. Therefore, HRMS is often used for non-targeted screening of unknowns, since compound to be monitored should not be established in advance. As a general remark, when natural toxins are considered, it must be noted that unknown compounds should be better defined as “expected unknowns” and “unexpected unknowns”, the former being modified forms of natural compounds that can be anticipated on the basis of biological pathways, and the latter novel compounds never described before. In addition, known compounds may occur in unexpected biological matrices, thus representing an unexpected known analyte. Examples of this definition are collected in Table 1.

In a more general meaning, a “non-targeted” analysis could be described as a screening against a large database of compounds, or a retrospective analysis of a dataset for compounds not specifically anticipated. This approach usually leads to a list of potential contaminants occurring in a sample that should be further confirmed by targeted analysis. The applicability of HRMS as a non-targeted approach indeed is based on the screening of an accurate mass of both precursor and fragments ions in one single run, by using data-independent analysis (DIA), without monitoring any preselected parent ions and based on general settings. This permits retrospective data analysis from the recorded HR-full-scan spectra; consequently, the presence of ‘newly discovered’ mycotoxins can be investigated with the data of prior-analyzed samples without the need for analytical standards.

As can be seen below (Figure 1), the untargeted workflow usually moves from seeking the exact masses of a list of compounds in the full scan spectra, to generating molecular formula, and comparing theoretical and experimental isotopic patterns and fragment spectra with those collected in the reference library, or available in on-line databases. Therefore, the full process can be described as the search of a limited number of compounds (those reported in the library) in an unlimited dataset (the stored data).

Table 1. Examples of parent and modified mycotoxins classified in known/unknown categories, according to the above mentioned definition.

Known/Unknown categories	Modified mycotoxin	Matrix	MS equipment	Identification based on	Analytical standard
Expected knowns	Aflatoxin M1	Cheese [39]	Q-Trap	authentic standards	Commercially available
	DON3Glc	Wheat and maize [22]	Q-Trap	authentic standards	Commercially available
	3/15Ac-DON	Wheat [23]	Q-Trap	authentic standards	Commercially available
Unexpected knowns	Aflatoxin M1	Feed [40]	QqQ	authentic standards	Commercially available
	enniatins, alternaria toxins, T-2/HT-2 toxins	Dietary supplements [41]	Q-Trap	authentic standards	Commercially available
	FB2	Culture media [42]	QqQ	authentic standards	Commercially available
Expected unknowns	T2-Glc	Wheat and oats [43]	LTQ Orbitrap	HRMS	in-house synthesized
	15Ac-DON-Glc	Wheat [44]	LTQ Orbitrap XL	authentic standards	in-house synthesized
	DON-oligoglycoside	Malt and Beer [38]	Exactive Orbitrap	HRMS	n.a.
	NIV-Glc	Wheat [45]	LTQ Orbitrap	HRMS/MS	n.a.
	Desmethyl Enn B1	Human liver [46]	Q-Tof	HRMS/MS	n.a.
ZEN-MalGlc	Wheat [47]	Q-trap	MS/MS	n.a.	
Unexpected unknowns	Feruloyl-T2	Barley [48]	Exactive Plus Orbitrap	HRMS/MS	n.a.
	DON-2H-glutathione	Wheat [44]	LTQ Orbitrap XL	HRMS/MS	n.a.
	Pentahydroxyscirpene (PHS)	Barley [49]	Q-Tof	MS/MS	in-house synthesized
	DON-3-Glc lactone	Wheat [50]	Exactive Orbitrap	HRMS	n.a.

Table columns: MS equipment = mass spectrometry instrument; identification based on = mycotoxin identification based on matching retention time, m/z and MS/MS fragment with that of authentic standards, or based on accurate mass (HRMS) and accurate fragments (HRMS/MS); analytical standard = mycotoxin standard commercially available, not available (n.a.) or in-house synthesized by research group.

Screening methods are usually based on a generic sample preparation (i.e., QuEChERS extraction procedure) [15,31], and thus data collected allow in principle the retrospective review of any potential compounds of interest. Nonetheless, the final detection and quantification of targeted compounds may be negatively affected by interferences from the matrix. In some cases, co-elution may impact on the mass accuracy by causing ion suppression. This may lead to the missing of suppressed compounds during the automated filtering process. Therefore, the improvement of sample preparation as well as the fit-for-purpose adjustment of software parameters are of great relevance for the analysis. A comprehensive description of the systematic workflow for quantitative target analysis, targeted screening of listed compounds, and untargeted screening of unknowns was reported by Krauss et al. [25].

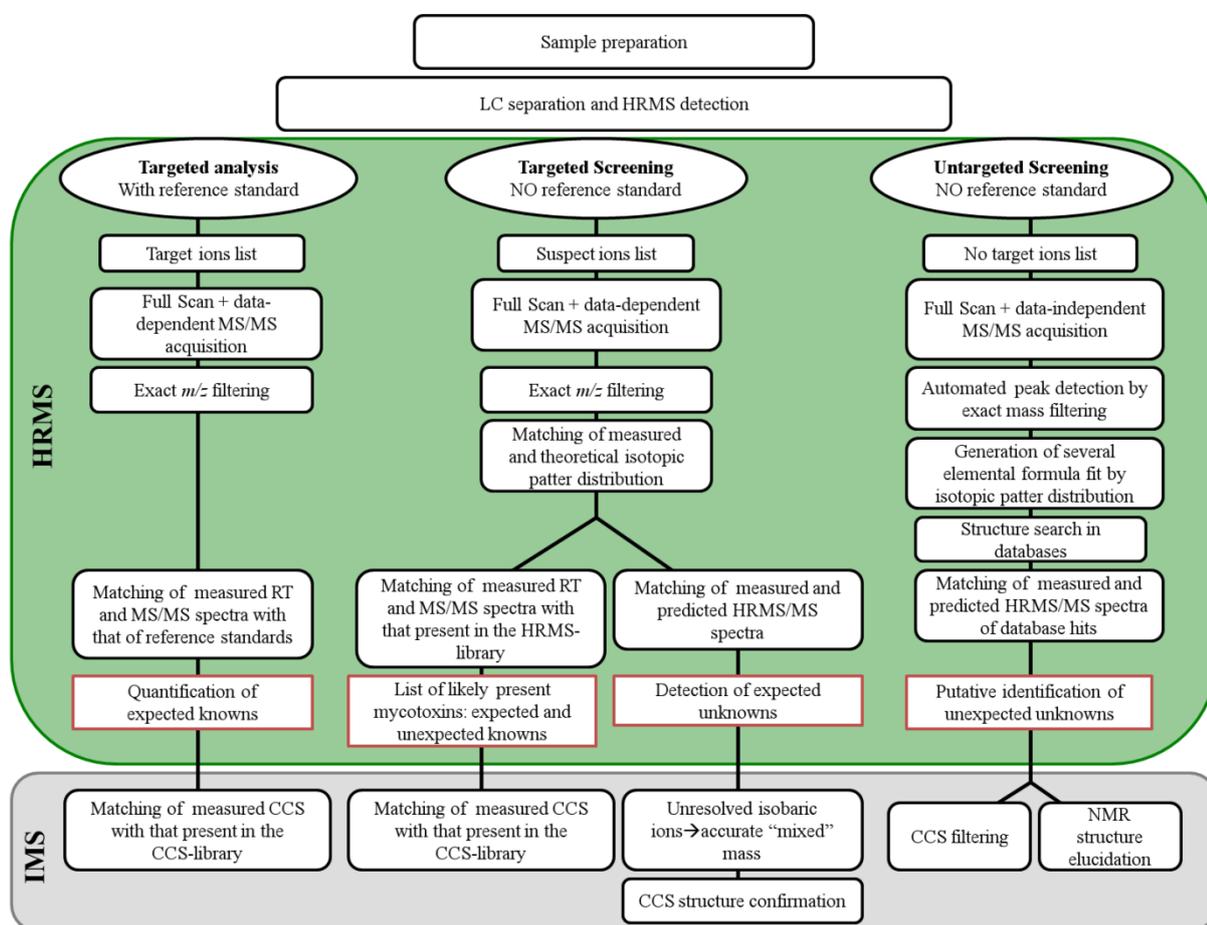


Figure 1. Workflow for targeted analysis with mycotoxin reference standards, targeted screening without analytical standards, and untargeted screening for unexpected unknowns (adapted from Krauss et al. [25]). CCS = Collision Cross Section.

Considering the large number of data generated by using HRMS, reliable bioinformatics tools for processing untargeted data are needed, as well as software packages for an automated compound detection. Therefore the development of a reliable bioinformatics workflow, providing a software solution from features extraction to unknown identification data is fundamental. The selection of

relevant compounds from a large data set still represents the bottleneck [51]. Recently developed approaches show that statistical analyses, in combination with untargeted screening, are useful methods to preselect relevant compounds [52]. In addition, there are different software tools that allow peak deconvolution and the removal of background noise [25] by comparing different chromatograms. As an example, the use of a blank matrix as a control sample can substantially reduce the number of compounds to be screened by the software algorithm. At this point, several elemental composition formula may be predicted from an accurate mass measurement using MS trademark packages. Subsequently, molecular formula candidates and MS/MS pathway may be jointly investigated by structure elucidation software. Nowadays, HRMS qualitative software tools are linked with on-line databases (i.e., Pubchem, Metlin) providing a comparison of accurate mass, isotopic pattern, and MS/MS fragment ions. In the end, the users may find a percentage of matches, which are interconnected with the unknown compound. However, it should be noted that the search for unknowns in on-line databases is limited to the recorded spectra of available reference standards. In this frame, a big step forward has been achieved with computer-based tools based on *in silico* strategies [51,52], since the unknown chemical structures may be putatively confirmed by matching measured and computational predicted MS/MS fragmentation.

5. Use of HRMS Methods for Structural Identification of Unknowns

Another relevant application of HRMS is the structural identification of unknown compounds, i.e., novel compounds identified for the first time in the considered matrix. Although the unequivocal structural elucidation of compounds still requires ¹H- or ¹³C NMR spectroscopy, in most cases there is already sufficient information to tentatively annotate and identify the unknowns. This is the case, for instance, of expected unknowns, modified mycotoxins where the possible modification pattern carried out by plants or microbes is well-established. As an example, the identification of new trichothecene conjugates such as FUSX-glucoside and NIV-glucoside [45], or acetyl-T2 [48,53] was an extrapolation of existing knowledge. The tentative identification of mycotoxin gluco-conjugates based on accurate mass, isotopic pattern distribution, and MS/MS fragmentation is indeed feasible. Accurate mass can be theoretically calculated and MS/MS fragments may be quite easily predicted since they generally lead to the loss of the glucosidic unit [M-H-glucose]⁻ and thus the detection of the aglyconic form. More challenging is the elucidation of the binding position of the sugar unit. As suggested by Dall'Asta et al. [54] the binding position in the DON molecule distinctly influences the stability of the molecular ion, thus leading to different fragments. In particular, the ion corresponding to [M-H-CH₂O]⁻ is reported to be characteristic of all 3-substituted trichothecenes; however that is not true for the other classes of mycotoxins. By

following this approach, also DON-oligoglycosides [38] were identified in malt and beer samples. Therefore, the molecular formula and exact masses of DON-di, tri, and tetra glucosides can be easily calculated by adding glucose units corresponding to 162.0523 Da, as summarized in Table 2, and thus many putative structures can be predicted. As a result, nine possible molecular structures were hypothesized for DON-di-Glc, corresponding both to di-glucoside conjugates, with one molecule of glucose conjugated to each of the hydroxyl groups of DON or oligosaccharides. Although chromatographic separation was optimized changing from a reverse phase to an HILIC column, isobar separation was not achieved. In addition, also considering the peaks that are not baseline-resolved, no more than four out of nine peaks were detected both in malt and beer samples, as is depicted in Figure 2.

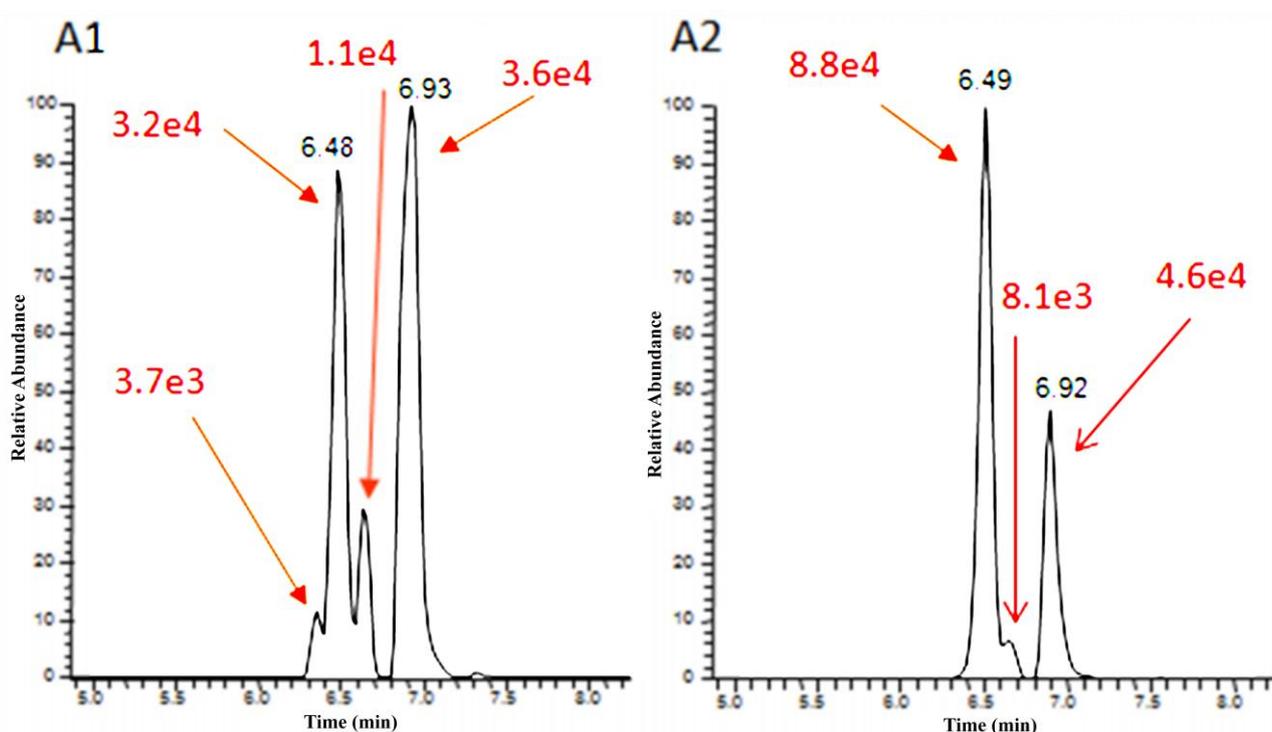


Figure 2. Extracted ion chromatogram for DON di glucosides determined in malt (A1) and beer (A2) by using HILIC phase chromatography coupled to HRMS (Orbitrap). (Reproduced with permission from [29], copyright (2016) American Chemical Society).

As well as gluco-conjugates, also mycotoxins conjugation with malonyl-glucoside frequently occurs. So far, occurrence of T2, HT2, ZEN, α and β ZEL, and DON malonyl-glucosyl (MalGlc) derivatives have been reported in artificially infected samples [44,47,48,53]. The extracted ion chromatogram (EIC) of tentative HT2-malonyl-glucoside showed two peaks that may result from the conjugation of malonic acid to different hydroxyl groups of glucose (four possible positions) or from conjugation of malonyl-glucoside to the two different position of the HT2 hydroxyl groups

[39]. As an example, the ZEL-MalGlc HRMS putative identification workflow is depicted in Figure 3.

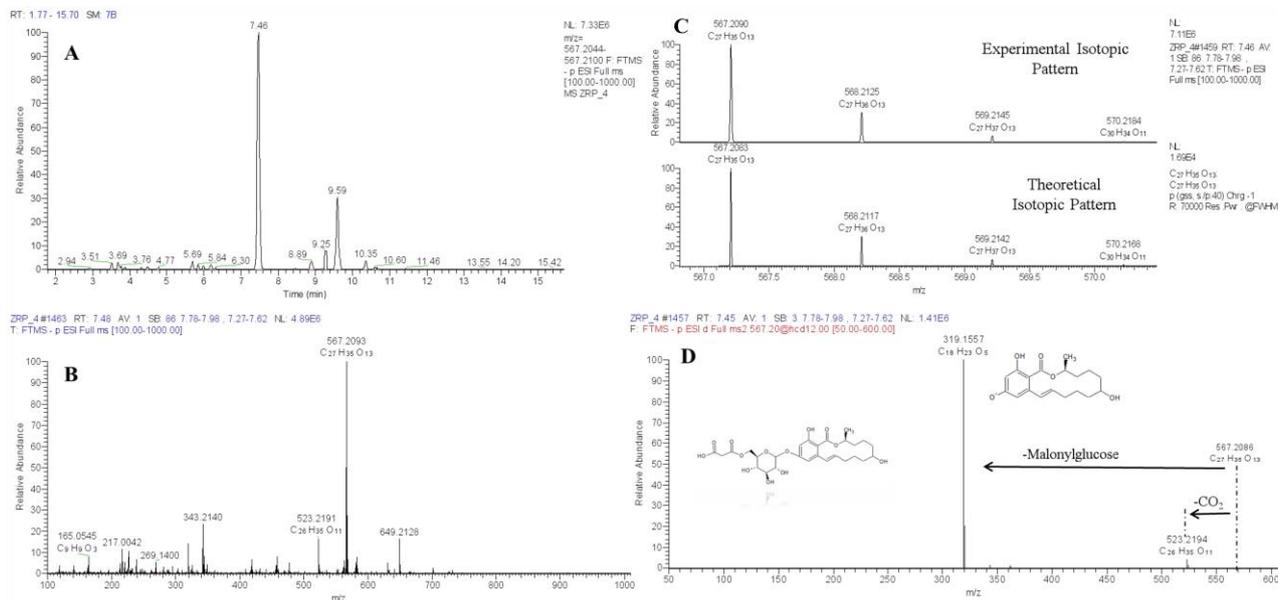


Figure 3. ZEL-MalGlc putative identification steps: UHPLC-Q-Exactive ion chromatogram (resolving power 70,000 FWHM, extraction window 5 ppm); **(B)** molecular formula assignment of parent ion; theoretical and experimental isotopic pattern comparison **(C,D)** high resolution fragmentation pathways obtained by using DDA acquisition.

Thus, also in these examples, it was not possible to achieve a high degree of certainty for the identification of expected unknowns and thus the authors concluded that NMR analysis was required. In addition, putative co-eluting isomers having the same m/z value can be present in the sample, considering the high number of theoretical structure compared to the detected peaks.

As far as modified mycotoxins, the metabolic modification occurring in plants usually follows phase I and phase II patterns. Therefore, the possible modification can be theoretically anticipated, and a list of expected unknowns can be used for peak annotation. As an example, a list of possible phase I and phase II modifications is reported in Table 2.

Table 2. Summary of calculated exact masses for putative phase I and phase II mycotoxin modifications.

Modification	Mass change (Da)	Molecula formula change
Hydrogenation	2.0151	H ₂
Hydroxylation	15.9944	O
Methylation	14.0151	CH ₂
Acetylation	42.0100	C ₂ H ₂ O
Glycine	57.0209	C ₂ H ₃ NO
Sulfate	79.9563	SO ₃
Sulfonation	102.9460	SO ₃ Na
Ferulic acid	176.0468	C ₁₀ H ₈ O ₃
Cysteine	119.0036	C ₃ H ₅ NO ₂ S
Acetyl-cysteine	161.0141	C ₅ H ₇ NO ₃ S
Glucose	162.0523	C ₆ H ₁₀ O ₅
Cysteine-glycine	176.0250	C ₅ H ₈ N ₂ O ₃ S
Glucuronic acid	176.0315	C ₆ H ₈ O ₆
Acetyl-glucoside	203.0550	C ₈ H ₁₁ O ₆
Malonyl glucoside	248.0527	C ₉ H ₁₂ O ₈
Glutathione	305.0682	C ₁₀ H ₁₅ N ₅ O ₆ S
Di-glucoside	324.1051	C ₁₂ H ₂₀ O ₁₀
Malonyl di-glucoside	410.1055	C ₁₅ H ₂₂ O ₁₃
Tri-glucoside	486.1579	C ₁₈ H ₃₀ O ₁₅
Di malonyl-di glucoside	497.1137	C ₁₈ H ₂₅ O ₁₆
Tetra-glucoside	648.2107	C ₂₄ H ₄₀ O ₂₀

Several expected unknowns of mycotoxins have been tentative identified so far thanks to the use of HRMS [55]. When only one isomer configuration is possible, the recorded HRMS/MS spectra allow a satisfactory compound structure elucidation. However it should be stated that many mycotoxins are characterized by more than one hydroxyl group, that can be further increased by phase I metabolism.

As a result, conjugation reactions may take place on several substituent groups and thus separation and characterization of the different isomers still remain challenging.

However, although it is frequently based on the search of expected unknowns, structural elucidation in complex samples is limited by the non-specific nature of the electrospray ionization process. It has been stated, indeed, that full scan spectra may contain up to 90% of noise compared to low concentration metabolites [56]. In such cases, the use of stable isotope labeling (SIL) may successfully assist metabolic profiling [57]. The software-driven correction of isotope pattern abundance errors resulted in better identification rates of the molecular formulas. In particular,

artifacts generated by solvents, matrix background or noise, can be filtered out, enabling better detection and annotation of novel and unexpected compounds.

Recently, the *in vivo* SIL approach was exploited for the identification of *Fusarium* toxin metabolites in grains, based on the comparison of natural and ¹³C-labelled patterns of metabolites showing identical chromatographic behavior but different (shifted) MS spectra [48,58]. The spectra comparison and metabolite identification was supported by the use of a dedicated software [59]. This powerful approach may simplify the identification of novel unexpected unknown compounds (i.e., feruloyl-T2 [48]) and successfully support the metabolic fingerprint.

6. Advantages and Challenges of HRMS in Modified Mycotoxin Analysis

When natural toxins are considered, the advantages derived from a qualitative use of HRMS overcome those obtained from a quantitative analysis. Retrospective data analysis, expected unknown screening, and novel compound identification are actually features that may strongly improve research in this field in the very near future. This is especially interesting in the field of emerging and modified mycotoxins, when analytical standards, calibrants, and reference materials are not commercially available. In addition, the urgent trend to base risk assessment on the combined toxicity derived from a pool of contaminants instead of a single compound, makes necessary the collection of (co)occurrence

data. In this frame, the information generated by HRMS identification of novel compounds can be added to parent toxins libraries, and further used for retrospective analysis of full-scan data. This can usefully concur to acquire qualitative occurrence data for better food quality controls, and more importantly, to ensure food safety.

Besides the advantages described above, HRMS still presents some critical points to be considered for its application in contaminants analysis. Among the possible disadvantages, the cost of instrumentation and the difficulties in big data management are often reported, but the improvement of software and storage systems as well as the introduction on the market of bench-top instruments favor broader diffusion LC-HRMS based methodologies. However, from a chemical point of view, issues such as isobars co-elution, and unknown molecule identification have to be solved to allow an effective use of HRMS for food safety purposes.

The innovative technique of ion mobility spectrometry (IMS) can be used to ascertain complementary information about analytes, adding a third dimension of separation based on size, shape, and charge of ions. The coupling of the two strategies, IMS and LC-HRMS platform, may act as a powerful tool to (i) improve the quality of mass spectral information obtained thanks to a filtered background; (ii) to increase the peak capacity to separate isomeric/isobaric compounds

which can neither be resolved by MS and sometimes nor by UHPLC; (iii) to enhance analyte identification thanks to structural information (size and shape) based on collision cross-section measurement.

7. The Potential Benefits of Ion Mobility Mass Spectrometry in Mycotoxin Analysis

In the first section, the many possibilities of HRMS as a tool for modified mycotoxin analysis were reviewed. However, it should be stated that confirmation and structure elucidation of new unknown molecules and the unambiguous identification of isomers still remains challenging when using conventional mass spectrometry methods. In comparison, NMR spectroscopy has been successfully employed with the goal of recognizing modified mycotoxins structure [22,60]. The NMR-based approach is efficient for evaluating isomeric heterogeneity, and for structural elucidation, but has the limitation of needing a considerable amount of analytes and obtaining a single molecular species following the purification steps.

Ion mobility spectrometry (IMS) is a promising approach that can overcome the above mentioned HRMS and NMR limitations, making it an ideal candidate for improving confidence in the identification and separation of structurally closely related isomers. IMS is a gas-phase electrophoretic technique that provides a new dimension (3D) of separation based on size, shape, and charge of ions. The ion mobility spectrometer consists of four main components that can be identified as: the sample introduction system (SIS), the ionization source, the drift tube region for separation and selection of ions, and the detection area [61,62]. In this review we mainly focus on the separation region, detailed information on the other components can be found elsewhere [61]. Once ionized, ions are directed to the drift tube region that contains an electric field, drift gas separates them according to their mobility. Ions moving in a gas phase medium and in the presence of an electric field are accelerated due to coulomb forces and slowed down due to collisions with molecules of the gas medium [61]. Thus small and compact ions travel faster and will reach the detector before large and heavy ions. In this way isobars are separated in the mobility spectrum, where the ion current is plotted as a function of the drift time or the compensation voltage [50]. Eight different types of IMS have been recently reviewed [61]. However, it should be noted that not all IMS devices are stand-alone instruments and for the purpose of the present review, only the IMS hyphenated with a mass spectrometer are discussed. A common hyphenated technique includes coupling IMS with MS (IMS-MS) in which IMS works as a pre-filter by confirming ion identities for the MS system. In addition, since IM separation typically occurs in a millisecond timeframe and MS detection, in a typical TOF instrument, takes only microseconds, additional separation techniques such as liquid chromatography (LC) can be hyphenated without compromising the speed

of MS detection. So far, four major IMS-MS separation approaches are currently commercially available coupled with MS: drift-time IMS (DT-IMS) [63], traveling-wave IMS (TW-IMS) [64,65], high field asymmetric waveform IMS (FAIMS) [66], also known as differential-mobility spectrometry (DMS), and trapped IMS (TIMS) [67–69]. In DTIMS and TWIMS all the ions pass through the mobility cell ions and are separated based on the time it takes to traverse the cell. Such devices are generally used for untargeted screening experiments. FAIMS/DMS devices separate ions by varying voltages, filtering ions in a space-dispersive fashion. TIMS-MS separates ions based upon differences in mobility, after trapping and selectively ejecting them. Readers interested in the principles behind these IMS technologies can refer to previous reviews [70].

Applications in the food analysis field, especially when analyzing contaminants, have been mainly addressed applying FAIMS technologies [71–73] probably because of the advantages offered by the filtering effect as well as for the possibility of the device being moved and placed at the front-end of the mass spectrometer. On the other hand, TWIMS applications are rapidly growing [74–76] because, enabling CCS values measurement, it has found application as both a separation device and a structural elucidation tool.

Considering the complexity of food and feed samples, the use of LC-IMS-MS hyphenated methods is starting to be considered vital for versatile applications. Indeed, LC-IMS-MS potentially provides three major benefits to modified mycotoxins detection compared to traditional approaches. First, LC-IMS-MS improves the peak capacity and signal-to-noise ratio of traditional analytical approaches providing cleaner mass spectra obtained from the filtered background [71,77,78]. Second, it allows the separation of co-eluting isobaric metabolites according to their mobility [79], simplifying the interpretation of mass spectra. Third, IMS enhances confidence in analyte identification thanks to the measurements of the collision cross section (CCS) [80], a physicochemical measure related to the conformational structure of ions (size, shape, and charge).

7.1. Peak Capacity and Signal-to-Noise Ratio Improvement

The coupling of IM with liquid chromatography (LC) and HRMS gives a degree of orthogonality to both techniques by separating co-eluting LC compounds in mobility space before mass analysis. Hence, the overall peak capacity of the method is increased [81,82] making IM-MS highly suitable for food safety control analysis. In this frame, a broad range of food contaminants such as herbicides [83], pesticides [75,84], mycotoxins [71,77,78], and veterinary drug residues [85] have been successfully detected by IMS.

Although mycotoxins have been scarcely analyzed by IMS-MS, three applications using low resolution mass spectrometry have been reported so far [71,77,78]. An unusual type of IMS, corona

discharge ion mobility spectrometry (CD-IMS) was applied to determine aflatoxin B1 (AFB1) and B2 (AFB2) in pistachio samples aiming to monitor spoilage status [77]. Sample extracts were directly introduced into the corona discharged ionization region via a headspace (HS) device without any chromatographic separation. As expected by the authors, IMS was not able to distinguish between AFB1 and AFB2 due to their similar chemical structure and their very close molecular weight. Thus they measured the total AFBs since it was demonstrated that their IMS response factors were identical [77]. The resulting LOQ and LOD (0.5 and 0.1 ng_{mL}⁻¹, respectively) were in line with those reported in literature obtained using different chromatographic and spectrometric techniques. In addition, pistachio samples were analyzed to demonstrate the capability of the method in detecting aflatoxins in real samples.

The same authors applied the proposed approach some years later [78] for the analysis of ochratoxin in licorice roots. Following extraction and purification by passage through an immuno-affinity column, the achieved LOD in real samples was compliant with established concentration limits for licorice roots (20 μ g/Kg). A significant improvement of the detection limits was also measured for ZEN and its metabolites α -ZEL, β -ZEL, and β -zearalanol (ZAL) in maize using FAIMS technology and direct infusion or flow injection [71]. In fact, compared to ESI-MS or ESI-MSMS, a five-fold improvement in the signal to noise ratio was reported. This result was attributed to the ability of FAIMS equipment to operate as an ion filter, focusing ions and reducing the chemical background attributable to the matrix. The achieved LODs for ZEN, α -ZEL, β -ZEL, and β -ZAL were 0.4 ng x mL⁻¹, 3.2 ng x mL⁻¹ and 3.1 ng x mL⁻¹, respectively. Thus, FAIMS filter step prior to ESI-MS analysis was able to selectively resolve and quantify species that otherwise cannot be selectively analyzed by ESI-MS alone. Additionally, since analytes are separated on account of their compensation voltages, reducing the time required for each sample run to about 1 min, the authors suggested that FAIMS might allow overstepping of the chromatographic separation [71]. LC-ESI-FAIMS was also used to develop a quantitative method for the determination of marine toxins in mussel tissue [73]. They investigated in depth how to improve the method sensitivity in relation to the number of CV values monitored at a given time. In fact, one of the limiting factors for analytical sensitivity is the duty cycle of the FAIMS device, which has a switching time between different CV values of about 100 ms, the time required to empty the device of ions that experience a particular CV. This means that limiting the number of CV values simultaneously monitored is an effective way of limiting sensitivity losses observed when using FAIMS in combination with LC. Two approaches were investigated for limiting the number of monitored CVs. The first one was developed using time periods with a limited number of optimized CV values at a given retention time, the second one reducing the number of monitored CVs to three values, which provided

coverage of all analytes close to, but not at their optimal CV. The latter method proved to be more robust but less sensitive due to the fact that toxins were not detected at their optimum CV values, but was more suitable for analysis of large sample sets where RT could be expected to drift slightly. The above mentioned IMS-MS approaches are pioneering, since they represent the first application of ion mobility spectrometry in the mycotoxin field. On the whole, the achieved limits of detection for these applications were in agreement with those required from the regulatory authorities, and confirming the method applicability to real samples and then the fitness for purpose, led to the expectation that IMS may help trace analysis control compliance. Thus, the development of further IMS-MS methods to extend the number of monitored parent and modified mycotoxins are encouraged. In this regard, more recently, a novel approach to screening multi-class pesticides by TW ion mobility time-of-flight mass spectrometry detection was successfully developed [75]. The authors demonstrated that combining full scan and mobility XIC (extracted ion chromatogram) it was possible to detect the mass spectrum of indoxacarb, that was masked by other co-eluting compounds in the scan spectra. This example demonstrated how drift times give a higher level of selectivity to the overall method as no interfering compound resulted at the same retention time, drift time, and measured exact mass. In addition, once the pesticide has been identified using its retention time, exact mass, and drift time, the resulting cleaned mass spectrum facilitates the identification process. In line with these findings, the cleaning effect due to ion mobility separation on MS/MS spectra was demonstrated [86]. Applying the DIA mode for the MS analysis of complex extracts could result in MS/MS spectra containing a mixture of product ions derived from co-eluting precursors, complicating interpretation of the spectra and the overall identification process (Figure 4). Combining IMS-MS with DIA might allow the separation of co-eluted precursor ions before fragmentation, resulting in a drift-time correlation of product ions with their respective precursor ions and thus cleaner MS/MS product-ion spectra.

This phenomenon might offer a straight benefit when analyzing mycotoxins in complex food and feed matrices and in particular modified mycotoxins, considering that they are usually present at a low concentration level. Despite the HRMS improvement, quite significant discrepancies when comparing relative intensities of fragment ions measured in pure solvent with those measured in matrix were reported [31]. Thus, these clean MS and MS/MS spectra might, in turn, facilitate compound identification and reduce false-positive assignments in complex food matrices.

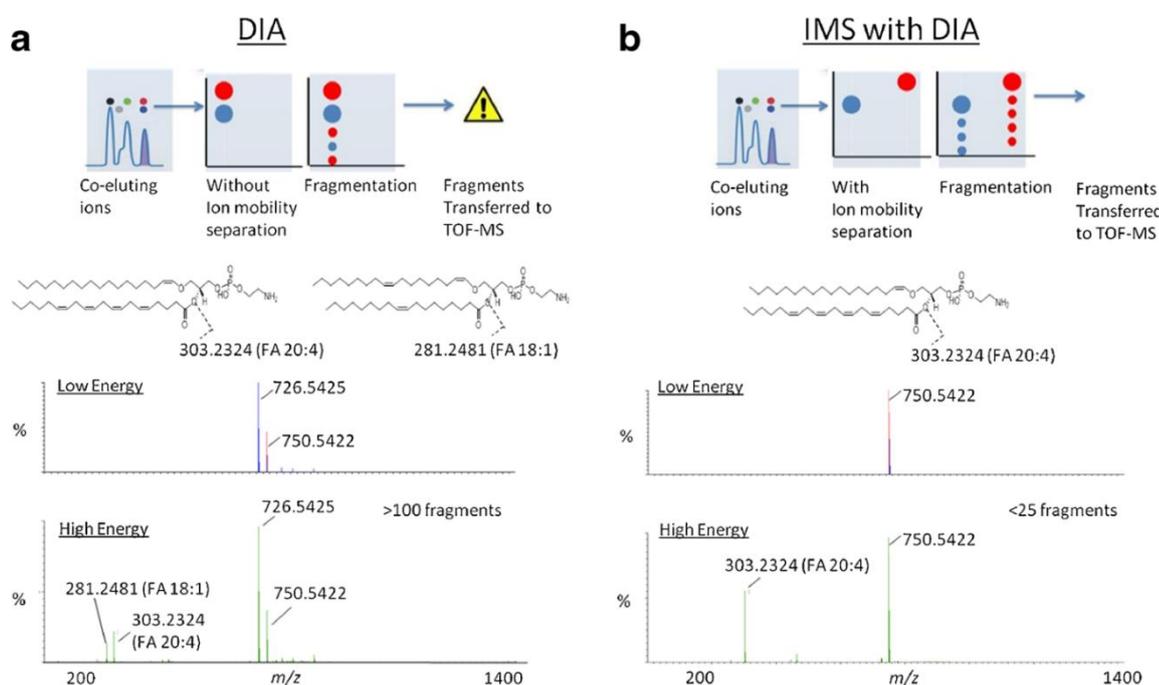


Figure 4. MS and MS/MS cleaner mass spectra obtained by using LC- IM- QTOF (**b**) compared to those obtained by LC- QTOF (**a**) (from Paglia et al. [86]).

7.2. LC-IMS-MS Enables the Separation of Isobar Molecules

Filtering out interferences, LC-IMS-MS may also allow the separation of co-eluting isobars and isomers that are difficult to separate by traditional LC-MS. Isobar co-elution may occur when expected unknowns are tentatively identified by LC-HRMS, as described in some examples reported in the previous sections. This issue is of a great relevance for mycotoxin analysis, above all when coeluting modified forms differ in their toxicological profile, as for the acetylated derivatives of DON. With regard to intestinal toxicity, 3Ac-DON was found to be less toxic than DON, which was less toxic than 15Ac-DON [87]. As a result, a precise quantification of the different isoforms has to be performed. However, considering that they differ only in the position of the acetyl group and thus similar polarities of these two mycotoxins, it has not been possible to achieve chromatographic separation so far [88]. Hence, different strategies have been developed to reach a correct quantification. By MS-single quadrupole detection, Biancardi et al. [89] calibrated the response for 15Ac-DON and 3Ac-DON separately and reported the results as the sum of 3- and 15Ac-DON (Ac-DONs). Afterwards, thanks to the selective detection power of MS/MS, separate identification was performed due to the difference in characteristic daughter ions in MRM mode, identified as m/z 339.5 >137.2 and 339.5 >321.1, and m/z 339.5 >213.0 and 339.5 >230.9 for 15Ac-DON and 3Ac-DON, respectively [88]. In a recently published multi-mycotoxin method [15], the two acetylated derivatives were detected by taking advantage of the detection polarity. In fact,

15Ac-DON was detected in positive mode (m/z 356.1, $[M+NH_4]^+$) and 3Ac-DON in negative mode as acetate adduct (m/z 397.1, $[M+CH_3COO]^-$).

Hence, it is evident that the potential of LC-IMS-MS to enhance isomer separation, would overcome challenges associated with modified mycotoxin isomers analysis that will not otherwise be achieved. In addition, the proper separation and then quantification of 3Ac-DON and 15Ac-DON is essential in order to collect further data and better characterize their potential contribution to the total exposure to DON. Even more challenging is the case of modified mycotoxins, whose analytical standards are not available, since the traditional quantification methods, represented by tandem MS, are not applicable. The detection and identification of these unknown modified forms is permitted by taking advantage of the accurate mass and the isotopic pattern distribution provided by HRMS. However, no information about putative co-eluting isomers having the same m/z value can be obtained with only LC-HRMS. When analyzing oligo-glycosides mycotoxins, indeed, information about binding position and configuration (α or β) between the sugar moiety and the mycotoxin, and the linkages, α/β 1-4, α/β 1-6, cannot be achieved. Regarding binding configuration, information available in the literature is quite contradictory. McCormick et al. [60] reported the occurrence of both T2- α -Glc and T2- β -Glc in naturally contaminated wheat and oat samples. By contrast, Meng-Reiter et al. [48], stated that since the UDP-glucosyltransferase is an inverting enzyme, the detection of the α -glucosyl isomer should be unexpected. In agreement, Zachariasova et al. [38] reported an increase of free DON after DON oligoglucosides incubation with fungal β -glycosidase, that was obviously caused by its release from the β -bound forms. However, also stating the glucosyl bound for DON-oligo-Glc, the number of possible theoretical isomeric structures was higher than the number of chromatographic peaks detected (see Figures 2 and 3) [38]. Thanks to the enhancement in isomer separation offered by applying DTIMS coupled with quadrupole time-of-flight spectrometer (Q-TOF), one more DON-di-Glc and two more DON-tri-Glc peaks separated by their drift time were detected [79]. These additional peaks could be due to the linkages, 1-4 or 1-6, between the sugar moieties and the mycotoxin, since the bounding position was confirmed by HRMS-MS/MS. Therefore, IMS-MS allowed the detection and subsequently the characterization of new isomeric DON-oligo-Glc, also increasing confidence in results. The same approach could be applied to resolve other modified mycotoxins such as oligo-glycosides forms of ZEN or α/β ZEL, that have already been detected [38] but whose structures have not yet been elucidated, giving new insight into the mycotoxin biotransformation that may occur in plants and/or animals.

It is evident from the examples given that a strong synergy arises between IMS and MS. IMS-MS can act as a tool to separate complex mixtures, to resolve ions that may be indistinguishable by mass spectrometry alone. This is vital in the modified mycotoxins field, since many different types

of isomers (diastereoisomers, epimers, anomers, protomers) may occur and need to be separated to achieve a correct quantification and subsequently to perform a reliable risk assessment. As for ADON isomers, also α/β -ZEL diastereoisomers present different toxicity. α/β -ZEL may undergo phase I and phase II metabolism [47], both in plants and in humans, leading to a wide range of metabolites having different configurations and potentially different toxicities. Thus, the separation and then elucidation of the binding configuration of the conjugated metabolites is highly advisable also for the toxicological outlook.

7.3. CCS Value: A New Unambiguous Molecular Descriptor

In addition to signal-to-noise improvement and enhancement in separation of co-eluting isobar molecules, IMS-MS has also been applied to more high-throughput analytical approaches for confirming compound identity, providing molecular structural and conformational information. Once the drift time is recorded, this can be converted into a CCS value, which represents the effective area for the interaction between an individual ion and the neutral gas through which it is travelling. Thus CCS is an important distinguishing characteristic of an ion in the gas phase and, being related to its chemical structure and three-dimensional conformation, can provide specific information on ionic configuration and potential structural confirmation.

Nowadays, CCS values can be routinely measured as an integrated part of the LC-HRMS experiment. In DTIMS instruments, CCS can be directly derived from the drift time. In TWIMS [90–92] instruments, CCS can be experimentally derived by using IMS calibration performed using compounds of known CCS under defined conditions (i.e., gas type and pressure, travelling wave speed or height). This allows CCS to be used alongside the traditional molecular identifiers of precursor ion accurate mass, fragment ions, isotopic pattern, and retention time as a confirmation of compound identity [93]. Indeed, Gosciny and colleagues [75] developed a TWIMS approach to screening multi-class pesticides and suggested the inclusion of the pesticide CCS values as a new identification point (IP) (Commission Decision 2002/657/EC [94]), to increase confidence in the results. Overall, they measured CCS values for 150 pesticides, using standard solutions, building an in-house CCS library with associated retention times, accurate masses, and diagnostic fragments. Thus, the CCS value may be included in the contaminants screening workflow, as reported in Figure 1, and it can be used as an additional means of filtering the screening data to significantly reduce the proportion of false positive and false negative detections. Therefore, the CCS tolerance of $\pm 2\%$ in combination with the traditional confirmation threshold filters of m/z ($\pm 5/10$ ppm) and retention time ($\pm 2.5\%$) will lead to a more definitive identification of the species of interest. A

further dataset providing CCS values for 200 pesticides has been recently reported by Regueiro et al. [84].

Population of databases with CCS values for pesticides and mycotoxins is pivotal in order to support the inclusion of CCS values as a new identification point (IP). In addition, since CCS measurements are undertaken in the gas phase, remotely from the ion source, their values are not affected by sample matrix and are consistent between instruments and across a range of experimental conditions [86,93]. Taking into account the analytical effort made in recent years for validating extraction and detection procedure depending on the sample matrix, the great advantage offered by the CCS measurement is evident. Moreover, it has been demonstrated that the concentration of the compound had no significant effect on the drift time values and thus on the CCSs [75]. This will help in avoiding false negative assignments in the screening confirmation procedure, mainly when analyzing contaminants close to method LOQs, since matching with HRMS/MS in libraries could be hard due to the low intensity of the fragmentation pattern [31]. In agreement, Paglia and co-workers [95] also confirmed the high reproducibility of CCS measurements of lipids classes in varying matrices. They created a CCS database for lipids that includes 244 CCS values, aiming to implement the ion-mobility derived CCS in routine lipidomics workflow. These findings raised the possibility that CCS can be used to help the identification process of targeted compounds, and, as for pesticides and lipids, CCS can be inserted in a routine workflow for parent and modified mycotoxin screening and used as an identification parameter. Data bases of mycotoxins can be created using CCS obtained by running standard compounds, providing an additional coordinate to support mycotoxin identification, and reducing the number of false positive and false negatives of the targeted analysis. In addition, at the end of the untargeted screening process (see Figure 1), CCS can be used to confirm the structure of expected unknowns by matching the theoretical and the experimental CCS values. In TWIMS devices, poly-DL-alanine is often used as IMS calibrant for deriving CCS. Since peptides have unique physical properties and gas-phase conformations, it might not be ideal to calculate the accurate CCS values for all metabolites and lipids classes. For instance, alternative calibrants have been proposed for specific lipids, which better reflect their chemical structure [96]. CCS values may also be estimated computationally if the 3D structure is known. A comparison of the theoretical and experimentally derived collision cross-sections can be utilized for the accurate assignment of isomeric metabolites. Recently, the CCS areas were used to elucidate the α and β epimeric forms of glycosylated T-2 and HT-2 toxins [80]. The two isomeric forms had already been separated by UHPLC-MS/MS [48] however, thanks to additional information provided by the CCS value, it was possible to confirm the bounding configuration between the toxin and the sugar moiety [80]. The aforementioned

application is the only one developed so far in the field of mycotoxins exploiting CCS potential; however IMS-MS, as a tool to gain insight into structural information, would expect to rise rapidly, offering a unique means of characterization. New modified forms, i.e., expected unknown mycotoxins, may be discovered and unequivocally characterized by matching theoretical and experimental rotationally averaged cross-sectional areas, despite the lack of analytical standards. Regarding unexpected unknown mycotoxins, even though HR-IMS reduces the number of possible candidates due to accurate mass, isotopic pattern, MS/MS fragment ions, and CCS values, the identification might still be challenging. In these particular cases, the use of NMR still represents the only approach for identification.

8. Conclusions and Future Trends

In the last few years, a significant increase in the number of studies reporting HRMS-based approaches for food contaminant analysis has been reported. The analytical potential of high resolving power, accurate mass, and acquisition in full scan permits a retrospective analysis using extensive databases of hundreds of analytes and enabling the investigation of ‘newly discovered’ mycotoxins in the data of prior-analyzed samples. Therefore, HRMS is undoubtedly going to redefine LC-MS workflow since targeted and routine quantification as well as qualitative research analysis can be performed with the same instrument. This situation is also facilitated by the launch from many MS companies of the latest generation of HRMS instrumentation designed for routine analysis and equipped with user-friendly dedicated data processing software.

On the other hand, ion mobility spectrometry is starting to be successfully employed in mycotoxin trace analysis with the aim of increasing signal-to-noise ratio, gaining higher sensitivity, and with longer dynamic range [71,77,78]. However, applications of IMS in separation and structure confirmation of mycotoxins has not been explored adequately so far, even though it offers great potential for gaining insight into the formation and characterization of new modified forms. In particular, the CCS values may be added in targeted and untargeted screening workflow, providing an additional coordinate to support mycotoxin identification, reducing the number of false positive and false negatives and confirming the structure of expected unknowns. In conclusion, all evidence points towards future growth in the number of applications of HRMS and HR-IMS in food safety, as the power of this technique gains wider recognition.

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Review II

Metabolomics approaches and their hidden potential for explaining the mycotoxin contamination problem

Metabolomics approaches and their hidden potential for explaining the mycotoxin contamination problem

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Abstract:

Food is essential to life. On the basis of the previous sentence, consumers have a right to expect that the foods they purchase and consume will be safe, authentic and of high quality. On these premises, target compounds, such as mycotoxins, pesticides or antibiotics have been commonly investigated on the food chain, and subsequently, were regulated by authorities. This raises the following question; may consumer be prevented to these risk exposures? Probably not, food chain is, step-by-step longer and more complex than ever before. Note that food chain is affected by globalized trade, culture, travel and migration, an ageing population, changing consumer trends and habits, new technologies, emergencies, climate change and extreme weather events which are increasing foodborne health risks, especially for mycotoxins. Because of the fact that mycotoxins are natural toxic compounds produced by certain filamentous fungi on many agricultural communities. In fact, these toxins have adverse effects on humans, animals, and crops that result in illnesses and economic losses. Nevertheless, so far mycotoxins and their modified forms have been mainly monitored in cereal and cereal-based products, however, may an early detection of mycotoxins be considered a reliable strategy? In this chapter, recent metabolomics approaches have been reviewed in order to answer this question and to understand future strategies in the field of mycotoxin contamination.

Keywords: food metabolomics, mycotoxins, plant metabolome, fungal pathogens

1. Introduction

Mycotoxins are secondary metabolites (300 – 800 Da) produced by filamentous fungi that colonize crops in field and upon storage, being among them cereals one of the most affected commodities [1]. Fungal colonization is strongly dependent on environmental conditions and agricultural practices. Climatic factors such as temperature, humidity, rainfalls, as well as the concomitant presence of other pests or insects may support the fungal infection. Therefore, climate change is significantly affecting the mycotoxin contamination of crops worldwide. As a consequence, fungal infection and related pathogenic diseases can cause significant yield losses, quality reductions and mycotoxins accumulation in crops, particularly grains [2]. Although regulations, adequate quality controls, and good agricultural practices have been implemented in many countries, the mycotoxin contamination represents a serious challenge for global trade in terms of animal and human health threat and economical losses. For this reason, the establishment of common standard procedure for fungal biocontrol and mycotoxin mitigation are under investigation.

From a toxicological perspective, mycotoxins can cause both acute and chronic effects for humans and animals. They are responsible for a broad spectrum of toxic activities, ranging from severe adverse effects on the liver, kidney, hematopoietic, immune system, foetal and reproductive systems, as well as significant contribution to carcinogenic and mutagenic developments [3]. In fact, The International Agency for Cancer Research (IARC) has formally classified a number of mycotoxins. For example, four aflatoxins are classified in Group 1 (AFB₁, AFB₂, AFG₁ and AFG₂) while ochratoxin A (OTA) is classified in Group 2B [4,5].

Among them, those produced by *Fusarium* spp. are often found in cereals, and are related to pathogenic diseases in plants, as well. In particular, Fusarium Head Blight (FHB) is recognized as one of the most destructive global diseases of wheat and barley [6]. FHB can cause, indeed, significant reductions in grain yield and quality, and is associated with the accumulation of mycotoxins, such as deoxynivalenol (DON). Thus, besides the severe economic impact, due to losses in productivity, FHB represents a serious health risk for consumers and livestock [3]. In order to reduce the economic and health impact of FHB, several cultural practices have been proposed so far. However, crop rotation, tillage, use of fungicides or other biocontrol agents are generally regarded as insufficient to tackle FHB and mycotoxins contamination alone [3]. This is mainly due to the fact that the breeding of grains for superior technological properties has led to a decrease of the genetic diversity, with a subsequent increase of susceptibility towards pathogenic diseases.

Therefore, the study of the plant response to fungal infection is crucial for developing possible strategies to counteract mycotoxin accumulation.

From a biological point of view, the role of mycotoxins in fungal colonization is still to be clarified. Some of them – such as deoxynivalenol (DON) have been proved to be virulence factor for fungal infection (7). However, the intense cross-talk among plant and pathogen affects the biological cascade, from genes to metabolites, and plays a significant role in mycotoxin accumulation. Fungal infection and mycotoxin contamination are commonly addressed with classical methods, from DNA-based techniques for fungal identification to analytical methodologies for mycotoxin detection. The residual DNA content of fungal pathogens was used to identify unequivocally fungal species, and they were associated with cereals and their mycotoxins [8], basically allowing for a toxigenic fungi monitoring. However, the main disadvantage of this technique is associated to relatively high cost and the fact that it is time-consuming. In addition, a poor correlation between fungal growth and mycotoxin accumulation has been pointed out.

For this reason, classical chromatographic methods are often used for mycotoxin determination in crops and products thereof [9-11]. Over the last decade, mass spectrometry-based methods have become the golden standard for mycotoxin analysis, being the multitoxin approach the most promising strategy to control the occurrence of multiple analytes in the same material [12]. As a complementation, rapid diagnostic methods are commonly based on immunochemical assays (i.e. lateral flow devices, dipsticks, etc.) for early detection at pre- and post-harvest [13]. More recently, non-destructive imaging methods have been proposed as well as rapid diagnostic tool [14]. In this context, the untargeted methodologies have started to be applied only recently, and only to meet specific needs. In particular, the –omics strategies have been applied to the mycotoxin issue to investigate the interaction between the plant and the pathogen in field, leading to mycotoxin accumulation [3, 15-19].

In a top-down view, genomics and transcriptomics studies have proposed to investigate the biosynthetic pathways for mycotoxin production, and their regulation upon biotic and abiotic stress. Similarly, proteomics has been often proposed for identifying enzymes and proteins responsive to pathogenic diseases, such as FHB [20], or responsible for mycotoxin modification in plant [21,22]. Over the last decade, however, the field of metabolomics has gained increasing interest across all disciplines, and has found a prominent role in mycotoxin related studies as well. Metabolomics is an emerging technique that can be considered complementary to the other ‘omics approaches and highlighting unique advantages. A metabolic fingerprint may generate thousands of data points, of which only a handful might be needed to describe the problem adequately [23, 24]. Extracting the

most meaningful elements of these data is thus key to generating useful new knowledge with mechanistic or explanatory power.

To date, however, in the vast majority of cases, mycotoxin contamination has been directionally explored. In this way, up to now, the mycotoxin contamination loop has not been properly closed and many issues are still open. One of the main challenges in mycotoxin analysis will be to improve our limited understanding of the roles of plant pathogen cross-talk at the molecular level. In this context, a multiomics global strategy may be able to identify chemical markers at the earliest stage, and to univocally characterize resistant varieties and the early detection of mycotoxins. The early detection of toxigenic fungi or of markers of the interaction between the pathogen and its host can be usefully exploited to limiting the enter of mycotoxins into the food/feed production chain.

2. Advanced analytical tools merged with chemometrics

The **multiomics** approach has been poorly compared to classical approaches described in the previous section. Initially, innovative spectral techniques (i.e. imaging analysis, near-infrared, Raman) have been proposed for the early detection of fungal pathogens [25, 26]. Since fungal growth is not strictly related to mycotoxin accumulation, and to the pattern of occurring mycotoxins, these techniques cannot provide a response on mycotoxin occurrence or chemical markers, mainly linked to the plant-pathogen interactions. In this framework, metabolomics may represent the golden tool for understanding the biological pathways involved in mechanisms of plant resistance. Nowadays, gas chromatography (GC) and liquid chromatography (LC), are commonly used for metabolomics approaches, mainly coupled to mass spectrometry (MS) [3]. In principle, LC-MS and GC-MS provide a high number of scans per peak, allowing peak picking and alignment (feature extraction), and if necessary quantification, as well as a large dynamic range in order to monitor low and high concentration levels of metabolites.

2.1. Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

LC-MS has been the most commonly used metabolic fingerprinting/profiling approach for understanding plant resistance mechanisms and the plant-pathogen cross-talk. For instance, Cajka et al. [27] have recently developed an analytical procedure based on the optimization of a solid-liquid extraction procedure using methanol/water (50:50, v/v), in order to isolate polar/medium-polar barley metabolites followed by ultra high performance liquid chromatography quadrupole-time-of-flight (UHPLC-QTOF) [27]. *Figure 1* shows unique and shared metabolites acquired by UHPLC-QTOF using both positive and negative ionization modes.

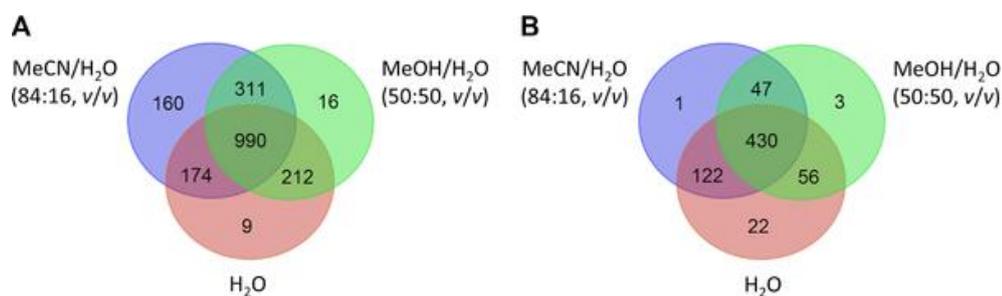


Figure 1. Venn diagrams illustrating shared and unique features in barley extracts prepared under the different extraction procedures and analysed by both positive (A) and negative (B) ionization modes UHPLC-QTOF.

The authors demonstrated how the carefully in-depth investigation of sample preparation could support the extraction of the broadest spectrum of metabolites isolated from the matrix, in this particular case barley. Obviously, UHPLC-QTOF chemical fingerprints differed significantly depending on the extraction solvent used (see *Figure 2*). For example, when deionized water was used a lower extraction efficiency of less polar compounds was exhibited. Nevertheless, sample preparation using a mixture of acetonitrile/water (84:16, v/v) or methanol/water (50:50, v/v) enhanced the extraction of less polar and polar compounds were also detected. The authors, as a compromise, chose methanol/water (50:50, v/v), since the extraction mixture permitted isolation of both highly polar and less polar metabolites. So far, various proportion of aqueous methanol as been mainly applied, as it can be seen in *Table 1*. In this way, the changes occurring both in primary carbohydrates and primary nitrogen metabolism upon plant infection has been partially elucidated. On the other hand, lipidomic approaches, applying more non-polar solvent (e.g. hexane, dichloromethane, ethyl acetate) have been exclusively used to investigate the plant-pathogen cross-talk in maize [28-30]. Increasing evidence indicates, indeed, that lipid signalling is an integrated part of the complex regulatory network in plant pathogen cross-talk.

Not only fingerprinting approaches, but also metabolic profiling strategy has been recently performed using a stable isotopic labelling approach in order to understand the metabolic fate of HT-2 toxin and T-2 toxin in wheat [31]. In general, untargeted metabolomics approaches are usually based on generic settings for sample preparation (which usually include a simple extraction without any purification step, or non-sample preparation), separation and detection. By contrast, if a particular group of metabolites is preselected, a metabolic profiling is carried out. Thereby, a more specific extraction procedure and chromatographic separation/detection has to be performed. In this way, this study was focused on Type A trichothecenes, such as HT2 and T2 toxins, and their detoxification pathways.

The stable isotopic labelling approach applied is really innovative since monitoring pairs of corresponding nonlabeled and labeled precursor allowed metabolome to be easier monitored and interpreted, providing further information. Liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) spectra of the observed metabolites of HT2 and T2 were compared with those obtained in wheat and were shown to be identical. *Figure 2* shows overlaid extracted ion chromatograms of all detected biotransformation products. In this frame, the authors demonstrated that the exposure of wheat to either HT2 or T2 toxins primarily activates biotransformations involving hydroxylation, (de)acetylation, and various conjugations. Furthermore, kinetic data revealed that detoxification progressed rapidly, resulting in the almost complete degradation of the toxins, within 1 week, after a single exposure.

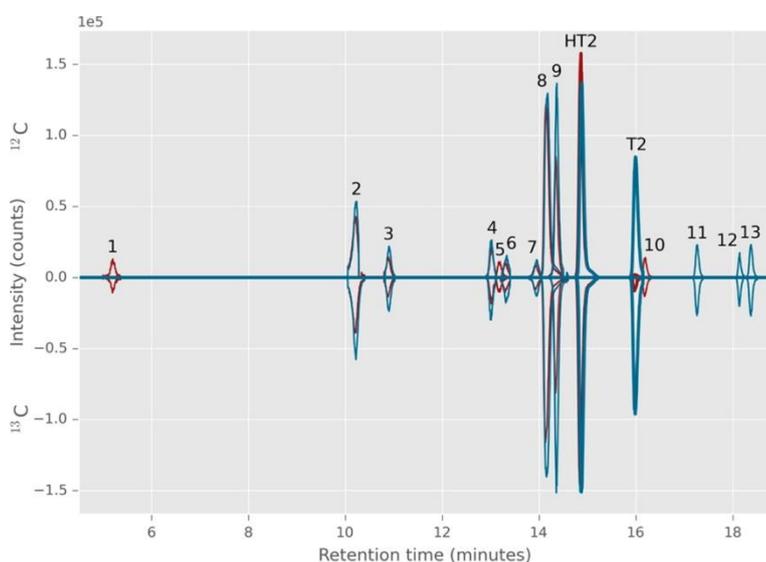


Figure 2. Overlaid extracted ion chromatograms (EICs) based on MetExtract data processing output showing the biotransformation products of a sample treated with a mixture of $^{12}\text{C}/^{13}\text{C}$ -HT-2 toxin (red trace) and one treated with a mixture of $^{12}\text{C}/^{13}\text{C}$ T-2 toxin (blue trace). EICs of nonlabeled metabolites were displayed with positive intensities; those of the corresponding labeled metabolites were displayed as negative intensities.

2.2. Gas Chromatography - Mass Spectrometry (GC-MS)

Surprisingly, GC coupled to high-resolution mass spectrometry (HRMS) has not been applied to *mycomics* strategies. As it was discussed above, the applicability of HRMS permitted metabolic pathways to be clearly described. Nevertheless, GC coupled to a single quadrupole shows selectivity and specificity for metabolomics approaches, since available databases containing mass spectra and retention indexes can tentatively identify metabolites due to the extensive and reproducible fragmentation pattern obtained in full-scan mode using electron ionisation (EI). A recent research work was focused on the applicability of GC–EI-MS in order to understand

deoxynivalenol (DON) accumulation in wheat [15]. In this research, the experimental design was nicely described, and similar to previous research describe above. Nevertheless, sample preparation took extra time compared to LC-MS, due to derivatization procedure based on silytation. Many metabolites contain polar functional groups and are thermally labile for separation by GC or present limited volatility, therefore, derivatisation often has to be applied. Oximation or silytation have been commonly applied due to their universality and versatility [24].

2.3. Data processing to extract meaningful markers

For processing massive information based on separation techniques and mass spectrometry, effective software tools capable of rapid data mining procedures have to be used. Note that data matrices contain thousands of variables (m/z , RT, intensity), and they have to be converted into more manageable information [24].

Data processing and data pre-treatment must be carried out in order to permit the identification of significant metabolites, which capture the bulk of variation between different datasets and may therefore potentially serve as biomarkers. Data processing usually involves four basic steps: deconvolution, alignment, filtering and gap filling. The features, defined by their m/z and retention time, and their intensities in different samples are used for the statistical analysis. Samples would be grouped and it can be observed using scores plots, heatmaps or hierarchical clustering. After data pretreatment, a statistical comparison can be performed using the multivariate (MVDA) data analysis. Usually this step involves unsupervised models (PCA) and supervised classification tools, such as PLS-DA and OPLS-DA. These supervised methods are performed to maximize differences between groups and to highlight potential biomarkers. When the experimental design is more complex, the use of t -test or other univariate data analysis (UVDA) tools represents the best choice [32].

Table 1. Putative metabolites involved in *Fusarium* Head Blight resistance, reported in the literature so far.

Instrumentation	Extraction	Plant	Chemical group	Markers class	Reference
LC-HRMS	water/methanol (45:65; v/v)	Barley	Fatty acids; flavonoid phenylpropanoids; amino acids; terpenoids; organic acids	RRC, PRr, PRs, RI	Bollina et al. (2010)
	water/methanol (50:50; v/v)	Barley	Fatty acids; flavonoid phenylpropanoids	RRI, RRC, RI	Bollina et al. (2011)
	water/methanol (50:50; v/v)	Barley	Fatty acids; phenylpropanoids	RR, RI	Cajka et al. (2014)
	water/methanol (40:60; v/v)	Wheat	Fatty acids; phenylpropanoids; terpenoids	RRI, RRC, RI, PRp	Guannaiah et al. (2012)
	water/methanol (40:60; v/v)	Wheat	Flavonoid phenylpropanoids; terpenoids; amino acids; carbohydrates	RRC, RRI, RI	Guannaiah et al. (2014)
GC-MS	water/methanol/formic acid (74:25:1; v/v)	Wheat	Poly-amines; amino acids; phenylpropanoids; carbohydrates	RR	Warth et al. (2015)
	water/methanol/formic acid (74:25:1; v/v)	Wheat	Amino acids; amines; carbohydrates	RR	Nussbaumer et al. (2015)
	water/methanol (50:50; v/v)	Wheat	Poly-amines; amino acids; phenylpropanoids; carbohydrates	RR	Paranidharan et al. (2008)
H NMR	methanol/water (40:60; v/v)	Wheat	Amines; amino acids, carbohydrates	RR, PR	Browne et al. (2007)
	methanol/water (40:60; v/v)	Wheat	Amines; amino acids, carbohydrates; phenylpropanoids	RR, PR	Cuperlovic-Culf et al. (2016)

RRC: resistance-related constitutive; RRI: resistance-related induced; RI: resistance indicator; PR: pathogenesis-related; PRr: pathogenesis-related resistant; PRs: pathogenesis-related susceptible; PRp: pathogenesis-related proteins

3. Metabolomics to decipher pathways involved in *Fusarium* Head Blight resistance

As it was already mentioned in the *Introduction*, mycotoxins and fungal pathogens, such as *Fusarium graminearum*, can cause global diseases of wheat (*Triticum aestivum*L.) and barley [6]. Nevertheless, up to now, all preventives techniques used have been pointless, such as fungicides or crop rotation. Breeding strategies for increasing pathogen resistance seem to be the most promising and environmentally safe strategy for controlling mycotoxin accumulation in grains. It is known, indeed, that plant resistance mechanisms may be controlled by several quantitative trait loci (QTLs) that contribute to overall pathogen resistance in three different ways classified as type 1, 2 and 3, and referred as resistance to initial infection of spikelets, spread of pathogen within spikes and accumulation of mycotoxins, respectively. The involved QTLs typically are linked to, or contain, the genes that control the phenotype. Over hundred of QTLs for FHB resistance in wheat have been already identified [3, 7, 15, 32]. However, fully resistant varieties are still to be identified or inbred. Thus, there is an urgent need to better understand the mechanisms of resistance against *Fusarium spp* in order to develop novel strategies and resistance varieties.

Nowadays, recent advances in metabolomics offer new opportunities to elucidate complex metabolic pathways involved in *Fusarium* resistance and potential FHB resistance biomarker metabolites in barley and wheat [3, 15-19, 32]. In fact, during the last decade, the applicability of metabolomics has significantly increased in this field. Nevertheless, knowledge remains still partial, and a long way have to be covered towards the development and understanding plant-pathogen interactions. This new scenario will provide a suitable knowledge related to plant metabolome, which was already explained by a few examples in the previous section.

Different strategies have been applied so far, NMR for polar metabolites [33,34], LC-QTOF for semi-polar metabolites [16-19, 32] and GC-MS for volatile compounds [15, 35-36]. However, we should keep in mind that a strategy able to simultaneously extract and detect the entire metabolome does not exist. Consequently, the data delivered by metabolomics studies only cover a fraction of the metabolome. In other words, the picture taken exclusively reveals one part of the metabolome. In addition, the resistance mechanism is a result of multi-interactions between biomolecules such as genes, proteins, metabolites and environmental factors. Therefore, a multiomics approach based on proteomics and metabolomics could overcome any limitation in the experimental design. For example, an integrated non-targeted metabolo-proteomics approach was recently published [18, 32]. This strategy demonstrated to be a powerful tool for a more comprehensive analysis in order to elucidate the mechanism, revealing successfully changes in the wheat primary metabolism, in response to *F. graminearum*.

4. Setting up of the experimental plan

Depending on the hypotheses to be tested, different combinations of plants and fungal pathogens can be employed to explore the system relationship. Up to date, the metabolomic approaches have been mainly restricted to study the resistance against *F. graminearum* and *F. culmorum* in wheat and barley [3]. Resistance mechanisms have been elucidated by using wheat/barley genotypes with various levels of resistance, classified as susceptible, intermediate and resistant. However, in most of the studies, unrelated germplasms are compared, leading to a confusing interpretation of the data delivered, since the differences in the metabolic profiles may actually result from the cultivar background [3]. Thus, the use of near isogenic lines (NILs) that differ in QTL conditioning FHB, is suggested to be the best approach to simplify the complexity, and allow to reach conclusive evidence related to resistance functions [18].

As for the comparison, mock-inoculated versus pathogen-inoculated plants is considered the best approach to highlight differences. Gunnaiah (et al. 2014) [19] instead, designed a different experiment in order to elucidate the host biochemical resistance to FHB spread in response to trichothecene producing and non-producing isolates of *F. graminearum*. The two *F. graminearum* strains differed in the loss of function of Tri5 gene [19]. In addition to *F. graminearum* inoculation, Warth et al. [15] also used DON injection into the middle florets of spikelets to decipher the mechanism of plant resistance to the toxin. Experiments have been performed in field conditions [27], under greenhouse [16-18, 33, 36] with computer-controlled settings for light, temperature and relative air humidity [15] and more recently, in environmental controlled growth chamber [34]. All these approaches are summarized in Table 1 together with the extraction and detection methodologies applied, the plants used and the main classes of metabolites identified by the authors so far.

5. Elucidating FHB resistance mechanisms by metabolomics

Plant resistance to *Fusarium Head Blight* and related mycotoxin accumulation has been described through five major types of mechanism, mainly described for wheat and further applied to other cereals. These mechanisms are often host-specific, thus requiring plant-specific elucidation studies. Type I resistance is related to initial infection of the floret in wheat and barley, and of the silk in maize [37]. The spreading of infection is then limited by type II and type III resistance. Type IV resistance is related with tolerance and ability to maintain yields, and type V resistance gathers all mechanisms of resistance to mycotoxin accumulation [38-40]. According to Boutigny et al. (2008) [41], type V-1 represents resistance to toxin accumulation operated by metabolic biotransformation [42, 43], while type V-2 corresponds to resistance due to the inhibitory effect of mycotoxin

biosynthesis exerted by plant endogenous compounds. Metabolomics has been exploited so far in this field for the comparison of metabolite composition of resistant and susceptible varieties upon *Fusarium* infection, allowing for the definition of a large set of compounds potentially involved in FHB modulation [3, 15-19]. Among those, fatty acids and compounds thereof have been found to be involved in the plant-pathogen signalling system, while terpenoids and phenylpropanoids take part to cell wall reinforcement, show antifungal properties, and may interfere with mycotoxin biosynthesis [3]. Generally, the workflow of markers identification comprises the following steps: (1) marker identification based on accurate mass (MS), isotopic pattern and MS/MS pathway, (2) off- or online database searching and (3) data interpretation. These markers can be tentatively identified without analytical standards, or unambiguously identified using analytical standards. The identification of markers usually represents the last step within metabolomics studies. This is crucial in order to understand the metabolite pathway, since they can be interesting intermediates or final secondary metabolites. In this particular topic of mycotoxin contamination, hundreds of metabolites related to FHB resistance have been putatively identified so far by metabolomics strategies [4]. It was already mentioned the number and chemical structures of metabolites significantly varies according to the experimental design and the applied analytical strategy.

Biomarker metabolites of resistance can be further sub-classified, according to their function. Those metabolites, whose abundance was increased in both resistant and susceptible cultivars, following pathogen inoculation as compared with those inoculated with water, were referred as pathogenesis-related (PR) metabolites [44]. Accordingly, metabolites that were significantly higher in resistant cultivars than in susceptible one, were designated as resistance-related (RR) metabolites.

Among RR metabolites, some of them have been demonstrated as constitutive, while other are induced upon fungal infection [16, 17]. Among them, resistance-indicator metabolites [3, 16, 17] include modified mycotoxins such as DON, DON-3Glc and the other DON-biotransformation products. Following wheat inoculation by *Fusarium*, DON is spread within spike, and the host counteracts mycotoxins by conjugating them to endogenous metabolites (i.e. by glucosylation, acylation, conjugation to amino acids and glutathione). Thus, all the modified forms are design as resistance indicators, since they indicates that the plant is reacting against the infection also by converting mycotoxins into their less toxic forms. According to the literature [19, 32], the chemical defense against fungal pathogens including DON producing *Fusarium* species is linked to three main mechanisms of resistance: cell wall reinforcement through the deposition of lignin, production of antimicrobial compounds and specific induction of defense signaling pathways. As reported by Gunnaiah et al. [18] among the metabolites reported as involved in plant response to FHB in soft

wheat, the main chemical groups are phenylpropanoids, and terpenoids, followed by amino acids derivatives. On the other hand, when functional properties are considered, the majority of resistance related metabolites showed an antimicrobial activity, followed by cell wall strengthening properties.

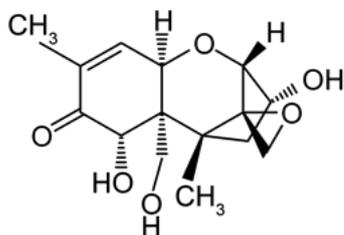


Figure 3. Chemical structures of deoxinivalenol (DON)

Phenylpropanoids such as flavonoids and phenolic acids, have been frequently described for their contribution to plant defense mechanisms. Their activity is exerted either through direct interference with the fungus, or through the reinforcement of plant structural components acting as a mechanical barrier [45, 46]. Flavonoids, especially flavones, flavonones and isoflavonoids, lignans and other phenolic compounds were induced in Sumai-3 as antimicrobial agents, following *F. graminearum* inoculation. This is mainly due to their antioxidant activity leading to the neutralization of ROS, produced under biotic stress. A similar profile was identified upon *F. graminearum* inoculation in barley cultivars [16, 47] and in wheat [18]. In addition, phenolic acids have been reported as inhibitory agents towards mycotoxin biosynthesis in vitro [48, 49]. Among phenolic acids, hydroxycinnamic acid (HCA) derivatives, such as ferulic and caffeic acids, have been reported as important contributors to FHB resistance [4], probably on account of the high antioxidant properties [50].

Among HCAs, chlorogenic acid has been reported as a potential resistance factor in different pathosystems [49, 51, 52]. Concerning the cell wall reinforcement, hydroxycinnamic acid amides (HCAAs) are deposited as cell wall appositions at the inner side of plant cell walls after cross-link with polysaccharides, lignin and suberin [27]. These HCAAs are synthesized by condensation of hydroxycinnamoyl-CoA thioesters with aromatic amines (e.g. spermidine, spermine, tyramine) originated from aromatic amino acids. Thus, the involvement of amino acids in resistance to *Fusarium* may also be related to their role as a precursor of cell wall-bound HCAAs. Among those identified so far, N-caffeoylputrescine, 4-coumaroyl-3-hydroxyagmatine and feruloyl-serotonin are significantly up-regulated upon *F. graminearum* infection in the resistant cultivar Sumai-3 [27]. With regards to the differences in terpenoid profile, Sumai-3 was characterized by an

higher amount of syringyl lignin precursors like sinapoyl alcohol and sinapaldehyde, and glucose conjugate of sinapoyl alcohol, syringing [27]. Lignin results from monolignol glucosides polymerizations and lead to a reinforced cell wall that is more resistant to fungal cell wall degradation enzymes [4].

Moreover, changes in the cell wall polysaccharides following infection were described by Cuperlovic-Culf et al. [24]. Large increase in concentration of sugars and inositols were found in all wheat varieties, particularly for Sumai-3, indicating an attempt at creation of cell wall barrier for *F. graminearum* penetration. In addition, fatty acids were also suggested to participate in resistance as physical barrier to pathogen ingress through their role in cuticle formation [4]. As far as the involvement of resistance related metabolites – mainly lipids - in the plant signaling pathways, significant results are summarized in the last part of this review.

6. The role of lipids in the plant-pathogen cross-talk

Increasing evidence indicates that lipid signalling is an integral part of the complex regulatory network in plant response to stress/infection. Modifications of membrane lipids produce different classes of signalling messengers, such as phosphatidic acid (PA), diacylglycerol (DAG), DAG pyrophosphate (DAGPP), lysophospholipids, free fatty acids (FFAs), oxylipins, phosphoinositides and inositol polyphosphates. Lipidomic approaches were developed to investigate in depth the plant-pathogen cross-talk, demonstrating a close relationship between the modification of the pathogen oxylipin profile with the mycotoxin synthesis [28].

Among metabolites associated with fatty acid metabolic pathways, a number of compounds have been identified for their potential contribution to cereal resistance towards FHB [53]. Fatty acids and their derivatives play significant role in plant defense against pathogens. Among their functions, they contribute to basal immunity, gene-mediated and systemic acquired resistance in plants. In addition, fatty acids are involved in the plant defense signalling pathway, through the formation of important mediators such as oxylipins and jasmonates. The unsaturated C18:1, C18:2 and C18:3 fatty acids, namely oleic, linoleic and linolenic acid, are often described as involved into defense mechanisms against fungal pathogens [47, 54, 55] and able to modulate mycotoxin production [55, 56]. The antimicrobial activity is probably due to their role in modulating ROS production, and in cuticle formation, which constitutes a physical barrier to pathogen infection [57]. In addition, they are precursors of the plant oxylipin pathway, which moves from the enzymatic formation of hydroperoxides, carried out by lipoxygenase (LOX) [58]. Distinct LOX isoforms, referred as 9-LOX and 13-LOX, preferentially add an hydroxyl moiety at C9 or C13 position of the fatty acid backbone, leading therefore to 9- and 13-hydroperoxides, respectively. These compounds

act then as substrates for the two distinct biosynthetic cascades, with the formation of approximately 150 known oxylipins including hydroxy-, oxo-, or keto-fatty acids, green leaf volatiles (GLV), and jasmonic acid (JA) [59]. Jasmonates originate from 13-LOXs products, while 9-LOXs products lead to less known metabolites known as defense factors in response to fungal attack [60]. Jasmonic acid and methyl jasmonate are well known for their roles as plant stress hormones. They cause programmed cell death activation, the production of ROS, and the deposit of wax layers on plant tissues [61]. Jasmonates play, in addition, an active role in the regulation of the phenylpropanoids pathway [62], exhibit antimicrobial properties towards toxigenic fungi [47, 60], and modulate mycotoxin accumulation [63, 64].

Besides these functions, jasmonates were proved to activate glucosyltransferase in *Arabidopsis thaliana* and barley [65]. This is a key enzyme activity involved in a DON detoxification pathway that transforms DON into less phytotoxic DON-3-Glc. Several metabolomic studies have highlighted the involvement of jasmonic acid [15 – 19, 33] in resistance to DON-producing *Fusarium* species. While the physiological function of jasmonates has been well describe over the last years, little is known about other 9-LOX derived compounds. Recent studies demonstrated that 9-oxylipins contribute to maize susceptibility or resistance to fungal pathogens, in a pathosystem-dependent way [61]. Several studies, indeed, suggested that mycotoxin accumulation is modulated by host oxylipins. In particular, linoleic acid and 9-oxylipins seem to be conserved signal molecules modulating mycotoxin biosynthesis, fungal sporulation and other aspects of fungal differentiation processes [54]. The effects of mutation of LOX gene were often studied in maize, observing that inactivation of the 9-LOX gene led to an increased susceptibility of maize to *Aspergillus flavus*, *A. nidulans* and *F. verticillioides* [66 – 68]. Similarly, modification of LOX genes lead to a modulation of fumonisin production in the maize – *F. verticillioides* pathosystem [69, 70]. The deep involvement of oxylipins in the intense cross-talk between host and pathogen have still to be clarified. Endogenous fungal oxylipins are known indeed for supporting host colonization, as well as mycotoxin biosynthesis. Some authors suggest the possible interaction between fungal oxylipins and plant GPCRs, transmembrane-proteins or receptor-like kinases, for host manipulation.

7. Conclusions

A metabolomics approach may support the quick growth of this relatively new field of research, allowing for a better understanding of the changes occurring in the plant and pathogen metabolites upon interaction. In principle, analytical methods developed have demonstrated significant advances in sensitivity, robustness, flexibility and discrimination power in order to build

successfully statistical models, and subsequent marker identification. Increasing evidence indicates that lipid signaling is an integral part of the complex regulatory network in plant response to stress/infection. Modifications of membrane lipids produce different classes of signaling messengers, such as phosphatidic acid, diacylglycerol pyrophosphate, lysophospholipids, free fatty acids, oxylipins, phosphoinositides and inositol polyphosphates. Lipidomic approaches can be developed to investigate in depth the plant-pathogen cross-talk, demonstrating a close relationship between the modification of the pathogen oxylipin profile with the mycotoxin synthesis. Therefore, metabolomics approaches will provide new solutions to old problems. In fact, the early detection of mycotoxins and smart detoxifications can be performed by metabolomics strategies for the first time, and these approaches can fill the gap in order to answer these questions and go a step further.

8. References

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2. AIMS OF THE THESIS

The **general objective** of this thesis was to investigate wheat integrity.

The **specific aims** of this thesis:

- **The assessment of wheat safety** by applying rapid immunochemical and sophisticated mass spectrometric multi-analytes methods (Section I, Chapter 1, 2, 3, 4).
- **The investigation of wheat quality**, focusing on different phenolic compounds among ancient and modern wheat varieties (Section II, Chapter 5 and 6).
- **The discrimination between common and durum wheat species** for an authenticity purpose (Section III, Chapter 7).

3. EXPERIMENTAL STUDIES

SECTION I: Wheat safety

One of the most relevant wheat safety aspects is related to its contamination with fungal pathogens, especially those of the *Fusarium* genera, which are closely connected with the production of secondary fungal metabolites, also called mycotoxins. These compounds elicit a wide spectrum of toxicological effects, representing a health risk for both humans and animals. The most worrying wheat disease is represented by *Fusarium* head blight (FHB) [1], which can cause the destruction of an entire wheat crop within a few weeks and yield losses may reach 50-60%, reducing nutritive and technological quality of the grain. It has been estimated that 10-30% of the harvested cereal grains are lost due to the fungal infection, whereas 25-50% of harvested crops have been contaminated with mycotoxins annually [2]. The widespread occurrence of these mycotoxins, as well as the presence of other food contaminants has been of increasing concern for consumers and producers, in part due to changes in eating habits, consumer behaviour, and the increased industrialization and globalization of food-supply chains. These changes have led to an increased demand for high standards in quality assurance and process control. In fact, the high demand requires appropriate analytical tools for food analysis. Rapid, sensitive, and accurate analytical methods for determination of these mycotoxins in unprocessed cereals and cereal-based products are highly required in order to properly assess both the relevant risk of exposure and the relevant toxicological risk for humans and animals, as well as to ensure that regulatory levels fixed by the EU [3-5] or other international organizations are met.

In order to deal with the increasing number of food matrices and contaminants to be monitored, two analytical approaches have become increasingly prevalent [6]. The first has been the development of rapid screening methods for a variety of analytes based on immunochemical techniques, such as ELISA or lateral flow devices. The second is the development of highly sophisticated multi-analyte methods based on liquid chromatography coupled with high resolution mass spectrometry for identification and simultaneous quantification of a wide range of contaminants. Rapid screening methods based on ELISA system are user friendly [7], enable testing of large numbers of samples and thus potentially represent a powerful tool for point-of sampling in all parts of the food processing chain providing semi-quantitative results. By contrast, the multi-contaminant mass spectrometric methods require highly skilled laboratory staff and enabling quantification with confirmation of the analytes of interest is spreading rapidly as a promising

SECTION I: Wheat safety

technique for simultaneous screening, identification and quantitative determination of a large number of mycotoxins [8].

Both approaches are useful when gathering surveillance data to determine the overall amount of both legislated and newly identified mycotoxins in cereals in order to estimate human daily intake for health risk assessment. Taking advantage of the improvement offered by high resolution mass spectrometry, also the untargeted methodologies have started to be applied recently. In particular, the omics strategies have been exploited to the mycotoxin issue to investigate the interaction between plant and pathogen [9] or to select resistant wheat varieties [10]. The increasing number of successful metabolomic approaches suggests that this new omics could soon play a major role in many aspects of wheat safety.

In the following section different analytical strategies to control mycotoxins occurrence, have been applied, starting from immunochemical method, continuing with innovative high resolution mass spectrometric application, including an untargeted metabolomics approach, and ending with a pioneering ion mobility spectrometry application. Therefore, chapter one will be focused on the development and validation of a multi-DON screening ELISA method by using wheat and maize naturally contaminated samples. Then, zearalenon biotransformation products were investigated by developing an *in vitro* plant model system; a targeted HRMS multi-mycotoxins screening method was applied and described in chapter two. Chapter three will report the development of a new untargeted metabolomic approach that could serve as an effective tool for the early detection of mycotoxins, and *Fusarium* disease prevention. Finally, in chapter four preliminary results obtained by applying drift tube ion mobility spectrometry (DT-IMS) to characterize new modified mycotoxins forms were described.

The first and third chapters were accepted for publication in Food Additives and Contaminants and Food Chemistry, respectively. On the other hand, the second chapter was submitted to Scientific Report and the fourth chapter describes preliminary data for further investigation. For additional details see section “Author”.

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Chapter 1
**Group detection of DON and metabolites
by an ELISA kit**

Group detection of DON and its modified forms by an ELISA kit

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Abstract.

Deoxynivalenol (DON) and its modified forms (3-, and 15-Acetyl-DON, DON-3-glucoside) are commonly analysed by chromatographic methods. Indeed, coupled with proper extraction and clean-up, LC-MS represents the best approach for multiresidual measurement of these mycotoxins. On the other hand, immunochemistry-based methods are possibly able to detect a family of structurally related compounds, although the determination of single contributions is not possible so far. However, ELISA methods often lead to an apparent overestimation of the mycotoxins content, because modified forms and matrix components can potentially cross-react with the antibodies (designed for the parent toxin).

Several data about the possible cross-reactivity of commercial DON-detecting ELISA kit are reported in the literature so far. Data are commonly obtained in buffer solutions or in matrix matched solutions, but comparison on a set of naturally incurred samples has never been reported.

In the present work the accuracy of a commercial DON-detecting ELISA kit was evaluated on naturally incurred soft wheat ($n = 15$) and maize ($n = 15$), taking into account the matrix effect.

Recovery was calculated considering the DON concentration found by LC-MS/MS and the total DON concentration, expressed as the sum of DON and its modified forms found by LC-MS/MS.

The obtained data clearly show that, when 3-modified forms of DON occur in the sample, the ELISA kit does actually detect them, thus returning an apparent overestimation if only DON content is considered. When the ELISA recovery is calculated on the total DON content, the accuracy of the analysis increases and the variability decreases.

According to our data, the ELISA kit seems to be a promising group detection tool for the accurate evaluation of DON and its modified forms, expressed as sum of DON, DON-3Glc and 3Ac-DON, for soft wheat and maize samples.

Abbreviations: DON, deoxynivalenol; 3-AcDON, 3-acetyl-deoxynivalenol; 15-AcDON, 15-acetyl-deoxynivalenol; DON-3Glc, deoxynivalenol-3-glucoside; ELISA, enzyme-linked immunosorbent assay

Introduction

Mycotoxins are secondary metabolites produced under field conditions by many species of *Fusarium*. Among them, Deoxynivalenol (DON), together with the other trichothecenes, is one of the major contaminants of cereals (e.g. corn, wheat, barley) and cereal-based products.

These toxins have been shown a variety of toxic effects in both animals and humans, including those gastrointestinal (vomiting, bowel inflammation) skin irritation, leukopenia and bone marrow aplasia (Eriksen and Petterson, 2004; Pestka et al. 2004).

DON also occurs in chemically modified forms after plants, animals and fungi metabolization. Up to date natural occurrence of its acetyl-derivatives, 3-acetyl-deoxynivalenol (3Ac-DON), 15-acetyl-deoxynivalenol (15Ac-DON) and glucoside-conjugate deoxynivalenol-3-glucoside (DON-3Glc) has been reported (Berthiller et al. 2013; De Boevre et al. 2013; Malachova et al. 2011). Chemical structures are reported in **Figure 1**.

In the last few years the problem of these modified forms has become more and more prominent since their presence could increase the total amount of toxins in food. This behaviour could be due to the possible release of the parent toxin during digestion (Dall'Erta et al. 2013; De Boevre et al. 2015).

Due to the frequent occurrence of DON in food and feed, guidance values were recommended at European level; through the Commission Recommendations 2006/1881/EC and 2007/1123/EC, the EU set as DON maximum levels (MLs) 1.250 mg kg⁻¹ and 0.750 mg kg⁻¹ in unprocessed cereals and food respectively. Additionally in 2010 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated the Provisional Maximum Tolerable Daily Intake (PMTDI) levels of 1 µg kg⁻¹ body weight for the sum of DON and its 3- and 15-acetyl-derivatives. In the same evaluation, the JECFA established a group acute reference dose (ARfD) of 8 µg kg⁻¹ b.w. for the sum of DON and its acetyl-derivatives. Because of lack of additional occurrence and toxicological data, DON-3Glc has not been covered into these limits.

In order to monitor and control the contamination levels of these toxins in cereals and feed, having rapid, easy-to-use, robust and reliable tools is essential. Due to their relative low cost and easy application, immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA), are widely used to quantify DON, especially during routine screening of large amount of samples (Berthiller et al. 2013; Zachariasova et al. 2014).

ELISAs are based on an antigen-antibody interaction and each antibody employed has its own pattern of cross-reactivities.. This means that the antibody could be able to bind both parental and modified mycotoxins with different degrees of affinity. The capability of the binding reagents to detect both parental and masked mycotoxins could lead to an ostensible overestimation when

screening results are compared to instrumental confirmatory analysis when native DON only is measured.

Cross-reactivities of DON antibodies and test kits are namely claimed by many commercial kits producers and have been investigated by several authors (Zachariasova et al. 2008; Tangni et al. 2010; Dzuman et al. 2013). Anti-DON antibodies showed strong affinities for DON-3Glc and 3Ac-DON, with cross-reactivity values ranging between 28-116% and 40-770% respectively. The most highest cross-reactivity was observed by the *AgraQuant*[®] kit (Romer Labs). On this regard, different studies demonstrated a great variability in the antigenic cross-reactivity with DON-3Glc and 3Ac-DON measured values of 45 and 392%, (Zachariasova et al. 2008), 52 and 770% (Tangni et al. 2010) 116 and 478% (Dzuman et al., 2013).

Cross-reactivity seems to be strongly dependent not only on the type of antibody used by the particular manufacturer (Tangni et al. 2010) but especially on the respective sample matrix. In the early study significant DON overestimation by ELISA kits was recognized to be associated with unspecified matrix components (Zachariasova et al. 2008). The hypothesis was demonstrated comparing ELISA response of two wheat samples just extracted and cleaned up on DON-dedicated immunoaffinity cartridges. At a later stage, Duzman et al. (2013) investigated the overestimation among different matrices, and in particular barley, malt and wheat; the last one gives the lowest overestimation (136 ± 3 %).

Taking into account that many laboratories are using ELISA kits to quantify mycotoxins in different matrices, further matrix-related research should be undertaken to improve a better knowledge of the method and a proper data management.

The purpose of this study was to evaluate one commercial enzyme-linked immunosorbent kit performances (Tecna *Celer*[®] DON v3, product code MD100), checking the contribution of DON-3Glc and acetylated derivative (3-Ac-DON) to the estimated concentration value.

Materials and methods

Chemicals

Analytical standards of DON (solution in acetonitrile $100 \mu\text{g mL}^{-1}$), 3-AcDON (solution in acetonitrile $1000 \mu\text{g mL}^{-1}$) and DON-3Glc (solution in acetonitrile $50.6 \mu\text{g mL}^{-1}$) were purchased from Romer Labs[®] (Tulln, Austria). Standard solutions of DON, 3-AcDON and DON-3Glc were prepared in acetonitrile at a concentration of $1000 \mu\text{g/L}$, were stored in a freezer at $-20 \text{ }^\circ\text{C}$ and brought to room temperature before their use.

HPLC-grade methanol, acetonitrile and acetic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA).

MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA), ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland), and NaCl from VWR International Ltd. (Ballycoolin, Blanchardstown, Dublin, Ireland) were used.

Enzyme-linked immunosorbent assay

ELISA kit

The ELISA test kit object of the investigation was *Celer*[®] DON v3, from Tecna S.r.l. (Trieste, Italy). The test is based on a direct competitive immunoassay. Briefly, the free toxin of the sample and a toxin-HRP conjugate compete for binding the anti-DON antibody that is coated on the microplate. After 10 minutes incubation, all unbound reagents are washed away. A colorless HRP substrate is then added to reveal the presence of bound conjugate: after 10 minutes incubation a blue color develops. The intensity of the color is inversely proportional to the DON content in the sample or standard. An acid solution is added to stop the reaction. The acid solution turns the blue color into yellow and so the absorbance values are measured at 450 nm.

ELISA kits were stored following the instructions recommended by the manufacturers (+2/+8 °C).

ChroMate reader (Awareness, USA) equipped with *ChroMate* Manager software was used for the absorbance reading. Sample concentration was obtained by means of a dedicated *Excel* spreadsheet (Tecna S.r.L., Trieste, Italy). For every determination, the absorbance signal (B) was related to the signal of the zero standard (Bo), thus obtaining the B/Bo (%) relative binding value. Samples B/Bo were interpolated onto the B/Bo vs concentration calibration curve using point-to-point algorithm in order to obtain samples concentration.

Matrices for which the test would be applicable are wheat, wheat products (bran, middlings, DDGS), durum wheat, maize and maize-by DDGS, feed.

ELISA sample preparation

Wheat and maize samples were homogenized by grinding and extracted according to manufacturer's instructions. 5 grams of sample and 1 gram of NaCl were mixed with 25 ml of a 70% methanol solution in distilled water. Samples were shaken vigorously for three minutes (manually or with shaker) and filtered through a Whatman No. 1 filter in order to obtain a clear extract.

Then the assay procedure was strictly followed according to the instruction provided. The measurement was recorded photometrically at 450 nm.

Matrix-matched calibration standards

Wheat blank matrices were previously checked using LC-MS technique. Individual matrix matched calibration standards of DON, 3Ac-DON, and DON-3Glc were prepared by dissolving aliquot portion of standard solution in the blank extract prepared according to the recommended extraction procedure. From the acetonitrile matrix solutions (1000 ng mL⁻¹) 5 diluted matrix solutions were prepared at 0, 8, 50, 250, 1000 ng mL⁻¹.

Cross-reactivity determination

Cross-reactivities were calculated as a ratio (%) between the concentration of 50% binding of DON curve and the same parameter of the cross-reagent curve. Buffer curves for 3Ac-DON and DON-3Glc were related to the buffer DON calibration curve of the test kit. Matrix-matched calibration curves were related to the homologous matrix-matched curve for DON.

U-HPLC-MS/MS analysis

Sample preparation

Samples were prepared according to Malachová et al. procedure (2014) with slight modifications. Briefly, 1 g of ground cereal was stirred for 90 minutes at 200 strokes/min on a shaker with 4 mL of acetonitrile/water (80/20, v/v) mixture acidified with 0.1% of formic acid. An aliquot of the extract was collected and centrifuged for 10 min at 14,000 rpm at room temperature, then 1 mL of supernatant was evaporated to dryness under a gentle stream of nitrogen. Finally, the residues were re-dissolved in 1 mL of water/methanol (80:20 v/v) prior to LC-MS injection.

U-HPLC-MS/MS parameters

UHPLC Dionex Ultimate 3000 separation system coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI) was employed.

For the chromatographic separation, a reversed-phase C18 Kinetex column (Phenomenex, Torrance, CA, USA) with 2.10×100 mm and a particle size of 2.6µm heated to 40 °C was used. 2 µl of sample extract was injected into the system; the flow rate was 0.350 ml/min.

Gradient elution was performed by using 5 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.2% acetic acid. Initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min; after an isocratic step (6 min), eluent B was

increased to 90% in 9 min; after a 3 min isocratic step, the system was re-equilibrated to initial conditions for 3 min. The total run time was 30 min.

MS parameters: the ESI source was operated in negative ionization mode (ESI⁻); spray voltage 3,000 V, capillary temperature at 270 °C, vaporizer temperature was kept at 200 °C, sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. S-Lens RF amplitude value and collision energies (CE) were optimized during infusion of analyte standard solutions (1 mg/kg, in methanol) employing an automatic function of X-calibur software (Thermo Fisher Scientific Inc., San Jose, CA, USA).

Detection was performed using multiple reaction monitoring (MRM) mode and monitoring the [M + CH₃COO]⁻ adducts. The following transitions were measured: DON m/z 355→295 (CE = 13 eV) and m/z 355→265 (CE = 17 eV); DON-3Glc m/z 517→457 (CE = 16 eV) and m/z 517→427 (CE = 23 eV); 3Ac-DON /z 397→337 (CE = 12 eV), m/z 397→307 (CE = 17 eV) and m/z 397→59 (CE = 12 eV).

Matrix-matched calibration curves (calibration range 100–2,500 µg kg⁻¹) were used for target analyte quantification. A good linearity was obtained for all the considered mycotoxins (R² > 0.99).

Results and discussion

Cross-reactivity in buffer and in matrix-matched solutions

The cross reactivity of DON antibody towards target modified forms was preliminary evaluated in buffer solution. Analysis were performed independently in two laboratories and results were compared. While NIV and 15Ac-DON were not detected by the assay (< 4%), both DON-3Glc and 3Ac-DON showed a significant cross-reactivity (68% and >100%, respectively).

This result was expected, considering that in the development of the antibodies the employed modified hapten had the molecular bridge to the protein carrier in the same position 3. The tested metabolites are even more similar to the immunogen than to the main target (DON).

On the other hand, the presence of an acetyl group in C15 instead of a hydroxyl group (15Ac-DON) or the presence of the hydroxyl group in C4 (NIV) determine a much lower affinity and so a very poor cross-reactivity.

Accordingly, the cross-reactivity of 3Ac-DON and DON-3Glc was evaluated in sample matrix, performing thus matrix-matched experiments in soft wheat. Again, DON-3Glc showed a cross-reactivity of 61%, while 3Ac-DON was found again to strongly interact with the antibody, giving a cross reactivity of 340%. Such data showed a non predictable conservation of the cross-reactivities when moving from the buffer environment to the real sample environment.

Accuracy of Celer[®] DON v3 on naturally incurred soft wheat

The accuracy of *Celer*[®] DON v3 was evaluated on naturally incurred soft wheat ($n = 15$). Wheat samples have been harvested in 2013 in Emilia Romagna, Italy. Samples were preliminary analysed by LC-MS/MS, and results are reported in **Table 1**.

Two independent sessions of ELISA analysis were performed. Recovery was calculated considering the DON concentration found by LC-MS/MS and the total DON concentration, expressed as the sum of DON and its modified forms found by LC-MS/MS. Data are reported in **Table 2**.

When samples are blank, the test kit doesn't show a significant matrix effect, thus not leading to any false positive result. When samples are contaminated more than $100 \mu\text{g kg}^{-1}$ of DON or total DON (DON plus metabolites), the assay always detects the contamination, according to the kit producer claim of a $125 \mu\text{g kg}^{-1}$ LOQ for soft wheat.

The data clearly shows that, when 3-modified forms of DON occur in the sample, the ELISA kit does actually detect them, thus returning an apparent overestimation if only DON content is considered. Still, the mean overall recovery could be considered satisfactory ($114 \pm 26\%$ for the first session and $120 \pm 24\%$ for the second), with less than 30% determination overstepping the recovery of 130%.

However, concerning single samples, recovery values may significantly vary between the two analytical section (i.e. #6250, #6103, #6185). The variability is higher when only DON is measured, while it decreases when total DON is considered. A possible explanation can be sought in the recognition mechanism of cross-reactive compounds that could be less accurate compared to main compounds.

When the ELISA recovery is calculated on the total DON content, the accuracy of the analysis increases and the variability decreases.

The recovery data calculated in both sessions considering DON and total DON were statistically compared (t-Student test, $\alpha = 0.05$), and a significant difference between the recovery sets was found.

In addition, while the cross reactivity towards DON-3Glc and 3Ac-DON was confirmed, the recoveries for samples #6167, #6245, #6048, #6022, #6140 containing DON only and #6250 and #6103, containing only DON and 15Ac-DON, were not overestimated. This is in agreement with the neglectable cross-reactivity of 15Ac-DON found in buffer solution.

A further elaboration of recovery data obtained within the two sessions of analysis is reported in **Figure 2**.

The mean recovery as DON was $117 \pm 25\%$ (CV 21%, $n = 24$), while the mean recovery as total DON was $94 \pm 15\%$ (CV 16%, $n = 24$).

According to our data, *Celer*[®] DON v3 seems to be a promising tool for the accurate evaluation of DON and its modified forms, expressed as sum of DON, DON-3Glc and 3Ac-DON, for soft wheat samples.

Accuracy of Celer[®] DON v3 on naturally incurred maize

The accuracy of *Celer*[®] DON v3 was evaluated on naturally incurred maize ($n = 15$). Maize samples for feed production have been harvested in 2014 in Emilia-Romagna, Italy. Samples were preliminary analysed by LC-MS/MS, and results are reported in **Table 3**.

Two independent sessions of ELISA analysis were performed. As already mentioned above, recovery was calculated considering the DON concentration found by LC-MS/MS and the total DON concentration, expressed as the sum of DON and its modified forms found by LC-MS/MS. Table 4 shows the results achieved, which are in line with those obtained analyzing wheat samples. Statistical comparison of recovery data calculated in both session (t-Student test, $\alpha = 0.05$) indicated a significant differences between DON and total DON results ($p < 0.001$). The mean recovery as DON was $109 \pm 40\%$ (CV 37%, $n = 24$), while the mean recovery as total DON was $86 \pm 31\%$ (CV 36%, $n = 24$). Mean recovery for total DON was lower than the one calculated for wheat, with a larger variance. This is could be due to a stronger matrix effect in maize that affects the recognition of cross-reactive compounds.

Sample #22 is contaminated more than $5,000 \mu\text{g kg}^{-1}$ that is maximum measurable concentration of the studied ELISA kit. Sample #22 was first extracted following the instruction and then diluted 5 times in methanol 70%, in order to obtain a dosage range of $200 - 2,500 \mu\text{g kg}^{-1}$ (see **Table 4**). In the former case, the sample was dosed $> 5,000 \mu\text{g kg}^{-1}$; the diluted extract, instead, was correctly dosed by the kit, that means overestimated as DON (recovery $152 \pm 9\%$) and accurate as total DON (recovery $109 \pm 6\%$).

Conclusions

Several data about the possible cross-reactivity of commercial DON-detecting ELISA kit are reported in the literature so far. Data are commonly obtained in buffer solutions or in matrix-matched solutions, but to our best knowledge comparison on a set of naturally incurred samples has never been reported before.

Data collected by different laboratory are not always matching for the same test kits, even for buffer experiments. This should suggest that the subject is not that obvious, and analysts adopting DON-detecting ELISA commercial kits should investigate critically the real accuracy of the method when naturally incurred materials are analysed.

This study reports the possible use of the ELISA kit as a promising group detection tool for the accurate evaluation of DON and its modified forms, expressed as sum of DON, DON-3Glc and 3Ac-DON, in soft wheat and maize. In particular, the collected data confirm that *Celer*[®] DON v3 has no matrix effect, and no false positive results leading to high accuracy when DON only is present.

The overestimation observed by the ELISA kit in some samples within this study is due to the presence of masked mycotoxins. Indeed, when the recovery is calculated for total DON, the goodness in accuracy and inter-sample variability are significantly increased. Therefore, the kits under study must be regarded as a group detection tool, measuring actually the sum of DON and three modified parent compounds.

Since no cross-reactivity is found for 15Ac-Don and NIV, even when these compounds are occurring at relevant concentration, this test kit cannot be considered as a “total DON” detection, but as a “group DON” detection.

Nonetheless, in consideration of the toxicological relevance of 3Ac-DON compared to 15Ac-DON (Pinton et al. 2012) and on account of the possible cleavage of DON-3Glc in the gut (Dall’Erta et al. 2013), this kit may be helpful in the routine detection of the overall amount of DON-related toxins in grains, as recommended by EFSA.

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Table 1: Occurrence of DON and its modified forms in naturally incurred soft wheat samples by LC-MS/MS

Sample code	DON (µg/Kg)	DON-3Glc (µg/Kg)	3Ac-DON (µg/Kg)	15Ac-DON (µg/Kg)	NIV (µg/Kg)
#6082	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
#6329	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
#6177	< LOQ	< LOQ	< LOQ	< LOQ	42±9
#6250	129 ± 29	< LOQ	75±12	53±11	< LOQ
#6057	124±22	48±9	< LOQ	< LOQ	< LOQ
#6167	251±45	< LOQ	< LOQ	< LOQ	< LOQ
#6103	259±47	162±29	41±8	59±11	< LOQ
#6245	286±52	< LOQ	< LOQ	< LOQ	< LOQ
#6056	284±51	43±8	< LOQ	< LOQ	< LOQ
#6048	466±84	< LOQ	< LOQ	< LOQ	< LOQ
#6137	460±83	112±20	< LOQ	< LOQ	< LOQ
#6022	561±101	< LOQ	< LOQ	< LOQ	< LOQ
#6014	574±103	141±25	51±10	40±9	< LOQ
#6140	892±161	< LOQ	< LOQ	< LOQ	< LOQ
#6185	846±152	117±21	< LOQ	< LOQ	< LOQ

Table 2: Recovery data expressed as DON or as total DON obtained on naturally incurred soft wheat samples within two analytical sessions.

Sample code	DON (µg/Kg)	Total DON ^b (µg/Kg)	B/Bo	DON (µg/Kg)	Recovery as DON	Recovery as total DON	B/Bo	DON (µg/Kg)	Recovery as DON	Recovery as total DON
<i>First analytical session</i>						<i>Second analytical session</i>				
#6082	-	-	111.7	< LOD ^a	-	-	93.1	< LOD ^a	-	-
#6329	-	-	134.9	< LOD ^a	-	-	86.3	< LOD ^a	-	-
#6177	-	42	116.0	< LOD ^a	-	-	89.3	< LOD ^a	-	-
#6250	129	257	46.6	224	174%	87%	59.4	177	137%	69%
#6057	124	172	51.3	172	139%	100%	60.0	169	136%	98%
#6167	251	251	47.7	210	84%	84%	86.6	272	108%	108%
#6103	259	521	40.8	314	121%	60%	48.3	403	156%	77%
#6245	286	286	44.9	247	86%	86%	55.2	242	85%	85%
#6056	284	327	42.7	281	99%	86%	52.3	299	105%	91%
#6048	466	466	33.0	496	106%	106%	44.3	541	116%	116%
#6137	460	572	30.7	568	123%	99%	46.0	478	104%	84%
#6022	561	561	32.0	527	94%	94%	45.4	499	89%	89%
#6014	574	806	25.7	762	133%	95%	39.2	787	137%	98%
#6140	892	892	24.8	803	90%	90%	36.1	992	111%	111%
#6185	846	963	21.2	993	117%	103%	32.7	1289	152%	134%
Mean ± SD					114±26%	91±12%	120±24%			97±18%

^aLOD: 40 µg/Kg; ^btotal DON expressed as the sum of DON, 3Ac-DON, DON-3Glc, 15Ac-DON and NIV.

Table 3: Occurrence of DON and its modified forms in naturally incurred maize samples by LC-MS/MS

Sample code	DON (µg/Kg)	DON-3Glc (µg/Kg)	3Ac-DON (µg/Kg)	15Ac-DON (µg/Kg)	NIV (µg/Kg)
#1	< LOD	< LOD	< LOD	< LOD	< LOD
#3	< LOD	< LOD	< LOD	< LOD	< LOD
#5	< LOD	< LOD	< LOD	< LOD	< LOD
#24	366±38	< LOD	< LOD	< LOD	< LOD
#14	507±21	< LOD	< LOD	< LOD	< LOD
#8	522±21	188±51	< LOD	< LOQ	< LOD
#28	536±31	< LOD	< LOD	< LOQ	< LOD
#12	538±19	222±17	< LOD	< LOD	< LOD
#41	765±145	< LOD	< LOD	< LOQ	< LOD
#11	808±14	225±6	< LOQ	< LOQ	< LOQ
#2	1112±3	351±47	< LOQ	< LOQ	< LOQ
#18	2,647±377	639±85	< LOQ	< LOQ	< LOQ
#19	3,212±184	1,170±18	329±11	< LOQ	< LOQ
#22	5,336±536	1,393±232	344±34	< LOQ	< LOQ

Table 4: recovery data expressed as DON or as total DON obtained on naturally incurred maize samples within two analytical sessions.

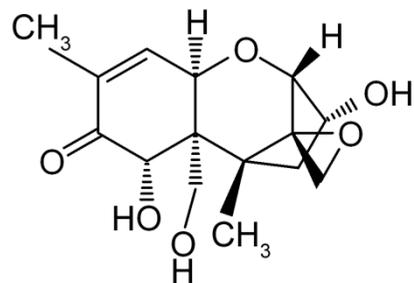
Sample code	DON (µg/Kg)	Total DON ^b (µg/Kg)	B/Bo	DON (µg/Kg)	Recovery as DON	Recovery as total DON				
							B/Bo	DON (µg/Kg)	Recovery as DON	Recovery as total DON
<i>First analytical session</i>							<i>Second analytical session</i>			
#1	< LOD	< LOD	80.7	< LOD ^a	-	-	78.9	< LOD ^a	-	-
#3	< LOD	< LOD	80.3	40	-	-	82.5	< LOD ^a	-	-
#5	< LOD	< LOD	81.0	< LOD ^a	-	-	82.7	< LOD ^a	-	-
#24	366	366	44.6	269	74%	74%	39.7	302	83%	83%
#14	507	507	30.3	689	136%	136%	29.3	606	119%	119%
#8	522	710	31.5	637	122%	90%	28.0	663	127%	93%
#28	536	536	30.0	702	131%	131%	30.2	571	106%	106%
#12	538	760	32.2	607	113%	80%	30.7	552	103%	73%
#41	765	765	27.6	824	108%	108%	23.9	873	114%	114%
#11	808	1,033	54.2	1,006	125%	97%	45.2	1,231	152%	119%
#2	1,112	1,463	61.8	1,391	125%	95%	30.9	1,324	119%	90%
#18	2,647	3,268	40.3	3,911	148%	120%	21.7	3,424	129%	105%
#19	3,212	4,711	51.4	2,295	71%	49%	15.4	2,786	87%	60%
#22	5,336	7,073	39.2	> 5,000 ^c	Na.	Na.	21.6	> 5,000	Na	Na
#22	5,336	7,073	19.0	8,431 ^d	158%	113%	17.3	7,724 ^d	145%	104%
Mean ± SD					119±27%	100±27%	117±22%		97±19%	

^aLOD: 40 µg/Kg; ^btotal DON expressed as the sum of DON, 3Ac-DON, DON-3Glc, 15Ac-DON and NIV; ^chigher concentration detectable with no further sample dilution; ^dthe sample was diluted 5x in 70% Methanol according to the kit insert prescription.

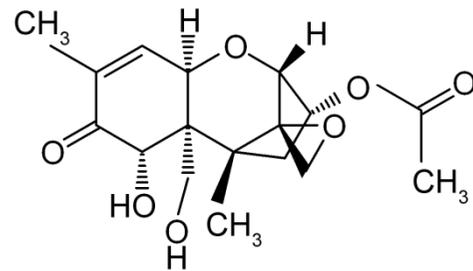
Table 5: Cross-reactivity data towards DON modified forms for commercially available ELISA kits, reported in the literature so far.

Kit	Solution	3ADON	15ADON	DON-3-Glc	NIV	Ref
Celer DON v3	buffer	470	2	64	n.d.	This study
Celer DON v3	buffer	n.r.	n.r.	76	< 4	This study
Celer DON v3	wheat	340	n.r.	61	n.r.	This study
Agraquant Romer	buffer	770	2	52	0	Tangni et al. (2010)
Agraquant Romer	buffer	392	10	45	n.r.	Hajslova (2008)
DON EIA EProxima	buffer	230	0	115	198	Tangni et al. (2010)
DON EIA EProxima	buffer	94	1	37	n.r.	Hajslova (2008)
Veratox Neogen	buffer	40	0	157	0	Tangni et al. (2010)
Veratox Neogen	buffer	103	0	32	n.r.	Hajslova (2008)

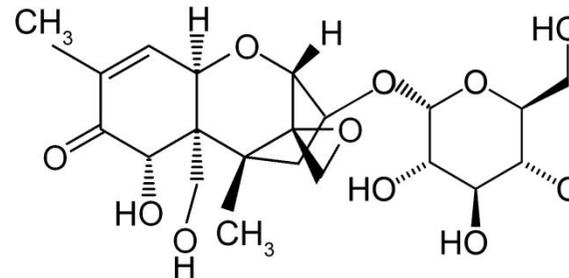
Figure 1



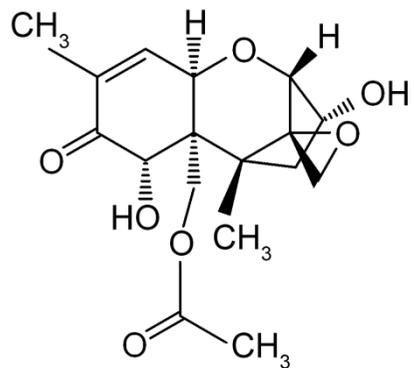
DON



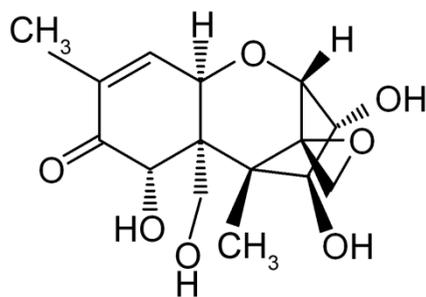
3-AcDON



DON-3Glc

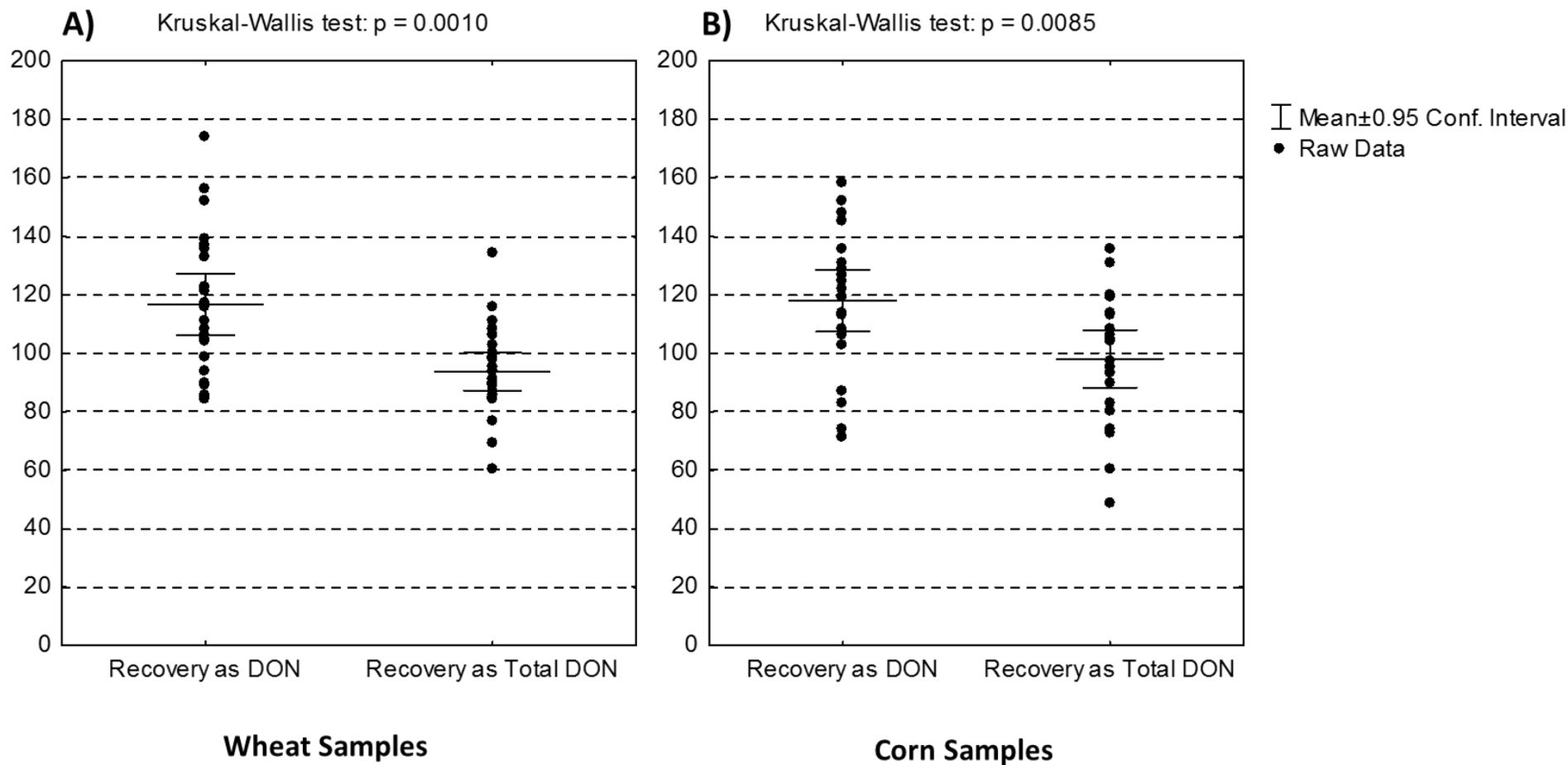


15-AcDON



NIV

Figure 2: Scattered dotplot of recoveries obtained as DON and as total DON ($n = 24$ determinations, each matrix) in wheat (plot A) and in corn (plot B).



Chapter 2

Are organ cultures suitable biofactories for masked mycotoxins? Deciphering the fate of zearalenone in micropropagated durum wheat roots and leaves.

Are organ cultures suitable biofactories for masked mycotoxins? Deciphering the fate of zearalenone in micropropagated durum wheat roots and leaves.

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ABSTRACT

To evaluate the physiological response of plants to xenobiotics, *in vitro* systems are often preferred to fields trials and greenhouse experiments despite their obvious distance from natural conditions. Compared to trichothecenes like DON and T-2, our knowlegde on *in planta* biotransformations of ZEN by wheat is limited. In the present study, an *in vitro* tissue culture technique to study the metabolic fate of zearalenone in durum wheat micropropagated roots and leaves was applied. Using a LC-HRMS approach, a complete, quick absorption of up to 100 µg of ZEN by uninfected plant organs was noticed and its biotransformation into a large spectrum of phase I and phase II metabolites has been depicted. A total of 64 chromatographic peaks were obtained, resulting in 9 putative phase I metabolites and 18 putative phase II compounds. The result obtained suggested that wheat organ tissue cultures can be used as replicable model for the investigation of masked mycotoxin formation. The same technology, however, has the potential to be applied as a biocatalytic tool for the production of masked mycotoxins. From a food safety point of view, this study underlines once again the potential occurrence of cocktails of mycotoxins in grains and, therefore, the urgency of considering them under a combined toxicity perspective.

1. Introduction

Modified mycotoxins have recently become a prominent issue in food safety research and risk assessment, due to the increasing awareness of possible toxic effects related to their (co)occurrence in food. In particular, “masked mycotoxins” *senso strictu* are those conjugates resulting from metabolic pathways activated by the interplay between pathogenic fungi and infected plants¹. Several masked mycotoxins have been described in cereals so far, including deoxynivalenol, nivalenol, T-2 and HT-2, alternariol and alternariol methyl ether^{1,2}. Enzymatic biotransformations are believed to be part of the plant detoxification system; xenobiotics carrying hydroxyl groups can be conjugated to a sugar moiety and further processed by addition of a malonyl, hexose or pentose moiety to facilitate translocation, compartmentation and storage, while further hydroxyls may be directly added³. These conjugates are not monitored in routine food control and their direct toxicity may be uncertain, but upon ingestion the parent form may be released in the digestive tract and absorbed, thereby increasing the total exposure to the original mycotoxin in both humans and animals⁴.

Zearalenone (ZEN) is produced by several *Fusarium* species found in both rhizosphere and phyllosphere of healthy cereal plants, where they act as soil saprophytes or behave as parasites or pathogens in both pre- and post-harvest stages^{5,6}. When occurring, the infection is actuated by colonizing parenchymatous and phloematic tissues, involving mycelia penetration from both the rhizoplane and stomata and by means of specialized infection cushions. Such process is accompanied by necrosis and by the biosynthesis of a wide array of toxic fungal secondary metabolites including ZEN, which is produced by *Fusarium* strains also during non-pathogenic growth. Although not exerting severe acute toxicity in plants and animals, ZEN is known for its strong estrogenic and hormone-like activity^{7,8}. Unlike other *Fusarium* mycotoxins, in living organisms ZEN undergoes reductive phase I metabolism with the formation of α - and β -zearalenol (ZELs), the saturated form zearalanone (ZAN), and its reduced metabolites α - and β -zearalanol (ZALs), which may possess an even higher endocrine disrupting behavior than their parent compound. Although ZEN largely occurs as *trans*-isomer, it has been reported that *cis*-ZEN may be formed upon light exposure and in the presence of some ionic species⁹. The *cis*-isomer is usually overlooked in food analysis, but its presence in edible plant matrices has been reported highlighting that in mammals *cis*-ZEN lead to metabolites of comparable estrogenicity as *trans*-ZEN^{10,11,12}. Given its potential accumulation in infected fields (it may exceed 5 g/ha in topsoil), ZEN could be potentially absorbed by healthy plant organs, but has been evaluated mostly for its involvement in a wide

array of biotransformation pathways operating during the infection⁶. In infected cereals, ZEN may undergo conjugation through glycosylation; zearalenone-14-glucoside (ZEN14Glc) is its most known masked form¹. Despite being the first masked mycotoxin ever described in the literature¹³, its biotransformation has been studied to lesser extent if compared to major trichothecenes such as deoxynivalenol (DON) and for instance the isomer ZEN16Glc was only recently elucidated¹⁴. Also sulfation products have been often reported in naturally infected cereals, but their structure is still to be univocally elucidated and it's not yet clear if they should be considered as plant or fungal metabolites of ZEN, given the fact that many *Fusarium* species are autonomously capable of their biosynthesis¹⁵. Recent reports highlighted the (co)occurrence of ZEN, its conjugated forms, its phase I metabolites α - and β -ZEL, and the conjugated forms thereof, in naturally infected cereals from Finland¹⁶. These findings are in agreement with the results obtained using uninfected *A. thaliana* as model system¹⁷. Healthy plants are known for their capability to biotransform a wide range of both natural and man-made xenobiotics and few papers have suggested that also mycotoxins may be absorbed by asymptomatic crops including rice, lettuce, sugarcane, asparagus and peanut¹⁸⁻²³. However, the biosynthesis of masked mycotoxins under physiological conditions has not been investigated so far.

To evaluate the physiological response of plants to xenobiotics and in consideration of lower cost and more controlled conditions, *in vitro* systems such as cell cultures are often preferred to fields trials and greenhouse experiments despite their obvious distance from natural conditions²⁴. An investigation of the interplay between ZEN and wheat by means of model systems based on organ cultures, may represent a fascinating but yet scarcely explored frontier to improve our understanding of masked mycotoxins. The biocatalytic potential of healthy plants and cultured cells on mycotoxins has been evaluated both recently and in the past, but ZEN has been mostly overlooked and the literature is focused on a very limited range of conjugated compounds, not taking full advantage of modern analytical tools.^{25,26} Usually, mycotoxins make usually their access to inner plant tissues via necrotic cells and, if compared to trichothecenes like DON and T-2, our knowlegde on *in planta* biotransformations of ZEN by wheat is limited. For instance, to comprehensively study the metabolism of mycotoxins *in planta*, stable isotope labelling (SIL) has been successfully proposed for the elucidation of T-2 biotransformation in wheat^{27, 28}. Nonetheless, such approach requires dedicated software for signal comparison and deconvolution, and the use of isotope-labelled standards in high amount. In particular, as a major advantage over targeted

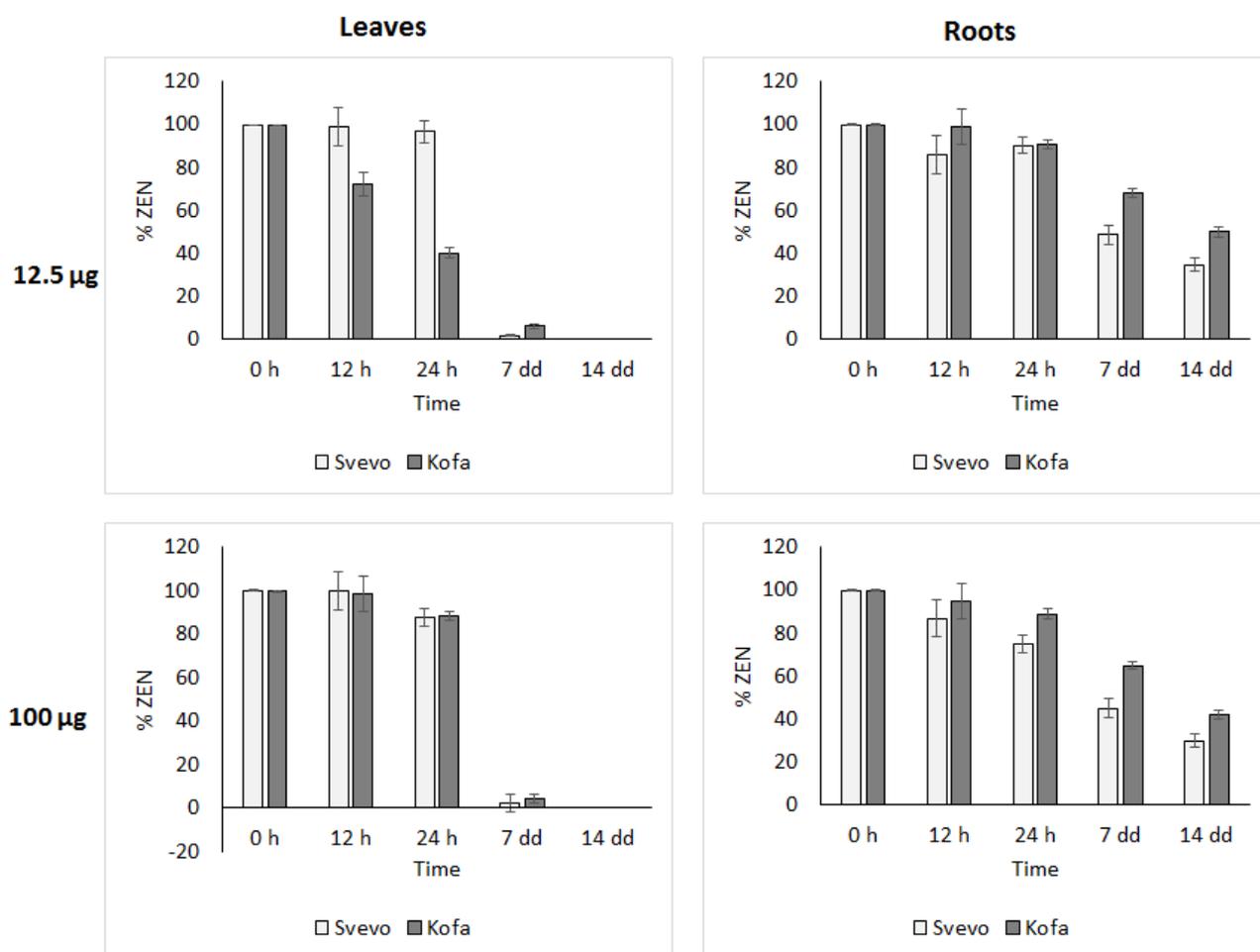
methods, untargeted metabolomics approaches based on mass-spectrometry have the potential to discover unknown biotransformation products originating from specific xenobiotics when limited previous knowledge is available²⁹. A targeted-untargeted metabolomics approach was therefore selected to investigate both phase I and II biotransformation of ZEN in healthy, micropropagated durum wheat roots and leaves, with the goal to assess their potential as masked mycotoxins biofactories and to evaluate the physiological response of the plant metabolism to ZEN exposure.

2. Results

2.1 Qualitative screening of ZEN conjugates. To focus on the effect of plant metabolism under physiological conditions, the qualitative screening of ZEN metabolites in roots and leaves cultures of durum wheat was performed by culture medium contaminated with calibrated amounts of pure ZEN. Two separate amounts were administered after previous checking the tolerance of cultured leaves and roots and in order to work under conditions far from those known for their toxicity in maize root cells³⁰. The final ZEN concentrations in the growing medium were 12.5 and 100 µg/L, respectively. In both Kofa and Svevo cultivars the mycotoxin was quantitatively absorbed after 7 days in leaves, with minor differences between the two administered amounts. When the lower amount was administered, the absorption was faster in Kofa than in Svevo, however differences were leveled up at higher amount administration. In roots, the absorption was slower and less efficient, although comparable at both amount of administered ZEN. The absorption was more efficient in Svevo than in Kofa, with a final residual amount of ZEN in the medium of 40% and 60% respectively. (Figure 1). As xenobiotics may be both modified by intracellular enzymes, by enzymatic pools secreted into the soil or also diffused back in the rhizosphere once biotransformed, and because light and ion exposure may induce ZEN isomerization, the growing media was carefully monitored. Our data show that no masked mycotoxins were produced or diffused in the growing media, and that no degradation occurred due to chemical and physical agents during the whole experiment. Previous reports have also hypothesized that ZEN may be in some occasions an endogenous product of plant metabolism, acting as a regulator of plant development and flowering, in particular during vernalization³¹. To avoid any interference on this regard, controls with untreated plants were set up and resulted negative both at the beginning and at the end of the experiment.

Targeted monitoring of ZEN, ZEN14Glc, ZEN16Glc, α - and β -ZEL returned comparable results in terms of amount detected in Svevo and Kofa cv. ($p = 0.794$), while organ-related differences have been found in both cultivars ($p = 0.027$). Therefore, following experiments have been performed keeping separates leaves and roots, but considering cultivars as biological replicates.

Figure 1: Absorption of ZEN from the growing medium (initial amount: 100 μg). Data are given in terms of residual ZEN% in the medium ($n = 4$). A) Leaves; B) roots.



Since few modified forms of ZEN are available as reference compounds (i.e. ZEN14Glc, ZEN16Glc, α - and β -ZEL), we followed an untargeted qualitative approach. A total of 64

chromatographic peaks were obtained, resulting in 9 putative phase I metabolites (**Table 1**) and 18 putative phase II compounds (**Table 2**). Of those, 16 feature groups were of sufficient intensity, enabling their structural elucidation by HR-MS/MS. In some cases, different peaks were assigned to one single putative metabolite, in consideration of the possible isomeric forms.

Table 1: Phase I metabolites of ZEN annotated from roots and leaves analysis

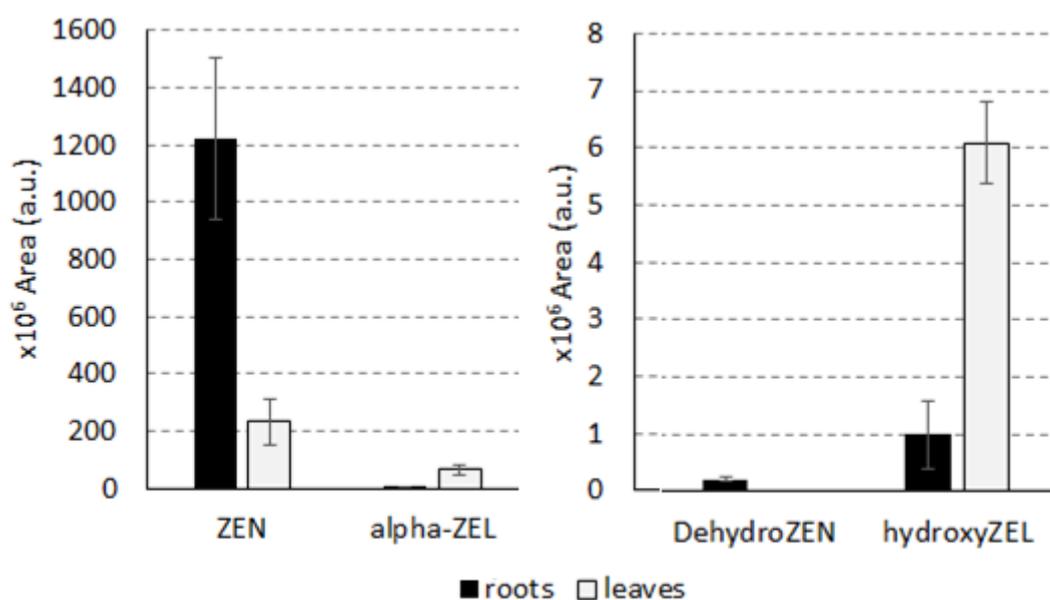
Peak no.	RT	Formula	Detected m/z [M-H] ⁻	Mass error ppm	Putative metabolite	Roots	Leaves
57	10.14	C ₁₈ H ₂₀ O ₅	315.1237	0.34	Dehydro-ZEN ^b	++	N.D.
	12.14	C ₁₈ H ₂₂ O ₅	317.1338	-1.97	ZEN*	++	+
	12.41	C ₁₈ H ₂₂ O ₅	317.1388	-1.75	<i>cis</i> -ZEN*	+	++
	11.94	C ₁₈ H ₂₄ O ₅	319.1544	1.50	α -ZEL*	+	++
	10.39	C ₁₈ H ₂₄ O ₅	319.1548	-0.08	β -ZEL*	+	++
37; 46	8.72; 9.26	C ₁₈ H ₂₀ O ₆	331.1185; 331.1185	-0.45; -0.45	Hydroxy-dehydro-ZEN ^b	++	++
16; 28; 38; 45; 54; 58	7.23; 8.09; 8.73; 9.26; 9.70; 10.24	C ₁₈ H ₂₂ O ₆	333.1338; 333.1338; 333.1343; 333.1342; 333.1344; 333.1340	1.66; 1.84; -0.15; -0.33; -0.20; -0.99	Hydroxy-ZEN or Hydroxy-dehydro-ZEL ^b		++
20; 59	7.51; 10.38	C ₁₈ H ₂₂ O ₆	333.1345; 333.1340	0.66; -0.81	Hydroxy-ZEN or Hydroxy-dehydro-ZEL ^b	++	N.D.
56	9.83	C ₁₈ H ₂₄ O ₆	335.1497	-0.15	Hydroxy-ZEL ^b	+	++
26	7.97	C ₁₈ H ₂₄ O ₆	335.1497	-0.15	Hydroxy-ZEL ^b	++	N.D.

N.D. = not detected metabolite. * Confirmation with standard by comparison of accurate mass, HRMS/MS and RT. ^a Annotation with accurate mass, elemental formula and HRMS/MS spectra. ^b Annotation with accurate mass and elemental formula.

2.2 Phase I metabolites. Few differences were noticed between the tested cultivars and many hydroxylated forms were identified. Those masked mycotoxins are ascribable not only to the reduction of the keto group, giving rise to the formation of α - and β -ZEL, but also to the hydroxylation on both the aromatic and the macrocyclic ring (Figure 3). In all the considered samples, ZEN co-occurs with its isomeric form *cis*ZEN, being the latter more abundant in leaves than in roots. As roots were grown in the dark and leaves under a calibrated photoperiodic illumination to simulate actual growth conditions, such behavior is only in partial agreement with the hypothesis of a light-mediated isomerization. Taking into consideration that *cis*ZEN was reported to undergo the same metabolic transformation than the *trans* isomer, the possible occurrence of phase I metabolites in both *cis* and *trans* isomeric forms, is likely. Besides reductive metabolism and hydroxylation, a putative compound originated by dehydrogenation of ZEN, has been annotated as well.

According to the collected results, the formation of phase I metabolites seemed to be organ-related, but in consideration of the lack of commercial standards, a quantification was not possible. However, comparing signals obtained for ZEN and other standard compounds in roots and leaves, matrix-related effects were not observed. Therefore, although matrix-related bias cannot be excluded for novel metabolites, a statistical comparison of the metabolite profile found in roots and leaves, based on chromatographic area, has been performed. Analysis pointed out the identification of those compounds able to discriminate between the two organs, namely ZEN, dehydro-ZEN, α -ZEL, and hydroxy-ZEL, as reported in **Figure 2**.

Figure 2: Compounds found to significantly differ in terms of area in roots and leaves (Kruskal-Wallis test, $\alpha = 0.05$)



In particular, ZEN and dehydro-ZEN were found to be more abundant in roots than in leaves, while signals related to α -ZEL and hydroxyl-ZEL are higher in leaves.

2.3 Phase II metabolites. ZEN14Glc, ZEN16Glc, and ZEN14Sulf were identified in both roots and leaves. Monoglycosyl and diglycosyl- conjugates of phase I metabolites were identified as well. Due to the lack of suitable standards, it was not possible to discriminate between the isomeric forms. Interestingly, mono- and di-malonyl conjugates of ZEN and ZELs were identified as well. Although occurring in different isomeric forms, their formation seemed to be more abundant in leaves than in roots.

Contrarily to phase I metabolites, the accumulation of conjugated compounds shared between both organs did not produce any statistical difference between leaves and roots. However, a

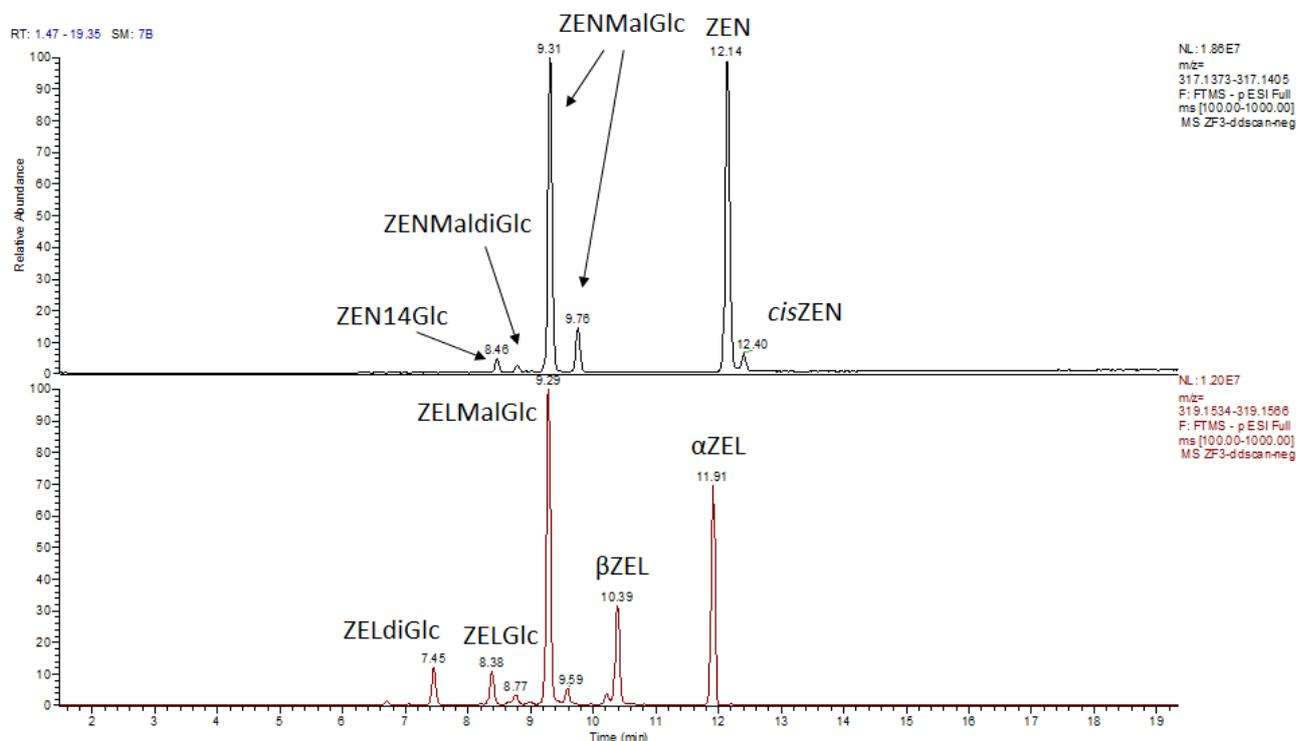
striking difference emerged for those isomers that are abundant in leaves and absent in roots and vice versa. In particular, isomers related to ZEL-Mal-di-Glc, Hydroxy-ZEN-Glc and ZEL-di-Glc showed an inverted distribution.

Table 2: Phase II metabolites of ZEN annotated from roots and leaves analysis

Peak no.	RT (min)	Formula	Detected m/z [M-H] ⁻	Mass error (ppm)	Putative metabolite	Roots	Leaves
ZEN-Sulf	7.83	C18H22O8S	397.0964	0.44		++	+
7; 24	6.22; 7.93	C18H24O8S	399.1120; 399.1121	0.29; 0.67	α - or β -ZEL-Sulf ^a	++	N.D.
ZEN-16-Glc*	6.30	C24H32O10	479.1915	-1.48		+	++
ZEN-14-Glc*	8.46	C24H32O10	479.1917	-1.04		++	+
50	9.32	C24H34O11	479.202	0.52	Hydroxy-ZEL-Glc ^a	N.D.	++
1; 4; 10; 33	5.46; 5.97; 6.70; 8.38	C24H34O10	481.2076; 481.2071; 481.2074; 481.2078	1.75; -0.89; -1.53; -1.60	ZEL-Glc ^a	+	++
15; 21; 40	7.18; 7.63; 8.97;		495.1860; 495.1857; 495.1857;	-0.09; -0.66; -0.72;		N.D.	++
5; 11	6.01; 6.76;	C24H32O11	495.1871; 495.1864;	2.10; 0.69;	Hydroxy-ZEN-Glc ^a	++	N.D.
2	5.47		495.1864;	0.81		++	+
19; 49	7.45; 9.31;		567.2074; 565.1918;	-1.61; -1.47;		++	+
48; 52; 55	9.29; 9.59; 9.75;	C27H34O13	567.2075; 567.2077; 565.1921;	-1.40; -0.97; -0.92;	ZEN-MalGlc ^a	N.D.	++
8; 34	6.30; 8.47	C29H40O14	611.2324; 611.2330	-0.75; -0.56	ZEN-HexPent ^a	N.D.	++
6; 17	6.18; 7.41		641.2446; 641.2445	-0.72; -0.81		++	N.D.
3; 32	5.61; 8.22	C30H42O15	641.2452; 641.2456	0.22; 0.80	ZEN-di-Glc ^a	N.D.	++
13; 18	7.13; 7.42	C30H44O15	643.2586; 643.2606	-1.51; -0.29	ZEL-di-Glc ^a	++	N.D.
31	8.19		643.2603	-0.56		N.D.	++
42; 51	9.01; 9.45	C33H44O18	727.2457; 727.2454	0.41; -0.01	ZEN-Mal-di-Glc ^b	+	++
41; 47	8.97; 9.27	C33H46O18	729.2611; 729.2610	1.46; 1.38	ZEL-Mal-di-Glc ^b	N.D.	++
23	7.91	C36H52O20	803.2984	0.60	ZEN-tri-Glc ^a	++	N.D.
44; 53	9.2; 9.6	C36H46O21	813.2452; 813.2464	-0.79; 0.71	ZEN-di-Mal-di-Glc ^b	+	++
9; 30; 36; 43	6.41; 8.18; 8.67; 9.17	C36H48O21	815.2603; 815.2606; 815.2612; 815.2606	-0.22; 0.30; 1.04; 0.22	ZEL-di-Mal-di-Glc ^b	N.D.	++
25; 29	7.94; 8.09	C39H54O24	889.2977; 889.2980	0.64; 0.91	ZEN-di-Mal-tri-Glc ^b	+	++
12; 14; 22; 39	7.02; 7.16; 7.89 8.65	C39H56O23	891.3123; 891.3129; 891.3134; 891.3137	-0.54; 0.14; 0.69; 0.96	ZEL-di-Mal-tri-Glc ^b	N.D.	++

A chromatogram of main conjugates of ZEN and ZELs are reported in **Figure 3**.

Figure 3: Separation of main conjugates of ZEN (above) and ZELs (below).



3. Discussion

Although the conditions applied within this study differ from those occurring in the field, the direct exposure to ZEN instead of fungal inoculation allows to define the full biocatalytic potential of phase I and phase II enzymatic pools in durum wheat. This cannot be obtained upon infection, because the cross-talk between the plant and the pathogen, involving the subsequent local necrosis as well as the different integrity of the involved tissues, may significantly modulate plant metabolism not allowing a discrimination between the plant and the fungi metabolism. The approach we used on this regard has been previously applied in a number of studies on the formation of masked mycotoxins³²⁻³⁴.

The possibility of an uptake of ZEN by maize or barley seedlings, rice or maize isolated cells or by entire soybean and wheat seeds was known, but never evaluated in wheat nor monitored in combination with the evolution of ZEN plant metabolites³⁵.

According to the data collected within this study, leaf culture was able to uptake ZEN almost quantitatively in 7 days, while its absorption in roots was only partial. Possible differences between leaves and roots in ZEN absorption, are probably to be found in the tissue

characteristics. While roots are designated organ for nutrient uptake, absorption in leaves may occur aspecifically based on the polarity of the compounds.

Previous studies in the literature showed a quantitative absorption of ZEN in 24 hours^{14, 17}. However, it must be underlined that in Kovalsky-Paris et al.¹⁴, barley seedlings were treated with an enormous amount of toxin compared to this study (5 mg vs 100 µg, respectively). In addition, DON was administered together with ZEN. In consideration of the well-known ribotoxic effect of DON and higher amount of administered toxins, the ability of plant tissue to absorb and biotransform both compounds could be altered. In our trial, on the contrary, the plant tissues were treated with lower amount of toxins to maintain for a longer time an healthy status of the cultures, and to observe the metabolic capability under no-stressed conditions.

According to Kavalsky-Paris¹⁴, conjugated metabolites of ZEN could be excreted in the growing medium. However, in the present study, modified forms have never been detected in the medium. This could be due again to the different conditions applied in these two studies. Barley seedling were actually treated with extremely high amount of toxins, and this might lead to a possible elimination of polar conjugates from plant tissues into the medium, to avoid toxic effects. In addition, possible species-specific differences may be taken into account.

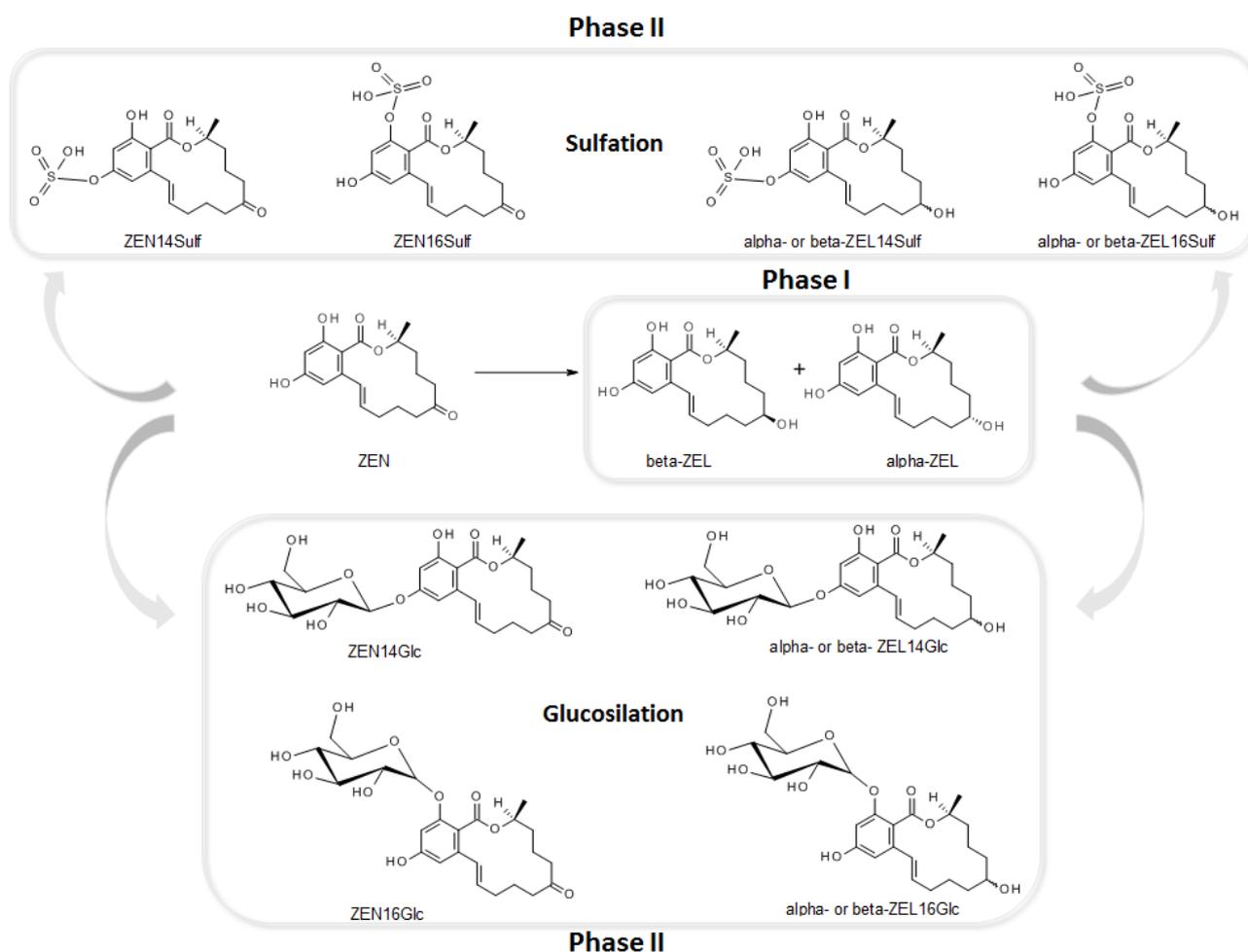
The formation of modified forms of ZEN in plants has been addressed in the past only using maize cells suspension cultures³⁶ and *Arabidopsis thaliana*¹⁷. The former study showed that, upon treatment with radiolabelled ¹⁴C-ZEN, more than 50% of the initial radioactivity was incorporated as insoluble residue in maize cell suspension cultures. Later, the use of *Arabidopsis* as model plant allowed the elucidation of the metabolic transformation of ZEN into its phase I and phase II modified forms¹⁷. In particular, the authors identified 17 compounds, among them α- and β-ZEL, ZEN14Glc, ZEN14Sulf, and the glucosidic conjugates of α- and β-ZEL, some of them apparently released in the culture media. In addition, several di-hexosides, hexose-pentosides and malonylglucosides of ZEN, α- and β-ZEL were described for the first time. A noticeable difference in our case is the complete lack of masked mycotoxins in the growing media during the whole course of the experiment in both leaves and roots, whereas both in barley and *Arabidopsis* seedlings such phenomenon was reported, albeit to a minor amount^{14, 17}. This different behavior may be related to plant-specific differences, to different levels of ZEN exposure or, more likely, to the peculiar conditions of organ culture. In particular, given the histological and biochemical differences between in mono- and dicotyledons, data acquired on *Arabidopsis* may not be automatically transferred to those crops preeminently involved in ZEN-related food safety issues, i.e. cereals³⁷.

Studies on masked mycotoxins are focused on one side on the occurrence of these forms under field conditions^{1,16,34,38}, and on the other side on the evaluation of their toxicological relevance^{8,39}. In this frame, micropropagated plants represent, indeed, a green and cost-effective model system for studying *in vitro* the biotransformation of mycotoxins, allowing for a strong decrease of the biological variability with respect to field trials, to tailoring the monitoring of different variables and endpoints.

In this study, we used micropropagated roots and leaves obtained from two varieties of durum wheat. While organ-specific differences in the formation of ZEN metabolites have been observed, the biotransformation of ZEN did not seem to be cultivar-specific, albeit the minimal pool may not be significant on this regard. Contrarily, slight differences were previously noticed between cultivars in barley-mediated biotransformation of ZEN into ZEN16Glc. Although in contrast from what reported for the same wheat varieties under greenhouse experiments performed with DON³⁸, this can be explained considering the peculiar role played by DON as virulence factor in wheat and the peculiar role of ZEN. Since DON is directly involved in FHB pathogenesis, its biotransformation to the less toxic DON3Glc should be regarded as a mechanism of resistance towards FHB in wheat⁴⁰. On the contrary a direct involvement in pathogenesis and/or in wheat resistance/susceptibility, has not been reported for zearalenone. At the same time, its putative role in plant physiology as a possible auxin-like substance may allow a less intense detoxification and a quicker systematic distribution in both radical and foliar tissues and therefore a high potential for bioaccumulation in plants.

Our data showed that the plant metabolism mainly leads to the formation of water-soluble phase II metabolites, in agreement with previous studies^{27,32}. Considering that conjugates are not released nor produced in the growing medium, the detoxification pathways activated in the plants probably lead to the compartmentation of ZEN modified forms. The main phase I and phase II metabolites reported in the literature so far, and found in our study as well, are reported in **Figure 4**.

Figure 4: Main phase I and phase II metabolites of ZEN



The content of untransformed ZEN in roots was higher. This may be in part related also to the different tissutal organization in these organs, where roots have a large amount of non-filtering parenchimatous tissue in which water diffuse via apoplastic route, therefore acting simply as a passive water container in which ZEN may remain dissolved and not actively exposed to cytoplasmatic enzymes. Relevant amount of *cis*-ZEN has been detected in both leaves and roots, albeit to a lower extent in the latter. This could be ascribed to the light exposure during growth conditions, similarly to what reported under field conditions¹¹. The leaf cultures were exposed to UV-lamp light to simulate the day/night cycle, while root cultures were grown in the dark to reproduce physiological conditions. The higher conversion rate in leaves may be related to their higher light permeation in comparison to roots, but at the same time the presence of the *cis*-isomer also in roots and the lack of conversion in the growing media may warrant further investigation in order to confirm or exclude an active role of wheat metabolism on this regard. Notably, *cis*-ZEN followed the same metabolic pathway

as *trans*-ZEN, with the formation of a large number of conjugates. In consideration of the similar estrogenicity of *cis*-isomer^{8,41}, its occurrence under natural conditions should be monitored within control plans.

According to our findings, reductive and oxidative hydroxylation, followed by glycosylation and malonyl-conjugation, are major biotransformation pathways of ZEN as response of plant detoxification also when a *Fusarium* infection is not occurring. Sulfation was identified as well, although at a minor extent. It must be underlined that sulfation seems to be a major pathway in microbes and animals, while it has been described as minor detoxification route in plants⁴². Overall, the modification pathways identified within this study, are consistent with those reported in *A. thaliana*¹⁷, an evidence that if confirmed may suggest a limited intra and interspecific variability between detoxification pathways of ZEN in plants. Notably, ZEN is biotransformed into its reduced forms α - and β -ZEL, and both of them may undergo further glycosylation or sulfation. This is a relevant observation, since recent studies showed that the possible occurrence of α -ZEL and its conjugates in food may represent a matter of toxicological concern⁴². In agreement with our findings, the formation of α - or β -ZEL conjugates in grains have been recently reported¹⁶. However, the authors did not reported the occurrence of phase I metabolites under field conditions, probably because conjugation pathways are strongly activated under these conditions, to allow the quick detoxification of mycotoxins through compartmentalization. This is in agreement with a quicker metabolic response in field, due to the pathogenic state of the plant under fungal infection.

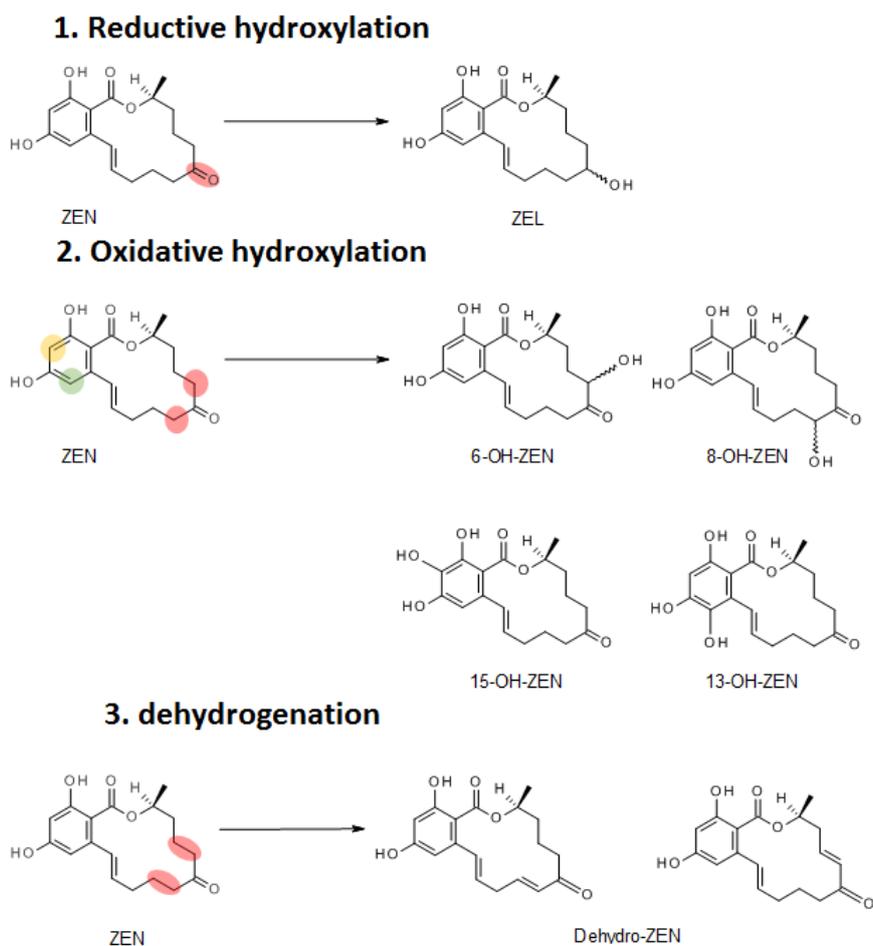
In our study, on the other hand, the use of HRMS on extracts from model plants clearly support the detection of compounds at trace level. Besides already known phase I metabolites such as α -ZEL and β -ZEL, several hydroxylated forms have been observed within this study. Although HR-MS spectra did not allowed the univocal structure elucidation, the preferential formation of several forms could be supported by Site Of Metabolism (SOM) methods⁴³. Previous studies have already depicted, besides reductive hydroxylation of ZEN to α - and β -ZEL, the formation of 6-OH- and 8-OH-ZEN via oxidative hydroxylation⁴⁴. A similar pathway may lead, in mammals, to the formation of 13-OH- and 15-OH- catechol forms⁴⁵. According to our data, dehydrogenated metabolites may be formed as well. As an example, peak 57 at t_R 10.47 min, is characterized by an accurate mass of 315.1227 Da, corresponding to the elemental formula $C_{18}H_{20}O_5$, that is consistent with a dehydrogenation of ZEN. Such compounds have never been reported in the literature so far, but their formation is in agreement with SOM analysis. Since this structural modification may affect the interaction

with the estrogen receptor, the isolation and further characterization of these metabolites is necessary to better define a possible bioactivation/deactivation.

In addition to phase I metabolites obtained by one of the major pathways, compounds carrying two or more modifications may be formed as well, according to the annotation. For instance, peaks at t_R 37 min and 46 min showed an accurate mass of 331.1185 Da, corresponding to the elemental formula $C_{18}H_{20}O_6$. This is consistent with a compound originated by dehydrogenation and hydroxylation of ZEN. Unfortunately, due to the low relative abundance, a further structural confirmation by HR-MS fragmentation spectra couldn't be obtained. In some cases, the annotation based on HR-MS returned the same elemental formula for many peaks, in account of the possible presence of isobaric and isomeric forms. As an example, the formula $C_{18}H_{22}O_6$ was attributed to 8 peaks eluting in the range 7.23 – 10.38 min. In particular, this formula can be referred to a hydroxylation of ZEN, or to a hydroxylation and dehydrogenation of ZEL. Unfortunately, due to the low abundance, further structural information cannot be achieved. Besides regio-isomers, it must be underlined that, since *cis*ZEN was found at relevant amount in both roots and leaves, it may enter biotransformation pathways, giving rise to the formation a range of *cis*-isomeric modified forms. Following SOM prediction, the main sites involved into phase I enzymatic biotransformation of ZEN are summarized in **Figure 5**.

The possible formation of hydroxylated forms of ZEN in healthy plants opens an important issue of concern, as these metabolites may be comparable or even more active than the parent compound towards the estrogen receptors in mammals⁴². It is known, in fact, that α -ZEL is much more active than ZEN towards ER α in susceptible mammals, such as pigs and humans⁴². Recently, the possible estrogenic activity of 15-OH-, 6-OH-, and 8-OH-ZEN was reported⁴⁶. On the basis of computation scoring functions, only 15-OH-ZEN was ranked as potent as ZEN. Although other oxidized compounds were estimated to raise low estrogenic concern, their occurrence in plants should be considered, and further isolation and structural elucidation of these compounds should be performed.

Figure 5: Main possible phase I metabolites according to Site of Metabolism (SOM) prediction.



According to the data collected within this study, the enzymatic pool of durum wheat is capable to biotransform ZEN into a wide spectrum of phase I metabolites, and such capability is not strictly connected to the presence of a *Fusarium* infection. These compounds may follow the conjugation pathways already in use for other secondary metabolites such as polyphenols and involved with the detoxification of many biotic and man-made xenobiotics⁴⁷. In particular, glycosylation, malonyl-conjugation, and sulfation seem to be the major route, originating a plethora of stereo- and regioisomers. For most of them, however, data on occurrence and toxicity cannot be collected due to the lack of proper reference compounds and to the cumbersome and demanding synthetic strategies needed to obtain pure compounds. In this context, wheat organ cultures may be successfully exploited as masked mycotoxins biofactories. Roots and leaves can uptake almost quantitatively ZEN from the growing medium, and biotransform it into a large spectrum of metabolites, following a cost-effective and green procedure. Taking into consideration that, in our assay, 100 μg of ZEN were uptaken almost quantitatively into 200 mg of leaf tissue, the isolation and purification of a wide array of modified compounds could be easily obtained. Therefore, following a proper

scale up, this approach may be exploited for the affordable in batch production of ZEN modified forms. Data reported herein suggest that an organ-related biotransformation may occur. If confirmed, this will enable the fine-tuning of organ-related strategies for the biotechnological production of masked mycotoxins. Furthermore, the recourse to cultured roots and leaves may represent at the same time an useful tool to evaluate the purported hormone-like effect of zearalenone that some authors have suggested in the past, for instance in the improvement of our knowledge regarding the differential distribution of ZEN in treated seeds and seedlings^{48,49}.

Despite the *in vitro* conditions and notwithstanding the evidence that ZEN may be degraded by soil microbiota⁵⁰, our work also suggest that wheat roots have a strong potential in the uptake of ZEN from soil. This phenomenon, recently described for other mycotoxins in healthy plants may be preset also in wheat for ZEN, for instance in *Fusarium* rich-soils or when FHB-spoiled plant material is incorporated in topsoil⁵¹. While most data available regards naturally infected plants, our data show in fact that healthy plants may have the potential to uptake and biotransform ZEN, albeit these findings should be confirmed by more comprehensive and specific evaluations.

Conclusion

The present study represents the first application of *in vitro* tissue culture technique to study the metabolic fate of zearalenone in durum wheat. Using an untargeted LC-HRMS approach, a complete, quick absorption of up to 100 µg of ZEN by uninfected plant organs was noticed and its biotransformation into a large spectrum of phase I and phase II metabolites has been depicted. Therefore, wheat organ tissue cultures can be effectively used as replicable model for the investigation of masked mycotoxin formation. The same technology, however, has the potential to be applied as a biocatalytic tool for the production of masked mycotoxins and for the investigation of the interplay between ZEN and wheat physiology. From a food safety point of view, this study underlines once again the potential occurrence of cocktails of mycotoxins in grains and, therefore, the urgency of considering them under a combined toxicity perspective.

4. Methods

4.1. Chemicals and reagents

Analytical standards of ZEN (100 µg mL⁻¹ in acetonitrile), α-ZEL (solution in acetonitrile 10 µg mL⁻¹) and β-ZEL (solution in acetonitrile 10 µg mL⁻¹) were obtained from Sigma-Aldrich

(Taufkirchen, Germany). ZEN14Glc and ZEN14Sulf were synthesised and purified in our laboratory⁴; *cis*-ZEN was obtained from *trans*-ZEN irradiated by UV-light ($\lambda = 350$ nm) as previously reported¹⁰, zearalenone-16-glucoside (ZEN16Glc) was kindly provided by Prof. Franz Berthiller (IFA-Tulln, University of Natural Resources and Life Science, Vienna). HPLC-grade methanol, acetonitrile and acetic acid, as well as dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using a Milli-Q System (Millipore, Bedford, MA, USA). MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland) were also used.

4.2 Experimental design

Two commercial durum wheat (*Triticum durum* L.) lines, namely Kofa and Svevo, were micropropagated conducting separately leaves and roots experiments. Two separate amounts of ZEN (12.5 and 100 μ g) were independently administrated to leaves and roots through culture medium, were it was dissolved. Liquid media were sampled five times (0, 12 h, 24 h, 7 days, 14 days) in both leaves and roots cultures and in flask containing solely liquid medium in order to monitor ZEN absorption. Whereas leaves and roots were collected once at the end of the experiment, that means after 14 days. All the experiments were carried out in triplicate and repeated three times.

4.4 Culture medium and ZEN solutions:

Cultures were carried out on MS medium, added with 3% (w/v) sucrose, (Murashige and Skoog 1962). The medium was solidified with 0.8% (w/v) phyto agar and pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 minutes. Liquid cultures were prepared as above without agar.

4.5 Plant material

Micropropagation

Two commercial durum wheat (*Triticum durum* L.) varieties, namely Kofa and Svevo, were selected for their previous different FHB resistance. Caryopsis were soaked 70% (v/v) ethanol for five minutes, rinsed 3 times in sterile distilled water. After rinsing, seeds were kept in the dark in distilled water for 5 hours at 28 °C. Surface disinfection was performed with 2.5% (v/v) sodium hypochlorite for 25 min, followed by six washes with sterilized distilled water. The sterilized caryopsis were cultured individually in glass culture tubes containing about 15

ml of ¼ strength MS medium. Cultures were maintained in a growth chamber at $25^{\circ} \pm 1^{\circ}\text{C}$ with a 16 h photoperiod under fluorescent tubes at a light intensity of $27 \mu\text{mol m}^{-2} \text{s}^{-1}$. One week after germination, seedlings grown above 5 cm in length were selected and segments of 10 mm containing apical meristems and leaves were obtained by transverse cuts with a scalpel blade. Explants were cultured on shoot multiplication medium (SM), containing MS basal salt medium, added with $8.88 \mu\text{M}$ N6-benzyladenine (BAP) and $2.2 \mu\text{M}$ 2,4-dichlorophenoxyacetic acid⁵². Multiple shoot clumps arising from the shoot apices were divided and subcultured in SM every 3 weeks. All the experiments were carried out in triplicate and repeated three times.

Root culture

For root induction, shoots were cultured on agarized MS medium *hormone free*. After 4 weeks, roots were excised and inoculated in liquid MS medium (50 ml) supplemented with $1 \mu\text{M}$ IBA in glass conical flasks (150 ml); Cultures were kept in the dark under continuous agitation at 100 rpm in an orbital shaker and maintained in climatic chamber for 4 wk. To improve root growth, $1 \mu\text{M}$ IBA, as auxin, was added in the roots culture

Leaf culture

Leaves (3 – 5 cm in length) were excised from 3 weeks old plants and placed in 50 ml test tube containing few milliliters of solid MS medium. The leaf base was immersed into the medium, then the tubes were filled with liquid medium (MS) added with $10 \mu\text{M}$ BAP, sealed and incubated in climatic chamber for two weeks. Cytokinin BAP at $10 \mu\text{M}$ was present in the leaf culture medium to prevent tissue senescence.

Sample preparation, ZEN administration, sampling and controls

ZEN was dissolved in an adequate amount of DMSO so that the final concentration of the solvent in culture medium did not exceed the one considered toxic (0.2%) with mycotoxin being at the final concentration of $12.5 \mu\text{g}$ and $100 \mu\text{g}$. Solutions were sterilized by $0.2\text{-}\mu\text{m}$ filters and dissolved in the liquid medium in leaves-containing tubes and in root-containing flasks. Leaves (approx. 200 mg for each tube), were anchored to the bottom of tubes by immersion of the basal part in a fine layer of solid medium, allowing a constant exposure of the emerging organ in liquid medium. Roots (approx. 600 mg for each flask) were instead suspended in liquid medium and kept in the dark under orbital shaking (100 rpm). Liquid medium without mycotoxin was used in all experiments as a control. To monitor the evolution of its absorption, ZEN presence in liquid media was determined five times in both leaves and roots cultures and in flask containing solely liquid medium at the following intervals: $t=0$,

t=12h, t=24h, t=7days and t=14days. At the end of the experiment neither leaves nor roots cultures exposed to 100 100 µg/l ZEN showed any visible degradation.

4.6 Sample extraction and UHPLC-HRMS analysis

Sample preparation

Plant samples were freeze dried for 24 h using a laboratory lyophilizer (LIO-5PDGT, 5Pascal s.r.l., Trezzano sul naviglio, Milano) and then milled. 50mg of homogenized plant material were extracted by adding 1500 µL of solvent mixture of acetonitrile/water/formic acid (79:20:1, v/v) and stirred for 90 min at 200 strokes/min on a shaker. The extract was centrifuged for 10 min at 1,4000 rpm at room temperature, then 500 µL of supernatant were evaporated to dryness under nitrogen and finally reconstructed by 500 µL of water/methanol (80:20, v/v) prior to LC-MS analysis.

All medium samples were diluted with water/methanol (80:20, v/v) to achieve a final ratio of 1:1 (v/v), vortexed for 1 min and then subjected to LC-MS analysis.

UHPLC-HRMS analysis

UHPLC Dionex Ultimate 3000 separation system coupled to a Q-ExactiveTM high resolution mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an electrospray source (ESI) was employed. For the chromatographic separation, a reversed-phase C18 Kinetex column (Phenomenex, Torrance, CA, USA) with 2.10×100 mm and a particle size of 2.6µm heated to 40 °C was used. 10 µl of sample extract was injected into the system; the flow rate was 0.4 ml/min. Gradient elution was performed by using 1 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.5% acetic acid. Initial conditions were set at 10% B followed by a linear change to 40% B in 4 min and to 90% B in 16 min. Column was then washed for 2 min with 90% B followed by a reconditioning step for 3 min using initial composition of mobile phases. The total run time was 25 min.

The Q-Exactive mass analyzer was operated in the full MS/data dependent MS/MS mode (full MS–dd-MS/MS) at following parameters: sheath and auxiliary gas flow rates 32 and 7 arbitrary units, respectively; spray voltage 3.3 kV; heater temperature 220 °C; capillary temperature 250 °C, and S-lens RF level 60. Following parameters were used in full MS mode: resolution 70,000 FWHM (defined for m/z 200; 3 Hz), scan range 100–1000 m/z, automatic gain control (AGC) target 3e⁶, maximum inject time (IT) 200 ms. Parameters for dd-MS/MS mode: intensity threshold 1e4, resolution 17,500 FWHM (defined for m/z 200; 12

Hz), scan range 50 – fragmented mass m/z ($m/z +25$), AGC target $2e^5$, maximum IT 50 ms, normalized collision energy (NCE) 35% with $\pm 25\%$ step.

Putative identification of ZEN metabolites

The full identification of ZEN, α -ZEL, and β -ZEL was obtained by comparison with commercial standards. Similarly, *cis*ZEN, ZEN14Sulf, ZEN14Glc, and ZEN16Glc were accurately identified by comparison with authentic standards, obtained by chemical or enzymatic synthesis^{4,10,14}.

For other metabolites, the annotation process involved the following items; (i) the measured accurate mass of $[M-H]^-$ must fit the theoretical accurate mass with a mass tolerance set at ± 5 ppm, (ii) isotopic pattern: the experimental and theoretical isotopic patterns shall correspond, (iii) MS-MS spectra: product ion of intact ZEN (m/z 317.1389) and ZOL (m/z 319.1550) and/or comparison of the fragments obtained with the fragmentation pathway of ZEN or other mycotoxins metabolites formerly found^{17,27,32}.

Only in few cases, fragmentation spectra could not be collected, due to parent ion abundance below the threshold. In this case, a tentative annotation based on accurate mass and elemental formula was performed, as already proposed by other authors^{27,32}.

4.7 Statistical analysis

All statistical analyses were performed using IBM SPSS v.23.0 (SPSS Italia, Bologna, Italy).

Data were analysed by Kruskal-Wallis test followed by Duncan post-hoc test ($\alpha = 0.05$).

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Author Contributions

LR was responsible for sample preparation and analysis, and performed spectral interpretation; ER designed and performed the micropropagation experiment; ER, GG and RB contributes to the experimental design; ER, RB and CD contributed to the biological interpretation of data; LR, MS, and RB prepared the manuscript; CD supervised and coordinated the study. All authors revised the manuscript.

Chapter 3

**Untargeted metabolomics based on
UHPLC-HRMS merged with
chemometrics: a predictable tool for an
early detection of mycotoxins.**

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Untargeted metabolomics based on ultra-high-performance liquid chromatography–high-resolution mass spectrometry merged with chemometrics: A new predictable tool for an early detection of mycotoxins.

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Abstract

In order to explore the early detection of mycotoxins in wheat three standardized approaches (*Fusarium* disease severity, PCR assays for *Fusarium spp.* identification and mycotoxin quantification) and a novel untargeted metabolomics strategy were jointly assessed. In the first phase of this research, standardized approaches were able to quantify mycotoxins and identify *Fusarium spp.* Then, an UHPLC-QTOF metabolic fingerprinting method was developed to investigate plant-pathogen cross-talk. At the same time, chemometrics analysis demonstrated to be a powerful tool in order to distinguish low and strong infection levels. Combining these results, the cross-talk plant pathogen related to the early detection of mycotoxins was discovered. As a rapid response to fungal infection an overexpression of phosphatidic acids was discovered. By contrast, when the infection became stronger an increase of oxilipins and diacylglycerols was revealed.

Keywords: alkylresorcinols; cereals; lipidomics; liquid chromatography-high resolution mass spectrometry; metabolomics; oxylipins; plant-pathogen cross-talk; wheat

1. Introduction

Cereals represent one of the most important commodities providing basic nutrients to human diet, since they are rich sources of carbohydrates, proteins, fats, minerals and vitamins. Among them, the average global annual production of wheat was estimated by FAO as 663 million tones (period 2004-2014) (FAO, 2016). In fact, wheat is a crop of many talents; wheat and wheat-based products are used in several sectors, such as food, feed, biofuel, cosmetics and bio-based plastics (Shewry, 2009). Nevertheless, the main sector is the food industry, where wheat is generally ground into flour and is used, among many others, for bread, pasta, and biscuits. Next to wheat flour, the milling process of the grains also produces bran, which is used as food and animal feed ingredients.

The need of specific characters in terms of nutritional and technological properties has increased the breeding pressure towards similar, high quality varieties. Unfortunately, this has led to an increase of susceptibility towards pathogenic diseases due to colonization by various toxicogenic fungi (i.e. *Fusarium* spp), and subsequent production of secondary metabolites, called mycotoxins (Kumar, Basu, & Rajendran, 2008). *Fusarium* Head Blight (FHB) is the most common fungal disease in small grains occurring worldwide, caused mainly by *F. graminearum* and *F. culmorum* infection (Bottalico, & Perrone, 2002; Müllenborn, Steiner, Ludwig, & Oerke, 2008). It is seen most commonly on spring and winter wheat, durum and barley. FHB can cause significant yield losses, quality reductions and accumulation of *Fusarium* mycotoxins, mainly those from the group of trichothecenes, enniatines, and zearalenones. In addition to production of mycotoxins as compounds causing various acute and chronic adverse health effects, the *Fusarium* pathogens also usually influence the qualitative and quantitative aspects of the crop yield (Richard, 2007). For this reason, legislated and modified mycotoxins are routinely monitored in cereal grains (Rubert, Dzuman, Vaclavikova, Zachariasova, Soler, & Hajslova, 2012; Nathanail et al., 2015; McCormick et al., 2015).

In order to reduce the crop loss, together with the costs of managing noncompliant batches has prompt the search for chemical markers able to identify possible contamination at the earliest stage, and to univocally characterize resistant varieties and infection. In this context, a question of potential early detection of this fungal pathogen on the wheat crop has been arisen.

The early detection of toxigenic fungi directly on cereals can be useful to put an end to the intake of these contaminated materials into the food and feed chain. Initially, these toxigenic fungi have been traditionally identified by microbiological and immunological methods or polymerase chain reaction (PCR) (Mishra, Fox, Culham, & 2003; Huet, Delahaut, Fodey, Haughey, Elliott, & Weigel, 2010). Besides classical microbiological and/or PCR-base methods, innovative spectral techniques (i.e. imaging analysis, near-infrared, Raman) have been proposed for the early detection

of colonizing fungi (Berardo, Pisacane, Battilani, Scandolara, Pietri, & Marocco, 2005; Del Fiore et al., 2010). Since fungal growth is not strictly related to mycotoxin accumulation, and to the pattern of occurring mycotoxins, these techniques – although very simple and effective – cannot provide an univocal response on mycotoxin occurrence. On the other side, the identification of specific chemical markers, mainly linked to the plant-pathogen cross-talk, could drive the selection of resistant wheat varieties, and thus support breeding programs. In this frame, metabolomics may represent the golden tool for understanding the biological pathways involved in mechanisms of plant resistance (Cajka, Vaclavikova, Dzuman, Vaclavik, Ovesna, & Hajslova, 2014; Rubert, Zachariasova, & Hajslova, 2015).

The plant-pathogen cross-talk leading to FHB and mycotoxin accumulation has been significantly studied over the last decade, but the scientific community is still far from a comprehensive scenario, in consideration of the complexity of genetic and environmental factors affecting this interaction (Cajka et al., 2014; Nathanail et al., 2015b; Gauthier, Atanasova-Penichon, Chéreau, & Richard-Forget, 2015; Warth et al., 2015). Recently, Cajka et al. (2014) have developed an analytical procedure optimizing a solid liquid extraction procedure using methanol/water (50:50, v/v) in order to isolate polar/medium-polar barley metabolites followed by ultra high performance liquid chromatography quadrupole-time-of-flight (UHPLC-QTOF). In this research, positive ionization data highlighted a superior discrimination power. In this way, control barley and *Fusarium* infected barley samples were successfully distinguished. In fact, plant stress-related metabolites such as jasmonic acid (JA) or dihydro-7-hydroxymyoporone showed up higher concentrations and correlated positively with increasing concentrations of deoxynivalenol (DON) and its modified forms. Focusing on wheat, a profiling metabolomics strategy has been performed using a stable isotopic labelling approach in order to understand the metabolic fate of HT-2 toxin and T-2 toxin in wheat (*Triticum aestivum* L.) (Nathanail et al., 2015b). The authors demonstrated that the exposure of wheat to either HT-2 toxin or T-2 toxin primarily activates metabolic reactions involving hydroxylation, (de)acetylation, and various conjugations. Furthermore, kinetic data revealed that detoxification progressed rapidly, resulting in the almost complete degradation of the toxins, within 1 week, after a single exposure. In parallel, DON accumulation and *Fusarium* infection in cereals have been recently reviewed by Gauthier et al. (2015) in order to interpret chemical defenses. In this review, the authors have clearly described that when mycotoxins were accumulated the major chemical defenses of the plant cell were related to carbohydrates and amino acid metabolism. These evidences have been recently confirmed by Warth et al. (2015) based on a GC-MS based metabolomics workflow. In this research, DON treatment modified both the primary

carbohydrate metabolism and the primary nitrogen metabolism of the plant, and amino acid levels were significantly increased.

Studies aimed at depicting the resistance/susceptibility of grains towards FHB are usually based on artificial grain inoculation in collection fields, in order to decrease natural variability and highlight significant effects. Giorni et al. (2015) reported, on the contrary, the identification of lipid markers of infection in maize naturally infected by *F. verticillioides* under open field conditions. Although the experimental plan involved only few maize varieties in a large number of replicates, the increased variability due to open field conditions affected positively the robustness of the statistical model (Giorni et al., 2015).

The main aim of this research work was to develop a novel metabolomics strategy exploitable for the early recognition of *Fusarium* disease, based on the detection of infection-related metabolites. For this purpose, a set of eighty-six naturally contaminated wheat samples was available. For the proper metabolomics data interpretation, determination of *Fusarium* disease severity was visually determined and *Fusarium spp.* were identified by PCR assays. Subsequently, targeted mycotoxins were quantified by a validated analytical method. In the second phase, an untargeted metabolomics strategy was optimized. First, several extraction solvents and mixtures of them were studied in order to extract the bulk of information, and then an UHPLC-QTOF method was developed to separate and detect metabolites isolated. Afterwards, advanced chemometric tools were used for wheat samples clustering, and metabolic pathways elucidation.

2. Material and methods

2.1 Chemicals and Reagents.

Polytetrafluoroethylene (PTFE) 50 mL centrifugation cuvettes were obtained from Merck (Praha, Czech Republic). HPLC grade methanol, ethanol, dichloromethane, 2-propanol and hexane were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were supplied by Sigma–Aldrich (St. Luis, MO, USA). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2 Plant material.

Altogether, 86 naturally contaminated winter wheat samples (harvest 2012) from the Czech Republic were analyzed within this study. All the samples were collected by the Central Institute for Supervising and Testing in Agriculture as a part of long-term study focused on FHB symptoms assessment and determination of mycotoxins (Chrpová et al., 2016). Regarding the sampling

strategy, 25 randomly selected wheat ears from different places of each field were collected and further analyzed.

2.3 Standardized approaches

2.3.1 Visual determination of *Fusarium* disease severity.

The extent of *Fusarium* disease severity was realized at the Crop Research Institute (Prague, Czech Republic). These experiments were visually determined using a 10-point scale (0 – 9; 0 – no symptoms up to 9 – severe symptoms) introduced by Schaller and Qualset (1980). Description of each level of *Fusarium* disease severity is described *Table 1*.

2.3.2 DNA extraction and PCR assays for species identification.

For the purpose of *Fusarium* species identification, PCR assays were used, as it was recently described by Chrpová et al. (2016). Nine pathogens associated with FHB were investigated: *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum*, *F. equiseti*, *F. langsethiae*, *F. tricinctum*, *F. sambucinum* and *F. sporotrichioides* (Oerke, Meier, Dehne, Sulyok, Krska, & Steiner, 2010).

2.3.3 Mycotoxin quantification.

Wheat samples were also analyzed by an ISO 17025 accredited method for 57 mycotoxins using UHPLC coupled with Q-Exactive system (Dzuman, Zachariasova, Lacina, Veprikova, Slavikova, & Hajslova, 2014; Dzuman, Zachariasova, Veprikova, Godula, & Hajslova, 2015). Mycotoxins were unambiguously identified, and subsequently were accurately quantified.

2.4. Untargeted metabolomics strategy

Three steps can be clearly distinguished within metabolomics analysis: (i) sample preparation, (ii) the chromatographic separation and detection conditions and (iii) data processing. In this research, UHPLC-QTOF untargeted metabolomics method and data processing have been performed based on previous works (Rubert, Lacina, Zachariasova, & Hajslova, 2016; Righetti et al., 2016).

2.4.1 Sample preparation and optimization.

Several extraction solvents and mixtures were initially tested in order to optimize an untargeted metabolomics extraction procedure: (a) methanol/water (50/50, v/v), (b) methanol/water (65/35, v/v), (c) methanol/water (80/20, v/v), (d) ethanol/water (65/35, v/v), (e) dichloromethane/methanol (50/50, v/v), (f) hexane/ethanol (70/30, v/v) and (g) hexane. Within each experiment, 1 g of wheat was extracted by hand shaking for 1 min with 10 mL of particular extraction solvents, and subsequently an automatic shaker (IKA Laborortechnik, Staufen,

Germany) was used for 30 min at 240 strokes/min. Wheat extracts were then centrifuged 5 min, 13,416g at 20 °C (Rotina 35 R, Hettich Zentrifugen, DJB Labcare, Newport, UK). These experiments were done in five repetitions. Extracts (a-d) were directly injected. By contrast, extracts (f-g) a prior to UHPLC-QTOF measurements, 1 mL of the extract was evaporated with a gentle stream of nitrogen and the residue was reconstituted to a final volume of 1 ml 2-propanol/methanol/water (65:30:5, v/v/v) prior to the analysis.

2.4.2 Optimized sample preparation procedure.

Wheat samples were ground into a fine powder using a ball mill (MM 301 Retsch, Haan, Germany). Then, 1 g wheat was extracted by hand shaking for 1 min with 10 mL of dichloromethane/methanol (50/50, v/v), and subsequently an automatic shaker (IKA Laborortechnik, Staufen, Germany) was used for 30 min at 240 strokes/min. Wheat extracts were then centrifuged 5 min, 13,416g at 20 °C (Rotina 35 R, Hettich Zentrifugen, DJB Labcare, Newport, UK). A prior to UHPLC-QTOF measurements, 1 mL of the extract was evaporated with a gentle stream of nitrogen and the residue was reconstituted to a final volume of 1 ml 2-propanol/methanol/water (65:30:5, v/v/v).

2.4.3 UHPLC-QTOF untargeted metabolomics method.

Dionex UltiMate 3000 RS UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with BEH C₁₈ (2.1x100 mm, 1.7 µm) analytical column and maintained at 60 °C was optimized. The mobile phases consisted of (A) 5 mM ammonium formate and 0.1% formic acid in water/methanol (95/5, v/v), and (B) 5 mM ammonium formate and 0.1% formic acid in 2-propanol/methanol/water (65/30/5, v/v/v). A multi-step elution dual-mode gradient was developed as follow: at 0.0 min (10% B; 0.40 mL min⁻¹) a gradient begun up to 1.0 min (50% B; 0.4 mL min⁻¹), and a second step was set to 5.0 min (80% B; 0.4 mL min⁻¹), then the third step 11.0 min reached 100% B and slightly increased the flow (100% B; 0.5 mL min⁻¹), subsequently an isocratic step was executed during four minutes and half, 15.5 min (100% B; 0.50 mL min⁻¹), 15.1 min (10% B; 0.40 mL min⁻¹) a reconditioning period up to 17.5 min (10% B; 0.40 mL min⁻¹) was used. The sample injection volume was 1 µL for both positive and negative ionization modes and the autosampler temperature was kept at 5°C.

TripleTOF[®] 5600 QTOF mass spectrometer (SCIEX, Concord, ON, Canada) was used for wheat metabolic fingerprints, as it was recently described by Rubert et al. (2016). The ion source was a Duo Spray[™]. Electrospray ionization (ESI) ion source was used for the measurement, while atmospheric pressure chemical ionization (APCI) probe worked as the second gas heater. The source ESI(+) settings were as follows: nebulizing gas pressure 55 psi; drying gas pressure 50 psi;

curtain gas 35 (arbitrary units); temperature 550°C; capillary voltage +5500 V and declustering potential 80 V. The capillary voltage in negative ESI was -4500 V, other source settings were the same as for ESI(+).

The method consisted of a full scan MS ranged from m/z 100 to 1200, followed by acquisition of product ion spectra, ranging from m/z 50 to 1200, for the ten most intensive ions of the survey spectra throughout the chromatographic run (MS/MS) with a collision energy of 35 V and collision energy spread of ± 15 V. Dynamic Background Subtraction was activated. The total cycle time of MS and MS/MS methods took 0.65 s. The APCI was used for exact mass calibration of the TripleTOF instrument. An automatic m/z calibration was performed by the calibration delivery system (CDS) every 5 samples using positive or negative APCI calibration solution (SCIEX, Concord, ON, Canada) according to the batch polarity. Each set of samples in each polarity was preceded by 3 blank controls, it was recently described by Rubert et al. (2016). The same MS approach was carried out by ESI(-) mode.

Instrument control and data acquisition were carried out with the Analyst 1.6 TF software (Sciex, Concord, ON, Canada), the qualitative analysis was performed using PeakView 2.2 (Sciex, Concord, ON, Canada) and LipidView (SCIEX, Concord, ON, Canada). Note that the in-batch sequence of the samples was random (random number generation). In order to evaluate overall process variability, metabolomics studies were augmented to include a set of six samples technical replicates and pooled quality control. In this way, repeatability, reproducibility, precision and mass accuracy of metabolites were successfully supervised (Rubert et al., 2015; Righetti et al., 2016; Rubert et al. 2016).

2.4.4 Data Processing and Chemometrics analysis.

MS data processing, filtering and multivariate data analysis have been performed based on previous works (Righetti et al., 2016; Rubert et al., 2016). Briefly, MarkerView software (version 1.2.1, SCIEX, Concord, ON, Canada) was employed in order to perform data processing of the UHPLC-HRMS records. Data mining was performed using an automated algorithm using retention time range (RT) (0.4 – 14 min), peak finding (m/z range was 100 – 1200). Subsequently, RT and m/z alignment of the respective peaks was executed using RT and m/z tolerances of 0.2 min and 0.02 Da, respectively. Two data matrices, positive and negative, containing lists of molecular features and characterized by (i) RT, (ii) m/z value, (iii) respective intensity and (iv) charge state, were automatically obtained. The variables were then filtered. Molecular features in at least 50% of the Quality Controls (QCs), with coefficients of variation less than 30% across the QCs, were selected, and models were built using SIMCA software (v. 13.0, 2011, Umetrics, Umea, Sweden; www.umetrics.com). In the last step, groups were compared using *t*-tests followed by Bonferroni

corrections to minimize false positives (corrected p value ≤ 0.05 ; MATLAB 7.10.0.499). Prior to PCA, the data were pre-processed using the pareto scaling. Orthogonal partial least squares discriminant analysis (OPLS-DA) was constructed using SIMCA. The quality of this unsupervised model was evaluated according to a previous work (Rubert et al., 2016) and a recent review (Rubert et al. 2015).

3. Results and discussion

3.1 Characterization and quality of wheat samples (*Fusarium* disease and mycotoxins content)

Toxigenic fungi activity has been traditionally reported by *Fusarium* disease severity, identification of pathogens associated with FHB and co-occurrence of mycotoxins (Chrpová et al., 2016). The extent of *Fusarium* disease severity was visually determined using a visual score scale (Table 1). A modified “Horsfall-Barrett” scale was used based on a 0-10 rating system (Schaller, & Qualset, 1980). The severity index was ranked according to visual inspection, performed by a trained person.

In the vast majority of wheat samples considered within this study, *Fusarium* infection level was low ranged from 0 to 1 marks (74% total). A medium-low severity level, ranged from 2 to 3 marks, was observed for 15% of wheat samples. In levels slightly above, 11% of wheat samples presented medium level (4-5 marks). In this study, wheat sample set did not show up a severity degree higher than 5.

In addition, pathogens associated with FHB were genetically identified (Table 1). Results obtained by PCR assays highlighted that *F. poae* was found in a significant number of samples, approximately 90% of total wheat collection, followed by *F. graminearum*, which was identified in 15% of cases. Overall, one quarter of wheat samples showed up co-occurrence of *Fusarium* spp. It should be noted that *F. culmorum* and *F. graminearum* are well known to be the most aggressive *Fusarium* species causing significant visual symptoms of FHB in wheat kernels. By contrast, *F. poae* infection is characterized by none significant visual symptoms on ears, resulting in a difficult FHB diagnosis (Stenglein, 2009).

As regards mycotoxins occurrence, the content range, together with % of positive samples, are summarized in Table 1. First, emergent *Fusarium* toxins, such as beauvericin and enniatins A, A₁, B and B₁ were detected in over 80% of wheat samples, with concentration levels ranging from 1 to 2,147 $\mu\text{g}/\text{Kg}$. Deoxynivalenol (DON), the major trichothecene commonly found in wheat, was detected in about 25% of samples, at a concentration varied from a few $\mu\text{g}/\text{Kg}$ up to 10 mg/Kg . Three out of 25 samples exceeded maximum tolerable level for unprocessed cereals of 1.250 $\mu\text{g}/\text{Kg}$ (EU, 2006, 2007). However, considering the DON contamination as the overall amount of DON-

related metabolites, as recommended by EFSA, 5 out of 36 samples would have been exceeded maximum tolerable level. The first goal of this research was successfully archived by three standardized methods, in the following step an untargeted metabolomics approach was explored in-depth.

3.2 Untargeted metabolomics and the early recognition of *Fusarium* diseases

3.2.1 Untargeted metabolomics method optimization

First, an UHPLC-HRMS metabolic fingerprinting method was optimized in order to detect as many metabolites as possible. The chromatographic run had to provide separation efficiency and good peak resolution. In this way, a BEH (Ethylene Bridged Hybrid) C₁₈ column was used. This column presented versatility and an excellent capacity to separate a diverse range of analytes based on a strong mobile phase (65 % of 2-propanol), modifiers (ammonium formate and formic acid) and temperature (60°C). As a result, polar or medium-polar metabolites, such as free fatty acids (FFA) or lysophospholipids (LysoPC) were nicely separated (time window 0-6 min), as well as late eluting compounds, such as triacylglycerol (TGs) (time window 10-12 min), showed an excellent chromatographic resolution, as it can be seen in the *Figure 1*. A good peak shape, chromatographic resolution and RTs stability are vital for rapid data mining procedures and alignment within metabolomics analyses (Rubert et al., 2015). A part from this, the QTOF system had to guarantee enough data point and linear dynamic range. In this research, the accumulation time took 0.65 s, under these conditions, more than 15 data points for 10-15 s chromatographic peaks were earned. In other words, detection of metabolites from low responses to high responses was enabled.

Consecutively, the extraction procedure was evaluated in-depth in order to extract the bulk of the information. In particular, the extraction capability of 7 solvent mixtures was compared, as described elsewhere (Section 2. Material and Methods). The extracts were measured by UHPLC-QTOF in both positive and negative ESI modes to perceive the number of ionizable/detectable metabolites under different experimental conditions. MarkerView software assessed the detected molecular features in the different wheat extracts (*Figure 2*). Among those tested, ethanol/water (65/35, v/v), dichloromethane/methanol (50/50, v/v) and hexane/ethanol (70/30, v/v) provided a superior number of molecular features using both ESI(+) and ESI(-) modes. The number of obtained features varied slightly 796, 718, and 696 using ESI(-) for ethanol/water (65/35, v/v), hexane/ethanol (70/30, v/v) and dichloromethane/methanol (50/50, v/v), respectively. On the other hand, evaluating the ESI(+) performance, dichloromethane/methanol (50/50, v/v) showed superior extraction efficiency. In agreement, dichloromethane/methanol (50/50, v/v) was chosen as extraction solvent for further experiments.

3.2.2 Data processing and statistical evaluation

Mycotoxin accumulation and *Fusarium* infection had to be understood in terms of cross-talk responses. Therefore, the UHPLC-QTOF untargeted metabolomics method had to detect simultaneously as many metabolites as possible in wheat, in order to understand metabolic pathways. In other words, hundreds of variables (m/z , RT, intensity) had to be converted into more manageable information. After data processing and data pre-treatment, multivariate data analysis was carried out based on a statistical comparison and molecular feature identification. Two steps can be clearly distinguished; (i) unsupervised model and (ii) supervised model (Rubert et al., 2015).

Principal component analysis (PCA) was initially employed, as the first step in the data analysis in order to detect sample clustering in the measured data. The same sample clustering was clearly observed using both ESI(+) and ESI(-) modes. Furthermore, in both cases, the sum of PC1 and PC2 was superior to 48%, in the same direction the goodness-of-fit parameter showed suitable values for ESI+ (65%) and ESI(-) (80%), therefore, nicely fitting the models. It can be seen in the *Figure 3A* and *3B*.

Nevertheless, initially, this grouping was not easily understood based on the sample description and results provided by classical methodologies (described above), and further evaluation was vital. Step-by-step, these two groups were successfully defined based on qualitative and quantitative data: (i) *Fusarium* infection level, (ii) *Fusarium spp.* and (iii) mycotoxin content range (*Table 1 supplementary material*). On the one hand, the first group was called “low infection” (LI) level. This group was characterized by infection levels from 0 to 1, in 90% of cases for both ESI(+) and ESI(-) modes, three *Fusarium spp.* detected; *F. poae*, *F. avenaceum* and *F. equiseti*, and a mycotoxin content ranged from 0 to 2.773 $\mu\text{g}/\text{Kg}$. On the other hand, the second group was called “strong infection” (SI) level. In this case, this cluster was defined by infection levels from 1 to 5, in 75% of cases for both ESI(+) and ESI(-) modes, *Fusarium spp.* detected; *F. poae*, *F. sporotrichoides*, *F. graminearum*, *F. culmorum*, *F. avenaceum*, and a mycotoxin content ranged from 13 to 10,510 $\mu\text{g}/\text{Kg}$. *Table 1 supplementary material* summarizes LI and SI levels.

Once the sample clustering was fully understood, supervised models, concretely OPLS-DA models, were validated. OPLS-DA scores plots showed successful discrimination between LI and SI levels, as it can be seen in the *Figure 3C* and *3D*. The statistical model parameters were $R^2X=0.63$, $R^2Y=0.91$ and $Q^2=0.83$ for positive ionization mode and $R^2X=0.65$, $R^2Y=0.91$ and $Q^2=0.86$ for negative ionization mode, in both cases using two components. The prediction ability and proportion of variance explained by the models justified the sample clustering defined above.

3.3 Marker interpretation.

As the last step, the most significant markers related to LI and SI levels were tentatively identified. These markers are summarized in the *Table 2*. The tentative identification was crucial in order to understand up and down regulated markers and metabolite pathways. These markers can be split into three categories, according to their chemical structure and their biological role; (i) oxylipins; (ii) alkylresorcinols; (iii) acyl glycerols.

Oxylipins are key signaling compounds that are involved in the plant-pathogen cross-talk. These molecules have been reported as able to regulate the expression of certain defense-related genes, modulating fungal sporulation, mycotoxin production, and the biosynthesis of the plant signaling molecule JA (Ludovici, Ialongo, Reverberi, Beccaccioli, Scarpari, & Scala, 2014). In our study, four oxylipins related to 13-lipoxygenase (13-LOX) pathway were up regulated for SI group (*Table 2*). The accumulation of 13-LOX pathway related oxylipins in SI group is in agreement with the literature, since studies suggested that the 13-LOX pathway is activated after pathogen assault as a defense response (Ross et al., 2003; Carrasco, & Mérida, 2006; Gao et al., 2007; Hong, Zhang, & Wang, 2010; Testerink, & Munnik, 2011; Dong, Lv, Xia, & Wang, 2012; Ludovici et al., 2014; Ciccoritti, Pasquini, Sgrulletta, & Nocente, 2015).

In this way, Ludovici et al. (2014) and Gao et al. (2007) reported that 13-LOX derived products related to linoleic acid, such as 13-HODE and 12,13-diHOME significantly increased in maize ears after *F. verticilloides* infection. Consistently, both markers were upregulated in SI samples with a significant relation with higher mycotoxin amount and higher fungal biomass. Among 13-LOX derived compounds, 12,13-diHOME revealed to be the most discriminant marker using ESI(-) ionization mode (*Table 2, Figure 4A*), having a VIP value higher than 4.5.

Another group of lipids that contributed significantly to the separation using ESI(-) records were alkylresorcinols (ARs). These particular phenolic lipids are 1,3-dihydroxybenzene derivatives with an odd numbered alk(en)yl chain at position 5 of the benzene ring and carbon chains of different lengths (i.e. C15:0, C17:0) (Ross et al., 2003; Ciccoritti et al., 2015). ARs are synthesized as secondary metabolites in the outer parts of grains and in the plant. They have been reported to act as protective agents against parasites like fungi and other microorganisms. Recently, Ciccoritti et al. (2015) confirmed that ARs showed a fungistatic activity against *Fusarium spp.* under *in-vitro* conditions, and highlighted that the antifungal activity was positively correlated to C21:0/C23:0 ratio. In agreement, our results showed that C21:0 and C23:0 were over-expressed in LI group, being the most significant discriminant compounds (*Table 2*). This evidence suggests that the localization of ARs at the surface of plant tissue and their amphiphilic structure could act as a chemical barrier against fungal infection.

In addition to oxylipins and phenolic lipids, different lipid signaling molecules can be produced as a consequence of membrane modifications, such as diacylglycerol (DAG) and phosphatidic acid (PA) (Carrasco et al., 2006). In this research work, one DAG and two PA compounds, were found to contribute significantly to ESI(+) sample clustering.

On the one hand, DAG (15:1/18:2) was exclusively found in the SI group, probably as a consequence of membrane alteration due to a pathogen attack. By contrast, PA (C18:2/C18:2), as it can be seen in the *Figure 4B*, and PA(C16:0/C18:2) were mainly found as significant up-regulated markers in LI group. Under physiological conditions, the DAG content of the plant cell is low and its production and clearance must be rigorously controlled to guarantee a permanent reservoir of this lipid, being, among others, an essential component of membranes (Dong et al., 2012). However, upon membrane alteration and glycerolphospholipid hydrolysis, DAG may accumulate in the apical domain of the plasma membrane (Testerink et al., 2011). The phosphorylation of DAGs by diacylglycerol kinase leads to the formation of PAs (Testerink et al., 2011), which are signaling lipids involved in the plant response to biotic and abiotic stress (Carrasco et al. 2006).

In short, we hypothesize that the exclusive occurrence of DAG (C15:1/C18:2) in SI group, and the strong accumulation of PAs in LI group may suggest that in low infected plants DAGs were immediately phosphorylated to PAs, as a rapid response to fungal infection. At the same time, high contents of ARs at the surface of kernels could act as a chemical barrier against fungal infection. Lastly, in plants where the infection was more aggressive, two metabolic pathways were activated; (i) PA hydrolysis, and (ii) 13-LOX signaling pathway, leading therefore to an increase of C18:2 derived oxylipins.

4. Conclusions

The outcome of our study strongly supports the key role played by lipid signaling compounds in the complex regulatory network. The undertaken study described the interconnection of metabolic pathways taking place in the *Fusarium* infected wheat, in other words, how the *Fusarium* infections influence mycotoxin and other metabolites formation. The main conclusions of this research can be summarized below:

- In the LI group of samples, an overexpression of PAs occurred. This suggests the hypothesis that DAGs are phosphorylated to PAs as a rapid response to fungal infection.
- The high content of ARs in the LI wheat samples could refer to the chemical barrier created against the fungal pathogen.
- The increased level of DAGs and oxylipins in the SI wheat samples reflects the activation of PA hydrolysis and 13-LOX signaling pathway.

The workflow developed here allows a significant simplification in future research focused on mycotoxins and plant-pathogen cross-talk. (Bio)monitoring of the most significant markers described here could serve as an effective tool for the early detection of mycotoxins, and *Fusarium* disease prevention.

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Table 1. Description and percentage of each level of *Fusarium* disease severity, pathogens associated with *Fusarium* head blight and mycotoxin co-occurrence are described.

<i>Fusarium</i> Infection Level (%) ¹	0 - Traces of <i>Fusarium spp.</i> infection.	45
	1 – Clear infection of either one spikelet or a slight infection of several spikelets.	29
	2 – Either stronger infection of 2 to 3 spikelets (the rest of the spikelets without infection) or a slight infection of several spikelets.	13
	3 – Approximately one third of spikelets are strongly infected or more spikelets show a slight infection.	2
	4 – A half of spikelets with clear symptoms with a slight expression of infection.	5
	5 – Either two thirds of spikelets with slight infection or a half of spikelets infected with several strongly infected spikelets.	6
<i>Fusarium spp.</i> (%) ¹	n.d. ²	11
	<i>F. poae</i>	89
	<i>F. sporotrichoides</i>	1
	<i>F. graminearum</i>	15
	<i>F. culmorum</i>	1
	<i>F. avenaceum</i>	6
Mycotoxin content range $\mu\text{g}/\text{Kg}^3$, and frequency of occurrence (%) ¹	<i>F. equiseti</i>	5
	Nivalenol	153-307 (3)
	Deoxynivalenol	51-10,034 (25)
	Zearalenone	2-76 (9)
	Deoxynivalenol-3-Glucoside	53-402 (11)
	Enniatin B	1-2147 (99)
	Enniatin B1	1-488 (91)
	Enniatin A	1-106 (80)
	Enniatin A1	1-148 (79)
	HT-2 toxin	50 (1)
	T-2 toxin	5-13 (5)
Beauvericin	1-105 (86)	

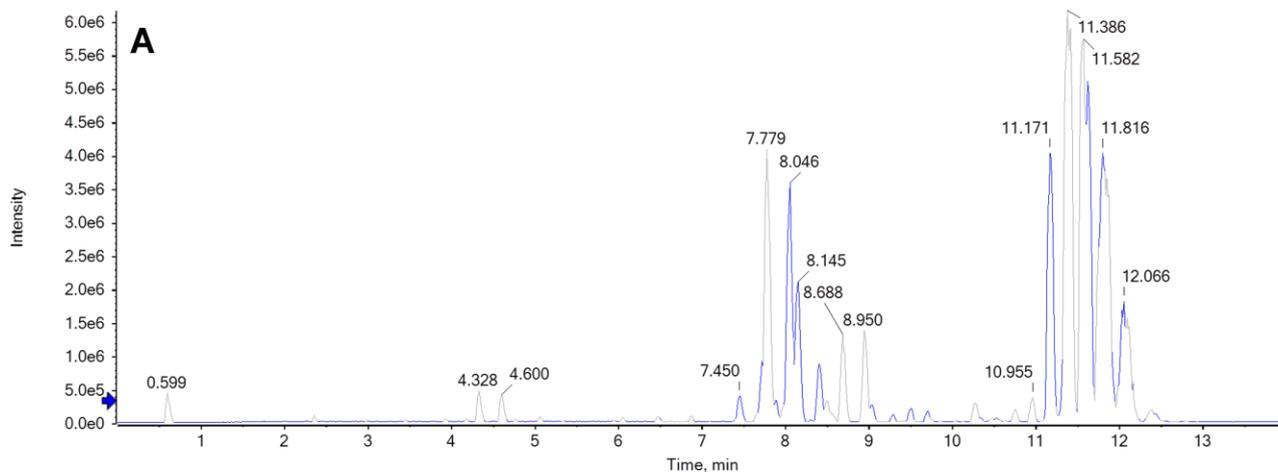
¹Overall percentage.

²non-detected.

³minimum-maximum range.

Figure 1. Base peak chromatograms of a wheat sample extract (dichloromethane/methanol (50/50, v/v)) are compared for positive ionization mode (A) and negative ionization mode (B). The chromatographic separation was carried out using BEH C₁₈ column, a multi-step elution dual-mode gradient and column oven temperature at 60°C.

BPC from 2015-09-15 ESI Pos AMICO GRANO Samples list 3 63.wiff (sample 1) - 2017, Experiment 1, +TOF MS (100 - 1200)



BPC from 2015-10-01 ESI Neg AMICO GRANO Samples list 3 63.wiff (sample 1) - 2017, Experiment 1, -TOF MS (100 - 1200)

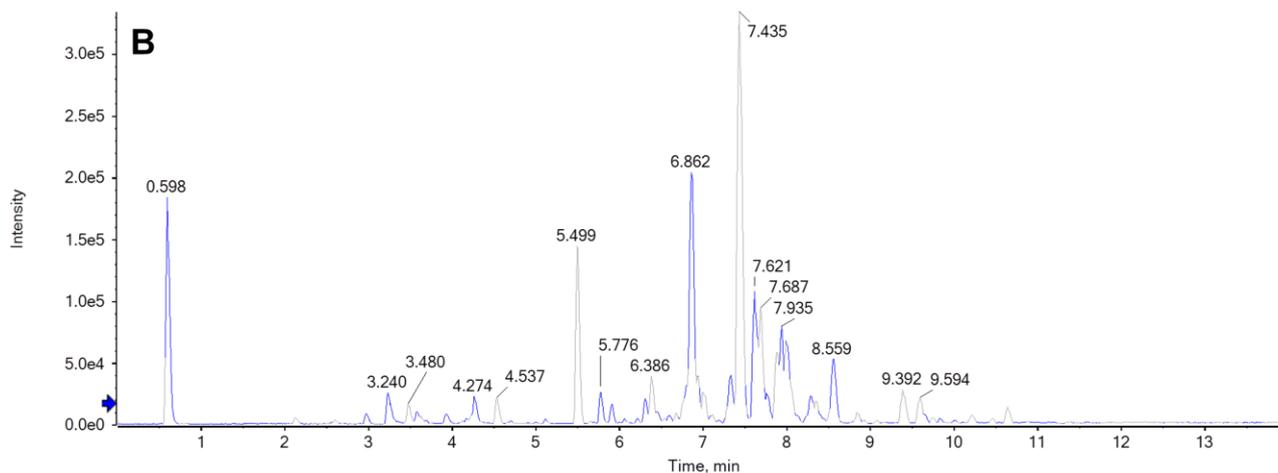


Table 2. Identification of the most significant metabolites related to low infection (LI) and strong infection (SI) groups. Pseudomolecular ions, m/z values, retention times (RT), molecular formula, mass errors, p-values, percentage of change and coefficient of variance (CV) in quality control (QC) are summarized.

Tentative identification	Pseudomolecular ion	m/z	RT (min)	Molecular formula	Mass error (Δ ppm)	p-value	Change SI vs LI [%] ³	CV in QCs [%]
13-keto octadecadienoic acid	[M-H] ⁻	293.2122	3.56	C ₁₈ H ₃₀ O ₃	0,0	3,7E ⁻²⁵ 1, 2	11	8
13-Hydroxy octadecadienoic acid	[M-H] ⁻	295.2282	3.57	C ₁₈ H ₃₂ O ₃	1,0	1,1E ⁻²³ 1, 2	93	4
12,13-Di-Hydroxy octadecadienoic acid	[M-H] ⁻	311.2228	2.69	C ₁₈ H ₃₂ O ₄	0,3	1,0E ⁻²² 1, 2	83	8
12, 13-Di-hydroxy octadecenoic	[M-H] ⁻	313.2385	2.93	C ₁₈ H ₃₄ O ₄	1,7	3,0E ⁻²² 1, 2	89	4
5-Nonadecanylresorcinol (C19:0)	[M-H] ⁻	375.3286	6.66	C ₂₅ H ₄₄ O ₂	4,5	4,5E ⁻²¹ 1, 2	-59	9
5-Heneicosylresorcinol (C21:0)	[M-H] ⁻	403.3601	7.20	C ₂₇ H ₄₈ O ₂	4,7	1,6E ⁻²⁰ 1, 2	-129	4
5-Tricosylresorcinol (C23:0)	[M-H] ⁻	431.3888	7.75	C ₂₉ H ₅₂ O ₂	1,6	1,3E ⁻¹⁸ 1, 2	-89	4
Diacylglycerol (C15:1/C18:2)	[M+H] ⁺	577.4825	6.59	C ₃₆ H ₆₄ O ₅	0,3	1,9E ⁻¹⁹ 1, 2	152	6
Phosphatidic acid (C18:2/C18:2)	[M+NH ₄] ⁺	714.5091	7.23	C ₃₉ H ₆₉ O ₈ P	1,6	1,2E ⁻¹⁹ 1, 2	-85	5
Phosphatidic acid (C16:0/C18:2)	[M+NH ₄] ⁺	690.5060	7.46	C ₃₇ H ₆₉ O ₈ P	1,5	6,3E ⁻²⁰ 1, 2	-82	6

¹: significant according to t-test (p value ≤ 0.05);

²: significant according to FDR correction

³: increased/decreased percentage of change in the first group (Strong Infection).

Figure 2. Venn [diagram](#) shows all possible relations between three extraction procedures selected, common molecular features of these extraction procedures being represented by intersections of the circles. Venn diagrams depicts shared and unique molecular features detected in the wheat extracts prepared under the different extraction procedures and analyzed using (A) UHPLC–ESI(+)-QTOF and (B) UHPLC–ESI(–)-QTOF.

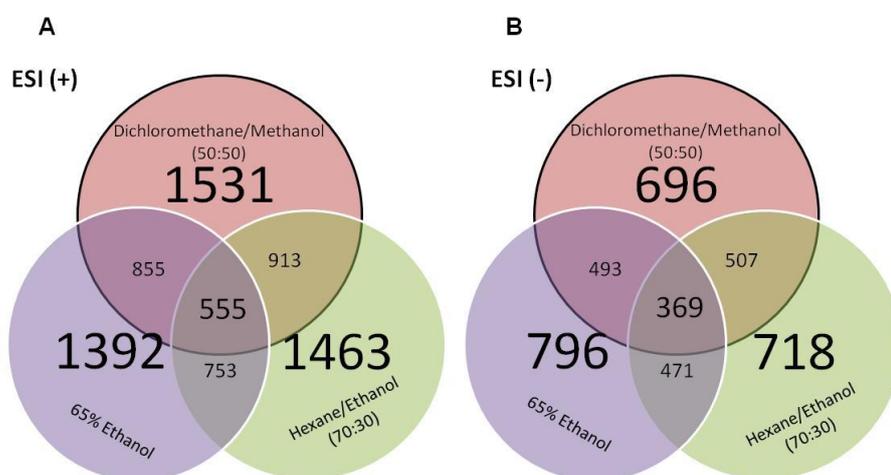


Figure 3. Unsupervised and supervised statistical models using principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The unsupervised models, PCA, are the first step in the data analysis in order to detect sample clustering in the measured data, based on linear combinations of their shared features. PCA scores plot for low (green) and strong infection (red) levels using positive ionization mode (A) and negative ionization mode (B). Discriminant models are based on building models for the known classes. In this case, OPLS-DA scores plot for low (green) and strong (red) infection levels using positive ionization mode (C) and negative ionization mode (D). The score plots of these statistical models, $n = 86$.

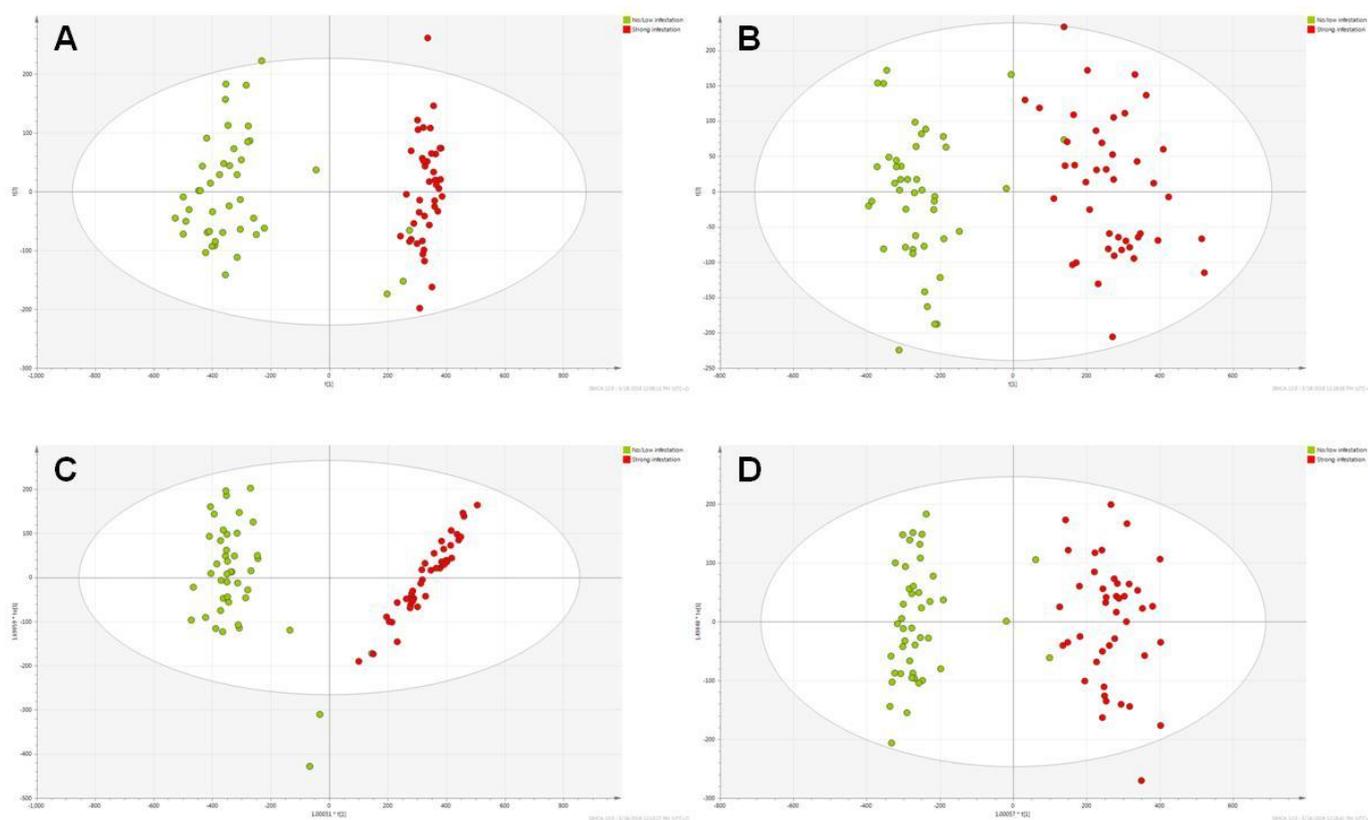


Figure 4. 12, 13-DiHome and PA (18:2/18:2) variable trend plots show up and down regulated markers for strong and low infection levels to confirm the **behavior** of selected variables across all samples. On the one hand, 12, 13-DiHome m/z 313.2385 retention time 2.93 min (A), SI level responses were higher than LI level. On the other hand, PA (18:2/18:2) m/z 714.5091 retention time 7.23 min (B), SI intensities have a negligible signal.

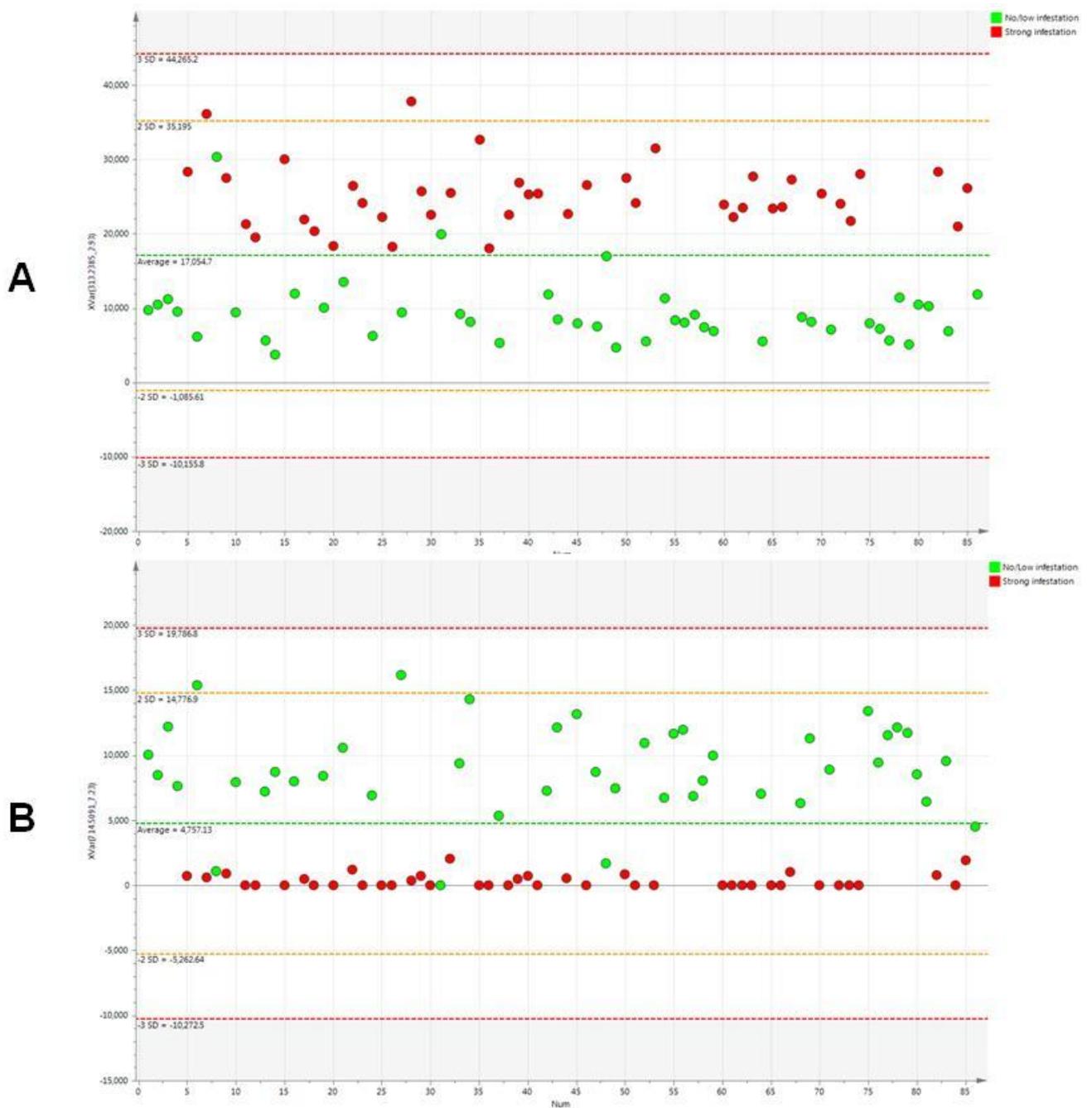


Table 1 Supplementary material. Summary of low infection and strong infection levels related to *Fusarium* infection level, *Fusarium* spp. and mycotoxin content range.

Groups	<i>Fusarium</i> infection level		<i>Fusarium</i> spp.	Mycotoxin content range
	ESI (+)	ESI (-)		
Strong infection (SI)	73% (1-5)	72% (1-5)	<i>F. poae</i> , <i>F. sporotrichoides</i> , <i>F. graminearum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i>	13-10510 µg/Kg
Low infection (LI)	86% (0-1)	90% (0-1)	<i>F. poae</i> , <i>F. avenaceum</i> , <i>F. equiseti</i>	0-2773 µg/Kg

Chapter 4

Structural characterization of glucuronidated mycotoxin metabolites by High Resolution-Ion Mobility Mass Spectrometry.

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by a large number of fungal species potentially infesting foodstuffs at all stages of food production, processing and storage. Therefore, humans and animals can be simultaneously exposed through the diet to different mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN), and emerging *Alternaria* toxins. Evaluation of the frequency and levels of human and animal exposure to these mycotoxins can be performed by measuring the levels of the relevant biomarkers in urine. Available data on the toxicokinetics of these mycotoxins in animals suggest that DON and ZEN glucuronidated metabolites (i.e. DON-3GlcA, DON-15GlcA, ZEN-14GlcA) can be used as urinary biomarkers [1-2]. Regarding alternariol (AOH) and alternariol-methyl ether (AME) it has been demonstrated that they are targets for oxidative as well as conjugative metabolism and the formation of glucuronides and sulfates of AOH and AME has been demonstrated in cultured human Caco-2 cells [3-4]. In addition, Pfeiffer et al. demonstrated that AOH and AME are glucuronidated during their absorption in the intestinal tract and the first pass through the liver [5]. As a consequence, the determination of these glucuronidated forms is essential to assess animal and human exposure.

Because of the absence of analytical standards, these glucuronidated metabolites are mainly chemical or enzymatic in house-synthesized by research groups. Incubation with different UDP-glucuronosyltransferase isoforms has been reported to be an efficient system to obtain DON, ZEN, AOH and AME glucuronides [3,6-8]. The rate of the glucuronidation process depends on UDPGT activity in the endoplasmatic reticulum and the accessibility of uridine diphosphate glucuronic acid (UDPGA). This justify the fact that glucuronidation is specie-dependent [3,6-8]. The obtained polar products generally are fractionated by semi-preparative HPLC and, when sufficient amount of toxins is obtained, their chemical structures are elucidated by nuclear magnetic resonance (NMR). The NMR-based approach is efficient for evaluating isomeric heterogeneity, and for structural elucidation, but has the limitation of needing a considerable amount of analytes and obtaining a single molecular species following the purification steps. In this frame, a new analytical technique, ion mobility spectrometry (IMS) is gaining wider recognition, since this promising approach can overcome the above mentioned NMR limitations, making it an ideal candidate for improving confidence in the identification and separation of structurally closely related isomers. IMS is a gas-phase electrophoretic technique that provides a new dimension (3D) of separation based on size, shape, and charge of ions [9-11]. So far, three major IMS-MS separation approaches are currently commercially available: drift-time IMS (DT-IMS), traveling-wave IMS (TW-IMS), and high field asymmetric waveform IMS (FAIMS), also known as differential-mobility spectrometry (DMS) [9-11]. In DT-IMS [12], ions move through a homogeneous, continuous electric field in a drift tube in

the presence of neutral gas molecules. DT-IMS consists of a series of stacked-ring electrodes where a near-uniform electric field is created along the axis of the drift tube. The carrier gas and the gaseous sample are introduced into the ionization region, while a counter current flow of a neutral gas (mostly nitrogen, helium or argon), called the drift gas, is introduced from the side of the detection region [9]. Thus, species with the same mass-to-charge ratio (i.e., isomers) can be separated according to their ability to pass through a tube filled with a gas under the influence of an electric field.

The synthesized glucuronides have the same mass-to-charge ratio, but a different three-dimensional (3D) conformation. Consequently, the time taken for each parent ion to drift through the tube will be significantly different. In addition, the recorded ions drift time, the time required for molecules to cross the drift tube, which depends principally on the collision frequency between the ions and the buffer gas [10], also allow the calculation of the collision cross section (CCS) according to the Mason-Schamp equation [12].

CCS is an important distinguishing characteristic of an ion in the gas phase, which represents the effective area for the interaction between an individual ion and the neutral gas through which it travels [10], being related to its chemical structure and 3dimensional conformation.

Therefore, the aim of this study was to structurally characterized and differentiated DON, ZEN, AOH and AME glucuronidated forms using high resolution mass spectrometry (HRMS) equipped with drift tube ion-mobility to provide another dimension of separation and measure their CCS values.

Materials and Methods

Chemicals and reagents

Mycotoxin standard AOH, AME and ZEN were purchased from Sigma (Stuttgart, Germany). Analytical standard of DON was purchased from Romer Labs[®] (Tulln, Austria). HPLC grade-solvents methanol, acetonitrile and acetic acid were obtained from Sigma (Stuttgart, Germany). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA). Human liver microsomes were purchased from Sigma Aldrich and stored at -80°C. Uridine 5'-diphosphoglucuronic acid, uridine 5'-diphospho-N-acetylglucosamine were purchased from Sigma-Aldrich (St. Louis, MO). UGT Reaction Mix Solution B containing 250 mM of Tris-HCl, 40 mM of MgCl₂, and 0.125 mg/mL of alamethicin in water was purchased from BD Biosciences (Woburn, MA).

Glucuronidation assay

Human liver microsomes were individually incubated with AOH, AME, ZEN and DON following the protocols already reported in literature [3,6-8]. Incubation mixtures were evaporated to dryness, reconstructed in methanol and analyzed by LC-MS.

UHPLC-IM-Q-ToF analysis

UHPLC Agilent 1290 Infinity LC system coupled to commercial prototype IM-MS, which incorporates a drift tube coupled to a quadrupole time-of-flight mass spectrometer (IM-Q-TOFMS, Agilent Technologies, Santa Clara, CA) was employed. An orthogonal electrospray ionization (ESI) source (Agilent Jet Stream) was used.

For the chromatographic separation, a reversed-phase C18 Acquity HSS T3 column (Waters) with 2.10×100 mm and a particle size of 1.8 μm heated to 40 °C was used. 5 μL of sample was injected into the system at the flow rate of 0.300 ml/min. The autosampler temperature was kept at 5 °C. Gradient elution was performed by using bidistilled water (eluent A) and acetonitrile (eluent B) both acidified with 0.5% acetic acid. From the initial conditions set at 20% B, eluent B was increased to 40% in 5 min and to 95% in 1 min; after an isocratic step (2 min), the system was re-equilibrated to initial conditions for 2 min. The total run time was 10 min.

The ESI source was operated in negative ionization mode (ESI) with a nitrogen sheet gas temperature at 400°C at a flow rate of 12L/min and the following voltages: capillary 4500V, nozzle 1700V. Nitrogen drying gas applied at the source entrance was heated at 150°C at a flow rate of 10 mL/min. The ion mobility drift gas pressure (nitrogen) was maintained at 4 Torr and 28.8 °C; the drift tube entrance voltages was set to 1700V.

The QTOF spectrometer operated in full scan mode from 50 to 1000 m/z and ions were targeted for collision induced dissociation (CID) fragmentation based on the previously determined accurate mass and retention time.

CCS calculation calculation

Drift tube Ion Mobility provides a direct accurate method to calculate the CCS (Ω) using the Mason-Schamp equation (May et al. 2014). IM-MS Browser (Agilent Technologies) was employed for data acquisition and processing. First, the drift ramp method with infusion based acquisition was applied with calibration solution. The method consists of time segments in a single acquisition, each segment with a different drift tube voltage (1000 – 1700 V with 100 V steps). The calibration factor was calculated based on the known calibrant CCS values and this factor was applied to the next sample analyzes. 5 μL of sample was injected onto the column and measured without any changes

of source conditions or within the ion optics following the drift tube. Instead of the time segmented acquisition, the analysis was sequentially repeated with the different drift tube voltage (1100, 1300, 1500 and 1700 V). Using the calibration factor and reference CCS (243 \AA^2) from the reference mass 922, the CCS values for the ions of interest were calculated. On the basis of a propagation-of-error analysis [12] incorporating the limits of precision for individual experimental parameters, it was estimate the accuracy of all CCS values to be better than 2%.

Computational volume calculation

The 3D structures for AOH and AME were retrieved by the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) [13]. All the glucuronidated metabolites were obtained by adding the glucuronide group computationally with the software sybyl version 8.1 (<http://tripos.org>). All the structures have were checked for atom- and bond-type assignments and were energy-minimized using the Powell algorithm with a coverage gradient of $\leq 0.05 \text{ kcal (mol \AA)}^{-1}$ and a maximum of 500 cycles. Volume and area were calculated by using the UCSF Chimera Software version 1.11 (<http://www.rbvi.ucsf.edu/chimera>) [14]. All images were obtained using the PyMol Software version 1.7 (<http://pymol.org>).

Result and Discussion

HRMS characterization

When ZEN was incubated with human hepatic microsomes in the presence of UDPGA and the incubation mixture analyzed by reversed-phase UHPLC, one product more polar than ZEN were observed. In UHPLC-Q-TOF analysis operated in the negative ESI mode, it had a quasimolecular ion of m/z 493.1720, which gave rise to fragment ions of 317.1395, corresponding to the loss of glucuronic acid (176.0315 amu) and m/z 175.0245 from the loss of the aglycone from quasimolecular ion (Table 1). This metabolite was assumed to represent ZEN-14GlcA, since the isomer ZEN-16GlcA differ in the fragmentation pathway, involving also the loss of carbon dioxide (44.0095 amu) [6].

Incubation of human microsomes with DON gave rise to two isomeric glucuronides, in agreement with previous literature [8]. The two glucuronidation products afforded primarily ions with m/z 471.1508 in negative ionization mode. However their MS/MS differ for the presence of m/z 441.1422 fragment that is likely to arise from cleavage of the CH_2OH moiety attached al C-6 [15], which supported that this peak was DON-3GlcA. In addition its low intensity agrees with results already obtained using human liver microsome [8], since DON-15GlcA was reported to be the predominant isomer compared to DON-3GlcA.

Table 1. Summary of HRMS mycotoxin metabolites characterization obtained from incubation with human liver microsomes.

Mycotoxin	Elemental composition	Detected m/z	Error (ppm)	MS/MS fragments
ZEN-GlcA	C ₂₄ H ₃₀ O ₁₁	493.1720	-1.0	317.1395; 175.0245
DON-3GlcA	C ₂₁ H ₂₈ O ₁₂	471.1510	-0.4	441.1422; 265.1109; 217.0890
DON-15GlcA		471.1513	-1.1	265.1092; 217.0890
AME-3GlcA	C ₂₁ H ₂₀ O ₁₁	447.0928	1.1	271.0609; 175.0237
AME-7GlcA		447.0938	-1.1	271.0611; 175.0242
AOH-9GlcA	C ₂₀ H ₁₈ O ₁₁	433.0779	-0.7	257.0456; 175.0239
AOH-3GlcA		433.0775	0.2	257.0459; 175.0241
AOH-7GlcA		433.0785	-2.1	257.0460; 175.0245

Two monoglucuronides with quasimolecular negative ions at m/z 447.0950 and fragment ions at m/z 271.0609 and 175.0237 were obtained when AME was incubated with human liver microsomes. The two HRMS/MS spectra were very similar, thus the assignment of the glucuronic acid moiety was not possible. However, considering that AME has only two hydroxyl groups the major glucuronide is proposed to be AME-3GlcA and the minor glucuronide AME-7GlcA, in agreement with previous findings [3], in which trimethylsilylation was applied to putative elucidate the isomer structures.

Regarding AOH, in our study, up to three glucuronide conjugates by microsomes assay were detected. The detection and identification of the third smaller alternariol glucuronides, representing only the 0.7% of the total AOH-glucuronides forms, was allowed, compared to previous works [3], by HRMS which measures the ions with accurate mass enabling the elemental composition calculation also at a low intensity level. Pfeiffer et al. (2009) in fact reported the formation of two AOH-glucuronides, involving position 9- and 3- of the AOH. However, conversely to AME, AOH has 3 hydroxyl groups where glucuronic acid can be linked, and also AOH-7GlcA may occur. HRMS/MS of the three glucuronides were almost identical, with a molecular ion of m/z 433.0779 and fragment ion corresponding to the loss of glucuronic acid (m/z 257.0456) and from the loss of the aglycone from quasimolecular ion m/z 175.0245. Therefore, it remained unknown to which of the three hydroxyl groups of AOH the glucuronic acid moiety was bound. Some assumptions regarding the more or less favourable position for conjugation may be done, but more detailed information is required in order to elucidate the chemical structure of each isomer. Chemical structure of the abovementioned mycotoxins glucuronides are reported in figure 1.

Measurement of the CCS values

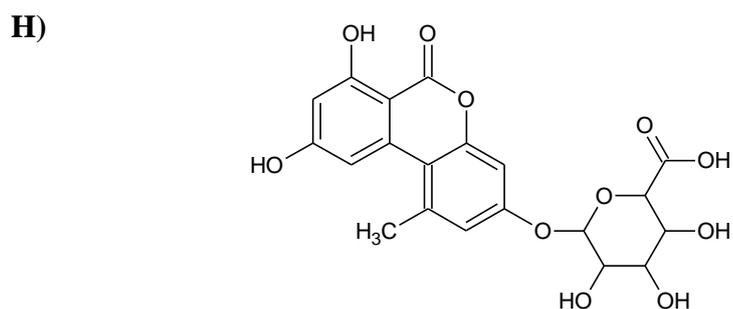
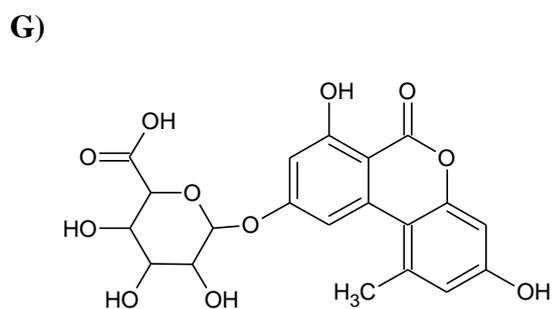
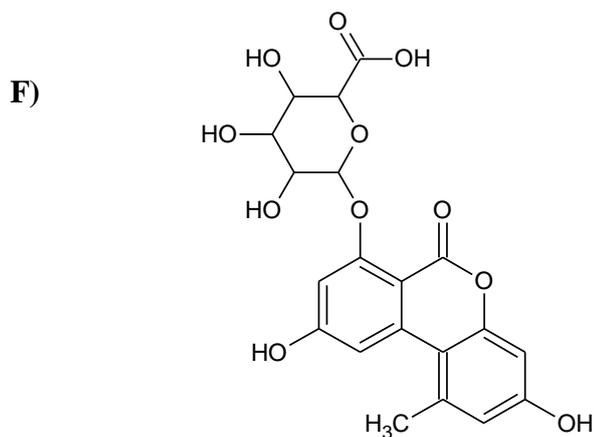
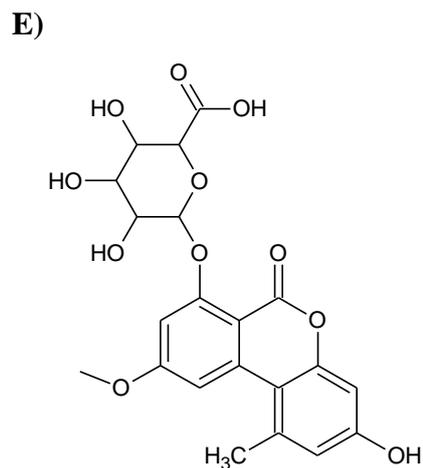
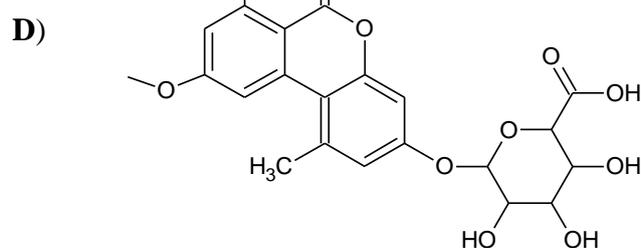
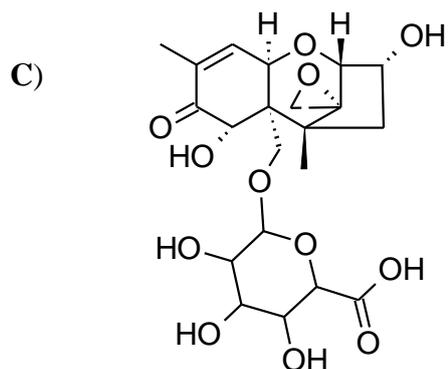
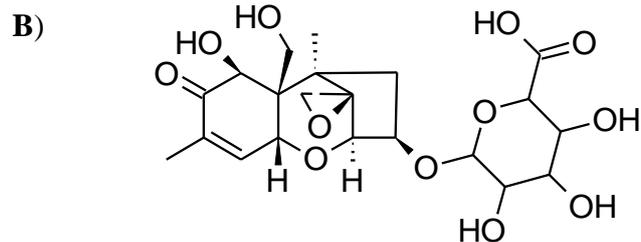
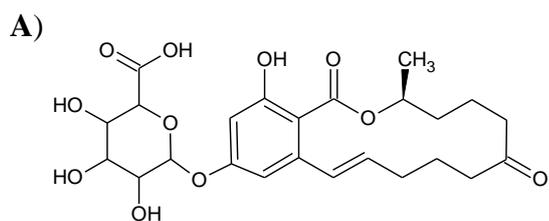
The CCS values of 11 parent and glucuronidated mycotoxins were measured using drift tube ion mobility system as shown in table 2. The correlation coefficient (R^2) between drift time and the corrected CCS was 0.994, which indicate that the method employed is able to measure CCS values of other molecules whit sufficient reliability.

Table 2. Summary of drift time and CCS values obtained for each parent and modified mycotoxin.

Mycotoxin	Drift Time (ms)	CCS (Å)	<i>m/z</i>
AOH	28.98	153	257.045
AOH-9GlcA	37.7	197.1	433.0779
AOH-3GlcA	38.9	203.8	433.0775
AOH-7GlcA	40.36	213.8	433.0785
AME	30.14	159	271.0606
AME-3GlcA	39.98	213	447.0928
AME-7GlcA	39.55	209	447.0938
ZEN-GlcA	41.89	218.8	493.1720
ZEN	34.02	178.2	317.1389
DON-3GlcA	38.29	200.7	471.151
DON-15GlcA	37.98	199	471.1513

The CCS values of the molecules were found to be strongly correlated with their respective molecular masses ($R^2 = 0.91$, Fig. 2), and it was found that the data set was described by a power-law relationship ($y = 0.0244x^{1.8416}$). Power-law equations are found to be descriptors for several phenomena related to mass-size scaling [12]. As earlier explained, the mobility of molecules strongly depends on their collision with the drift gas. Therefore, bigger molecules would experience greater collision and thus would arrive at the detector slower than smaller molecules. This explains the high correlation between the mass and the CCS of the molecules. The same observation was found in earlier studies [10-11, 16] wherein the mass and number of measured CCS values was higher than 100, and also the range of m/z values was larger, thus allowing a better correlation. However, although this correlation is high, relying on mass alone to predict CCS is insufficient as several metabolites, such as glucuronidated isomers, have the same mass but differ in the spatial arrangement of the molecule, thus having different CCS values as shown in Fig. 3., where the three different AOH-glucuronide stereoisomers differing in their glucoroidation sites are represented.

Figure 1. Chemical structure of ZEN-14GlcA (A), DON-3GlcA (B), DON-15GlcA (C), AME-3GlcA (D), AME-7GlcA (E), AOH-7GlcA (F), AOH-9GlcA (G), AOH-3GlcA (H).



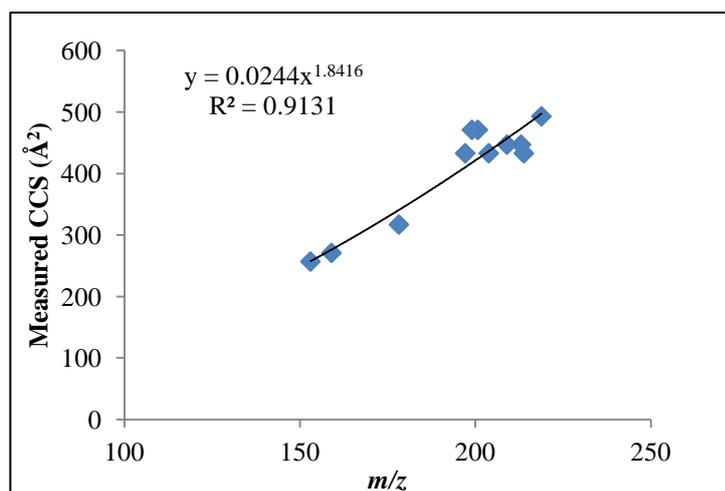


Figure 2. Correlation between detected m/z and experimental CCS values ($n = 11$).

Since only the attachment differs, these 3 molecules have exactly the same mass and MS/MS fragmentation patterns and are therefore difficult to distinguish without comparing their LC retention times to isolated standards.

As shown using a soft surface, AOH-3GlcA and AOH-9GlcA have more compact structures compared to AOH-7GlcA. On the other hand, AOH-9GlcA seems to have a more elongated structure compared to AOH-3GlcA. As molecules continually rotate in the ion mobility cell during DT separation, this elongation creates a bigger surface area available for interaction with the drift gas, hence having a longer drift time. These results indeed show the power of ion mobility in separating and distinguishing mycotoxins stereoisomers, which could therefore provide an additional tool for structural characterization and identification.

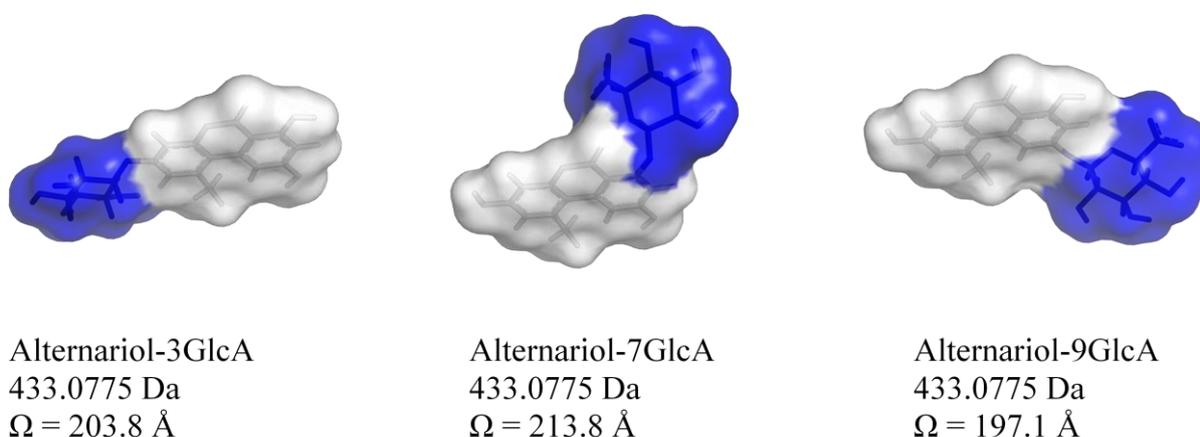


Figure 3. 3-D structure and CCS values of alternaria-glucuronide isomers. Soft surface was added to emphasize the overall shape of the molecule; the blue area represents the glucuronide linked to 3-, 7-, 9- alternariol positions.

Thus, CCS can be considered an important distinguishing characteristic of an ion in the gas phase, being related to its chemical structure and 3dimensional conformation. In addition, since CCS measurements are undertaken in the gas phase, remotely from the ion source, their values are not affected by sample matrix and are consistent between instruments and across a range of experimental conditions [17]. Taking into account the analytical effort made in the last years for validating extraction and detection procedure depending on the sample matrix, the great advantage offered by the CCS values as molecular descriptor is evident. Moreover, it has been demonstrated that neither the concentration of the compound had significant effect on the drift time values and thus on the CCSs [16]. These two aspects represent a great advantage for modified mycotoxins analysis considering that, in general they are present in a low amount, and these glucuronidated metabolites are detected in complex matrices, such as urine and blood, characterized by an higher matrix effect.

The mobility of an ion (K_0) is defined as the ratio of the ion velocity to the magnitude of electric field [9] and is dependent on characteristic properties of a sample, including size, charge and mass of the ion. When two isomers with the same m/z differ in their volume, they will reach the detector separately, resulting in a different drift time. The time taken for each AOH-GlcA parent ion isomers to drift through the tube was significantly different, as shown in figure 4.

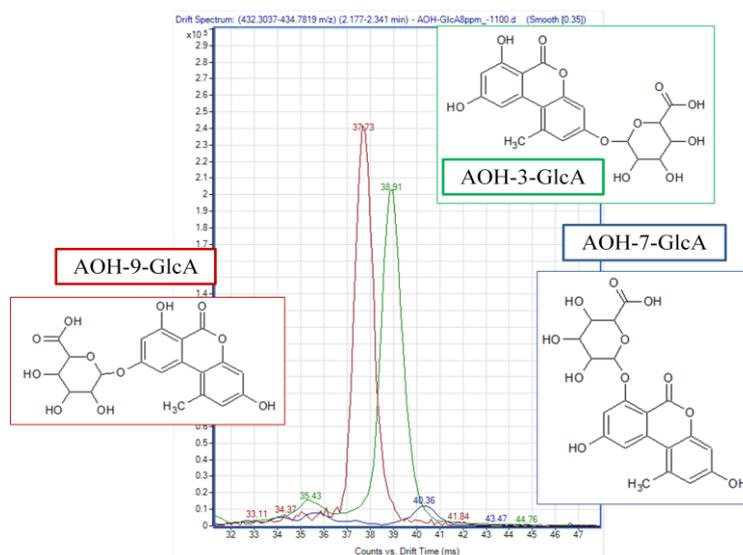


Figure 4. Drift time chromatogram of AOH-GlcA isomers.

Even if the intensity of the slower isomer was really low compared to the other two isomers, it was possible to detect its peak in the drift time chromatogram. Its late arrival to the detector (DT 40.36) could be explained considering its 3D structure as well as its molecular volume. Indeed, its calculated volume was found to be higher (331.5) compared to the other two isomers (328.5,

329.1). By plotting the calculated volume of the 11 mycotoxins considered in this study with their drift time, a good correlation was found ($R^2 = 0.9588$, $y = 1.7391x^{0.5331}$, figure 5).

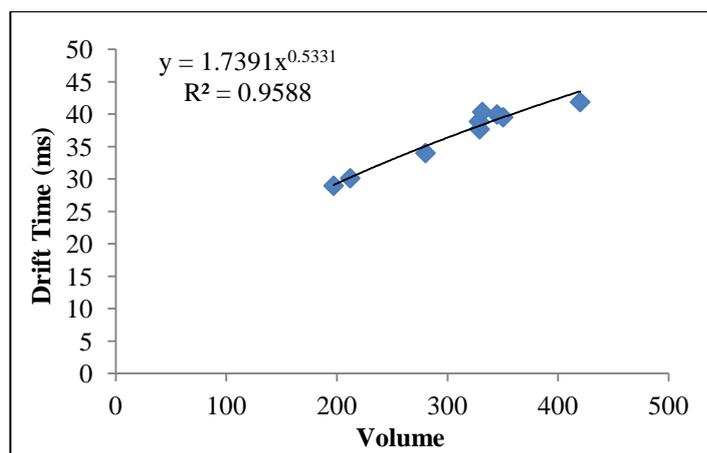


Figure 5. Correlation between molecule volume and the time needed to pass through the drift tube (n = 11).

Therefore, due to the aforementioned considerations, it was possible to putatively assign the structure of AOH-7GlcA to the third small peak obtained after incubation of AOH with human liver microsomes. Even though further studies are needed to unequivocally confirm the molecular structures of mycotoxins glucuronide, our results suggest the power of ion mobility spectrometry as a tool to aid structure assignment of positional isomers.

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SECTION II: wheat quality

Wheat quality assessment attributes are the subject of several viewpoints, depending mainly on the eye of the beholder and the end-use. To decipher “quality” several disciplines are necessary [1]. Starting from breeders, for century’s crops improvement programs focused on the yield, resistance to biotic and abiotic stress to provide quality wheat for the increasing population. Thus the quality for the farmer means productivity and this concept is linked to the need to obtain high yields in order to maximize profits. Later attention has been focused on technological wheat quality to respond to quality industrial requirements. Whole-grain physical properties such as size and shape influence milling yield and screening losses, which determine the processing efficiency and value of the grain. As an example, high quality pasta begins with good quality grain that lead, considering its protein content, to high pasta cooking quality. Thus, wheat quality may be defined by a range of physical and compositional properties where threshold values are set according to end-use requirements [2]. In addition, grain quality is influenced, among other, by genetics, crop management and environment [3].

Also for the consumers, quality represents an important issue and they have become accustomed over the years to demanding grain with particular quality attributes mainly connected to its chemical composition. In this frame, nutritional aspects and health issues are associated with consumption of wheat [4]. In the first case, the still large use of wheat as the main source of nutrients, makes necessary to investigate and enhance the nutritional value of wheat based-foods. Regarding health issue, the increasing consumption of calorie and fat-rich foods which is causing health problems, is also leading to a promoting increases in consumption of fiber-rich foods, such as whole-grain wheat, to reduce health risks. Among other, nutritional benefit might be connected with a relatively high concentration of antioxidant compounds.

During the following chapters, particular focus is point towards alkylresorcinols (AR), phenolic lipids found in high levels in the outer layers of wheat kernels [5] that exert their biological effects [6] when wheat is consumed in the form of whole grain. Their homologues composition, together with other lipid metabolites, has been characterized across three different ancient grains and described in chapter five by applying a metabolomic approach. This analytical strategy was selected since allowing the simultaneous characterization of large numbers of small metabolites in wheat matrix, it actually offers a more detailed and comprehensive molecular picture of its composition [7]. Metabolomics can detect small molecules such as amino acids, organic acids,

SECTION II: wheat quality

sugars metabolites and many other health-related compounds such as phenolic compounds, carotenoids or anthocyanins. Such molecules are known to play important roles in quality of wheat. In addition, these antioxidants compounds (e.i. polyphenols and phenolic lipids) represent a valuable source not only for humans, but also for the wheat plant itself. In chapter six, indeed, their direct role in modulating accumulation of mycotoxins in ten wheat lines has been reported.

The fifth chapter was published on International Journal of Molecular Science and the sixth chapter will be submitted on Journal of Agricultural and Food Chemistry.

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Chapter 5

Characterization and discrimination of ancient wheats: a metabolomic approach

Characterization and Discrimination of Ancient Grains: A Metabolomics Approach

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Abstract: Hulled, or ancient, wheats were the earliest domesticated wheats by mankind and the ancestors of current wheats. Their cultivation drastically decreased during the 1960s; however, the increasing demand for a healthy and equilibrated diet led to rediscovering these grains. Our aim was to use a non-targeted metabolomic approach to discriminate and characterize similarities and differences between ancient *Triticum* varieties. For this purpose, 77 hulled wheat samples from three different varieties were collected: Garfagnana *T. turgidum* var. *dicoccum* L. (emmer), ID331 *T. monococcum* L. (einkorn) and Rouquin *T. spelta* L. (spelt). The ultra high performance liquid chromatography coupled to high resolution tandem mass spectrometry (UHPLC-QTOF) metabolomics approach highlighted a pronounced sample clustering according to the wheat variety, with an excellent predictability (Q^2), for all the models built. Fifteen metabolites were tentatively identified based on accurate masses, isotopic pattern, and product ion spectra. Among these, alkylresorcinols (ARs) were found to be significantly higher in spelt and emmer, showing different homologue composition. Furthermore, phosphatidylcholines (PC) and lysophosphatidylcholines (lysoPC) levels were higher in einkorn variety. The results obtained in this study confirmed the importance of ARs as markers to distinguish between *Triticum* species and revealed their values as cultivar markers, being not affected by the environmental influences.

Keywords: small grains; non-targeted metabolomics; phenolic lipid compounds; lipidomics; foodomics

1. Introduction

Cereals represent one of the most important commodities providing basic nutrients to human diet, such as corn, rice, sorghum, or wheat, whose starchy grains are used as food. Cereals are annual plants, and cereal crops must be reseeded for each growing season. These cereal grasses, domesticated during the Neolithic period, formed the basis of systematic agriculture. In the particular case of *Triticum* species, they have been classified into hulled and free-threshing (“naked”) forms. Among the latter, bread and durum wheat are the most important *Triticum* species cultivated worldwide [1].

On the one hand, “hulled wheats”, which means that the kernel retains its husk during harvest, were the earliest domesticated wheats by mankind and are the ancestors of current wheats. Ancient wheat cultivation drastically decreased during the 1960s due to dietary and economic changes, as well as the introduction of bread and durum wheat, which are both higher yielding [2]. However, during the past years, the increasing demand for natural and organic products led to the rediscovery of ancient wheat species such as spelt (*Triticum spelta* L.), emmer (*Triticum dicoccum* L.), and einkorn (*Triticum monococcum* L.) [3]. This renewed interest is associated with the desire for a healthy and equilibrated diet, such as the Mediterranean diet. In fact, hulled wheat has been recognized as a dietetic and healthy cereal, and it is recommended in treatment of disease related to high blood cholesterol, colitis, and allergies [3]. A comparison of ancient and standard wheat highlighted that the ancient grains are characterized by a higher content of soluble dietary fiber, proteins, and lipids (mostly unsaturated fatty acids) [4]. In addition, ancient wheats provide a much greater proportion of rapidly digestible starch (RDS) and higher starch digestion index (SDI) compared to bread wheat [5,6]. Concerning trace elements, emmer, einkorn, and spelt mainly differed from wheat cultivars for higher contents of Li, Mg, P, Se, and Zn [7].

Another additional benefit might be connected with a relatively high concentration of antioxidant compounds, which can contribute to the excellent nutritional properties of the hulled wheats. Among these phenolic compounds, alkylresorcinols (ARs) represent one of the major groups that are found in high levels in the outer layers of the kernels [8]. The impact of ARs have been studied for wholegrain wheat and rye, because these layers are mostly removed during flour production [9]. Furthermore, the C17:0/C21:0 ARs homologue ratio has been proposed to differentiate between common and durum wheats [8,9]. Recently, the concentration of saturated ARs allowed the differentiation of *Triticum* species according to their degrees of ploidy [10]. In particular, the levels of all ARs homologues significantly differed between hexaploid (bread wheat and spelt), tetraploid (durum and emmer), and diploid (einkorn) species.

Up to now, targeted methods, developed for quantification of a given class of metabolites, have been exclusively applied to investigate differences between ancient *Triticum* varieties [9,10]. Nevertheless, nowadays, advanced analytical tools have permitted the simultaneous analysis of hundreds of metabolites, allowing a better characterization of small molecules (up to 1200 Da), therefore, the composition of complex plant matrices can be investigated in-depth [11]. In fact, in the last decade, the applicability of metabolomics to food science and nutrition research has strongly emerged [11–16].

In the present study, a metabolomic untargeted method was developed to investigate a broad spectrum of ancient wheats compounds in order to determine the relative roles of genotype and environment in determining the metabolites composition. Identifying similarities and differences that permit to distinguish between ancient *Triticum* varieties may be useful for the determination of nutritional aspects and adulterations, since emmer and einkorn are more expensive than spelt. For this purpose, 77 hulled wheat samples were analyzed using a non-targeted metabolomics approach based on solid liquid extraction followed by a reversed phase liquid chromatography separation coupled to quadrupole-time-of-flight mass spectrometer (LC-QTOF), and multivariate data analysis.

2. Results

2.1. Multivariate Modeling

To perform sample classification, at first all 77 chromatograms were independently aligned for both polarities (see Figure 1). This returned a primary dataset with 4191 and 3253 features for positive and negative modes, respectively. Afterward, data reduction was performed based on previous work [12]. The primary filtering step excluded the background peaks present in blank samples. Then, in order to remove signal redundancy, only monoisotopic peaks were considered. The third filtering step was performed by choosing all the molecular features present in at least 50% of the samples in one group. This last step removed 2051 peaks for positive mode and 1666 for negative mode, representing approximately 50% of the original dataset, leaving 686 and 490 peaks for positive and negative, respectively.

At this point, the principal components analysis (PCA) models were built to investigate the metabolome, and therefore, differences between all three classes of wheat. The mechanism, already explained elsewhere [13], is based on the ability of the PC model to cluster samples in an unsupervised approach, since no information on group identity is used to construct the model. The PCA score plot obtained for positive and negative ionization modes are summarized in Figure 2.

The first two principal components (PC) explained 50% of the total variance of the ESI(+) (32.9% and 17.1% for the PC1 and PC2, respectively) and 47.2% of the ESI(−) model (25.8% and 21.4% for the PC1 and PC2, respectively).

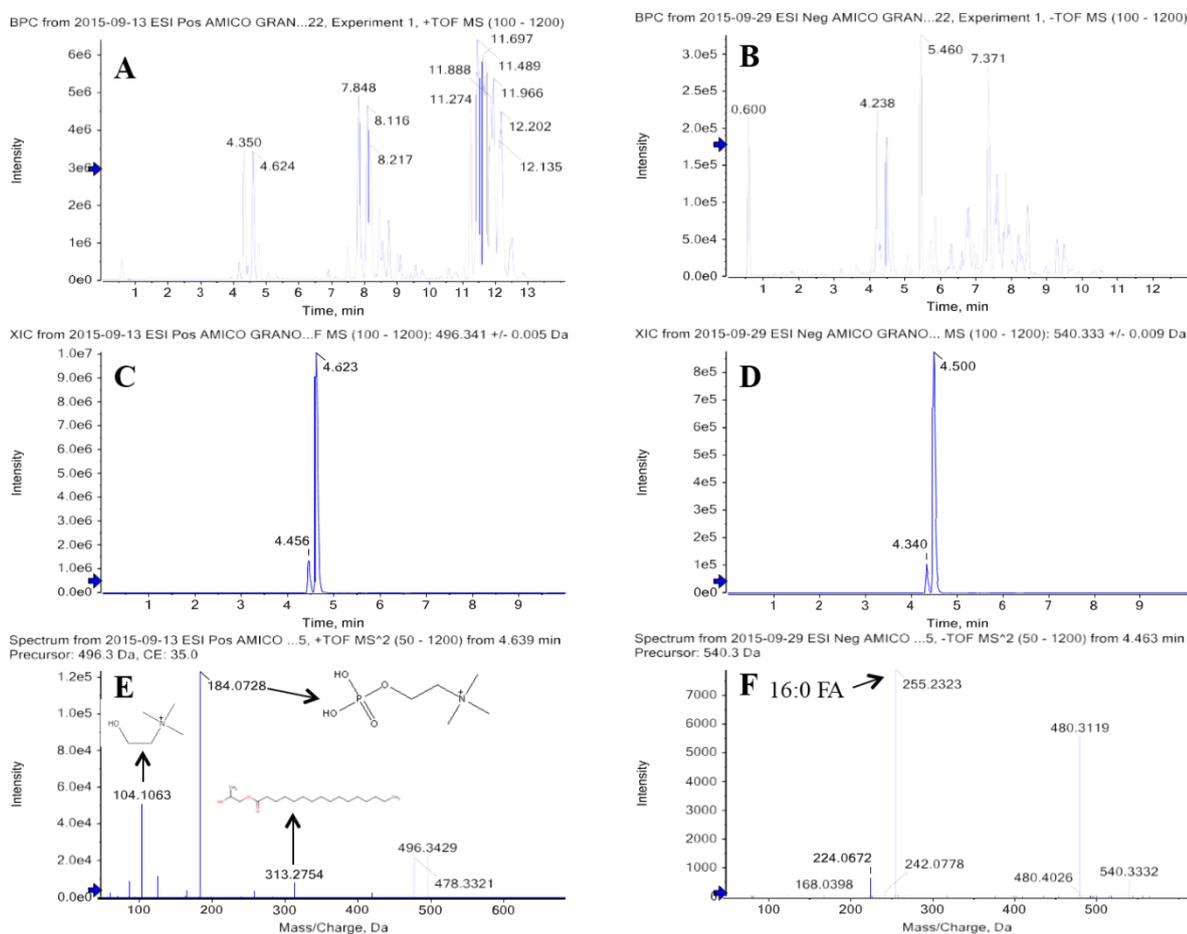


Figure 1. Ultra high performance liquid chromatography coupled to high resolution tandem mass spectrometry base peak chromatograms of ancient wheat extract obtained using positive (A) and negative (B) ionization modes. Extracted ion chromatogram (XIC) of Lyso PC 16:0 ionized in positive ($[M + H]^+$ m/z 496.3399) (C) and negative ($[M + HCOO]^-$ m/z 540.3332) (D) modes. Product ions acquired automatically by the information-dependent acquisition (IDA) method for the m/z 496.3399 (E) and m/z 540.3332 (F) parent ions. Blue arrows are thresholds and indicators in terms of RT and m/z values.

Samples were arranged in three major groups, indicating a sample clustering according to the varieties: emmer, einkorn, and spelt. A more pronounced clustering, among sample classes, was obtained in the ESI(+) data, as it can be seen in the PCA score plot (Figure 2A), even if one sample from ID331 is mixed up with Garfagnana variety. One out of 77 samples fell outside the 95% confidence ellipse, as it is shown in the ESI(−) PCA score plot (Figure 2B). This is considered a

“moderate” outlier, while samples out of the confidence interval value of 99% (critical limit) are “strong” outliers. For this reason, this outlier was kept into the data set. No clustering according to vegetative year, growing location, and farming condition was found.

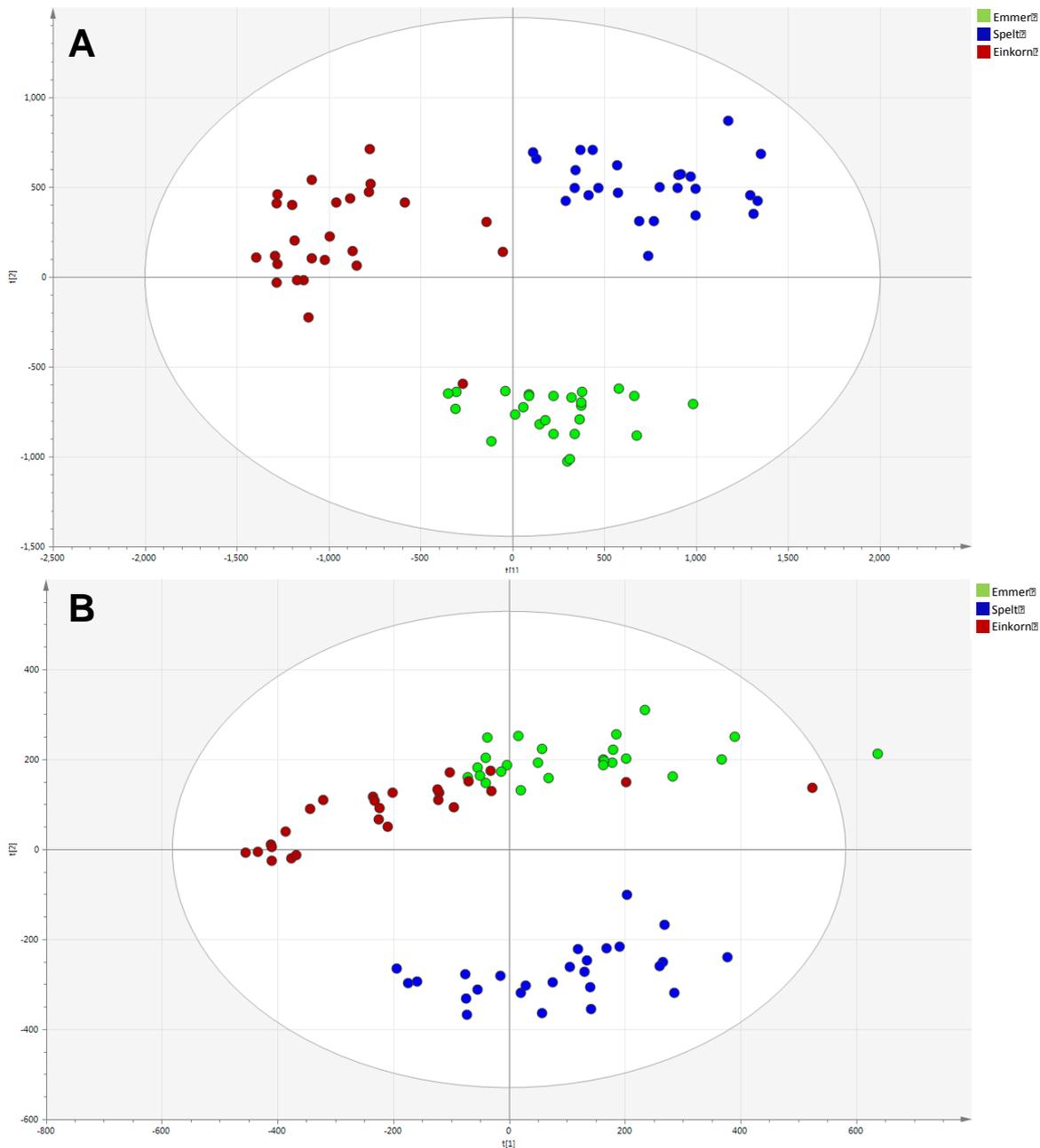


Figure 2. Unsupervised principal components analysis (PCA) models built from positive (A) and negative (B) ionization data set. Red dots: Einkorn (ID331). Green dots: Emmer (Garfagnana). Blue dots: Spelt (Rouquin).

The differences between these three varieties were confirmed when partial least squares discriminant analysis (PLS-DA) (see Figure 3) and orthogonal partial least squares discriminant analysis (OPLS-DA) models were constructed. PLS-DA was performed to maximize differences

and OPLS to highlight key variables and potential biomarkers. The quality of the models was excellent as shown in Table 1, where all the goodness of fit (R^2) and the prediction ability (Q^2) parameters are summarized. PLS-DA models highlighted highly quality parameters that were not significantly improved to OPLS-DA models, suggesting a low “structure noise” in the data set. OPLS-DA has the capacity to improve prediction ability because it separates out the structured noise, which is modeled separately.

The high Q^2 values obtained for both supervised models indicated excellent predictabilities and suggested that the metabolomics approach applied was able to reveal differences between the grain varieties studied.

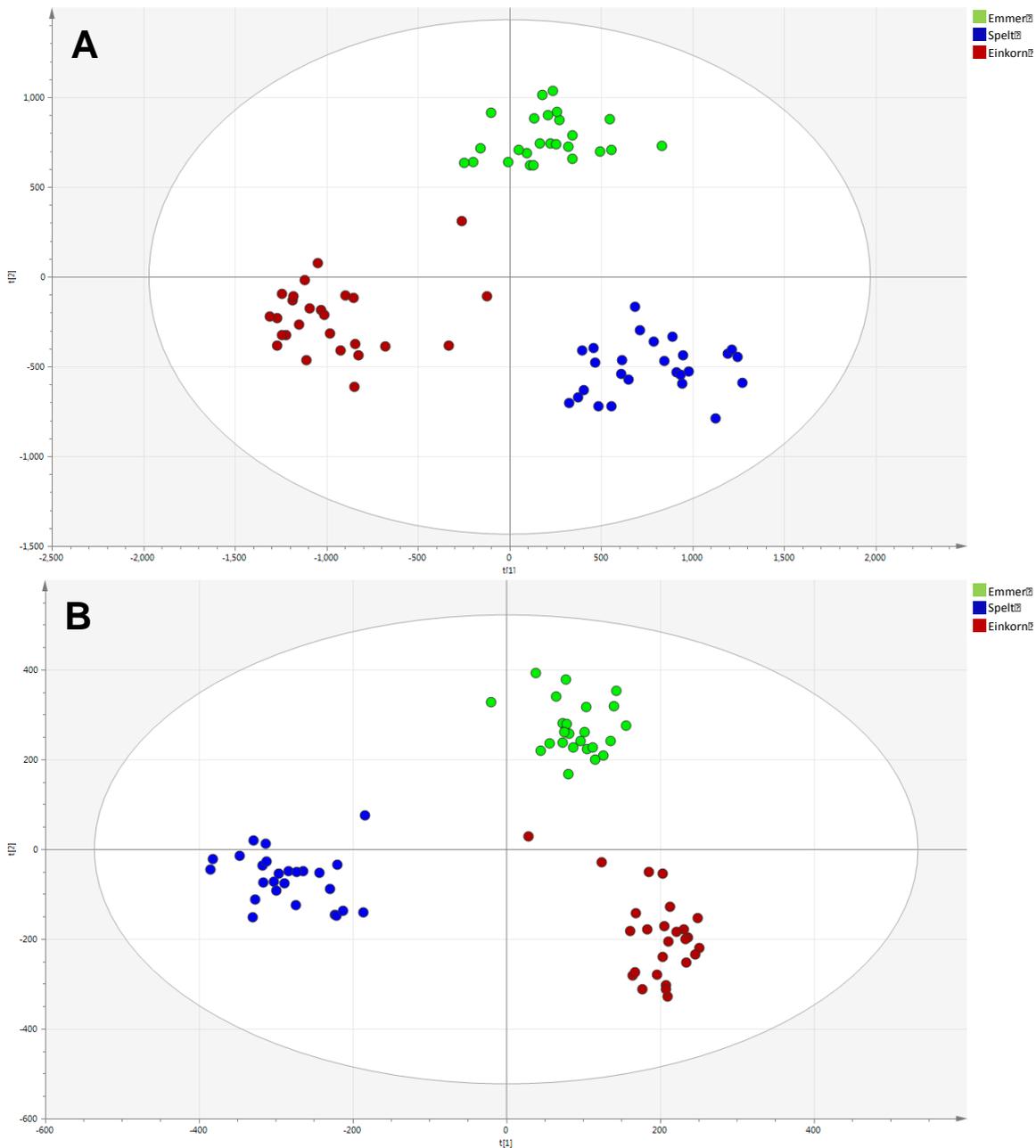


Figure 3. (A,B) PLS-DA model built with positive ionization data ($R^2X = 0.578$, $R^2Y = 0.942$, $Q^2 = 0.916$) and negative ionization data ($R^2X = 0.709$, $R^2Y = 0.967$, $Q^2 = 0.944$). In both ionization modes these three varieties were clearly separated. Red dots: Einkorn (ID331). Green dots: Emmer (Garfagnana). Blue dots: Spelt (Rouquin).

Table 1. Statistical values for PCA, PLS-DA, OPLS-DA models. R^2X (cum) and R^2Y (cum) represent the variance of the x and y variables explained by the model, while Q^2 is the cumulative predicted variation in the Y matrix.

Statistical Parameters	ESI(+) Models			ESI(-) Models		
	PCA	PLS-DA	OPLS-DA	PCA	PLS-DA	OPLS-DA
R^2X (cum)	0.81 6	0.578	0.579	0.89	0.709	0.709
R^2Y (cum)	-	0.942	0.942	-	0.967	0.967
Q^2 (cum)	0.66 3	0.916	0.917	0.77 8	0.944	0.956

Principal components analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

Moreover, in order to avoid the risk of overfitting, each generated model was validated by cross-validation tool [14], using the leave 1/3 out approach. Misclassification tables (see Table S1) indicate that 100% of ancient wheat lines (three out of three) were correctly classified in the ESI(-) data, while in ESI(+) OPLS-DA model the percentage of total correct classification was 98.7%, as one sample was not correctly predicted.

2.2. Discriminant Metabolites Identification

In order to obtain relevant information regarding the metabolic differences between the varieties, a limited set of statistically meaningful metabolites had to be selected. In the present study, discriminant markers selection was performed merging the metabolites resulting from the PLS-DA loadings plot with those obtained using the Variable Influence in Projection (VIP threshold > 1.5). The identity of compounds that were found to be significant in class separation was confirmed by ultra high performance liquid chromatography coupled to high resolution tandem mass spectrometry (UHPLC-HRMS) analysis based on accurate MS and MS/MS data, as well as theoretical and experimental isotopic patterns were evaluated in-depth. Features were searched against the METLIN, KEGG, LIPIDMAPS and HMDB online databases [11]. At the same time, empiric formulae of the unknown compounds were calculated by Formula Finder option in Peak View software (version 2.2, SCIEX, Concord, ON, Canada) aiding to confirm or refuse potential structures. Subsequently, comparison of the fragmentation pathway of the proposed compound,

found in the above-mentioned databases, with the fragments experimentally obtained confirmed the identity.

All metabolites identified are summarized in Table 2 including tentative identification, pseudomolecular ion, retention time, mass error (ppm), higher metabolite intensities associated with ancient grain varieties, and VIP values. For all metabolites identified calculated mass error (Δ ppm) was lower than 4 ppm.

In the present work, seven statistically significant markers, belonging to the resorcinol's class, were tentatively identified. The seven ARs were detected in negative ionization mode producing both a $[M - H]^-$ and the $[M + HCOO]^-$. Since these metabolites are commonly detected by GC-MS [17] or HPLC-UV [18] techniques, MS/MS spectra were not available in the online database. Thus, we tentatively identified them checking the exact mass (mass error less than 1.7 ppm), the match of experimental and theoretical isotope pattern in terms of spacing and relative intensities, and the most abundant fragment ion $[M - C_2H_2O]^-$ yielded from the resorcinol ring, resulting from the neutral loss of 42 Da (see Figure S1) [19].

For the lipid identification, LipidView software (version 1.3 beta, SCIEX) was employed. Diacylglycerols (DGs) and triacylglycerols (TGs) were detected in positive mode as ammonium adducts, giving a pseudomolecular ion $[M + NH_4]^+$. Identification of 1-palmitoyl-2-linoleoyl glycerol was based on the accurate m/z 610.5405 $[M + NH_4]^+$, theoretical and experimental isotopic patterns and on the product ions m/z 337.2737 and m/z 313.2737 corresponding to the loss of palmitic and linoleic acid, respectively. The mass spectrum of a TG contained two different fatty acids; 1,2-dipalmitoyl-3-linoleoyl glycerol (m/z 848.7708), and two DG ions (m/z 551.5034). Similarly, the MS/MS spectrum of 1-palmitoyl-2-oleoyl-3-eicosenoyl-glycerol (m/z 904.8339), as it contains three different fatty acid species, exhibited three DG ions (m/z 631.5660, m/z 605.5503 and m/z 577.5190) [15,16].

Phospholipids were detected in both ionization modes and confirmed by ESI(+) with a characteristic fragment ion of m/z 184.0739 for phosphatidylcholines (PC) and m/z 184.0739, m/z 104.1078, m/z 86.0974 m/z for lysophosphatidylcholines (lysoPCs) [20]. Lyso PC (16:0) fragmentation pattern that allows identification of the compounds, is depicted in Figure 1.

Table 2. Identification of discriminant metabolites between the three wheat varieties.

Biochemical Category	Biochemical Class	Tentative Identification	Pseudomolecular Ion	<i>m/z</i>	RT (min)	Elemental Formula	Mass Error (Δ ppm)	Higher Metabolite Intensity in	VIP Value
Phenols	Resorcinols	Heptadecylresorcinol (C17:0)	[M - H] ⁻	347.2956	6.3	C ₂₃ H ₄₀ O ₂	1.7	spelt	1.5
		Nonadecanylresorcinol (C19:0)	[M - H] ⁻	375.3269	6.9	C ₂₅ H ₄₄ O ₂	1.5	spelt	4.2
		Nonadecenyl-resorcinol (C19:1)	[M - H] ⁻	373.3112	6.3	C ₂₅ H ₄₂ O ₂	1.4	spelt	2.2
		Heneicosylresorcinol (C21:0)	[M - H] ⁻	403.3582	7.4	C ₂₇ H ₄₈ O ₂	1.4	spelt	2.9
		Heneicosenyl-resorcinol (C21:1)	[M - H] ⁻	401.3425	6.9	C ₂₇ H ₄₆ O ₂	1.3	spelt	1.5
		Tricosylresorcinol (C23:0)	[M - H] ⁻	431.3895	8	C ₂₉ H ₅₂ O ₂	1.3	emmer	3.2
		Pentacosylresorcinol (C25:0)	[M - H] ⁻	459.4208	8.5	C ₃₁ H ₅₆ O ₂	1.2	emmer	3.1
Glycerophospholipids (GLP)	Lysophosphatidylcholines (LysoPC)	LysoPC 16:0	[M + H] ⁺	496.3399	4.5	C ₂₄ H ₅₀ NO ₇ P	3.2	einkorn	4.3
		LysoPC 18:2	[M + H] ⁺	520.3392	4.2	C ₂₆ H ₅₀ NO ₇ P	1.2	einkorn	3.1
	Phosphatidylcholines (PC)	PC 16:0/18:1	[M + H] ⁺	760.5851	8.2	C ₄₂ H ₈₂ O ₈ NP	1.6	einkorn	2.9
		PC 16:0/18:2	[M + H] ⁺	758.5712	7.9	C ₄₂ H ₈₀ NO ₈ P	2.3	einkorn	3.9
Phosphatidylinositols (PI)	PI 16:0/18:1	[M + H] ⁺	835.5478	7.7	C ₄₃ H ₈₁ O ₁₃ P	1.6	emmer	1.6	
Glycerolipids (GL)	Diacylglycerols (DG)	DG 16:0/18:2	[M + NH ₄] ⁺	610.5405	8.8	C ₃₇ H ₆₈ O ₅	1.5	emmer	4.9
	Triacylglycerols (TG)	TG 16:0/16:0/18:2	[M + NH ₄] ⁺	848.7708	11.4	C ₅₃ H ₉₈ O ₆	1.8	spelt	3.1
		TG 16:0/18:1/20:1	[M + NH ₄] ⁺	904.8339	12	C ₅₇ H ₁₀₆ O ₆	1.9	einkorn	3.8

Table columns: pseudomolecular ion = positive and negative ionization adduct; *m/z* = mass-to-charge ratio in daltons; RT = ion retention time in minutes; elemental formula = elemental composition of the neutral molecule; mass error ppm = Δ in ppm between the detected *m/z* and the theoretical *m/z*; higher metabolite intensity in = ion spectral intensity higher in emmer, einkorn, or spelt as indicated; VIP value = Variable Influence in Projection values.

3. Discussion

3.1. Phenolic Compounds

According to our results, ARs composition significantly differs between the studied varieties. In particular, two ARs, C21:0 and C19:0, turned out to be the most useful homologues to discriminate spelt from emmer and einkorn, as illustrated in the variable trend plot (Figure 4A). This is consistent with the results reported in the HEALTHGRAIN study [21], since, among the *Triticum* spp., spelt showed higher maximum values of ARs content ranging from 490 to 741 $\mu\text{g/g}$ with C21:0 (approximately 47%) and C19:0 (36%) as the predominantly homologues found. Spelt wheat, Rouquin, showed a distribution of AR homologues similar to that of common wheat, in agreement with earlier studies [21,22], being both hexaploid species [10]. By contrast, C23:0 (see Figure 4B) and C25:0 had the highest influence to discriminate emmer variety, Garfagnana, showing the same homologue pattern of durum wheat, characterized by the influence of the longer homologues. These longer AR homologues, which were isolated from a cereal bran-milling fraction, have been found efficient inhibitors of 3-phosphoglycerate dehydrogenase. Note that 3-phosphoglycerate dehydrogenase is a key enzyme of triglyceride synthesis, in adipocytes [23]. Also for this reason, the intake of ARs is considered beneficial as it reduces the absorption of cholesterol, regulate metabolism of triacylglycerols and affect levels of lipid-soluble vitamins [24].

ARs with modified alkyl chains are also present in cereals. These are believed to differ from ARs only in side-chain unsaturation or oxidation. On average, 15%–20% ARs contain unsaturated hydrocarbon chains as well as ketone and hydroxyl groups [21–23]. In the present study, two alk(en)yl resorcinols, nonadecenyl-resorcinol, and heneicosenyl-resorcinol, were identified and contributed to the clustering and differentiation of spelt, Rouquin. These AR analogues are suggested to be more bioactive than normal saturated ones [24].

ARs are found mainly in the outer layers (bran fraction) of cereal grains, which means that they are largely missing in refined cereal flour and conventional cereal products. Taking into account that these ancient grain varieties are mostly consumed in the form of whole grain, ARs may be present in food in high enough concentrations to have a bioactive effect.

ARs are absorbed in the small intestine of pig with an ileal recovery that varies between 21% and 40%, with no major difference between different chain-length homologues [25]. In fact, their metabolized forms have been found in human plasma and urine [26] suggesting that ARs might exert their biological effect in human after whole grain intake.

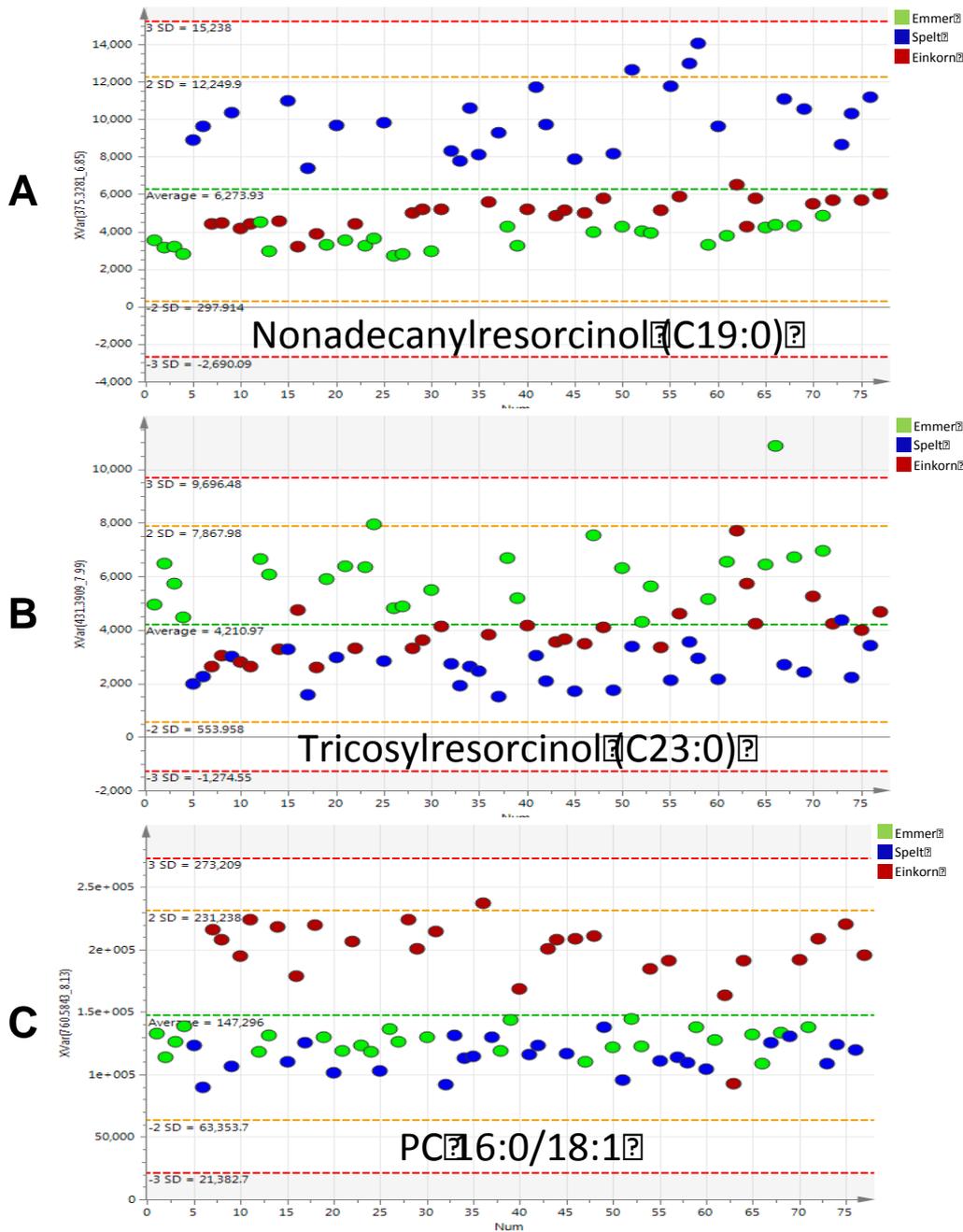


Figure 4. Variable trend plots of the most discriminant markers: nonadecanylresorcinol (C19:0), overexpressed in the spelt variety Spelt (Rouquin) (A), tricosylresorcinol (C23:0) marker having the highest influence to discriminate emmer variety (Garfagnana) (B) and PC (16:0/18:1), significantly higher in the einkorn variety (ID331) (C).

Interestingly, our data suggests that sample clustering was not affected by growing location, organic or conventional farming and vegetative year, as it was previously reported [27]. Thus, the level of ARs metabolites was identified as a cultivar marker, strongly influenced by the genetic background, which is partially in line with Ziegler et al. [10]. In fact, they reported significant

difference in the AR content of spelt grown in different location, whereas einkorn content did not differ among different location.

This outcome indicated a strong genetic influence on the AR homologue profile, suggesting that the metabolomics approach applied could potentially allow the determination of ancient wheat adulterations.

3.2. *Glycerophospholipids and Glycerolipids*

Among the statistically significant phospholipids, four molecular species (m/z 496.3399, m/z 520.3392, m/z 760.5851, m/z 758.5712) were found responsible for the separation of einkorn variety.

Two PCs, PC (16:0/18:2) and PC (16:0/18:1) were tentatively identified and trend plot of PC (16:0/18:1) is illustrated in Figure 4C. These results are consistent with a previous study [4], as einkorn was reported to show a richer lipid profiling among the ancient varieties, a lipid content 50% higher than those of bread wheat. In fact, PC 34:2 together with lysoPC 16:0 and lysoPC 18:2 are the major PC species detected, representing 60%–70% of the total wheat PC [28]. Acyl carbon and double-bond configurations in phospholipids are probably combination of the major fatty acids, that in einkorn are reported to be linoleic (18:2), oleic (18:1), and palmitic (16:0) acids [4]. In bread wheat, linoleic acid is the prevalent fatty acid too, however palmitic acid is more abundant than oleic acid. Consequently, einkorn lipids profile has a higher content of monounsaturated fatty acids (MUFA), lower content of polyunsaturated fatty acids (PUFA), and lower saturated fatty acids (SFA) that, from a nutritional point of view, contribute to the prevention of cardiovascular diseases, since MUFA and PUFA reduce thrombosis and atherosclerosis risk, influencing lipid and cholesterol synthesis [4].

4. Materials and Methods

4.1. *Chemicals*

The deionized water used for the LC mobile phase was obtained from a Milli-Q[®] Integral system supplied by Merck (Darmstadt, Germany). High-performance LC (HPLC)-grade methanol, 2-propanol, dichloromethane, formic acid, and ammonium formate were supplied by Sigma-Aldrich (St. Louis, MO, USA).

4.2. *Plant Material*

For this study, three ancient wheat species have been chosen: Garfagnana *T. turgidum* var. *dicoccum* L. (emmer), ID331 *T. monococcum* L. (einkorn), and Rouquin *T. spelta* L. (spelt).

The most extensively cultivated species is *T. turgidum* ssp. *dicoccum*, which was largely grown in the hills and low mountain areas in Central and Southern Italy until the 19th Century, as reported by local tradition. The three varieties were cultivated in two locations in Emilia Romagna region, Parma and Bologna, in plots of 8.25 m² with four replications. Grains were grown over two consecutive years (2013/2014 and 2014/2015) under two agricultural conditions: conventional ($n = 23$) and organic farming ($n = 30$) in Parma, whereas only conventional farming was applied in Bologna ($n = 24$). After harvesting, the whole grains were dried at ca. 10% humidity, stored at -20 °C and kept refrigerated until the analysis. Overall, 77 wheat samples were collected.

4.3. Metabolite Extraction

Wheat samples were ground into a fine powder using a ball mill (MM 301 Retsch, Haan, Germany). An amount of 1 g of ground wheat was weighed into a 50 mL polypropylene centrifugation tube, followed by the addition of 10 mL of a mixture of methanol/dichloromethane (50:50, v/v). After brief shaking, the content was stirred for 30 min at 240 strokes/min by a shaker (IKA Laborartechnik, Stufen, Germany). The tube was centrifuged (13,416 g) for 7 min (Rotina 35 R, Hettich Zentrifugen, Germany), then 1 mL of the extract was evaporated to dryness under a gentle stream of nitrogen. Finally, the residues were re-dissolved in 1 mL of isopropanol/methanol/water (60:30:5, v/v) prior to UHPLC-Q-TOF analysis. During the sample preparation blanks were also prepared for analysis consisting of all the steps mentioned above except for the addition of sample.

4.4. Quality Control (QC) Samples Preparation

In order to measure performance and system stability and assess the reproducibility of the sample treatment procedure, Quality Control samples (QC) were injected during the analyses.

QCs ($n = 2$) were obtained by mixing equal volumes (50 μ L) of all 77 sample extracts and following the same procedure as for the other samples. QCs were injected at the beginning of the run and after every 10 real samples.

4.5. Ultra-High-Performance Liquid Chromatography-High Resolution Mass Spectrometry

UHPLC Dionex UltiMate 3000 RS system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TripleTOF[®] 5600 quadrupole time-of-flight (TOF) mass spectrometer (SCIEX) was employed for untargeted analysis of wheat.

The chromatographic separation was performed using an Acquity BEH C18 column (Waters, Milford, MA, USA) 100 mm × 2.1-mm inner diameter, 1.7- μ m particle size maintained at 60 °C. The mobile phases for metabolic analysis were the same for negative and positive electrospray ionization (ESI) modes. Gradient elution was performed by using 5 mM ammonium formate in Milli-Q water/methanol (95:5, *v/v*) (solvent A) and 5 mM ammonium formate in isopropanol/methanol/Milli-Q water (65:30:5, *v/v*) (solvent B) both acidified with 0.1% formic acid.

The following multistep elution gradient was used with both electrospray ionization (ESI) polarities: 0.0 min (10% solvent B; 0.40 mL·min⁻¹) to 1.0 min (50% solvent B; 0.40 mL·min⁻¹), subsequently 1–5 min (80% solvent B; 0.40 mL·min⁻¹), 11.0 min, (100% solvent B; 0.50 mL·min⁻¹). After a 4.5 min isocratic step, the system was re-equilibrated to initial conditions for 2.5 min (10% solvent B; 0.4 mL·min⁻¹). The sample was permanently kept at 5 °C.

The ion source was a DuoSpray™ with a separated ESI ion source and APCI. ESI was used for the sample measurement and APCI was used for exact mass calibration of the TripleTOF instrument. In ESI(+) mode, the source parameters for metabolic analysis were as follows: capillary voltage, +4500 V; nebulizing gas pressure, 60 psi; drying gas pressure, 50 psi; temperature, 550 °C; and declustering potential, 80 V. The capillary voltage in ESI(-) mode was -4000 V, and the other source settings were the same as for ESI(+).

At the same time, a TOF MS method and information-dependent acquisition (IDA) method were used to collect MS and MS/MS spectra. The method consisted of a survey TOF MS experiment ranged from *m/z* 100 to 1200, in parallel, Product Ion (PI) spectra for the eight most intense ions of the survey spectra throughout the chromatographic run were recorded. Dynamic background subtraction was activated to acquire PI spectra of real eluted compounds, avoiding background ions. PI spectra were collected for ions ranging from *m/z* 50 to 1200. The PI spectra were recorded with collision energy of 35 V and collision energy spread of ± 15 V was also set. In this way, both low-energy and high-energy fragment ions were present in a single spectrum. The total cycle time of the TOF MS and IDA methods was 0.55 s.

An automatic *m/z* calibration was performed by the calibration delivery system for every five samples using a positive or negative APCI calibration solution (SCIEX) according to the batch polarity. Each set of samples for each polarity was preceded by three blank controls: Milli-Q water, methanol and a blank (extraction procedure without the sample). Finally, the same MS approach was applied in ESI(-) mode. The resolving power achieved was greater than 31,000 (*m/z* 321.0192)

full width at half maximum (FWHM) with both polarities. The PI spectra were measured in high-sensitivity mode, which provides half resolving power.

Instrument control and data acquisition were performed with Analyst 1.6 TF (SCIEX), and the qualitative analysis was performed using PeakView 2.2 (SCIEX) equipped with MasterView and Formula Finder and directly linked to the ChemSpider database, and LipidView software (version 1.3 beta, SCIEX) for lipid evaluation. The in-batch sequence of the samples was random (established on the basis of random number generation) to avoid any possible time-dependent changes during UHPLC-HRMS analysis, which would result in false clustering. To address overall process variability, metabolomics studies were augmented to include a set of eight sample technical replicates (10% of the samples set). Reproducibility analysis for compounds detected in these replicates provided a measure of variation for extraction, injection, retention time (RT), and mass accuracy.

4.6. Data Processing and Chemometrics Analysis

Data processing has been performed based on previous work [12]. Briefly, MarkerView software (version 1.2.1, SCIEX) was used for data processing (data mining, alignment, filtering, normalization, and Principal Component Analysis (PCA)) of the UHPLC-HRMS records. Data mining was performed based on an automated algorithm using RT window and peak finding; retention time (RT) range 0.4–13 min and m/z range 100–1200. In the next step, RT and m/z alignment of the respective peaks was carried out using RT and m/z tolerances of 0.2 min and 0.02 Da, respectively. Two separate positive and negative ionization data matrices, comprising lists of molecular features (called also peaks by MarkerView) characterized for each sample by (i) RT; (ii) m/z value; (iii) respective intensity and (iv) charge state (monoisotopic and isotopic), were automatically obtained using MarkerView, and subsequently total area sum normalization was performed for each sample. Prior to the actual PCA, data matrices were pre-processed using the Pareto scaling (the square root of the standard deviation is used as the scaling factor).

Orthogonal partial least squares discriminant analysis (OPLS-DA) employing the software SIMCA (v. 13.0, 2011, Umetrics, Umea, Sweden) was performed. The quality of the models was evaluated by the goodness-of-fit parameter (R_2X), the proportion of the variance of the response variable that is explained by the model (R_2Y) and the predictive ability parameter (Q_2), which was calculated by a seven-round internal cross validation of the data using a default option of the SIMCA software. R_2X and R_2Y represent the fraction of the variance of X matrix and Y matrix, respectively, while Q_2 suggests the predictive accuracy of the model. R_2X , R_2Y , and Q_2 values close to 1 indicate an excellent model, and thus, from values higher than 0.5 indicate good quality of

OPLS-DA models. In order to select the most significant and reliable variables, variable importance in the projection (VIP) was used. This parameter summarizes the importance of the *X*-variables, both for the *X*- and *Y*-models. In this research, VIP with the threshold >1.5 was used for selection of the most significant markers. VIP-values larger than 1 indicate important *X*-variables.

To avoid risk of overfitting, as the results found after Multivariate Data Analysis (MVDA) are sensitive to chance-correlations, statistical models have to be validated. For this reason, supervised models, OPLS-DA and PLS-DA, were validated by cross-validation, using the leave one-third out approach. The data set was divided into three parts and one-third of samples were excluded to build a model with the remaining two-thirds of samples. Excluded samples, one-third of samples, were then predicted by this new model and the procedure was repeated until all samples had been predicted at least once. Each time the percentage of correctly classified samples was calculated by generating a misclassification table.

5. Conclusions

In conclusion, differences in the metabolome of ancient grains were successfully detected using an untargeted UHPLC-HRMS metabolomics approach. Discriminant metabolites including alkylresorcinols, glycerophospholipids, and glycerolipids were identified allowing a metabolic characterization of ancient wheat grains.

The results obtained in this study confirmed the importance of different AR homologues as markers to distinguish between *Triticum* species. Furthermore, all the 15 identified molecules were revealed to be cultivar markers, strongly influenced by the genetic background, since their abundance was not significantly affected by growing location, organic or conventional farming, and vegetative year.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/17/8/1217/s1>.

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Author Contributions: Chiara Dall’Asta and Gianni Galaverna designed the experiments and were responsible for the metabolomics study setup. LC-MS conducted analysis, statistical analysis of the data, metabolites identification: Laura Righetti and Josep Rubert. Interpretation of the data: Laura Righetti, Josep Rubert, Milena Stranska-Zachariasova, and Chiara Dall’Asta Sample collection: Silvia Folloni, Roberto Ranieri. Drafting the manuscript for important intellectual content: Laura Righetti, Josep Rubert, Gianni Galaverna, Milena Stranska-Zachariasova, Jana Hajslova, and Chiara Dall’Asta.

Conflicts of Interest: The authors have declared no conflict of interest.

Abbreviations

ARs	alkylresorcinols
QTOF	quadrupole time of flight
HRMS	high resolution mass spectrometer
QC	quality control
PCA	principal component analysis
PLS-DA	projection on latent structure-discriminant analysis
OPLS-DA	orthogonal projection on latent structure-discriminant analysis
DG	diacylglycerol
TG	triacylglycerol
PC	phosphatidylcholine
Lyso PC	Lysophosphatidylcholine

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Chapter 6

**5-n-Alkylresorcinols are directly related to
a lower accumulation of mycotoxins in
grains**

5-n-Alkylresorcinols but not hydroxycinnamic acids are directly related to a lower accumulation of deoxynivalenol and its glucoside in wheat grains

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Abstract

This study was designed to investigate the possible correlation between 5-n-alkylresorcinols and hydroxycinnamic acids on mycotoxin accumulation in grains under field conditions. At this purpose, ten different lines characterized by different degree of ploidy (hexaploid, tetraploid, diploid) were chosen. The contamination, expressed as the sum of DON and DON3Glc, was found to be significantly lower in soft wheat varieties than in durum wheat and small grains. The mycotoxins content negatively correlated with the total 5-n-alkylresorcinols and to the ratio C21:0/C23:0, recently reported as an indicator of antifungal activity. Interestingly, DON3Glc was negatively correlated with the ratio C17:0/C21:0, reported as a genetic-related indicator, in agreement with its biological role, which is formed in plant as detoxification product. On the contrary, no correlation was pointed out with total HCA and free HCA. The results obtained indicated that only the lipophilic phenolic fraction in wheat could exert an inhibitory effect towards mycotoxin accumulation.

Keywords: 5-n-alkylresorcinols; hydroxycinnamic acids, deoxynivalenol; wheat, antifungal activity.

Abbreviations

ARs, alkylresorcinols; HCA, hydroxycinnamic acids; DON, deoxynivalenol; DON3Glc, deoxynivalenol-3-glucoside;

1. Introduction

According to FAO, the cereal crop production worldwide in 2016 stand at 2,571 million tonnes, with a forecast for utilization in 2016/17 of about 2,562 million tonnes¹. It is therefore evident how this scenario may be easily unbalanced by climate change and related phenomena.

Commercial wheat cultivars are mainly hexaploid (*Triticum aestivum*; common wheat) or tetraploid (*Triticum turgidum* ssp *durum*; durum wheat) species originated from natural hybridizations between diploid ancestors². Current varieties are the result of an intensive breeding program carried out over the last 70-80 years to increase agronomic yield and technological properties for the production of bread, pasta, and bakery products. Ancient wheat varieties, i.e. *Triticum monococcum* (einkorn) or *T. turgidum* ssp *dicoccum* (emmer), are still cultivated although as a niche crop in small area in Europe. However, due to their peculiar characters, such varieties are of growing interest for organic cultivation in marginal area²⁻⁴.

One of the most important threat for food security is represented by fungal diseases, due to the infection of pathogenic fungi in field and subsequent accumulation of mycotoxins in crops. Besides possible toxicological implications, this may lead to enormous economic losses⁵.

Among fungal diseases, Fusarium Head Blight (FHB) strongly affects common and durum wheat, as well as other small grain cereals, worldwide⁶. FHB is caused by toxigenic fungi belonging to *Fusarium* spp., such as *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*. Under supportive conditions, infected grains may accumulate *Fusarium* mycotoxins, such as trichothecenes and zearalenone⁷. Among them, deoxynivalenol (DON) and its glucosylated form DON3Glc have been recognized as strongly related with the plant resistance/susceptibility towards the pathogen⁸.

Recent studies have focused on the role played by plant secondary metabolites in mediating defense mechanisms in field⁹⁻¹⁰. Among different classes, phenolic compounds have been often suggested as related to *Fusarium* infection and trichothecene accumulation in wheat and barley, as mechanism of resistance¹¹⁻¹².

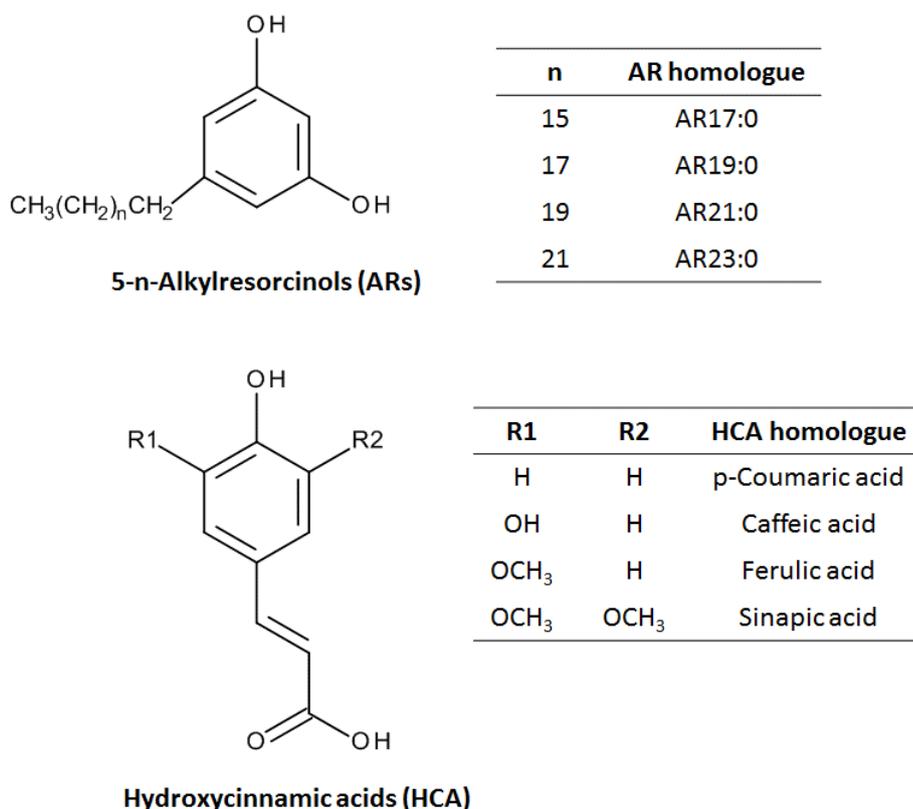
In particular, benzoic acids and hydroxycinnamic acids were found to modulate trichothecene biosynthesis in vitro¹³⁻¹⁵. This is in agreement with the biological role of phenolic acids, which are ubiquitous constituents of plants acting as signaling and defense compounds¹⁶.

However, the modulation of trichothecens biosynthesis in vitro by phenolic compounds is controversial. It has been reported, indeed that some phenolic acids stimulate the accumulation of *Fusarium* toxins¹⁷. On the other hand, ferulic acid is known to inhibit trichothecenes production in vitro¹⁸.

Studies by Ponts et al. (2011)¹⁷ identified hydroxycinnamic acids as elicitors of trichothecene production in vitro. Recently, similar results were reported by Etzerodt et al. (2016)¹⁹. In the same study, benzoic acids are able to affect DON accumulation in a wheat-type dependent manner. The same authors reported on the possible salicylic acid accumulation in wheat as a response to *Fusarium* infection, and on the subsequent induction of phenolic acid biosynthesis. Therefore, this lead to a positive correlation between DON contamination and phenolic acids content.

According to Ponts et al. (2011)¹⁷, the inhibitory effect on fungal growth and mycotoxin accumulation increases with the lipophilic behavior of the phenolic compounds. Among secondary plant metabolites, alkylresorcinols (ARs) norisoprenoid phenolic lipids, which have been reported as phytoanticipins and allochemicals²⁰⁻²¹. Structurally, 5-n-alkylresorcinols are characterized by two hydroxyl groups at C1 and C3 of the aromatic ring, and a lipophilic alkyl chain at C5 position (**Figure 1**).

Figure 1: Structures of the main 5-n-alkylresorcinols and of the main hydroxycinnamic acids considered within this study.



Due to their amphiphilic nature, ARs may interact with biological membranes, nucleic acids, and enzymes, being responsible of a wide spectrum of biological activities, such as antimicrobial, antifungal, and antiparasitic activity^{20, 22-23}. Therefore, recent studies are mainly focused on their nutritional role as active component in wholegrain food²⁴⁻²⁵.

In cereal grains, resorcinolic lipids are localized only in an intermediate layer of caryopsis, while they cannot be found in endosperm and germs²². From a biological perspective, the localization on the surface of the kernel could enhance their protective effect towards pathogens²⁶. It has been proven, in fact, that their biosynthesis can be triggered by fungal infection²⁷.

Recently, Cicoritti et al. (2015)²⁸ demonstrated that ARs may inhibit *Fusarium* growth in vitro. The authors proposed the exploitation of ARs as biopesticides, and suggested the inclusion of ARs in cereal breeding programs to obtain new genotypes with increased resistance and improved nutritional properties. However, the direct effect of 5-n-alkylresorcinols towards pathogen infection and mycotoxin accumulation in field was never reported so far.

The present study aims at investigating the possible correlation between hydroxycinnamic acids as well as 5-n-alkylresorcinols, and mycotoxin accumulation in grains. At this purpose, ten different lines were chosen, belonging to the species *T. aestivum* (3 lines – common wheat), *T. turgidum* ssp *durum* (3 lines – durum wheat), *T. monococcum* (1 line - emmer), *T. turgidum* ssp. *Dicoccum* (1 line – einkorn), *T. spelta* (1 line – spelt), and *T. turgidum* ssp. *turgidum* (1 line – rivet).

2. Materials and Methods

2.1. Chemicals

Analytical standards of DON (solution in acetonitrile 100 µg mL⁻¹ and Deoxynivalenol-3-glucoside (solution in acetonitrile 50.6 µg mL⁻¹) were purchased from Romer Labs[®] (Tulln, Austria). Standard solutions of DON and DON-3Glc were prepared in acetonitrile at a concentration of 2500 µg/L, were stored in a freezer at -20 °C and brought to room temperature before their use. Ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid, sodium hydroxide, citric acid, 5-heptadecyl-resorcinol (C17:0), 5-nonadecylresorcinol (C19:0), 5-heneicosyl-resorcinol (C21:0) and 5-tricosylresorcinol (C23:0) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade methanol, acetonitrile, ethyl acetate and acetic acid were purchased from Sigma-Aldrich (Steinheim, Germany); bidistilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA). MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA), ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland), and NaCl from VWR International Ltd. (Ballycoolin, Blanchardstown, Dublin, Ireland) were used.

2.2. Sampling plan

Ten lines of wheat were collected, among them hexaploid, tetraploid, and diploid species (**Table 1**). Grains were cultivated in 2015 in Parma, in two different fields. After harvesting, the whole grains were dried at ca. 10% humidity. Four biological samples were collected for each field,

and pooled in a single batch. Batches were stored at $-20\text{ }^{\circ}\text{C}$ and kept refrigerated until the analysis. Each batch was analysed in triplicate.

For simplicity, data have been elaborated following a commercial classification, i.e. common wheat, durum wheat, and small grains (emmer, einkorn, and spelta). Grano del Miracolo is an old *T. turgidum* ssp *turgidum* line still in use in Emilia Romagna region. It has durum wheat like characteristics, therefore it has been classified within durum wheat for this study.

Table 1. Description of wheat lines used for this study

Species	Wheat line	Ploidy level	Genome	Classification
<i>Triticum aestivum</i> L.	Bologna	hexaploid	AABBDD	Common wheat
	Virgilio	hexaploid	AABBDD	
	Blasco	hexaploid	AABBDD	
<i>Triticum turgidum</i> ssp <i>turgidum</i>	Grano del miracolo*	tetraploid	AABB	Rivet, cone or English wheat
<i>Triticum turgidum</i> var. <i>durum</i> Desf.	Senatore Cappelli	tetraploid	AABB	Durum wheat
	Timilia	tetraploid	AABB	
	Odisseo	tetraploid	AABB	
<i>Triticum turgidum</i> ssp. <i>dicoccum</i>	ID331	tetraploid	AABB	Emmer
<i>Triticum monococcum</i>	Garfagnana	diploid	A _M A _M	Einkorn
<i>Triticum spelta</i>	Rouquin	hexaploid	AABBDD	Spelt

Table columns: Wheat line = common field identifier; Ploidy level = number of sets of chromosomes; Genome = nucleotide diversity assigned to the A, B and D genomes.

*An ancient line, still in use in Emilia Romagna region, considered as a precursor of durum wheat

2.3 Extraction and analysis of hydroxycinnamic acids

Hydroxycinnamic acids were extracted and analysed according to Zaupa et al. (2014)²⁹. Briefly, for the extraction of free phenolic compounds, 50 mg of sample was extracted with 6 mL of water under agitation for 20 min at room temperature, followed by centrifugation at 9200g for 10 min, and the supernatant was collected. For the extraction of the bound phenolic, the residue was further hydrolyzed with 1.5 mL of 2 molL⁻¹ sodium hydroxide at room temperature for 1 h. After alkaline hydrolysis, the pH of the mixture was adjusted to 3 by adding 1.35 mL of 3 molL⁻¹ citric acid. The bound phenolic samples were then extracted with 6 mL of ethyl acetate. After evaporation to dryness, the residue was dissolved in methanol.

The analysis was carried out by UHPLC-MS/MS, under negative ionization mode. The MS worked with a capillary temperature equal to 275 °C, while the source heater temperature was set to 45 °C. The sheath gas flow was 40 units, while auxiliary and sweep gases were set to 5 and 2 units,

respectively. The source voltage was 4 kV. The capillary voltage and tube lens were -21.00 and -57.71 V, respectively. For separation of the analytes, phase A was aqueous formic acid (0.1% v/v) and phase B was methanol/water (98:2 v/v). The mobile phase, pumped at a flow rate of 0.3 mL/min, was kept for 10 min in linear gradient of 7% to 40% of B. Analyses were carried out using Selected Reaction Monitoring, using the following transitions: p-coumaric acid 163 \rightarrow 119 (CID 30); caffeic acid 179 \rightarrow 135 (CID 29); ferulic acid 193 \rightarrow 149 (CID 28); sinapic acid 223 \rightarrow 179 (CID 25).

2.4. Extraction and determination of 5-n-alkylresorcinols

For determination of AR by gas chromatography (GC), 2.5 g of wholemeal was extracted by continuous shaking at room temperature with 50 mL ethyl acetate containing for 2 h. The extracts were then filtered through Whatman No 42 paper and analysed by GC-MS.

AR relative homologue composition was determined by GC analysis using methyl behenate as internal standard (IS). An aliquot (1 mL) of AR extract was dried under nitrogen and a mixture (200 μ L) of TMCS and HDMS (exametildisilazan) (20:80, v/v) was added. The sample was then shaken to dissolve the extract in the mixture, heated at 60 °C for 45 min. An aliquot of 1 mL was dried and reconstructed by 1 mL of ethyl acetate containing 50 μ L of methyl behenate and immediately analysed.

GC analysis was performed on a Thermo Scientific Trace 1300 gas-chromatograph coupled to a Thermo Scientific ISQ MS equipped with an electronic impact (EI) source (Thermo Fisher Scientific Inc.). The separation was performed on a BP-5 fusesilica capillary column, 25 m in length, inner diameter 0.32 mm, film thickness 0.25 μ m (Chebios, Rome, Italy). Temperature increase The injector and transfer line temperatures were set at 270 °C. Full scan mode was chosen as acquisition mode in the range of 50–600 m/z . The homologue pattern was determined by comparing the relative retention times with those obtained for a mix of homologue standards: C17:0, C19:0, C21:0, C23:0, C25:0.

2.5. Extraction and determination of mycotoxins

Samples were prepared according to Malachová et al. procedure (2014)³⁰ with slight modifications. Briefly, 1 g of ground wheat was stirred for 90 minutes at 200 strokes/min on a shaker with 4 mL of acetonitrile/water (80/20, v/v) mixture acidified with 0.1% of formic acid. An aliquot of the extract was collected and centrifuged for 10 min at 14,000 rpm at room temperature, then 1 mL of supernatant was evaporated to dryness under a gentle stream of nitrogen. Finally, the residues were re-dissolved in 1 mL of water/methanol (80:20 v/v) prior to LC-MS injection.

UHPLC Dionex Ultimate 3000 separation system coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI) was employed.

For the chromatographic separation, a reversed-phase C18 Kinetex column (Phenomenex, Torrance, CA, USA) with 2.10×100 mm and a particle size of 2.6µm heated to 40 °C was used. 2 µl of sample extract was injected into the system; the flow rate was 0.350 ml/min.

Gradient elution was performed by using 5 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.2% acetic acid. Initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min; after an isocratic step (6 min), eluent B was increased to 90% in 9 min; after a 3 min isocratic step, the system was re-equilibrated to initial conditions for 3 min. The total run time was 30 min.

MS parameters: the ESI source was operated in negative ionization mode (ESI⁻); spray voltage 3,000 V, capillary temperature at 270 °C, vaporizer temperature was kept at 200 °C, sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. S-Lens RF amplitude value and collision energies (CE) were optimized during infusion of analyte standard solutions (1 mg/kg, in methanol) employing an automatic function of X-calibur software (Thermo Fisher Scientific Inc., San Jose, CA, USA).

Detection was performed using multiple reaction monitoring (MRM) mode and monitoring the [M + CH₃COO]⁻ adducts. The following transitions were measured: DON m/z 355→295 (CE = 13 eV) and m/z 355→265 (CE = 17 eV); DON-3Glc m/z 517→457 (CE = 16 eV) and m/z 517→427 (CE = 23 eV).

Matrix-matched calibration curves (calibration range 50–2,500 µg kg⁻¹) were used for target analyte quantification. A good linearity was obtained for all the considered mycotoxins (R² > 0.99).

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS v.23.0 (SPSS Italia, Bologna, Italy). Data were analysed by MANOVA followed by Duncan post-hoc test ($\alpha = 0.05$). Data correlation was evaluated by Pearson's correlation test ($\alpha = 0.05$).

3. Results

3.1 Mycotoxin accumulation in grains

The mycotoxin content of selected wheat cultivars was determined by UHPLC-MS/MS. In order to evaluate differences in terms of resistance/susceptibility, DON and its major masked form DON3Glc were considered (**Table 2**). The overall contamination was in the range 236 – 2167

µg/Kg for DON, and LOD – 201 µg/Kg for DON3Glc (median value: 436 µg/Kg and 148 µg/Kg, respectively).

The bioconversion ratio expressed as DON3Glc/DON, was in the range 0.09 – 0.69 with a mean value of 0.26.

Considering different species, the total contamination – expressed as the sum of DON and DON3Glc - was significantly lower in soft wheat (*T. aestivum*; mean value: 506 µg/Kg) than in durum wheat (*T. durum*; mean value: 1478 µg/Kg) and small grains (*T. monococcum*, *T. spelta*, *T. turgidum v. dicoccum*; mean value: 1766 µg/Kg).

Among small grains, the highest contamination was found in emmer (*T. turgidum v. dicoccum*), while einkorn (*T. monococcum*) and spelt (*T. spelta*) were comparable in terms of mycotoxin content.

3.2 Phenolic compounds

Free and total hydroxycinnamic acids were measured by LC-MS/MS (**Table 2**).

The overall content of total HCA was in the range 4.1 – 30.0 mg/Kg for CaA, 8.3 – 244.8 mg/Kg for CouA, 76.1 – 490.6 mg/Kg for FeA, and 3.1 – 19.2 mg/Kg for SiA. Concerning free HCA, concentrations ranged between 0.04 – 1.08 mg/Kg for CaA, 0.09 – 2.33 mg/Kg for CouA, 0.93 – 3.08 mg/Kg for FeA, and 0.01 – 0.15 mg/Kg for SiA. Data are reported in **Figure 2**.

Data were analysed by ANOVA test, showing that content of free HCA is significant higher in durum wheat than in common wheat and other small grains ($p < 0.0001$).

The presence of 5-n-alkylresorcinols (AR17:0 – AR23:0) in selected grains was measured by GC-MS (**Table 2**). Besides concentrations, the ratio AR21:0/AR23:0 was calculated as well, because it was reported as an indicator of antifungal activity (Ciccoritti et al. 2015).

The overall content of 5-n-alkylresorcinol was in the range 1.2 – 23.9 µg/Kg for AR17:0, 19.8 – 191.6 µg/Kg for AR19:0, 171.4 – 660.1 µg/Kg for AR21:0, and 64.4 – 175.3 µg/Kg for AR23:0.

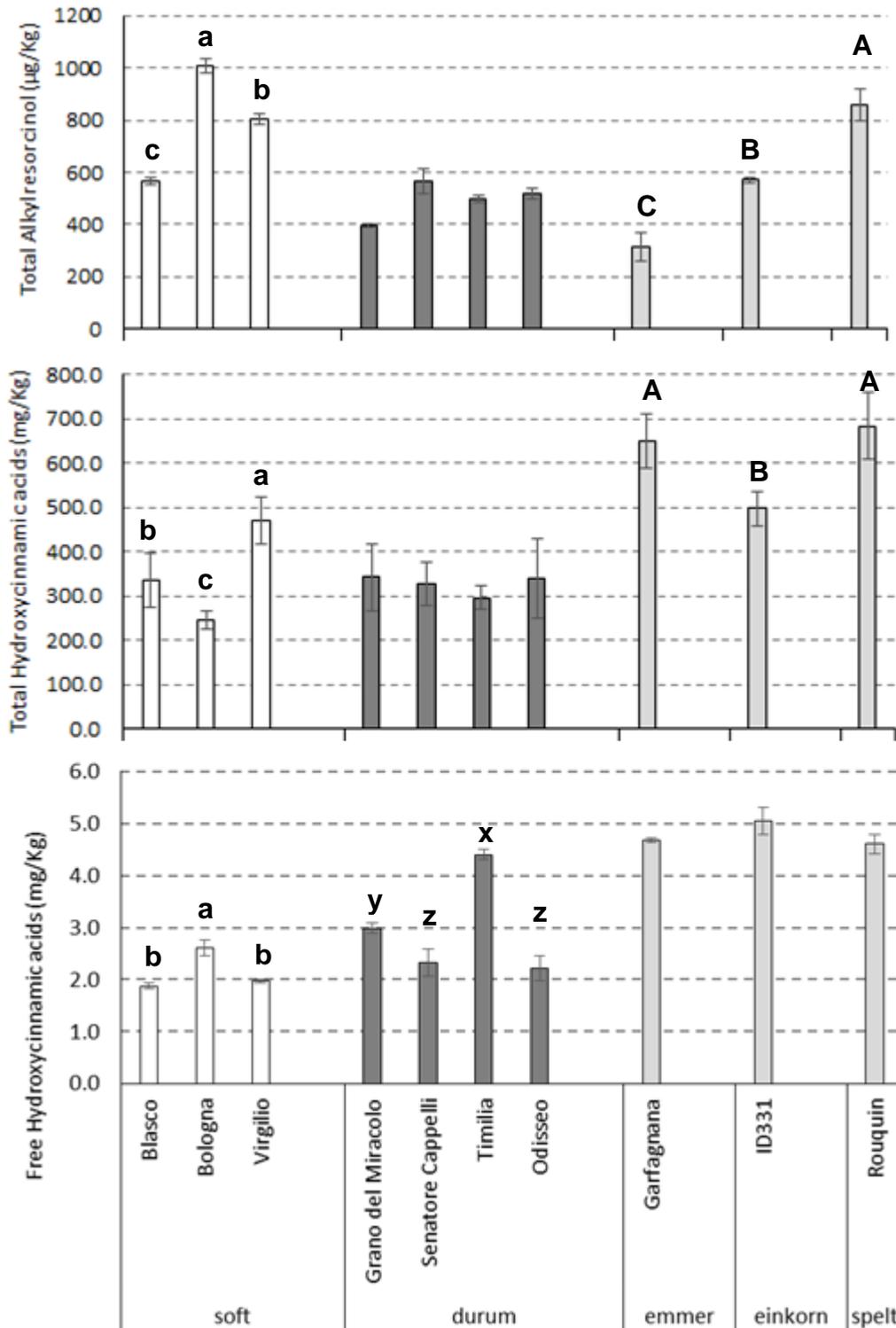
The ratio AR21:0/AR23:0 was in the range 1.9 – 5.4.

When total alkylresorcinols are considered, differences between soft and durum wheat, and small grains, are not statistically significant. However, cultivar related differences could be observed, as reported in **Figure 2**.

Table 2: Occurrence of mycotoxins and alkylresorcinols in selected wheat cultivars. Two biological replicates were considered for each cultivar; each analysis was performed in triplicate.

Cultivar	Genotype	Mycotoxins		Alkylresorcinols				Free Hydroxycinnamic acids				Bound Hydroxycinnamic acids (mg/Kg)			
		(µg/Kg)		(µg/Kg)				(mg/Kg)							
		DON	DON3Glc	AR 17:0	AR 19:0	AR 21:0	AR 23:0	CaA	CouA	FeA	SiA	CaA	CouA	FeA	SiA
Blasco	<i>T. aestivum</i>	236	< LOD	21.4	144.5	335.0	65.4	0.07	0.16	0.98	0.62	6.31	17.12	295.91	15.89
		56	-	0.7	5.2	4.1	1.4	0.03	0.05	0.01	0.00	0.40	9.44	47.11	4.67
Bologna	<i>T. aestivum</i>	344	< LOD	23.3	187.9	653.1	141.2	0.78	0.12	1.06	0.62	10.24	12.34	211.59	10.55
		5	-	0.9	5.2	9.9	11.6	0.06	0.04	0.07	0.01	6.61	3.44	191.60	5.12
Virgilio	<i>T. durum</i>	369	193	15.0	135.3	537.7	116.7	0.24	0.17	0.94	0.60	20.74	28.49	408.17	12.67
		23	60	0.3	3.8	25.5	10.3	0.03	0.08	0.01	0.00	7.43	6.65	64.95	1.58
Senatore Cappelli	<i>T. durum</i>	1374	177	2.6	32.5	364.9	164.4	0.25	0.22	1.21	0.63	18.19	46.41	252.85	8.83
		383	32	1.6	13.2	21.3	10.0	0.04	0.11	0.08	0.03	16.81	4.89	229.26	8.05
Timilia	<i>T. durum</i>	1441	182	1.6	24.7	337.5	133.5	0.63	0.87	2.14	0.68	10.37	15.06	257.55	13.03
		777	35	0.3	1.4	16.5	4.9	0.00	0.68	1.33	0.03	0.86	1.61	23.76	1.09
Odisseo	<i>T. durum</i>	451	141	1.5	27.2	351.2	138.1	0.15	0.18	1.18	0.66	7.37	17.03	297.16	16.89
		72	7	0.0	2.6	13.5	1.8	0.12	0.09	0.07	0.01	4.62	12.38	71.56	2.30
Garfagnana	<i>T. turgidum</i>	2167	201	1.2	20.4	196.5	96.9	0.60	2.08	1.25	0.72	18.14	195.64	425.42	11.04
	<i>v. dicoccum</i>	1689	18	n.d.	0.8	35.5	19.3	0.12	0.34	0.21	0.06	5.40	69.56	85.55	0.31
Grano del Miracolo	<i>T. turgidum</i>	876	< LOQ	2.6	27.6	244.3	123.3	0.15	0.50	1.65	0.64	8.74	23.18	296.99	13.02
	<i>v. turgidum</i>	524	-	0.2	0.5	5.8	0.4	0.06	0.16	0.04	0.05	3.12	13.17	91.55	1.68
ID331	<i>T. monococcum</i>	889	212	3.2	49.6	343.2	174.6	0.95	2.14	1.06	0.81	19.28	183.67	284.30	9.44
		459	57	0.4	4.8	13.6	0.9	0.05	0.21	0.09	0.02	2.72	11.61	25.77	0.52
Rouquin	<i>T. spelta</i>	410	183	18.6	137.0	591.5	110.9	0.97	1.55	1.08	0.91	24.22	189.58	456.42	12.76
		30	53	3.0	22.4	32.7	5.2	0.16	0.18	0.07	0.13	0.46	28.54	48.36	1.33

Figure 2: Content of 5-n-alkylresorcinols, free hydroxycinnamic acids, and total hydroxycinnamic acids in the considered grain varieties. Different letters, when reported, indicate significant differences (ANOVA test, $\alpha = 0.05$).



Data analysis and interpretation

This work focused on the possible correlation between the phenolic component and the mycotoxin content in wheat. Samples were collected in the harvest season 2015, from two collection plots located in Parma, Emilia Romagna. Wheat was grown under field conditions, therefore natural infection occurred. The aim of the study was to evaluate the different content of phenolic compounds in wheat samples, and investigate if there was a possible correlation with mycotoxin content. The cultivar-specific resistance toward *Fusarium* was neither considered nor investigated. Recent studies involving different wheat species indicated that the accumulation of phenolic compounds in wheat, among them hydroxycinnamic acids and 5-n-alkylresorcinols, is regulated by both the genetic background and the environmental factors³¹. However, since our samples were harvest from the same location and in the same year, it can be assumed that they underwent comparable environmental conditions. Therefore, ANOVA analysis was carried out on phenolic compounds, considering only species and/or ploidity as factors.

Table 3: Results of the analysis of variance (ANOVA) and Duncan post-hoc test, considering species as factor. The variables were: total HCA, free HCA, total ARs, ratio C17:0/C21:0, ratio C21:0/C23:0, total mycotoxins. Only significant variables were reported in the table, along with the ploidity level of each species (H: hexaploid; T: tetraploid; D: diploid).

	Total ARs	C17:0/C21:0	C21:0/C23:0	ploidity
common	792 ab	0.043 a	4.80 a	H
durum	531 cd	0.010 b	2.71 b	T
spelt	863 a	0.032 a	5.34 a	H
emmer	576 bc	0.009 b	1.97 b	T
einkorn	315 d	0.006 b	2.04 b	D

According to the ANOVA analysis, both free and total HCAs does not significantly differ among wheat species, indicating a possible level-effect of the environment on the genetic contribution.

On the other hand, total ARs are more abundant in spelt and common wheat, followed by emmer, durum wheat, and einkorn ($p < 0.001$). Consistently, the ratios AR21:0/AR17:0 and AR21:0/AR23:0, both already reported as a genetic-related indicator³² and an antifungal activity indicator²⁸ respectively, significantly decrease according to hexaploid > tetraploid \geq diploid. This suggests an important role of the genetic background on the accumulation of 5-n-alkylresorcinols in

wheat, in agreement with evidence in the literature reporting on the remarkable heritability of AR content, and the differences in AR profile between hexaploid, tetraploid, and diploid wheat lines³². It must be observed that all the samples collected within this study were naturally infected by *Fusarium* spp., without a clear trend in mycotoxin accumulation among wheat species. Therefore, this may suggest that ARs biosynthesis is genetic-driven and not fungi-induced, indicating their role as phytoanticipins³³.

Possible correlations between data were then investigated using Pearson's correlation test (**Table 4**).

Table 4: Pearson's correlation calculated for DON and DON3Glc towards phenolic compounds and related parameters.

		Total ARs	Free HCA	Total HCA	C17:0/C21:0	C21:0/C23:0
DON	r	-0.491	n.s.	n.s.	n.s.	-0.488
	<i>p-value</i>	0.033				0.034
DON3Glc	r	n.s.	n.s.	n.s.	-0.545	n.s.
	<i>p-value</i>				0.015	

Concerning 5-n-alkylresorcinols, a negative correlation with mycotoxins was highlighted, in agreement with those from the literature, reporting that 5-n-alkyl-resorcinols exert a potential inhibitory effect towards mycotoxin accumulation. On the contrary, no correlation was pointed out with total HCA and free HCA.

In order to further explore the possible role of hydroxycinnamic acids, in particular ferulic acid, Pearson's correlation test was run again considering single compounds, both as free and total forms. Again, no significant correlation was pointed out.

Studies in the literature reported controversial information in the role played by phenolic compounds in mycotoxin modulation. Phenolic compounds have been extensively studied with regard to *Fusarium* Head Blight and have been shown to be involved in the disease resistance^{19, 34-35}. In general, phenolic acids with greater antioxidant capacities resulted in a higher toxin accumulation, probably on account of a modulatory effect on the transcriptional control of the *TRI5* gene¹⁷. In particular, among HCA, ferulic acid seemed to exert an effect on the accumulation of DON. However, this finding was not confirmed in our study.

Intriguingly DON but not DON3Glc accumulation, was related to total 5-n-alkylresorcinol content and to the ratio C21:0/C23:0, already reported as an indicator of antifungal activity²⁸. Since DON is

known as a virulence factor for FHB in wheat, this suggests a direct effect of the resorcinolic fraction on the spread of fungal infection, and subsequent DON production. In addition, considering the localization of ARs at the surface of plant tissue and their amphiphilic structure, their key role as chemical barrier against fungal infection and in cell wall reinforcement³³ appears plausible.

On the other hand, DON3Glc is negatively related with the ratio C17:0/C21:0, reported as a genetic-related indicator. This is consistent with the biological role of DON3Glc, which is formed in plant as detoxification product³⁶. Its formation rate can be actually seen as a genetical-related mechanism of resistance towards FHB in wheat³⁷.

Altogether, our data indicated that only the lipophilic phenolic fraction in wheat could exert an inhibitory effect towards mycotoxin accumulation. α -Tocopherol, another lipophilic antioxidant compound, has been reported to inhibit DON accumulation in wheat¹⁹. This could indicate a possible interaction of lipophilic compounds with fungal membranes, contributing thus to an increased cellular uptake of low-polarity phenolic compounds, as already described for BHT¹⁷. However, the mechanism of action of 5-n-alkyl-resorcinols leading to a decrease in DON accumulation in wheat, requires an in-depth investigation with a focus on the species-related differences. In particular, minor species at different ploidity should be carefully investigated, as a source of genetic biodiversity leading to the highlight of possible genetic resistance factors.

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SECTION III: Wheat authenticity

Making fraudulent profit from misrepresentation of food has been a feature of society from historical times [1]. Adulteration with intent to deceive is usually carried out by admixture of cheaper products and materials which are usually difficult to detect not only by consumers but also by routine analytical techniques, with high-priced commodities being usually the main target for adulteration. Thus, the determination of food authenticity is a crucial issue for food quality and safety, demonstrating, once again that these three aspects are strongly linked one to each other, contributing to the assessment of food integrity.

Wheat adulteration is relatively common [2] as it is very difficult to detect visually, especially when durum wheat is grounded into semolina. About 95% of the wheat that is grown and consumed globally is common wheat (*Triticum aestivum*), while most of the remaining 5% is tetraploid durum wheat (also called pasta wheat) (*T. turgidum* var *durum*) which is more adapted to the dry Mediterranean climate [3]. Durum wheat price is 25% higher than common wheat, for this reason, raw material or pasta in industrial food production, may be adulterated by common wheat, generating more money. It should be note that so far, this adulteration has been reported to have a huge impact on quality rather than safety.

Alongside the traditional targeted techniques, omics approaches, and in particular the study of metabolites is growing up rapidly in food authenticity studies, enabling the detection of hundreds of small molecules in one run. The general application of metabolomics in food authentication has been recently reviewed [4-7]. Metabolomics studies in this particular field are mainly discriminative, aiming to find differences between sample populations, and predictive, aiming to create statistical models to predict class memberships [4]. Few studies are also informative [8-9], aiming to identify markers to understand the reason of samples discrimination.

In the following chapter, untargeted metabolomics has been applied to differentiate between common and durum wheat varieties. Subsequently markers were identified, enabling also to authenticate adulterated admixture samples both in whole-grain and refined durum wheat flour. Compared to the classical genetic methods, the new proposed approach is characterized by a more rapid and less laborious sample preparation and shorter time for the analysis is required.

The seventh chapter will be submitted to Food Chemistry.

SECTION III: Wheat authenticity

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Chapter 7

**Untargeted lipidomics based on UHPLC-
HRMS reveals differences in the lipid
pattern among durum and common wheat**

Untargeted lipidomics based on UHPLC-HRMS reveals differences in the lipid pattern among durum and common wheat.

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Abstract

In the present work the possibility of using an untargeted metabolomic strategy to discriminate between common and durum wheat lipidome for an authenticity purpose was explored. A first study was conducted by analyzing 52 samples from two durum and common wheat varieties. Afterwards, an extended and independent sample set (172 samples and five varieties) was used for as a confirmatory study to verify the stability and consistency of the models obtained. Putatively identified markers were evaluated applying ROC curves resulting in individual marker AUC >90% both in preliminary and confirmatory study. In addition, the untargeted analysis was shown to be an effective approach differentiating between authentic durum wheat and its adulterated admixture down to 3% adulteration level, which is the maximum contamination level allowed by Italian legislation. The results demonstrate that untargeted lipidomics, in conjunction with chemometric tools has potential as a screening tool for the detection of wheat fraud.

Keywords: Authenticity; Common wheat; Durum wheat; Lipidomics; Untargeted metabolomics; Wheat; High-Resolution Mass spectrometry.

1. Introduction

Pasta constitutes a dominant portion of a standard Mediterranean diet, supplying a large fraction of the needs for energy-rich materials, such as considerable amounts of carbohydrates, proteins, fiber or minerals (Shewry et al., 2009; Pauly et al., 2013). There are many forms to cook pasta, and for this reason, pasta has been regularly voted in the top favorite dishes for many years, for almost everyone. In fact, 14.3 million tons of pasta are produced worldwide according to the survey carried out by the Associations of Pasta Manufacturers of the European Union (UN.A.F.P.A, 2015). In other words, it's clear that pasta is big business, but where there is big business there is the potential for fraud (Everstine et al. 2013).

The most important wheat species are durum wheat (*Triticum turgidum* spp. *durum*), also called pasta wheat to reflect its major end-use, and common wheat (*Triticum aestivum*), which is usually employed to make bread or other baked goods (Shewry et al., 2009). In Italy, dried pasta must be exclusively made of durum wheat, allowing a maximum common wheat flour contamination of 3% (Ministero Dell'Interno, 2001), considering that accidental contamination of semolina with bread wheat during harvesting, transport or storage remains possible. Europe's national governments, on the contrary, permit the production of dried pasta using common wheat (<http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A61985CJ0407>).

Durum wheat is the preferred raw material for pasta due to its technological properties. The high level of carotenoids in durum wheat gives pasta its desired yellow color, and the higher protein content is the primary factor associated with superior pasta cooking quality (Pauly et al. 2013). However, the price of durum wheat is 25% more expensive compared to common wheat. For this reason, raw material or pasta in industrial food production, which may be adulterated by common wheat, could easily generate more money. This has not been the first time that food was slightly diluted, to give only two examples, the melamine incidents in 2008 (Gossner et al. 2009) and the horsemeat scandal in 2013 (Abbots & Coles 2013).

During the last decade, food authenticity has become more and more important and different “Omics” techniques have been gradually employed (Cevallos–Cevallos et al. 2009, Cubero-Leon et al. 2014, Rubert et al. 2015, Sørensen et al. 2016). Throughout the biological cascade, durum and common wheat have been verified. Initially, common and durum wheat were authenticated by DNA-based methods (Woolfe et al. 2004), taking advantage of the different ploidy levels of common (ABD) and durum wheat (AB). Amplification with end-point PCR of DNA sequences belonging to the DD genome has been also investigated (Arlorio et al. 2003). Nevertheless, DNA degradation may occur during technological processing, generating false negative results. A part from this disadvantage, DNA approaches are relatively expensive and time-consuming.

Subsequently, proteins, a step down in the biological cascade, are of great importance, since different genomic structures, such as common and durum wheat, may affect their protein expression. In this frame, the aleurone layer of *Triticum aestivum* and *Triticum durum* were manually dissected and analyzed using two-dimensional gel-based proteomics (Meziani et al. 2012). The comparison between species revealed that only 12.7% differed between the two species, mainly globulin type storage proteins, which were involved in carbohydrate metabolism and in stress pathways (Alary et al. 2002). The absence of the D genome from durum wheat was also investigated by a bottom-up proteomics strategy. In this case, common and durum wheat samples were treated with pepsin and chymotrypsin, and a peptide with a molecular weight of 3909 Da was exclusively found in common wheat samples (Prandi et al. 2012).

Coming to the end of the biological cascade, the study of metabolites is growing up rapidly. Since these small molecules (<1200 Da) are generated by enzymatic reaction that result from gene expression, the metabolome can be considered the final downstream product of genome, transcriptome and the proteome, linking together genotype and phenotype. Therefore, some most relevant differences in the genetic background (i.e. common and durum wheat) may be detected and amplified investigating differences in the metabolome (Gieger et al., 2008). Up to now, differences in the small molecule composition of common and durum wheat have been scarcely reported (Matteheews et a. 2012; Knödler et al. 2010). As an example, the alkylresorcinol (AR) composition, and in particular the C17/C21 homologues ratio, has been used to estimate the adulteration of durum wheat (Knödler et al. 2010). Unfortunately, alkylresorcinols are present only in the hyaline layer, outer layer, limiting the analysis to whole-grain products. Nevertheless, step-by-step metabolomics emerged as the combination of advanced analytical techniques merged with chemometric pattern recognition, providing a powerful approach for food metabolomics, and it served as a new solution to old problems (Cevallos–Cevallos et al. 2009, Cubero-Leon et al. 2014, Rubert et al. 2015, Sørensen et al. 2016).

The main aim of this research was to investigate common and durum wheat lipidome in order to identify significant markers for wheat verification strategies. A first study was conducted by analyzing 52 samples from two wheat varieties Odisseo (durum wheat) and Blasco (common wheat). Afterwards, the preliminary statistical model was validated by applying two strategies: (i) the analysis of further samples, 173 samples of 5 different wheat varieties (common and durum wheat), and subsequently (ii) the use of statistical tests for a continuous diagnostic markers and the preparation of admixtures at different concentration levels were employed. These novel validation approaches were performed in order to confirm the stability and consistency of the models obtained and the applicability of markers for the authentication purpose.

2. Material and methods

2.1 Chemicals and Reagents.

Polytetrafluoroethylene (PTFE) 50 mL centrifugation cuvettes were obtained from Merck (Praha, Czech Republic). HPLC grade methanol, dichloromethane and 2-propanol were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were supplied by Sigma–Aldrich (St. Luis, MO, USA). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2 Study design

The experimental design is the plan to perform data-gathering studies in order to provide a realistic strategy, which can catch the variation related to biological observations rather than process variation. This study had to be sufficiently powered to produce meaningful measures of specificity and sensitivity. For this reason, two complementary studies were carried out (i) the preliminary study and (ii) the confirmatory study.

The preliminary study was initially conducted by analyzing 52 samples from Odisseo (durum) and Blasco (common) wheat lines. In parallel, multivariate data analysis (MVDA) and univariate data analysis (UVDA) strategies were performed in order to build unsupervised and supervised statistical models and to discriminate markers. At this point, in order to confirm that the changes observed in Blasco and Odisseo were not attributed to these specific varieties and could be considered as a general change occurring between common and durum wheat, an extended sample set (172 samples and five varieties) was used for the confirmatory study. The confirmatory study was performed repeating in a separate chromatographic run and applying the same analytical and data treatment procedure. In the end, in order to determine the method sensitivity and specificity two approaches were evaluated: (i) receiver operating characteristic (ROC) curves and (ii) admixture samples test. The overall study design scheme is depicted in *Figure 1*.

2.3 Plant Material

Blasco (common, n = 26) and Odisseo (durum, n = 26) varieties were chosen among genotypes currently cultivated and used for food products in Italy. Samples were cultivated in Parma.

For the confirmatory study, 173 samples for five varieties of durum (*Triticum durum* Desf.) and common wheat (*Triticum aestivum* L.) were collected (*Table 1 supplementary material*). Grains were cultivated in two locations in Emilia Romagna region, Parma and Bologna, in plots of 8.25 m² with four replications. Samples were grown over two consecutive years (2013/2014 and 2014/2015)

under two agricultural conditions: conventional ($n = 58$) and organic farming ($n = 58$) in Parma, whereas only conventional farming was applied in Bologna ($n = 57$). After harvesting, the whole grains were dried at ca. 10% humidity, stored at $-20\text{ }^{\circ}\text{C}$ and kept refrigerated until the analysis. Overall, seven wheat varieties were collected resulting in 225 wheat samples, considering both preliminary and confirmatory study.

In order to determine the method sensitivity limit, a set of artificial samples with known content of adulterant were constructed in duplicate. The percentage values of common wheat in the mixtures (15%, 10%, 5%, 3%, 2%, 1%) were calculated on flour wheat weight.

2.4 Untargeted lipidomics workflow

Three steps can be clearly distinguished within lipidomics analysis: (i) sample preparation, (ii) the chromatographic separation and MS conditions and (iii) data treatment and statistics. In this research, sample preparation and ultra-high performance liquid chromatography quadrupole-time of flight (UHPLC-QTOF) untargeted lipidomics method have been based on our previous research works (Righetti et al., 2016; Rubert et al. 2017). By contrast, data treatment and statistics have been partially carried out by works and complemented by new strategies (detailed below).

2.4.1 Data treatment and statistics

MarkerView software (version 1.2.1, SCIEX, Concord, ON, Canada) was employed in order to process UHPLC-HRMS records. Data mining was performed using an algorithm using retention time range (RT) (0.4 – 14 min) and peak finding (m/z range was 100 – 1200). Subsequently, retention time and m/z alignment of the respective molecular features were executed using retention time and m/z tolerances of 0.2 min and 0.02 Da, respectively. Two separate positive and negative ionization data matrices, were automatically obtained using MarkerView, and subsequently processed.

In order to avoid systematic bias due to analytical variation, all samples were injected under a randomized sequence and two QC samples were prepared and analyzed at regular intervals through the analysis every ten “test samples”. The robustness of the analytical procedure was demonstrated by the tight clustering of QC samples obtained by mixing equal volumes of all the samples (Godzien, Alonso-Herranz, Barbas, & Armitage, 2014). In addition, QCs located in the center of the plot when sent to be classified by the model, ensure that separation between groups is not random but due to real variability. After QCs check, the variables were filtered retaining entities present in at least 50% of the samples in one group, with coefficients of variation less than 30% across the QCs. At this point, UVDA was performed comparing groups using the Mann-Whitney

U-test followed by Benjamini-Hochberg post-hoc corrections (corrected *p* value ≤ 0.05 ; *q* value set at 0.01) to minimize false positives by using IBM SPSS v.23.0 (SPSS Italia, Bologna, Italy). In parallel, MVDA has been applied: data were pre-processed using the pareto scaling and unsupervised principal components analysis (PCA) and supervised model, orthogonal partial least square discriminant analysis (OPLS-DA) models were built using SIMCA software (v. 13.0, 2011, Umetrics, Umea, Sweden; www.umetrics.com). Statistically significant markers with variable influence in projection (VIP) value threshold > 1.5 were selected. Putatively markers identification was performed choosing the most significant metabolites resulting common to both univariate and multivariate data treatment.

Finally, to evaluate the discriminatory capability of potential markers, ROC curves of each marker were exploited using the SPSS Statistics software (v.23.0, SPSS Italia, Bologna, Italy). (Xia et al., 2013, Picò et al., 2015, Garcia-Aloy et al., 2015, Alonso et al., 2015). The global performance of each marker was evaluated using the area under the ROC curve (AUC) evaluating both preliminary and confirmatory sets. Based on the ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate ($=100$ —specificity, %) for different cutoff points of a targeted marker (Xia et al., 2013, Picò et al., 2015, Garcia-Aloy et al., 2015).

3. Results and discussion

3.1 Data processing and statistics within lipidomics analysis

3.1.1 Preliminary sample set

The robustness of the analytical procedure was assessed by the clustering of quality control samples obtained by mixing equal volumes of all the samples. Initially, raw data PCA was constructed in order to detect sample clustering in the measured data and to have an overview of the trend, including the determination of putative outliers. After data quality assessment check, data were filtered in two steps. First, by choosing the entities present with a rate of 50% in at least one group of samples. Secondly, metabolites with large measurement error represented by coefficient of variation in QC samples higher than 30% were removed from the data set. PCA models based on filtered data (*Figure 1 A and B*) clearly indicate a scenario where common and durum wheat were successfully separated both positive and negative ionization modes. The first two PC components, ESI(+) and ESI(−) models, described more than 52% of variance. At the same time, supervised models, such as OPLS-DA were constructed, to maximize differences and to highlight key variables and potential markers. The quality of the models for both positive and negative ionization modes were excellent as shown in *Figure 1 E* where all the goodness of fit (R^2) and the prediction ability (Q^2) parameters are summarized.

In parallel to MVDA also UVDA was performed to select significant variables. The number of entities filtered out through each statistical analysis steps are listed in *Table 2 supplementary material*.

3.1.2 Confirmatory study

The same data treatment, which has been explained above, was applied to the confirmatory study. Initially, PCA score plots (*Figures 1 A and B supplementary material*) of UHPLC–MS records of unfiltered raw data demonstrated that the QCs were tightly clustered close to the plot center across the entire sequence, suggesting a high quality of data acquisition. Above all when large set of samples are analysed resulting in a long length metabolomic sequence, QC injections distribution is the most popular, pragmatic and reliable solution for controlling the quality and validating the repeatability of the untargeted metabolomics dataset (Godzien et al., 2014).

In the filtered PCA models (*Figure 1 C and D*) the five varieties were arranged clustered in two major groups according to their degree of ploidy, confirming the sample clustering obtained in the preliminary set. The quality of both positive and negative models were increased since the sum of PC1 and PC2 explained more than 55% of the total variance (ESI(+)) and ESI(-)). In fact, a more pronounced clustering was obtained using ESI(-) data, since the common and durum wheat groups resulted less spread into the 95% confidence ellipse, indicating less inter-group variability. In addition, it should be note that sample clustering was not affected by vegetative year, growing location, and farming condition, suggesting the strong influence of the genetic background more than environment on the sample differentiation.

At this point, sample clustering was fully understood and models were validated. Nevertheless, lastly, metabolomic studies in food authenticity are mainly discriminative, aiming to find differences(between sample groups) and predictive , aiming to create(statistical models to predict class memberships (Cevallos et al., 2009). Understanding the reason of the classification by the identification and quantification of metabolites responsible for this classification (informative metabolomics) is not considered as essential aim. In our opinion, however, the identification of metabolites is vital to obtain information regarding sample classification, as well as markers which may potentially use for authenticity purpose .

3.2 Markers evaluation

3.2.1 Data interpretation

In this way, taken together, 73 metabolites were cross-selected based on the preliminary and confirmatory studies using MVDA and UVDA analysis. Six-teen markers were tentatively identified following the next steps: marker identification based on accurate mass, isotopic pattern and MS/MS pathway, and off- or online databases. Even different databases were used, including

Lipid View, several metabolites remained unknown, suggesting that the wheat lipidome is much more complex than currently is known and deserves further investigation. The confirmatory analysis revealed a large number of statistically and significant markers in the vast majority of cases, markers were shared between both preliminary and confirmatory studies. The 16 markers putatively identified in the first study, were also significant in the confirmation study. In other words, the same tendency of variation between common and durum wheat was observed in both studies. Basically, the most significant markers were related to alkylresorcinols (AR), triacylglycerols (TAGs) and galactolipids. All metabolites identified are summarized in *Table 1.*, describing pseudomolecular ions, m/z values, retention times (RT), molecular formula, mass errors, adjusted p -values in both preliminary and confirmatory study and coefficient of variance (CV) in quality control are summarized.

In the present food authenticity work, seven statistically significant markers, belonging to the resorcinol's class, were tentatively identified. These ARs were detected in negative ionization mode producing both a $[M - H]^-$ and the $[M + HCOO]^-$ and tentatively identified based on information available in literature (Righetti et al., 2016). By contrary, TAGs and galactolipids were detected in positive ionization mode as ammonium adducts, giving a pseudomolecular ion $[M + NH_4]^+$ and protonated species. The putative identification was performed comparing experimental MS/MS spectra with the literature reported (Bird et al., 2011, Brewer et al., 2016) and LipidView off-line database. On the other hand, ESI(-) sample clustering was clearly influenced by different ARs homologues composition of the two wheat species. Level of ARs characterized by shorted alkylic chain, including C17:0, C19:0, C19:1, C21:0, C21:1, was found to be significantly higher in common wheat compared to durum wheat, which is consistent with the results reported in the literature (Andersson et al., 2008). By contrast, as depicted in figure *Figure 3*, our results suggested that durum wheat is characterized by a higher level of longer homologues such as C23:0 and C25:0, in agreement with information available in literature (Andersson et al., 2008). Among the major cereals, ARs have been reported to be present in high levels ($>500 \mu\text{g/g}$) in wheat (Ross et al., 2003), mainly in the outer layers of the kernels (Landberg et al., 2008), that means not in the edible part of cereals, since they are lost during flour refining steps. Therefore, they are linked only with products containing or enriched by bran fractions and can be used as markers for whole grain wheat cereal products (Knodler et al. 2010). Furthermore, the C17:0/C21:0 ARs homologue ratio has already been proposed to differentiate between common and durum wheats (Landberg et al. 2008, Knodler et al. 2010).

On the other hand, concentration of TAGs and galactolipids increased pearling and milling fractions. A recent study has demonstrated that TAGs, the major form of storage lipid in wheat,

accounted for 55% of the total lipids in the inner oil-rich tissues such as aleurone fraction, together with galactolipids (10% of the total lipids) (Gonzalez-Thuillier et al., 2015). Taken together, TAGs and galactolipids are responsible for the excellent separation of common and durum wheat in positive ionization mode. It should also be noted that the high signal intensity and the intra-group reproducibility, suggested by the standard error of the mean (SEM), makes these compounds excellent markers. This was also demonstrated by the lower coefficient of variation in QCs (<10%) and by the fact that no missing values were found for these markers through all the 225 samples. In our study, an opposite tendency of intra-class variation of these two lipid species was observed. Lower levels of monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) and higher levels of TAGs have been found in tetraploid wheats compared with those in hexaploid wheats. While most of the research effort has been expended in studying common wheat lipid profile (Finnie et al. 2010a, Finnie et al. 2010b, Gonzalez-Thuillier et al., 2015), a few studies have been focused on the comparison between common and durum lipids content. In agreement with our results, Brewer et al. (2016) reported a higher content of TAGs in Svevo cultivar (durum wheat) compared to a common wheat line N11. However since that was not the aim of the study, the chain lengths of the fatty acids composing triglycerides were not specified.

Regarding galactolipids, their higher amounts in common wheat have been previously reported (Hernandez-Lucas et al. 1976) with DGDG as major component, followed by MGDG. This difference was attributed to their genetic diversity (*T. turgidum* AABB, *T. aestivum* AABBDD) (Shewry et al. 2009), leading to the hypothesis that DGDG could be the lipid factor controlled by the D chromosomes (Hernandez-Lucas et al. 1976). These results were further supported by the difference in bread-making quality between the two types of wheat, since DGDG contributes to baking quality by improving the retention of fermentation gases (Pareyt et al. 2011).

3.2.2 Roc curves

DGDG 36:4 was found to be one of the most discriminant common marker (adjusted *p*-value 0.000397) with a mean value three times higher compared to durum wheat. The marker SEM variation reported in the confirmatory study was 3.5% along common samples (n=52) and of 6.5% for durum wheat sample (n= 69). This suggested that its presence is strongly influenced by the genetic background, since other wheat varieties, growing locations and vegetative did not provided significant differences. The same trend was highlighted for AR C17:0, detected in negative ionization mode. For this particular marker, the common wheat intensities were almost 8 times higher compared to durum varieties.

For an authenticity purpose, these two markers could be considered ideal since their intensities are higher in common wheat compared to durum wheat. Thus, they can be used as markers for the presence of common wheat. In order to examine the potential of these two metabolites for food fraud, especially distinguishing borderline adulterated samples, receiver operating characteristic (ROC) curves with AUC (area under the curve) value were constructed. ROC curves have been used for the evaluation of the sensitivity and specificity based on the false positive/negative rate (Xia et al., 2013, Picò et al., 2015, Garcia-Aloy et al., 2015, Alonso et al., 2015). In fact, ROC AUC can be calculated for the robustness of the statistical analysis, being the measure of how well a marker can distinguish between two groups. *Figure 4* shows ROC curves of DGDG 36:4 and AR C17:0. These two markers can be considered perfect classifiers having an AUC > 95% in both preliminary and confirmatory sets (*Figure 4*). ROC curves for all the putatively identified common wheat markers were constructed independently, and their AUC values, ranged from 65-100%, are reported in *Table 3 Supplementary material*. Note that the performance of the confirmatory set remained excellent, even though five more varieties, two agricultural conditions, two harvest years and two growing locations were added as additional sources of variability.

3.2.3 Admixture test

Six adulterated samples by mixing 1%, 2%, 3%, 5%, 10%, 15% of common wheat in durum wheat samples were prepared. *Figure 5* shows the intensity of the marker DGDG 36:4 in 100% durum wheat and in different admixture levels. This marker is significantly higher in common wheat, therefore, the adulterated samples should discriminate increasing the content of DGDG 36:4 compared to 100% durum wheat (mean value resulted from the 69 durum wheat samples of the confirmatory study).

First of all the differences in this marker among 100% durum wheat and admixtures were significant (p -value < 0.05) (*Figure 5*). However, 2% and 1% adulteration levels were not achieved. It should be noted that the applied methodology was untargeted, that means that the chromatographic separation and MS detection were not properly optimized for the above-mentioned marker, an individual optimization could significantly improve the limit of detection. Based on the changes in the intensity of this marker, the limit of detection was fixed at 3%. Sooner or later, untargeted metabolomics approaches must accurately quantify markers in order to report the content of these markers for the scientific community.

4. Conclusion

In the current study an untargeted lipidomics strategy was performed to discriminate common and durum wheat varieties. This approach revealed statistically significant metabolites,

which potentially can differentiate common wheat and durum wheat. Among the metabolites resulted statistically significant in both preliminary and confirmatory study, alkylresorcinols, and in particular heptadecyl-resorcinol, could be further used for the discrimination of common and durum whole grain flour, being present in the outer layer of the kernel and thus lost during refine process. By contrast, digalactosyl diglyceride 36:4, an abundant membrane-forming lipid mainly concentrated in the common wheat inner layer, may be distinguish durum wheat adulteration also in refined flours.

On the other hand, the untargeted analysis was shown to be an effective approach differentiating between authentic durum wheat and its adulterated admixture down to 3% adulteration level, which is the maximum contamination level allowed by Italian legislation. To further develop and investigate such a strategy, more different common and durum wheat varieties must be analyzed and markers have to be unambiguously identified by using analytical standards. In conclusion, the results demonstrate that untargeted lipidomics, in conjunction with chemometrics, has potential as a screening tool for the detection of wheat fraud.

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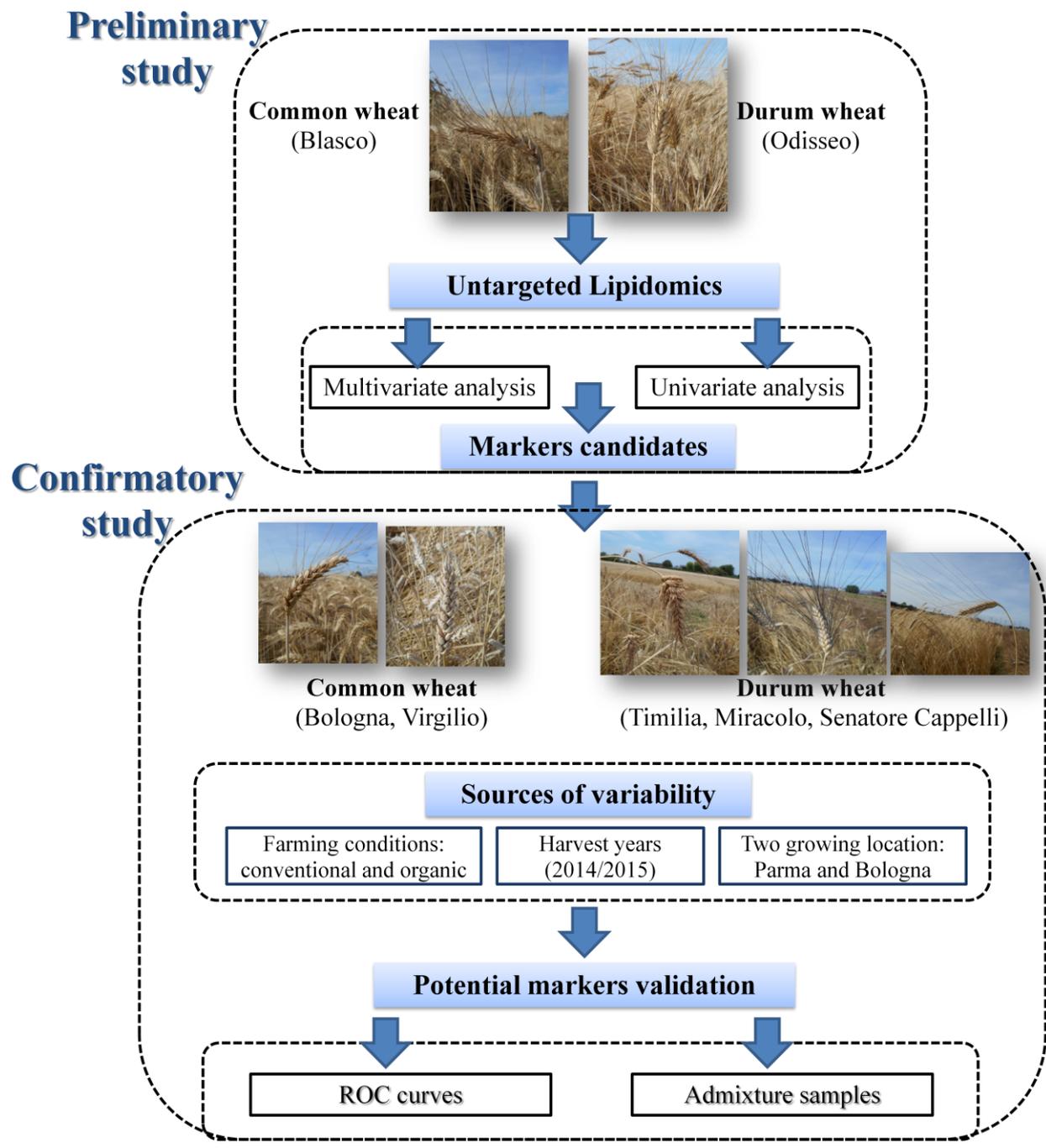
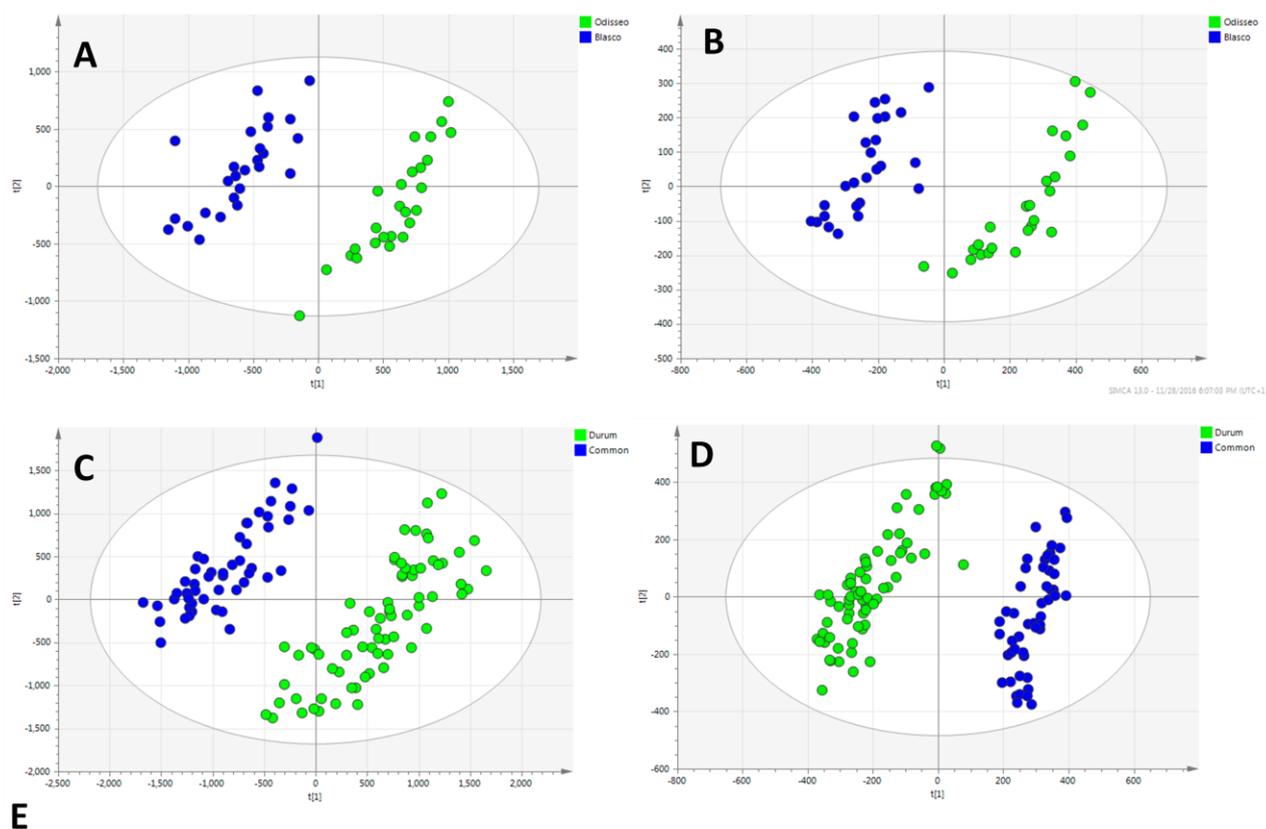


Figure 1. Schematic illustration of the study design.



E

Ionization mode	ESI(+) Models				ESI(-) Models			
	Preliminary		Confirmatory		Preliminary		Confirmatory	
Study	PCA	OPLS-DA	PCA	OPLS-DA	PCA	OPLS-DA	PCA	OPLS-DA
Statistical Parameters								
R^2X (cum)	0.847	0.77	0.844	0.638	0.903	0.756	0.883	0.554
R^2Y (cum)	-	0.989	-	0.978	-	0.96	-	0.976
Q^2 (cum)	0.737	0.982	0.708	0.967	0.858	0.954	0.797	0.962

Figure 2. Unsupervised principal components analysis (PCA) models built with preliminary sample set (positive (A) and negative ionization data (B)) and confirmatory study data (positive (C) and negative ionization data (D)). Green dots represent common wheat varieties and blue dots durum wheat. In both studies, the two *Triticum* species were clearly separated already in the unsupervised models. Statistical values for PCA and OPLS-DA models, including the variance of the x and y variables explained by the model (R^2X (cum) and R^2Y (cum)), and cumulative predicted variation in the Y matrix (Q^2) are summarized (E).

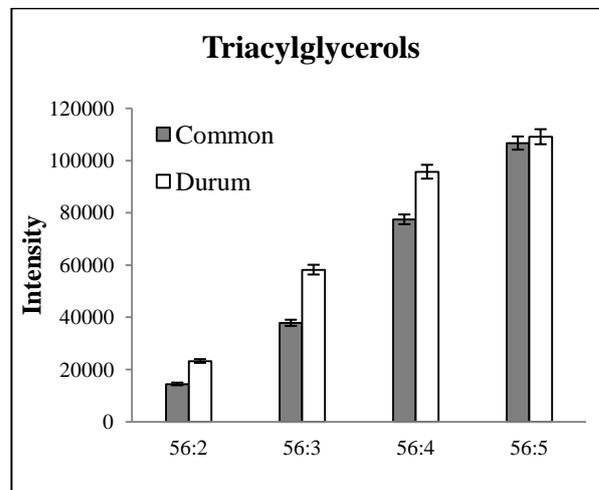
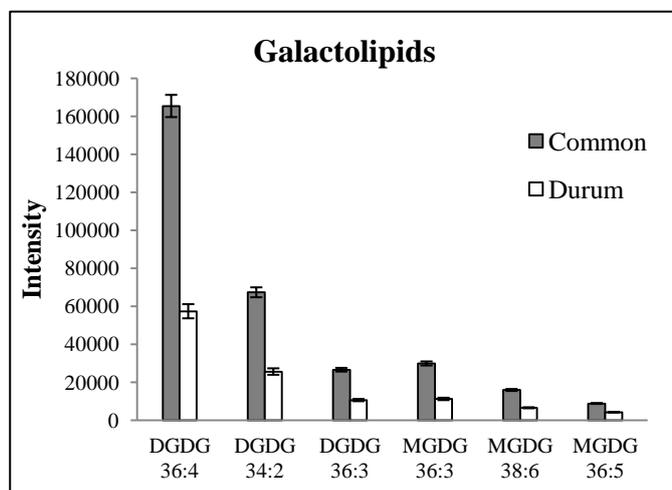
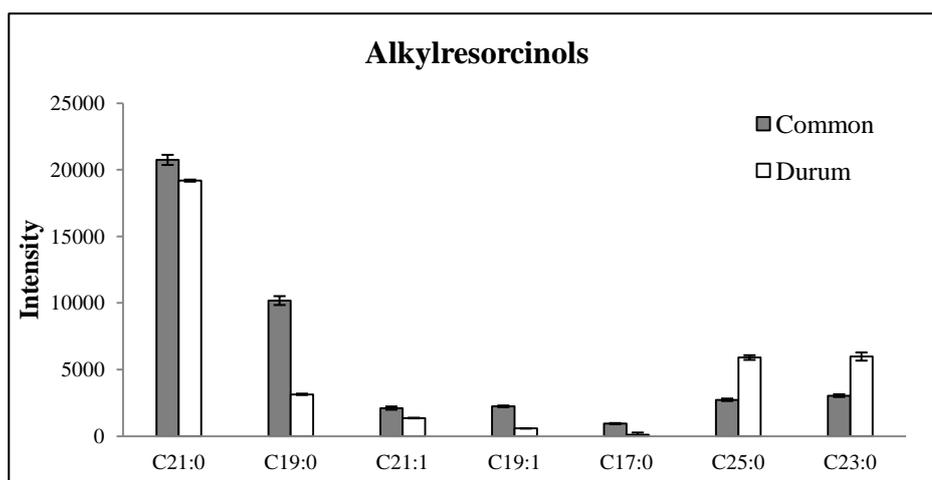
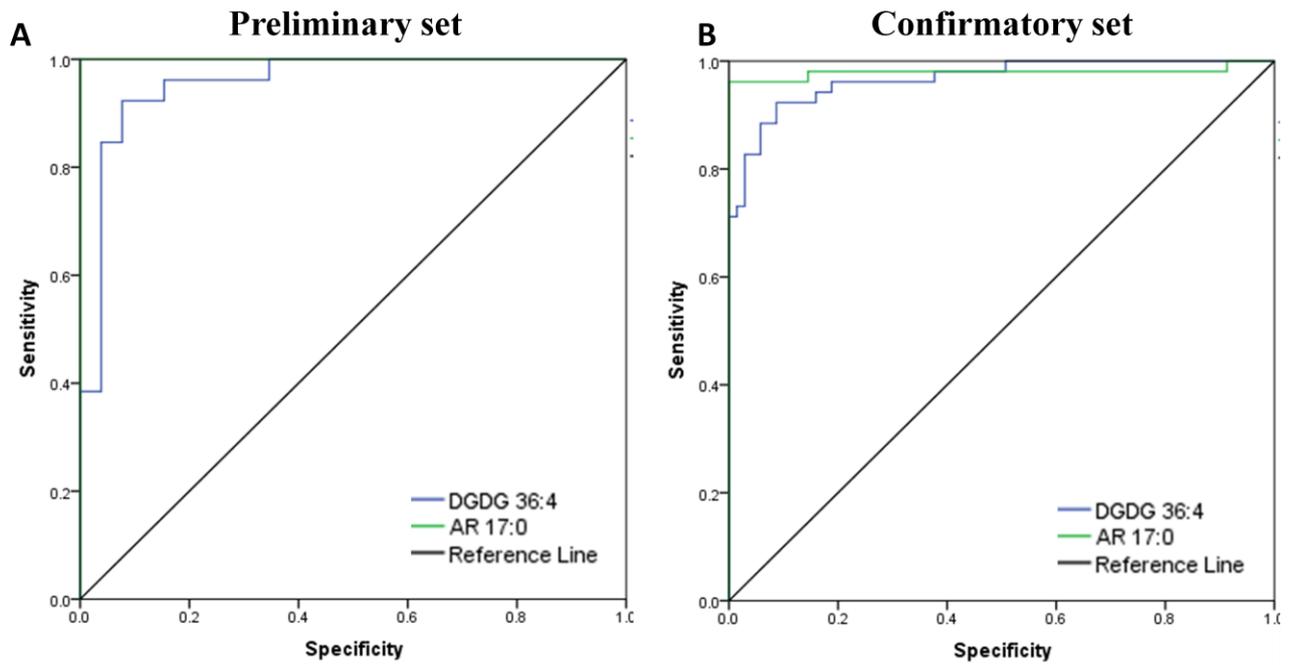


Figure 3. Lipid class bar graphs showing intensity of statistically significant markers in both common and durum wheat varieties: alkylresorcinols (A), galactolipids (B) and triacylglycerols (C). Intensities are given as mean values \pm SEM (standard error of the mean) of 69 durum and 52 common wheat samples (confirmatory study).



AUC (CI 95%)

Marker	DGDG 36:4	AR 17:0
Preliminary set	95.7 (90.3-100)	100 (100-100)
Confirmatory set	96.7 (93.8-99.5)	98.0 (94.5-100)

Figure 4. operating

Receiver characteristic

(ROC) curves of DGDG 36:4 and AR C17:0 metabolite in the training and validation sets.

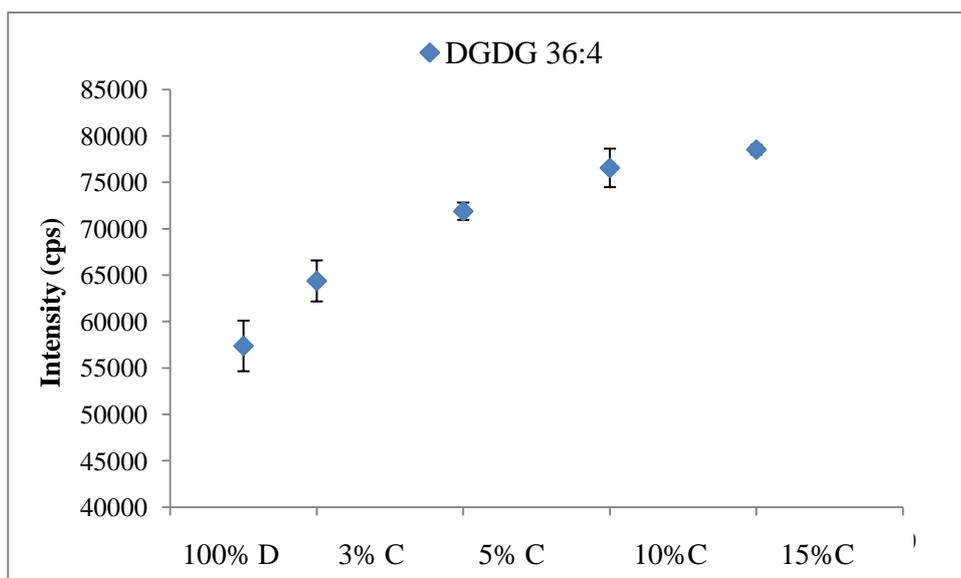


Figure 5. Variable trend plot for digalactosyl diglyceride 36:4 found in the authentic durum wheat (100%D) and durum wheat samples adulterated with common wheat at 3%, 5%, 10%, 15%. Data points represent mean intensity values \pm standard deviation of two replicates for the admixture samples and of 69 samples (durum wheat samples in confirmatory study) for the authentic durum wheat sample.

Table 1. Identification of the most significant metabolites when comparing common and durum wheat lipidome.

Tentative identification	Pseudomolecular ion	Detected m/z	RT (min)	Elemental formula	Mass error (Δ ppm)	<i>p</i> -value		CV in QCs [%]
						Preliminary set	Confirmatory set	
5-Heptadecylresorcinol (C17:0)	[M-H] ⁻	347.2958	6.26	C ₂₃ H ₄₀ O ₂	0.7	2.70E-05	4.14E-04	15
5-(12-Nonadecenyl)-resorcinol (C19:1)	[M-H] ⁻	373.3133	6.35	C ₂₅ H ₄₂ O ₂	0.9	8.10E-05	6.21E-04	13
5-Nonadecanylresorcinol (C19:0)	[M-H] ⁻	375.3274	6.85	C ₂₅ H ₄₄ O ₂	1.5	1.08E-04	2.66E-04	13
5-(Heneicosenyl)-resorcinol (C21:1)	[M-H] ⁻	401.3433	6.88	C ₂₇ H ₄₆ O ₂	2	4.80E-03	9.02E-03	6
5-Heneicosylresorcinol (C21:0)	[M-H] ⁻	403.3589	7.38	C ₂₇ H ₄₈ O ₂	1.8	6.59E-03	7.99E-03	12
5-Tricosylresorcinol (C23:0)	[M-H] ⁻	431.3910	7.99	C ₂₉ H ₅₂ O ₂	1.2	7.86E-04	8.88E-04	13
5-Pentacosylresorcinol (C25:0)	[M-H] ⁻	459.4236	8.47	C ₃₁ H ₅₆ O ₂	2.3	2.44E-04	5.90E-05	8
MGDG 36:5	[M+NH ₄] ⁺	794.5769	7.63	C ₄₅ H ₇₆ O ₁₀	-1	8.21E-04	2.55E-04	5
MGDG 36:3	[M+NH ₄] ⁺	798.6091	8.18	C ₄₅ H ₈₀ O ₁₀	0.2	1.22E-03	3.40E-04	6
MGDG 38:6	[M+H] ⁺	803.5654	8.27	C ₄₇ H ₇₈ O ₁₀	-1.7	1.14E-03	1.13E-04	10
DGDG 34:2	[M+NH ₄] ⁺	934.6485	7.82	C ₄₉ H ₈₈ O ₁₅	2.5	2.99E-03	6.23E-04	6
DGDG 36:4	[M+NH ₄] ⁺	958.6456	7.57	C ₅₁ H ₈₈ O ₁₅	-0.6	1.74E-03	3.97E-04	6
DGDG 36:3	[M+NH ₄] ⁺	960.6629	7.81	C ₅₁ H ₉₀ O ₁₅	1.1	7.16E-03	4.82E-04	6
TAG 56:5 (18:2/18:2/20:1)	[M+NH ₄] ⁺	926.8174	11.79	C ₅₉ H ₁₀₄ O ₆	0.3	1.00E-04	6.16E-03	5
TAG 56:4 (18:2/18:1/20:1)	[M+NH ₄] ⁺	928.8336	11.94	C ₅₉ H ₁₀₆ O ₆	0.9	4.98E-04	5.50E-03	4
TAG 56:3 (18:1/18:1/20:1)	[M+NH ₄] ⁺	930.8491	12.18	C ₅₉ H ₁₀₈ O ₆	0.7	1.24E-04	2.32E-03	8
TAG 56:2 (18:1/18:1/20:0)	[M+NH ₄] ⁺	932.8630	12.36	C ₅₉ H ₁₁₀ O ₆	0.7	2.61E-03	1.64E-03	8

Supplementary information

Table 1. Information for seven wheat lines used for the authenticity study.

Species	Wheat line	Ploidy level	Genome	Classification
<i>Triticum aestivum</i> L.	Bologna	hexaploid	AABBDD	Common
	Virgilio	hexaploid	AABBDD	wheat
	Blasco	hexaploid	AABBDD	
<i>Triticum turgidum</i> ssp <i>turgidum</i>	Grano del miracolo*	tetraploid	AABB	Rivet, cone or English wheat
<i>Triticum turgidum</i> var. <i>durum</i> Desf.	Senatore Cappelli	tetraploid	AABB	Durum wheat
	Timilia	tetraploid	AABB	
	Odisseo	tetraploid	AABB	

Table columns: Wheat line = common field identifier; Ploidy level = number of sets of chromosomes; Genome = nucleotide diversity assigned to the A, B and D genomes.

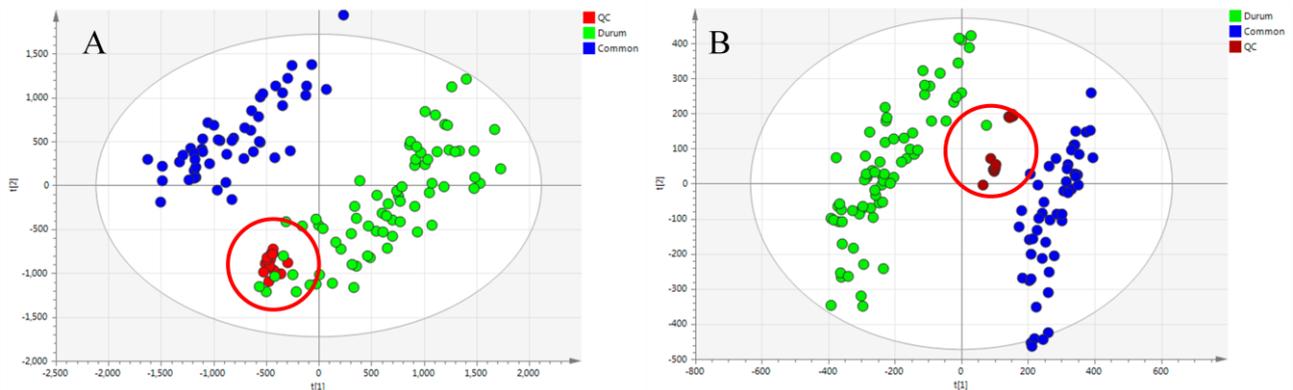


Figure 1. Quality of the methodology assessed using PCA modelling in the confirmatory study (positive (A) and negative (B) ionization data), showing QCs clustering.

Table 2: Entities resulting after different step of data treatment and statistical analysis for preliminary and confirmatory study.

Data analysis	Stages	Common/Durum wheat preliminary study		Common/Durum wheat markers confirmation study	
		ESI+	ESI-	ESI+	ESI-
Data set creation	Alignment	2397	3459	2741	4087
QCs assessment	Filter by Frequency 50%	1051	1093	1034	1271
	Filter by Variability 30% in QCs	392	358	493	464
Data treatment	Mann-Whitney <i>U</i> -test				
UVDA	(<i>p</i> -value ≤ 0.05)	388	358	353	338
	FDR (<i>q</i> *0.01)	388	356	353	338
MVDA	VIP>1.5	35	38	74	73
	Common in both statistics	35	38	59	64
	Identified	10	7	-	-

Table 3. Area under the curve (AUC) values for common wheat markers.

Marker	AUC (CI 95%)	
	Preliminary set	Confirmatory set
5-(12-Nonadecenyl)-resorcinol (C19:1)	100 (100-100)	97.8 (94.4-100)
5-Nonadecanyl-resorcinol (C19:0)	100 (100-100)	98.0 (94.3-100)
5-(Heneicosenyl)-resorcinol (C21:1)	15.5 (5.3-15.8)	76.3 (67.8-84.7)
5-Heneicosylresorcinol (C21:0)	25.9 (12.6-39.1)	65.1 (55.5-74.9)
MGDG 36:5	99.1 (97.2-100)	97.3 (94.0-100)
MGDG 36:3	97.9 (94.9-100)	97.0 (94.3-99.7)
MGDG 38:6	98.5 (96.0-100)	98.1 (96.2-99.9)
DGDG 34:2	90.2 (81.9-98.6)	95.2 (91.7-98.7)
DGDG 36:3	76.6 (63.1-90.1)	96.1 (93.1-99.9)

4. CONCLUSIONS and FUTURE PRESPECTIVES

In the present Ph.D thesis the integrity of wheat has been investigated in depth, evaluating aspects related to safety, quality and authenticity. In the first section different analytical techniques have been successfully applied for ensuring wheat safety: from immunochemical screening, such as ELISA, to sophisticated high resolution mass spectrometry instrumentation, as well as from the targeted quantification of three mycotoxins (DON, DON-3Glc, 3Ac-DON) to the untargeted analysis of thousands of metabolites. The current trend, indeed, still continues for the validation of multi-mycotoxins method providing information both on regulated and less legislated mycotoxins, and thus providing a more realistic occurrence scenario. Therefore, the results obtained confirmed that HRMS is going to redefine LC-MS workflow since targeted and routine quantification as well as qualitative research analysis can be performed with the same instrument. In addition, metabolome differences between *Fusarium* contaminated wheat samples were successfully investigated and significant markers were identified allowing a better understanding of wheat metabolic pathways involved in plant-pathogen upon interaction. As a future perspective, stability and consistency of obtained multivariate models should be proven by the analysis of further samples in order to confirm the validity of the models. Then a bio-monitoring of the most significant markers identified could serve as an effective tool for the early detection of mycotoxins, and *Fusarium* disease prevention.

In the second section, untargeted analysis was shown to be an effective approach, used to differentiate between ancient wheat varieties. Multivariate models were validated and the high R^2 and Q^2 obtained for all the unsupervised and supervised models indicate an excellent predictability when different harvest year sample sets were jointly considered. The results obtained confirmed the importance of different alkylresorcinol homologues as cultivar markers, being strongly influenced by the genetic background, since their abundances were not significantly affected by growing location, organic or conventional farming, and/or vegetative year. Such molecules were shown to play important roles in quality of wheat but also for the wheat plant itself, exerting an inhibitory effect towards mycotoxin accumulation in ten wheat lines. Chapter 5 can be considered borderline between quality and authenticity assessment. Indeed, the identified variety-related markers might be used also to detect fraudulent practices.

In the last section, common and durum wheat lipidome were studied and compared by applying an untargeted metabolomic approach. Several markers have been tentative identified aiding to detect the durum wheat adulteration with common wheat down to 3% adulteration level, which is the maximum contamination level allowed by Italian legislation. In light of these results, a future

perspective could be further validated by analyzing more varieties and then markers could be unambiguously identified using analytical standards. At this point, simpler analytical methods (HPLC-QqQ or immunochemical methods) could be applied to provide control authorities with applicable routine methods.

Summarizing, the result obtained highlighted that untargeted methods as a powerful tool, which could be merged with chemometrics as a valuable approach to address different questions in food science, including the assessment wheat integrity. In addition,

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Studies

Laura Righetti got the Master degree in Pharmaceutical Chemistry (University of Modena, Italy) in November 2013, project titled “HPLC-QTOF-MS based metabolomic fingerprinting on acute respiratory distress syndrome by H₁N₁ influenza in humans: patient discrimination and biomarkers discovery”. Her master degree’s thesis focused on untargeted metabolomic approaches took place at the “Center for metabolomics and Bioanalysis” (University CEU San Pablo, Madrid, Spain) for a period of 6 months (2013). She volunteered in a pharmacy for 6 months and got the professional title of “pharmacist” in December 2013. Subsequently, January 2014, Laura Righetti started her PhD in Food Science (University of Parma, Italy), under the supervision of Prof. Chiara Dall’Asta. The PhD research work has been associated with a strong collaboration with the Department of Food Science (University of Parma, Italy) and University of Chemistry and Technology, Prague (Czech Republic) where she carried out a short-term visit, ten months exploiting new advanced mass spectrometry techniques, such as high resolution and ion mobility spectrometry. Her doctoral research aimed to ensure safety, authenticity and quality of cereals, using advanced mass spectrometry techniques. She has been also involved in national projects dealing with cereals contaminants, mainly mycotoxins and their metabolites.

Scientific activity

Review

Recent advance and future challenging in modified mycotoxins analysis: why HRMS has become a key instrument in food contaminant research.

Laura Righetti, Giuseppe Paglia, Gianni Galaverna, Chiara Dall'Asta.
Toxins, 2016, 8, 361.

Metabolomics approaches and their hidden potential for explaining the mycotoxin contamination problem.

Laura Righetti, Chiara Dall'Asta, Jana Hajšlová, Josep Rubert.
Book chapter in "Metabolomics" ISBN 978-953-51-4891-3 (*In press*).

Original papers

Group detection of DON and metabolites by an ELISA kit.

Laura Righetti, Gianni Galaverna, Chiara Dall'Asta.
Food Additives and Contaminants (*In press*).

Are organ cultures a suitable biofactory for masked mycotoxins?

Laura Righetti, Enrico Rolli, Gianni Galaverna, Michele Suman, Renato Bruni, Chiara Dall'Asta.
Scientific Report (*Submitted*).

Untargeted metabolomics based on UHPLC-HRMS merged with chemometrics: a predictable tool for an early detection of mycotoxins?

Josep Rubert, Laura Righetti, Milena Stranska-Zachariasova, Zbynek Dzuman, Chiara Dall'Asta, Jana Chrpova, Jana Hajslova.
Food Chemistry (*In press*).

Characterization and discrimination of ancient wheats: a metabolomic approach.

Laura Righetti, Josep Rubert, Gianni Galaverna, Silvia Folloni, Roberto Ranieri, Milena Stranska-Zachariasova, Jana Hajšlová, Chiara Dall'Asta.
International Journal of Molecular Science, 2016, 17, 1217.

5-n-Alkylresorcinols are directly related to a lower accumulation of mycotoxins in grains.

Laura Righetti, Martina Cirlini, Terenzio Bertuzzi, Paola Giorni, Silvia Folloni, Roberto Ranieri, Paola Battilani, Gianni Galaverna, Chiara Dall'Asta.
Journal of Agricultural and Food Chemistry (*Submitted*).

Untargeted lipidomics based on UHPLC-HRMS reveals differences in the lipid pattern among durum and common wheat.

Laura Righetti, Josep Rubert, Gianni Galaverna, Silvia Folloni, Roberto Ranieri, Milena Stranska-Zachariasova, Jana Hajšlová, Chiara Dall'Asta.
Food Chemistry (*Submitted*).

Communications

Routine analysis of DON, 3AcDON and DON3Glc by an immunoassay.

Laura Righetti, Giulia Rosar, Gianni Galaverna, Maurizio Paleologo, Chiara Dall'Asta.
Vendor Seminar Tecna, 7th RAFA, Praga, Czech Republic, 3-6 Novembre 2015.

A novel approach for authentication of durum/common wheat based on liquid chromatography high-resolution tandem mass spectrometry merged with chemometrics.

Josep Rubert, Laura Righetti, Kamila Hurkova, Milena Zachariasova-Stranska, Gianni Galaverna, Jana Hajšlová, Chiara Dall'Asta. Vendor seminar SCIEX, Food Integrity, Praga, Czech Republic, 6-8 April 2016.

Safety and quality of grains: new insights through –omics fingerprinting.

21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, Italy, 14-16 September 2016.

Characterization and authentication of ancient Triticum varieties: a lipidomic approach.

Laura Righetti, Josep Rubert, Gianni Galaverna, Milena Zachariasova-Stranska, Jana Hajšlová, Chiara Dall'Asta.

XI Congresso italiano di Chimica degli Alimenti, Cagliari, Italy, 4-7 October 2016.

Characterization and discrimination of ancient wheat varieties: a metabolomics approach.

Laura Righetti, Josep Rubert, Gianni Galaverna, Milena Zachariasova-Stranska, Jana Hajšlová, Chiara Dall'Asta.

Wageningen – Parma PhD day. Meeting of PhD students from Food Quality and Design chair (Wageningen UR) and the PhD Course in Food Science (University of Parma) Parma, Italy, 18 th October 2016.

Poster presentation

Catabolic fate of masked mycotoxins

Laura Righetti, Martina Cirlini, Letizia Bresciani, Gianni Galaverna, Arnaldo Dossena, Chiara Dall'Asta.

X Chimali, Firenze, Italy, 6-10 July, 2014.

Influence of pearling process on level of deoxynivalenol and deoxynivalenol-3-glucoside in wheat

Laura Righetti, Martina Cirlini, Chiara Dall'Asta, Jean Daniel Coisson, Marco Arlorio, Gianni Galaverna.

8th World Mycotoxin Forum Conference. Vienna, Austria, 10-12 November, 2014.

Transfer and metabolism of the modified mycotoxin Zearalenone-14-glucoside in human intestinal CACO-2 cells.

Martina Cirlini, Amelia Barilli, Laura Righetti, Valeria Dall'Asta, Chiara Dall'Asta.

13th European Fusarium Seminar, Martina Franca, Italy, 10-14 May, 2015.

Determinazione di deossinivalenolo e forme modificate mediante kit ELISA.

Laura Righetti, Giulia Rosar, Maurizio Paleologo Oriundi, Chiara Dall'Asta.

V Congresso nazionale micotossine, ISS, Roma, Italy, 28-30, September 2015.

Group detection of DON and metabolites by an ELISA kit

Laura Righetti, Giulia Rosar, Maurizio Paleologo Oriundi, Chiara Dall'Asta.

7th RAFA, Prague, Czech Republic, 3-6 November, 2015.

A metabolomic strategy to discriminate ancient Triticum varieties

Laura Righetti, Josep Rubert, Gianni Galaverna, Milena Zachariasova-Stranska, Chiara Dall'Asta, Jana Hajšlová.

Food Integrity Conference, Prague, Czech Republic, 6-7 April, 2016.

A novel approach for authentication of durum/common wheat based on liquid chromatography high-resolution tandem mass spectrometry merged with chemometrics

Josep Rubert, Laura Righetti, Kamila Hurkova, Milena Zachariasova-Stranska, Gianni Galaverna, Jana Hajšlová, Chiara Dall'Asta.

Food Integrity Conference, Prague, Czech Republic, 6-7 April, 2016.

Development of in vitro model plant system to study the detoxification ability of different wheat varieties

Laura Righetti, Enrico Rolli, Chiara Dall'Asta

38th Mycotoxin Workshop, Berlin, Germany, May 02-04, 2016

A novel lipidomic approach for characterization and authentication of ancient Triticum varieties

Righetti Laura, Rubert Josep, Stranska Milena, Hajšlová Jana, Galaverna Gianni and Dall'Asta Chiara

1st Incubatore Scientifico di Spettrometria di massa-IMass, Torino, Italy, July 20, 2016.

Awards and distinctions

Poster Prize Winner:

A novel lipidomic approach for characterization and authentication of ancient Triticum varieties

Righetti Laura, Rubert Josep, Stranska Milena, Hajšlová Jana, Galaverna Gianni and Dall'Asta Chiara

1st Incubatore Scientifico di Spettrometria di massa-IMass, Torino, Italy, July 20, 2016.

Participation to Phd School and workshop

Scientific Writing Training Course, Parma Italy, 6-10 October, 2014.

ILSI Workshop on "Hazard vs. Risk Based Approaches in Food Safety Assessment", Brussels, 2 December, 2014

"TripleTOF in Food Analysis" –AB SCIEX, UCT Prague, Czech Republic, 1 April, 2015.

Workshop: Contaminants Screening-Waters Corporation, Modena, Italy, 16-17 February, 2016.

Can you speak MATLAB? Parma, Italy, 16 March, 2016.

Food and Environmental European Seminars 2016-Thermo Fisher, Bologna, Italy, 23 June, 2016.

Hard skills for your academic success, Parma, Italy, 4-8 July, 2016.

1st IMaSS Data Analysis School-Torino, Italy, 21-22 July, 2016.

21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, Italy, 14-16 September, 2016.