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

Food Chemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

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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ARTICLE INFO

Article history:
Received 2 September 2016
Received in revised form 17 November 2016
Accepted 24 November 2016
Available online xxxx

Keywords:
Alkylresorcinols
Cereals
Lipidomics
Liquid chromatography-high resolution mass spectrometry
Metabolomics
Oxylipins
Plant-pathogen cross-talk
Wheat

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 oxylipins and diacylglycerols was revealed.

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, enniatins, and zearalenones. In addition to production of mycotoxins as compounds causing various acute and chronic adverse health effects,

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http://dx.doi.org/10.1016/j.foodchem.2016.11.132
0308-8146/

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 (McCormick et al., 2015; Nathanail & Syvähuoko et al., 2015; Rubert et al., 2012).

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) (Huet et al., 2010; Mishra, Fox, & Culham, 2003). 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 et al., 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 et al., 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; Gauthier, Atanasova-Penichon, Chéreau, & Richard-Forget, 2015; Nathanail & Varga et al., 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 & Varga et al., 2015). 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 Merci (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).

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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.

Fusarium infection level (%)[1]	0 – Traces of *Fusarium* spp. infection	45
	1 – Clear infection of either one spikelet or a slight infection of several spikelets	29
	2 – Either stronger infection of 2–3 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
Fusarium spp. (%)[1]	n.d.[2]	11
	F. poae	89
	F. sporotrichioides	1
	F. graminearum	15
	F. culmorum	1
	F. avenaceum	6
	F. equiseti	5
Mycotoxin content range μg/kg[3], and frequency of occurrence (%)[1]	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)

[1] Overall percentage.
[2] Non-detected.
[3] Minimum-maximum range.

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 et al., 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, Veprikova, Godula, & Hajslova, 2015; Dzuman et al., 2014). 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 (Righetti et al., 2016; Rubert, Lacina, Zachariasova, & Hajslova, 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 Laboratortechnik, Staufen, Germany) was used for 30 min at 240 S/min. Wheat extracts were then centrifuged 5 min, 13,416*g* 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 Laboratortechnik, Staufen, Germany) was used for 30 min at 240 S/min. Wheat extracts were then centrifuged 5 min, 13,416*g* 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 C_{18} (2.1 × 100 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

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(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* 100–1200, followed by acquisition of product ion spectra, ranging from *m*/*z* 50–1200, 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 (Righetti et al., 2016; Rubert et al., 2015, 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 $\leqslant 0.05$; 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, A_1, B and B_1 were detected in over 80% of wheat samples, with concentration levels ranging from 1 to 2.147 μg/kg. Deoxynivalenol (DON), the major trichothecene commonly found in wheat, was detected in about 25% of samples, at a concentration varied from a few μg/kg up to 10 mg/kg. Three out of 25 samples exceeded maximum tolerable level for unprocessed cereals of 1.250 μg/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 provided separation efficiency and good peak resolution. In this way, a BEH (Ethylene Bridged Hybrid) C_{18} 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 a 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 Fig. 1. A good peak shape, chromatographic resolution and RTs

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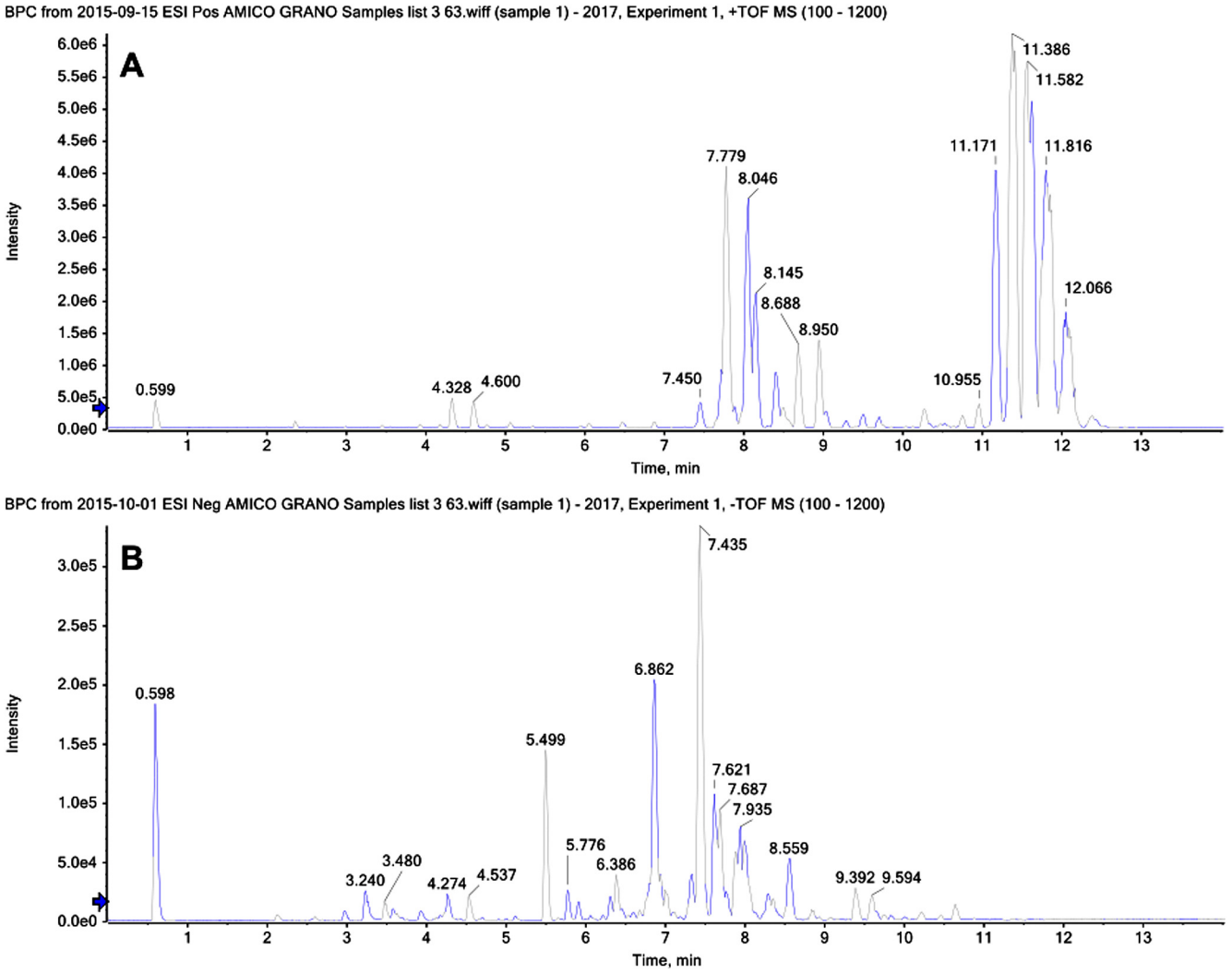

Fig. 1. Base peak chromatograms of a wheat sample extract (dichloromethane/methanol (50/50, v/v)) are compared for positive ionization mode (A) and negative ionization mode (B). The chromatographic separation was carried out using BEH C_{18} column, a multi-step elution dual-mode gradient and column oven temperature at 60 °C.

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 (Fig. 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 pretreatment, 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 Fig. 3A and B.

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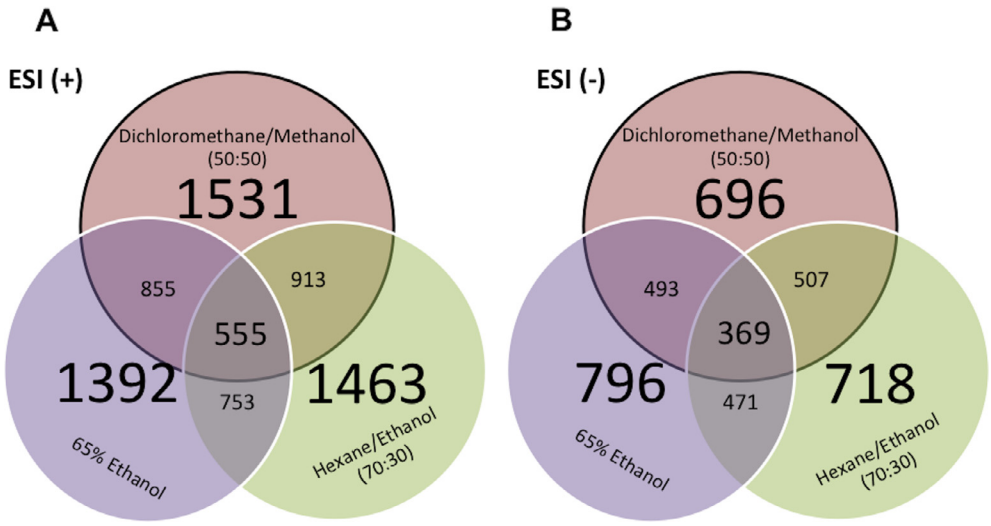

Fig. 2. Venn diagram shows all possible relations between three extraction procedures selected, common molecular features of these extraction procedures being represented by intersections of the circles. Venn diagrams depicts shared and unique molecular features detected in the wheat extracts prepared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

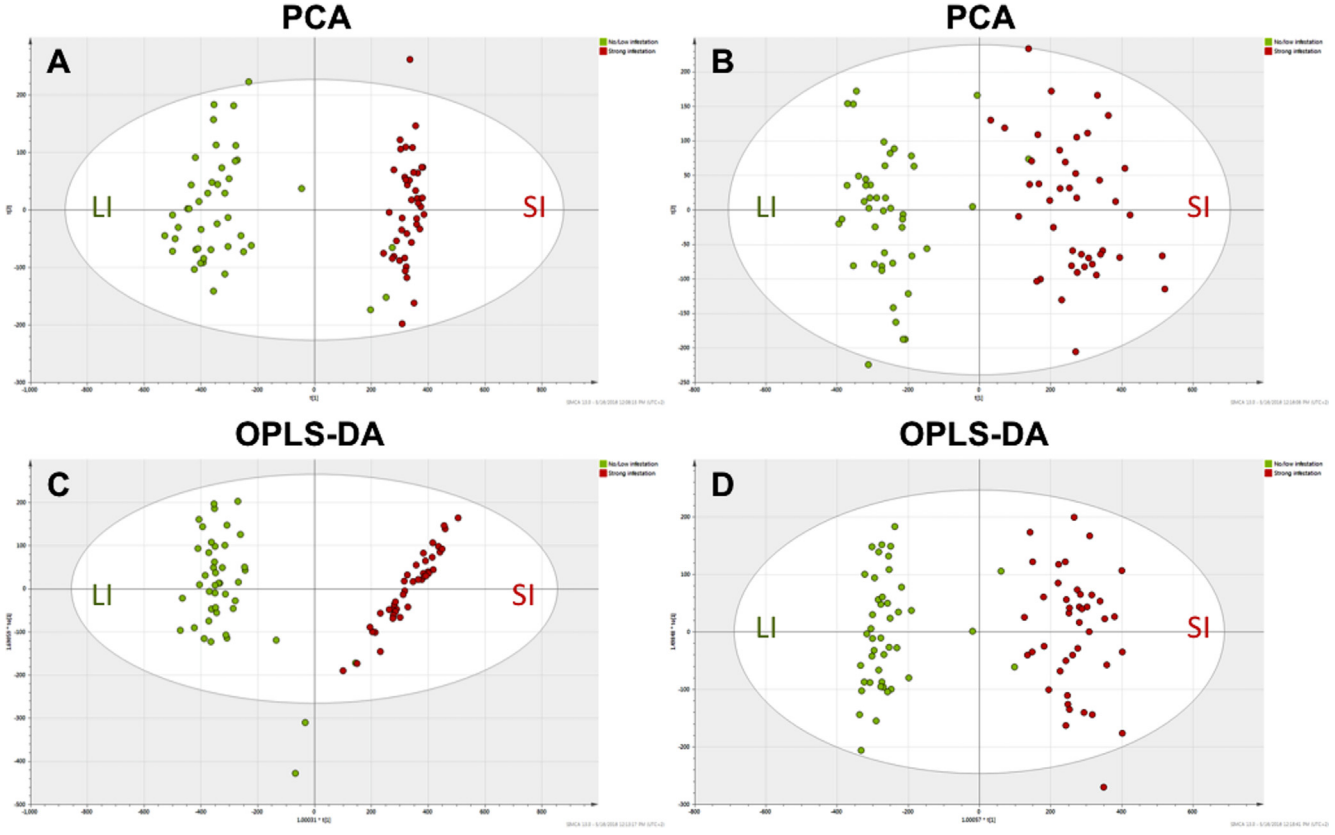

Fig. 3. Unsupervised and supervised statistical models using principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The unsupervised models, PCA, are the first step in the data analysis in order to detect sample clustering in the measured data, based 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 are depicted (D). The score plots of these statistical models, n = 86. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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*,

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and a mycotoxin content ranged from 0 to 2.773 μg/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 μg/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 Fig. 3C and D. 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 et al., 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 (Carrasco & Mérida, 2006; Ciccoritti, Pasquini, Sgrulletta, & Nocente, 2015; Dong, Lv, Xia, & Wang, 2012; Gao et al., 2007; Hong, Zhang, & Wang, 2010; Ludovici et al., 2014; Ross et al., 2003; Testerink & Munnik, 2011).

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, Fig. 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) (Ciccoritti et al., 2015; Ross et al., 2003). 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 & Mérida, 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 Fig. 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 & Munnik, 2011). The phosphorylation of DAGs by diacylglycerol kinase leads to the formation of PAs (Testerink & Munnik, 2011), which are signaling lipids involves in the plant response to biotic and abiotic stress (Carrasco & Mérida, 2006).

In short, we hypnotize 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.

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 (Δppm)	*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	$C_{39}H_{69}O_8P$	1,6	$1{,}2E^{-19}$[1,2]	−85	5
Phosphatidic acid (C16:0/C18:2)	$[M+NH_4]^+$	690.5060	7.46	$C_{37}H_{69}O_8P$	1,5	$6{,}3E^{-20}$[1,2]	−82	6

[1] Significant according to *t*-test (p value $\leqslant 0.05$).
[2] Significant according to FDR correction.
[3] Increased/decreased percentage of change in the first group (Strong Infection).

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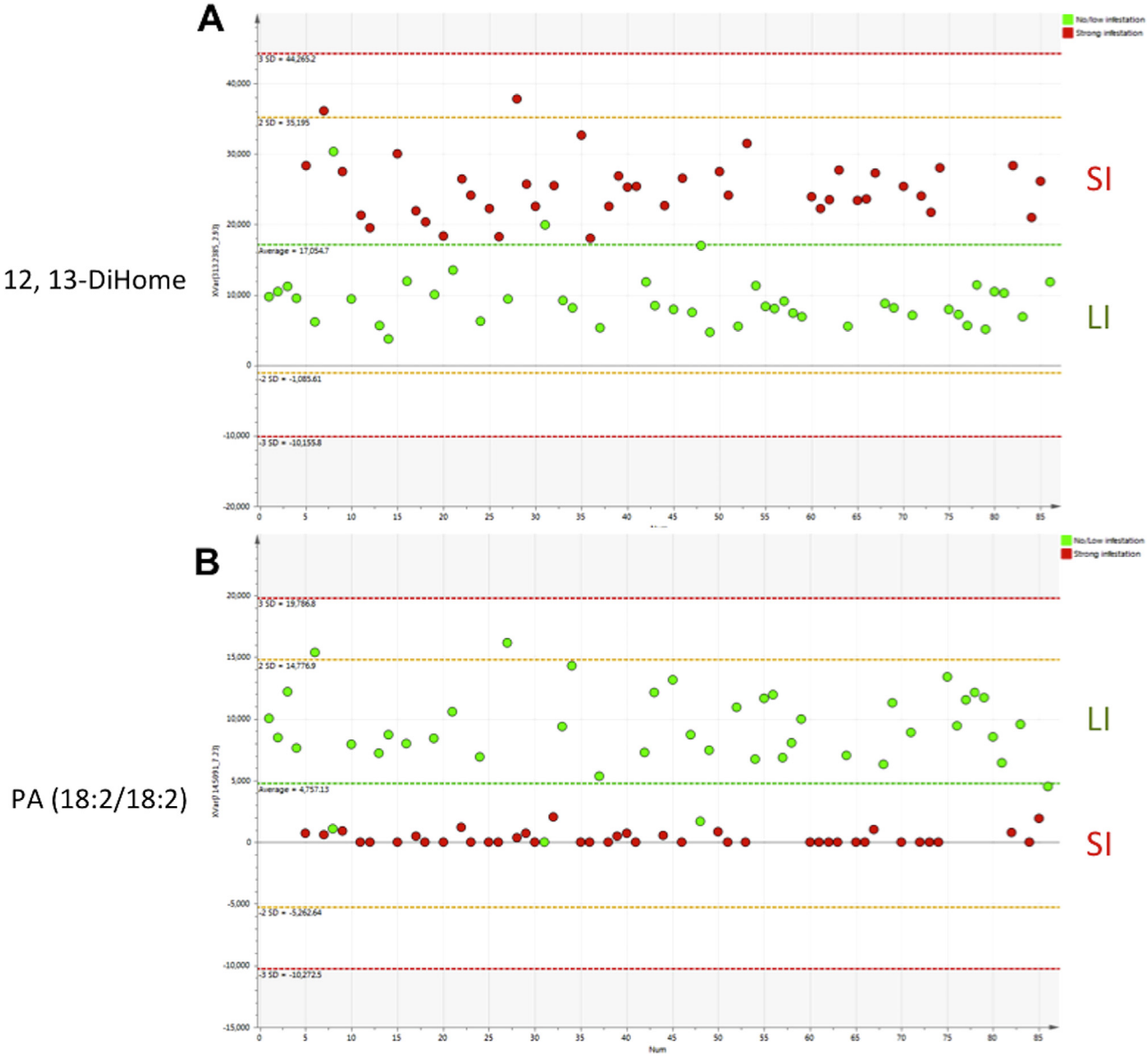

Fig. 4. 12,13-DiHome and PA (18:2/18:2) variable trend plots show up and down regulated markers for strong and low infection levels to confirm the behavior of selected variables across all samples. On the one hand, 12,13-DiHome *m/z* 313.2385 retention time 2.93 min (A), SI level responses were higher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

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.

Acknowledgments

Josep Rubert thanks the Generalitat Valenciana (Conselleria d'Educació, Cultura i Esport), for the VALi+d postdoctoral fellowship 'Contractació de personal investigador en formació en fase postdoctoral 2014' [grant number APOSTD/2014/120]. This study

was financially supported by the specific university research (MSMT No 20/2013), Ministry of Agricultural of the Czech Republic within the project QI111B044, Operational Programme Prague – Competitiveness (CZ.2.16/3.1.00/21537 and CZ.2.16/3.1.00/24503) and by the "National Program of Sustainability I" – NPU I (LO1601 – No.: MSMT-43760/2015).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.132.

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