Development and validation of a UPLC-MS/MS method for the simultaneous
determination of free and conjugated *Alternaria* toxins in cereal-based foodstuffs

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ABSTRACT

A UPLC-ESI⁺/-MS/MS method for the simultaneous determination of free (alternariol, alternariol monomethyl ether, altenuene, tenuazonic acid, tentoxin, alternatin-I) and conjugated (sulphates and glucosides of alternariol and alternariol monomethyl ether) Alternaria toxins in cereals and cereal products (rice, oat flakes and barley) was developed. Optimization of the sample preparation protocol was achieved through experimental design, using full factorial design for extraction solvent composition optimization and fractional factorial design to identify the critical factors in the protocol. Subsequently, the method, applying isotopically labelled internal standards ([2H₄]-alternariol monomethyl ether and [13C₆,¹⁵N]-tenuazonic acid), was validated for several parameters such as linearity, apparent recovery, limit of detection and quantification, precision, measurement uncertainty and specificity (in agreement with the criteria mentioned in Commission Regulation N° 401/2006/EC and Commission Decision N° 2002/657/EC). During validation, quality of the bioanalytical data was improved by counteracting the observed heteroscedasticity through the application of weighted least squares linear regression (WLSLR). Finally, 24 commercially available cereal-based foodstuffs were analyzed, revealing the presence of tenuazonic acid in both rice and oat flake samples (<LOQ - 68 ± 7 µg.kg⁻¹) and tentoxin in rice samples (<LOQ - 10.9 ± 2.0 µg.kg⁻¹).

KEYWORDS: Alternaria, (masked) mycotoxins, Ultra high performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS), survey.
Mycotoxins are naturally occurring toxic secondary metabolites produced by fungi. The most important mycotoxin producing fungi are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*.\(^1^-^3\) The fungal genus *Alternaria* contains numerous species that can contaminate a wide variety of crops in the field and cause post-harvest decay of various fruits, grains, and vegetables. In addition to causing economic losses, several of these species (of which *A. alternata* is regarded as the most predominant one) are able to produce a number of mycotoxins with toxic properties, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins (ATX), tenuazonic acid (TeA) and tentoxin (TEN). TeA is toxic to several animal species, e.g. mice, chicken and dogs. AOH, AME, ALT and ATX-I are not acutely toxic. There are several reports on the mutagenicity and genotoxicity of AOH and AME. Also, AOH has been identified as a topoisomerase I and II poison which might contribute to the impairment of DNA integrity in human colon carcinoma cells.\(^4\) Natural occurrence of *Alternaria* toxins in food in European countries has been previously reviewed by Bottalico and Logrieco\(^5\), Scott\(^6\), Ostry \(^4\) and Fernandez-Cruz et al.\(^2\). The highest levels of AOH and AME were found in lentils, oilseeds, tomato paste, followed by fruit and vegetable juices, wines, cereals and vegetables (tomatoes and carrots). The highest concentration of TeA was reported for cereals, followed by commercial beers\(^7\). Recent data (Call for scientific data on mycotoxins, EFSA, 2010) demonstrated that AOH, AME and TeA mainly occur in cereal (oats, rice, barley and wheat), in tomato (products), in fruit and vegetable juices, in wines (AOH, AME) and in beer (TeA).\(^8\) Maximum levels of *Alternaria* mycotoxins reported in marketed products were in the range of 1-10\(^3\) µg.kg\(^-1\), whereas higher levels were found in samples visibly infected by *Alternaria* rot, i.e. in products obviously not suitable for human consumption.\(^4\)
There are currently no guideline limits set for *Alternaria* mycotoxins by regulatory authorities. At the request of the European Commission, The European Food Safety Agency (EFSA) provided a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food.\(^8\) Risk assessment results proved to be inconclusive due to limited representatitive occurrence and toxicity data. Nevertheless, application of the threshold of toxicological concern (TTC) approach\(^9\)–\(^11\) indicated that there might be a possible risk for human health related to the presence of *Alternaria* toxins in foodstuffs.\(^8\) Additionally, mycotoxins can as other xenobiotics partly be metabolised, e.g. the formation of conjugated toxins in living plants.\(^12,13\) In a recent definition, for structurally altered mycotoxins the term “modified” has been suggested with reserving the historical term "masked" mycotoxins to conjugates generated by plants \(^14\). In the latter, the toxin is usually bound to a more polar substance such as glucose, and these compounds are of concern as it has been shown that they are able to release their native precursors after enzymatic hydrolysis in the digestive tract of organisms (zearalenone-4-sulphate (ZEN4S) to zearalenone (ZEN) in swine\(^15\). Historically, they are referred to be “masked” mycotoxins because they have the ability to escape established analytical methods due to both differences in polarity between the native precursors and their metabolites and the unavailability of reference standards, and their allledged contribution to the total mycotoxin content.\(^14\) In the last 2 decades, several conjugated metabolites, such as deoxynivalenol-3-glucoside (DON3G) and ZEN4S, were determined in various cereals and plants\(^12,16\)–\(^18\), and these observations have marked a shift towards multi-mycotoxin methods in which toxin conjugates are also included [19, 20]. With regards to *Alternaria* toxins, Ostry\(^4\) stated that the occurrence of glucoside conjugates of AOH and AME in foodstuffs is a distinct possibility.
Therefore, it is of paramount importance to develop analytical methodologies to detect and quantify these “masked” conjugates in various matrices.

In this research article, the development and validation of reliable, sensitive and selective UPLC-ESI-MS/MS methods for the simultaneous determination of free and conjugated Alternaria toxins in cereals and cereal based foodstuffs (rice, oats and barley) is reported. Additionally, the occurrence of these (masked) toxins was assessed in a number of commercially available rice and oat flakes samples. To the best of our knowledge, this is the first analytical methodology aiming at the detection and quantification of both free and conjugated (sulphates and glucosides of AOH and AME) Alternaria toxins in any food matrix whatsoever.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

AOH (1 mg, solid standard) was supplied by Fermentek (Jerusalem, Israel) and dissolved in 1 mL of methanol:dimethylformamide (60:40, v/v). Certified reference standards of AME (100.6 µg, dried down), TeA (100.0 µg, dried down) and TEN (102.0 µg, dried down) were purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria). TeA and TEN were reconstituted in 1 mL of acetonitrile, whereas AME was reconstituted in 1 mL of methanol:dimethylformamide (60:40, v/v). ALT (1 mg.mL⁻¹, in methanol) was purchased from the Institut für Organische Chemie (KIT, Karlsruher Institut für Technologie), whereas ATX-I (200 µg.mL⁻¹, in acetonitrile) was kindly provided by Dr. Michele Solfrizzo (ISPA-CNR, Bari, Italy). Reference standards of conjugated Alternaria toxins (AOH-3-sulphate [AOH3S], AOH-3-glucoside [AOH3G], AME-3-glucoside [AME3G], AME-3-sulphate [AME3S]) were synthesized and provided by the Institute of Applied Synthetic Chemistry (University of
Technology, Vienna, Austria). Stock solutions of AOH3S (1 mg.mL⁻¹) and AME3S (1 mg.mL⁻¹) were prepared in methanol, whereas stock solutions of AOH3G (1 mg.mL⁻¹) and AME3G (1 mg.mL⁻¹) were prepared in acetonitrile. Stock solutions in acetonitrile were stored at 4°C, whereas stock solutions in other solvents were stored at -18°C. The isotopically labeled internal standards [²H₄]-AME and [¹³C₆,¹⁵N]-TeA were synthesized and provided by the Chair of Analytical Food Chemistry (Technische Universität München (TUM), Freising, Germany). Stock solutions of 40 µg.mL⁻¹ were prepared in methanol and stored at -18°C.

Working solutions of TEN, TeA, ATX-I, AOH3G and AME3G (10 µg.mL⁻¹) were prepared in acetonitrile and stored at 4°C; whereas working solutions of AOH, AME, ALT, AOH3S and AME3S were prepared in methanol and stored at -18°C. All working solutions were renewed monthly. Working solutions of [²H₄]-AME and [¹³C₆,¹⁵N]-TeA (1 µg.ml⁻¹) were prepared in methanol and stored at -18°C.

Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol and acetonitrile (both absolute, LC-MS grade) and glacial acetic acid (ULC/MS) were purchased from BioSolve BV (Valkenswaard, The Netherlands), whereas methanol, acetonitrile and n-hexane (all HiPerSolv Chromanorm HPLC grade) were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%), ammonium acetate and ammonium carbonate were supplied by Merck (Darmstadt, Germany). Acetone (99.5+% for analysis) was purchased from Acros Organics (Geel, Belgium). Disinfectol® (denaturated ethanol with 5% ether) was supplied from Chem-Lab NV (Zedelgem, Belgium).

2.2 Synthesis of masked Alternaria mycotoxins: sulfates and glucosides of AOH and AME

Following the procedure recently reported by Mikula et al., sulfates and glucosides of AOH and AME were prepared by total synthesis. Starting from commercially available compounds, the synthetic intermediates alternariol-7,9-dimethyl ether (ADME) and alternariol-7,9-
dibenzyl ether (ADBE) were obtained applying palladium-catalyzed C-H-activation as key step (Figure 1a). Furthermore, the mono-silylated AOH derivative ASE was prepared by silylation and subsequent demethylation of ADME. Glucosylation of all intermediates was performed using Königs-Knorr conditions (Aceto-bromo-α,D-glucose, Ag₂O) followed by deprotection of acetyl, benzyl and/or silyl groups, respectively, to afford the desired conjugates AOH-9-glucoside, AOH-3-glucoside and AME-3-glucoside (Figure 1b). Sulfation of ADME and ADBE using the sulfuryl imidazolium salt SIS followed by deprotection of benzyl groups or the 7-methyl group, respectively, and subsequent cleavage of the 2,2,2-trichoroethyl group of the sulfate moiety led to the successful preparation of AOH-3-sulfate and AME-3-sulfate (Figure 1c), both as ammonium salts. All compounds were obtained in milligram quantities as white solids, characterized by NMR spectroscopy and HPLC and stored under argon at 20°C.

2.3. Synthesis of labeled alternariol monomethyl ether ([2H₄]-AME) and tenuazonic acid ([¹³C₆,¹⁵N]-TeA).

Deuterium-labeled AME was synthesized by palladium-catalyzed protium-deuterium exchange from the unlabeled toxin. After stirring an aliquot of AME in a mixture of [²H₈]-dioxane and deuterium oxide for 4 days at 160 °C in the presence of palladium on barium sulfate, a mixture of isotopologues [²H₃₋₇]-AME was recovered, of which [²H₄]-AME was the predominant form (59%). Further details about the synthesis and the nuclear magnetic resonance spectroscopic and mass spectrometric characterization of the internal standard can be found elsewhere.²²

The internal standard [¹³C₆,¹⁵N]-TeA was synthesized from [¹³C₆,¹⁵N]-isoleucine by Dieckmann intramolecular cyclization after acetoacetylation with diketene. This three-step synthesis involved the preparation of the methyl ester of [¹³C₆,¹⁵N]-isoleucine with thionylchloride in methanol, the subsequent N-acetoacetylation of the protected amino acid
with the diketene-releasing reagent 2,2,6-trimethyl-4H-1,3-dioxin-4-one in toluene, and the 

Dieckmann intramolecular cyclization with sodium methoxide in methanol to yield labeled 

TeA together with its 5R epimer. Further details about the synthesis and the characterization 

of the internal standard can be found elsewhere.23

2.4 LC-MS/MS methodology

Analysis was performed on a Waters Acquity UPLC - Quattro Premier XE mass spectrometer 
(Waters, Milford, MA, USA), equipped with MassLynx and QuanLynx® version 4.1. software 
(Micromass, Manchester, UK) for data acquisition and processing.

Chromatographic separation was achieved using an Acquity UPLC HSS T3 (1.8 µm, 2.1 x 100 
mm) column (Waters, Milford, MA, USA). Column and autosampler temperature were set at 
35°C and 10°C, respectively. A mobile phase consisting of eluents A [ultrapure water/acetic 
acid (99:1, v/v)] and B [acetonitrile/acetic acid (99:1, v/v)] was used at a flow rate of 0.4 
ml.min⁻¹. A gradient elution was applied as follows: 95% mobile phase A for 0.5 min, 
followed by a linear increase from 31 to 38% mobile phase B between 0.6 and 4.25 min. An 
isocratic phase of 90% mobile phase B was initiated at 4.35 min and maintained for 0.85 min, 
after which column reequilibration took place, resulting in a total run time of 7 min.

In order to achieve optimal sensitivity and selectivity of the MS conditions, data acquisition 
was performed applying multiple reaction monitoring (MRM) with an optimized dwell time 
(0.07 to 0.25 s). For each target analyte, two precursor to product ion transitions were 
determined. In accordance with Commision Decision (EC) N° 657/2002, laying down the 
performance criteria of analytical methods, the use of two transitions allows determination 
of the ratio between these two transitions (relative ion intensity). This ratio, together with 
the relative retention time and signal-to-noise ratio (S/N), allows the confirmation of the 
identity of the detected compound.23 MS/MS instrumental parameters were optimized via
direct infusion (flow rate of 10 $\mu$L.min$^{-1}$) of a tune solution (10 ng.$\mu$L$^{-1}$ of each analyte, dissolved in methanol/ultrapure water (50/50; v/v, 0.1% formic acid). MRM transitions, the optimum cone voltages and collision energies selected for each transition are given in Table 1, as well as the indicative retention times on the column. The first transition, which corresponds to the most abundant product ion was used for quantification, and the second one for confirmation purposes.

The mass spectrometer was operated both in the positive (ESI$^+$) and negative (ESI$^-$) electrospray ionization mode. Instrumental parameters were set as follows: ESI source block and desolvation temperatures: 125 and 350°C, respectively; capillary voltage: 3.2 kV (ESI$^+$) and 2.95 kV (ESI$^-$); argon collision gas pressure: 9.1E$^{-3}$ bar; cone nitrogen gas flow and desolvation gas flow: 50 and 800 L.h$^{-1}$, respectively.

2.5. Collection of samples of commercial foodstuffs

A total of 24 commercially available cereal based food products (rice, $n=16$ and oat flakes, $n=8$) were obtained from several supermarkets in Belgium between February and July 2013. In accordance with Commission Regulation (EC) N° 2006/401, laying down the method of sampling for the official control of the maximum levels established for aflatoxins, ochratoxin A and Fusarium toxins in cereals and cereal products, the weight of the aggregate sample (which represents the combined total of all the incremental samples) at retail stage must be at least 1 kg.$^{24}$ Therefore, in this case, several retail units were combined to obtain a total sample size of at least 1 kg. After thorough homogenization and grinding, a 2,5000g ($\pm$ 0,0025) laboratory sample was stored (4°C) until further analysis.

2.6. Sample preparation and extraction methodology

Rice, oat flakes and barley samples were thoroughly ground using the M20-grinder (Ika Werke, Staufen, Germany). After each milling step, the device was thoroughly cleaned and
decontaminated using water, soap and disinfectol. Before weighing, the ground material was vigorously homogenized.

Homogenized sample (2,5000g ± 0,0025) was spiked with labeled internal standards [²H₄]-AME and [¹³C₆,¹⁵N]-TeA at concentrations of 12 µg.kg⁻¹ and 8 µg.kg⁻¹, respectively. After 20 sec of vortex mixing, the samples were allowed to equilibrate in the dark for 15 min. Samples were extracted for 60 min with 10 mL of extraction solvent (acetonitrile/water/acetic acid [79/19.5/1.5, v/v/v]), combined with a hexane defatting step using an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). Sample extracts were centrifuged (12 min, 3000g) and after removal of the upper hexane layer, the supernatant was filtered through a paper filter (MN 617, Ø 110mm, Machery Nagel, Germany). Subsequently, the filtrate was evaporated to dryness under a gentle nitrogen stream at 40°C using an evaporator module (Grant Instruments Ltd, Cambridge, UK). Finally, the residue was redissolved in 100 μL of injection solvent, consisting of mobile phase A and mobile phase B (70/30, v/v), vigorously vortexed and subjected to ultracentrifugation (Ultrafree®-MC centrifugal device; Millipore, Bedford, MA, USA) for 10 min at 10000g prior to LC-MS/MS analysis. An LC-MS/MS chromatogram of a spiked (40 µg.kg⁻¹) oat flakes sample is shown in Figure 2.

2.7. Method validation

Due to the unavailability of certified reference material, optimization and validation of the multi-mycotoxin analytical methods for cereal (barley) and cereal products (rice, oat flakes) were performed using spiked blank samples. Performance criteria that were in compliance with Commission Decision (EC) N° 2002/657 and Commission Regulation (EC) N° 401/2006 were assessed for the following validation parameters: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), apparent recovery, repeatability (intraday
precision; RSDr), reproducibility (intermediate precision; RSDr) and expanded measurement uncertainty. Limit of detection (LOD) and limit of quantification (LOQ) were assessed according to ICH harmonized guidelines [26]. All validation parameters were calculated using the response which is calculated as the ratio of the peak area of the analyte to the peak area of the isotopically labeled internal standards ([13C6, 15N]-TeA was used for AOH3G, ALT, AME3G, TeA, ATX-I, AOH, TEN and AME3S, whereas [2H4]-AME was used for AME).

2.7.1. Specificity, matrix effect, linearity, LOD and LOQ

For every matrix, specificity and selectivity were tested by the analysis of 10 representative blank samples and 3 blank samples fortified with chemically related compounds (fumonisin B1 and deoxynivalenol at 100 µg.kg⁻¹, and ochratoxin A and aflatoxin B1 at 20 µg.kg⁻¹). Matrix effects were assessed according to Sulyok et al. [17]: for each analyte in each investigated matrix, calibration curves in neat solvent were constructed by plotting the signal intensity versus the analyte concentration. Likewise, signal intensities obtained from spiking the samples before and after extraction were plotted against the actual spiking levels. Subsequently, using the slopes of the resulting functions, the efficiency of the extraction step (EE) and the signal suppression/enhancement (SSE) due to matrix effects were calculated.

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank samples at six concentration levels (5, 10, 20, 40, 60 and 80 µg.kg⁻¹) for the three different matrices. In addition to the commonly reported regression coefficients (R²), a lack-of-fit test (IBM SPSS 21) was also performed to assess the adequacy of the linear model. Furthermore, it is recognized that the condition of equal variances, termed homoscedasticity (homogeneity of variance), should be tested in any linear regression analysis. When the assumption of homoscedasticity is not met for analytical data, an effective way to
counteract the greater influence of the greater concentrations on the fitted regression line is
to use weighted least squares linear regression (WLSLR).27–29

LOD and LOQ were determined using MMC curves (in triplicate), by spiking blank samples at
8 concentrations levels (0.1 to 10 µg.kg\(^{-1}\)) for each investigated matrix. For every analyte in
each matrix, the standard error of the \(y\)-intercept, as well as the slope of the corresponding
calibration curve (lower level equals concentration for which \(S/N \geq 3\) for both product ions,
and upper concentration level equals 10 µg.kg\(^{-1}\)) were calculated using the linest function
(Microsoft Excel 2010). LOD equaled the concentration corresponding to the blank response
plus three times the standard error of the \(y\)-intercept, whereas LOQ equals two times the
LOD value.

2.7.2 Apparent recovery, repeatability, reproducibility and measurement uncertainty

Apparent recovery, repeatability, intermediate precision and expanded measurement
uncertainty were all calculated from a combined sample plan, which comprised of the
analysis of blank samples of each matrix spiked in triplicate with the different mycotoxins at
concentration levels of 5, 40 and 80 µg.kg\(^{-1}\) on three consecutive days.
The apparent recovery was determined using MMC curves (six concentration levels, 5 to 80
µg.kg\(^{-1}\)). The measured concentration of the spiked blank samples (for each matrix) was
determined by plotting the observed signal (expressed as the relative peak area) into the
corresponding MMC curves. IUPAC defines the apparent recovery as the ratio of the
predicted value obtained from the MMC curves divided by the actual/theoretical value.17

Repeatability (RSD\(_r\)) and intermediate precision (RSD\(_p\)) relative standard deviations were
calculated using one-way analysis of variance (ANOVA).
The combined standard uncertainty (\(u_c\)) is an estimated standard deviation equal to the
positive square root of the total variance obtained by combining the intralaboratory
reproducibility \( (\text{RSD}_n) \) and the bias of the analytical method, which consists of the uncertainty associated with the purity of the standards \( (U|C_{ref}|) \), the accuracy of the bias \( (S_{bias}) \) and the root mean square of the bias \( (\text{RMS}_{bias}) \). The expanded measurement uncertainty \( (U) \) is obtained by multiplying \( u_c \) by a coverage factor of 2, which gives a level of confidence of approximately 95%.

2.8. Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2010 and IBM SPSS Statistics 21.

3. RESULTS AND DISCUSSION

3.1. Optimization of the MS/MS conditions

MS/MS instrumental parameters were optimized via direct infusion (flow rate of 10 \( \mu \text{L.min}^{-1} \)) of a tune solution (10 ng.\( \mu \text{L}^{-1} \) of each analyte) in the inlet of the mass spectrometer. During 30s, both in positive and negative ionization mode, a diagnostic MS spectrum was obtained, from which a precursor ion for each analyte was selected. After optimization of the cone voltage, the precursor ion for each analyte was mass-selected by the first quadrupole and subjected to collision-induced dissociation (CID) by applying different collision energies, yielding a product ion mass spectrum. To ensure high specificity and selectivity, for each analyte, two product ions were selected and inserted in the final MS method. The optimized parameters for every analyte (ionization mode, cone voltage, collision energy, precursor and product ions) are shown in Table 1. Generally, it is recognized that operating in the negative electrospray ionization mode (ESI⁻) leads to lower signal intensity, but is also less prone to matrix interferences, yielding a lower background signal in sample matrix. ESI⁻ mode was selected for all but one of the Alternaria toxins present in the LC-MS/MS method.\textsuperscript{22,23,30–32}
However, for ALT, ESI⁺ mode was selected, because CID in ESI⁻ mode did not yield a satisfactory product ion mass spectrum.

### 3.2. Optimization of the LC conditions

Optimization of the LC conditions aimed at both retention and separation of 10 analytes in a fast chromatographic run. Due to differences in polarity of the analytes, the selection of a suitable stationary phase was imperative. For all analytes, preliminary experiments with various gradient elution programmes of ultrapure water and MeOH or ACN pointed out that ACN led to narrower peak shapes compared with MeOH. The performance of 1 HPLC (Zorbax Eclips XDB-C₁₈ [3.5 μm, 2.1x100 mm]) and 2 UPLC (Acquity UPLC HSS T3 [1.8 μm, 2.1x100 mm]; UPLC BEH C₁₈ [1.7 μm, 2.1x100 mm]) chromatographic columns was assessed.

As previously shown, the Eclips XDB-C₁₈ column column proved to be suitable for the simultaneous determination of the polar conjugates and less polar mycotoxins. Chromatographic separation was achieved in an analytical run of 14 minutes. However, UPLC, which is characterized by column packing material with sub 2 micron particle size, leads to a significant increase in speed, resolution and sensitivity as compared to HPLC. Indeed, for both UPLC columns, chromatographic separation was achieved in less than 10 minutes. Furthermore, an improved peak shape for TeA was observed with the HSS T3 column, making this the column of choice for further optimization. Ten mobile phase modifiers (no modifier; formic acid (FA) (0.1-0.3%); acetic acid (AA) (0.5-1%); 0.1% FA/ammonium formate (2mM-5mM); 0.5% AA/ ammonium acetate (2mM); 1% AA/ammonium acetate (5mM); ammonium hydrogen carbonate (5mM)) were tested in triplicate. In the absence of any modifier, the highest absolute intensity for the majority of analytes (especially the sulphates) was observed, but the resulting pH (close to neutral) led to an impaired peak shape for TeA. For all analytes, average overall results (intensity, peak shape) were satisfactory with 1%
glacial acetic acid (pH 2.9) as a modifier. A flow rate of 0.400 ml.min\(^{-1}\) and a column temperature maintained at 35°C resulted in retention times ranging from 1.81 (AOH3G) to 5.25 min (AME). Finally, to increase the sensitivity and selectivity of the MS conditions, the MRM method was ultimately divided into 6 data acquisition windows (based on these retention times), and the dwell time for each window was optimized (0.07 to 0.25 s).

### 3.3. Optimization of the sample preparation

Starting from an in-house developed sample preparation and extraction methodology\(^{19}\), design of experiments (DOE) was applied for optimization of the sample preparation for rice and oat flakes matrices. The intuitive approach (Changing one separate factor at a time, COST) to experimental work often does not lead to the real optimum, and gives different implications with different starting points. The solution is to construct a carefully prepared set of representative experiments, in which all relevant factors are simultaneously varied. In contrast with COST, DOE enables the scientist to deal with factor interactions and differentiate between systematic (real effects) and unsystematic (noise) variability. Furthermore, DOE allows an interpretation of the results through the generation of response contour plots (MODDE\(^{\circledR}\) software).\(^{31}\)

In a first phase, for both matrices, the extraction solvent composition was optimized using a three-level full factorial design (\(3^3\)) with three factors, i.e. type of organic solvent (methanol, acetonitrile or acetone), percentage of organic solvent (50, 70 or 90) and pH (3, 6 or 9). Interpretation of the generated response contour plots (data not shown) indicated that the type of organic solvent proved to be a critical factor for multiple analytes, both in rice and oat flakes matrices. With the exception of ALT, TeA and AME, acetonitrile resulted in far higher absolute responses for all analytes. The percentage of organic solvent also proved to be a critical factor. For 2 analytes, i.e. AME and ALT in both matrices, 50% of acetonitrile
resulted in the highest absolute areas. However, for all other analytes, highest absolute areas were observed using 90% of acetonitrile. In case of conflicting outcomes, which is to be expected in the development of multi-mycotoxin analytical methodologies, a compromise was sought to determine the favourable level of a factor. Finally, an acidic pH resulted in the highest absolute areas for ALT, TeA, AOH and AME in both matrices, whereas pH proved to be a non-critical factor for the other analytes. Ultimately, for both rice and oat flake matrices, considering the wide range of polarities of the analytes, the best compromise for the extraction solvent composition was as follows: acetonitrile/water/acetic acid (79/19.5/1.5, v/v/v), which is in good agreement with previously reported studies.¹,¹³,¹⁸,²⁰,³⁴,³⁵

Next, using the optimal extraction solvent composition, the whole extraction procedure for both matrices was subjected to screening using a three-level fractional factorial design with resolution IV (³⁻⁵). These designs allow γ factors to be examined on x levels with \( x^{γ-p} \) experiments, where p is the number to which factors or interactions are confounded, i.e. cannot be estimated independently. When a factor proved to be significant after statistical analysis (\( p=0.05 \)), the level of the factor corresponding to the highest absolute areas was built in to the protocol. If a factor proved to be non-critical, the least time-consuming or cheapest level of the factor was built into the protocol. Fractional factorial designs give little to no insight in possible interactions between the factors. For this reason, full factorial designs were set up with the factors that proved to be critical in the fractional factorial designs. Investigated factors were: extraction solvent volume, liquid-liquid extraction (LLE) with different hexane volumes, duration of vortex-mixing, duration of the extraction, duration of centrifugation, evaporation temperature and duration of ultracentrifugation. For the majority of analytes in both matrices, extraction solvent volume, LLE with hexane and duration of the extraction were factors that proved to be critical, and were subsequently
optimized using a three-level full factorial design ($3^3$). No interaction effects were observed from the full factorial designs with the critical factors. Finally, applying the optimized sample preparation and extraction methodology for rice and oat flakes to another cereal-based matrix, i.e. barley, also generated satisfactory results.

3.4. Method validation


**Specificity** concerns the ability of the analytical method to distinguish between the analyte being measured and other substances or interferences.$^{23}$ In order to determine the specificity/selectivity, for every matrix, 10 representative blank samples and 3 blank samples fortified with chemically related compounds were subjected to analysis, revealing no interfering peaks ($S/N \geq 3$) in the 2.5% margin of the relative retention time for all target analytes.

**Matrix effects** result from co-eluting residual matrix components affecting (suppression or enhancement) the ionization efficiency of target analytes. In extreme cases, these matrix effects can result in complete suppression of the analytical signal.$^{17}$ Since matrix effects may exert a negative effect on important method performance parameters and subsequently lead to erroneous quantitative results, they have to be tested for and evaluated during method development and validation. $EE$ values varied from 35% (TeA in all three matrices) to 98% (AME in oats matrix). Strong ion suppression (<10%) was observed for the glucosides (AOH3G, AME3G), TeA and AME in oats and barley matrix, while only limited ion suppression (>70%) could be observed for the sulphates (AOH3S, AME3S) in all three matrices.
Furthermore, comparison by visual inspection of the slopes confirmed the presence of matrix effects, and indicated the need to use matrix specific (since, for all target analytes, construction of a calibration curve in rice, oats or barley matrix resulted in significantly different slopes, i.e. non-parallelism of the curves confirmed by t-test\textsuperscript{25}) MMC curves for quantification purposes (Figure 3).

**Linearity** was evaluated using MMC curves (in triplicate), by spiking blank samples at six concentration levels (5-80 µg.kg\textsuperscript{-1}) for each investigated matrix. In addition to the commonly reported regression coefficients ($R^2$\textsuperscript{34}, the lowest observed value being 0.951 for AME3G in oats (Table 3), a lack-of-fit test was also performed to assess the adequacy of the linear model\textsuperscript{35}. The linearity of the relative peak areas for all analytes in each investigated matrix was excellent in the applied range, with $p$-values greater than 0.05, which demonstrated no lack of fit and thus good adequacy of the models in predicting linearity.

Since analyte concentrations in unknown rice and oat flake samples will be evaluated by using the regression results obtained from calibration curves, a well-designed and interpreted calibration curve is essential, and contributes to the quality of bioanalytical data.\textsuperscript{26} Since the condition of equal variances, termed **homoscedasticity** or homogenity of variance, is frequently not met for bioanalytical data, larger deviations present at larger concentrations tend to influence the regression line more than smaller deviations associated with smaller concentrations, and thus the accuracy in the lower end of the range is impaired.\textsuperscript{25-27} Therefore, the homoscedasticity assumption should be tested in any linear regression analysis. It can be performed by plotting residuals versus concentration and by applying an $F$-test in accordance with the following statistics:

$$F_{exp} = \frac{s_2^2}{s_1^2} \leq F_{tab}(f_1, f_2; 0.99)$$
where the experimental $F$-value is expressed as the ratio between the variances obtained at the lowest ($s_1^2$) and at the highest ($s_2^2$) concentration level of the calibration range, and the tabled $F$-value is obtained from the $F$-table at the confidence level of 99% for $f_1=f_2=(n-1)$ degrees of freedom. If variance is constant over the whole calibration range, residuals will fall more or less randomly around the x-axis and $F_{exp}$ will be lower than $F_{tab}$, indicative for a situation of homoscedasticity. As an example, the residual versus concentration plot of TEN in oat flakes clearly shows that the error is not randomly distributed around the concentration axis (Figure 4). The $F$-test (data not shown) also revealed a significant difference between the variances, when the experimental $F$-value ($F_{exp} = 31.90$) was compared to the tabled one ($F_{tab} = 6.03$).

An effective way to counteract the observed heteroscedastic situation is to use weighted least squares linear regression (WLSLR). The first step in WLSLR is the choice of the weighing factor, $w_i$, and possible empirical weighing factors to be studied are $1/x$, $1/x^2$, $1/x^{1/2}$, $1/y$, $1/y^2$ and $1/y^{1/2}$. The best weighing factor is chosen according to a percentage relative error (%RE), which compares the regressed concentration ($C_{found}$) calculated from the regression equation obtained for each $w_i$, with the nominal standard concentration ($C_{nom}$):

$$\%RE = \left( \frac{C_{found} - C_{nom}}{C_{nom}} \right) \times 100$$

Calculating $\sum$%RE, defined as the sum of absolute %RE values, is a useful and sensitive indicator of goodness of fit in the evaluation of the effectiveness of a weighing factor for WLSLR. In Table 2, the sum of the relative errors ($\sum$%RE) and accuracy (in terms of bias, %) at low (5 µg.kg$^{-1}$), medium (40 µg.kg$^{-1}$) and high (80 µg.kg$^{-1}$) concentration level obtained by using unweighted ($w_i = 1$) and weighted ($w_i = 1/x^2$) linear regression for all target analytes in rice and oats matrix are displayed. Compared to the other possible empirical weighing factors...
factors \(\frac{1}{x}, \frac{1}{x^{1/2}}, \frac{1}{y}, \frac{1}{y^2} \text{and} \frac{1}{y^{1/2}}\), the weighing factor \(\frac{1}{x^2}\) produced the least \(\Sigma \%RE\) for this data set, providing the most adequate approximation of variance. Additionally, the weighing factor \(\frac{1}{x^2}\) drastically improved the accuracy (expressed as bias, in \%) at the lowest concentration level of the calibration curve (Table 2).

**Limit of Detection (LOD) and Limit of Quantification (LOQ)** were determined using MMC curves, by spiking blank samples at 8 concentrations levels (0.1-10 \(\mu g.kg^{-1}\)) for each investigated matrix. Although no regulatory limits have been set for *Alternaria* toxins in cereals and cereal-based foodstuffs, the developed UPLC-MS/MS methodology allowed for the determination of all target analytes at a low parts per billion (ppb) level. In Table 3, LOD and LOQ values are represented for every analyte in each investigated matrix. LOQ values ranged from 0.93 to 4.99 \(\mu g.kg^{-1}\) except for AME3S in rice (8.32 \(\mu g.kg^{-1}\)). For all analytes in each investigated matrix, the calculated LODs and LOQs were also verified by the S/N ratio, which should be more than 3 and 10 according to the IUPAC (International Union of Pure and Applied Chemistry) settings\(^{36,37}\). EFSA states that the database concerning toxicological effects of *Alternaria* toxins in experimental animals and/or in humans is currently too limited to be used as a basis for identification of reference points for different toxicological effects (EFSA). In the absence of established tolerable daily intake (TDI) values, a risk assessment based on TDI values for T-2/HT-2 toxins\(^{38}\) was performed, indicating that when the incorporated amount of *Alternaria* toxins in grains and grain-based products would result in a concentration at LOQ level, the dietary exposure to humans, based on standard food basket consumption figures\(^{39}\), would be negligible with regard to toxicity.

A combined sample plan, which comprised of the quantitative analysis (using MMC curves with six concentration levels, 5 to 80 \(\mu g.kg^{-1}\)) of spiked blank samples (concentration levels of 5, 40 and 80 \(\mu g.kg^{-1}\)) in triplicate on three consecutive days for each investigated matrix,
was used to determine the apparent recovery, repeatability, intermediate precision and expanded measurement uncertainty. Results for each investigated matrix are displayed in Table 4.

The **Apparent recovery** \( (R_A) \) at and above LOQ level ranged from 94.4% to 106.5% for all analytes in all three matrices (Table 4). These results are in good agreement with the guideline ranges (80-110%) imposed by Commission Decision N° (EC) 2002/657²³. **Repeatability** \( (RSD_r) \) and **intermediate precision** \( (RSD_R) \), calculated using 1-way ANOVA, ranged from 2.0% to 14.1% and 2.2% to 14.1%, respectively (Table 4). According to Commission Decision N° (EC) 2002/657, repeatability and intermediate precision values for the repeated analysis of fortified material, under repeatability and reproducibility conditions, shall not exceed the level calculated by the Horwitz Equation \( RSD_r = \frac{3}{\sqrt{2(1-0.5 \log C)}}; \ RSD_R = 2^{(1-0.5 \log C)} \). However, for mass fractions lower than 100 μg.kg\(^{-1}\) the application of the Horwitz Equation gives unacceptable high values.²³ Therefore, according to an in-house developed **standard operating procedure** on analytical method validation, \( RSD_r \) and \( RSD_R \) values for concentrations lower than 100 μg.kg\(^{-1}\) should be lower than 20% and 25%, respectively.

Uncertainty of the measurement is defined as a parameter, which is associated with the results of a measurement and characterizes the dispersion of the values that could reasonably be attributed to the measurand.⁴⁰,⁴¹ The **expanded measurement uncertainty** \( (U) \) is an estimated standard deviation equal to the positive square root of the total variance obtained by combining the intralaboratory reproducibility (intermediate precision; \( RSD_R \)) and the bias of the analytical method, multiplied by a coverage factor of 2, giving a level of confidence of approximately 95%. \( U \) was calculated at three concentration levels (5, 40 and 80 μg.kg\(^{-1}\)) for all analytes in all three matrices, and ranged from 6.1% to 52.3% (Table 4).
general, lower $U$ values were obtained for AOH, AME, TeA and TEN (values ranging from 6.1 % to 22.4%) except for AME and TEN at medium (40.5% and 30.2% respectively) and high (26.7% and 32.0% respectively) concentration levels in barley matrix. The availability of reference standards with a certificate of analysis for these analytes indeed leads to a significantly lower value for the $U/[C_{req}]$ term in the calculation of $U$.

3.5. Analysis of commercially available cereal based foodstuffs

Subsequent to the optimization and validation of these analytical methods, commercially available rice ($n=16$) and oat flake ($n=8$) samples were sampled (between March and June 2013) and subsequently subjected to analysis. Analytical results are reported as $x \pm U$ whereby $x$ is the analytical result and $U$ is the (concentration dependent) expanded measurement uncertainty. A total of 75% (12/16) of rice samples, and 38% (3/8) of oat flake samples were contaminated with TeA, with concentrations in the range of 1.9 ± 0.1 to 68 ± 7 $\mu$g.kg$^{-1}$ and 2.1 ± 0.2 to 39 ± 5 $\mu$g.kg$^{-1}$, respectively. Furthermore, 25% (4/16) of rice samples were contaminated with TEN (4.1 ± 0.7 to 10.9 ± 2.0 $\mu$g.kg$^{-1}$), yet no TEN was found in oat flake samples. Although the prevalence of TeA and TEN in these samples is higher, the observed levels are in good agreement with the mean lower bound (LB) - upper bound (UB) concentrations for TeA and TEN in grain and grain-based products reported by EFSA$^8$. None of the other target analytes (AOH, AME, ALT, ATx-I) nor AOH and AME conjugates (AOH$_3$G, AOH$_3$S, AME$_3$G, AME$_3$S) were detected in any of the samples analyzed.

4. CONCLUSIONS

A UPLC-ESI$^+/−$-MS/MS method for the simultaneous determination of both free and conjugated *Alternaria* toxins in cereal and cereal based foodstuffs was successfully developed and validated, using Regulation 401/2006/EC and Decision 2002/657/EC as
Accordingly, these methods, applying isotopically labeled internal standards, were validated for several parameters and proved to be fit-for-purpose. Subsequently, a mini-survey, conducted between March and June 2013, revealed the presence of TeA in both rice and oat flake samples and TEN in rice samples. The validated analytical methodologies will be further used to analyze cereal-based foodstuffs within the framework of a research project funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (Contract RF 12/6261 ALTER). The principal aim of this project is to gather quantitative occurrence data on free and conjugated Alternaria toxins in various foodstuffs available on the Belgian market.

ACKNOWLEDGMENTS

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (Contract RF 12/6261 ALTER).
Figure 1 - (a) Chemical structures of AOH derivatives, (b) chemical glucosylation and deprotection, (c) chemical sulfation and deprotection.
Table 1 - Optimized LC-MS/MS instrumental parameters for all analytes, including the isotopically labeled internal standards $[^{13}\text{C}_6,^{15}\text{N}]$-TeA and $[^2\text{H}_4]$-AME.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion (m/z)</th>
<th>Molecular ion</th>
<th>Cone voltage (V)</th>
<th>Product ions (m/z)</th>
<th>Collision energy (eV)</th>
<th>Retention time (min)</th>
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</thead>
<tbody>
<tr>
<td>AOH3G</td>
<td>419.0</td>
<td>[M-H]</td>
<td>65</td>
<td>256.1 228.1</td>
<td>32 40</td>
<td>1.81</td>
</tr>
<tr>
<td>AOH3S</td>
<td>337.1</td>
<td>[M-H]</td>
<td>34</td>
<td>213.0 257.1</td>
<td>38 24</td>
<td>2.40</td>
</tr>
<tr>
<td>ALT</td>
<td>292.9</td>
<td>[M+H]$^+$</td>
<td>23</td>
<td>257.1 239.0</td>
<td>14 11</td>
<td>2.62</td>
</tr>
<tr>
<td>AME3G</td>
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<td>63</td>
<td>270.1 227.1</td>
<td>31 45</td>
<td>2.97</td>
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<tr>
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<td>[M-H]</td>
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<td>139.0 112.0</td>
<td>20 24</td>
<td>3.28</td>
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<tr>
<td>ATX-I</td>
<td>351.2</td>
<td>[M-H]</td>
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<td>315.1 333.1</td>
<td>18 12</td>
<td>3.73</td>
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<td>AOH</td>
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<td>24 26</td>
<td>3.97</td>
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<tr>
<td>TEN</td>
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<td>[M-H]</td>
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<td>141.0 271.2</td>
<td>24 18</td>
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</tr>
<tr>
<td>AME3S</td>
<td>351.1</td>
<td>[M-H]</td>
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<td>271.1 256.1</td>
<td>24 38</td>
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<tr>
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<td>271.1</td>
<td>[M-H]</td>
<td>48</td>
<td>256.0 228.0</td>
<td>24 30</td>
<td>5.25</td>
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<tr>
<td>$[^{13}\text{C}_6,^{15}\text{N}]$-TeA</td>
<td>202.9</td>
<td>[M-H]</td>
<td>52</td>
<td>141.9 112.9</td>
<td>20 24</td>
<td>3.28</td>
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<tr>
<td>$[^2\text{H}_4]$-AME</td>
<td>274.9</td>
<td>[M-H]</td>
<td>57</td>
<td>260.0 232.0</td>
<td>24 30</td>
<td>5.25</td>
</tr>
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</table>


(b) The underlined product ion is the most abundant and thus used for quantification purposes.
Figure 2 - LC-MS/MS chromatogram (quantifier ions displayed) of a 40 μg.kg\(^{-1}\) spiked oat flakes sample.

Figure 3 - Comparison of the slopes of calibration curves of altertoxin-I in rice, oats, barley and standard solution, revealing the presence of varying (matrix dependent) signal suppression.
Figure 4 - Residuals plotted against TEN concentrations (oat flakes) for the inter-day validation assay. The residual plot clearly shows that the error is not randomly distributed around the concentration axis, indicative of heterogeneity of variance.

Table 2 - Sum of the relative errors ($\sum$RE) and accuracy at low (5 $\mu$g.kg$^{-1}$), medium (40 $\mu$g.kg$^{-1}$) and high (80 $\mu$g.kg$^{-1}$) concentration level obtained by using unweighted ($w_i = 1$) and weighted ($w_i = 1/x^2$) linear regression for all target analytes in rice and oats matrix.

<table>
<thead>
<tr>
<th></th>
<th>RICE</th>
<th>Bias (%)</th>
<th>OATS</th>
<th>Bias (%)</th>
</tr>
</thead>
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<td>$w_i$</td>
<td>$\sum$RE</td>
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<td>medium</td>
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<tr>
<td>AOH3G</td>
<td>1</td>
<td>218.1</td>
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<td></td>
<td>$1/x^3$</td>
<td>197.1</td>
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<td>0.9</td>
</tr>
<tr>
<td>AOH3S</td>
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<td>398.4</td>
<td>12.4</td>
<td>5.7</td>
</tr>
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<td></td>
<td>$1/x^3$</td>
<td>271.6</td>
<td>1.4</td>
<td>1.3</td>
</tr>
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<td>9.9</td>
<td>3.5</td>
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<tr>
<td></td>
<td>$1/x^3$</td>
<td>199.0</td>
<td>1.9</td>
<td>0.3</td>
</tr>
<tr>
<td>AME3G</td>
<td>1</td>
<td>233.1</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>$1/x^3$</td>
<td>199.9</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>TeA</td>
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<td>4.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>$1/x^3$</td>
<td>149.8</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>ATX-I</td>
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<td>247.4</td>
<td>10.8</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>$1/x^3$</td>
<td>212.9</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>AOH</td>
<td>1</td>
<td>266.4</td>
<td>13.6</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>$1/x^3$</td>
<td>209.6</td>
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<td>0.9</td>
</tr>
<tr>
<td>TEN</td>
<td>1</td>
<td>222.4</td>
<td>6.9</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>$1/x^3$</td>
<td>172.0</td>
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<td>0.9</td>
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<td>AME3S</td>
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<tr>
<td></td>
<td>$1/x^3$</td>
<td>187.4</td>
<td>0.1</td>
<td>1.9</td>
</tr>
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</table>

Table 3 - $R^2$ values and ranges of the matrix-matched calibration curves in rice, oats and barley, supplemented with limits of detection (LOD) and limits of quantification (LOQ) for all analytes ($\mu$g.kg$^{-1}$)

<table>
<thead>
<tr>
<th></th>
<th>Rice</th>
<th>Oats</th>
<th>Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range $R^2$ LOD LOQ</td>
<td>Range $R^2$ LOD LOQ</td>
<td>Range $R^2$ LOD LOQ</td>
</tr>
<tr>
<td>AOH3G</td>
<td>5-80 0.992 0.82 1.65</td>
<td>5-80 0.992 0.74 1.49</td>
<td>5-80 0.986 2.11 4.23</td>
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<tr>
<td>AOH3S</td>
<td>5-80 0.989 1.54 3.08</td>
<td>5-80 0.991 1.24 2.48</td>
<td>5-80 0.988 2.41 4.82</td>
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<td>5-80 0.991 1.63 3.26</td>
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<td>5-80 0.993 0.77 1.53</td>
<td>5-80 0.951 2.46 4.91</td>
<td>5-80 0.984 2.50 4.99</td>
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<tr>
<td>TeA</td>
<td>5-80 0.996 0.93 1.86</td>
<td>5-80 0.996 0.61 1.21</td>
<td>5-80 0.995 1.18 2.35</td>
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<td>5-80 0.983 1.81 3.62</td>
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<tr>
<td>TEN</td>
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<tr>
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<tr>
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<td>5-80 0.991 1.25 2.50</td>
<td>5-80 0.975 1.32 2.52</td>
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</table>

Table 4 - Repeatability ($RSD_r$), intermediate precision ($RSD_i$), apparent recovery ($R_{re}$, %) and expanded measurement uncertainty (U, %) values for all analytes on low, medium and high concentration level in rice, oats and barley

<table>
<thead>
<tr>
<th></th>
<th>AOH3G</th>
<th></th>
<th></th>
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<tr>
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<td>$R_{re}$</td>
<td>$U$</td>
<td>$RSD_r$</td>
<td>$RSD_i$</td>
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