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Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice

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ABSTRACT

A simple and efficient QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) sample preparation method was modified to provide good analytical results for 14 mycotoxins in rice. The method involved mixing sample with acidified aqueous acetonitrile, followed by salt-out liquid partitioning using $MgSO_4$, NaCl, and citrate buffer salts. The extract was cleaned-up by dispersive solid-phase extraction with $MgSO_4$, PSA, C_{18}, and alumina-neutral. The analysis was performed using ultra-high performance liquid chromatography coupled to triple-quadrupole tandem mass spectrometry (UHPLC–MS/MS). Throughout the validation experiments, 70–98% overall recoveries were achieved with RSDs $\leqslant$7% for most analytes at concentrations 10–100 μg kg^{-1}. Limit of detections were 0.5–15 μg kg^{-1}. Inter-laboratory precision was performed by proficiency testing, $|z| \leqslant 2$ was considered satisfactory. We compared our modified QuEChERS method against sample preparation using an immunoaffinity column; the recovery and specificity were comparable for the two methods, but the QuEChERS approach was more time- and cost-effective.

1. Introduction

Rice is a staple food for more than half of the human population. It is valued as a source of nutrients, vitamins, minerals, and complex carbohydrates. Continuously changing environmental and temperature patterns have disrupted natural containment systems, and seen the emergence of the mycotoxins as a food safety concern. Many countries that import foodstuffs have defined new standards and strengthened existing regulations to ban imports if foods are found to pose a threat to health (Siegel & Babuscio, 2011). According to the Thailand Foreign Agricultural Trade Statistics, 10.7 million metric tons of rice were exported during 2011(OAE, 2012). Therefore, producers and exporters need to be aware of many regulations, and ensure the safety and quality of rice before shipment.

Mycotoxins are secondary metabolites produced by fungi, and thus are natural contaminants that can infect food and other commodity crops at every step in the supply chain. The most important mycotoxins are subjected in positive lists and often occur in rice; these include aflatoxins, trichothecenes, fumonisins, zearalenone, citrinin, and ochratoxins, and are produced by *Aspergillus*, *Penicillium*, and *Fusarium* genera (Tanaka, Sago, Zheng, Nakagawa, & Kushiro, 2007). These compounds are very stable and heat-resistant; they can even be found in fully processed foods and have the potential adverse effect on human health (Shephard, 2008). The European Union has set maximum levels (MLs) for the mycotoxin contents permitted in foodstuffs, including cereal grains and cereal products (EC, 2006b, 2007) (Table 1).

To support the enforcement of regulations, protect consumers' health, and facilitate domestic and international food trade, analytical method must yield high quality results at the desired concentrations in a short time and at an affordable cost. To meet these challenges, a number of methods for describing sample preparation and instrumentation for the determination of single and multiple mycotoxins in foods have been reported (Turner, Subrahmanyam, & Piletsky, 2009). In recent years, developments in mass spectrometry (MS) technology have improved selectivity and sensitivity, and provided the capacity to distinguish residues and contaminates from co-extractives. Thus, instrumental analysis now requires minimal sample preparation using simple methods, with or without clean-up. The triple-quadrupole tandem MS (MS/MS) is considered the gold standard for quantitative analysis of multiple compounds. "Dilute-and-shoot" based approaches (Mol et al., 2008), based on modern liquid chromatography (LC) combined with MS/MS, became a popular analytical method with many applications, including mycotoxins analysis (Oueslati, Romero-González, Lasram, Frenich, & Vidal, 2012; Sulyok, Krska, & Schuhmacher, 2010; Zachariasova et al., 2010). The Dilute-and-shoot method has several practical benefits, such as minimising analyte losses during purification, while increasing detection limits. In some situations, sample complexity can exceed the

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Table 1
Average results of retention times (t_R), ion ratios, linear regressions (R^2) in matrix, matrix effects, and analytical limits for the 14 mycotoxins in the method. The maximum limits (MLs) are according to 2006/1881/EC.

Analyte		Avg. t_R ± tolerance value[a] (min)	Avg. ion ratio ± tolerance value[b]	Avg. R^2 in matrix	Avg. matrix effects (%)	Analytical limit (μg kg^{-1})		ML (μg kg^{-1})
						LOD	LOQ	
Nivalenol	NIV	3.75 ± 0.09	0.97 ± 0.19	0.9960	−15	15	50	nc
Deoxynivalenol	DON	4.00 ± 0.10	0.51 ± 0.10	0.9971	8	5	16.7	1250
Fumonisin B1	FUM-B1	4.29 ± 0.11	0.99 ± 0.20	0.9933	33	1	3.3	Sum FUM-B1 and B2 = 4000
Aflatoxin G2	AF-G2	4.65 ± 0.12	–	0.9972	34	1	3.3	Sum AFs = 4 (only AF-B1 = 2)
Aflatoxin G1	AF-G1	4.70 ± 0.12	0.48 ± 0.12	0.9946	34	0.5	1.7	
Aflatoxin B2	AF-B2	4.76 ± 0.12	0.83 ± 0.17	0.9920	27	0.5	1.7	
Aflatoxin B1	AF-B1	4.86 ± 0.12	0.91 ± 0.18	0.9970	44	0.5	1.7	
Citrinin	CIT	4.93 ± 0.12	0.08 ± 0.04	0.9949	50	1	3.3	nc
HT-2 toxin	HT-2	5.08 ± 0.13	0.91 ± 0.18	0.9970	33	5	16.7	–
Ochratoxin B	OTB	5.15 ± 0.13	0.31 ± 0.08	0.9974	78	0.5	1.7	nc
Fumonisin B2	FUM-B2	5.20 ± 0.13	0.57 ± 0.11	0.9958	43	0.5	1.7	Sum FUM-B1 and B2 = 4000
T-2 toxin	T-2	5.45 ± 0.14	0.76 ± 0.15	0.9978	33	1	3.3	–
Ochratoxin A	OTA	5.64 ± 0.14	0.56 ± 0.11	0.9966	29	0.5	1.7	3
Zearalenone	ZEA	5.72 ± 0.14	0.75 ± 0.15	0.9932	−16	0.5	1.7	75

nc = not controlled.
[a] According to 2002/657/EC: avg. t_R ±2.5% tolerance.
[b] According to 2002/657/EC: avg. ion ratio ±20% tolerance for NIV, DON, FUM-B1, FUM-B2, AF-B1, AF-B2, HT-2, T-2, OTA, and ZEA; avg. ion ratio ±25% tolerance for AF-G1and OTB; and avg. ion ratio ±50% tolerance for CIT.

capability of MS/MS instrumentation and significantly impact the accuracy of a determination and ruggedness of method. This is especially true for mycotoxins, which are generally present at very low concentration. Therefore, methods with greater selectivity, such as high-resolution MS, accurate mass MS, or two-dimensional chromatography–MS, that can analyse samples with a minimum of pre-treatment, are necessary for the determination of these toxins. Moreover, the continuous advances in direct sample introduction or novel ambient desorption ionisation techniques of MS, such as direct analysis in real time (DART), desorption electrospray ionisation (DESI), and the atmospheric-pressure solids analysis probe (ASAP), have been reported for mycotoxins analysis (Hajslova, Cajka, & Vaclavik, 2011; Nielen, Hooijerink, Zomer, & Mol, 2011; Vaclavik, Zachariasova, Hrbek, & Hajslova, 2010). These techniques require no chromatographic separation and minimal or no sample preparation, thus providing high-throughput analysis. However, the instruments are expensive and are demanding for targeted-analyte monitoring during routine operations. Additionally, the equipment requires expertise to operate and maintain and to process the results. For these reasons, we believe that a simple extraction with proper clean-up is still needed and a combination with ultra-high performance (UHP) LC–MS/MS is an alternative to provide good performance and robustness for routine operation.

The extraction of mycotoxins from solid samples usually involves solvent extraction, ultrasonic extraction, accelerated solvent extraction (ASE), matrix solid-phase dispersion (MSPD), or in-tube solid-phase microextraction methods. Appropriate clean-up methods involving liquid–liquid partitioning, solid-phase extraction (SPE), or an immunoaffinity column (IAC) (Senyuva & Gilbert, 2010) are commonly used for removing matrix interferants from sample extracts. SPE methods using different bonded-phases, such as C_{18}, ion exchange, polymeric reversed-phase, or molecular imprinted polymers, are employed for the clean-up of various foods (Khayoon et al., 2010; Pérez-Ortega, Gilbert-López, García-Reyes, Ramos-Martos, & Molina-Díaz, 2012). Molecular recognition based IACs are routinely and successfully used in many mycotoxins analyses (Ren, Zhang, Lai, Han, & Wu, 2011; Soleimany, Jinap, Faridah, & Khatib, 2012). Target mycotoxins selectively bind to antibodies immobilized on a solid support in the column, while structurally different co-extractives pass through the column. The analyte is released from the column using solvent to break the analyte-antibody bonds. This feature of the IAC method results in low matrix effects (low ion suppression), low detection limits, and increased analyte recovery. These sample clean-up methods are suited to automation, additionally, they use less solvent and are faster to carry out than liquid–liquid partitioning. However, there are several major disadvantages such as non-simultaneous extraction, multiple steps, and possible cross-reactivity. Additionally, disposable materials have a high cost.

"Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS) sample preparation approaches (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003; EN 15662, 2008; Lehotay, 2007) have been proposed for analysis of a wide range of matrices and analytes. Many procedures can provide high quality results and other benefits for the analysis of mycotoxins (Desmarchelier et al., 2010; Lacina et al., 2012; Sospedra, Blesa, Soriano, & Mañes, 2010; Vaclavik et al., 2010; Zachariasova et al., 2010).

We previously reported a method for analysis of phenoxy acids in rice using a citrate-QuEChERS based method coupled with UHPLC–MS/MS analysis (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2008). The method allowed us to detect and identify polar herbicides with good results at low concentrations, and is currently being used for routine analysis of phenoxy acids in rice for export. As with phenoxy acids, mycotoxins are polar substances, and we wondered if our previous method could be successfully applied to the analysis of mycotoxins in rice.

In this work, we aimed to develop an effective method for the analysis of 14 mycotoxins in rice by adapting our previous citrate-QuEChERS method coupled with UHPLC–MS/MS. We strived to optimise sample preparation conditions to accommodate a variety of analyte polarities and to determine the necessary performance criteria for the application. We also examined and compared our proposed method with current IAC methods to identify where replacements might be made.

2. Experimental

2.1. Chemicals and materials

Mycotoxin reference standards, ⩾97% or higher purity, were obtained from Sigma–Aldrich (St. Louis, MO, USA), Supelco (Bellefonate, PA, USA), and WAKO (Richmond, VA, USA). MeCN and methanol (MeOH), of high purity grade for residue analysis, were obtained from Baker (Denver, Holland). Analytical grade glacial

acetic acid (HOAc) and formic acid (FA) were obtained from BDH (Briare, France).

For extraction and partitioning, anhydrous $MgSO_4$ was purchased from Panreac (Barcelona, Spain). Sodium citrate dibasic sesquihydrate (di-Na) and ammonium formate were obtained from Fluka (Steinem, Germany). Sodium citrate tribasic dehydrate (tri-Na) was purchased from Riedel-deHaën (Austria). NaCl was purchased from Merck (Darmstadt, Germany). For clean-up using d-SPE, PSA bulk powder was obtained from Varian (Oxfordshire, UK). Octadecyl (C_{18}) was obtained from Merck. Alumina-neutral (Al-N) was purchased from Waters Corp. (Milford, MA, USA). For method validation, mixtures of salts and mixtures of d-SPE sorbents were pre-weighed and stored in glass vials. Water for sample swelling in the extraction step and for LC mobile phase preparation, was prepared using the Milli-Q ultrapure water system (Millipore, Billerica, MA, USA).

Samples of different types of white rice were collected from local food stores and milled to a flour consistency. The milled and thoroughly homogenised samples were transferred into airtight plastic bags, sealed, and stored in desiccators at room temperature until required for sample preparation and analysis.

For method comparison, AFLAPREP, DONPREP, and ZEAPREP IACs were purchased from R-Biopharm Rhöne Ltd. (Glasgow, Scotland). AflaTest P, DonTest, OchraTest, and ZearalaTest IACs were purchased from Vicam L.P. (Watertown, MA, USA). AflaStar, DonStar, OchraStar, and ZearaStar IACs were purchased from Romer Labs Inc. (Stylemaster Drive Union, MO, USA). Phosphate buffered saline (PBS) was purchased from R-Biopharm Rhöne Ltd.

Liquid nitrogen and ultra-high purity (99%) argon gas used in the LC–MS/MS interface were supplied by TIG (Bangplee, Samutplakarn, Thailand).

2.2. Standard solution preparation

Stock standard solutions of each analyte were prepared at concentrations of approximately 200 mg L^{-1} in MeCN or MeOH, depending on solubility, and stored in amber-glass vials at −10 °C. A working standard mixture of all analytes (Mix-1) was prepared at 5 mg L^{-1} in MeCN, except for a mixture of NIV and DON (Mix-2), which was separately prepared at 50 mg L^{-1} in MeOH. These working standard solutions were appropriately diluted to provide 11-point calibration standards in mobile phase A–B (1:1, v/v). For the recovery and precision experiments, Mix-1 and Mix-2 served as high spiking solution. Middle and low spiking solutions were prepared by dilution of the high spiking solution in MeCN.

2.3. Sample preparation

(1) Weigh 10 ± 0.05 g finely milled sample into a 50 mL Teflon centrifuge tube, use water for the reagent blank; (2) for a spiked sample, add the required volume of spiking standard solution; (3) add water (10 mL) and 10% formic acid in MeCN (10 mL), then vortex the tube for 30 s; (4) shake the tube with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample; (5) add anhydrous $MgSO_4$ (4 g) + NaCl (1 g) + tri-Na (1 g) + di-Na (0.5 g); (6) shake the tube immediately and vigorously by hand for 1 min; (7) centrifuge the tube at 3400 rpm (approximately 2171 rcf) for 5 min; (8) for d-SPE clean-up, transfer 8 mL of the MeCN extract (upper layer) into a 15 mL centrifuge tube containing anhydrous $MgSO_4$ (1.2 g) + C_{18} (0.25 g) + Al-N (0.25 g) + PSA (0.4 g); (9) shake the tube vigorously by hand for 1 min; (10) centrifuge the tube at 3400 rpm for 5 min; (11) transfer 5 mL of the extract to a glass tube; (12) evaporate to dryness at 40 °C under a stream of N_2; (13) add 1 mL of mobile phase A–B (1:1, v/v) to reconstitute the extract, and then vortex for 1 min; (14) filter the extract using 0.2 μm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into an autosampler vial.

2.4. UHPLC–MS/MS conditions

For LC–MS/MS, we used an Acquity Ultra Performance Liquid Chromatography (UPLC) system interfaced with a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Waters Corp., MA, USA). MassLynx software version 4.1 (Waters Crop.) was used for instrumental control, and data acquisition and processing. LC separation was performed using an Acquity UPLC BEH C_{18} column (2.1 mm i.d. × 100 mm; 1.7 μm particles), which was integrated with a Vanguard pre-column (2.1 mm i.d. × 5 mm; 1.7 μm particles) (both from Waters Corp.). The column and autosampler tray temperature were controlled at 40 and 20 °C, respectively. The injection volume was 5 μL and the mobile phase flow rate was 0.25 mL min^{-1}. The mobile phase composition was (A) 0.5% formic acid in 5 mM aqueous ammonium formate and (B) MeCN–MeOH (1:1, v/v). A mobile phase gradient program was started at 5% B (0–2 min), 70% B at 3 min, and held for 2.5 min, 95% B at 6 min, and held for 2 min, and finally, 5% B at 8.5 min. The initial column equilibration step was carried out for 2.5 min before the next injection.

The MS instrument was used in electrospray (ESI) positive mode for all analytes, except for ZEA, which was only detected in negative mode. Analyte-specific MS/MS parameters were obtained by direct infusion of each standard solution (5 mg L^{-1}) into the ESI source and using signal optimisation software. The two most abundant product ions generated from each precursor ion were chosen as the MRM transitions of each analyte. The collection time segment and optimal dwell time for each transition were set up by the instrument's software. The retention times (t_R), fixed ESI conditions, and optimised MS/MS conditions for each analyte are shown in Table S-1 (Supplementary data).

2.5. Method validation

The European Commission (EC, 2002, 2006a) and Document N° SANCO/12495/2011 were used as for guidelines and criteria to assess the method validation. Selectivity was determined from t_R, ion ratios, and identification-points (IP) for each analyte.

Calibration standards were prepared by combining standard solutions into the neat solvent and blank matrix extracts (matrix-matching) to yield the desired concentrations in the range of 10–500 μg L^{-1} for each analyte. The NIV and DON concentrations were 5-fold greater than they were for the other analytes in the range of 50–2500 μg L^{-1}.

Matrix effect of each analyte was estimated by calculating the difference of the linear best-fit slope, obtained from the matrix-matched calibration curve and solvent-based standards calibration curve, divided by the slope of the solvent-based standards calibration curve.

Recovery and precision studies were conducted by spiking rice samples with 10 replicates at each spiking level: 10 (low), 50 (middle), and 100 μg kg^{-1} (high), on three separate days. For NIV and DON, which generally showed low precursor ion signal intensities in ESI^+, we used spiking levels of 100 (low), 500 (middle), and 1000 μg kg^{-1} (high) throughout the quantitative validation. To check for possible interferences and carry-over, the first and the last injections were reagent blanks. Recoveries were calculated from matrix-matched standard calibrations.

We tested several commercial IACs that are selective for detection of various mycotoxins. Recovery experiments were carried out in duplicate extractions at different spiking levels for each IAC-mycotoxin type. Spiked samples were extracted following the instructions for each IAC method, and then analysed using the

same UHPLC–MS/MS conditions as described above. Recoveries were calculated by comparison with matrix-matched standard calibrations.

3. Results and discussion

3.1. UHPLC–MS/MS optimisation

Our goal was to simplify the analysis of mycotoxins in rice using up-to-date instruments. We started with optimising analyte-specific MS/MS conditions both in positive and negative ion detection modes. The base peak of $[M+H]^+$ in the full scan mass spectra of all analytes, except for the T-2 and HT-2 toxins, which formed more stable $[M+NH_4]^+$ ions, served as the precursor ion for monitoring in ESI^+ mode. ZEA produced a high intensity of $[M-H]^-$ peak with less interference and chemical noise in ESI^- mode. The most intense fragment ion generated from each precursor ion was chosen as the quantifier ion for quantification, and the second most intense ion was used as qualifier ion for identification. The instrument's software automatically set the collection-time window, dwell time, and inter-scan delay for each ion transition, to maximise analyte responses (number of data points per peak, sensitivity, and selectivity). Promising ion transitions (Table S-1) were tested for selectivity and sensitivity in various rice extracts.

MeCN, MeOH, water, and buffers are commonly used as the mobile phase in LC analysis. We chose ammonium formate buffer (mobile phase A) because its ionisation efficiency and solubility in the presence of MeCN are greater than those of ammonium acetate buffer. We evaluated a range of modifiers. Using MeCN as mobile phase B, the first eluting analyte NIV showed a deteriorating peak shape (fronting) because of its high-polarity and poor solubility in MeCN. ZEA is less polar than other analytes, and elutes more slowly. ZEA produced split peaks in its LC chromatogram when eluted with MeOH. MeOH has insufficient solvent strength to fully replace the analyte molecule that interacted well with the stationary phase, and two discrete interactions occur simultaneously between the stationary phase and mobile phase. We tried a mixture of MeCN–MeOH (1:1, v/v) to ameliorate these effects, and this produced an improvement in peak shape for both NIV and ZEA. Other analytes, which were not affected by the mobile phase composition, consistently provided good signal responses. Although using the MeCN-MeOH solvent mixture resulted in lower analyte intensities, it provided good peak shapes and consistent retention times, and thus could be considered a good method ruggedness. Fig. S-1 (Supplementary data) shows the influence of mobile phase composition on NIV and ZEA chromatographic peak shapes.

Various mobile phase gradient conditions were experimented with to maximise the numbers of chromatographic results obtained with reasonable run times. Ultimately, we chose (A) 0.5% formic acid in 5 mM aqueous ammonium formate, and (B) MeCN–MeOH (1:1, v/v) as the mobile phase. The total run time was 11 min, which included 2 min at 95% B to prevent carry-over effects, plus another 2.5 min at 5% B for column re-equilibration.

3.2. Sample preparation

Recent advances in separation and detection provided by the UHPLC–MS/MS instrument permit analysis at desired detection limits without intensive sample preparation. We were not only interested in the analysis of mycotoxins in rice, but considered that the method could be applied to foods with different matrix compositions. A good sample preparation method is essential to ensure long-term system performance.

Our modified QuEChERS method was previously developed for analysis of phenoxy acids in rice (Koesukwiwat et al., 2008). Briefly, in the previous method, milled rice (10 g) was swelled with water (5 mL), then extracted with 5% formic acid in MeCN (10 mL) and shaken for 1 h. The MeCN and aqueous phases were separated by addition of anhydrous $MgSO_4$ (4 g) + NaCl (1 g) + tri-Na (1 g) + di-Na (0.5 g) to the solution; 5 mL of the MeCN extract was then cleaned-up using anhydrous $MgSO_4$ (1.5 g) + C_{18} (0.25 g) + Al-N (0.1 g), and evaporated to dryness. Finally, the residue was re-constituted in LC mobile phase.

Mycotoxins have greater polarity than the herbicides analysed in our previous method, and most mycotoxins cannot be completely extracted and partitioned in MeCN phase. Therefore, we modified that method to increase its effectiveness for the analysis of mycotoxins in rice. The structures of studied mycotoxins are given in the Table S-2 of the Supplementary data.

We investigated the optimisation of sample (10 g) and water (5–20 mL) ratios, simultaneously varying the acid modifiers by 0–10% for both formic and acetic acid in MeCN. The addition of 10 mL water was sufficient for sample swelling and shaking, and resulted in a slight improvement in the extraction efficiency. In the case of MeCN, the recovery of acidic mycotoxins CIT, FUM-B1, FUM-B2, OTA, and OTB greatly improved with increasing acid content. CIT, FUM-B1, and FUM-B2 gave the best recoveries at 10% formic acid in MeCN. We attributed this to acid stabilization of the acidic mycotoxins in their neutral forms in the MeCN phase. The remaining of analytes (neutral mycotoxins) exhibited recoveries that were consistently 80% or better, regardless of solution pH. Only NIV, which was the first eluting analyte, had a recovery of $\leqslant$60%, because of its low signal response and the effects of polar matrix co-extractives. However, the system showed better sensitivity towards NIV, and good recovery was obtained (70%) when extracting with 10% acetic acid in MeCN. Fig. 1a and b demonstrates the influence of extraction solvents on the recoveries of acidic and neutral mycotoxins. We chose to use 10% formic acid in MeCN as the extraction solvent for the following experiments.

For sample clean-up step, we tried our previous d-SPE based method as mentioned above, but it proved inadequate for removing co-extractives in this application. The use of formic acid with a buffer led to convert a number of fatty acids to their neutral forms, and thus, resulted in greater partitioning in the MeCN phase. The 0.1 g Al-N was overwhelmed by the presence of high amount of formic acid in the extraction solvent. This reduced the effectiveness of the removal of the increased fatty acid contents (mainly palmitic acid and linoleic acid) in the final extracts. Al-N is a polar sorbent used for removal of polar matrix components. It has a neutral surface that allows interaction of aluminum metal clusters with molecules that contain N, O, P, and S. Increasing the amount of Al-N can remove a greater amount of polar interferants, however, Al-N also retains polar analytes in the method.

Rather than optimising the amount of Al-N alone, we simultaneously evaluated the addition of PSA, which QuEChERS methods typically use to remove fatty acids. To improve sensitivity of the method, we increased the volume of the MeCN extract to approximately 8 mL, and mixing this with anhydrous $MgSO_4$ (1.5 g) + C_{18} (0.25 g) + Al-N (0–0.9 g) + PSA (0–0.9 g). The amounts of anhydrous $MgSO_4$ and C_{18} were maintained for removing residual water and non-polar components without affecting the analytes. As shown in Fig. 2, the use of an optimal amount of PSA (0.4 g) produced significant improvements in recovery at $\geqslant$80% for all analytes, except for NIV (58%) and CIT (53%). NIV had low recovery due to low sensitivity. CIT is a small molecule that contains a carboxylic group, its recovery was the most affected by PSA compared to other acidic mycotoxins and by the presence of fatty acids co-extractives. The use of Al-N (0.4 g) produced a cleaner extract, with lower fatty acid responses present in the full scan ion GC–MS/MS chromatograms compared to those seen for PSA; however, its use resulted in the retention of a significant quantity of mycotoxin analytes. An

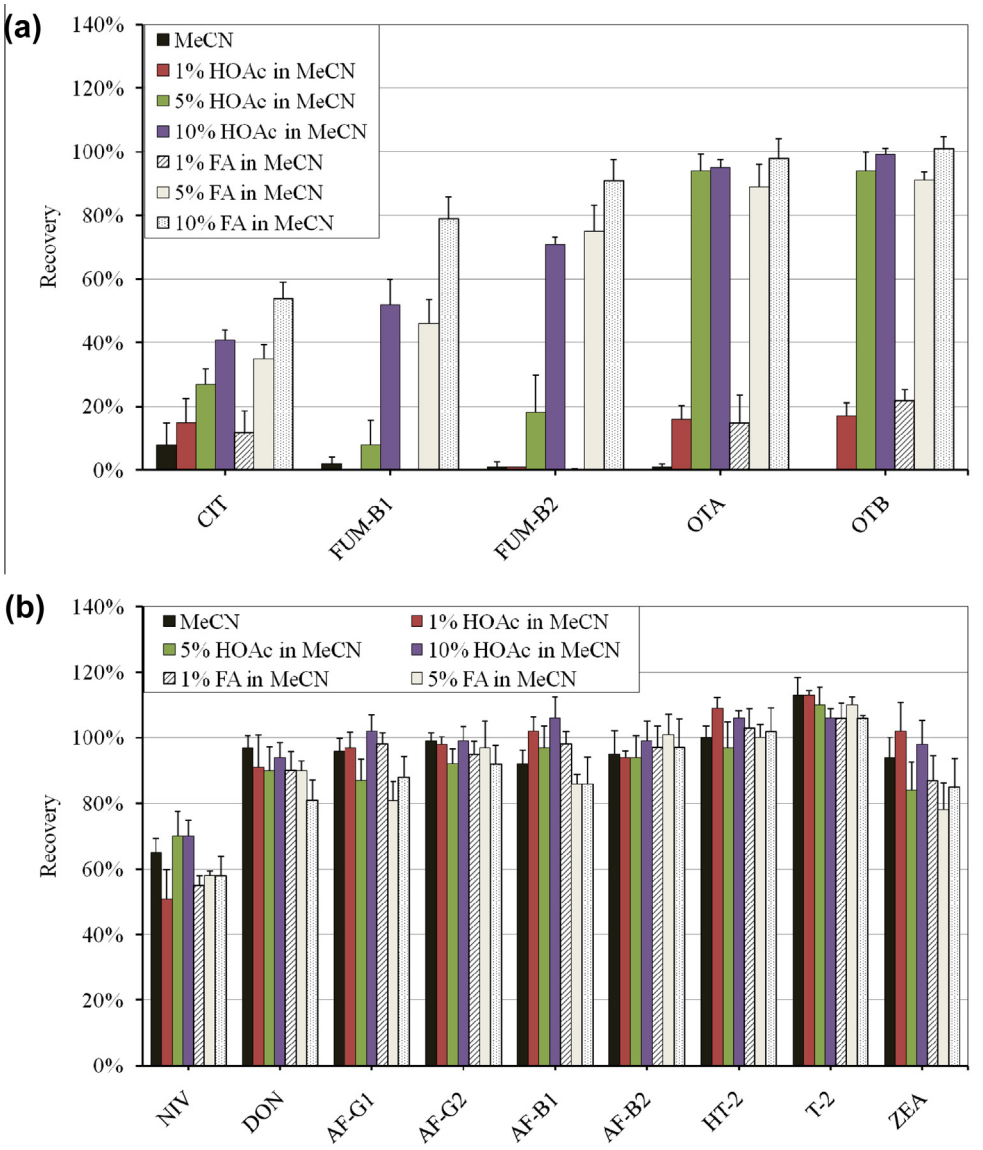

Fig. 1. Effect of extraction solvents in the QuEChERS method on recoveries of: (a) acidic mycotoxins and (b) neutral mycotoxins at spiking level 100 μg kg^{-1} for NIV and DON, and 20 μg kg^{-1} for the rest of analytes ($n = 4$). Optimal UHPLC–MS/MS conditions are described in Section 2.

optimal combination of Al-N (0.25 g) + PSA (0.4 g), NIV and CIT gave acceptable recoveries of 64% with ⩽10% RSD values ($n = 5$). Cleaner extracts were achieved in the order Al-N + PSA > Al-N > PSA. Overall, our rugged method performance and instrumentation provided good recoveries of 64–104% with ⩽10% RSDs, and produced good analyte peak shapes.

During testing of the optimised method, we unintentionally reduced the amount of anhydrous $MgSO_4$ to 1.2 g; however, after several tests, we found that this change did not significantly affect any of our results. We determined the optimum sorbent combination to be anhydrous $MgSO_4$ (1.2 g) + C_{18} (0.25 g) + Al-N (0.25 g) + PSA (0.4 g) for d-SPE clean-up of 8 mL of the MeCN extract in this application. The final optimised procedure for sample preparation is summarised in the Section 2.

3.3. Method performance

3.3.1. Qualitative identification criteria

Several criteria and guidelines for MS/MS-based identification and confirmation of chemicals and contaminants in food analysis have been discussed in the literature (Document N° SANCO/12495/2011; EC, 2002; Heller, Lehotay, Martos, Hammack, & Fernandez-Alba, 2010; Lehotay et al., 2008). In accordance with 2002/657/EC, we monitored two ion transitions for each analyte to obtain 4 IPs, exceeding the EU minimum requirement. With the exception of AF-G2, only one ion transition could be detected using this method. Other identification criteria, which we included for qualitative aspects of the proposed method were that: (1) the t_R of the sample peak must fall within ±2.5% of the average t_R of the calibration standards analysed in the same sequence, (2) the t_R and chromatographic peak shapes of the quantifier and qualifier ions should be similar, (3) the intensity of the quantifier ion should have an S/N ratio ⩾3, and (4) the ion ratio for 2 transitions must match within ±50%, ±30%, ±25%, and ±20% for relative ion intensities of ⩽10, >10–20, >20–50, and >50%, respectively, with those ratios obtained from the calibration standards analysed in the same sequence.

To define acceptable t_R values and ion ratios, percentage tolerances were entered into the instrument's software for automate qualitative analyte screening during quantitative validation and further applications with real samples. Table 1 shows the average and range of variation for the t_R values and ion ratios.

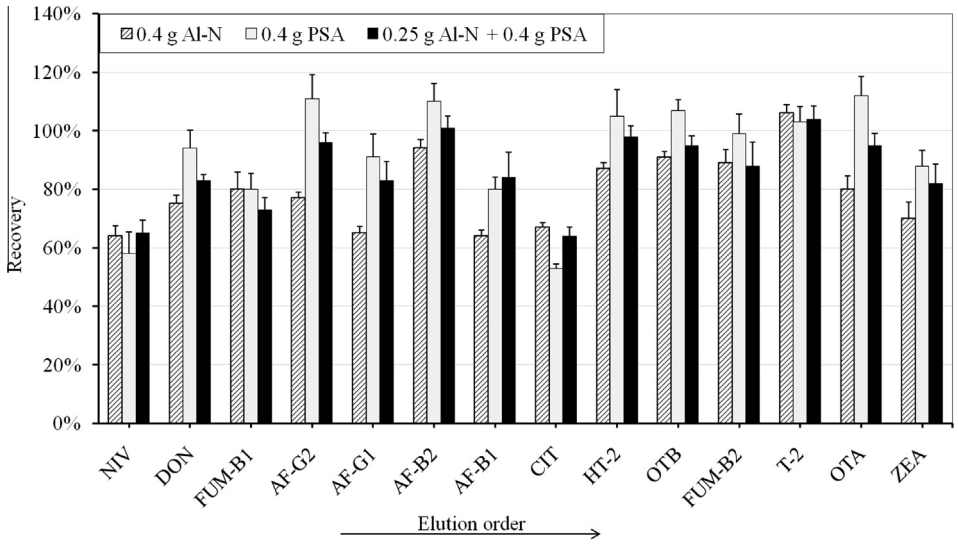

Fig. 2. Comparison of different sorbents for d-SPE clean-up in the QuEChERS method for the 14 mycotoxins at spiking level 100 μg kg^{-1} for NIV and DON, and 20 μg kg^{-1} for the rest of analytes. Optimal UHPLC–MS/MS conditions are described in Section 2.

3.3.2. Selectivity and detectability

We examined selectivity of the method by analysing samples of 20 different rice varieties. To compare t_R values and ion ratios, we injected a system blank (0 μL, only mobile phase), a reagent blank, standard (10 μg L^{-1}) in neat solvent, samples of each of the 20 rice extracts (a matrix blank and a spiked matrix blank (10 μg L^{-1})) for analysis. No interfering signals from co-eluting compounds appeared with the analyte ion peaks of the verified samples. All negative rice samples were combined for use in the development and validation experiments.

For the detectability study, we used quantifier ion of each analyte (which met the four identification criteria described earlier). LODs and LOQs were obtained from spiked rice samples at the lowest spiking level of 10 μg kg^{-1} by measuring the concentrations that gave S/N ratios of 3 and 10, respectively. The obtained results are listed in Table 1. The LOD and LOQ values ranged from 0.5 to 15 μg kg^{-1} and from 1.7 to 50 μg kg^{-1}, respectively. NIV, DON, and HT-2 gave slightly higher values than the other analytes because of low signal responses (NIV and DON) and indirect matrix effects (HT-2). Nonetheless, these values were still below the EU maximum limits.

3.3.3. Linearity and matrix effects

The linearity of response *vs.* concentration was evaluated using 11 matrix-matched calibration points (not including zero concentration) over the range of 50–2500 μg kg^{-1} for NIV and DON, and 10–500 μg kg^{-1} for the remaining analytes. Most analytes gave average linear regression (R^2) values of >0.990 over the range of interest (Table 1).

Matrix effects are unavoidable and cannot be eliminated in the analysis (Heller et al., 2010). Table 1 shows our estimated matrix effects, calculated from the differences between the matrix-matched standards and solvent-based standards calibrations. Analytes that were ionised in positive mode showed similar trends of signal enhancement (up to 78%), with the exception of NIV, which was the first eluting analyte and its ionisation efficiency was affected by polar matrix co-extractives, thus the NIV signal is suppressed by 15%. Conversely, ZEA was ionised in negative mode and its signal shows a suppression of 16% because it undergoes different ionisation mechanism in the presence of matrix components. Moreover, matrix effects are depended on concentration, on analyte–matrix interactions, and on the competition during the ionisation process between analytes and co-injected components. These results indicate the necessity of using matrix-matched standards to quantify analytes, in order to compensate for matrix effects.

3.3.4. Recovery and precision

Recovery and precision results of the studied mycotoxins are summarised in Table 2. Most analytes show average recoveries in the range of 70–98%, falling within an acceptable concentration range (EC, 2006a, 2002). CIT and FUM-B1 showed relatively low overall recoveries at 56% and 66% respectively, but their RSD values were 5%. This small variation implies that losses occurred during the extraction. Possible causes of these losses are insufficient extraction with solvent, the use of PSA and Al-N sorbents in the d-SPE format, the process of evaporating the extract to dryness, and losses during extract transferring steps. Adding an isotopically-labeled internal standard to the sample before extraction is recommended for correcting the possible errors during sample preparation, and so improving the accuracy of the results.

For precision, the repeatability for all analytes was consistently ⩽7% RSD_R for each spiking level (n = 10) and overall concentration (n = 30). For within-laboratory reproducibility, all analytes gave excellent RSD_R values of ⩽10%, which were lower than the acceptable limits of 23% for spiking levels of ⩽100 μg kg^{-1}, and 16% for 1000 μg kg^{-1} spiking level (EC, 2002). These results demonstrate reliability of the method for quantification.

We independently verified the method performance by participating in proficiency testing (PT) under the Food Analysis Performance Assessment Scheme (FAPAS). Check samples of rice, oat, breakfast cereal, and maize containing mycotoxins were prepared by the FAPAS, while the analysts were blind to the mycotoxin doping of samples. To perform the inter-laboratory comparison, each check sample was prepared in duplicate and analysed using the proposed method, as described in Section 2. Our obtained z-score values were all between −1.8 and +0.6, and so were within the satisfactory limit of ±2.0. These results (Table 3) were all in compliance with the regulations, confirming the efficiency and suitability of the proposed method for quantitation of mycotoxins (within the scope of the present work) in a rice matrix.

3.4. Immunoaffinity column

In addition to the parameters investigated during the validation process, we also compared the performances of commercial IACs

Table 2
Percentage recoveries and RSD values of the 14 mycotoxins in the method.

Analyte	% Recovery (% RSD)												
	Repeatability, $n = 10$								Within-laboratory reproducibility, $n = 30$				
	Low (10 μg kg^{-1})		Middle (50 μg kg^{-1})		High (100 μg kg^{-1})		Overall, $n = 30$		Low (10 μg kg^{-1})		Middle (50 μg kg^{-1})		High (100 μg kg^{-1})	
NIV[a]	70	(2)	73	(4)	71	(4)	71	(2)	72	(6)	66	(6)	64	(7)
DON[a]	75	(3)	76	(2)	79	(3)	77	(3)	82	(6)	76	(3)	75	(4)
FUM-B1	69	(5)	62	(4)	67	(2)	66	(5)	69	(9)	66	(7)	63	(5)
AF-G2	96	(3)	97	(1)	87	(2)	93	(6)	94	(4)	93	(3)	85	(3)
AF-G1	94	(4)	86	(2)	98	(1)	93	(7)	87	(6)	90	(4)	95	(5)
AF-B2	94	(5)	92	(2)	94	(3)	93	(1)	94	(7)	96	(4)	103	(6)
AF-B1	93	(3)	97	(1)	104	(1)	98	(6)	88	(5)	89	(5)	99	(7)
CIT	57	(4)	53	(1)	58	(1)	56	(5)	58	(10)	57	(7)	57	(4)
HT-2	85	(4)	84	(5)	84	(3)	84	(1)	89	(6)	86	(6)	87	(6)
OTB	94	(2)	90	(1)	96	(1)	93	(3)	93	(4)	93	(4)	94	(3)
FUM-B2	82	(4)	82	(3)	85	(3)	83	(2)	81	(6)	83	(4)	85	(3)
T-2	97	(3)	95	(2)	98	(2)	97	(2)	97	(3)	96	(4)	95	(3)
OTA	100	(2)	93	(2)	96	(1)	96	(4)	98	(3)	94	(3)	98	(3)
ZEA	95	(2)	90	(2)	88	(1)	91	(4)	90	(4)	87	(3)	88	(4)

[a] Spiking levels: low = 100, middle = 500, and high = 1000 μg kg^{-1}.

Table 3
Recoveries and *z*-score values of mycotoxins in the check samples obtained from the proficiency testing.

Test material	Material No.	Analyte	Assigned value (μg kg^{-1})	% Mean recovery[a]	Result (μg kg^{-1})	*z*-Score
Rice	T4151	Total AFs	5.09	94	5.76	0.6
Cereal (oat)	T1776	OTA	5.92	95	3.58	−1.8
Breakfast cereal	T2257	ZEA	69.5	100	76.07	0.4
Maize	T2262	DON	1714	88	1513	−0.8

[a] The values obtained from lab's participants.

Table 4
Recoveries and LOD values of mycotoxins obtained from different IAC methods.

Analyte	IAC	Spiking level (μg kg^{-1})	% Mean recovery	LOD (μg kg^{-1})
Total AFs	Romer	400	108	112
	R-Biopharm	400	95	72
	Vicam	100	89–118	AF-B1 = 0.3 AF-B2 = 2 AF-G1 = 0.05 AF-G2 = 3
DON	Romer	800	74	75
	R-Biopharm	1600	103	30
	Vicam	800	101	20
OTA + OTB	Romer	200	82	3
OTA	Vicam	200	71	0.3
ZEA	Romer	400	89	1
	R-Biopharm	800	103	3
	Vicam	250	108	0.3

vs. the proposed QuEChERS method for the analysis of 14 mycotoxins. Each type of IAC was chosen to provide high selective detection of specific mycotoxins. Spiked samples were extracted and then analysed using the optimised UHPLC–MS/MS method. Table 4 provides the mean recoveries and LODs of the analytes obtained from the IAC methods. Overall, good recoveries of 71–118% were achieved, but the LODs obtained by the commercial IACs were greater than those obtained from our modified QuEChERS method (Table 1). The exceptions were AF-B1, AF-G1, OTA, and ZEA, which gave lower LODs when using the Vicam-IAC extraction method.

The IAC methods provided acceptable extraction efficiency with high specificity; however, the lack of sensitivity for some mycotoxins, non multi-analysis, possible cross-reactivity, and practical limitations (such as short antibody shelf life, time consuming, tedious, and personnel and materials costs) were important considerations. However, these IAC methods are useful for confirmatory purposes at high-levels of contamination. As apparent from the results, the modified QuEChERS method provides several benefits over the IACs, including using less solvent, simple (no technical skills are required), multi-analysis, high sample throughput, high sensitivity, and in particular, low cost (approximately a 5–6-fold cost reduction per sample).

3.5. Analysis of incurred samples

We performed analyses of 6 representative commodities, 11 of feed samples, 5 of corn starch, 5 of cocoa, 2 of maize flour, 12 of rice, and 2 of malt powder obtained from local markets. Samples were prepared following the procedures described in the Section 2. All identification criteria (t_R, peak shape, S/N, and ion ratio) were used to identify the mycotoxins in these samples.

Of the 37 samples tested, 27 were positive for mycotoxins contamination. No toxins were found in corn starch samples, possibly because the contaminate concentrations were less than the LOD values. The detected mycotoxins were ZEA (5 feed, 5 cocoa, 2 rice, and 1 malt powder samples), DON (3 feed samples), FUM-B1 (3

feed and 2 maize flour samples), and AF-B1 (6 rice samples). The results are shown in Supplementary data, Table S-3. Although the contamination levels were below the MLs prescribed by the EU for typical foods and feeds, these findings illustrate the current state of food contamination, and more attention needs to be paid towards hygienic practices to ensure consumer safety.

4. Conclusions

A previously reported QuEChERS method with minor modifications was developed and evaluated for the analysis of 14 mycotoxins in rice using UHPLC–MS/MS. Our modified method is simple, and is more time- and cost-effective compared to IAC methods. The method was validated according to the European Communities 2002/657/EC and SANCO/12495/2011 guidelines and met acceptability criteria in all cases. Good analytical results were obtained, including recovery, precision (repeatability, within- and inter-laboratory reproducibility), linearity, and analytical limits (LOD and LOQ). The method is currently being used to replace the IAC for routine multiple mycotoxin analysis in rice for export and some related matrices (feed, maize, and wheat flour).

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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.2013.12.029.

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