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A single-step solid phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A simple and rapid extraction procedure for the simultaneous determination of eight mycotoxins (*Alternaria* toxins, ochratoxin A, patulin, citrinin) in a variety of fruit matrices has been developed using ultra high performance liquid chromatography coupled to tandem mass spectrometry. The procedure involves a one-step cleanup using homemade solid phase extraction (SPE) cartridges. By comparative evaluation among six various adsorbents (C18, PSA, HLB, MCX, Silica, NH_2), the combination of MCX and NH_2 was found to provide the most effective cleanup, removing the greatest number of matrix interferences and also allowing the quantification of all analyzed mycotoxins in fruits. The optimized extraction conditions including acidified aqueous acetonitrile and an additional salt-out step using NaCl were employed before SPE cleanup. Method validation was performed by analyzing samples spiked at three levels (LOQ, 2 LOQ and 10 LOQ). Four fruits including apple, sweet cherry, tomato and orange fruits were selected, and accuracy (recovery%), precision (RSD%), limits of quantification (LOQ), linearity and matrix effect were evaluated during validation. Matrix-matched linearity with correlation coefficients $\geq$0.9921 was established in the range of 5–200 ng mL^{-1} for patulin and 1–200 ng mL^{-1} for other mycotoxins, respectively. Recoveries between 74.2% and 102.4% and relative standard deviations lower than 4.7% were obtained for all tested fruits. The matrix effect observed was low ($\leq\pm$17%) in all three fruit matrixes with the exception of orange, for which strong ion suppression was observed for alternariol (25.3%), ochratoxin A (31.6%) and citrinin (40.3%). Therefore, matrix-matched calibration was used for a correct quantification in order to compensate for matrix effect. The limits of quantification (LOQ), ranging from 1 to 5 μg kg^{-1} depending on mycotoxins type, were always lower than maximum permitted levels for every regulated mycotoxin by the current European legislation.

1. Introduction

Mycotoxins are secondary metabolites produced by many species of filamentous fungi, which grow on various agricultural commodities in the field site, and during postharvest (transport, processing and storage) [1]. Fruits are plant products especially susceptible to be infested by fungi and in consequence, several classes of mycotoxins can simultaneously contaminate fruits. The most common mycotoxins associated with fruits are ochratoxin A (OTA), patulin (PAT), citrinin (CIT) and *Alternaria* toxins, which are produced by *Aspergillus*, *Penicillium* and *Alternaria* genera [2,3]. The growth of fungi and consequent production of mycotoxins occur after damage to fruit skin. Although the visible rotten areas are usually removed or avoided by the consumer, mycotoxins can be transferred from the rotten part to the surrounding tissues [4]. Moreover, mycotoxins contamination of fruit products is linked to the use of improper technological processing steps [5]. Therefore, mycotoxins can be found in fresh fruit and processed products with low concentrations. Considering a large consumption of these products and the concurrent intake of different contaminated food and drinks by the population, the presence of mycotoxins could have the potential adverse effect on human health, especially hepatic, gastrointestinal, and carcinogenic diseases [6].

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To protect consumer health, maximum levels for mycotoxins in foodstuffs have been established worldwide. Particularly, the European (EU) legislation often considered as being the most comprehensive for mycotoxins in foods [7]. However, the information for mycotoxins in fresh fruit or fruit-based products is limited. Only the presence of PAT in apple products and OTA in grape products is regulated in EU, and the strict limit is 2 and 10 $\mu g\,kg^{-1}$ for OTA and PAT, respectively [8]. Although the other toxins have not been regulated, their toxigenic potential has been evaluated by EFSA [9]. Therefore, it is necessary to develop more rapid, accurate and highly sensitive methods for the routine analysis of mycotoxins to better understand their global contamination impact, as well as to assess the toxicological risk.

To date, numerous methods have been developed for the simultaneous determination of multiple mycotoxins in cereals and foods [6,7,10,11], but in fresh fruits, a single mycotoxin or multiple mycotoxins belonging to the same group or class was widely investigated, such as the determination of PAT in fruits [12] and *Alternaria* toxins in tomato and citrus [13,14]. Furthermore, the same fruit matrix might be contaminated by more than one mycotoxin belonging to various groups or classes. It was observed that the co-occurrence of *Alternaria* toxins with other mycotoxins is likely to occur [9]. It was reported that OTA and ZEN were simultaneous detected in breakfast cereals at low concentrations [6]. In addition, the analytical procedure for detection of PAT is rather limited [12], especially for simultaneous detection with other mycotoxins. Owing to the different physical and chemical properties of these prevalence mycotoxins in fruits, the determination of trace amounts of them in samples represents an extremely challenging task.

For multiclass mycotoxin analysis, in recent years there is a growing tendency to develop rapid LC–mass spectrometry (MS) methods with minimum sample treatments. In many works the QuEChERS methodology (quick, easy, cheap, effective, rugged and safe) has also been recently employed for the determination of multiclass mycotoxin analysis in different matrices [10,11,15,16], but its effectiveness for removing matrix interference is still under examination. Also, the QuEChERS method was less attractive because we have to weight all the different reagents for each sample. Molecular recognition based immunoaffinity column (IAC) are routinely and successfully used in many mycotoxins analyses [7,11]. However, the IAC is not generally appropriate for multi-mycotoxin analysis, due to the high degree of specificity of the antibodies comprising them. Solid phase extraction (SPE) cartridges are by far the most popular technique used for analysis of mycotoxins in food [13,14,17]. Nevertheless, the conventional SPE-based procedures take considerable time and extraction; it is not convenient enough in its application, especially when large amounts of samples need to be analyzed.

The aim of our study was the development of a sensitive and reliable confirmatory multiclass procedure, based on SPE cleanup followed by UPLC–MS/MS, for the simultaneous determination of prevalence mycotoxins in fruits. An important advantage of the proposed method is using an in-house made 'MCX + NH_2' SPE cartridge as filters. As results, the extraction procedure is simple and fast. Also, a significant amount of matrix effect was substantially reduced. The proposed SPE-UPLC–MS/MS methodology was validated for apple, sweet cherry, tomato and orange fruits according to EU guidelines [18,19].

2. Experimental

2.1. Reagents

Acetonitrile (MeCN) and formic acid of HPLC grade were purchased from Thermo Fisher Scientific (New Jersey, US), and ammonium acetate (NH_4AC, HPLC grade) was from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was obtained by a Milli-Q-System (Millipore, Bedford, MA, USA). Acetic acid, citric acid, magnesium sulfate ($MgSO_4$), sodium chloride (NaCl) and sodium citrate tribasic dehydrate (NaCA) were of analytical grade. Octadecylsilyl (C-18), primary secondary amine (PSA), aminopropyl (–NH_2), silica gel, mixed-mode cationic exchange (MCX), hydrophilic–lipophilic balanced (HLB) sorbents with 40–60 μm of particle size were obtained from Guangpuda Technologies (Beijing, China).

2.2. Standards

Certified standards of Alternaria toxins, namely alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN) and tenuazonic acid (TeA), were purchased from Sigma–Aldrich (St. Louis, MO, USA); ochratoxin A (OTA) and patulin (PAT) were purchased in solution from Romer Labs Inc. (Union, MO, USA). Citrinin (CIT) was acquired from Fermenteck Ltd. (Jerusalem, Israel).

Solid portions of each standard were weighed and dissolved directly in MeCN to prepare 100 $\mu g\,mL^{-1}$ stock solutions, which were stored in the dark at −20 °C. Different standard mix solutions of mycotoxins were prepared from the stock standard solutions at 10 $\mu g\,mL^{-1}$ in MeCN and stored in the dark at −20 °C for one month. The spiking solution (1 $\mu g\,mL^{-1}$) was freshly prepared from the mix solutions, and the working solutions with concentrations of 1–200 $ng\,mL^{-1}$ were freshly prepared just before use with the blank matrix.

2.3. Samples

Apple, orange, sweet cherry and tomato fruits were purchased from local markets and all samples were checked to be free of targeted mycotoxins. Approximately 1 kg of apple, orange, sweet cherry and tomato samples were cut into small portions with a knife and comminuted at room temperature to a mash with a blender. All samples were stored in a freezer at −20 °C until thawed for extraction.

2.4. Sample preparation and solid-phase extraction procedures

2.4.1. SPE preparation

SPE cartridges were prepared by placing the six different adsorbents (C18, PSA, –NH_2, silica gel, MCX, HLB) inside 6 mL polypropylene tubes between two polyethylene frits. 500 mg of C18, PSA, –NH_2 or silica gel was packed into the cartridge, respectively; and the two other sorbents (HLB or MCX) was 100 mg. For a combination of MCX and –NH_2 adsorbents, 100 mg of MCX was packed into an empty SPE tube, and 500 mg of –NH_2 was set at the top. The frits were placed at the two sides and between the different adsorbents, respectively. After that, the cartridges were treated with 5 mL of MeCN and 5 mL of Milli-Q water with a Supelco Visiprep vacuum manifold (Bellefonte, PA).

2.4.2. Extraction method

An amount of 5.0 g of fine homogenized fruits were placed into a 50 mL polypropylene centrifugation tube and dilute with Milli-Q water to 5 mL, and then 20 mL of MeCN containing 100 mM citric acid was added. The tube was mixed well and placed to a laboratory shaker at 150 rpm for 30 min. Subsequently, 2.0 g of NaCl was added and followed by centrifugation (Sigma, Steinheim, Germany) at 10,000 rpm for 5 min at 10 °C. A 4.0 mL aliquot of upper MeCN layer was passed through the homemade SPE cartridge and collected. During this process, large matrix interferences were retained on SPE column. Finally, the cleanup extract was evaporated to dryness

at 50 °C under a gentle nitrogen stream, and reconstituted with 1 mL of MeCN/water (3:7, v/v) containing 5 mM NH_4AC, and then the obtained solution was forced through a 0.22 μm PTFE membrane filter (Pall, MI, USA). A 3 μL of the final solution was analyzed by UPLC/ESI-MS/MS.

2.5. UPLC–MS/MS analysis

A Waters ACQUITY UPLC™ (Milford, MA, USA) system was interfaced to a triple quadrupole MS (TQ-S, Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray ionization (ESI) interface. The LC separation was performed on an ACQUITY CORTECS UPLC C18 column (1.6 μm particle size; 2.1 mm × 100 mm, Waters), maintaining the column temperature at 40 °C. As the mobile phases, 5 mM NH_4AC in Milli-Q water (A) and MeCN (B) were used. The separation was performed at a flow rate of 0.3 mL min^{-1}, with a gradient elution starting at 0.1% of phase B and held for 1 min, rising linearly to 100% phase B over 3 min and the column was washed for 0.5 min with 100% organic phase (B). Then, the mobile phase composition was return to the initial condition in 0.1 min and this composition was held for 1.4 min for reequilibration, resulting in a total run time of 6 min. The autosampler was maintained at 10 °C.

Based on the structural properties of analytes, both the positive and negative ionization modes were applied. The parameters were as follows: capillary voltage, +2.5 kV/−1.5 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L h^{-1}; and desolvation gas flow, 1000 L h^{-1}. Detection was carried out in multiple reactions monitoring (MRM) mode. All analyte dependent parameters are summarized in Table 1 of the supplementary materials. The MassLynx™ 4.1 software (Waters) was used for data acquisition and processing.

2.6. Validation study

The analytical method was in-house validated by spiking apple, sweet cherry, tomato and orange at three concentration levels of the target mycotoxins ($n = 6$). Method validation was performed according to performance criteria established in the Commission Regulation EC No 401/2006 [18] and the European SANCO guideline 12571/2013 [19] for determination of the following validation parameters: specificity, linearity, matrix effects, accuracy (recovery), precision (%RSD), as well as limits of quantifications (LOQ). Specificity was checked looking at any interference peak in the MS/MS transition at the retention time of each mycotoxin. No peak could be observed at a level higher that the LOQ. The LOQ was based on the signal to noise observed in a sample spiked at 1 ng mL^{-1} for most of mycotoxin or in a sample spiked at 5 ng mL^{-1} for PAT showing a lower response.

Seven calibration levels ranging from 1 to 200 ng mL^{-1} in solvent and in matrix have been prepared for the investigation of the linearity and the matrix effects. It was considered satisfactory when correlation coefficients were higher than 0.99 with residuals lower than 20%. Matrix effects were assessed for each analyte by comparing the slope of the standard calibration line ($a_{standard}$) with that of the matrix-matched calibration line (a_{matrix}), for the same concentration levels, according to the formula $[1 - (a_{matrix}/a_{standard})] \times 100$.

Recoveries and precision were determined intra-day by analyzing spiked blank samples in six replicates. Each sample was spiked 10–12 h before the extraction and left at 4 °C. Recovery studies have been performed at three levels: at the LOQ, at 2 times the LOQ and at 10 times the LOQ. Spiked samples were extracted and analyzed using the same UPLC–MS/MS conditions as described above. Analytical recovery was calculated by comparison with matrix-matched standard calibrations. The EC No 401/2006 criteria have been applied for the relative standard deviation (maximum 20%) and for the mean recoveries (in the 70–120% range).

2.7. Statistical analysis

To determine the optimization of extraction procedures for target mycotoxins, all data were analyzed by one-way analysis of variance (ANOVA) using SPSS 15.0 for Windows statistical software (SPSS Inc., Chicago, IL, USA). Statistical significance was determined at the level of $P \leq 0.05$.

3. Results and discussion

3.1. Optimization of MS/MS conditions

MS/MS parameters were optimized by chromatography using individual standard solutions of each mycotoxin at concentrations of 100 or 500 ng mL^{-1} depending on the sensitivity of the compound. Experiments were carried out in both positive and negative ionization modes. Full-scan acquisitions were performed to select the precursor ion and optimum cone voltage. Once the precursor ion was selected, daughter scan acquisitions were performed at different collision energies (CE) in order to select product ions and optimum CE. Product ions resulting from non-specific losses (such as H_2O or CO_2 losses) were avoided. To ensure high specificity and selectivity, for each analyte, two transitions were selected and inserted in the final MS method. All selected compounds presented higher sensitivity in positive ionization mode (ESI^+) with the exception of PAT and AME, which showed more satisfactory results in negative mode (ESI^-). Generally, it is recognized that operating in the ESI^- mode should be less prone to matrix interferences, yielding a lower background signal in sample matrix. AOH and TEN also produced a high intensity of $[M-H]^-$ peak with less interference and chemical noise in ESI^- mode. Also, some authors have reported negative ionization mode for them [20], and therefore in this work negative mode was selected for further experiment. The optimized parameters for every analyte (ionization mode, cone voltage, collision energy, precursor and product ions) were shown in Table 1.

Table 1
Optimized MRM parameters for mycotoxins analyzed.

Mycotoxins[a]	Ionization made	Retention time (min)	Precursor ion (m/z)	Quant ion (CE)	Qual ion (CE)	Cone voltage
AOH	ESI−	3.33	257.0	147.0 (28)	212.7 (32)	40
AME	ESI−	3.83	271.0	256.0 (35)	213.0 (20)	32
TeA	ESI+	2.41	198.2	125.0 (18)	139.0 (20)	22
ALT	ESI+	3.15	293.1	257.0 (14)	239.1 (20)	16
TEN	ESI−	3.40	413.2	141.0 (24)	271.1 (17)	30
PAT	ESI−	2.48	153.0	108.9 (7)	81.0 (11)	16
OTA	ESI+	3.08	404.1	239.1 (26)	358.2 (20)	24
CIT	ESI+	2.82	251.2	205.2 (24)	191.1 (26)	22

[a] AOH: alternariol; AME: alternariol monomethyl ether; TeA: tenuazonic acid; ALT: altenuene; TEN: tentoxin; PAT: patulin; OTA: ochratoxin A; CIT: citrinin.

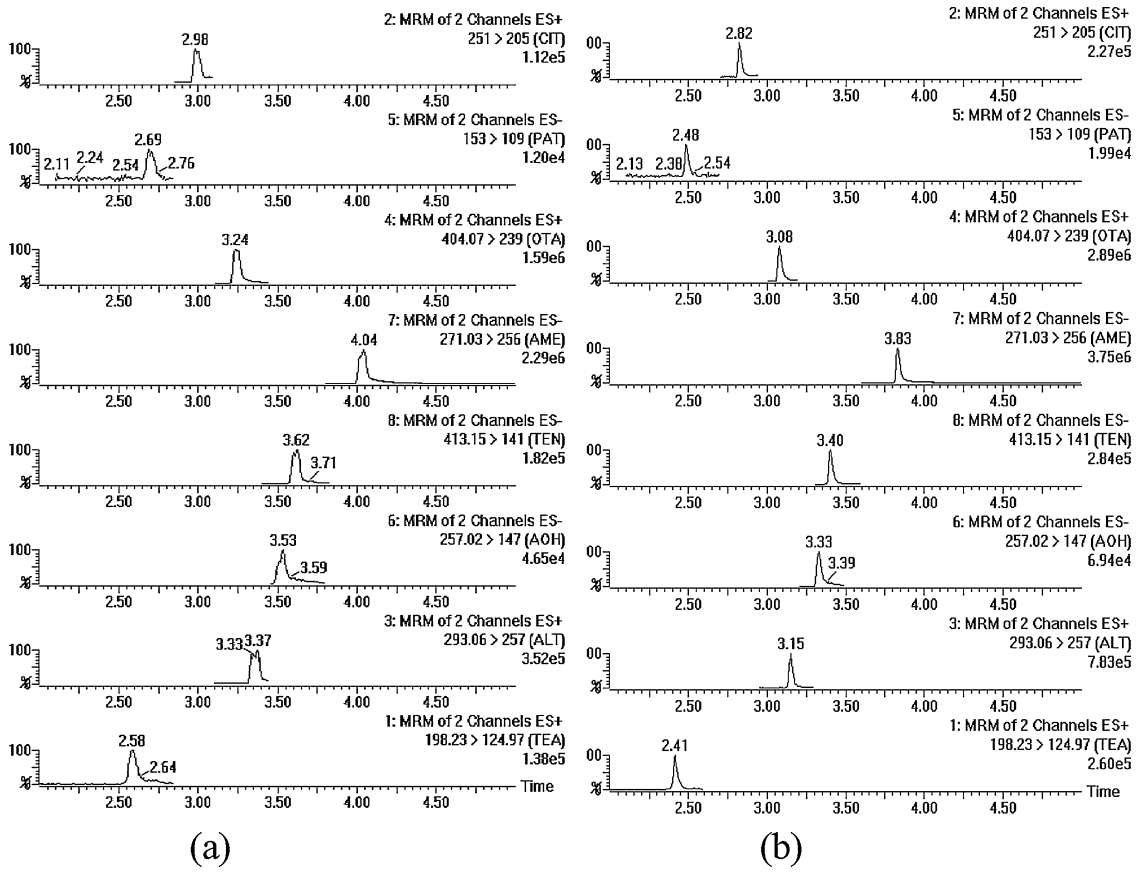

Fig. 1. UPLC–MS/MS chromatograms for mycotoxins at a level of 10 ng mL^{-1} validated in solvent using different UPLC C18 columns: (a) BEH. (b) CORTECS.

3.2. Optimization of the LC conditions

In order to optimize the chromatographic separation, different organic mobile phases (methanol and MeCN) with NH_4AC at various concentrations were tested. Narrower peak shapes were obtained when using MeCN as the eluent. This observation was in line with the previous studies on mycotoxins [14,20]. In addition, in the presence of a modifier, the best results were obtained with 5 mM NH_4AC, as ionization suppression occurred at higher concentrations of this salt (10 mM). Therefore, a gradient elution using a mobile phase containing both MeCN and 5 mM NH_4AC in water was used.

Reversed phase UPLC–MS/MS system employing a C18 separation column represents a 'gold standard' in multi-mycotoxin analysis [21]. Two different UPLC chromatographic columns, BEH C18 (1.7 μm, 2.1 mm × 100 mm) and CORTECS C18 (1.6 μm, 2.1 mm × 100 mm), were tested to obtain an efficient separation of the analytes. After testing the same gradients in both columns, the CORTECS C18 column proved to be suitable for the simultaneous detection of several mycotoxins with different polarity. As shown in Fig. 1, the sharper chromatographic peaks therefore higher sensitivity for all analytes was observed with the CORTECS column. In addition, the effect of the flow rate was tested in the range of 0.1–0.5 mL min^{-1}, and 0.3 mL min^{-1} was selected as working flow rate as separations could be achieved in a short analysis time, without a loss of sensitivity.

3.3. Optimization of the extraction procedures with clean-up step

3.3.1. clean-up procedures

Matrix effects result from co-eluting residual matrix components affecting (suppression or enhancement) the ionization efficiency of target analytes, and could lead to lower sensitivity for some mycotoxins and subsequently erroneous quantitative results [22]. Therefore, matrix effects have to be tested and evaluated during method development and validation. To minimize the matrix effects, a rapid and simple solid phase extraction (SPE) cleanup was used and developed in this work. The difference with conventional SPE procedures is that only a single step was needed for removing co-extractive compounds. The step is that a defined volume of sample extracts was passed through the column and collected. The SPE cartridge thus served as chemical filters, retaining the matrix co-extracting interferences while allowing the target mycotoxins to pass through, this purification is rapid and convenient.

Six homemade SPE columns with different adsorbents including octadecylsilyl (C18), primary secondary amine (PSA), aminopropyl (–NH_2), silica gel, mixed-mode cationic exchange (MCX) as well as hydrophilic–lipophilic balance (HLB) were tested for the cleanup efficiency of the target mycotoxins. Briefly, 5.0 g of fruits was diluted with water to 5 mL, and 20 mL of MeCN containing 100 mM citric acid was added. The mixture was shaken for 30 min and centrifuged. A 5 mL aliquot of the extract that passed through the homemade SPE cartridges collected, evaporated to dryness under a gentle nitrogen stream. Finally, the residue was reconstituted with 1 mL of MeCN/water (3:7, v/v) containing 5 mM NH_4AC, and then forced through a 0.22 μm PTFE membrane filter. A 3 μL of the final solution was analyzed by UPLC–MS/MS. The recovery was obtained by spiking, before and after the SPE step, analyte-free samples with 20 ng mL^{-1} solution. Sweet cherries were selected for investigations of matrix effects impact on the electrospray ionization of eight mycotoxins. The main reason is not only more complex matrix than apple and tomato, model selected fruits used in mycotoxins by previous studies [12,14,23], but also the cherries were easily infected

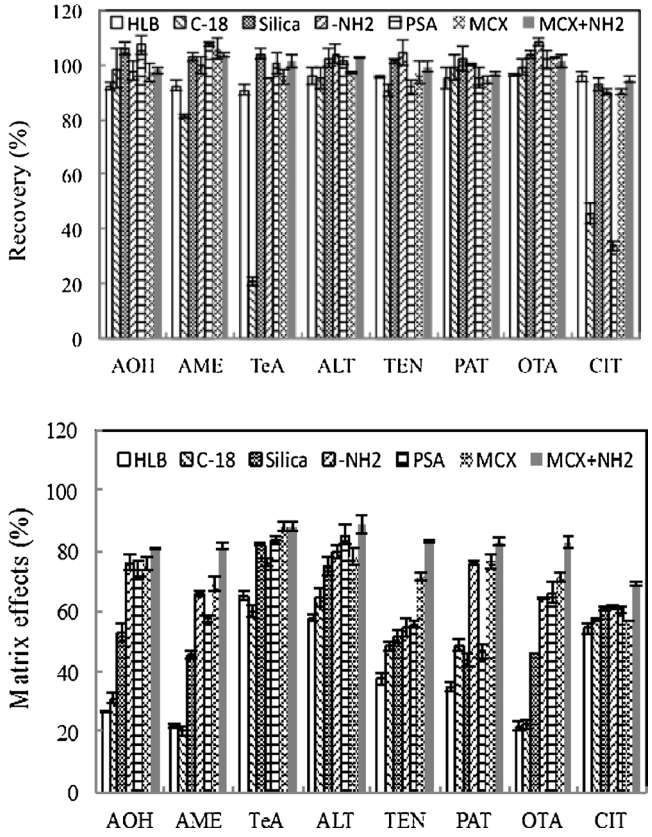

Fig. 2. Effects of SPE adsorbents on the recoveries (upper) and matrix effects (lower) of mycotoxins. Vertical bars indicate ± standard errors.

by *Penicillium* and *Alternaria* spp. [24], which are common causes of mycotoxin contamination. At this step matrix effects were calculated for each mycotoxin as matrix effects (%) = $(1 - A_m/A_s) \times 100$, where A_s is the peak area of the standards in solvent and A_m is the peak area of standards in matrix-matched solution at the same concentrations.

The relative cleanup achieved with the various SPE columns was evaluated in UPLC–MS/MS. As shown in Fig. 2, the MCX was the most effective in removing the matrix interferences detected by MS ($P < 0.05$), and the analyte signals were suppressed by the matrix ranging from 12.0% up to 43.2%. Furthermore, from the standpoint of visual appearance, the most pigments of the extracts that had been subjected to a MCX cleanup were removed. It may be due to its two different chromatographic separation mechanisms, reversed phase and ion exchange. MCX exhibits the advantage to be an adsorbent suitable for both polar and non-polar compounds, such as protein, peptides and inorganic salts [25]. The bonded normal phase SPE columns (–NH_2 and PSA) provided a better cleanup than the other columns (Fig. 2). As formerly reported [26], PSA and –NH_2 interact with chemicals by hydrogen bonding, and removed similar types of compounds, including fatty acid, other organic acid, and to some extent various sugars and pigments such as anthocyanidins. But unfortunately using PSA led to an absorption effect for CIT due to the presence of the secondary as well as the primary amine, while using –NH_2 could achieve the acceptable recoveries for all analytes (Fig. 2). Silica also provided satisfactory recovery, but the matrix effects were significantly higher than those using MCX and –NH_2, especially for PAT and AME ($p < 0.05$). In contrast, although C18 and HLB are most commonly used for mycotoxins purification [13,14,17], the cleanup efficiency for these adsorbents was significantly lower than other adsorbents, except for CIT ($p < 0.05$). As shown in Fig. 2, five out of 8 mycotoxins using C18 and HLB were suppressed by 62.5% or above, and AME signal was suppressed the most by matrix, with an ion suppression percentage of 79.7% and 77.9% using C18 and HLB, respectively (Fig. 2). Moreover, TeA and CIT still did not have acceptable recoveries (20.5–45.8%) when C18 adsorbent was selected for purification in our study (Fig. 2).

Based on these results, it can be said that MCX or –NH_2 adsorbents was suitable for cleaning-up the extract before LC–MS/MS determination of mycotoxins. Further experiments were carried out to find the cleanup efficiency and efficacy by combination of different adsorbents, since previous studies had shown the combination of two or three adsorbents would provide the additional cleanup [26,27]. This view was further supported by the present study. The combination of –NH_2 and MCX is an excellent cleanup for removal of a variety of matrix interferences. Seven out of 8 mycotoxins were slightly suppressed by 19.2% or lower and only CIT was suppressed by 31.2% (Fig. 2). However, the matrix effect for CIT by a combination of –NH_2 and MCX was improved by 10.8% and 17.4% compared with –NH_2 column and MCX column, respectively. In addition, there was no significant difference of mycotoxins recoveries between combination and single adsorbents ($p < 0.05$; Fig. 2). After these results, the combination of MCX and NH_2 column was employed for subsequent experiments due to the better selectivity and lower matrix effects for fruits. To best of our knowledge, this was the first time to use SPE purification in a simple and timesaving way, and the results of recoveries and matrix effects for eight mycotoxins provide a good basis to investigate a suitable quantitative method for mycotoxins.

3.3.2. Optimization of extraction procedures

During method development, choosing MeCN as the extraction solvent was based on its frequency of use by other authors for mycotoxins [1,10,20,21]. And also, previous study was shown some mycotoxins cannot be extracted or have a low recovery when using methanol as extraction solvent [1]. The extraction efficiency was carried out by spiking the target analytes in blank cherries at 20 ng mL^{-1} before the extraction. Our result had shown that 80% acidified aqueous MeCN was effective extraction solvent for the target mycotoxins. This observation was in good agreement with previous studies on cereals and food matrices [17,20,21]. As shown in Fig. 3, all targets extracted by 80% MeCN were achieved satisfactory recoveries (84.5–97.8%) except for CIT, which showed an acceptable recovery of 74.5%. Furthermore, the higher organic solvent did not significantly improved recoveries for all analytes ($p < 0.05$), but reduced MeCN concentration (70%) led to a low recovery for most mycotoxins (data not shown). The reason for these results may be associated to SPE procedures employed in our study, resulting in that MeCN played roles as both extraction and elution solvents, and finally the elution was insufficient by a low organic solvent.

In general, addition of electrolytes to the sample solution can increase their distribution into the organic phase and improve the recoveries according to the salting-out effect [26,28]. Here, the effect of salts on extraction efficiency was studied by adding 2 g of NaCl, $MgSO_4$ or sodium citrate (NaCA) to acidified MeCN before centrifugation. Afterwards, a 4 mL aliquot of the upper MeCN layer was passed through homemade 'MCX + NH_2' SPE column and followed by evaporation-reconstitution steps as described above. By comparison, the addition of NaCl to the solution gave the best improvement in extraction efficiency ($p < 0.05$), especially for CIT and TeA (Fig. 3). For example, the recovery of CIT after adding NaCl was 19.5% and 23.5% higher than those without salt out, calculated from the matrix-matched standards and solvent-based standards, respectively (Fig. 3). Moreover, the column cleanup after using NaCl was transparent and almost colorless from the standpoint of visual appearance (Fig. S1 in Supplementary data). Although

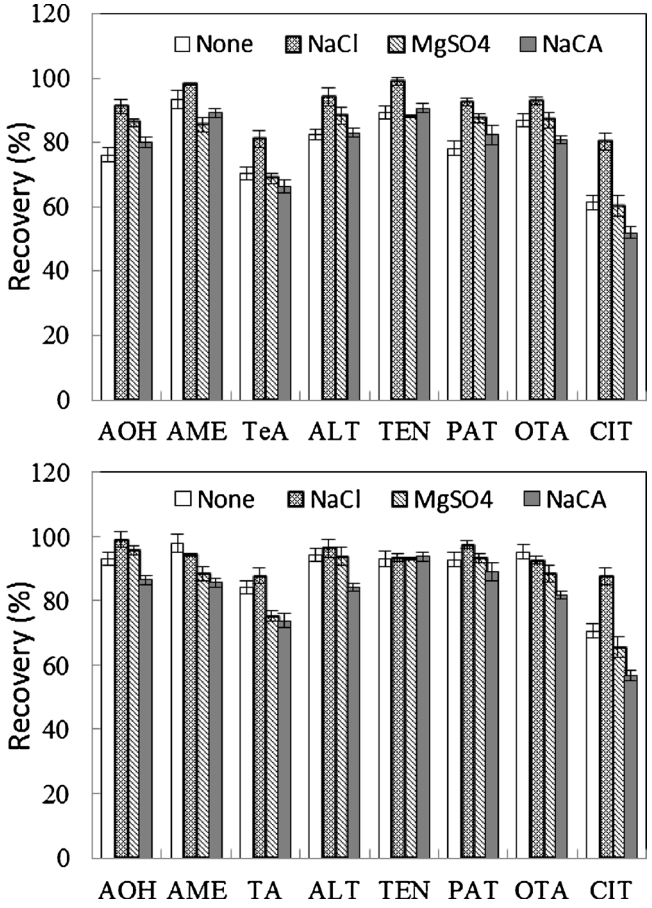

Fig. 3. Recovery of mycotoxins in sweet cherries, calculated from the solvent-based standards (upper) and matrix-matched standards (lower), with and without salt-out step. Vertical bars indicate ± standard errors.

Anastassiades and Lehotay [26] suggested that $MgSO_4$ was the best choice as the salting out reagent, due to the strong chelate effect of TeA and CIT on Mg^{2+} [29,30], the use of $MgSO_4$ to improve recoveries of target mycotoxin was not suitable. The addition of NaCA also reduced the recoveries of CIT and TeA (Fig. 3). A possible explanation might be associated to pH value of extracts. As previously reported, adding citrate buffer elevated the pH of rice sample and as a consequence dramatic increase in the amount of co-extracts in the raw extracts. These co-extracts might strongly bind with the analyte molecules resulting in decreased recoveries [31].

To our knowledge, formic acid and acetic acid were usually applied to improve the extraction efficiency for mycotoxins. However, in this work citric acid was selected due to its strong chelate effect with heavy metal, and thus it plays a protective role on mycotoxin. Comparing with formic acid and acetic acid, citric acid to extraction mixture led to slightly higher recoveries for some mycotoxins, especially for PAT (Fig. 4). Generally, the residues were dissolved using a mobile phase with an initial ratio of the gradient elution profile to reduce the solvent effects, particularly for those polar components. A high content of water in the dissolving solution significantly improved the peak shape. The organic solvent was evaluated for dissolving the sample residues, and a minimum of 30% MeCN was necessary to recovery all target analytes in our study. Furthermore, the effect of four of membrane filters (PTFE, PVDF, nylon and PES) on mycotoxins was studied by using solvent-based standards. Based on recoveries, PTFE membrane filter was the best choice for target mycotoxins in our study (Fig. S2 in Supplementary data).

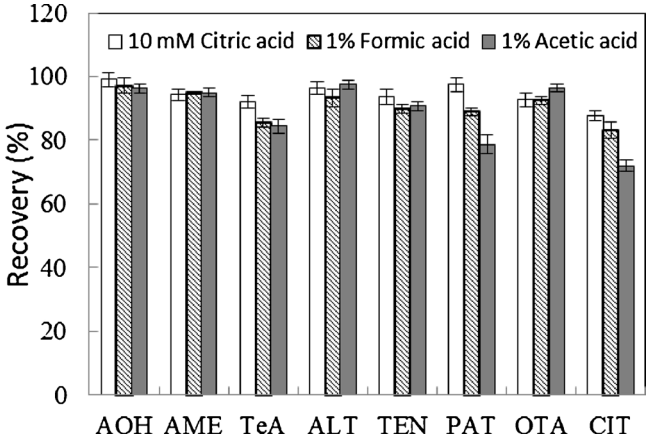

Fig. 4. Effect of extraction mixture on recoveries of mycotoxins in sweet cherries. Vertical bars indicate ± standard errors.

To sum up, the optimized extraction procedure using home-made SPE cartridge was developed (see Section 2.4.2). This procedure gave low time consuming, as well as, it was the most efficient and effective extraction procedure.

3.4. Method validation

Method validation was performed in terms of linearity, accuracy, precision and limit of quantifications (LOQ) for all analyzed mycotoxins in four selected model fruits, including apple, sweet cherries, tomato and orange. These fruits have been added to the list of products exposed to mycotoxin contamination according to previous reviews [2,3].

Matrix-matched standard calibration showed good linearity in the studied range (1–200 ng mL^{-1} for Alternaria toxin, OTA and CIT; and 5–200 ng mL^{-1} for PAT), with correlation coefficients ≥0.9921 in all the studied fruit matrices (Table 2). Matrix effects are unavoidable and cannot be eliminated in the analysis [32]. As shown in Table 2, only slight ion suppression or enhancement (≤±17%) could be observed for target mycotoxins in all three fruit matrixes with the exception of orange, for which strong ion suppression was observed for AOH (25.3%), OTA (31.6%) and CIT (40.3%). These results indicated that using self-developed SPE column is effective to remove matrix interference for most fruits, but matrix-matched standards are still necessary for correct quantification.

Calculated LOQs below the first calibration point have been set to 1 ng mL^{-1}, and also gave signal/noise ratios of 10 or more. LOQ were obtained from spiked blank samples at three concentrations levels (1, 2 and 5 ng mL^{-1}) for each investigated matrix. All LOQs are reported in Table 2. Five mycotoxins are quantified at 1 ng mL^{-1}, PAT is quantified at 5 ng mL^{-1} because of low signal responses in all studied matrixes, and the different LOQs with fruits were observed for ALT and CIT due to high background noise and indirect matrix effects. Nonetheless, all of the LOQs meet the requirements of the EU regulations, suggesting that the method is sensitive enough for measuring trace amounts of mycotoxins in fruits.

The accuracy and precision of the method were evaluated by means of recovery experiments at the spiking levels, with six replicates at each level ($n = 6$). Table 3 showed the summarized data on recoveries and RSD for the studied mycotoxins, which were spiked at three different levels: LOQ, 2 times the LOQ and 10 times the LOQ. Spiked samples were subjected to extraction and home-made SPE cleanup before analysis using the UPLC–MS/MS method. Overall, good recoveries in the range of 74.2–102.4% with a RSD

Table 2
Validation parameters of the proposed UPLC–MS/MS method for selected mycotoxins in fruits.

Mycotoxins	Linear range (ng mL $^{-1}$)	Sweet Cherries			Apples			Tomato			Orange		
		r^2[a]	ME[b] (%)	LOQ (ng mL $^{-1}$)	r^2	ME (%)	LOQ (ng mL $^{-1}$)	r^2	ME (%)	LOQ (ng mL $^{-1}$)	r^2	ME (%)	LOQ (ng mL $^{-1}$)
AOH	1–200	0.9942	16.7	1	0.9972	13.8	1	0.9966	10.7	1	0.9936	25.3	1
AME	1–200	0.9978	15.2	1	0.9932	11.3	1	0.9948	3.3	1	0.9978	2.3	1
TeA	1–200	0.9926	−2.3	1	0.9948	8.6	1	0.9940	−8.3	1	0.9972	3.3	1
ALT	1–200	0.9986	16.2	1	0.9988	12.1	1	0.9942	−10.0	2	0.9974	16.7	1
TEN	1–200	0.9992	17.0	1	0.9928	9.6	1	0.9974	9.4	1	0.9980	14.6	1
PAT	5–200	0.9972	0.2	5	0.9924	2.5	5	0.9954	−0.7	5	0.9921	15.6	5
OTA	1–200	0.9974	6.1	1	0.9994	16.1	1	0.9982	10.6	1	0.9996	31.6	1
CIT	1–200	0.9974	7.9	1	0.9998	16.3	1	0.9968	8.4	1	0.9930	40.3	2

[a] Coefficient of determination of matrix-matched calibration regression line.
[b] Matrix effects (ME%): $[1 - (\text{slope}_{\text{matrix-matched calibration}}/\text{slope}_{\text{solvent calibration}})] \times 100$.

Table 3
Accuracy and precision of the UPLC–MS/MS method for determining mycotoxins in fruits spiked at three different concentration levels.

Mycotoxins	Spiked level	Sweet Cherries		Apples		Tomato		Orange	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
AOH	LOQ	90.2	2.9	86.5	2.8	89.6	3.8	88.1	1.8
	2 LOQ	95.1	3.6	95.8	3.7	91.8	3.8	91.7	2.7
	10 LOQ	94.1	3.5	93.5	1.4	95.0	4.4	93.8	1.3
AME	LOQ	95.8	2.6	93.1	3.3	92.0	3.0	94.8	2.6
	2 LOQ	97.1	4.5	97.1	2.4	100.5	2.5	98.4	4.5
	10 LOQ	102.3	1.3	98.9	1.4	98.7	1.7	97.3	1.5
TeA	LOQ	90.5	0.8	90.1	2.1	90.8	3.3	94.9	2.6
	2 LOQ	93.5	4.7	92.7	2.3	92.0	1.8	95.8	1.8
	10 LOQ	93.6	2.4	90.4	2.3	87.4	4.1	90.1	1.9
ALT	LOQ	93.5	2.5	95.3	4.6	95.4	2.8	96.3	2.0
	2 LOQ	95.8	3.6	94.2	3.6	95.6	3.0	97.1	2.6
	10 LOQ	98.4	2.1	97.4	3.1	96.8	3.4	95.9	2.3
TEN	LOQ	95.6	2.5	94.2	4.0	94.5	3.2	95.8	3.0
	2 LOQ	98.1	2.1	97.2	3.5	95.4	3.0	98.4	3.1
	10 LOQ	101.2	1.6	95.7	3.9	95.9	1.9	99.8	3.2
PAT	LOQ	91.4	2.0	91.0	2.2	87.9	2.4	90.7	2.1
	2 LOQ	98.8	3.3	95.7	2.6	93.7	3.1	92.8	1.9
	10 LOQ	97.3	1.2	97.0	1.4	96.7	2.0	96.9	1.6
OTA	LOQ	95.9	2.6	101.5	3.6	94.8	2.7	96.4	2.4
	2 LOQ	97.2	3.0	100.4	3.0	102.4	0.7	99.4	1.7
	10 LOQ	98.3	3.8	101.1	2.8	101.0	1.8	99.0	3.3
CIT	LOQ	77.4	2.0	76.0	1.5	79.6	2.4	74.2	2.1
	2 LOQ	81.2	3.2	80.8	3.8	82.6	2.1	82.3	2.8
	10 LOQ	83.6	2.2	81.8	1.7	81.8	2.8	84.7	1.9

For each concentration level, mean recovery and RSD were calculated on $n = 6$.

below 4.7% were achieved. To our knowledge, limited information is available on simultaneous detection and quantification of all major mycotoxins found in fruits. Van de Perre et al. [23] developed an UPLC–MS/MS method for the detection of mycotoxins in fruits. It was reported that the tomato components induce suppression of AME and OTA signal by 38% and 34%, respectively. The PAT contamination in fruits was extracted by the combination of QuEChERS and MycoSep multifunctional cleanup column [12]. Recoveries and matrix effects for fresh apples were 83% and 42%, respectively. *Alternaria* mycotoxins in tomatoes were extracted by using traditional SPE protocols [14]. Recoveries for TEN and AME were 56% and AOH signals were severely suppressed by 70%. According to our results, significantly reduced matrix effects and improved recoveries were obtained for the same matrix and mycotoxins. Therefore, the method presented herein is highly accurate and reliable.

4. Conclusions

Highly sensitive, rapid and reliable method has been developed for the simultaneous determination of 8 mycotoxins in fruits using UPLC–MS/MS. ESI positive mode was selected to analyze 4 mycotoxins including TeA, ALT, OTA and CIT, and ESI negative mode was selected to analyze the others. The optimization of the LC conditions was shown that the CORTECS C18 column gave a better performance for the target mycotoxins than BEH C18 column, so a further validation with the CORTECS C18 column was carried out.

In addition, an important advantage of the proposed method is a single-step cleanup using a homemade 'MCX + NH_2' SPE cartridge instead of expensive immunoaffinity columns and MycoSep multifunctional cleanup column. This one-step method can significantly shorten the sample preparation time with superior recoveries and minimum matrix effects over the conventional method. The

optimized extraction conditions including acidified aqueous acetonitrile and an additional salt-out step using NaCl were employed before SPE cleanup. The method has been validated in apple, sweet cherry, tomato and orange fruits with excellent accuracy and precision for all mycotoxins studied at three spiking level. Finally, the proposed method was proven to be a simple and accurate method for determining the mycotoxins in a variety of fruit matrices, and may also be applied to the determination of all the selected mycotoxins found in cereal and food system.

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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.chroma.2015.12.004.

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