

A fully automated method for simultaneous determination of aflatoxins and ochratoxin A in dried fruits by pressurized liquid extraction and online solid-phase extraction cleanup coupled to ultra-high-pressure liquid chromatography–tandem mass spectrometry

Luca Campone · Anna Lisa Piccinelli · Rita Celano ·
Mariateresa Russo · Alberto Valdés · Clara Ibáñez ·
Luca Rastrelli

Received: 28 October 2014 / Revised: 18 January 2015 / Accepted: 26 January 2015 / Published online: 19 February 2015
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Abstract

According to current demands and future perspectives in food safety, this study reports a fast and fully automated analytical method for the simultaneous analysis of the mycotoxins with high toxicity and wide spread, aflatoxins (AFs) and ochratoxin A (OTA) in dried fruits, a high-risk foodstuff. The method is based on pressurized liquid extraction (PLE), with aqueous methanol (30 %) at 110 °C, of the slurried dried fruit and online solid-phase extraction (online SPE) cleanup of the PLE extracts with a C18 cartridge. The purified sample was directly analysed by ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) for sensitive and selective determination of AFs and OTA. The proposed analytical procedure was validated for different dried fruits (vine fruit, fig and apricot), providing method detection and quantification limits much lower than the AFs and OTA

maximum levels imposed by EU regulation in dried fruit for direct human consumption. Also, recoveries (83–103 %) and repeatability (RSD < 8, $n=3$) meet the performance criteria required by EU regulation for the determination of the levels of mycotoxins in foodstuffs. The main advantage of the proposed method is full automation of the whole analytical procedure that reduces the time and cost of the analysis, sample manipulation and solvent consumption, enabling high-throughput analysis and highly accurate and precise results.

Keywords Aflatoxins · Ochratoxin A · PLE · Online SPE · UHPLC–MS/MS · Dried fruit

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8518-4) contains supplementary material, which is available to authorized users.

L. Campone · A. L. Piccinelli (✉) · R. Celano · L. Rastrelli
Dipartimento di Farmacia, Università di Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, SA, Italy
e-mail: apiccine@unisa.it

M. Russo
Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, loc. Feo di Vito, 89122 Reggio Calabria, RC, Italy

A. Valdés · C. Ibáñez
Laboratory of Foodomics, Institute of Food Science Research (CIAL), CSIC, Nicolas Cabrera 9, Cantoblanco Campus, 28049 Madrid, Spain

Introduction

Mycotoxins are toxic substances produced by secondary metabolism of several fungal species (*Aspergillus*, *Penicillium* and *Fusarium*), which grow on a large number of commodities, in the field and during storage, under a wide range of climatic conditions [1–3]. It was demonstrated that these food contaminants generate, in different ways, a frightening toxicity for animals and humans with genotoxicity, carcinogenic, immunotoxicity, mutagenicity, nephrotoxicity and teratogenicity characteristics [1–3]. However, only in a few cases is there a marked causal–effect relationship among the presence of mycotoxin and a pathological event: endemic diseases correlated to acute mycotoxin intoxication are kwashiorkor, Reye's syndrome and Balkan endemic nephropathy, caused

by aflatoxins and ochratoxin A, respectively [1–3]. The immunosuppressant effect is probably the most insidious, as it induces other diseases or consequences hardly evaluable. In fact, many observed symptoms in animals are secondary events arising from opportunistic diseases resulting from exposure to immunosuppressant mycotoxins [4]. Hence, mycotoxins are presently considered as the most important chronic dietary risk factor, higher than synthetic contaminants, food additives or pesticide residues [5].

Among the known mycotoxins, aflatoxins (AFs) and ochratoxin A (OTA) are of greatest concern due to their severe effects on animal and human health and their frequent occurrence in foods. AFB1, the main AF detected, is the most potent hepatocarcinogen known [6], and the aflatoxins are classified by the International Agency of Research on Cancer (IARC) as human carcinogen (group 1) [7], whereas OTA is considered carcinogenic to animals and possibly to humans (group 2B IARC) [8]. AFs have a high presence in tropical and subtropical regions where climatic conditions are optimal for toxin production, whereas the invasion of OTA-producing fungal species has been reported worldwide. AFs and OTA naturally occur in a wide range of important agricultural commodities including cereals, tree nuts, dried fruit, wine, coffee and spices.

Dried fruit is one of the food matrices more prone to mycotoxin (mainly AFs and OTA) contamination [9]. The Rapid Alert System for Food and Feed (RASFF) reported a significant increase of notifications on the presence of mycotoxins in dried fruit in the last decade—4 % of mycotoxin notifications for the product category “fruit and vegetables” in 2003 compared to 30 % in 2012. These notifications are mainly related to the presence of AFs in dried figs and OTA in dried vine fruit (<https://webgate.ec.europa.eu/rasff-window/portal/>), but these mycotoxins have been also reported in dried apricots, plums and dates [9]. On the basis of these occurrence data, it is clear that dried fruit represents a high risk of contaminated matrix and an increased frequency of controls related to AF and OTA contamination in dried fruit is strongly recommended.

National and international institutions and organizations recognized the potential health risks to animals and humans posed by mycotoxins and addressed this problem by adopting regulatory limits for major mycotoxin classes. The European Commission set maximum levels (MLs) for some mycotoxins in certain foodstuff [10, 11] and laid down the methods of sampling and analysis for the official control [12]. For enforcement purposes, it is essential to have available accurate, sensitive and selective analytical methods for the identification and quantification of mycotoxin at part-per-billion and part-per-trillion levels in a large variety of food matrices [13, 14].

The determination of trace contaminants in complex matrices, as foods, requires a complex protocol of sample treatment. Conventional methods are usually multi-step processes

based on exhaustive extraction of analytes from the matrices and the subsequent removal of co-extracted components by different cleanup techniques prior to instrumental analysis [15]. These procedures are expensive, in terms of time and material consumption, and the sample throughput is too low to meet the current concerns of food safety and the public health protection. Sample preparation is also the weakness of the analytical methods as regards the uncertainty and accuracy of analytical data. This even becomes more critical at the low concentrations required for ultra-trace-level analysis, such as mycotoxins [15]. Therefore, it is necessary to minimize the number of steps to reduce both the time of analysis and the error sources so the development of automated preparative techniques could reduce the time of analysis and would make more reproducible and accurate the analyses [16]. Although significant efforts have been made to enable a fast and reliable analysis of a large number of food samples for surveillance and monitoring studies of mycotoxins, growing concern over food safety requires accurate analytical methods and fast and automated procedures to take into account the constant increase in the number of samples to be tested. Thus, there is the need of new faster methodology, suitable for automated sample preparation and instrumental analysis. Among the extraction techniques, pressurized liquid extraction (PLE) is considered the most suitable for automation and/or online coupling with the purification and detection systems. PLE provides significant advantages over competing techniques as regards time saving, solvent use, automation and efficiency [16–18] and has been rapidly accepted for routine analysis of environmental and food contaminants. PLE has been also applied to the analysis of several mycotoxins [19–22], demonstrating excellent analytical performance. Regarding purification processes, online solid-phase extraction (online SPE) technology coupled to liquid chromatography allows to automate and make faster the cleanup and chromatographic steps, and it is particularly suitable when large numbers of samples have to be analysed routinely. As compared to offline SPE, it offers a series of advantages affecting mainly the precision and sensitivity of the analytical method [23–25]. Online SPE has found increasing application in the analysis of environmental contaminants although its use in safety food has been still very limited.

The aim of this study was the development of a fully automated analytical procedure, based on PLE combined with online SPE, for the simultaneous determination of AFs and OTA in dried fruit. The experimental parameters affecting the extraction efficiency of PLE (temperature, solvent extraction, pH, number of cycles and flush volume) and the selectivity of online SPE (SPE sorbent, type and flow of wash solvent, injection volume) were studied in detail and optimized. Ultra-high-pressure liquid chromatography tandem coupled to tandem mass spectrometry (UHPLC–MS/MS) was chosen

as a selective and sensitive detection method. Moreover, matrix effects, which could affect the accuracy and precision of the method, were carefully evaluated in the optimization of sample preparation and analysis processes. Finally, the analytical performance of the whole analytical procedure was determined for different dried fruits (dried vine fruit, fig and apricot) and its trueness was evaluated on quality control materials.

Experimental

Standards and materials

Reference standard solutions in acetonitrile of aflatoxins (AFB1 and AFG1 $2 \mu\text{g mL}^{-1}$; AFB2, AFG2 and AFM1 $0.5 \mu\text{g mL}^{-1}$) and OTA ($10 \mu\text{g mL}^{-1}$) were obtained from LGC Promochem GmbH (Wesel, Germany), whereas OTA-d₅ reference standard solution ($100 \mu\text{g mL}^{-1}$, acetonitrile) was purchased from Fluka (Milwaukee, WI, USA). Mycotoxin standard solution mixture (AFs 400 ng mL^{-1} and OTA 2000 ng mL^{-1}) and internal standard (IS) mixture (AFM1 500 ng mL^{-1} and OTA-d₅ $2 \mu\text{g mL}^{-1}$) were prepared in acetonitrile and stored in amber glass vials at $-20 \text{ }^\circ\text{C}$. Mycotoxin standard solution mixture was used for the spiking procedure and for the preparation of working calibration solutions. IS mixture, added to PLE extracts, was used as volumetric internal standard and to compensate the matrix effects (AFM1 for all AFs and OTA-d₅ for OTA). Quality control materials (CQ material) of figs [T04235QC: AFB1 (assigned value $1.31 \mu\text{g kg}^{-1}$, ± 2 z-score 0.73–1.88), AFB2 (assigned value $0.86 \mu\text{g kg}^{-1}$, ± 2 z-score 0.48–1.24), AFG1 (assigned value $0.90 \mu\text{g kg}^{-1}$, ± 2 z-score 0.50–1.29) and AFG2 (assigned value $0.87 \mu\text{g kg}^{-1}$, ± 2 z-score 0.49–1.26)] and dried vine fruit (T17101QC: OTA assigned value $6.89 \mu\text{g kg}^{-1}$, ± 2 z-score 3.86–9.92) were obtained from the Food and Environment Research Agency (FAPAS, York, UK).

Analytical-grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Carlo Erba (Milan, Italy). Ultrapure water (18 M Ω) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). MS-grade MeCN and MeOH were supplied by Romil (Cambridge, UK), and MS-grade ammonium formate (NH₄CO₂H) and formic acid (HCOOH) were provided by Sigma-Aldrich (Milan, Italy). Diatomaceous earth (DE) from Dionex (Sunnyvale, CA, USA) was used as dispersing agent for PLE extraction. Three SPE cartridges were evaluated during the online SPE optimization: Oasis HLB (20 mm \times 2 mm, I.D., 30 μm particle size) from Waters Corporation (Milford, MA, USA), Strata-C18-E and C8 On-Line Extraction cartridges (20 mm \times 2.0 mm I.D., 20 μm particle size), both provided by Phenomenex (Bologna, Italy).

Safety considerations on mycotoxin handling

Mycotoxins are highly toxic compounds; their handling requires strict safety precautions in order to guarantee the better protection of research workers. To avoid any risks, all stock solutions were prepared under a fume hood with laminar air flow, and an absorbent paper was used to protect the work surfaces. All disposable materials that were in contact with the mycotoxins were treated with an aqueous solution of sodium hypochlorite (5 %) to degrade the analytes and minimize the health risks due to mycotoxin contamination.

Samples

Dried vine fruit, figs and apricot destined for human consumption were obtained from local supermarkets in the Campania region (Italy). Samples were finely blended and homogenized with water in ratio 2:1 (slurry) using a knife mill Grindomix GM 200 (Retsch, Haan, Germany) to achieve representative samples. Slurried samples were dispensed into a plastic vessel and stored at $-20 \text{ }^\circ\text{C}$ until the analysis. Samples used for optimization and validation experiments were earlier analysed by IAC followed by HPLC–FLD [26] to verify the absence of mycotoxins contamination. Spiked samples were prepared by adding specific volumes of mycotoxin standard solution mixture to 4 g of slurry. The mixture was stirred with a spatula for 1 min and then incubated at $30 \text{ }^\circ\text{C}$ for 2 h before analysis to ensure an intimate contact between mycotoxins and samples.

PLE procedure

Mycotoxin extraction was performed using an automated Dionex ASE 200 system equipped with 3 mL stainless steel cells both purchased from Dionex. For collection of the extracts, 60-mL glass vials with Teflon septa were used. Nitrogen was supplied to assist the pneumatic system and to purge extraction cells.

Under optimal conditions, the PLE procedure was performed on 4 g of dried fruit slurry mixed with 1 g of DE before the extraction. Slurried samples with dispersing agent were introduced into the extraction cell, and two circular PTFE frits (Sigma-Aldrich), 20 μm porosity, were placed at each end of stainless steel extraction cells. The PLE extraction conditions were extraction solvent, MeOH 30 %; temperature, $110 \text{ }^\circ\text{C}$; pressure, 1500 psi; static time, 5 min; flush volume, 50 %; purge, N₂ 50 s; and number of cycles, 3. Finally, the extracts were spiked with ISs before the online SPE cleanup.

Online SPE–UHPLC–MS/MS analysis

Online SPE and chromatographic analysis were carried out using an Ultimate 3000 UHPLC system (Thermo Scientific, Milan, Italy) which constituted a degasser, a dual pump, a

column compartment and an autosampler fitted with a 100- μL injection loop. A programmable Rheodyne[®] ten-port two-position valve was used to switch between the load and inject modes. The chromatographic system was interfaced to an Ultimate 3000 UV detector and a TSQ Quantum Ultra (Thermo Scientific) triple quadrupole mass spectrometer. The mobile phase of both pumps (left and right) consisted of H_2O (A) MeOH (B) and MeCN/ H_2O 99:1, v/v (C), all buffered with 2 mM $\text{NH}_4\text{CO}_2\text{H}$ and 0.1 % HCOOH. In the optimal conditions, a Strata C18-E online cartridge was used to remove the matrix and retain analytes from PLE extracts and fitted into a ten-port switching valve. UHPLC analyses were performed with a Kinetex PFP column (100 \times 2.1 mm I.D., 2.6 μm , Phenomenex) held at 30 $^\circ\text{C}$ for all chromatographic runs. The online SPE–UHPLC system setup consists of three steps (I–III): I (–2.5–0.0 min), SPE cartridge was conditioned with 2.5 mL of 15 % MeOH (2 mM $\text{NH}_4\text{CO}_2\text{H}$ 0.1 % HCOOH); II (0.0 min), 50 μL of PLE extract was injected into the cartridge followed by a washing step (0.0–5.0 min) consisting of 5 mL of 15 % MeOH (2 mM $\text{NH}_4\text{CO}_2\text{H}$ 0.1 % HCOOH); and III (5.0–13.5 min), the extracted analytes were transferred in the back-flush mode to the UHPLC column using the gradient responsible for the analytical separation. The detailed online SPE and UHPLC conditions, time valve and chromatographic gradient are reported in Table 1.

The triple quadrupole mass spectrometer was equipped with a heated electrospray ionization (H-ESI) source and operated in positive ionization mode. MS/MS transitions and operative parameters of the mass spectrometer were selected by infusing individual standard solutions (1 $\mu\text{g mL}^{-1}$, MeOH/ H_2O 1:1, 0.1 % HCOOH) at a flow rate of 5 $\mu\text{L min}^{-1}$. Ion source conditions were optimized by flow-injection analysis of a mixed solution of analytes at 10 ng mL^{-1} . The optimal conditions were spray voltage, 3500 V; capillary temperature, 300 $^\circ\text{C}$; vaporizer temperature,

80 $^\circ\text{C}$; tube lens 130; sheath and auxiliary gas pressure, 20 and 5 units, respectively; and collision gas pressure 1 bar. Nitrogen (99.9 % purity) was used as auxiliary and sheath gas in the H-ESI source and argon (99.9999 % purity) as collision gas in the collision cell. For identification, confirmation and quantification of the analytes, selected reaction monitoring (SRM) mode was applied using two characteristic SRM transitions for each analyte. The SRM values for all scan transitions were scan width (m/z), 0.200; scan time (ms), 20; and Q1 and Q3 resolution (FWHM), 0.7. Quantification of the target analytes was carried out using both SRM transitions. Identification was accomplished by comparing the retention time and SRM signals of the analytes in the matrix with those of standard solutions. Positive identification was achieved when retention time agreement was within 1 % and when the relative abundance of the two selected ion transitions was within a margin of ± 20 % of the expected ratio. Excalibur software version 2.2 was employed to collect and process the data. Final MS–MS conditions and precursor ion and product ions, selected for identification and quantification of each mycotoxin, are summarized in Table 2.

Experimental design

In order to obtain the best PLE parameters, a chemometrics approach was applied, using the Statgraphic Centurion XVI version 16.1 software (Statistical Graphics, Rockville, USA). Optimization of the PLE procedure was performed on 4 g of dried vine fruit slurry spiked at 4 $\mu\text{g kg}^{-1}$ with AFs and 20 $\mu\text{g kg}^{-1}$ OTA, and successively mixed with 1 g of DE before the extraction. Initially, a screening design was used to select the parameters statistically significant on PLE efficiency, and then a response surface was used to get the best extraction parameters.

In the screening study, a Plackett–Burman $2^6 \times 3/1$ fractional factorial design (eight degrees of freedom) was used to evaluate the influence of PLE parameters on the extraction efficiency of the target mycotoxins from dried fruit. The experimental factors tested in this design were temperature, pressure, number of cycles, %MeOH, static time and pH. The UHPLC–MS response (determined as normalized analyte/IS area ratio) was considered as the response variable. A total of 15 experiments (three centre points to consider the experimental errors) were conducted in a randomized order. Low and high levels of each factor, the experimental conditions of design and the values for the response variable are listed in Table S1 of the Electronic Supplementary Material (ESM).

The values of the most significant parameters identified by screening design were successively optimized by response surface design. A Box–Behnken design 2-factor interactions with six degrees of freedom, four centre points and 16 randomized experiments was applied. High and low levels of

Table 1 Timetable of online SPE and UHPLC system and switching valve position

| Time (min) | SPE (pump right) | | | UHPLC (pump right) | | | Valve position |
|------------|--|------------|------------|--|------------|------------|----------------|
| | Flow rate ($\mu\text{L}/\text{min}$) | Solv B (%) | Solv C (%) | Flow rate ($\mu\text{L}/\text{min}$) | Solv B (%) | Solv C (%) | |
| –2.5 | 1000 | 0 | 15 | 600 | 0 | 15 | Load |
| 0.0 | 1000 | 0 | 15 | 300 | 0 | 15 | |
| 5.0 | 1000 | 0 | 15 | 300 | 0 | 15 | Inject |
| 7.0 | 10 | 45 | 45 | 300 | 0 | 50 | |
| 11.0 | 10 | 45 | 45 | 300 | 15 | 60 | |
| 13.0 | 10 | 45 | 45 | 300 | 15 | 60 | |
| 13.5 | 10 | 45 | 45 | 300 | 80 | 15 | |
| 14.0 | 500 | 45 | 45 | 600 | 80 | 15 | Load |
| 19.0 | 500 | 45 | 45 | 600 | 80 | 15 | |

Table 2 UHPLC/ESI–MS/MS parameters for the analysis of AFs and OTA

| Mycotoxin | t_R (min) | Precursor ion | SRM transitions (m/z) | Collision energy (eV) | Tube lens (V) | $I_1/I_2 \pm \text{tol}^b$ |
|---------------------------------|-------------|--------------------|---------------------------|-----------------------|---------------|----------------------------|
| AFG2 | 11.15 | [M+H] ⁺ | 331.1→217/245 | 40/30 | 130 | 1.48±1.1 |
| AFG1 | 11.35 | [M+H] ⁺ | 329.1→199/243 | 40/35 | 130 | 1.49±1.2 |
| AFB2 | 11.54 | [M+H] ⁺ | 315.1→243/259 | 40/37 | 130 | 1.20±1.4 |
| AFB1 | 11.72 | [M+H] ⁺ | 313.1→241/285 | 42/30 | 130 | 1.28±1.2 |
| OTA | 13.21 | [M+H] ⁺ | 404.1→239/257 | 20/20 | 72 | 1.09±0.9 |
| AFM1 ^a | 10.11 | [M+H] ⁺ | 329.1→273 | 25 | 130 | – |
| OTA-d ₅ ^a | 13.21 | [M+H] ⁺ | 409.1→239 | 20 | 72 | – |

^a Internal standards^b Intensity ratio SRM1/SRM2±maximum tolerance; average ratio calculated over the calibration range

factors (temperature, number of cycles and %MeOH) were chosen according to preliminary experiments (see ESM Table S2). Extraction recovery (ER) and matrix effect (ME) of each analyte were considered as response variables, and they were determined processing spiked and unspiked samples by the experimental conditions (see ESM Table S2). The

values of pressure, static and purge time were fixed at 1500 psi, 5 min and 30 s, respectively. An aliquot of PLE extracts of unspiked samples was spiked with AFs and OTA at the concentration corresponding to the fortification level (post-extraction spiked sample). ER and ME were calculated according to the following equations:

$$\text{ER} = (\text{Response pre-extraction spiked sample} / \text{Response post-extraction spiked sample}) \times 100$$

$$\text{ME} = (\text{Response post-extraction spiked sample} / \text{Response standard solution}) \times 100$$

Assessment of matrix effect

For the evaluation of matrix effect, three independent PLE extracts of blank dried fruit matrices (dried vine fruits, figs and apricots) were spiked with AFs and OTA at the level of 0.4 and 2 ng mL⁻¹, respectively, corresponding to the EU MLs established for dried fruit [10] and ISs (AFM1 10 ng mL⁻¹ and OTA-d₅ 40 ng mL⁻¹). Analyte responses (peak area or analyte/IS area ratio) of spiked post-extraction extracts were compared with those of a mixture standard solution in 30 % MeOH at the same concentration. Matrix effects were calculated using the equation reported in the previous section.

Method performance

In the validation study, ISs were added (AFM1 10 ng mL⁻¹ and OTA-d₅ 40 ng mL⁻¹) to the final extracts and calibration levels as volumetric internal standard and to compensate the matrix effects of corresponding analytes.

Calibration solutions were prepared by diluting appropriate volumes of the mycotoxin standard solution mixture (AFs 400 ng mL⁻¹ and OTA 2000 ng mL⁻¹) with 30 % MeOH (solvent curve) or with the PLE extracts of blank dried vine fruit, fig and apricot samples (matrix-matched curves). Linearity of the solvent and matrix-matched curves was estimated

in the working range of 0.05–20 ng mL⁻¹, corresponding to 0.25–100 µg kg⁻¹, with six calibration levels, each injected in triplicate. Statistical analysis (ANOVA) was performed to check the goodness of fit and linearity.

Method detection limits (MDLs) and method quantification limits (MQLs) were established using analyte-free dried fruit samples fortified at low mycotoxin levels (0.05, 0.10, 0.25 and 0.50 µg kg⁻¹). Each level was processed in triplicate by the optimized analytical procedure, and the MDLs and MQLs were calculated by extrapolation of the concentrations giving a signal-to-noise ratio (S/N) of 3 and 10, respectively, from a linear regression (S/N versus concentration).

Recovery experiments were performed with three different dried fruit matrices (dried vine fruit, fig and apricot) each spiked at three levels: 0.2 (0.5 AFG2), 2 and 4 µg kg⁻¹ of AFs and 1, 10 and 20 µg kg⁻¹ of OTA (three experiments for each level). The mycotoxin levels were determined by matrix-matched curve of the corresponding matrices. The intra-day precision (expressed as relative standard deviation, RSD) was determined with the same experiments.

FAPAS QC materials (figs T04235QC and dried vine fruit T17101QC) were analysed ($n=3$) by the developed method to check its trueness. For these samples, AFs and OTA levels were established by the standard addition method. Briefly, aliquots (1.0 mL) of the PLE extract were added with a known amount of mycotoxin standard solution mixture to obtain three spiking levels.

Result and discussion

The analysis of low levels of toxic contaminants in complex matrices as foods requires a sample treatment before instrumental analysis, based on exhaustive extraction of analytes and a further purification and/or concentration procedure to achieve low levels of detection and high selectivity. Furthermore, more rapid and automated procedures were required to increase the number of analysed samples and reduce as much as possible the human contact with these highly toxic compounds during the analysis.

In this study, a fully automated method was developed for the determination of AFs and OTA in dried fruit, using PLE as a rapid and automatic extraction technique and online SPE coupled to UHPLC–MS/MS for the simultaneous purification of the PLE extracts and determination of the target analytes.

UHPLC–MS/MS analysis

LC–MS methods require an efficient separation and a sensitive detection of the analytes to minimize the matrix effects and improve the sensitivity of developed method. In order to obtain good peak resolution and the most intense ionization of analytes, several LC eluents (MeOH/H₂O, MeCN/H₂O and MeOH/MeCN/H₂O) and buffers (0, 1 and 2 mM of acetic acid, formic acid and ammonium acetate) were investigated. When buffers were added to the mobile phase, a reduction of the Na-adduct at advantage of the ionization of the analytes was observed. Moreover, adding formic acid at mobile phases which caused a splitting of the OTA peak, due to the acidic characters, was avoided. Regarding the selection of the organic phase, better sensitivity (around two times) and the complete resolution of AF peaks were obtained when methanol was used to elute AFs. On the other hand, a MeOH/MeCN mixture was preferred to elute OTA in a short time and achieve a good peak shape. Globally, the sternaly eluent system reported in the “[Experimental](#)” section provided a better response and chromatographic resolution and the selected gradient elution (Table 1) was able to purify and separate the mycotoxins in just 13 min.

For MS detection, positive ionization mode was selected for all analytes and selected reaction monitoring (SRM) was used to monitor the selected mycotoxins. According to the 2002/657/EC regulation [27], two characteristic MS/MS transitions for each analyte were selected to confirm their identity. The ratio between the signal intensities of two SRM transitions was used as additional identity confirmation. Optimal UHPL/ESI–MS/MS parameters selected for the identification and quantification of AFs and OTA are compiled in Table 2.

PLE optimization

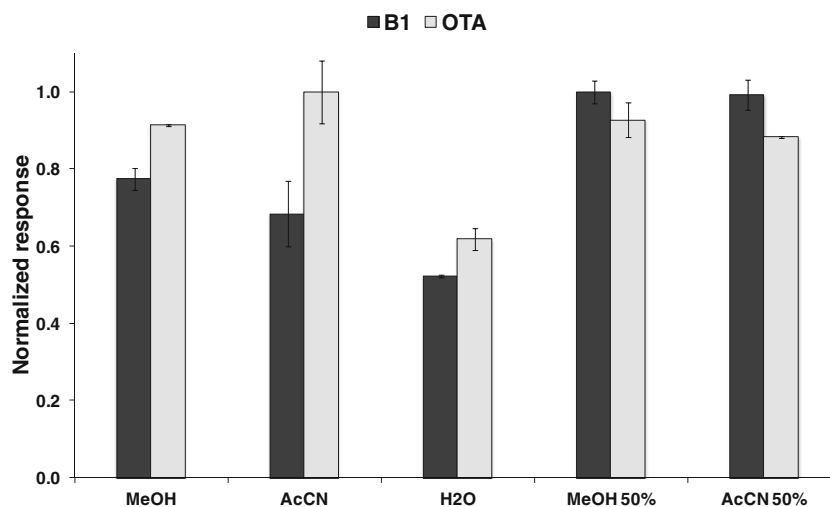
In this step, the slurry of vine dried fruit was selected as test material for higher complexity than dried fig and apricot. Experiments were carried out in triplicate using 4 g of slurried sample spiked with concentrations of AFs (4 µg kg⁻¹) and OTA (20 µg kg⁻¹) corresponding with twice the MLs fixed by EU for dried fruit [10]. Since during the study of the PLE process similar results were obtained for all AFs, only the data of AFB1 have been reported in order to simplify the discussion of results.

Optimization of the PLE process generally begins with the selection of an appropriate extraction solvent, which must be able to extract exhaustively the analytes from the matrix, reducing, as much as possible, the co-extraction of the matrix interferents [17, 18]. Usually, methanol or acetonitrile and their aqueous mixtures were used for the extraction of mycotoxins from foods [14]. Furthermore, PLE also allows the use of solvents that are not suitable for the conventional extraction methods using high temperature and pressure that increase the extraction efficiency [17, 18]. So, ACN, MeOH, H₂O, MeOH/H₂O 1:1 and ACN/H₂O 1:1 were preliminarily evaluated as PLE extraction solvents. Starting experimental conditions of PLE used to evaluate the extraction efficiency (EE) of these solvents were temperature, 150 °C; pressure, 1500 psi; static times, 5 min; flush volume, 10 %; purge, 50 s; and cycle number, 1. As shown in Fig. 1, all tested PLE solvents were able to extract AFB1 and OTA with higher EE when organic solvents and their aqueous mixtures were used. In particular, a significant improvement of EE was observed with organic solvents, indicating that their presence in solvent composition is necessary. However, an increase in colour and in cloudy suspension in PLE extracts, which indicate the presence of a high amount of matrix interference, was noticed varying the extraction solvent from H₂O to organic solvents. Quite dirty extracts were obtained when MeCN was used, whereas water and aqueous mixtures provided much cleaner extracts. Lastly, the selection of PLE solvent was based on the extraction efficiency, the extract cleanliness and the compatibility with the following online SPE process. According to these needs, the MeOH/H₂O mixture was selected as extraction solvent.

Once the extraction solvent composition was selected, a screening design was used as preliminary tool to determine the statistically significant experimental factors affecting PLE efficiency. In the screening study, the significance of six experimental factors (temperature, pressure, number of cycles, %MeOH, static time and pH) was simultaneously evaluated using a Plackett–Burman design. Low and high levels of each factor, the experimental conditions of design and the values for the response variable are listed in ESM Table S1. A low percentage of MeOH in aqueous mixture (range 0–30 %) was selected considering the extraction selectivity of water observed in the previous experiments. pH was evaluated as an

Fig. 1 Effect of the extraction solvents on PLE efficiency.

Experimental conditions: temperature, 150 °C; pressure, 1500 psi; cycle number, 1; heat and static times, 5 and 5 min, respectively; flush volume, 20 %; purge (N₂), 100 s (*n*=3)



experimental factor that could affect the extraction of OTA because it plays a crucial role in the water solubility of this ionisable analyte [28]. Figure 2 shows the standardized Pareto charts of the screening design, illustrating the importance and the statistical significance of the evaluated experimental factors. As can be seen, temperature, number of cycles and %MeOH are the only PLE experimental factors that significantly affect the extraction efficiency of AFB1 (Fig. 2a) and OTA (Fig. 2b). The effects of these factors were positive for both mycotoxins, meaning an increase on the analyte extraction at high values. The observed positive influence of %MeOH is in agreement with the results of the experiments performed to select the extraction solvent. Effects of the temperature and number of cycles agree with the literature data and PLE theory [17, 18].

Subsequently to the recognition of the significant PLE parameters affecting PLE efficiency (temperature, number of cycles and %MeOH), a response surface design was applied to optimize their experimental values in order to obtain the best extraction efficiency of AFs and OTA from dried fruits. The remaining factors (pressure, pH, static and purge time) were fixed at convenient values according to operating aspects

and instrumental limitation and in order to reduce the time analysis and the final volume of PLE extracts. Therefore, a middle value of pressure (1500 psi) was used to reduce possible instrumental problems and at the same time allow the penetration of solvent through the matrix. The static and purge times were fixed at the low values, 5 min and 30 s, respectively, and the extraction solvent pH was not adjusted in order to reduce time analysis.

The experimental factors temperature, number of cycles and %MeOH were optimized by a Box–Behnken design 2-factor interactions design. Table S2 of the ESM shows the field definition for these factors (chosen according to preliminary experiments), the experimental conditions of design and the response variables. Matrix effect (ME) was considered as response variable, in addition to the extraction recovery (ER), since the matrix effect primarily influences the accuracy and sensitivity of the quantitative LC–MS methods [29, 30]. Therefore, the design experiments were carried out also with unspiked samples to determine these response variables (ER and ME; see ESM Table S2). Statistical significance of the experimental factor contributions, and their first-order interactions, were determined by analysis of variance (ANOVA), and

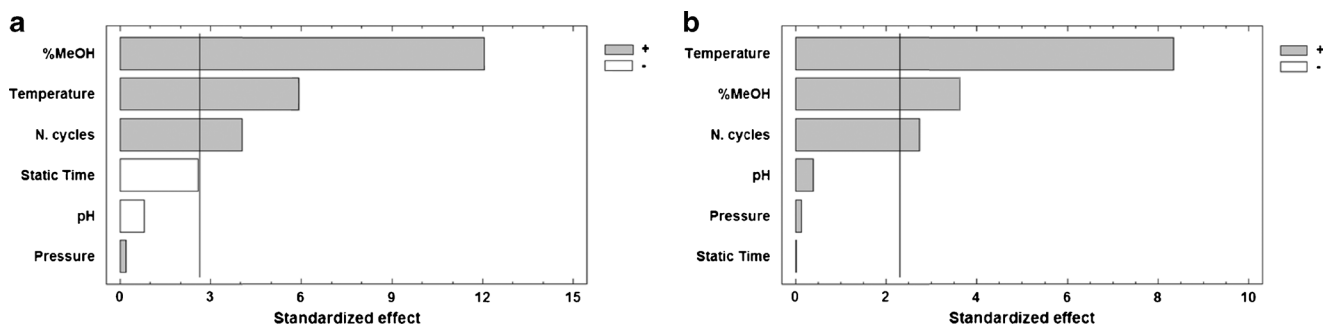


Fig. 2 Standardized effects Pareto charts of AFB1 (a) and OTA (b) for the screening design. The difference in the bar shadings indicate positive and negative effects of the experimental factors on the response variable,

and the vertical line corresponds to the statistically significant with 95 % confidence level

the estimated standardized effects for ER and ME are summarized in Fig. 3. Temperature and/or %MeOH showed statistical significance for ER and ME, while the standardized values of the number of cycles, and the first-order interactions, resulted below the statistically significant threshold. Specifically, AFB1 and OTA recoveries were statistically affected by %MeOH and temperature, respectively, whereas the temperature was the only factor with a statistical significance for ME of both analytes.

As can be observed in the response surface plots of Fig. 4, an increase of ER of AFB1 directly proportional to the composition of extraction solvent (%MeOH) was observed (Fig. 4a). ER of OTA instead improved, increasing the temperature of extraction (Fig. 4a). Regarding ME, the positive effects of temperature on ME of AFB1 and OTA (Fig. 4b) were clearly appreciated: these responses are linearly enhanced with the increase of the extraction temperature. This behaviour is related to the amount of interferents that increase raising temperature, affecting negatively the MS ionization of the analytes.

The optimal values of the experimental factors, extrapolated from chemometric analysis (temperature, 110 °C; %MeOH, 30; number of cycles, 3) showed desirability levels calculated on ER and ME of 75 and 98 %, respectively. These most favourable conditions were experimentally corroborated by recovery experiments, and the obtained results are illustrated in Fig. 5. Since the ER values of all analytes were still low, the influence of flush volume (initial condition, 10 %) was evaluated to improve extraction yield. An improvement of

the analyte recoveries was noticed when the flush volume was increased from 10 to 50 % (Fig. 5), and an exhaustive extraction of the analytes, with a small increase of PLE extract volume (9, 11 and 13 mL for 10, 30 and 50 %, respectively), was achieved using a flush volume of 50 %. Thus, the PLE conditions were set as follows: temperature, 110 °C; solvent, 30 % MeOH; number of cycles, 3; pressure, 1500 psi; static time, 5 min; flush volume, 50 %; and purge, 50 s.

Online SPE optimization

The first parameter to be investigated in the optimization of online SPE procedure was the type of sorbent. Its selection depends on the nature of the target analytes and the matrix. Initially, three disposable cartridges, Oasis HLB, Strata C8 and C18, were evaluated in terms of the extraction efficiency of analytes. In these experiments, a mycotoxin solution mixture (0.4 ng mL⁻¹ of AFs and 2 ng mL⁻¹ of OTA) in 30 % MeOH was used. The injection volume was preliminary set at 25 µL, and after loading, the cartridges were washed with 5 mL of 5 % MeOH before the elution of the analytes with the UHPLC gradient. Recoveries ($n=3$) of each SPE cartridge were calculated from the peak areas obtained in the online analysis as percentages of the peak areas obtained in the direct chromatographic injection. As shown in Fig. 6a, the Oasis HLB cartridge showed strong retention of AFs, resulting in a very broad peak, probably due to a too strong interaction of the analytes with the SPE stationary phase. Regarding the Strata C8 cartridge, poor extraction efficiency was observed

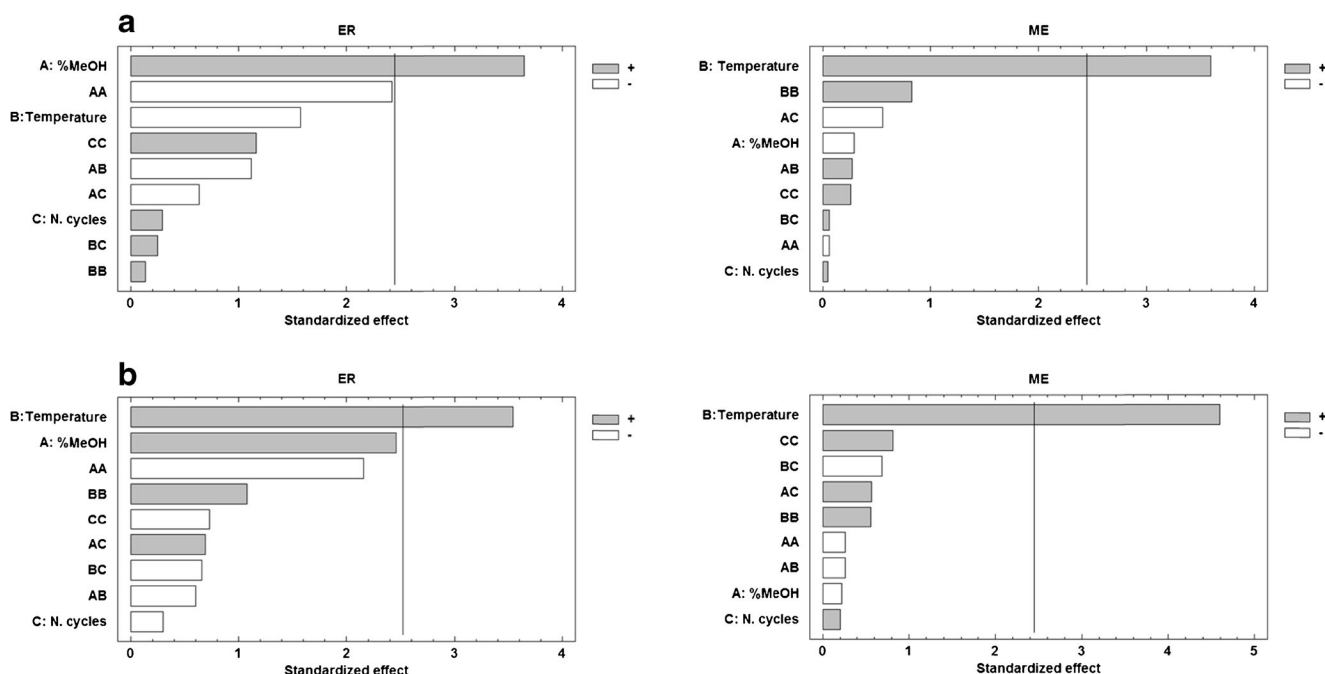


Fig. 3 Standardized effects Pareto charts of AFB1 (a) and OTA (b) for the response surface design. The difference in the bar shadings indicate positive and negative effects of the experimental factors on the response

variable, and the vertical line corresponds to the statistically significant with the 95 % confidence level

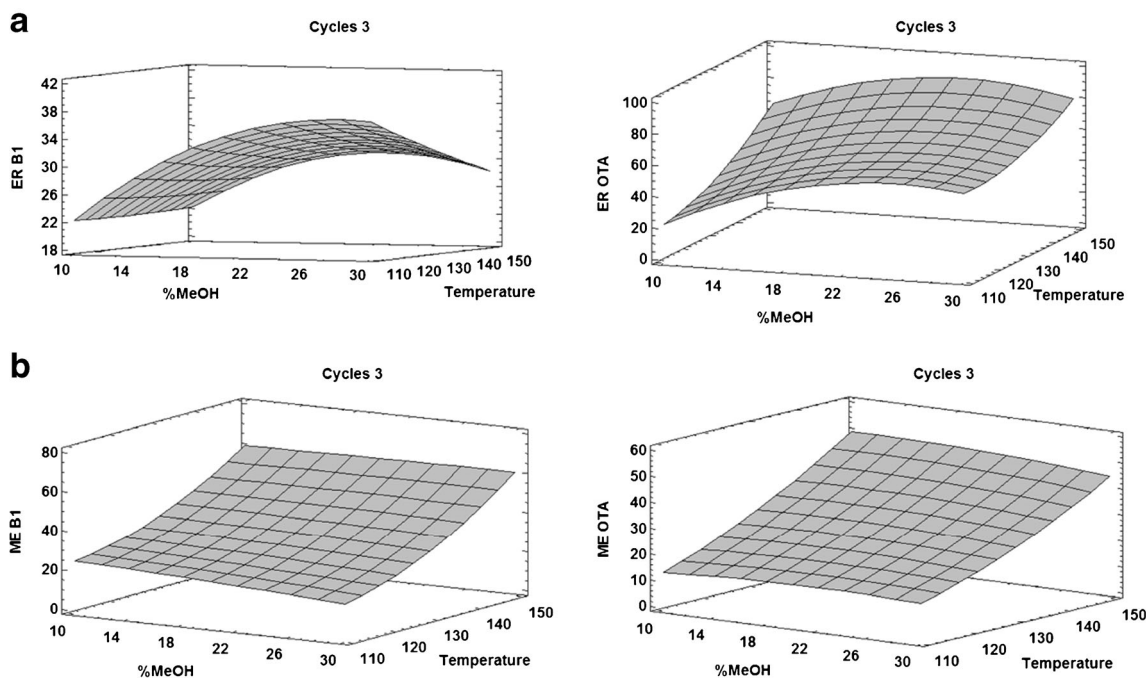


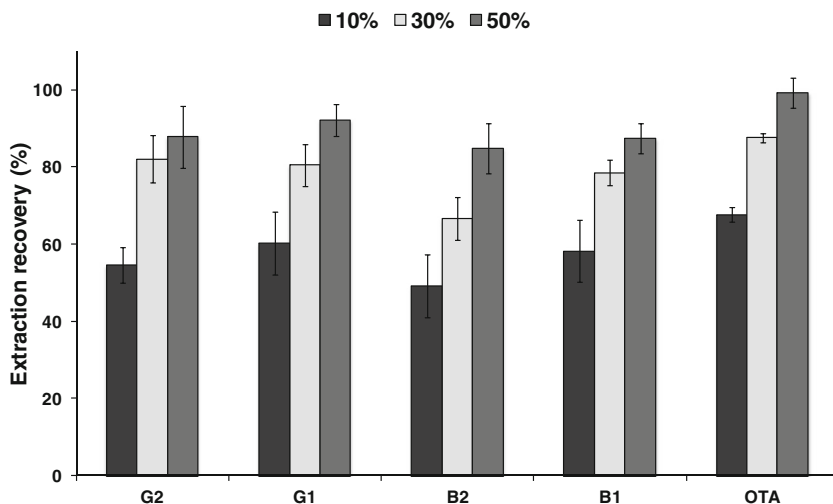
Fig. 4 Response surface plots of the two response variables, ER (a) and ME (b), depending on the temperature and the solvent composition (number of cycles=3)

following the loss of the analytes (10–33 %) in the loading and washing steps. Whereas, acceptable recovery (>97 %) and good peak shapes were obtained when the Strata C18 cartridge was used as SPE stationary phase (Fig. 6b). Thus, the Strata C18 cartridge was chosen as SPE stationary phase.

In any online SPE–LC–MS method, the removal of matrix interferences is very important, especially when complex food samples are analysed. In our preliminary experiments, an easy obstruction of the ion transfer capillary of mass spectrometry was observed, due to the sugar contents of the extracts, when a washing step was not performed. So, in order to safeguard the instrument lifetime and improve the sensitivity of the mass spectrometer, in the next optimization step, the wash

conditions were studied to remove/reduce the interfering compounds without any loss of the analytes. Therefore, the composition of wash solvent and its flow rate were investigated considering analyte recoveries and UV (220 nm) chromatographic profiles. The experiments were performed using a free-analyte vine dried fruit extract spiked with AFs and OTA, corresponding to the MLs established by EU for dried fruit [10] (2 µg kg⁻¹ of AFs and 10 µg kg⁻¹ of OTA), and different MeOH/water mixtures (0–50 % MeOH, 5 mL at flow rate of 0.5 mL min⁻¹) were tested. Results demonstrated that the polar compounds (mainly sugars) were totally eluted from the SPE cartridge in the washing step regardless of the composition of the wash solvent (see ESM Fig. S1). In the case of

Fig. 5 Effect of the flush volume on PLE efficiency. Experimental conditions: temperature, 110 °C; pressure, 1500 psi; solvent, 30 %MeOH; cycle number, 3; static times, 5 min; purge (N₂), 50 s (n=3)



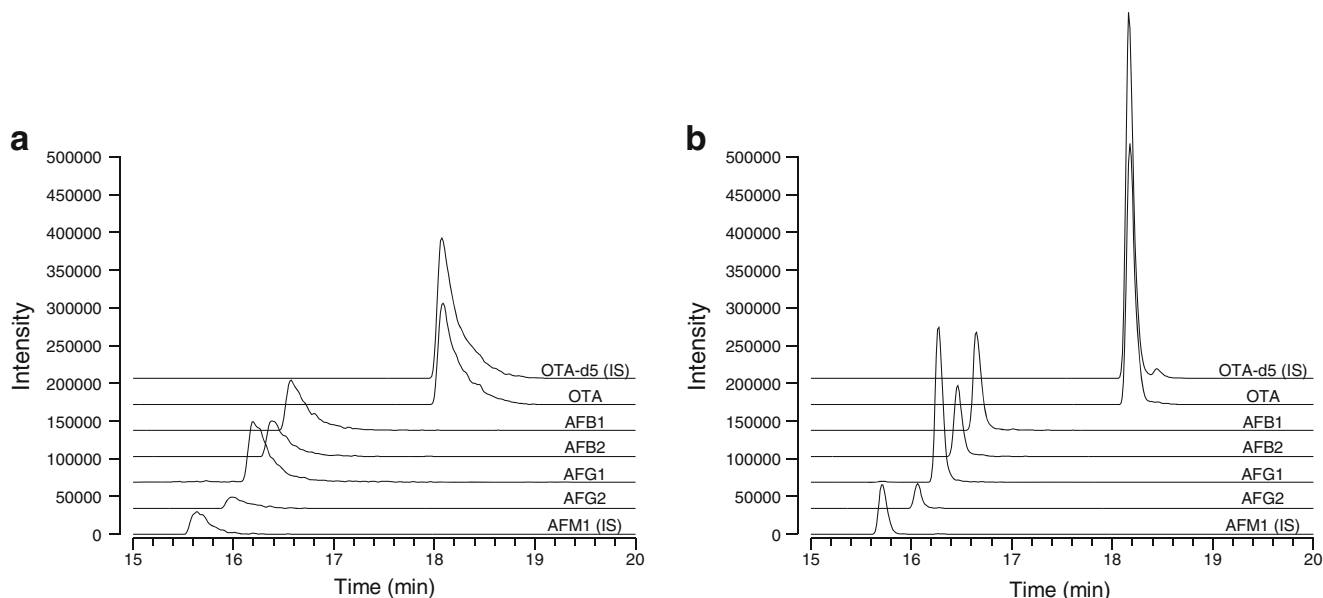


Fig. 6 Online SPE–UHPLC–MS/MS profiles using **a** OASIS HLB and **b** Strata C18 cartridges. Injection of 10 pg of each AF and 50 pg of OTA

the interfering compounds of medium polarity, the increase of the elutropic strength of the wash solvent provided a significant removal, as indicated by their UV signal (220 nm) (see ESM Fig. S1). However, over 15 % MeOH, AF recoveries started to decline, while OTA was retained on the cartridge without any losses up to 50 % MeOH. On the basis of these results, a wash step with 5 mL of 15 % MeOH was selected to reduce the amount of the matrix interferences with medium polarity, avoiding losses of AFs.

Regarding the flow rate of the wash solvent, no loss of analytes and excessive column backpressure was observed in the tested range (0.5–2 mL min⁻¹). This is also confirmed after 50 injections of the real sample at 1 mL min⁻¹ flow rate, which means that the matrix impurities are removed effectively by online SPE without clogging the cartridge. Based on these data, the flow rate of the washing step was set at 1 mL min⁻¹.

An advantage of the online SPE over the offline procedure is higher sensitivity due to the transfer and analysis of a greater amount of the extracted analytes to the column. In this study, to improve the sensitivity of the proposed procedure, the injection volume was investigated. A range of injection volume of 10–300 µL (PLE extract of vine dried fruit spiked at the level of 2 and 10 µg kg⁻¹ of AFs and OTA, respectively) was assessed to evaluate the impact of the applied sample extract volume on the absolute MS signal response of target analytes. A good linearity ($R^2 > 0.999$) of the signal response was obtained for AFB1 and AFG1 up to 100 µL, for AFG2 up to 75 µL and for AFB2 and OTA up to 50 µL (see ESM Fig. S2). Over these values, the MS response became not linear and a significant increase of the signal-to-noise ratio (*S/N*), especially for AFG2, was observed. Probably at higher injection

volumes, the co-eluting matrix constituents caused an important signal suppression that compromises the accurate determination of the analytes. Finally, an injection volume of 50 µL was chosen as a good compromise among sensitivity and accuracy.

Matrix effect

In the development of quantitative LC–MS methods, the evaluation of the possible occurrence of matrix effects (ME) is very important. Co-extracted and co-eluting matrix substances can seriously affect the analyte response by suppression or enhancement of the analyte signals. Some matrix components, sometimes at trace amounts, are inevitably present in analysed samples even after a cleanup procedure. ME primarily influences the accuracy and precision of the method [29, 30], and therefore, it should be assessed carefully for each type of matrix. In order to evaluate the degree of ion suppression or enhancement for each target mycotoxin and to establish the best quantification method, ME in different dried fruit matrices, dried vine fruits (DVF), dried figs (DF) and dried apricots (DA), was investigated by comparing the analyte responses of post-extraction spiked PLE extracts of free-analyte samples with those of a standard solution in 30 % MeOH. Figure 7 displays the ME of AFs and OTA for DVF, DF and DA. In the case of AFs, a strong suppression of signals was observed for all matrices (Fig. 7a). For the purpose of evaluating the eventual correction and/or minimization of ME through the use of IS, AFM1 and OTA-d₅ were tested as surrogate of AFs and OTA, respectively. In the case of OTA, ME was effectively corrected by the use of the correspondent labelled IS (Fig. 7b),

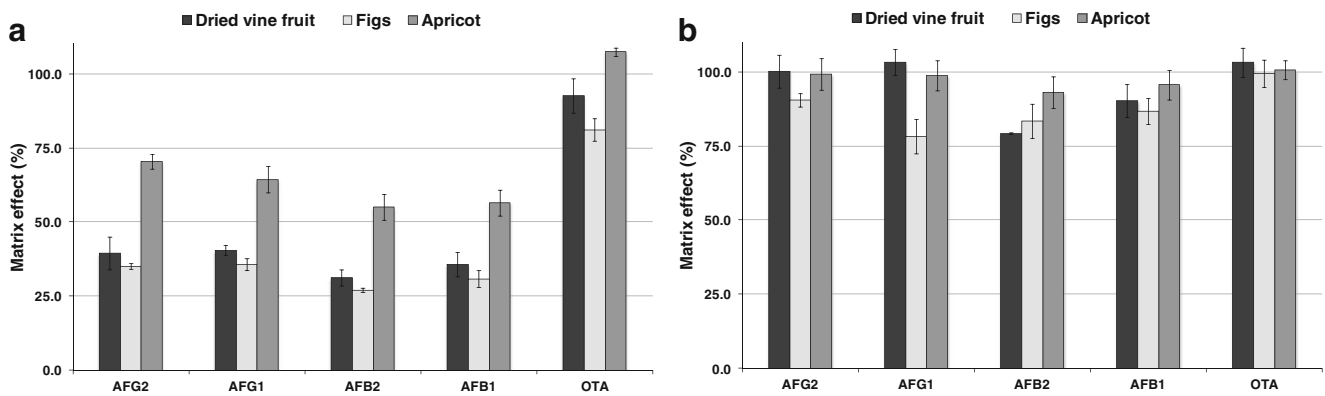


Fig. 7 Matrix effect (ME) for dried vine fruit, fig and apricot samples. **a** ME calculated by comparing the absolute analyte signals and **b** by comparing analyte/IS signal ratios. PLE extracts spiked at AFs and OTA

levels corresponding to the EU MLs established for dried fruit (0.4 and 2 ng mL⁻¹, respectively)

whereas AFM1 led to a significant reduction of the signal suppression of AFs (ME > 78 %).

Nevertheless, the evaluation of the relative ME (difference in response between various lots of the same matrix) [31] revealed a statistically significant RSD difference (data not shown) due to the type and concentration of matrix interferences that change between different lots of the same matrix, creating a negative influence on the precision of the developed method. Although matrix-matched calibration curves could compensate the observed MEs, in this case, the variability of the sample matrix makes this practice unfeasible. Therefore, the use of the standard addition method or

isotopically labelled compounds is nearly indispensable for the accurate and precise quantification of AFs.

Analytical performance

Analytical performance of the proposed method was evaluated according to the European Commission Decision 657/2002 [27] by the determination of the parameters selectivity, sensitivity, linearity, accuracy and precision. To evaluate the applicability of the method, the method validation was performed on three different dried fruit matrices (DVF, DF and DA).

Table 3 Analytical performance in various dried fruit matrices

| Matrix | AFG2 | AFG1 | AFB2 | AFB1 | OTA |
|------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| MDLs (µg kg ⁻¹) | | | | | |
| Dried vine fruits | 0.11 | 0.03 | 0.004 | 0.01 | 0.03 |
| Dried figs | 0.14 | 0.03 | 0.004 | 0.02 | 0.02 |
| Dried apricots | 0.06 | 0.02 | 0.002 | 0.004 | 0.01 |
| MQLs (µg kg ⁻¹) | | | | | |
| Dried vine fruits | 0.37 | 0.11 | 0.01 | 0.03 | 0.09 |
| Dried figs | 0.49 | 0.09 | 0.01 | 0.06 | 0.07 |
| Dried apricots | 0.19 | 0.06 | 0.01 | 0.01 | 0.05 |
| Recovery ± RSD (%) (n=3) | | | | | |
| Level (0.1 MLs) ^a | 0.5 µg kg ⁻¹ | 0.2 µg kg ⁻¹ | 0.2 µg kg ⁻¹ | 0.2 µg kg ⁻¹ | 1.0 µg kg ⁻¹ |
| Dried vine fruits | 87 ± 3 | 90 ± 4 | 93 ± 4 | 93 ± 2 | 95 ± 1 |
| Dried figs | 88 ± 2 | 103 ± 8 | 93 ± 6 | 92 ± 1 | 94 ± 1 |
| Dried apricots | 91 ± 4 | 90 ± 3 | 92 ± 2 | 93 ± 2 | 96 ± 3 |
| Level (MLs) | 2.0 µg kg ⁻¹ | 2.0 µg kg ⁻¹ | 2.0 µg kg ⁻¹ | 2.0 µg kg ⁻¹ | 10 µg kg ⁻¹ |
| Dried vine fruits | 87 ± 1 | 91 ± 5 | 87 ± 5 | 88 ± 4 | 92 ± 3 |
| Dried figs | 83 ± 4 | 101 ± 3 | 93 ± 4 | 90 ± 1 | 98 ± 3 |
| Dried apricots | 91 ± 7 | 85 ± 4 | 87 ± 6 | 84 ± 4 | 98 ± 3 |
| Level (2 MLs) | 4.0 µg kg ⁻¹ | 4.0 µg kg ⁻¹ | 4.0 µg kg ⁻¹ | 4.0 µg kg ⁻¹ | 20 µg kg ⁻¹ |
| Dried vine fruits | 88 ± 1 | 92 ± 1 | 85 ± 5 | 88 ± 2 | 97 ± 1 |
| Dried figs | 83 ± 1 | 99 ± 4 | 96 ± 3 | 94 ± 1 | 95 ± 3 |
| Dried apricots | 95 ± 3 | 84 ± 3 | 84 ± 2 | 86 ± 1 | 97 ± 2 |

^a 0.25 MLs for AFG2

Data acquisition by SRM mode with two characteristic transitions precursor/product ions makes the proposed method highly selective and meets EU guidelines [27] with four identification points for the confirmation of analytes with LC–MS/MS detection. Additionally, the SRM1/SRM2 intensity ratio was used as additional identification criterion with a tolerance of less than 20 % of the expected ratio. Selectivity was experimentally evaluated by analysing blank and spiked samples [10]. No interfering peaks in the chromatographic region of the mycotoxins were observed for all tested matrices.

The method sensitivity was experimentally estimated by the analysis of dried fruit samples spiked at low levels and the evaluation of the signal-to-noise ratio (S/N). The calculated method detection and quantification limits (MDLs and MQLs) are listed in Table 3. For all dried fruit matrices, MQLs were lower than the MLs imposed by current EU regulation for dried fruit intended for direct human consumption or use as an ingredient in foodstuffs ($2 \mu\text{g kg}^{-1}$ of AFB1, $4 \mu\text{g kg}^{-1}$ of total AFs and $10 \mu\text{g kg}^{-1}$ of OTA) [10]. MDLs and MQLs were in the range of 2–30 and 10–110 ng kg^{-1} , respectively. Only, in this case of AFG2, a high S/N observed around its retention time decreases the sensitivity of this mycotoxin (MDLs 60–110 ng kg^{-1} and MQLs 190–490 ng kg^{-1}).

The linearity range was estimated by solvent- and matrix-matched standard calibration curves. Except for AFG2, the MS/MS responses of the analytes were found to be linear in the concentration range of $0.05\text{--}20 \text{ ng mL}^{-1}$ (corresponding to $0.25\text{--}100 \mu\text{g kg}^{-1}$ of dried fruits) with a correlation coefficient greater than 0.99 for a linear model of the calibration curve (ANOVA test). Good linearity of AFG2 ($R^2 > 0.99$) was observed in the working range of $0.1\text{--}20 \text{ ng mL}^{-1}$ ($0.5\text{--}100 \mu\text{g kg}^{-1}$ of dried fruits).

Accuracy and precision of the whole analytical procedure were established processing the blank dried fruit samples (DVF, DF and DA), each spiked at three concentration levels (0.1 MLs, MLs and 2 MLs). The results of the recovery and intra-day precision (expressed as RSD) experiments ($n=3$ independent analysis) are reported in Table 3. The recovery values (within the range 70–110 % for concentration between 1.0 and $10 \mu\text{g kg}^{-1}$ and between 50 and 120 % for levels $< 1.0 \mu\text{g kg}^{-1}$) and RSD (< 10 %) were in agreement with EU regulation [11] regarding the performance criteria of the analysis methods for the official control of the levels of mycotoxins in foodstuffs.

Finally, the proposed procedure was applied for the analysis of AFs and OTA in naturally contaminated samples (figs and dried vine fruit QC materials, $n=3$) to check its trueness. Obtained mycotoxin levels (figs, 1.27 ± 0.06 , 0.78 ± 0.03 , 1.04 ± 0.03 and $0.92 \pm 0.05 \mu\text{g kg}^{-1}$ of AFB1, AFB2, AFG1 and AFG2, respectively, and dried vine fruit, $7.34 \pm 0.22 \mu\text{g kg}^{-1}$ of OTA) were in agreement with assigned values by a proficiency test (see “Experimental” section), confirming the

validity of the proposed method for the determination of AFs and OTA in dried fruits.

Conclusions

A new fully automated PLE SPE–LC–ESI–MS–MS method has been developed for the accurate analysis of AFs and OTA in dried fruits. With the whole analytical procedure (extraction, cleanup and chromatographic analysis), the presence of target analytes can be assessed in less than 40 min, achieving trace-level detection with good method precision and accuracy. The proposed method was validated using different dried fruit matrices (dried vine fruit, fig and apricot), and its analytical performance fulfils the criteria required for methods of analysis of mycotoxins in foodstuffs (EC Decision 657/2002 and Regulation 401/2006).

The main advantages of the proposed method are the quick and fully automated analytical procedure (from the extraction till determination), enabling high-throughput analysis. In addition, the method allows a reduction of sample manipulation, reducing the costs and potential risks of analysis. Thus, the presented method represents a valid alternative to the conventional IAC methods and it is suitable for screening and quality control programmes in food chain and occurrence studies.

Acknowledgments The project was funded by the Italian Ministry of the University and Research (MIUR) with a FIRB “Futuro in Ricerca” Project n. RBFR10GSJK “Tecniche Analitiche Avanzate per l’Analisi dei Contaminanti negli Alimenti”.

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