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# Comprehensive assessment of different extraction methodologies for optimization and validation of an analytical multi-method for determination of emerging and regulated mycotoxins in maize by UHPLC-MS/MS

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ABSTRACT

A multi-analyte method for identification and quantification of 23 regulated and emerging mycotoxins was developed in maize samples by ultra high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Several extraction methodologies were evaluated, including solid-liquid extraction (SLE), solid-phase extraction (SPE), and modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) protocols. The final method consisting of an extraction by QuEChERS using C18 sorbents, was in-house validated in terms of linearity, repeatability, reproducibility, recovery, Limits of Detection (LOD) and Limits of Quantification (LOQ). Acceptable performance characteristics were obtained according to specific and general European regulations for regulated and emerging mycotoxins, respectively. The developed method proved to be specific and selective for all mycotoxins, with LODs and LOQs lower than 21.10 and 37.49 ng $g^{-1}$, respectively; and recoveries ranging from 55.25 to 129.48% with precisions, expressed as relative standard deviations, below 15.03%. The method was successfully applied to real maize samples.

## 1. Introduction

Maize (*Zea mays* L.) is a worldwide cereal product used as stable food for human consumption and as animal feed in grain or silage form, presenting widely recognized health benefits due to its rich composition in essential nutrients, vitamins, and minerals (Leite et al., 2020). According to the latest report from the International Grains Council (2021), global maize production and consumption will climb 2.2 and 1.9%, respectively, in the next five years, with an overall increase for this consumption in all its applications, namely as food, feed, industrially and for ethanol production. Global feed use is forecast to increase 1.6% per year, which is equivalent to 59% of the total grain maize uptake.

The diverse routes of maize processing and usage leads to different end food and feed products, in which the quality and safety can be endangered by the presence of mycotoxins. These natural low-molecular weight toxins produced by filamentous fungi, mainly from the genera *Alternaria, Aspergillus, Fusarium* or *Penicillium*, can contaminate feed and food chains at different stages due to their ability to be carried-over through the different levels, reaching end consumer products (Escrivá et al., 2017; Giorni et al., 2019). More than 400 mycotoxins have been identified until date, with aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), fumonisins (FBs), and trichothecenes (TCTs), namely deoxynivalenol (DON), T-2 and HT-2 toxins representing the main fungi toxins in cereal crops (Lago et al., 2021).

*Abbreviations:* ACN, acetonitrile; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; AFM1, aflatoxin M1; AFs, aflatoxins; BEA, beauvericin; CIT, citrinin; DHCS, disodium hydrogen citrate sesquihydrate; DON, deoxynivalenol; EC, European commission; EU, European union; EFSA, European food safety authority; ENNA, enniatin A; ENNB, enniatin B; ENNs, enniatins; $ESI^+$, electrospray in positive mode; $ESI^-$, electrospray in negative mode; FB1, fumonisin B1; FB2, fumonisin B2; FBs, total fumonisins (B1 e B2); LC, liquid chromatography; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; $MgSO_4$, magnesium sulfate; MON, moniliformin; MPA, mycophenolic acid; $Na_2SO_4$, sodium sulfate; NaCl, sodium chloride; NIV, nivalenol; OTA, ochratoxin A; PA, penicillic acid; PAT, patulin; QuEChERS, quick, Easy, cheap, effective, rugged and Safe; SLE, solid-liquid extraction; SPE, solid phase extraction; TCTs, trichothecenes; TEA, tenuazonic acid; TLC, thin layer chromatography; TSCD, tri-sodium citrate dihydrate; TTX, tentoxin; UHPLC-MS/MS, ultra-high performance liquid chromatography coupled to tandem mass spectrometry; ZEA, zearalenone.

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Negative effects to human and animal health, such as hepatotoxicity, genotoxicity, immunosuppression, nephrotoxicity, teratogenicity, and/or carcinogenicity due to mycotoxin exposure led to the establishment of regulatory threshold values by the European Commission, including in maize (Agriopoulou et al., 2020). Nonetheless, EU maximum guidance levels have only been defined for a reduce set of mycotoxins, with recent reports showing the importance of non-regulated and emerging mycotoxins due to high occurrence patterns and concentrations levels in maize grains for food and feed (Abdallah et al., 2017; Dorn et al., 2011; Ekwomadu et al., 2020; Goertz et al., 2010; Jajic et al., 2019; Kovalsky et al., 2016; Oliveira et al., 2017). Amongst them, beauvericin (BEA), enniatins (ENNs), moniliformin (MON), nivalenol (NIV), citrinin (CIT), and *Alternaria* toxins, such as tenuazonic acid (TEA), and tentoxin (TTX), can be accounted for its exposure risk (EFSA, 2011, 2012, 2013, 2014, 2018).

Development of improved methods for multi-mycotoxin analysis, since sample preparation and extraction to detection parameters, has been an increasing research field due to co-occurrence processes and newly rising mycotoxins, while responding to the wide range of physicochemical properties and low residue levels found in different matrices. Sample preparation strategies for mycotoxin analysis include Solid-Liquid Extraction (SLE), Solid Phase Extraction (SPE), multifunctional and immunoaffinity columns, and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technology (Leite et al., 2020). Parameters such as time, solvent consumption, simplicity, selectivity, and sensitivity are crucial when considering an appropriate extraction/clean-up strategy. Conventional SPE is a rapid and economical procedure for mycotoxin extraction when comparing to multifunctional and immunoaffinity columns, though SPE columns can have limiting selectivity and, recovery rates may differ depending on the complexity of the matrices. On the contrary, multifunctional and immunoaffinity columns are highly specific techniques due to their selective sorbent composition, allowing to obtain cleaner extracts and lower detection limits, which, nonetheless, becomes a disadvantage for multi-analyte purposes, since this specificity lowers the range of analytes to be targeted. Expensive cost, cross-reactivity and low tolerance to organic solvents are also some of the disadvantages of these type of procedures. On the other hand, QuEChERS has been increasingly used in mycotoxin analysis since it provides simultaneous extraction and clean-up of samples, while allowing the analysis of several groups of compounds in a single extraction and in different matrices (González-Jartín et al., 2019). Mycotoxin detection is based on liquid chromatography (LC) for quantification, and thin layer chromatography (TLC) or immunoassays for qualitative or semi-quantitative determination. However, the golden method for the multi-compound analysis has been liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) due to its ability to detect and quantify several compounds simultaneously at very low concentrations in complex matrices.

It is important to have effective and reliable analytical methods for the determination of mycotoxins at the legislated levels in representative samples not only to perform accurate risk assessments, but also to enforce the regulatory limits established by EC. Emerging mycotoxins should also be included in this process since their toxicity and co-occurrence effects have already been acknowledged. Therefore, the aim of this study was to develop and optimize a sensitive, precise, effective, and robust multi-mycotoxin method by ultra high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the analysis of 23 regulated, non-regulated, and emerging mycotoxins in maize grain matrices. In this matter, a comprehensive comparison of the efficiency of several extraction procedures and parameters were evaluated to achieve this goal, namely the main extraction procedures used in analytical chemistry: solid-liquid extraction, solid-phase extraction and, more recently, QuEChERS protocol. To ensure the adequate analysis of these mycotoxins in maize grain samples, a validation process was ultimately performed for the most efficient extraction procedure. Finally, application of the method was assessed through the analysis of eight maize grain samples.

## 2. Materials and methods

### 2.1. Materials and reagents

All reagents used were of analytical grade, except the solvents used as mobile phase which were of high-performance liquid chromatography (HPLC) grade. Analytical standards of AFB1 (from *Aspergillus flavus*, ≥ 98% purity), AFB2 (≥ 98% purity), AFG1 (from *Aspergillus flavus*, ≥ 98% purity), AFG2 (≥ 98% purity), AFM1 (from *Aspergillus flavus*, ≥ 98% purity), BEA (≥ 97% purity), CIT (from *Penicillium citrinum*, ≥98% purity), DON (≥ 98% purity), ENNA (from *Gnomonia errabunda*, ≥ 95% purity), ENNB (from *Gnomonia errabunda*, ≥ 95% purity), FB1 (from *Fusarium moniliforme*, ≥ 98% purity), FB2 (from *Fusarium moniliforme*, ≥ 96% purity), HT-2 toxin (≥ 98% purity), Moniliformin sodium salt (from *Fusarium proliferatum*, ≥ 98% purity), OTA (from *Petromyces albertensis*, ≥ 98% purity), PAT (≥ 98% purity), T-2 toxin (from *Fusarium* sp., ≥ 98% purity), TEA (≥ 98% purity), TTX (from *Alternaria tenuis*, ≥ 95% purity) and ZEA (≥ 99% purity) were purchased from Sigma-Aldrich (Steinheim, Germany). MPA (≥ 98.5% purity) and NIV (≥ 98% purity) were obtained from Supelco (Pennsylvania, USA); and PA from Santa Cruz Biotechnology (Texas, USA). Ultrapure water was obtained from Millipore System (France). Primary-Secondary Amine (PSA) and SPE C18 sorbent were purchased from Agilent Technologies (California, USA). Anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl) and methanol (MeOH) were supplied by Honeywell (Seelze, Germany). Acetonitrile (ACN) was purchased from Carlo Erba (Val de Reuil Cedex, France), formic acid from Chem-Lab (Zedelgem, Belgium), acetic acid from Panreac (Barcelona, Spain), and acetone from Merck (Darmstadt, Germany).

Oasis® HLB 6cc (500 mg), Oasis® PRiME HLB 6 cc (200 mg), Sep-Pak Vac C18 6 cc (xx) and 3 cc (xx) polymeric sorbent cartridges were purchased from Waters (Milford, MA, USA); and Bond Elut C18 3 cc (XX) from Agilent (Santa Clara, CA, USA). ACQUITY UPLC® HSS T3 1.8 $\mu$m (2.1×100 mm i.d.) and ACQUITY UPLC® BEH C18 1.7 $\mu$m (2.1×100 mm i.d.) separation columns were purchased from Waters (Milford, MA, USA); ZORBAX Eclipse Plus C18 1.8 $\mu$m (2.1×50 mm i.d.) and ZORBAX Eclipse Plus C18 5.0 $\mu$m (2.1×150 mm i.d.) from Agilent Technologies (California, USA); Gemini NX C18 110 Å 3.0 $\mu$m (100×2.0 mm i.d.) and Kinetex Biphenyl 100 Å 1.7 $\mu$m (2.1×50 mm i.d.) from Phenomenex (California, USA). HPLC vials and Syringeless Device Mini UniPrep filters (0.45 µm PVDF, polypropylene) were obtained from Whatman (Maidstone, England).

### 2.2. Instrumentation

For identification and confirmation of mycotoxins in maize samples, a liquid chromatographic system coupled to a tandem mass detector (UHPLC-MS/MS) was used. In the chromatographic system, a Gemini NX C18 110 Å 3.0 $\mu$m (100×2.0 mm i.d.) separation column was used for analyte separation. The UHPLC Nexera X2 Shimadzu system (AB Sciex, Foster City, USA) consisted of binary pumps, a variable-volume autosampler with refrigeration system for the samples and a thermostatic column compartment. Mycotoxins were identified and quantified in maize samples using a QTRAP 5500+ detector (AB Sciex, Foster City, USA) coupled to the prior chromatographic system. The QTRAP-MS was operated using an electrospray interface (Turbo Ion Spray) in positive and negative ion mode in a single run (ESI+/ESI-). Data acquisition was controlled by the Analyst® software (AB Sciex, Foster City, USA). Multiple Reaction Monitoring (MRM) was used to measure the target compounds. MRM parameters for each compound and ion transitions were optimized, as summarized in Supplementary Material. Chromatographic conditions were: sample injection volume, 20 $\mu$L; flow rate, 0.2 mL $min^{-1}$; column temperature, 30ºC; autosampler

temperature, 10ºC; mobile-phase solvents, (A) 0.1% formic acid and (B) acetonitrile; gradient elution protocol, 95% A to 30% A (15 min.), 30% A to 0% A (5 min, 2-min. hold), 0% A to 95% A (3 min.); total run time, 25 min. Identification and data processing were performed through MultiQuant™ software (AB Sciex, Foster City, USA).

### 2.3. Maize samples

Harvested maize grain samples were obtained from Portuguese farmers and homogenised by grinding in the laboratory. Prior to the optimization procedures, samples were tested for blank matrices to be used as Quality Control (QC) samples and for spiking purposes.

For method application, real samples ($n$ = 8) were collected from several agricultural maize producers in the Centre and North region of Portugal. Sampling methods were compliant with Commission Regulation (EC) nº 401/2006 (European Commission, 2006). Ten kilograms of aggregate sample were completely and finely grinded with sieves of 1 mm size on a Retsch mill (Düsseldorf, Germany). Laboratory samples ($n$ = 50g) were taken from the grinded aggregate sample, in triplicate, and stored at -20±2ºC until further analysis.

### 2.4. Preparation of calibration standards and fortified samples

Stock solutions were prepared in ACN 100% (v/v), with the exception of AFB1, AFG2, and OTA prepared in MeOH 100% (v/v) and, FB1 and FB2 in ACN:$H_2O$ (50:50, v/v). Stock solutions were prepared in a concentration of 1 mg/mL, except for T-2 toxin (2.5 mg/mL). Working solutions were prepared by successive dilution process of the stock solutions with ACN and later used to prepare a multi-standard solution in ACN:$H_2O$ (80:20, v/v) for fortification of QC samples. All standard solutions were stored in amber vials at -20±2ºC, protected from light. Blank maize samples (2.0 ± 0.1g) were fortified by adding appropriate amounts of the multi-standard solution for method optimization and validation, in a matrix-matched approach. All optimization experiments were performed on blank ($n$ = 3) and fortified QC samples ($n$ = 3).

### 2.5. Extraction of mycotoxins from maize samples

Grinded maize grain matrices were weighed (2.0 ± 0.1g) into a 50-mL centrifuge tube, and homogenised with 20 mL of ACN:$H_2O$ (80:20, v/v), using a rotary shaker, during 60 min., at room temperature. A mixture of 0.5 g of NaCl and 2.0 g of $MgSO_4$ (1:4, w/w) was added to the solution, and the tube was vortexed for 1 min. After centrifugation at 4500x g for 10 min., at 4ºC, 10 mL of the organic supernatant were used for the clean-up step. The collected extract was added to a dSPE tube containing 150 mg C18, and 900 mg $MgSO_4$. The tube was vortexed for 1 min., and centrifuged at 4500x g, for 10 min. The final extract was evaporated to complete dryness under nitrogen, at 40ºC, using a Turbovap Zymark Evaporator system (Hopkinton, MA, USA), and reconstituted in 500 µL of 40% ACN. The 500-µL extract solution was filtered to HPLC vials, and 20-µL of the reconstituted extract was injected in the UHPLC-MS/MS system.

### 2.6. Method validation

The present method was validated for the determination of 23 regulated and emerging mycotoxins in maize grain samples according to the guidelines established by European Commission (EC), European Medicines Agency (EMA), and Food and Drug Administration (FDA) (European Commission, 2006, 2014, 2021; European Medicines Agency (EMA), 2012; Food & Drug Administration (FDA), 2018). Therefore, the performance criteria evaluated encompassed linearity, repeatability, reproducibility, recovery, Limits of Detection (LOD) and Limits of Quantification (LOQ). SSE was not validated since no specified performance criteria range has yet been regulated.

Fifteen blank samples were analysed for peak interference with other analytes to assess specificity and selectivity of the method. Spiked control samples were studied at ten concentration levels, for linearity purposes, which was evaluated by calculation of the regression equations through the method of least squares for each curve, and the correlation coefficients obtained. Quality Control (QC) blank samples ($n$ = 15) were assayed for the determination of limits of detection (LOD) (signal-to-noise 3:1) and limits of quantification (LOQ) (signal-to-noise 10:1). Linearity was re-evaluated for integration of LOQ values lower than minimum calibration points. Trueness was assayed based on extraction recoveries by analysing spiked QC samples at low concentration level (LL), medium concentration level (ML) and high concentration level (HL) for each mycotoxin ($n$ = 6). These parameters were determined by the following equations:

$$\text{Recovery (\%)} = A_{ex} / A_{th} \times 100$$

$A_{ex}$ – average concentration of 3 replicates; $A_{th}$ – theoretical concentration assayed

Spiked QC samples at LL, ML, and HL concentration levels for each mycotoxin were assayed against a standard calibration curve to determine intraday ($n$ = 6), and interday ($n$ = 3) precision. Coefficient of variation (CV) was calculated according to the following equation:

$$\text{CV (\%)} = \sigma / \mu \times 100$$

$\sigma$ – standard deviation at each calibration level; $\mu$ – mean concentration.

## 3. Results and discussion

Multi-analyte LC-MS/MS methods have become fundamental tools for assessment of co-occurrence of mycotoxins in single samples. Therefore, the aim of this work was to optimize, develop and validate a powerful and reliable multi-analyte method by UHPLC-MS/MS that would allow the simultaneous extraction and detection of 23 mycotoxins, from regulated to emerging toxins, in maize grain samples. In this matter, several experiments encompassing three major extraction procedures in analytical chemistry, solid-liquid extraction (SLE), solid phase extraction (SPE), and QuEChERS, were singly evaluated for different intrinsic parameters, and compared amongst them. All optimization and validation experiments were done by matrix-match of each separate clean-up material and, simultaneously, blank samples without fortification were submitted to the same clean-up materials and conditions.

The compounds selected for the study encompass a wide range of chemical properties, including different polarities, and are known to be present in the maize value chain or have been recently reported in such matrices. Eleven mycotoxins present maximum levels set by the European Commission in this matrix and, subsequently, the optimized method needs to be able to detect and quantify such analytes at those levels.

### 3.1. Optimization of UHPLC-MS/MS conditions for determination of mycotoxins

MS/MS conditions were optimized in full scan mode by direct injection of individual standards for each mycotoxin at concentrations between 1 and 5 µg $mL^{-1}$ in both positive and negative modes. Most of the analytes exhibited higher signal intensities and signal-to-noise (S/N) ratios in the ESI positive mode, except for DON, MON, NIV, OTA, PAT, and TEA. DON, and OTA were effective in both ESI modes, but due to lower signals in ESI positive mode, as well as a higher noise background, the negative mode was the final choice for further mycotoxin identification. MON and NIV gave no peaks in the positive mode. These findings are in agreement with previous reports (Sulyok et al., 2006). Detection was mainly performed with protonated $[M+H]^+$ and $[M-H]^-$ adducts, depending on the ESI mode, except for DON and T-2 toxin which were

detected as formate ($[M+HCOO]^-$) and sodium ($[M+Na]^+$) adducts, respectively. Mycotoxins were identified and confirmed by their retention time (RT), ion transitions, and mass spectrum. In Table S1, conditions used for the confirmation and quantification of analytes are described. RT and ionic transitions (precursor > ion product) presented are those who produce the most intense signal in MRM mode. To fulfil the identification criteria set by European Commission (2021), two ionic transitions corresponding to the main ion fragmentation were used, with the most intense for quantification purposes, with the exception of MON which, due to its low molecular weight, showed only one product ion. Since LC-MS/MS has the possibility of polarity switching, both ionization modes ($ESI^+$ and $ESI^-$) were used and selected for each mycotoxin for a more sensitive and effective identification of this wide range of chemical compounds, in a single run.

Preliminary studies were assayed for chromatographic-spectrometric conditions using different UHPLC columns, LC gradient programs, flow rates and injection volumes. Mobile phases described in literature consist of water and methanol (MeOH) or acetonitrile (ACN), with or without addition of a buffering solution (acetic or formic acid) to improve chromatographic separation and detection (Leite et al., 2020). Several authors highlighted the use of MeOH instead of ACN as mobile phase solvent due to the protic nature of MeOH, which enhances the response of $[M+H]^+$ in the positive mode (Dagnac et al., 2016). On the other hand, MeOH has been reported to negatively affect FBs and DON (Li et al., 2018). Acidification of the mobile phase, mostly performed with acetic or formic acid, allows to improve the ESI ionization efficiency and to obtain better peak resolution of the analytes. In the present study, mobile phase composition was defined as previously established in the laboratory for regulated mycotoxins, which is characterized by (A) 0.1% formic acid and (B) acetonitrile (Silva et al., 2019). Elution gradient was redefined and optimized to the final program with a run time of 25 min., as defined in Section 2. Materials and reagents, to include non-regulated and emerging mycotoxins.

For UHPLC separation, studies were firstly performed concerning LC columns and injection solvents. Six different reverse-phase columns were tested, namely ACQUITY UPLC® HSS T3 1.8 $\mu$m (2.1×100 mm i.d.), ACQUITY UPLC® BEH C18 1.7 $\mu$m (2.1×100 mm i.d.), ZORBAX Eclipse Plus C18 1.8 $\mu$m (2.1×50 mm i.d.), ZORBAX Eclipse Plus C18 5.0 $\mu$m (2.1×150 mm i.d.), Kinetex Biphenyl 100 Å 1.7 $\mu$m (2.1×50 mm i.d.) and GEMINI NX-C18 100 Å 3 $\mu$m (2.0×100 mm i.d.). Results expressed as peak area (Fig. 1a and 1b) and as signal-to-noise ratio (Fig. 1c and 1d) revealed comparable data between all the columns with the defined elution gradient, with good separations and high intensities for the majority of the 23 mycotoxins analysed. Nonetheless, for both ZORBAX Eclipse Plus C18, low resolution peaks were observed for PAT, and low signal-noise (S/N) ratio was verified for BEA. With Kinetex Biphenyl column, AFBs and FBs also presented very low S/N ratios when compared to other columns, and no peak signal was observed for TEA. ACQUITY UPLC columns presented elution of ENNB at a RT of 20.89 and 21.62 min., respectively, with no peak signal for ENNA for the established elution program (25 min.), which elutes later than ENNB. Overall, GEMINI NX-C18 presented the best chromatographic separation towards the identification and quantification of all the target mycotoxins in a single run, regarding peak area and S/N ratios data. Lattanzio et al. (2007) also performed several studies in what concerns chromatographic columns, all of them reversed-phase columns (Synergi Hydro®-RP; Synergi Fusion®-RP; Gemini® C18), being the Gemini® C18 (150 × 2 mm, 5.0 $\mu$m particle size) the most suitable for separation of the analytes in their study, with a good separation of the analytes eluting near the polarity switching. Scarpino et al. (2019) also observed reasonable peak shapes for this UHPLC column for the analysis of emerging and regulated mycotoxins. In the present study, solvent used for injection was also optimized by using dilutions or evaporation and reconstitution, namely with (i) ACN 40%, (ii) ACN 80%, (iii) ACN 100%, and (iv) ACN:0.1% formic acid (95:5) (initial proportion of LC mobile phase). For all mycotoxins, ACN 40% was the most suitable injection solvent, with lower background noise.

### 3.2. Efficiency evaluation of extraction and clean-up procedures

#### 3.2.1. Solid-liquid extraction (SLE)

Extraction solvent selection is one of the most crucial steps for extracting compounds from complex solid matrices due to the chemical diversity of mycotoxins. Compounds such as AFs, OTA, and ZEA are best extracted by using high organic solvents; on the other hand, aqueous solvents and/or acidic solvents are the most suitable for extraction of FBs and MPA (Lattanzio et al., 2007; Sulyok et al., 2006). The first step of this work was to identify the solvent proportion and amount of acidified solvent needed to maximize the extraction of the analytes in this study. Blank maize samples were firstly spiked with 1 mL of multi-standard solution, with final concentrations of 1 ng $g^{-1}$ for AFB1; 2 ng $g^{-1}$ for AFB2, AFG1 and AFG2; 0.05 ng $g^{-1}$ for AFM1; 100 ng $g^{-1}$ for BEA, ENNA, ENNB, CIT, HT-2, T-2, MPA, NIV, TTX and TEA; 1.5 ng $g^{-1}$ for OTA; 750 ng $g^{-1}$ for DON; 10 ng $g^{-1}$ for PAT; 200 ng $g^{-1}$ for ZEA; 1000 ng $g^{-1}$ for FB1, FB2 and MON; and 300 ng $g^{-1}$ for PA. Different solvent mixtures were tested by using ACN:$H_2O$ (80:20, v/v), ACN:MeOH (80:20, v/v), and Acetone:$H_2O$ (80:20, v/v). Acetonitrile phase used as first extraction solvent was then analysed by acidifying the organic phase with formic acid or acetic acid at 0.1 and 1.0% (v:v) each. Fig. 2a and 2b summarize the results obtained for maize grain samples, expressed as recoveries, for solvent composition and acidification, respectively. From these results, it can be observed that FBs showed the lowest recoveries with a mixture of organic solvents, being the best results obtained with ACN:$H_2O$ composition. TEA also present the lowest recovery rates with ACN:MeOH, with the highest rates obtained with acidified organic phase, especially with formic acid. This mycotoxin is soluble in most organic solvents, and it is also an acidic toxin (pKa = 3.5). The lower the pH value is comparing to the compound pKa, more favourable is the compound distribution in the organic phase. Formic acid (pKa = 3.75) is stronger than acetic acid (4.75), allowing a higher recovery of TEA. On the other hand, OTA (pKa = 4.4) is also an acidic compound, with better recoveries by acidifying the extraction solvent with acetic acid. Nonetheless, acidification of organic phase negatively affects CIT, either with acetic or formic acid. Overall, combination of ACN:$H_2O$ without acidification, presents the most appropriate extraction for the majority of the analytes. This combination was, therefore, chosen as the best compromise for the extraction of the 23 mycotoxins present in this work, in the first step of the extraction methodology.

Optimization of the extraction time is also an important step not usually addressed in the development of multi-mycotoxin methods, which reveals itself as an important parameter since metabolites can be present inside or outside the matrices in analysis (Rasmussen et al., 2010). An optimization of the time of extraction was performed by adding 10 + 10 mL of ACN:$H_2O$ (80:20, v/v) to blank maize samples submitted to a spiking procedure as previously described, and by homogenising the samples in a rotary shaker for a total period of 120 min. (E1), 90 min. (E2), 60 min. (E3) and 30 min. (E4), respectively, in a double extraction procedure. Results are shown in Fig. 2c and are represented as percentage of recovery. Comparable results were obtained for each time period analysed, except for CIT with a recovery rate obtained with 120 min. of homogenisation with ACN:H2O (80:20, v/v), twice higher than the other time extractions. It was observed that most of the content of CIT is extracted after the second extraction (56.80%) (Fig. 2d). Nonetheless, and to maximize the extraction protocol in terms of recovery and, also, time of extraction, a total of 60 min. for SLE was selected for comparison purposes with other extraction procedures. Analysis of single extracts after first (60 min.) and second extraction (60 min.) also revealed that most of the mycotoxin content is extracted at the first step, in a range between 60 and 88% of the total content obtained for all mycotoxins, except for CIT, as shown in Fig. 2d.

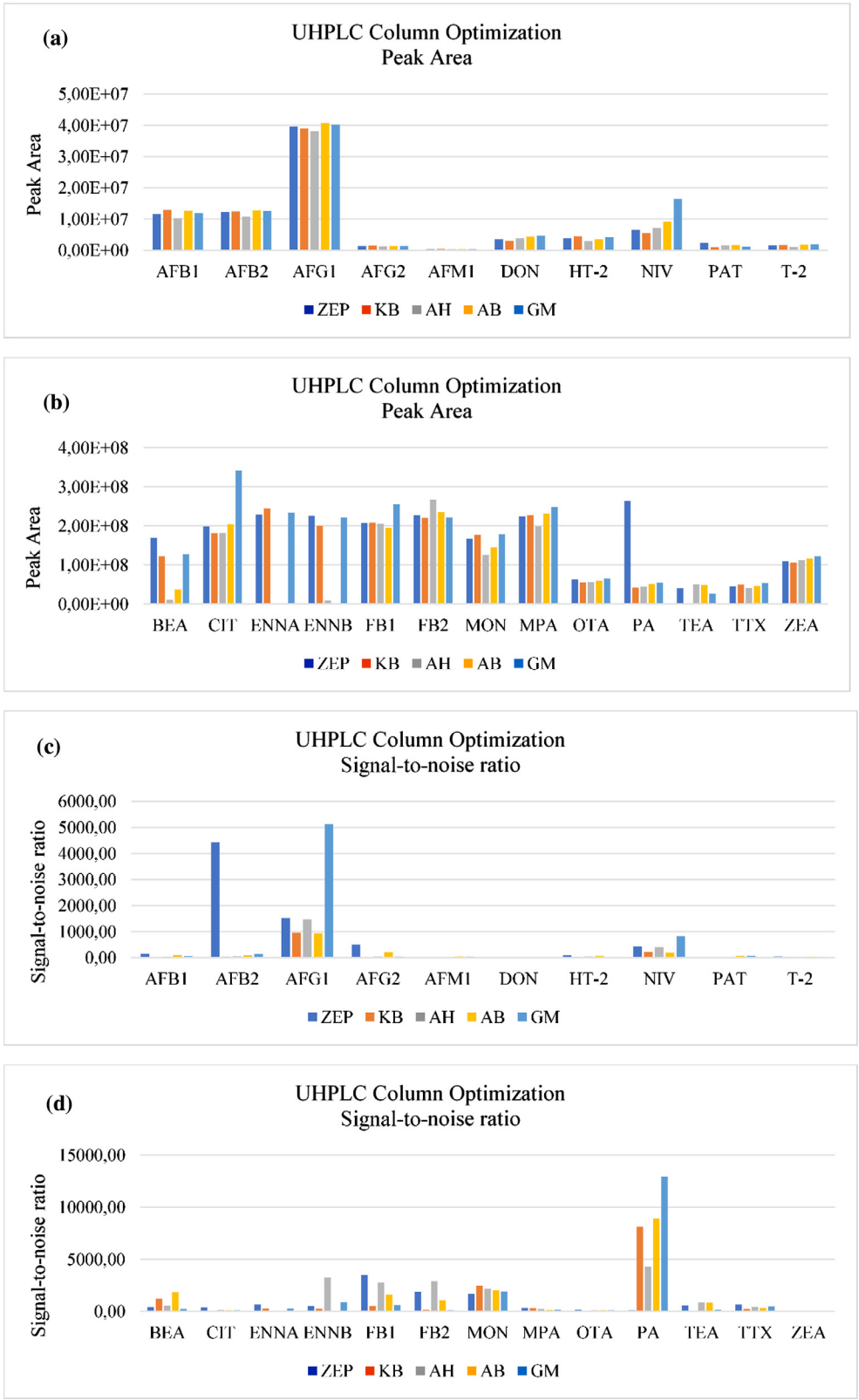


**Fig. 1.** Data values for UHPLC column optimization for chromatographic separation of (a) AFs, DON, HT-2 and T-2 toxins, NIV, PAT, (b) BEA, CIT, ENNs, FBs, MON, MPA, OTA, PA, TEA, TTX, and ZEA, in peak area; and (c) of AFs, DON, HT-2 and T-2 toxins, NIV, PAT; (d) BEA, CIT, ENNs, FBs, MON, MPA, OTA, PA, TEA, TTX, and ZEA, in signal-to-noise ratio.

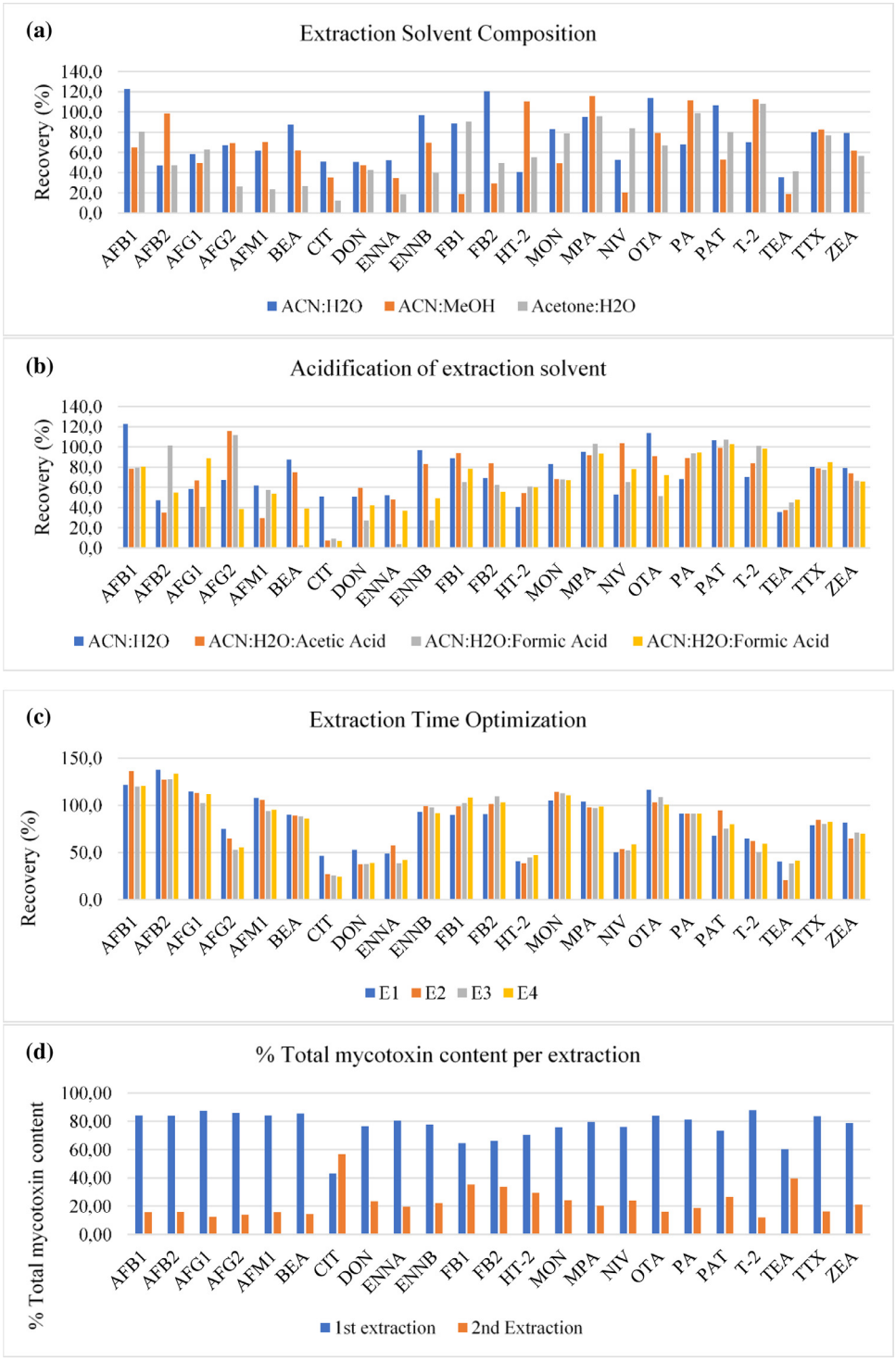


**Fig. 2.** Results data in recovery (%) for (a) solvent composition, (b) solvent acidification, (c) extraction times, and (d) total content per extraction, in double SLE.

### *3.2.2. Solid-phase extraction (SPE)*

Solid-Phase Extraction (SPE) is a conventional sample preparation procedure widely used as an extraction method for liquid samples, or as a clean-up and concentration step, following a prior extraction procedure, for solid samples, such as maize. A wide range of SPE sorbents are available, with different chemical retention mechanisms that allow to minimize matrix effects. Selection of the appropriate cartridge is dependent on the sample matrix and the chemical properties of the analytes. Most methods concerning SPE for mycotoxin clean-up procedures in food samples include reverse phase columns with C18 as sorbents (Pamel et al., 2011; Scarpino et al., 2019; Wang et al., 2013). A survey on C18 sorbents for the analysis of regulated and emerging mycotoxins was, therefore, performed in order to compare recovery efficiency of different SPE columns.

For SPE experiments, blank maize samples were spiked according to the previous extraction test and subjected to the optimized SLE procedure. 5-mL of the 20-mL extracts were applied to different C18 SPE cartridges, namely (1) Oasis PRiME HLB 6cc, (2) Oasis HLB 6cc; (3) Sep-Pak Vac C18 6cc; (4) Sep-Pak Vac C18 3cc; and (5) Bond Elut Varian 6cc. Bond Elut C18 column is a proprietary silica-based ion

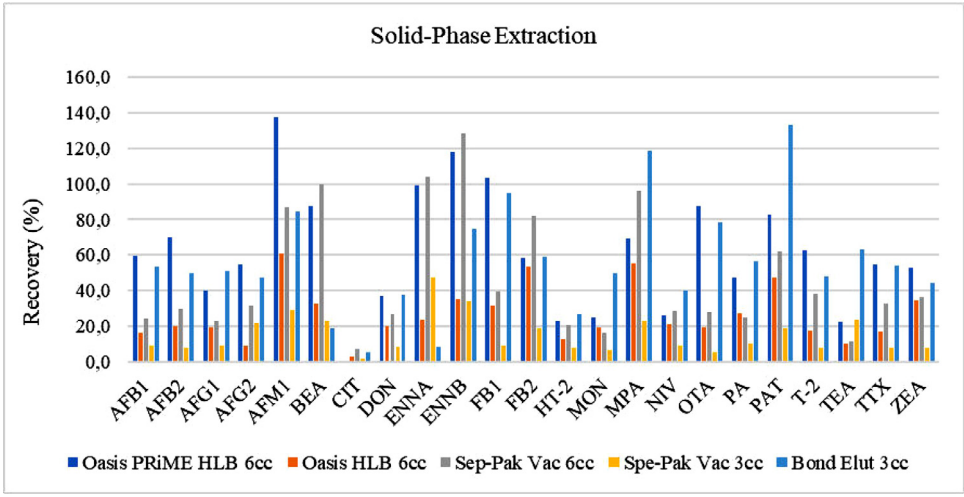


**Fig. 3.** Recovery values, in percentage, for analysis of different SPE C18 sorbents.

exchange sorbent characterized by non-retention mechanisms. On the other hand, Sep-Pak Vac and Oasis HLB cartridges function on a selective retention mechanism, which means both toxins and matrix components are retained in these columns, and the analytes of interest are then eluted with an appropriate elution solvent. Therefore, Oasis HLB and Sep-Pak Vac columns were firstly preconditioned with MeOH and ACN 80%, and the mycotoxins were eluted with 10 mL of MeOH:ACN (50:50, v/v). For Oasis PRiME and Bond Elut Varian columns, 0.5-mL extract was applied to the cartridges, without preconditioning, and discarded; and then 5-mL extract passed through the C18 columns, and finally collected for further analysis. The final extracts were evaporated to dryness under nitrogen, at 40ºC, and the dry residue reconstituted in 500 µL of ACN 40%. Recovery rates were obtained and are shown in Fig. 3.

Best recovery results were observed with Oasis PRiME and Bond Elut cartridges for most of the mycotoxins, due to their ability to retain compounds with different physicochemical properties. For the emerging mycotoxins BEA and ENNs, highest recovery rates were obtained with the Sep-Pack Vac 6cc, though at comparable rates from the ones obtained with Oasis PRiME HLB. Scarpino et al. (2019) also reported the use of Oasis PRiME HLB clean-up columns for the determination of mycotoxins in maize with satisfactory results for multi-analyte analysis. Comparable data was obtained concerning the SLE approach, similarly to the findings of the present work. Bond Elut columns were reported for the analysis of TCTs and ZEA in maize with validated recovery values between 88 and 113%. Pamel et al. (2011) and Wang et al. (2013) also developed SPE based on Oasis HLB cartridges, with reasonable mean recoveries from 68.3 to 94.3%, and 61 to 116%, respectively. In this study, however, Oasis HLB, as well as Sep-Pak Vac 3 cc, presented the lowest values of recovery for most mycotoxins. Overall, this study showed that both Oasis PRiME and Bond Elut are effective in recovering chemically different mycotoxins.

### 3.2.3. QuEChERS

QuEChERS method is a known clean-up procedure for analysis of pesticides, characterized by a solvent extraction with ACN and a salting-out step for water removal by magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) followed by a dispersive SPE (dSPE) with salts and sorbent materials for compound purification. Nowadays, QuEChERS protocols have been increasingly adapted for mycotoxin analysis due to its ability of rapid extraction, purification characteristics, and multi-analyte extraction.

Development and optimization procedures were performed in order to evaluate different salting-out reagents, and sorbent materials, such as C18, PSA, and combined C18/PSA. Blank samples were spiked as previously described, and initially extracted with ACN 80% for 60 min. in a rotary shaker. General protocol consisted of (1) salts addition to the solid-liquid mixture, (2) manual homogenisation (1 min.), (3) centrifugation for 10 min., at 4ºC, (4) supernatant (organic phase) removal and, (5) addition of purifying agent to the organic phase. Concerning the dSPE step, different sorbents were studied, namely with C18, PSA, and a combination of C18 + PSA. Several combinations were used in the salting-out process, namely $MgSO_4$ and NaCl (4:1), $MgSO_4$ and sodium sulfate ($Na_2SO_4$) (4:1), and an adapted in-laboratory QuEChERS protocol for pesticide residue analysis, composed of $MgSO_4$, NaCl, tri-sodium citrate dihydrate (TSCD) and disodium hydrogen citrate sesquihydrate (DHCS) (4:1:1:0.5, w/w/w/w). All combinations were analysed with and without the addition of 1% formic acid (FA) to the initial extraction solvent. Fig. 4a shows the data obtained for the comparison of different salt combinations and solvent acidification, used in this study. Very low analyte signals for BEA, CIT, and MON were observed with the salting-out compounds of the original QuEChERS method due to an inefficient extraction of the compounds to the organic layer. On the other hand, an improvement of this results occurs with the use of $MgSO_4$ and sodium compounds as salting-out reagents. Best mixtures were obtained with $MgSO_4$/NaCl and $MgSO_4$/$Na_2SO_4$, in the range of 42.6 to 122.3% and of, 26.1 to 129.0%, respectively. Acidification of the extraction solvent shows an approximately 2-fold increase in the recovery of FBs; nonetheless, as previously observed, this extraction solvent acidification is critical for NIV, MON and, especially, CIT detection. Decrease in recoveries of CIT are of a 17-, 11- and 13-fold magnitude for $MgSO_4$ / NaCl, $MgSO_4$ / $Na_2SO_4$, and $MgSO_4$ / NaCl / TSCD / DHCS, respectively. The mixture $MgSO_4$/NaCl in a proportion of 4:1 was the selected salting-out reagent for further optimization studies.

Effects on the purification process and recoveries of two purifying agents (PSA and C18) were then compared individually and in combination at different amounts (25 and 50 mg, each). Results, expressed as recovery rates, are shown in Fig. 4b. It was observed the high negative effect of PSA sorbents on several mycotoxins. For instance, the removal of FBs from samples due to ion exchange mechanisms is evident and has been stated by other authors (Yan et al., 2016b). OTA also gave very low recoveries, possibly due to the ionic affinity between carboxyl groups of OTA and amine groups of PSA. Nonetheless, good recovery rates were similarly obtained with no dSPE and C18 sorbent for both FBs (ranging from 83.7 to 102.9%) and OTA (79.4 and 72.3%). MON, MPA, and PA also presented very low or no signals with all the experiments containing PSA, which can be due to the fact that these mycotoxins are also absorbed by this dSPE reagent. On the other hand, recoveries of 84.4 to 105.8%, and 72.3 to 79.4%, were obtained for these mycotoxins, respectively, with no dSPE and with C18 sorbent. On

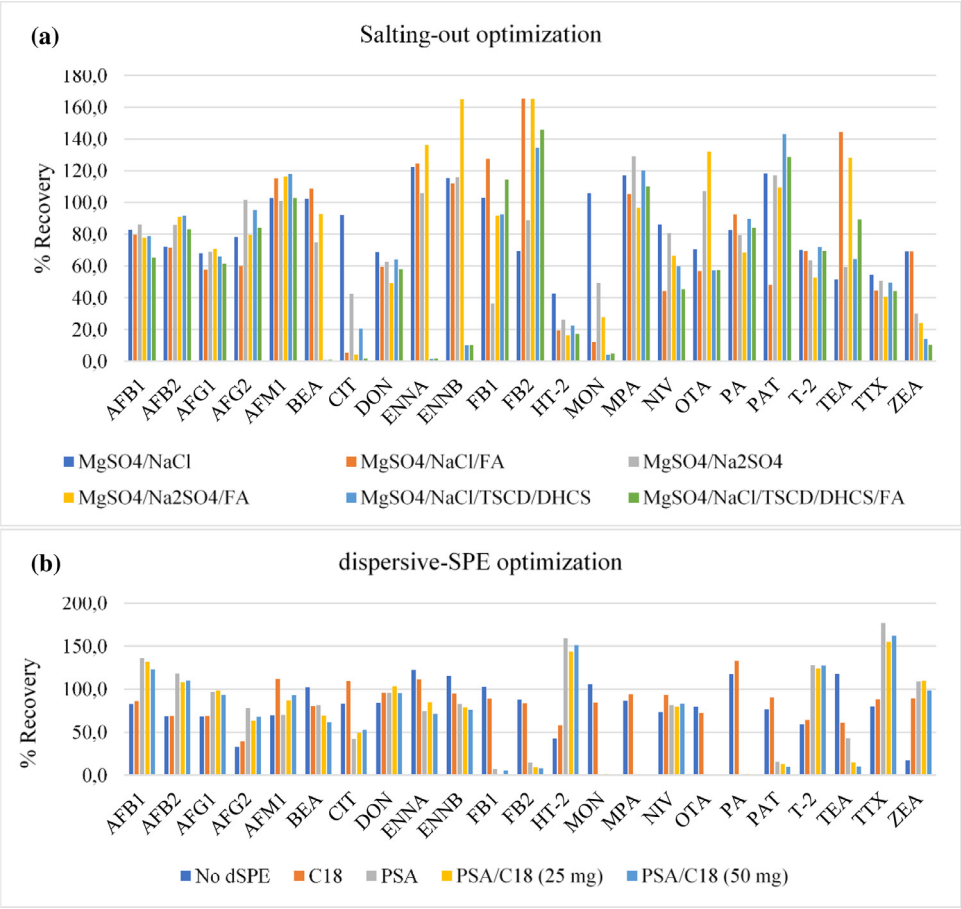


**Fig. 4.** Recovery values, in percentage, for analysis of different (a) salting-out reagents, and (b) modified-QuEChERS methods, concerning dispersive-SPE step.

the contrary, AFs present better results with PSA, and combinations of PSA and C18, although with a slight decrease on recovery rates for the latter. A decrease factor of 1.5,1.6 and 1.4 for AFB1, AFB2, and AFG1, respectively, is observed when comparing experiments with PSA and experiments without this purifying agent. AFG2 presents a higher factor of 1.9 decrease. HT-2, T-2, TTX and ZEA also present better results with PSA, and combinations of PSA and C18. Wang et al. (2016) observed similar behaviour with AFs and ZEA when comparing both sorbents individually. It also obtained comparable recoveries with both purifying agents for DON, as in accordance with our data. AFM1 presents the exception in the AFs group with the best percentage of recovery obtained with C18 sorbent (112.1%). Matrix materials, such as starch, fat and sugar, can be effectively removed by C18 sorbent, with no apparent adsorption for most of the mycotoxins in analysis, being therefore, the most effective as a multi-analyte clean-up regarding the selected compounds.

### *3.2.4. Comparison of extraction/clean-up procedures*

The previous optimization procedure regarding intrinsic parameters of different extraction/clean-up methods, allowed to select the method most suitable and providing best performance for the mycotoxins in this study in a global point-a-view. In Fig. 5, a comparison between SLE, SPE with both Bond Elut and Oasis PRiME HLB, and QuEChERS with C18 is performed.

The simple and non-time consuming SLE procedure reveals better extraction, in significantly different recovery rates, for AFs, except AFM1, MON, and OTA. For most mycotoxins, the use of SPE and QuEChERS as clean-up procedures after SLE, presents itself as an additional step to improve analytical results in what concerns the removal of matrix components. Comparable results can be found with both clean-up methods, especially with Oasis PRiME HLB cartridge. As previously described, Scarpino et al. (2019) concluded that SLE and SPE based on Oasis PRiME HLB are both efficient in reducing analytical time and costs regarding routine analysis of co-occurring regulated, emerging, and masked mycotoxins, being comparable methods for multi-mycotoxin analysis. However, for mycotoxins CIT, DON, NIV, PA, TTX and ZEA it is clear the much higher rates of recovery with the modified QuEChERS protocol when comparing to the SPE procedure. Therefore, the optimized QuEChERS method comprising $MgSO_4$ and NaCl as salting-out reagents, and C18 as purifying agent for the dSPE step, was the method chosen for further validation purposes. Its ability for multi-analyte analysis allied to its application for multi-matrices purposes, is also a unique feature for future method extension to other food and feed matrices. In this regard, several studies have been successfully applied. Pantano et al. (2021) developed an effective d-SPE approach for mycotoxin detection in cereal products, namely maize, and in spices, with all the results being compliant with the proficiency tests performed for the validated QuEChERS extraction method. For the detection of 9 mycotoxins in rice, a solvent extraction with 10% (v/v) acetic acid-acetonitrile in the presence of four salts and d-SPE using C18, PSA and silica sorbents was used by Jettanajit & Nhujak (2016) which allowed good performance criteria, including detection limits below the maximum limits of EU regulations. On the other hand, Aguilera-Luiz et al. (2011) tested three methodologies for mycotoxin analysis in milk, including SPE, DaS and QuECheERS. A combined QuEChERS and SPE Oasis HLB initially revealed the best results when using a skimmed milk matrix, though the recovery rates significantly decreased when using a more complex milk matrix. Recently, González-jartín et al. (2021) obtained acceptable performance characteristics for the quantification of 40 mycotoxins in raw milk, including regulated, emerging and modified mycotoxins based on a QuEChERS

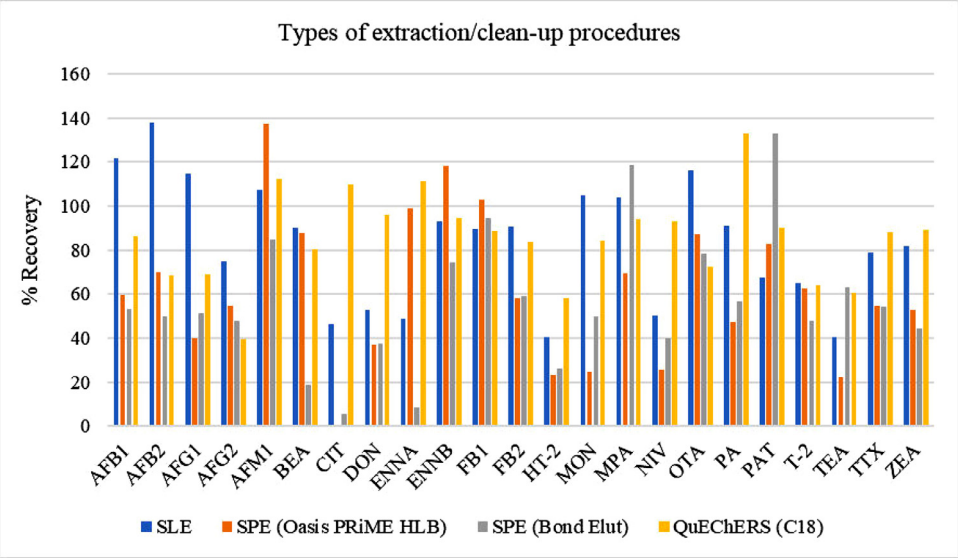


**Fig. 5.** Comparison between the extraction/clean-up procedures in terms of recovery (%).

approach with $MgSO_4$ and NaCl for phase partitioning, followed by a d-SPE step with C18.

### 3.3. Method validation

Several official validated methods for the determination of regulated mycotoxins in maize are already available (Leite et al., 2020). To guarantee the verification of compliance with feed and food law, regulations on official controls of mycotoxins have also been established, namely performance criteria for sample preparation and methods of analysis in foodstuffs by the Commission Regulation (EC) Nº 401/2006 (European Commission, 2006). This includes AFM1, AFB1, AFB2, AFG1, AND AFG2, OTA, PAT, DON, ZEA, FB1 and FB2. Later, Commission Regulation (EU) Nº 519/2014 (European Commission, 2014), amending the previous regulation, also included the establishment of performance criteria towards T-2 toxin, HT-2 toxin and citrinin (CIT). Nonetheless, the development of innovative methods and, consequently, their validation are still a need in order to enforce the established limits introduced by the EC and, to include other compounds, such as emerging mycotoxins.

Previously optimized QuEChERS method was validated according to performance criteria defined for regulated mycotoxins, as previously stated, and the general guidelines on the performance of analytical methods (EMEA, 2009; European Commission, 2021; Food & Drug Administration (FDA), 2018). For linearity evaluation, ten-point calibration curves were prepared by spiking blank maize samples in defined work ranges for each mycotoxin; and re-evaluated by preparing spiked blank samples in working ranges comprising LOQ values. In Table 1, linearity data, expressed through the method of least squares ($R^2$), is presented, with values ranging from 0.95 to 0.99. Good linearities were achieved with the broad concentrations' range analysed, with the best results obtained with AFs. To evaluate the sensitivity of the method, LODs and LOQs were calculated on the basis of S/N ratios by analysing the intensity of the background of twenty blank samples at the respective RT for each mycotoxin. Values of LOD and LOQ are also represented in Table 1, in a range of 0.13 (AFM1) to 21.10 (NIV) ng $g^{-1}$, for LODs; and in a range of 0.31 (BEA and ENNA) to 37.49 (DON) ng $g^{-1}$. According to the maximum permitted and guidance levels established for regulated mycotoxins in maize, namely AFB1, total AFs, DON, total FBs, OTA, T-2 and HT-2 toxins, and ZEA, the limits obtained for quantification purposes are all below the regulated levels. For example, EU maximum permitted levels for AFB1 is 5 ng $g^{-1}$ for maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs, and of 5 to 20 ng $g^{-1}$, for animal feed. In what concerns AFs (sum of AFB1, B2, G1 and G2), regulation is only available in foodstuffs, with an EU maximum permitted level of 10 ng $g^{-1}$. LOQs obtained in the present study were of 1.40 ng $g^{-1}$ for AFB1, and of 4.70 ng $g^{-1}$ for total AFs. DON, which presented the highest LOQ (37.49 ng $g^{-1}$), is regulated for maize commodities between 750 and 1750 ng $g^{-1}$ for foodstuffs in processed for direct human consumption and unprocessed cereals, respectively. Considering the use of maize grains for animal feed, regulations established guidance values ranging from 900 (compound feed for pigs) to 12000 (feed materials: maize by-products) ng $g^{-1}$. HT-2 and T-2 toxins, with LOQs of 15.39 and 7.62 ng $g^{-1}$, have EU maximum guidance levels, as sum of both, of 100 ng $g^{-1}$ for maize grains for direct human consumption; 200 ng $g^{-1}$ for unprocessed maize; and of 250 and 500 ng $g^{-1}$ for cereal products and compounds for animal feed. A LOD of 3.30 ng $g^{-1}$ was obtained for ZEA, which is regulated in a range of 100 to 350 ng $g^{-1}$ for maize-based foodstuffs; and between 100 and 3000 ng $g^{-1}$ for products intended for animal feed, such as maize grains.

In a study performed by Rasmussen et al. (2010) using QuEChERS extraction followed by LC-MS/MS analysis of 27 mycotoxins, LOD values ranged from 1 to 739 ng $g^{-1}$, with unsatisfactory performance criteria obtained for CIT and FBs. On the other hand, Zachariasova et al. (2010) reported LODs and LOQs ranging from 5 to 50 ng $g^{-1}$ and, 10 to 100 ng $g^{-1}$, respectively, by using a modified QuEChERS approach with UPLC-ToF-MS detection for the detection of 11 major *Fusarium* mycotoxins. Better results were obtained by Wang et al. (2016) for the validation of 9 mycotoxins through the dispersive SPE approach in maize. LOQs obtained in the study ranged between 0.1 (AFs) and 200 ng $g^{-1}$ (DON). Overall, the data obtained concerning these parameters are in agreement with other published reports (Lago et al., 2021; Wang et al., 2016; Zachariasova et al., 2010). Nonetheless, the present method is sensitive enough to determine regulated and emerging mycotoxins in maize grain samples, either for human consumption or animal feed purposes.

To evaluate the trueness of the method fortified blank matrices were used, since no certified reference material was available (European Commission, 2021). This parameter was analysed by means of recovery rates, obtained from the analysis of three concentration levels (low, medium, and high), each in triplicate. As shown in Table 1, recovery values ranged from 55.25 and 129.48% for AFG2 and HT-2, respectively. The method developed presents good extraction efficiency with reproducible

**Table 1**
Performance criteria for method validation of 23 mycotoxins in maize.

| Micotoxin | Range (ng $g^{-1}$) | Linearity ($R^2$) | Level (ng $g^{-1}$) | Repeatability (%) | Reproducibility (%) | Recovery (%) | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| AFB1 | 1 | 0.9987 | 1 | 7.1 | 8.4 | 99.9 | 0.4 | 1.4 |
| | to | | 15 | 3.9 | 8.2 | 102.5 | | |
| | 40 | | 40 | 3.9 | 6.4 | 101.5 | | |
| AFB2 | 1 | 0.9980 | 1 | 6.3 | 7.4 | 122.8 | 0.3 | 0.4 |
| | to | | 15 | 1.3 | 9.4 | 101.5 | | |
| | 40 | | 40 | 2.3 | 5.1 | 101.4 | | |
| AFG1 | 1 | 0.9939 | 1 | 1.4 | 8.4 | 96.7 | 0.2 | 0.6 |
| | to | | 15 | 0.5 | 1.8 | 101.0 | | |
| | 40 | | 40 | 0.8 | 3.5 | 101.1 | | |
| AFG2 | 1 | 0.9953 | 1 | 3.7 | 12.2 | 55.3 | 0.9 | 2.2 |
| | to | | 15 | 3.0 | 9.2 | 96.8 | | |
| | 40 | | 40 | 2.3 | 3.2 | 103.5 | | |
| Sum | - | - | 1 | - | - | 93.1 | - | - |
| of | | | 15 | - | - | 100.6 | | |
| AFs | | | 40 | - | - | 90.2 | | |
| AFM1 | 0.0125 | 0.9908 | 0.0125 | 6.9 | 10.6 | 85.3 | 0.1 | 0.4 |
| | to | | 0.1875 | 8.5 | 5.3 | 93.1 | | |
| | 0.5 | | 0.5 | 6.1 | 1.6 | 97.0 | | |
| BEA | 20 | 0.9733 | 20 | 3.4 | 5.4 | 89.7 | 1.8 | 3.8 |
| | to | | 300 | 4.4 | 14.1 | 96.7 | | |
| | 800 | | 800 | 2.5 | 5.7 | 106.1 | | |
| CIT | 50 | 0.9852 | 50 | 4.2 | 10.8 | 95.4 | 7.1 | 12.7 |
| | to | | 750 | 4.5 | 9.0 | 89.0 | | |
| | 2000 | | 2000 | 3.3 | 4.8 | 114.1 | | |
| DON | 75 | 0.9942 | 75 | 5.8 | 9.5 | 76.7 | 3.2 | 37.5 |
| | to | | 1125 | 2.9 | 5.7 | 115.5 | | |
| | 3000 | | 3000 | 3.5 | 7.4 | 90.7 | | |
| ENNA | 15 | 0.9959 | 15 | 7.1 | 12.8 | 73.0 | 0.2 | 0.3 |
| | to | | 225 | 3.4 | 14.8 | 97.1 | | |
| | 600 | | 600 | 2.5 | 6.0 | 98.9 | | |
| ENNB | 15 | 0.9773 | 15 | 2.7 | 4.6 | 116.1 | 0.5 | 1.4 |
| | to | | 225 | 1.1 | 5.0 | 82.4 | | |
| | 600 | | 600 | 2.0 | 5.7 | 100.4 | | |
| FB1 | 50 | 0.9907 | 50 | 3.3 | 5.2 | 83.3 | 6.3 | 13.7 |
| | to | | 750 | 1.5 | 5.3 | 103.9 | | |
| | 2000 | | 2000 | 2.4 | 4.3 | 96.4 | | |
| FB2 | 50 | 0.9830 | 50 | 7.9 | 10.6 | 116.9 | 5.9 | 11.9 |
| | to | | 750 | 4.4 | 14.5 | 80.8 | | |
| | 2000 | | 2000 | 1.6 | 8.3 | 103.9 | | |
| HT- | 10 | 0.9870 | 10 | 9.1 | 14.6 | 129.5 | 4.8 | 15.4 |
| 2 | to | | 150 | 1.5 | 12.5 | 97.3 | | |
| | 400 | | 400 | 1.2 | 7.0 | 104.7 | | |
| MON | 50 | 0.9641 | 50 | 7.4 | 8.0 | 99.3 | 10.7 | 17.7 |
| | to | | 750 | 6.8 | 12.4 | 107.2 | | |
| | 2000 | | 2000 | 1.4 | 9.7 | 98.7 | | |
| MPA | 20 | 0.9791 | 20 | 5.3 | 7.1 | 122.8 | 6.9 | 16.6 |
| | to | | 300 | 3.9 | 4.8 | 85.1 | | |
| | 800 | | 800 | 4.9 | 5.6 | 97.4 | | |
| NIV | 40 | 0.9809 | 40 | 8.5 | 14.5 | 106.9 | 21.1 | 33.9 |
| | to | | 600 | 8.5 | 12.4 | 109.3 | | |
| | 1600 | | 1600 | 4.9 | 5.1 | 95.0 | | |
| OTA | 2 | 0.9566 | 2 | 9.5 | 13.6 | 75.8 | 3.4 | 3.7 |
| | to | | 30 | 2.8 | 10.2 | 98.1 | | |
| | 80 | | 80 | 3.5 | 6.6 | 110.2 | | |
| PA | 30 | 0.9954 | 30 | 8.1 | 9.3 | 116.9 | 10.5 | 19.9 |
| | to | | 450 | 1.9 | 4.1 | 119.7 | | |
| | 1200 | | 1200 | 2.2 | 3.1 | 97.5 | | |
| PAT | 2.5 | 0.9933 | 2.5 | 7.1 | 15.0 | 66.9 | 2.9 | 4.5 |
| | to | | 37.5 | 3.4 | 5.0 | 77.9 | | |
| | 100 | | 100 | 3.4 | 5.3 | 105.8 | | |
| T- | 10 | 0.9874 | 10 | 5.4 | 6.6 | 71.2 | 5.5 | 7.6 |
| 2 | to | | 150 | 5.4 | 14.6 | 106.3 | | |
| | 400 | | 400 | 3.3 | 6.5 | 104.2 | | |
| TEA | 10 | 0.9761 | 10 | 4.4 | 14.7 | 119.2 | 6.8 | 14.8 |
| | to | | 150 | 3.9 | 13.7 | 87.6 | | |
| | 400 | | 400 | 2.3 | 8.3 | 96.2 | | |
| TTX | 2.5 | 0.9919 | 2.5 | 6.3 | 8.4 | 117.2 | 0.2 | 0.4 |
| | to | | 37.5 | 2.5 | 5.3 | 116.8 | | |
| | 100 | | 100 | 1.2 | 4.4 | 98.4 | | |
| ZEA | 20 | 0.9756 | 20 | 4.3 | 6.2 | 74.7 | 2.5 | 5.1 |
| | to | | 300 | 0.9 | 3.8 | 100.1 | | |
| | 800 | | 800 | 5.8 | 9.7 | 93.4 | | |

AFB1 – Aflatoxin B1; AFB2 – Aflatoxin B2; AFG1 – Aflatoxin G1; AFG2 – Aflatoxin G2; AFM1 – Aflatoxin M1; AFs – Aflatoxins (B1 and B2); BEA – Beauvericin; CIT – Citrinin; DON – Deoxynivalenol; ENNA – Enniatin A; ENNB – Enniatin B; FB1 – Fumonisin B1; FB2 – Fumonisin B2; MON – Moniliformin; MPA – Mycophenolic acid; NIV – Nivalenol; LOD – Limit oif Detection; LOQ – Limit of Quantification; OTA – Ochratoxin; PA – Penicillic Acid; PAT – Patulin; TEA – Tenuazonic acid; TTX – Tentoxin; ZEA – Zearalenone.

percentages of recovery, with values for regulated mycotoxins within the performance criteria set for this parameter by European Commission (2006). Lowest recoveries were obtained for AFG2 (55.25%), T-2 toxin, (71.17%) and ENNA (72.95%) at the low concentration level (1, 10 and 15 ng $g^{-1}$, respectively). Except for the latter, all non-regulated mycotoxins gave recovery values within the range according to the general guidelines for confirmatory methods, namely -50% to +20% for concentrations ≤ 1 ng $g^{-1}$, -30% to +20% for concentrations from > 1 ng $g^{-1}$ to 10 ng $g^{-1}$, and -20% to +20% for concentrations ≥ 10 ng $g^{-1}$ (European Commission, 2021). The data of the present study concerning this performance criteria is in line with other studies (Rasmussen et al., 2010; Wang et al., 2016; Zachariasova et al., 2010), though the use of internal standard would be an improvement in the results, as demonstrated by Zachariasova et al. (2010).

Table 1 also shows the values of coefficient of variation (CV) obtained for intraday and inter-day analyses. For this parameter, spiked samples of maize grains were evaluated in the same day ($n$ = 6) during 3 consecutive days ($n$ = 3), for 3 concentration levels (LL, ML, and HL). The present data reveals low variation between individual assays in different days, demonstrating the repeatability and reproducibility of the methodology, since the values obtained are within the control limits considered acceptable for non-regulated mycotoxins, and fulfil the performance criteria for regulated mycotoxins. For intra-day precision, variation coefficients ranged from 0.90 to 9.51%; for inter-day precision, all values were below 15.03%.

The optimized and developed method for the analysis of 23 mycotoxins in maize grain samples is suitable with good performance parameters, in accordance with the established regulations, providing the capability of routine analysis of regulated, non-regulated and emerging mycotoxins, simultaneously.

## 4. Application to real samples

To assess the applicability of the validated method for routine analysis, eight samples from maize producers were collected according to mycotoxin sampling guidelines (European Commission, 2006) to ensure precision in the determination of mycotoxins due to its heterogeneous distribution in a lot. In the present study, regulated, non-regulated and emerging mycotoxins were found, namely FBs, DON, ZEA, ENNs, BEA, CIT, and TTX. Regarding regulated mycotoxins, 87.5% of samples were contaminated with ZEA, followed by 62.5% for both fumonisins (FB1 and FB2). Two samples were contaminated with DON. It is known that ZEA and DON are highly crop-associated mycotoxins, and maize grains for animal and human consumption present high contamination levels of FBs (Leite et al., 2021). For non-regulated and emerging mycotoxins, the highest occurrence found was BEA (87.5%), with equal percentage of positive samples as ZEA. The data obtained reveals the need for monitoring of such mycotoxins and, therefore, of methods including both groups of mycotoxins (regulated and emerging).

## 5. Conclusion

The maize food chain is one of the most complex chains that provides directly or indirectly several highly consumed end products, in all population group ages. The presence of mycotoxins in these food chain needs constant monitoring in order to evaluate the risk to which each population group is exposed to. Development and validation of new improved rapid multi-detection methods towards these fungi toxins is crucial to allow this continuous assessment ultimately pointing to human health protection.

A unique comprehensive assessment of several extraction methodologies and evaluation of specific parameters in each methodology was, therefore, performed, to the best of the authors knowledge, for the first time on a specific selected group of mycotoxins that (potentially) threatens the food safety of the maize value chain. An analytical methodology based on QuEChERS extraction with C18 sorbent, followed by UHPLC-MS/MS analysis for the determination of regulated, non-regulated, and emerging mycotoxins in maize grain samples was developed in this sense, since the analytical conclusions aimed at better performances of this method for the selected mycotoxins in comparison to solid-liquid extraction and solid-phase extraction. The proposed extraction protocol was finally optimized to maximize recovery rates and, subsequently, validated for maize grain samples, proving to be specific and selective. Good performance criteria were also obtained regarding linearity, repeatability, reproducibility, and recovery, in compliance with Commission Regulation (EC) Nº 401/2006 and Commission Regulation (EU) Nº 519/2014 for regulated mycotoxins; and Commission Implementing Regulation (EU) 2021/808, for non-regulated and emerging mycotoxins. LODs and LOQs demonstrated the method's capacity to determine concentrations below the maximum residue levels for such samples, as established in Commission Regulation (EC) Nº 1881/2006, for AFs, FBs, T-2 and HT-2 toxins, OTA, DON, and ZEA.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Marta Leite:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Andreia Freitas:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Jorge Barbosa:** Conceptualization, Writing – review & editing. **Fernando Ramos:** Conceptualization, Writing – review & editing, Writing – original draft.

## Data Availability

Data will be made available on request.

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## Supplementary materials

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