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Luca Campone[a,b,*], Anna Lisa Piccinelli[a], Rita Celano[a], Imma Pagano[a], Mariateresa Russo[b], Luca Rastrelli[a]

a Dipartimento di Farmacia, Università degli studi di Salerno, Via Giovanni Paolo II, 84084 Fisciano, SA, Italy
b Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, loc. Feo di Vito, 89122 Reggio Calabria, RC, Italy

Abstract
This study reports a fast and automated analytical procedure based on an on-line SPE-HPLC–MS/MS method for the automatic pre-concentration, clean up and sensitive determination of OTA in wine. The amount of OTA contained in 100 μL of sample (pH ≅ 5.5) was retained and concentrated on an Oasis MAX SPE cartridge. After a washing step to remove matrix interferents, the analyte was eluted in back-flush mode and the eluent from the SPE column was diluted through a mixing Tee, using an aqueous solution before the chromatographic separation achieved on a monolithic column. The developed method has been validated according to EU regulation N. 519/2014 and applied for the analysis of 41 red and 17 white wines. The developed method features minimal sample handling, low solvent consumption, high sample throughput, low analysis cost and provides an accurate and highly selective results.

Keywords: Ochratoxin A, On-line SPE, HPLC–MS/MS, Red wine and white wine, Food safety, Oasis MAX

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species, such as *Aspergillus* and *Penicillium* genera (van der Merwe, Steyn, Fourie, Scott, & Theron, 1965). These fungi grow spontaneously in different kinds of agricultural products. In particular, it is most commonly found in cereals (Wheat, barley, maize, and oats). Fungal species also grow in other kinds of foods, such as beans, coffee, and dried fruits (Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011). Furthermore, OTA has also been detected in many beverages, such as wine, beer, and grape juices (Di Stefano et al., 2015, Mariño-Repizo et al., 2016, Mateo et al., 2007). In the year 1996, scientists found for the first time that wine samples contained OTA as trace contaminants (Zimmerli & Dick, 1996) and was further authenticated by several authors in recent times (Campone et al., 2011, Otteneder and Majerus, 2000, Pietri et al., 2001, Soufleros et al., 2003). The incidence of OTA in wine especially in the Mediterranean basin, is very high (>50%) (El Khoury & Atoui, 2010). It is important to note that OTA is a highly toxic compound, and it mainly impairs the normal functioning of kidney and liver in humans (Pfohl-Leszkowicz & Manderville, 2007). In a previous study, OTA exhibited strong carcinogenic properties in rats and mice; it also exhibited immunosuppressive, teratogenic, genotoxic activities, which impaired blood coagulation and disrupted carbohydrate metabolism (O'Brien and Dietrich, 2005, Pfohl-Leszkowicz and Manderville, 2007). Furthermore, the development of tumors is stimulated by OTA in the urinary tract of humans (Maaroufi et al., 1993, Nikolov et al., 1995). In the year 1993, the International Agency for Research on Cancer (IARC) included OTA in the group 2B, implying that it is a possible human carcinogen. To minimize the public health risk caused by OTA intake, the European Commission (EU) stipulated that the maximum permissible limit (ML) of OTA should be 2 ng mL^{-1} in wine (Commission Regulation

(EC) 1881/2006 Off J Eur Union). Nowadays, all analytical methods used for detecting OTA in wine samples must be sensitive enough to detect trace levels of OTA and determine if they do not exceed the ML (2 ng mL^{-1}) value stated by the EU. The performances of these methods should be compliant with the established legislation pertaining to OTA limits in wine samples (Commission Regulation (EC) 519/2014 Off J Eur Union).

Presently, the most common analytical methods for quantification of OTA in foodstuffs are based on HPLC separation coupled to fluorescence detection (FLD). However, according to the EC Decision 657/2002 (Commission Decision 2002/657/EC (2002) Off J Eur Comm): in addition to FLD determination a mass spectrometry confirmatory method must be performed because it provides full or complementary information pertaining to the unambiguous identification of OTA in the sample.

As far as the sample preparation, different extraction and clean-up techniques for the analysis of OTA in foods have been reported. Most of them, including the official method (Visconti, Pascale, & Centonze, 2001) described by the European standard for analysis of OTA in wine samples, are based on SPE using immunoaffinity columns (IACs) (Visconti et al., 2001). Although IACs exhibit high selectivity during the isolation of OTA from wine, they are very expensive both in terms of time and material consumption. Therefore, other sample preparation methods for the analysis of OTA in complex foods such as wine have been also used. The other sample preparation techniques are as follows: i) solid-phase extraction (SPE) with various alternative stationary phase, including silica gel, octadecylsilane, and molecularly imprinted polymer (Campone et al., 2015, Cigić and Prosen, 2009); ii) dispersive liquid-liquid microextraction (DLLME) (Campone et al., 2012, Campone et al., 2011); iii) solid phase microextraction (SPME) (Aresta, Vatinno, Palmisano, & Zambonin, 2006); iv) packed in-tube SPME (Andrade & Lanças, 2017); v) quechers (Mariño-Repizo et al., 2016) and vi) on-line SPE using a C18 cartridge (Bacaloni et al., 2005)

Most of these methods are expensive in terms of time and material consumption. In addition, only specially trained personnel can perform these sample preparation techniques and the sample throughput is too low to meet the current standards of food safety and public health protection (Cigić & Prosen, 2009). Therefore, it is necessary to develop novel, automated procedures that overcome the main limitations of conventional techniques; the novel method must not only be rapid but also accurate enough to minimize the number of errors in order to obtain reproducible responses. On-line SPE (Rodriguez-Mozaz, de Alda, & Barceló, 2007) is a good alternative method for sample preparation performed during the determination of OTA in wine samples. The on-line SPE technique does not have the drawbacks of conventional methods, and the sample manipulation errors of this technique are reduced. An automated on-line SPE-HPLC system consists of the following components: an autosampler, two HPLC pumps, and a six or ten-port switching valve. After the injection, performed by autosampler, the sample is transported by one of the pumps and concentrated in the SPE cartridge, while the matrix interferences are eliminated out to the waste. After completing the sample loading and washing steps, the position of the valve change, in order to drive the mobile phase from the second HPLC pump into SPE cartridge; this mobile phase elutes the analytes from the SPE cartridge into the chromatographic column. In the HPLC column, the interferents are separated and target analytes are detected. The on-line SPE is a suitable alternative technique for sample preparation, because the sample pretreatment procedure is minimal and the analysis time is sharply reduced (Campone et al., 2016, Rodriguez-Mozaz et al., 2007, Rogeberg et al., 2014). This study, describes the optimization of an on-line SPE-HPLC–MS/MS method for the analysis of OTA in wine samples; To carry out sample extraction, a mixed mode anion exchange cartridge (Oasis MAX) was used coupled with a triple quadrupole mass spectrometer through a two-position 10 port switching valve for OTA quantification: the aforementioned configuration performs automatically the loading, washing, and elution steps of analysis. We cautiously optimized all parameters of the on-line SPE procedure affecting the extraction efficiency paying particular attention to the removal of matrix effect. Under the optimal conditions, we performed validation of the method according to EU regulation 519/2014 (Commission Regulation (EC) 519/2014 Off J Eur Union), and finally, applied at 58 different wine samples (red and white).

2. Experimental

2.1. Standards and materials

OTA Reference standard solution (10 μg mL^{-1}) in acetonitrile was purchased from LGC Promochem GmbH (Labservice Analytica, Bologna, Italy). The stock solutions of OTA (200 ng mL^{-1}), used for spiking procedure and for preparation of working calibration solutions, was prepared in EtOH 15% to simulate the alcohol composition of wine, and stored in amber glass vials at −20 °C. Ultrapure water (18 MΩ) was preparing using a Milli-Q purification system (Millipore, Bedford, USA). MS-grade water (H_2O), methanol (MeOH), and acetonitrile (CH_3CN) were supplied by Romil (Cambridge, UK), ammonia solution 30% (NH_4OH) and ethanol absolute (EtOH) were supplied by Carlo Erba reagents (Milan, Italy), MS-grade formic acid (HCOOH) was provided by Sigma-Aldrich (Milan, Italy). Reference material (RM) of white wine T1755 (OTA assigned values 1.63 μg L^{-1}; satisfactory range 0.91–2.34 μg L^{-1}) was purchased from Fapas (York, UK). The following on-line cartridges: Oasis HLB (20 × 5 mm, 25-μm particle size, 80-Å pore diameter; Waters, Milford, MA, USA), copolymer of divinybenzene and vynil pyrrolidone column; Oasis MAX column (20 mm × 2.1 mm, 12 μm particle diameter, 175 Å pore size; Waters), mixed-mode, reversed-phase/strong anion-exchange; and Strata C18 silica column reversed-phase (25 × 4 mm, 25 μm particle size, 60-Å pore diameter, Phenomenex, Bologna, Italy), were tested in the optimization of the on-line SPE procedure.

2.2. Samples

Wine samples (alcohol content from 10 to 15%) from Mediterranean countries were purchased from different local stores and supermarkets (Salerno and Naples, Italy) and stored at room temperature. Before analysis, the pH of the samples was adjusted to pH ≅ 5.5 (Reinsch, Töpfer, Lehmann, & Nehls, 2005) using NH_4OH 0.1 M and then centrifuged for 5 min at 10,000 rpm (IEC-CL30R, Thermo Electron Corporation, Milan, Italy).

Samples used in optimization and validation studies, were earlier analyzed to verify the absence of OTA contamination (Campone et al., 2011). Spiked samples were prepared by adding specific volumes of OTA stock solutions (200 ng mL^{-1}) to 10 mL of OTA-free wine to achieve the required contamination levels. After spiking, the fortified samples were stirred for 1 h left at room temperature and then stored in the dark at 4 °C for a maximum of three days.

2.3. On-line SPE and chromatographic conditions

The system used for both on-line preconcentration and chromatographic separation was an Ultimate 3000 (Thermo Electron Corporation) equipped with dual ternary gradient pumps, a thermostated column compartment that includes a Rheodyne® 10-port two position (load/inject) switching valve and an autosampler with a 5000 μL injection loop. The chromatographic system was connected to an Ultimate 3000 UV detector (280 nm) to monitoring the matrix components during the washing step and a TSQ Quantum Ultra (Thermo Electron Corporation) triple quadrupole mass spectrometer, for the detection of analyte. The mobile phases in the left pump were as follows: H_2O (A_L), H_2O/MeOH/HCOOH 79.5:20:0.5 v/v/v (B_L) and MeOH (C_L). The mobile phases of right pump were: H_2O/MeOH/HCOOH 19.5:80:0.5 v/v/v (A_R) H_2O with 0.1% HCOOH, v/v, (B_R) and CH_3CN % with 0.1 HCOOH, v/v, (C_R). The chromatographic separation was performed in gradient elution mode using a monolithic column (Chromolith® FastGradient 50 × 2 mm I.D. Merk); the column temperature was maintained constant at 25 °C during the entire chromatographic run. To perform sample enrichment and removal of matrix components the automated on-line SPE was performed in a mixed-mode (reversed-phase/strong anion-exchange) Oasis MAX cartridge. The cartridge was fitted into the load position with a 10 port-switching valve. Before injection, cartridge and column were conditioned for 2 min and then 100 μL of wine sample was loaded into SPE cartridge at high flow rate (1 mL min^{-1}) using 2 mL of solvent A_L. Thereafter SPE column was washed with 2 mL of B_L and 2 mL of C_L (both at 1 mL min^{-1}) sequentially. Thus the interfering species of the matrix were

flushed out as wastes and the analyte was retained on the SPE cartridge. After washing, the valve was switched back in the inject position in order to connect the SPE cartridge with the chromatographic column, and the right pump was used to elute the analyte in back-flush with 3.5 mL of A_R at 0.5 mL min^{-1} performing the desorption of analyte. During elution, the SPE cartridge was connected to a stainless steel Tee-mixer (VICI Valco, Houston, TX, USA) and 7 mL of A_L (1 mL min^{-1}) was used to dilute the elution solvent and to reduce its organic content, in order to perform the refocusing the analyte on top of the analytical column. After 7 min, the analyte was transferred completely on top of the HPLC column, the position of the valve was changed again, and the chromatographic gradient was used to perform the separation of OTA from the matrix components. At the end of each run, both the HPLC column and SPE cartridge were washed before the next analysis. The Chromleon software version 7.1.2 was used to control LC pumps, 10 ports valve and UV detector. Table 1 presents the details of on-line SPE conditions, mobile phase composition, switching times of valve and chromatographic gradients.

2.4. Mass spectrometry analysis

For detection of OTA, the analytical column was coupled to a TSQ Quantum Ultra (Thermo Electron Corporation) triple quadrupole mass spectrometer; this device was equipped with a heated electrospray ionization source (HESI) and operated in a positive ionization mode; The operating parameters of mass spectrometry were as follows: spray voltage 2.4 kV, tube lens 130 V and vaporized temperature of 250 °C. The ion transfer tube temperature was set to 300 °C and nitrogen (purity > 99.98%) was used as a sheath gas and auxiliary gas at flow rates of 30 and 5 (arbitrary units), respectively. For tandem mass spectrometry the protonated molecule $[M+H]^+$ was used as precursor ion. The optimization of HESI working parameters and MS/MS transitions was achieved by infusing 5 μg mL^{-1} stock solution of OTA (MeOH/H_2O 1:1 v/v, 0.1% HCOOH), at a flow-rate of 10 μL min^{-1} using the syringe pump integrated into the TSQ instrument. For identification and quantification of the analyte, the instrument was operated in a selected reaction monitoring (SRM) mode. The parent/product ion transitions were as followed: *m*/*z* 404.1 → 239.1 (I_1); 404.1 → 341.1 (I_2). Argon (99.9999% purity) was used as collision-induced-dissociation gas (CID) at 1.0 mTorr and the collision energy (CE) for each transition was 25 (V) and 20 (V) respectively. The SRM parameters for all the transitions were as follows: scan width (*m*/*z*), 1; scan time (s), 0.100; Q1 and Q3 peak width, 0.7 (FWHM). The Xcalibur software version 2.2 (Thermo Electron Corporation) was used to control the MS system and to process the data. OTA quantification was carried out using both the SRM transitions and the intensities ratio (I_1/I_2) was used as an additional identification criterion, with a tolerance of less than 10% of the expected ratio. The tolerance of intensities ratio (I_1/I_2) was calculated by comparing the SRM OTA transition ratio (I_1/I_2) of the analyte obtained in the matrix with those obtained in the standard solutions.

2.5. Method performance and matrix effect evaluation

Calibration solutions were prepared by diluting appropriate volumes of OTA stock solution with EtOH 15% (solvent curve) or with wine OTA-free sample (matrix-matched curves). The linearity of the solvent and the corresponding matrix-matched curves were evaluated in the working range of 0.5–5 ng mL^{-1} using six calibration levels, each sample was analyzed in triplicates. The statistical analysis of variance (ANOVA) was performed to determine the goodness-of-fit and linearity of the curves.

To determine the matrix effect (signal suppression or enhancement), we analyzed the solvent and matrix-matched (red and white wines) curves (OTA peak area versus concentration). The analysis was carried out by performing on-line SPE HPLC–MS/MS in the same concentrations range and the matrix effect was determined by comparing the slope of each curve.

Method detection limits (MDL) and method quantification limits (MQL) were calculated using analyte-free wine samples fortified at low levels (0.1; 0.05 and 0.025 ng mL^{-1}). Each level was processed in triplicate and the MDL and MQL were calculated by extrapolating 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 carried out on red and white wine samples that were spiked with OTA at three different contamination levels (0.5; 2 and 5 ng mL^{-1}), each sample was analyzed in triplicate. The intra-day precision was expressed as relative standard deviation (RSD), and it was calculated with the same experiments. Because the matrix effect was not observed, OTA was quantified using solvent calibration curve. The trueness of method was evaluated processing six aliquots of RM of white wine T1755 (FAPAS).

3. Results and discussion

3.1. Optimization of on-line SPE

The performances of the procedure was mainly assessed by evaluating the capability of on-line SPE process in removing the interfering species from the matrix. At the same time, the on-line SPE process must minimize losses of analytes. To achieve high recovery of the analyte and to ensure maximum sensitivity for OTA in wine samples, we optimized the following parameters i) SPE sorbent, ii) the composition, volume and flow rate of both washing and elution solvents, and iii) the injection volume.

3.1.1. Selection of extraction cartridge

In the optimization of an on-line procedure the first parameter to be investigated is the selection of SPE sorbent that depends on the nature of the matrix and the physical-chemical properties of the target analytes. Based on the results of our previous studies (Campone et al., 2015), three cartridges with different retention mechanisms were selected: an Oasis HLB (copolymer of divinybenzene and vynilpyrrolidone), an Oasis MAX (mixed-mode, reversed-phase/strong anion-exchange) and a Strata C18 silica column.

Initially, the capability of each cartridge in retaining OTA was studied by evaluating the extraction efficiency, which was calculated by comparing the peak area of the analyte injected into the SPE cartridge after the elution using MeOH 2% HCOOH with those obtained through direct injection of OTA standard solution in HPLC column and eluted with the same solvent and flow rate. For this purpose, the SPE cartridge was connected directly to the mass spectrometer. Following the injection (50 µL) of OTA standard solution (10 ng mL^{-1}) the analyte was loaded onto the cartridges with 1 mL of H_2O. Then we carried out two consecutive elution steps using 1 mL of MeOH and 1 mL of MeOH 2% HCOOH (flow rate of 0.5 mL min^{-1}) respectively. The results of the analysis show that when Oasis HLB or Strata C18 cartridges were used, most of the OTA (>95%) eluted with pure MeOH, conversely, when the Oasis MAX cartridge was used only a small amount of the analyte (<10%) was eluted with MeOH; therefore, the remaining part of OTA was eluted using MeOH 2% HCOOH (≈90%). This could be probably attributed to the interaction of OTA carboxylic group (pKa 3.3) (Campone et al., 2012) with the quaternary ammonium groups of sorbent material. Based on these results, we selected the Oasis MAX cartridge for further experiments in order to eliminate matrix interferents to the maximum possible during the washing step.

3.1.2. Selection of washing solvent

After selecting the extraction cartridge, we determined whether it could reduce the matrix components during the washing step, avoiding the loss of OTA. For this purpose, to select the best composition, volume and flow rate of the washing solvent, we evaluated the reduction of matrix interferents (UV trace) and OTA (MS signal) extraction efficiency % (EF%). To select the washing solvent, the cartridge was used as the chromatographic column; red wine (100 µL) was injected and the profile of interfering species eluted out during the washing step was monitored continuously in a UV detector (λ_{max} = 280 nm). The OTA extraction efficiency was calculated comparing the OTA peak area, eluted using MeOH containing 2% HCOOH, v/v, after the washing step with those obtained without the washing step, in order to evaluate the loss of analyte for each washing condition. MeOH and its different aqueous solution with 0.5% HCOOH (2.5 mL at flow rate of 0.5 mL min^{-1}) were tested as washing solvents. As shown in Fig. 1, the matrix band corresponding to the unretained components

were eluted completely from the SPE cartridge within the first 4 min of the loading step (2.5 mL H_2O at 0.5 mL min^{-1}) without any losses of OTA (Fig. 2). When MeOH was used as the washing solvent, the unionized and non-polar interfering species were eluted from SPE cartridge without causing significant losses of analyte (Fig. 2). In the case of the ionized interfering species, a significant removal of such species was achieved by increasing the percentage of acidified MeOH (0.5% HCOOH) in the washing solvent, as indicated by the UV signal (Fig. 1). Unfortunately, when the washing solvent composition exceeded the 20% MeOH (0.5% HCOOH), the extraction efficiency starts to decrease, become drastically over 60% MeOH (0.5% HCOOH) (Fig. 2). Based on these results, after loading of OTA (2 mL of H_2O), two washing steps (2.0 mL of MeOH and 2.0 mL of 20 % MeOH 0.5% HCOOH) were performed prior to the elution of analyte to reduce the significant amount of the interfering species of the matrix (Fig. 1A Supplementary Material) and the loss of the analyte was small (15% ± 7).

After carrying out the optimization of washing solvents, we evaluated the influence of the flow rate, in the range of 0.5–2 mL min^{-1} to reduce the analysis time. The results indicated that there was no observable loss of analyte when the flow rate was increased from 0.5 up to 1 mL min^{-1}; however, the extraction efficiency of OTA decreased slightly when the flow rate was increased within this range. Based on these factors, 1 mL min^{-1} was selected as the optimal condition for reducing washing time and avoiding losses of OTA (Fig. 1B Supplementary Material).

3.1.3. Selection of elution solvent and peak focusing optimization

After optimizing the washing step, we used the same gradient elution and analytical column of our previously application (Campone et al., 2015). Compared to the chromatographic peak obtained in our previous study (Fig. not shows), a significant difference was observed in the OTA peak shapes. With the use of Oasis MAX cartridge, the OTA peak shape was broader, this could probably be attributed to the fact that the analyte is eluted using a mobile phase containing a high concentration of acidified MeOH. Under such conditions, HPLC column was not able to elute OTA in a narrow and well-defined peak shape. Several analytical columns were tested to optimize chromatographic conditions but they did not produce any viable result. To overcome this problem, we studied the best elution solvents of OTA in order to improve the analyte refocusing on HPLC column. For this purpose, 100 µL of standard solution (50 ng mL^{-1}) was injected into the SPE cartridge, connected directly to the mass spectrometer using a 10-port switching valve. After completing the washing steps, the 10 port-valve was switched into inject position. Then, different aqueous mixtures of MeOH 0.5% HCOOH (0.5 mL min^{-1}), in the range from 0 to 100%, were used in back-flush elution mode to elute OTA from the SPE cartridge. After conducting 10 min of isocratic elution, the SPE cartridge was eluted with 4 mL MeOH 2% HCOOH to estimate the residue of analyte that was not eluted. As shown in Fig. 3, the affinity between OTA and the sorbent material is very strong; therefore, MeOH (0.5% HCOOH) solution concentrations that were lower than 60% could not efficiently elute the analyte from the cartridge in an acceptable time. By increasing the eluotropic strength of solvent elution (>60% MeOH 0.5%HCOOH) the analyte eluted effectively. The elution of OTA from the cartridge was highly efficient when the mobile phase contained more than 80% MeOH (0.5%HCOOH). Based on these results, 3.5 mL of 80% MeOH 0.5% HCOOH was selected as optimal elution solvent at flow rate of 0.5 mL min^{-1} was selected as good compromise to efficiently elute OTA in an acceptable time (7 min) avoiding that hydrophobic interferents that were still present in the cartridge were transferred into the analytical column. On the other hand, when the solvent containing high organic content is used to elute OTA, the refocusing of the analyte on top of the HPLC column does not produce a narrow band, resulting in broad and badly resolved peak. To overcome this limitation and to improve the focusing of analyte on top of chromatographic column, a stainless steel Tee mixer was inserted between the SPE cartridge and HPLC column. Water (A_L, flow rate of 1 mL min^{-1}) was mixed with the elution solvent (3.5 mL 80% MeOH 0.5% HCOOH) to reduce the organic content from 80% to ≈26%. With this configuration, the analyte could be refocused on the HPLC column successfully.

The modified configuration produces symmetric and sharp peak for OTA in less than 20 min (Fig. 4).

3.1.4. Optimization of injection volume

After optimizing the condition of the online SPE-HPLC analysis, we investigated whether the sensitivity of this method, could be improved by increasing the sample injection volume (Yan, Li, Zhao, & Lin, 2009). In this study, the impact of injection volume on the sensitivity and accuracy of the method was evaluated by carrying out analyses with sample volume in the range 100–400 μL. The slope of each curve, showed a good linearity ($R^2 > 0.999$) with solvent and matrix-matched in the evaluated injection volume range (Fig. 2 Supplementary Material). The analyte response increased proportionally with the injection volume and no matrix effects were observed (Fig. 2 Supplementary Material). These results indicate that the sensitivity of the proposed method could be increased without inducing the phenomena of matrix effects. Thus, we proved that the method has high efficiency in eliminating of matrix components during the washing step. However, OTA could be detected at levels lower than MLs when the sample injection volume was 100 μL, and an overload of SPE cartridge could also be avoided. Finally the carry over problems, occurring during the analysis of contaminate samples were investigated by processing spiked samples containing a high concentration of OTA (50 ng mL^{-1}). After three consecutive injections a blank solvent was processed and no signal in the retention time of OTA was obtained in the LC– MS/MS chromatograms.

3.2. Optimization of HPLC–MS/MS

As reported in our previous study, we selected an RP column (Fused-Core TM) with small particle size (2.6 μm) because it has high efficiency and enables the analysis time to be completed in a short time. Unfortunately, due to the strong interaction between the analyte and sorbent material of the SPE cartridge, a solvent with a high organic content is required to elute the analyte out of the cartridge. As a result, OTA could not be effectively focused on the UHPLC column, resulting in a broad peak shape. As described in Section 3.1, the "peak focus" configuration (diluting of elution solvent before HPLC column) was employed to overcome this problem. However, the main limitation of this strategy is to avoid an overpressure of the system during the dilution of elution solvent. This limitation is more pronounced in chromatographic columns having small particles in the stationary phase.

To maintain the backpressure of the system in an acceptable range, we replaced the column used in our previous study (Kinetex PFP 2.6 μm Fused-Core) (Campone et al., 2015) with a monolithic column, considering its efficiency to retain the analyte and its capability to work at a high flow rate. After selecting the chromatographic column, we optimized the solvent composition and the gradient for LC-MS/MS. Several gradients with different eluent mixtures (MeOH/H_2O, CH_3CN/H_2O and MeOH/CH_3CN/H_2O) and buffers (0, 1, and 2 mM of acetic acid, formic acid and ammonium acetate) were investigated. Solvent composition and the gradient reported in Table 1 provided, narrow peak, short analysis time and better ionization response.

3.3. Matrix effect evaluation

Matrix effects, such as the enhancement or suppression of analytical signals, are frequently observed in the quantitative LC–MS/MS analysis. This phenomenon either positively or negatively influences the ionization of the analyte in the HESI source (Cappiello et al., 2008, Niessen et al., 2006). To calculate the degree of ion suppression or enhancement, we evaluated the absolute matrix effects by comparing matrix-matched curves with the solvent curve (15% EtOH) in the same concentration range. The results are presented in Fig. 3 of the Supplementary Material. These results indicate that significant differences were not observed between the matrix-matched curves and solvent curve (ME 95–108%). It can be concluded that the absolute matrix effect was not observed. Furthermore, the relative ME (difference in the response between various samples of red wine) were evaluated, but the results did not indicate statistically significant RSD difference (data not shown). This implies that the developed method had the capability of removing co-eluted compounds during the washing step

avoiding the signal suppression or enhancement. Therefore, quantification can be performed using standard solutions, instead of matrix-matched curve.

3.4. Analytical performance

The proposed analytical procedure was validated in terms of selectivity, linearity, sensitivity, recovery, accuracy and precision. The validation of various parameters was done according to the guidelines mentioned in the European Commission Decision 657/2002 (Commission Decision 2002/657/EC 2002). Data acquisition was carried out in SRM mode that produced two characteristic fragments of $[M+H]^+$ ion of OTA. Furthermore, the SRM intensities ratio (I_1/I_2) was used as an additional identification criterion, with a tolerance of less than 10% of the expected ratio.
Selectivity was evaluated experimentally by monitoring the SRM transition of OTA in different non-contaminate samples, and no interfering peaks were observed in the elution region of OTA for all the tested matrices. The linearity range was estimated in solvent calibration curves (EtOH 15%) and in matrix-matched curves and OTA response was found to be linear in the concentration range from 0.5 to 5 ng mL^{-1} in both the samples, representing a linear model of the calibration curve, with a correlation coefficient >0.99 (ANOVA test). The sensitivity of method was experimentally estimated by analyzing spiked wine samples at the signal-to-noise ratio (S/N) of 3 and 10 for MLD and MQL, respectively. As shown in Table 2, the calculated values of MDL and MQL were much lower than the limit imposed by CE (Commission Regulation EC 1881/2006).

Recoveries were determined by processing wine samples spiked with three OTA levels (0.5, 2 and 5 ng mL^{-1}); each level was analyzed in triplicate, and the results were reported in Table 2. The recovery values and RSD (<7%) were in agreement with EU regulation (Commission Regulation (EC) 519/2014 Off J Eur Union) laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Precision and accuracy of the developed method were evaluating by processing reference material (RM) T1755 white wine in an optimized procedure. The results exhibit a good agreement between the value obtained (1.38 ng mL^{-1}, accuracy of 84.6% ± 4) and the certified value. In Fig. 4 were reported two representative chromatograms of naturally contaminated white wine sample (black line) reference material (FAPAS T1755 OTA 1.63 μg L^{-1}) and non-contaminated red wine respectively (red line).
Finally, the proposed procedure was used to analyze 58 commercial wines samples (41 red and 17 white wines) that were purchased from different local markets in the Campania region of Italy. The values obtained for OTA concentrations in the analyzed wines were listed in Table 1 of Supplementary material, and they are in agreement with the literature data (Dachery et al., 2016, Otteneder and Majerus, 2000):. White wines (17 samples) are generally less contaminated than red wine, which often contains a detectable OTA contents that is below the MQL. Among the 58 analyzed samples, no sample contained an OTA level higher than the maximum limit established by EU for wine (2 ng mL^{-1}). The highest value of OTA found in a sample was 0.270 ng mL^{-1}, which is approximately seven times lower than the EU limits.

4. Conclusions

The rapid and automated on-line SPE-HPLC–MS/MS that was developed and validated in this study allowed the sensitive, selective and reliable quantification of OTA in wine samples. By optimizing the main parameters, affecting extraction efficiency and the cleaning step of the SPE on-line cartridge, we made consistent efforts to increase the reproducibility of results in a short analysis time. The proposed method was validated using different wine matrices (red and white) in accordance with the guideline of CE. Its analytical performance fulfills the criteria of method recommended for the determination of mycotoxins in foodstuffs (EC Decision 657/2002 and Regulation 519/2014). The main advantages of the proposed method are the short analysis time enables a high-throughput analysis and the complete automation of analytical procedure, thereby reducing the manual procedures. In addition, the method showed better analytical performances when compared to other

conventional methods requiring hours for the completion of extraction and the sample treatment. In this method, the accurate determination of OTA was completed in less than 20 min. We conclude that the presented method can be considered a valid alternative to the conventional IAC methods.

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The authors declare that there is no conflict of interest.

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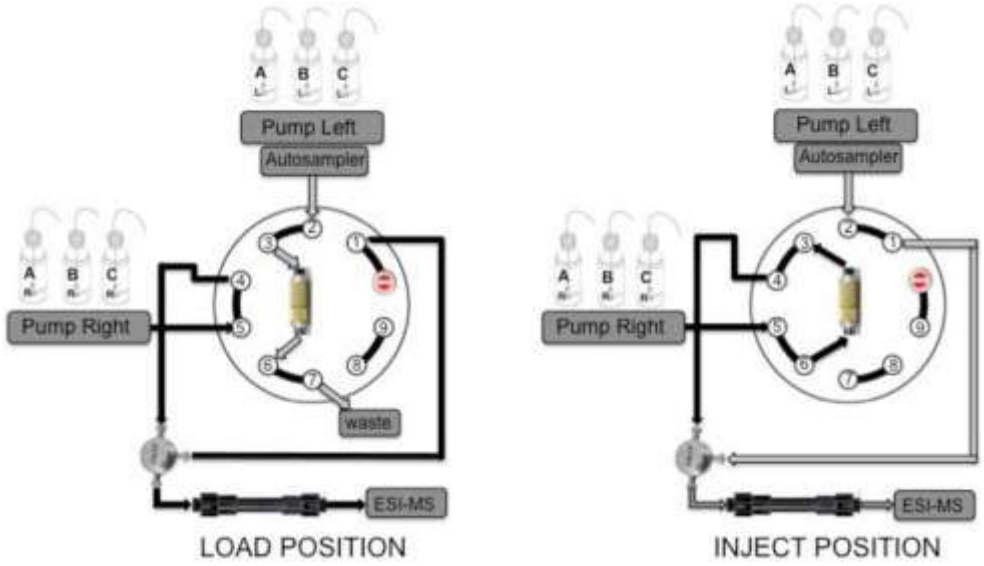
Table 1. Schematic diagram of on-line SPE-HPLC system with timetable of solvents and switching valve set up.



Pump left					**UHPL pump right C**					**Switching valve**	
Time (min)	**Flow (mL/min)**	**Solvent AL (%)**	**Solvent BL (%)**	**Solvent CL (%)**	**Time (min)**	**Flow (mL/min)**	**Solvent AR (%)**	**Solvent BR (%)**	**Solvent CR (%)**	**Time**	**Position**
−2.0	1.0	100	0	0	-2.0	0.5	0	98	2	0	Load
0.0	1.0	100	0	0	0.0	0.5	0	98	2		
2.0	1.0	100	0	0	7.5	0.5	0	98	2		
2.5	1.0	0	0	100	7.6	0.5	100	0	0	7.5	Inject
4.5	1.0	0	0	100	14.5	0.5	100	0	0		
5.0	1.0	0	100	0	15.0	0.5	0	98	2	15.5	Load
7.0	1.0	0	100	0	15.5	0.5	0	98	2		
7.5	1.0	100	0	0	17.0	0.5	0	65	35		
15.0	1.0	100	0	0	19.0	0.5	0	5	95		
16.0	0.1	100	0	0	22.0	0.5	0	5	95	19.5	Inject
19.0	1.0	100	0	0							
22.0	1.0										

A_L = H_2O A_R = 80%MeOH 0.5% HCOOH

B_L = 20%MeOH 0.5% HCOOH B_R = H_2O 0.1% HCOOH

C_L = MeOH C_R = CH_3CN 0.1% HCOOH

Ù

Table 2. Analytical performance of proposed method in solvent solution and in wine samples.

	MDL	**MQL**	**Recovery ± SD[a] (intra-day precision, RSD[b]) (n = 3)**		
Samples			**Level (ng mL^{-1})**		
	ng mL^{-1}		**0.5**	**2.0**	**5.0**
Red wine	0.014	0.045	80 ± 2 (5)	87 ± 5(3)	82 ± 5 (3)
White wine	0.012	0.041	85 ± 4 (6)	82 ± 6 (6)	88 ± 4 (5)
EtOH 15%	0.010	0.030	83 ± 2 (3)	83 ± 3 (5)	85 ± 2 (5)

a. SD: standard deviation.
b. RSD: relative standard deviation.

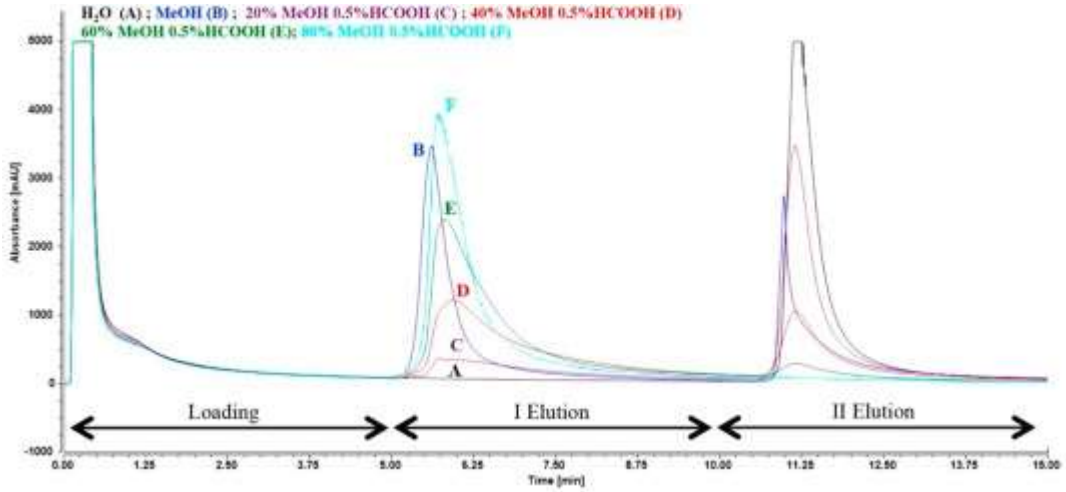


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Fig. 1. UV profile (280 nm) of matrix interferences using different washing online-SPE solvent compositions. Experimental conditions: injection volume, 100 µL of red wine; flow rate, 0.5 mL min^{-1}; loading: 2.5 mL H_2O (0–5 min); washing: 2.5 mL (5–10 min); elution; 2.5 mL MeOH with 2% HCOOH (10–15 min).

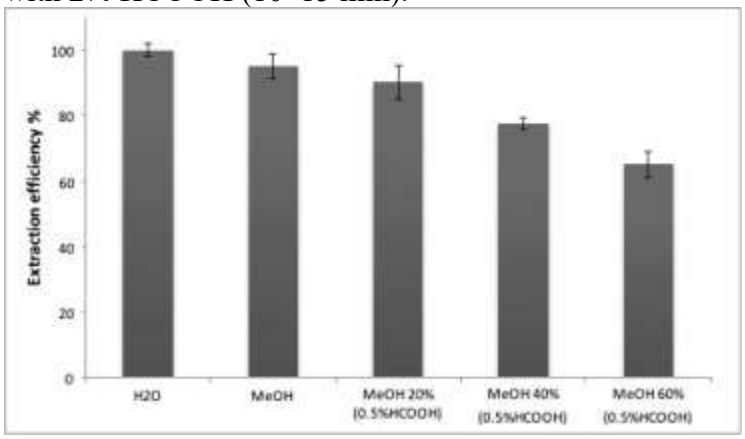

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Fig. 2. OTA extraction efficiency after different washing solvents. Experimental conditions: injection volume, 100 µL OTA 10 ng mL^{-1}; flow rate, 0.5 mL min^{-1}; loading: 2.5 mL H_2O; washing: 2.5 mL; elution; 2.5 mL MeOH with 2% HCOOH.; Error bars mean ± SD (n = 3).

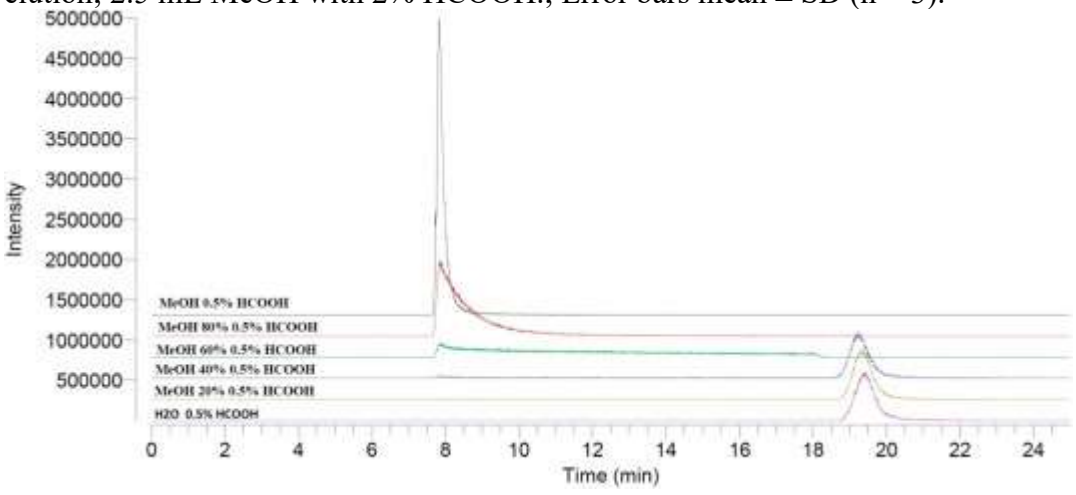

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Fig. 3. Mass spectrometry signal of OTA using different elution solvents composition. Experimental conditions: injection volume, 100 μL OTA 50 ng mL^{-1}; flow rate, 0.5 mL min^{-1}; optimized washing, 0–7 min at flow rate 1 mL min^{-1}; elution I, 7–17 min at flow rate 0.5 mL min^{-1}; elution II, 17–25 min, MeOH 2% HCOOH at flow rate 0.5 mL min^{-1}.

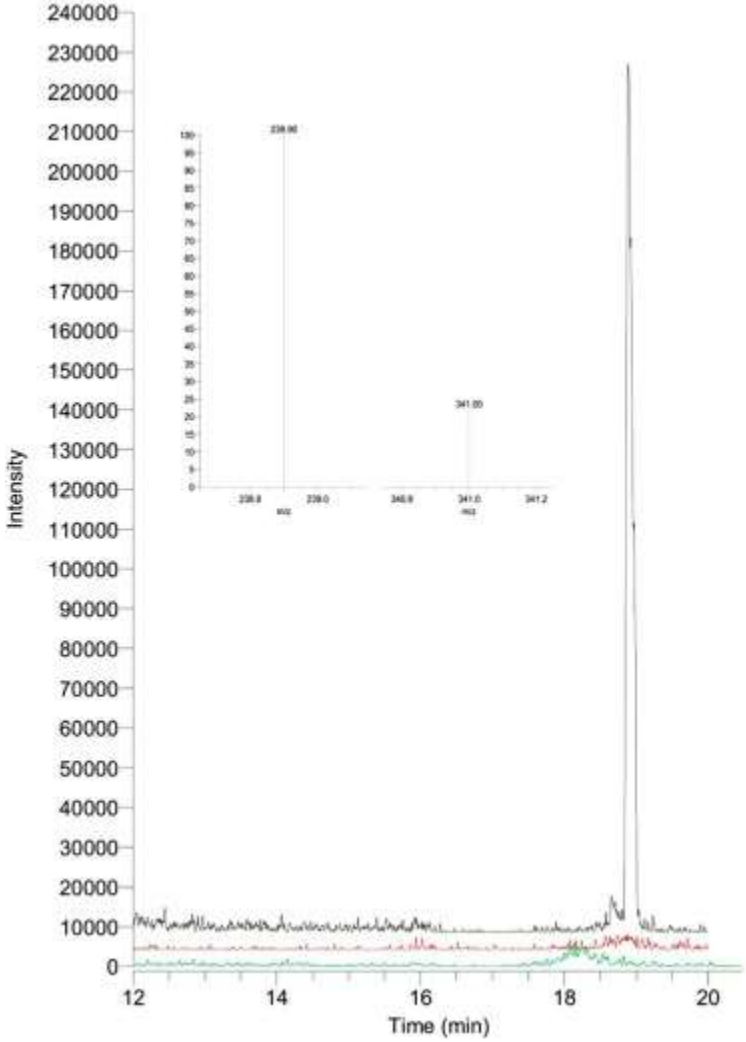

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Fig. 4. Selected reaction monitoring chromatograms of on-line SPE–HPLC of naturally contaminated wine sample near to EU ML for white wine samples (RM Fapas 1.63 μg L^{-1}) (black line), non-contaminated red wine (red line) and blank sample after three repeated injection of OTA at high concentration (50 ng mL^{-1}; green line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)