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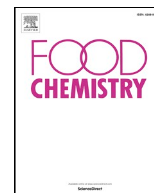
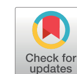


Determination of mycotoxins in beer by multi heart-cutting two-dimensional liquid chromatography tandem mass spectrometry method



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ABSTRACT

Beer is one the most consumed alcoholic beverage in the world and its contamination with mycotoxins is of public health concern. This study reports a fast and automated analytical procedure based on a multi-heart-cutting two-dimensional liquid chromatography tandem mass spectrometry method using electrospray ionization for the determination of seven mycotoxins (aflatoxins B1, B2, G2 and G1, ochratoxin A, fumonisins B1 and B2) in beers. The developed method was based on the heart-cutting 2D- HPLC technique in which only the specific portions of the first dimension, in the retention time of analytes, were transferred into the second dimension for the further separation and successive determination. The method uses two different chromatographic columns; in the first dimension, 50 µL of sample was injected on first column, and mycotoxins elution regions were collected in a loop and transferred into the second column for the separation of analytes. Each column operated in gradient elution mode in order to eliminate interfering compounds and improve separation and peak shape. After the optimization, the method has been validated according to EU regulation and finally applied for the analysis of forty beer samples collected from Italian supermarkets. Among all mycotoxins studied, fumonisins B1 was the most widely distributed in analysed beers (> 21%) in the range from 0.6 to 12.3 ng mL^{-1}. The automated methodology developed was able to determine accurately and simultaneously seven mycotoxins in beer. This provided a significant reduction of sample handle and, consequently of analysis time.

1. Introduction

Mycotoxins are a heterogeneous group of toxic secondary metabolites with a low molecular weight produced by several filamentous fungi as *Aspergillus*, *Penicillium and Fusarium* genera (Murphy, Hendrich, Landgren, & Bryant, 2006). These natural compounds are synthesized under particular climatic conditions and, after ingestion, can cause several diseases in animals and humans. Among all mycotoxins, those that have the highest distribution in foods and greatest influence on agroeconomic and public health are mainly aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON), nivalenol (NIV), fumonisins B1 and B2 (FB1; FB2) and zearalenone (ZEN) (Murphy et al., 2006). Mycotoxins have been associated with human and animal diseases and, for this high toxicity, AFB1 has been classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (group 1) (IARC, 1993) while OTA and FB1 into group 2B as a possible human carcinogen (IARC, 2012). Other toxic effects related to mycotoxins intake include: hepatotoxicity, nephrotoxicity, neurotoxicity, immunosuppressive effects endocrine dysfunction, weight loss and malnutrition (Marroquín-Cardona, Johnson, Phillips, & Hayes, 2014). Mycotoxins have been reported in several commodities and products used as food and feed ingredients such as cereals, milk and derivatives, coffee, wine, beer, vegetables, and dried fruits (Marin, Ramos, Cano-Sancho, & Sanchis, 2013). Beer is one of the most alcoholic beverages consumed worldwide (Organization, 2004); recent available data reported an average annual consumption per capita of about 70 l in Europe, up to over 100 l in some countries (Organization, 2004). Therefore, due to its high consumption, beer can be considered an important dietary mycotoxins intake; nonetheless, maximum residue limit for mycotoxins in beer has not yet been defined. The European Commission has established maximum limits only for raw materials used for beer production (Commission Regulation (EC) 1881/2006). In particular, the maximum

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limits allowed for cereal based products including beer are: 400 μg kg^{-1} for the sum of FB1 and FB2, 5 μg kg^{-1} for OTA, 2 μg kg^{-1} for AFB1 and 4 μg kg^{-1} for total AFs (Commission Regulation (EC) 1881/2006). Many studies have been carried out on the fate of mycotoxins during the malting and brewing processes (Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Piacentini et al., 2019; Scott, 1996), and results demonstrate that mycotoxins could be transmitted from contaminated raw materials: barley, malt, hops into beer, as a consequence of their moderate stability during food processing (Bullerman & Bianchini, 2007; Milani & Maleki, 2014) and good water solubility (Inoue et al., 2013). The occurrence of mycotoxins in beer and brewing product have been frequently reported by several authors; in particular, several studies have been published concerning fusarium toxins (DON FB1 and FB2) (Piacentini et al., 2017, 2018; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). OTA has been repeatedly detected in beer samples (E. M. Mateo, Gil-Serna, Patiño, & Jiménez, 2011; Mateo, Medina, Mateo, Mateo, & Jiménez, 2007), whereas the information regarding AFs contamination are still not clear. Common analytical methods for determination of mycotoxins in complex matrices are based on the isolation of the analytes using immobilized antibodies, the so-called immunoaffinity columns (IACs), followed by an HPLC separation coupled to fluorescence detection (FLD). The immunoaffinity procedures are highly selective but unfortunately they are not able to analyse different class of mycotoxins in a single analysis. In order to overcome this limitation the analytical techniques have been made great progress. Recently, several sample preparation techniques such as QuEChERS, (Tamura, Uyama, & Mochizuki, 2011) dispersive liquid-liquid microextraction (DLLME) (Antep & Merdivan, 2012), solid phase extraction (SPE) (Romero-González, Martinez Vidal, Aguilera-Luiz, & Garrido Frenich, 2009; Ventura et al., 2006), and enzyme-linked immunosorbent assay (Kuzdraliński, Solarska, & Muszyńska, 2013) have been employed for the analysis of multi-mycotoxins in beer, obtaining satisfactory results. However, these methods demand highly qualified personnel and have numerous disadvantages in terms of time, cost and materials consumption. In addition, they are impossible to automate and consequently, they have low sample throughput, generating a bottleneck of whole analytical procedure. Therefore, in order to increase occurrence data and clarify mycotoxins distribution in beers, the development of quickly, sensitive, accurate and automated analytical methods for the analysis of mycotoxins became necessary (Rodríguez-Carrasco et al., 2015). Among them, on-line solid phase extraction methods are currently the main automated samples preparation used for the analysis of mycotoxins in complex matrices. In our previous study, the automated on-line SPE has been successfully applied for the analysis of AFM1 in milk (Campone et al., 2017, 2016), AFs and OTA in cereal products (Campone et al., 2015), and OTA in wine (Campone et al., 2018). These methods allowed to increase samples throughput and, at same time, improve sensitivity if compared to the off-line method. Although automated on-line SPE is quite universal and capable to multiple analyte analysis, this technique mainly uses the first dimension (cartridge) to pre-concentrate analytes, improving the enrichment factor. A good alternative which fully utilizes the selectivity and separation efficiency of both chromatographic columns is the two-dimensional liquid chromatography (2D-LC), in which the sample is subject to two different separations (Mondello et al., 2008; Tranchida, Franchina, Dugo, & Mondello, 2016; Tranchida, Dugo, Dugo, & Mondello, 2004). When first and second dimension use totally different separation mechanisms (Orthogonal), the main advantage is the potential elimination of co-eluting components. Depending on the number of fractions transferred from the first to the second dimension, 2D-LC can be classified as comprehensive two-dimensional liquid chromatography (LC × LC) and heart-cutting liquid chromatography (LC–LC). In comprehensive liquid chromatography (LC × LC), every part of the sample is subjected to both separations (Mondello, Dugo, Kumm, Cacciola, & Dugo, 2010), on the other hands, in heart-cutting chromatography (LC-LC), just some selected number of 1D-fractions is subject to separations in second dimension (Breidbach & Ulberth, 2015). In both approaches, two independent HPLC pumps connected two chromatographic columns by using a six or ten-port switching valve. On the basis of the method used to transfer the analytes from the first to the second dimension, three main methods can be distinguished: (i) direct transfer, (ii) loop transfer, (iii) cartridge trapping transfer (León-González, Rosales-Conrado, Pérez-Arribas, & Guillén-Casla, 2014). Among the aforementioned methods, loop transfer is actually the most widely used for its high versatility; in this setup, the fractions from the first dimension are eluted into one or several loop(s) before being transferred into the second column. This configuration has been commonly used in LC-LC for trace analysis, where only few fractions of the first dimension are usually of interest.

In this study, the development of on-line heart-cutting chromatography MS/MS method for the analysis of four aflatoxins (AFB1, AFB2, AFG2, and AFG1), two fumonisins (FB1 and FB2), and ochratoxin A, in beer samples at the sub-ng L^{-1} level was reported. The developed procedure is based on an automated LC-LC, achieved using a C8 column in the first dimension and a kinetex biphenyl column in second dimension, coupled with a triple quadrupole mass spectrometer through a two-position 10 port switching valve. This configuration allows to perform automatically the determination of target analytes in less than 30 min. After the optimization of the method, a validation study was carried out according to EU regulation 401/2006 (Commission Regulation (EC) 401/2006) and finally applied to forty beer samples. The developed methodology could be used in routine analysis as a valid alternative to conventional methods commonly used for the analysis of aflatoxins (AFB1 AFB2 AFG1 and AFG2), ochratoxin A (OTA), and fumonisins (FB1, FB2) in beers. The main advantages of the developed method are the improvement of analytical sensitivity, accuracy, and precision, owing to the automated online process with less human errors. This reduces consumption of organic solvent, analysis time, and samples manipulations, avoiding cross contamination of real samples.

2. Experimental

2.1. Standards and materials

MS-grade water (H_2O) and methanol (MeOH), were supplied by Romil (Cambridge, UK). HPLC-grade ethanol (EtOH) was obtained from Sigma-Aldrich (Milan). Ammonium formate (NH_4HCO_2, purity > 99.0%) and formic acid (HCOOH) were purchased from sigma Sigma-Aldrich (Milan). 18.2 MΩ ultrapure water was obtained by a Milli-Q purification system (Millipore, Bedford, USA). Reference standard solution of aflatoxin mixture (AFB1 and AFG1 2 μg mL^{-1}; AFB2 and AFG2 0.5 μg mL^{-1}) and OTA (10 μg mL^{-1}) were obtained from LGC promochem GmbH (Labservice analytica, Bologna Italy). Fumonisins mixture (FB1 and FB2) (50 μg mL^{-1}) was supplied by Riedel-de Haën (Germany). Stock solution of mycotoxins mixture used in spiking procedure and in calibration solutions was prepared in EtOH 10% w/w in order to simulate the alcohol composition of beer, and stored in a glass vial at −20 °C.

A comparative study of several chromatographic columns, used in first and second dimension, was carried out; Biobasic C8 (150 × 2.1 mm, 2.6 μm), Fusion RP (75 × 2.00 mm, 4 μm), Gemini (50 × 2.00 mm, 3 μm) Kinetex C18 (50 × 2.1 mm, 2.6 μm Phenomenex, Bologna, Italy) were tested in the first dimension. Kinetex HILIC (50 × 2.10 mm, 1.7 μm), Kinetex PFP (50 × 2.1 mm, 2.6 μm), Kinetex C8 (50 × 2.10 mm, 1.7 μm), Kinetex C18 (50 × 2.10 mm, 1.7 μm) Kinetex Biphenyl (50 × 2.1 mm, 2.6 μm) were tested in the second dimension.

2.2. Samples

A total of 75 samples of commercial beer cans and bottles (alcohol content from ≅5 to 10%) consisting of several commercial brand,

produced in different countries (see Table 1S) were analysed. These samples were collected between July 2018 and December 2019 from Italian markets (supermarkets, restaurants and pubs) and stored at room temperature until the analysis. Before the injection, each sample was degassed in ultrasonic bath for approximately 5 min in order to remove CO_2 and, if necessary was centrifuged for 3 min. at 14,000 × g to remove foam and particulates (IEC-CL30R, Thermo Electron Corp., Milan, Italy). To ensure the absence of target compounds in beers utilized during the development and validation studies, samples were previously analysed used method by Nakajima, Tsubouchi, and Miyabe (1999) for aflatoxins and ochratoxin A, whereas the determination of fumonins were carried out according to Scott and Lawrence (1995). Spiked samples were prepared by adding specific volumes of stock solutions mixtures (AFB1 and AFG1 200 ng mL^{-1}; AFB2 and AFG2 50 ng mL^{-1}; OTA 200 ng mL^{-1}; FB1 and FB2 200 ng mL^{-1}) to achieve the required contamination levels.

2.3. Equipment

The on line 2D system used for chromatographic separation consisted of an Ultimate 3000 (Thermo Electron Corp.) equipped with dual ternary gradient pumps, a vacuum degasser, an autosampler, column thermostat, and UV detector. The two columns used for bi-dimensional separation were connected via an electronically 10 port two position valve Rheodyne®. An additionally, six port two position switching valve was used to deliver chromatographic flow to the H-ESI mass spectrometry only during the elution of analytes. The chromatographic system was coupled to an UV detector at 220 nm in order to monitor the interfering compounds, whereas the detection of analytes was obtained using a TSQ Quantum Ultra (Thermo Electron Corp.) triple quadrupole mass spectrometer. In the optimized procedure, the Biobasic C8 (150 × 2.1 mm, 2.6 µm particle size) kept at 50 °C was employed in the first dimension, and the Kinetex Biphenyl (50 × 2.1 mm, 2.6 µm) was used as column in the second dimension. The mobile phase of the left pump, and the right pump consisted of water (A_L and A_R) and MeOH (B_L and B_R), both with 0.1%, v/v, HCOOH and 2 mM NH_4HCO_2. After the sample injection (50 µL), the regions containing the analytes were eluted into the loop (850 µL), and then, by changing the position of the valve, the loop was connected to the second column, and the analytes were transfer on top of the second column. In the multiple heart-cutting mode, two segments of the 1-dimension eluate were transferred into the 2-dimension column. A schematic diagram of system and a detailed time schedule for column 1 and column 2, flow rate switching vale position and chromatographic gradient are reported in Fig. 1. Chromaleon software (ver. 7.1.2) was used to manage the Ultimate 3000 system.

2.4. HESI-MS/MS conditions

A TSQ Quantum Ultra (Thermo Electron Corp.) triple quadrupole mass spectrometer equipped with a heated electrospray ionization source (H-ESI) operated in positive ionization mode was used for the detection of analytes. In order to optimize H-ESI parameters and to select MS/MS transitions, an infusion of each standard solution (5 µg mL^{-1}; flow rate of 25 µL min^{-1}) was carried out using syringe pump integrated in mass spectrometer. The optimized MS conditions were as follow: spray voltage 2.4 Kv, vaporized temperature 250 °C, ion transfer tube temperature 300 °C. Nitrogen (purity > 99.98%) was used as sheath gas and auxiliary gas at flow rates of 30 and 5 (arbitrary units) respectively. The mass spectrometer analyser operated in multiple reaction monitoring (MRM) mode, using argon (99.9999% purity) as collision gas (CID) at 1.0 mTorr. The selected MS/MS transitions, tube lens, and optimized collision energy (CE) for each analyte are reported in Table 1. Instrument control and data analysis were performed using Xcalibur software (version 2.2 Thermo Electron Corp.).

2.5. Method performance and matrix effect evaluation

The recovery of the whole procedure was evaluated by analysing beer samples spiking with standard mixture of analytes at three concentration, each of them was analysed in triplicate. The calibration curve obtained by plotting mycotoxins peak area versus concentration (ng mL^{-1}) were constructed diluting appropriate volume of stock solution mixture, in EtOH 10% (solvent curve) and in non-contaminate beer (matrix-curve). The linearity of the solvent curve and of the matrix curve were evaluated at six levels in range of 1–50 ng mL^{-1} for the aflatoxins B1, and G1, fumonisins FB1, FB2 and ochratoxin A OTA; whereas for aflatoxins B1 and G1 in range of 0.25–12 ng mL^{-1}. The ANOVA test was carried out in order to check the linearity.

Method detection limits (MDL) and method quantification limits (MQL) were estimated using analyte-free beer spiked at low levels (0.05, 0.1 e 0.5 ng mL^{-1}). Each level was processed through the optimized analytical procedure; the MDL and MQL were calculated through the extrapolation of the concentrations, giving a signal-to-noise ratio (S/N) of 3 and 10, respectively from a linear regression (S/N versus concentration). The matrix effect (signal suppression or enhance) was evaluated by comparing the slope of solvent curve with the matrix curve, in the same concentration range, obtained using the developed method.

3. Results and discussion

3.1. HPLC-MS/MS conditions

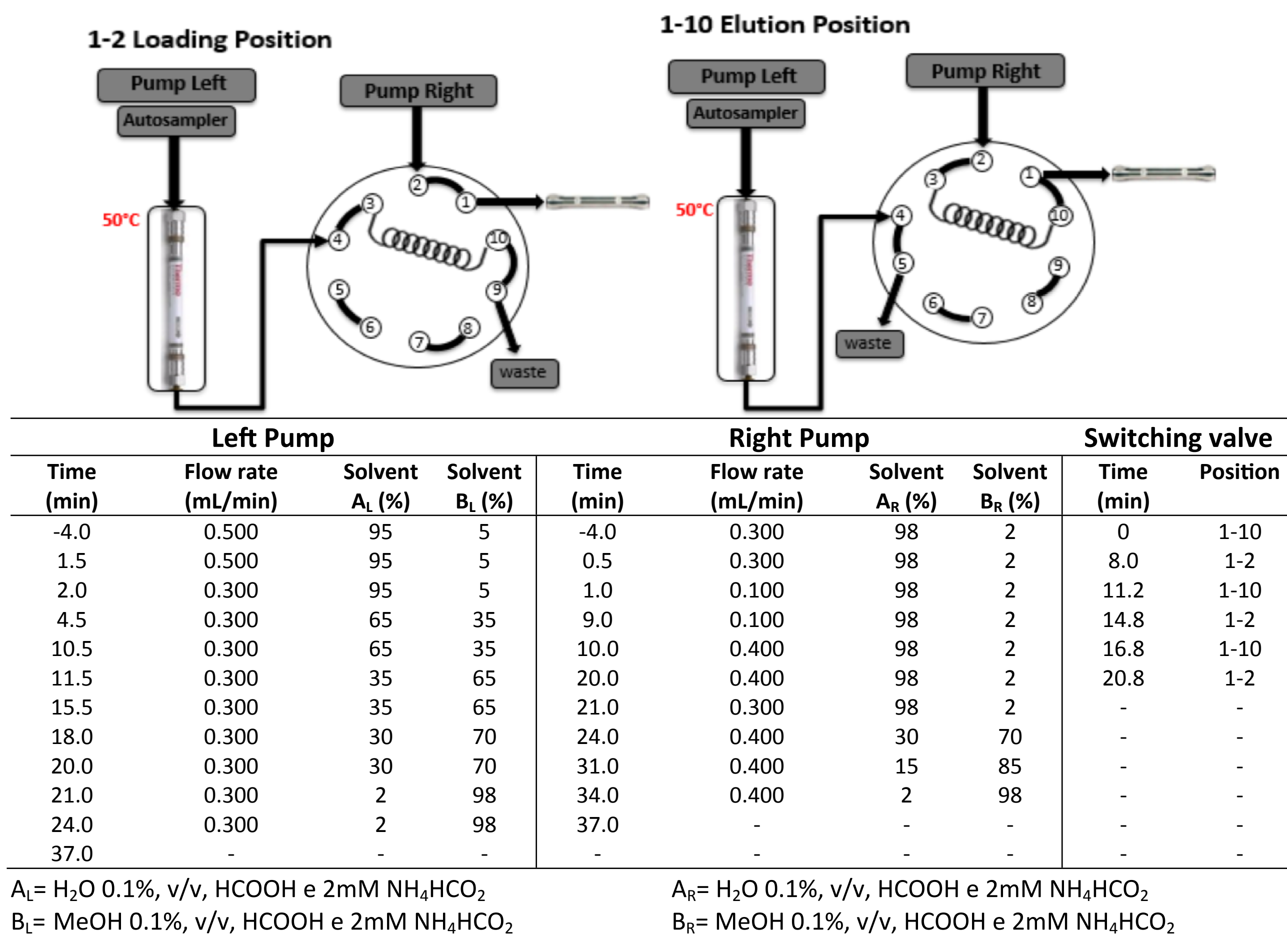
In order to obtain the most intense ionization response, detection of mycotoxins was tested in positive and negative mode. Since in the negative mode de-protonated molecule $[M-H]^-$ ion was slightly intense in case of OTA, the use of positive ionization mode was preferred for all the analytes. This allowed to avoid the switching of mass spectrometry polarity during the acquisition time. The selection of H-ESI parameters and of MS and MS/MS product was carried out following the same procedure used for our previous study (Campone et al., 2018). Briefly, the selection of best H-ESI conditions were carried out through the infusion of standard solution of each analyte at concentration of 5 µg mL^{-1}. Optimized H-ESI parameters and MRM transitions are summarized in Table 1. Subsequently, the influence of the mobile phase composition on the ionization efficiency of analyte was studied. The experiments were performed through the injection of a standard mixture (10 ng mL^{-1}) on a Kinetex C18 (50 × 2.1 mm, 2.6 µm) using a linear gradient. Among all the tested mobile phases and buffer combinations, the better result on the ionisation efficiency of the H-ESI ion source for all analytes was obtained using water and MeOH both with 0.1% formic acid and 2 mM ammonium formate (data not show).

3.2. Selection of first dimension column 1D-LC

The selection of the most appropriate chromatographic column to use in the first dimension (1D) was carried out considering three main characteristics:

(i) High load capacity, in order to allow the injection of high sample volumes without losing the separation efficiency of the analytes from the interferents;
(ii) Particle size > 2 µm, to avoid the clogging of the chromatographic column after the injections of few samples, with an increase of the back pressure;
(iii) Retention factor of the analytes (k') lower than the second dimension, in order to facilitate the focusing of the analytes in the second dimension, generating narrow and symmetrical peaks.

On the basis of these characteristics, four columns: Biobasic C8 columns (150 × 2.10 mm, 2.6 µm), Fusion RP (75 × 2.00 mm, 4 µm),



Left Pump				Right Pump				Switching valve	
Time (min)	Flow rate (mL/min)	Solvent A_L (%)	Solvent B_L (%)	Time (min)	Flow rate (mL/min)	Solvent A_R (%)	Solvent B_R (%)	Time (min)	Position
-4.0	0.500	95	5	-4.0	0.300	98	2	0	1-10
1.5	0.500	95	5	0.5	0.300	98	2	8.0	1-2
2.0	0.300	95	5	1.0	0.100	98	2	11.2	1-10
4.5	0.300	65	35	9.0	0.100	98	2	14.8	1-2
10.5	0.300	65	35	10.0	0.400	98	2	16.8	1-10
11.5	0.300	35	65	20.0	0.400	98	2	20.8	1-2
15.5	0.300	35	65	21.0	0.300	98	2	-	-
18.0	0.300	30	70	24.0	0.400	30	70	-	-
20.0	0.300	30	70	31.0	0.400	15	85	-	-
21.0	0.300	2	98	34.0	0.400	2	98	-	-
24.0	0.300	2	98	37.0	-	-	-	-	-
37.0	-	-	-	-	-	-	-	-	-

A_L= H_2O 0.1%, v/v, HCOOH e 2mM NH_4HCO_2
B_L= MeOH 0.1%, v/v, HCOOH e 2mM NH_4HCO_2
A_R= H_2O 0.1%, v/v, HCOOH e 2mM NH_4HCO_2
B_R= MeOH 0.1%, v/v, HCOOH e 2mM NH_4HCO_2

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

Table 1
Mass spectrometry optimized parameters for determination of mycotoxins in beer.

Mycotoxin	Parent ion $[M+H]^+$	MS/MS	CE	Tube Lens
AFB1	313.09	241.22	42	130
		285.00	30	130
AFB2		243.02	40	130
	315.10	259.07	37	130
		287.00	32	130
AFG1		243.13	35	130
	329.09	282.90	25	130
		311.00	20	130
AFG2		245.10	35	130
	331.10	257.00	35	130
		313.00	30	130
OTA	404.13	238.90	40	130
		358.00	20	130
FB1	722.50	334.40	39	130
		352.40	39	130
FB2		318.40	36	167
	706.50	336.40	36	167
		354.50	31	167

Gemini (50 × 2.00 mm, 3 µm), and Kinetex C18 (50 × 2.10 mm, 2.6 µm) were tested, by performing a linear gradient from 2 to 98% of mobile phase B (MeOH 0.1%, v/v, HCOOH and 2 mM of NH_4HCO_2) in 30 min. These columns provided the same elution order FB1, FB2, AFG2, AFG1, AFB2, AFB1, and OTA but different retention factors (K'). As showed in Table 2, the Biobasic C8, due to the lower lipophilicity of the stationary phase, provided the lowest retention factors k' for all target analytes. This property enables to elute the analytes with a lower percentage of organic solvent, allowing a better refocusing into the second dimension, an essential feature in the development of two-dimensional method. Based on this result and considering its high load capacity and the totally porous particles, Biobasic C8 was selected as 1D column and used for further experiments.

Subsequently, the influence of 1D column temperature on analyte separation has been optimized in order to further reduce the organic solvent required to elute the analyte. This ensured a better refocus in the second dimension, generating narrower and symmetrical peaks. For this purpose, the same linear gradient previously used in the selection of 1D column was performed by changing the temperature from 25 to 50 °C. As expected, increasing the temperature of the 1D column, a lower organic content to elute the analytes was required. Consequently, a further decreasing of retention factors for all analytes occurred. This phenomenon decreases the analysis time and promotes the focus of analytes on top of the column used in the second dimension, whereby the column temperature of 1D was kept constant at 50 °C.

3.2.1. Selection of second dimension column 2D

The selection of column used in second dimension is the key factor in the development of the 2D multiple heart cutting chromatography method. The main aim of the second dimension is, not only the removal of rest matrix components from analytes, which the first dimension was not able to remove, but also the separation of analytes from each other for an accurate qualitative and a quantitative analysis. The choice of the 2D column was performed considering the following characteristics:

(i) High efficiency and narrow particle size (< 3µm), in order to

Table 2
Mycotoxins retention factors k′ of tested chromatographic column under the same elution conditions.

	1D Column				2D Column			
Mycotoxin	Biobasic C8*	Fusion RP	Gemini	Kinetex C18*	Kinetex PFP	Kinetex C8**	Kinetex C18**	Kinetex Biphenyl
AFB1	7,8	13,2	19,5	15,4	24,2	21,6	22,9	35,7
AFB2	7,4	12,8	18,7	14,9	23,4	20,7	22,1	34,2
AFG1	7,2	12,3	18,0	14,2	22,8	19,9	21,2	33,7
AFG2	6,7	11,9	17,2	13,7	21,9	19,0	20,3	31,9
OTA	10,2	16,6	25,6	19,9	29,0	28,9	30,3	35,3
FB1	9,8	15,8	22,8	18,6	27,6	27,2	28,8	27,5
FB2	10,9	17,7	25,6	20,7	30,2	30,5	32,2	30,5

* Particle size 2.6 µm.
** Particle size 1.7 µm.

separate the analytes and matrix interferents
(ii) Different separation mechanism compared to 1D column (orthogonal)
(iii) Retention factor k' higher than the one of first-dimension column, to allow the focusing of the analytes, avoiding asymmetric and broad peak shape.

On the basis of these characteristics, five columns were tested: Kinetex HILIC (50 × 2.10 mm, 1.7 µm), Kinetex PFP (50 × 2.1 mm, 2.6 µm), Kinetex C8 (50 × 2.10 mm, 1.7 µm), Kinetex C18 (50 × 2.10 mm, 1.7 µm), and Kinetex Biphenyl (50 × 2.1 mm, 2.6 µm). The experimental conditions were the same performed for the selection of first column. The results show that, when a Kinetex HILIC column was used, analytes were not retained by stationary phase and, eluted in dead time. Among all the other columns that were used, the Kinetex Biphenyl gave higher values of retention factor k' compared to the other columns (Table 2); this result points out that the interaction of analyte with the stationary phase was very strong. Consequently, a high percentage of organic solvent was necessary to elute analytes. This strong interaction facilitates the re-focusing of polar analytes on top of the 2D column, generating narrow and symmetrical peaks; thus, kinetex biphenyl was selected as 2D column for further experiments.

3.3. Selection of chromatographic gradient

Once selected and coupled the two columns, using a 10-port switch valve as show in Fig. 1, the optimization of the chromatographic gradients for both pumps were carried out. The optimization of chromatographic gradient of both dimensions is extremely important in order to achieve the analytes separation, avoid the co-elution with matrix interferents, and reduce the analysis time. Moreover, quantitative transfer of analytes, peak symmetry, and the reduction of matrix effects phenomena have been also considered. For this purpose, the optimization of chromatographic gradients, in first and second dimension, were carried out evaluating simultaneously, the matrix effect phenomena and interfering compounds, monitoring the UV chromatogram at 220 nm (Fig. 2). Matrix effect experiments were conducted in triplicate by comparing the peak area obtained analysing a standard mixture solution (EtOH 10%, v/v) and free analyte beer sample spiked at same concentration (5 ng mL^{-1}). The results showed that the optimization of chromatographic gradients in the first and second dimension strongly affected the shift of matrix interferents from the elution zone of the analytes. In particular, looking at the UV profile obtained performing linear gradients (2–98% of MeOH) in both dimensions (Fig. 2a), most of the interfering compounds with medium polarity elute in the retention time zone of analytes, generating an intense signal suppression (data not shown). On the other hand, the optimization of chromatographic conditions provides a significant reduction in the coelution of interfering compounds (Fig. 2b) which results in a considerable reduction of matrix effect.

Once the chromatographic gradients and flow rate of both dimensions have been optimized, the multiple heart cutting time windows of the switching valve was carefully selected considering, simultaneously, the retention time of analytes and loop volume. The selection of an optimal heart-cutting window reduces the amount of interfering compounds transferred into the second column and, at same time, avoids the analytes loss.

Under optimized conditions, narrow and symmetrical peaks of target analytes were obtained (Fig. 3), and no loss of analytes and no retention time changes were observed, even after more than 300 injections. Finally, another great advantage of the developed method was, that ESI source of mass spectrometer did not get dirty due to elution of matrix interfering even after 100 injection compared to mono dimensional analysis (Fig. 1S).

3.4. Selection of injection volume

One of advantage of the on-line sample preparation techniques, compared to the off-line methods, is the possibility to increase the method sensitivity by increasing the injection volume. In this regard, the influence of different injection volumes on analytes peak area response, for solvent and spike beer (blonde beer) at concentration of 5 ng mL^{-1}, was investigated in the range of 25–200 µL. The results showed that the analyte response increases linearly ($R^2 > 0.99$) by increasing the injection volume in the range from 25 to 100 µL, for target analytes, in both solvent and spiked beer. Increasing the injection volume over 100 µL, a decrease of MS peak area response for AFB2, AFG2, FB1 and FB2 were noticed in spiked samples ($R^2 < 0.96$), whereas in the solvent MS peak area response still remained linear (Fig. 2S). These results demonstrate that most of the matrix compounds were removed in first dimension. However, increasing the injected matrix over 100 µL the 1D column was not able to remove all the interferences, generating a signal suppression. Furthermore, a high sample load decreases both the performance and column lifetime, with a negative impact on reproducibility of analytes retention time. On the basis of this results, injection volume of 50 µL was selected as the best compromise to obtain good sensitivity, avoiding matrix effect phenomena.

3.5. Matrix effect evaluation

During the development of quantitative LC-MS/MS method for the analysis of trace contaminants in complex samples, the matrix effect is considered one of the major drawbacks (Cappiello et al., 2008; Niessen, Manini, & Andreoli, 2006). The effect of co-eluting compounds arising from the matrix can result in signal enhancement or suppression; this affects the reproducibility and accuracy of the developed method. In order to evaluate the possible occurrence of matrix effects, MS/MS peak area of solvent curve EtOH 10% (1–50 ng mL^{-1} for AFB1, AFG1, OTA, FB1, FB2, and 0.25–12.50 for AFB2 and AFG2) was compared to those

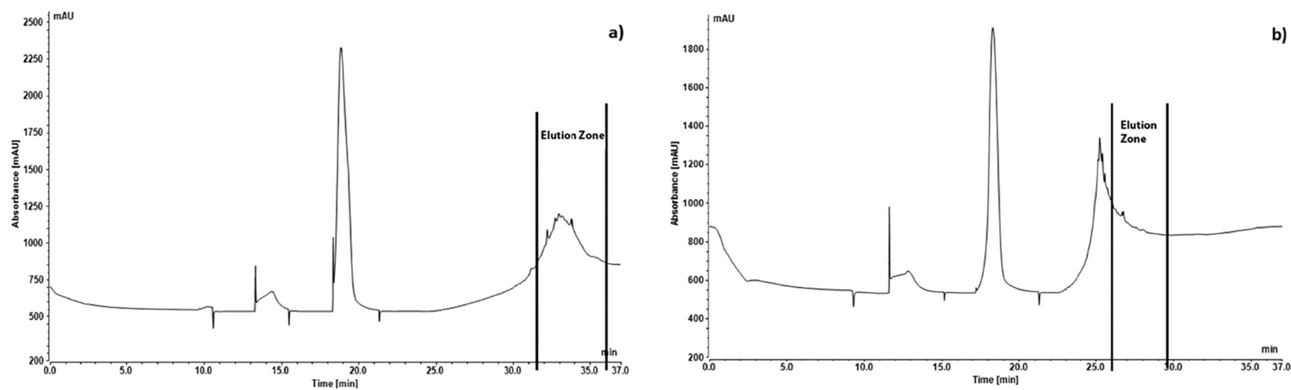


Fig. 2. Monitoring of matrix interferent compounds, by UV profile (220 nm) using: (a) the linear gradient for both pumps and (b) the optimized gradient. The region among black lines show the portion of interferents co-eluted in the retention time of analytes that were not removed by two-dimensional separation.

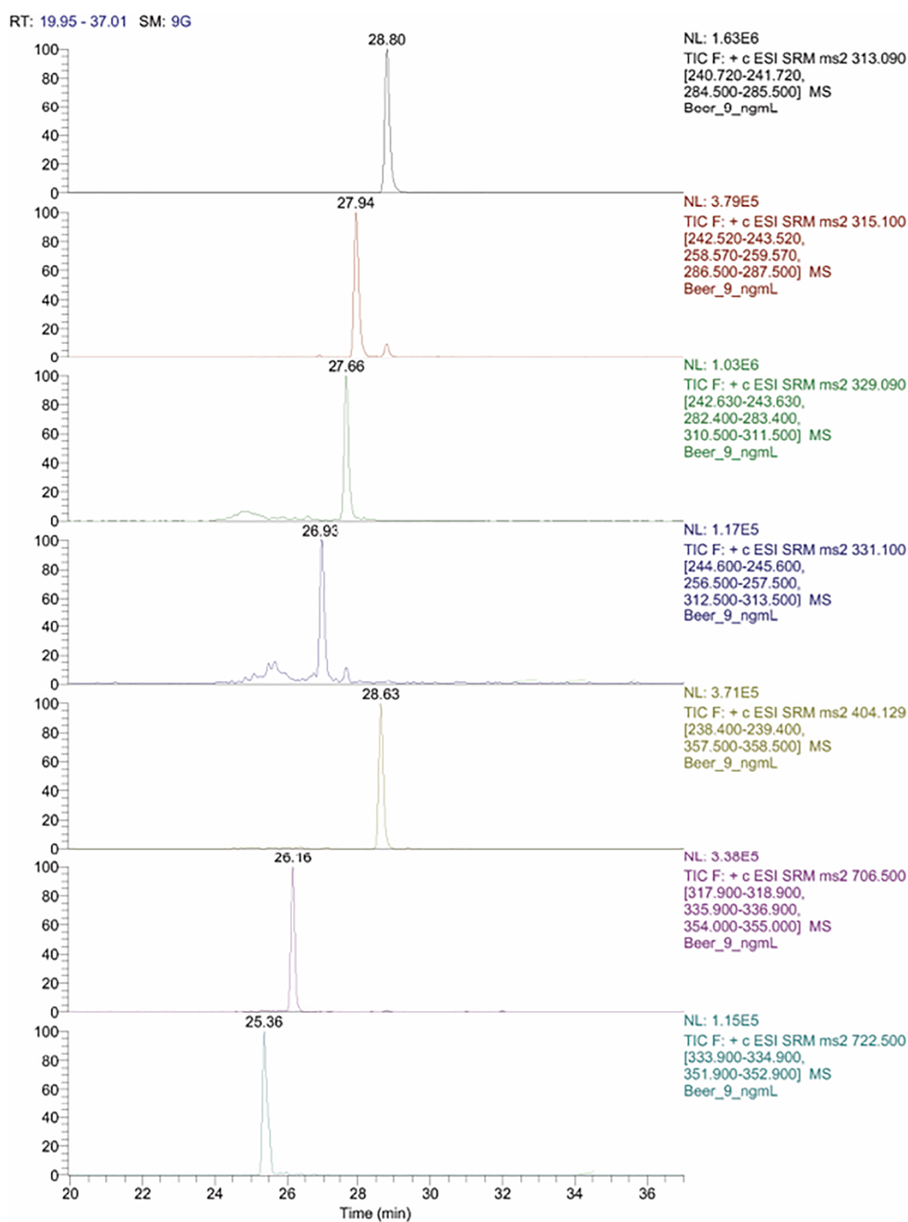


Fig. 3. Multi hear cutting HPLC-MS/MS chromatograms of spiked beer sample at concentration of 9 ng mL^{-1}.

Table 3
Analytical performance of proposed method in beer samples.

	AFB1	AFB2	AFG1	AFG2	OTA	FB1	FB2
MLD (ng mL^{-1})							
Bloond beer	0.005	0.071	0.040	0.055	0.005	0.079	0.052
Red beer	0.006	0.068	0.039	0.25	0.005	0.043	0.075
MLQ (ng mL^{-1})							
Bloond beer	0.017	0.238	0.135	0.185	0.017	0.263	0.172
Red beer	0.020	0.227	0.131	0.833	0.017	0.143	0.250
Recovey ± RSD (%)							
Level (2.9 ng mL^{-1})*	93 ± 4.5	94 ± 2.6	88 ± 7.5	86 ± 9.1	100 ± 0.8	99 ± 8.2	110 ± 1.2
Level (5.1 ng mL^{-1})**	111 ± 3.5	110 ± 1.3	104 ± 4.1	100 ± 8.2	107 ± 8.2	115 ± 2.5	119 ± 1.1
Level (9.9 ng mL^{-1})***	107 ± 2.0	107 ± 0.7	104 ± 2.9	105 ± 2.4	97 ± 0.2	100 ± 6.9	96 ± 0.7
Linear Range (ng mL^{-1})	1–50	0,25–12	1–50	0,25–12	1–50	1–50	1–50
r^2	0,9979	0,9978	0,9969	0,9997	0,9992	0,9991	0,9995

* 0.725 ng mL^{-1} (AFB2 e AFG2).
** 1.275 ng mL^{-1} (AFB2 e AFG2).
*** 2.475 ng mL^{-1} (AFB2 e AFG2).

measured in matrix-matched curves of different beer types (blonde, brown and red), at same concentration level. The results presented in Fig. 3S of the supplementary material show that no significant differences were obtained comparing matrix-matched curves to solvent curves, for all target analytes in blonde and brown beer. However, in the case of red beer, a considerable signal suppression (< 20%) for the aflatoxins was noticed. Therefore, no matrix effect was observed for blonde and brown beers; this is probably due to their simpler chemical composition, whereas, in the case of more complex beer, such as red beer, ion suppression for AFs occurred. Therefore, the quantification of ochratoxin A and fumonisins can be carried out using a standard calibration curve, whereas AFs quantification in beer samples should be performed using matrix matched curve, even if matrix effect was only noticed in red beer.

3.6. Analytical performance and real samples analysis

The proposed analytical procedure was validated according to the European Commission Decision 657/2002 (Commission Decision 2002/657/EC). Beer samples used in optimization and validation procedure were previously examined to verify the absence of target analytes. Parameters of validation such as recovery, selectivity, precision, limit of detection and limit of quantification are reported in Table 3.

The calibration curve of each analytes was estimated in solvent (EtOH 10%) and in spiked blonde beer. The analytes response showed good linearity in the concentration range from 0.5 to 50 ng mL^{-1} for AFB1, AFG1, OTA, FB1, FB2, and 0.25–12.50 for AFB2 and AFG2 in solvent and in matrix with correlation coefficient over 0.996 for all the analytes.

In order to evaluate the recovery of the developed method, beer samples (blonde) spiked at three different levels were analysed in triplicate. Recovery values ranged from 86 to 119% (Table 3), fulfilling the European Commission Decision 657/2002. The precision of the developed method, expressed as RSD%, was evaluated by processing spiked beer samples over linear range using optimized procedure; results ranged from 2 to 7%. The selectivity was experimentally evaluated monitoring the MRM transition of analytes in several beer quality of non-contaminate samples; no interfering peaks were eluted in the retention time of target analyte. The method sensitivity was estimated through the analysis of beer samples spiked at the signal to noise ratio (S/N) of 3 and 10 for MLD and MQL respectively and were reported in Table 3.

After validation, the optimized method was applied to the analysis of 75 beers, purchased in different supermarkets or Italian stores (supermarket, pub and restaurants). Before the analysis, beers were sonicated for about 5 min in order to remove gas and prevent effervescence during the injection. The results obtained from the analysis of 75 samples are summarized in Table 1S. Among the analysed samples, sixteen samples (21% of total samples) contain FB1; the lowest amount found was 0.6 ng mL^{-1} and the highest was 12.3 ng mL^{-1}. Regarding the two beers contaminated by FB2 (5% of total samples), the amount found for both samples was 0.7 ng mL^{-1}. AFs and OTA were not detected in any analysed sample.

4. Conclusions

In this study, we have successfully developed and validated a rapid and automated multiple heart cutting 2D-HPLC-MS/MS method for the simultaneous determination of aflatoxins (AFB1, AFB2, AFG2 and AFG1), fumonisins (FB1 and FB2), and ochratoxin A (OTA) in beer. The use of the two-dimensional liquid chromatography procedure allowed an automated sensitive and accurate analysis of seven mycotoxin in a total run time of 37 min, eliminating tedious manual sample preparation techniques, such as IAC-SPE. The method was validated according the guideline of CE, using different beer types (blonde brown and red white), and its analytical performance fulfils the criteria of the method for determination of mycotoxins in foodstuffs (EC Decision 657/2002 and Regulation 401/2006). The advantages of this method are the rapidity and the full automation of analytical procedure, that reduces the manual procedures. The developed method clearly demonstrates a significant decrease of interferents compared to the one-dimensional technique, with a substantial reduction of the matrix effect phenomenon. Overall the result of this study do not raise concerns for moderate consumer health as regards exposure to mycotoxins from beer in Europe, in fact mycotoxins contamination is limited to fumonisins group. It is important to underline that despite is extensively reported the mycotoxins contaminations of beer there is a lack of regulation. For these reasons further studies are needed to increase the evaluation of mycotoxins contamination in beers in order to understand intake and risk to the population and force the international authority to set maximum levels permitted of mycotoxins in beer.

CRediT authorship contribution statement

Luca Campone: Conceptualization, Writing - review & editing, Supervision. **Serena Rizzo:** Investigation. **Anna Lisa Piccinelli:** Validation. **Rita Celano:** Software. **Imma Pagano:** Formal analysis. **Mariateresa Russo:** Project administration, Funding acquisition, Supervision. **Massimo Labra:** Methodology. **Luca Rastrelli:** Project administration, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126496.

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