PAPER

PRESENCE OF DESTRUXIN A AND BEAUVERICIN IN CEREALS

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ABSTRACT

A LC-MS/MS method for the detection of destruxin A (DTX A) and beauvericin (BEA) in cereals was developed, validated and applied to commercial products collected in Italian markets in the years 2015-2016. Results showed that BEA contaminated 59 % of the samples even if only 15 of them (34%) showed quantifiable residues (comprised between 0.11 and 7.51 ng/g). The sample of red rice contaminated with the highest BEA level was also contaminated with DTX A (0.28 ng/g). Finally, no significant differences were detected between contaminated samples based on the production year and the agronomic technology used (organic or conventional farming).

Keywords: LC-MS/MS, mycotoxins, organic, conventional

1. INTRODUCTION

Cereals supply and demand have been steadily increasing in recent years (USDA, 2017), however these products are exposed to pre-/post-harvest fungal infections potentially dangerous for humans and animals and responsible for economic losses (WHO-IARC, 2002; PERAICA *et al*., 1999; ZAIN, 2011).
Fungal toxicity is mainly due to the production of mycotoxins. These secondary metabolites, produced by molds as natural protection, are generally thermostable and resistant to food transformation processes (KARLOWSKY *et al*., 2016). For these reasons, mycotoxins are considered the main chronic dietary risk factors and therefore a correct evaluation of the real contamination and co-occurrence of these products is required. Among all mycotoxins, European Union has set a maximum level in food only for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, T-2 toxin and HT-2 toxin (Commission REGULATION 1881/2006; COMMISSION RECOMMENDATION 2013/165/EU).
Recently, a particular attention was paid in EC to enniatins and beauvericin (BEA). EFSA's Panel on contaminants has reported the occurrence of enniatins and BEA in European foods and feeds in 2014 in the food chain (EFSA, 2014). Even if no concerns for human health have been related to the acute exposure to these mycotoxins, given the lack of relevant *in vivo* toxicity data, no reliable conclusions can be drawn on chronic exposure to these compounds (contam, 2014).
Many fungi, such as *Beauveria bassiana* and *Fusarium* spp., produce BEA and, in the last period, *B. bassiana* is widely used as entomopathogenic mycoinsecticide alone or in combination with *Metarhizium anisopliae* (WANG and XU, 2012).
The *Metarhizium* spp. and other ubiquitous soil fungi, produce a family of cyclic peptide toxins termed destruxins (DTXs). To date, a number of DTXs have been identified and placed in five major subgroups (A-E) with DTX A, B and E as the most predominant one (HSIAO and KO, 2001; WANG *et al*., 2009; ibraim and asker, 2012). They are known to possess cytotoxic and cytostatic effects on mammalian and insect cells with DTX A and E being the most toxic (SKROBECK and BUTT, 2015).
For some authors, mycotoxins from mycoinsecticides have limited ways to enter in environment and the risks of contaminating foods are negligible (HU and ZHANG, 2016). Differently from BEA, no analytical data are currently available on the occurrence of DTXs in food chain and the present work aimed to investigate of the real occurrence of DTX A, along with BEA, in cereals purchased from the Italian market in 2015-2016 period. Based on our previous experience with these two analytes and on the extraction solvents reviewed in literature (HSIAO and KO, 2001; WANG *et al*., 2009; CITO *et al*., 2014 and 2016; BUTT *et al*., 2009; TAIBON *et al*., 2015; BLESA *et al*., 2012; SØRENSEN *et al*., 2008), a validated LC-MS/MS method was optimized in order to determine simultaneously both analytes in commercial organic or conventional farming cereals samples.

2. MATERIALS AND METHODS

2.1. Chemicals

The standards of DTX A and BEA were obtained from Sigma-Aldrich S.r.l (Milano, Italia). All reagents were obtained by Sigma unless stated otherwise. Acetonitrile, dichloromethane and ethyl acetate, used for the mycotoxins extractions, were of analytical grade while acetonitrile used for chromatographic analysis was of HPLC grade. Milli-Q quality water (Millipore, Milford, MA, USA) was used.

2.2. Standard solutions

Standard solutions were prepared by dissolving each compound with methanol in a volumetric flask and then diluted with methanol to make the working solutions.

2.3. Sample extraction

The cereals samples (maize, barley, oat, rice, red rice, amaranth, millet, wheat and spelt) were purchased in local supermarkets. In the first step, a representative portion of the cereal samples (100 g) was mixed well with a food chopper. An accurately weighed portion of all the samples (10 g) was placed in a centrifuge tube and 25 mL of acetonitrile or dichloromethane: ethyl acetate (1:1, v/v) was added. The extractions were carried out using an IKA Labortecnhik homogenizer model T25 basic (IKA WERKE GmbH & Co., Staufen, Germany) for 5 min at 13500 rpm. The supernatant was transferred after centrifugation, and another aliquot of extraction solvent was added to the residue and homogenized. The organic fractions were pooled and evaporated to dryness under vacuum by rotary evaporation (temperature of the bath, 20°C), and the residue redissolved in 500 μL of acetonitrile. The sample was filtered with 0.45 μm Minisart SRP 4 (Sartorius: Goettingen, Germany) and used for the LC/MS-MS analysis.

2.4. Quantification and recovery

The quantitative analysis of BEA and DTX A was based on calibration curves obtained analysing spiked samples (10 g of equimolar mixture of barley, oat, maize, rice, wheat and spelt) at different concentrations ranged between 0.1 and 100 ng/g. For the equations, six points with different concentrations were used. Extraction recoveries were determined by spiking untreated powdered equimolar mixture of cereals with standard solutions to obtain tree different final concentrations (0.1, 10 and 100 ng/g for each investigated compound). After the solvent evaporated, the samples were extracted, as reported above. Recovery values were calculated as the ratio of the peak area obtained from the extraction of the fortified samples to the corresponding peak area determined by a single-point calibration standard.

2.5. LC–ESI-tandem MS analysis

Chromatography-mass spectrometry system consisted of a Varian apparatus (Varian Inc.) including a vacuum solvent degassing 20 unit, two pumps (212-LC), a Triple Quadrupole MSD (Mod. 320-LC) mass spectrometer with ESI interface and Varian MS Workstation System Control Ver. 6.9 software. The chromatographic separation was performed by using a Kinetex 2.6μm C18 100Å column (Phenomenex) (100 mm×4.6 mm). The sample was injected (5 μL) after filtration. Chromatographic analysis was carried out by using acetonitrile and aqueous solution of formic acid (0.05%) (3:97 v/v). The flow rate was 0.1 mL/min.

The instrument operated in positive mode and ESI parameters were: detector voltage 1250 V, drying gas pressure 18.0 psi, desolvation temperature 300.0°C, nebulizer gas 42.0 psi, needle voltage 6000 V and shield voltage 250 V. Nitrogen was used as nebulizer and drying gas. Collision induced dissociation was performed using argon as the collision gas at a pressure of 1.8 mTorr in the collision cell. The selected reaction monitoring (SRM) transitions as well as the capillary voltage and the collision energy are summarized in Table 1. Quantitative analysis was performed in SRM to maximize sensitivity. For each investigated compound the $[M+H]^+$ species were selected as precursor ions. Two SRM

transitions (Table 1), the first one for quantification and the second one for confirmation purpose, were acquired by using the experimental conditions described above.

Table 1. Chromatographic and selected reaction monitoring (SRM) parameters used in the analysis (retention time (t_R), quantification and confirmation transitions, collision energy and capillary voltage).

Compound	t_R (min)	Quantification transition (*m/z*)	Collision energy (eV)	Confirmatory transition (*m/z*)	Collision energy (eV)	Capillary voltage (V)
DTX A	3.52±0.08	578.1→465.1	-28.5	578.1→436.8	-22.5	86.29
BEA	4.32±0.06	784.2→244.0	-25.0	784.2→262.0	-24.5	140.00

2.6. Validation procedure and evaluation of the matrix effect

The specificity of the method was assessed by analysing blank samples (one sample for each analysed cereals) and blank samples spiked with the investigated compounds, according to the procedure reported above. Assay selectivity was defined by evidence of non-interference at retention times and ion channels identical to those of BEA and DTX A in the blank samples.

In order to determine the linearity of the method, calibration curves (obtained from five replicate experiments) were constructed by analysing spiked cereal samples (10 g of equimolar mixture of barley, oat, maize, rice, wheat and spelt fortified before extraction) ranged between 0.1 and 100 ng/g. The linearity was evaluated by linear least-squares regression analysis.

The detection limit (LOD) was defined as the concentration for which a signal-to-noise ratio equal to 3 was obtained. The quantification limit (LOQ) was defined as the lowest concentration for which an accuracy between 80% and 120% and a precision with a coefficient of variation of±20% or less was obtained over six measurements, with a signal-to-noise ratio superior or equal to 10.

Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying added blank cereal samples, six replicates set at the same concentration (0.1, 10 and 100 ng/g), during the same day. The between-day precision was studied by assaying added blank cereals samples, six replicates set at the same concentration (0.1, 10 and 100 ng/g), on different days (5 days). The accuracy of the method was also evaluated at the same concentration levels and expressed as relative error % (RE).

The recovery data were determined by spiking blank cereal samples with standard solutions (three concentrations analysed in triplicate). After spiking, the samples were extracted as previously described. Recovery values were calculated by comparing the analytical results of the samples through overall extraction procedure with those obtained from blank samples fortified after extraction.

In order to study the matrix effect (ME), blank samples were processed and spiked later to obtain three final concentration levels (set B: six samples with final concentrations of 0.1, 10 and 100 ng/g). The response (peak area) was compared with directly injected standard solutions (set A: six samples prepared in methanol at the same concentration levels). The matrix effect (ME) was evaluated by comparing the mean peak area of the spiked samples (post-extraction addition) with corresponding standard solutions at equivalent concentrations prepared in methanol. The ME values were then calculated as follows: ME (%) = A/B×100 (MATUSZEWSKI *et al.*, 2003).

3. RESULTS AND DISCUSSION

3.1. LC–ESI-tandem-MS optimization

The selected reaction monitoring (SRM) was performed to enhance sensitivity and specificity of the analysis. The MS/MS dissociation study was optimized, for each single standard compound, by varying the cone voltage and collision energy, using the flow injection analysis (FIA) of working standard solutions at a flow rate of 0.01 mL/min directly through the electrospray probe. $[M+H]^+$ ions were found to be the most abundant ones and selected as precursor ions for the target compounds.
The MS–MS breakdown for DTX A showed a fragmentation pattern similar to that reported by other authors (WANG *et al*., 2009; BUTT *et al*., 2009). As expected the DTX A ion $[M+H]^+$ at 578.1 *m/z* represented the most abundant ion without any adduct. The collision-induced dissociation (CID) experiments showed common losses of amino acids following ring opening.
As previously described by other authors, BEA tends to be readily ionized via ESI to form $[M+H]^+$ ion. As determined in this work, $[M+H]^+$ ion was found to be the most abundant one and selected for BEA analysis. The MS/MS tuning experiments displayed product ions scan spectra of BEA in accordance with those reported in literature (SØRENSEN *et al*., 2008; SONG *et al*., 2009). Most intense fragments were selected for DTX A and BEA quantification and confirmatory purposes (Table 1).

3.2. Method optimization and validation procedure

The method was validated for accuracy, precision, specificity, linearity and sensitivity. In order to control for variability in recovery from biological samples and factors that can affect the instrumental response, various cereals samples were assayed. Cereals used for calibration and for recovery studies were analysed to verify the absence of each investigated compound before performing the analysis. The analysis of blank samples showed the absence of interfering endogenous compound peaks at the same ion channel or retention time of DTX A or BEA.
Two extraction methods were tested in order to identify a unique system to quantify contemporarily both mycotoxins. Satisfactory mean recoveries for BEA (89 and 72 % respectively) and only with binary mixture for DTX A (56 %) were obtained by using both selected extraction solvents (acetonitrile and dichloromethane: ethyl acetate 1:1 v/v) (Table 2).

Table 2. Mean extraction recoveries obtained with the two extraction procedures (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat) (n=3).

	Spiked concentration (ng/g)	Extraction with Acetonitrile		Extraction with CH_2Cl_2:ethyl acetate (1:1 v/v)	
		Recovery (mean±SD)	RSD%[a]	Recovery (mean±SD)	RSD%[a]
DTX A	0.1	38.6±7.8	20.3	66.7±6.66	10.4
	10	43.1±6.4	14.8	68.2±5.52	8.10
	100	39.2±5.8	14.9	79.9±3.46	4.3
BEA	0.1	88.4±5.0	5.9	55.8±6.7	12.0
	10	88.7±3.1	3.4	54.9±6.2	11.2
	100	89.4±2.9	3.2	57.5±4.7	8.1

[a]Relative standard deviation.

The matrix-induced effects, such as signal enhancement or suppression, were also evaluated according to MATUSZEWSKI *et al*. (2003), and the results obtained in presence of an extract of equimolar mixture of barley, maize, oat, rice, spelt and wheat are summarized in Table 3. An enhancement of the absolute response was observed for both analytes with both the extraction systems. Very close results were obtained for all matrices when tested separately (results not shown), therefore, calibration curves were generated from blank constituted of an equimolar-mixed cereals sample (barley, oat, maize, rice, wheat and spelt) spiked before extraction to avoid and minimize any uncertainty related to the matrix-induced effects.

Table 3. Matrix effects obtained with the two extraction procedures (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat) (n=3).

	Spiked concentration (ng/g)	Matrix effect (mean±SD)	
		Acetonitrile	CH_2Cl_2:ethyl acetate (1:1 v/v)
DTX A	0.1	146.1±11.3	120.6±10.6
	10	137.8±9.9	130.6±9.6
	100	136.9±7.9	124.8±9.0
BEA	0.1	147.9±8.5	156.6±10.7
	10	148.7±4.7	137.2±8.7
	100	143.1±8.5	140.8±8.5

Based on the results obtained in these preliminary stages, the binary extraction mixture was selected for the continuation of the work, ensuring satisfactory recovery values for both analytes. Calibration curves (five replicate experiments) were constructed and the method was found to be linear within the range 0.1–100 ng/g with correlation coefficient above 0.9994. The equations of the curves, obtained by a least squares fit, are reported in Table 4.

Table 4. Regression plot parameters for DTX A and BEA quantification in mixed cereals (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat).

	Range (ng/g)	Regression plots parameters	R^2	LOQ[a] (ng/g)	LOD[b] (ng/g)
DTX A	0.1-100	y = 24423x+6127	0.9994	0.1	0.03
BEA	0.1-100	y = 46020x+7695	0.9997	0.1	0.03

[a]Quantification limit.
[b]Detection limit.

The selected method was validated in term of precision and accuracy (results are reported in Table 5). Intra-day and inter-day precision, expressed as the relative standard deviation (RSD %) values, were always less than 15 % (n = 6) for both the analytes. The relative errors (RE %) ranged from −12.00 % for BEA to +14.00 % for DTX A obtained at LOQ levels. The reported results indicated that the developed method is precise, accurate, reproducible and utilizable for determination of the two compounds in cereal-based foods. Compared to the LOQ present in literature our values are comparable for both analytes (TAIBON *et al*., 2015; BLESA *et al*., 2012; Sørensen *et al*., 2008; TOLOSA *et al*., 2017; MALACHOVÁ *et al*., 2014).

Table 5. The intra-day and inter-day precision and accuracy of the method (n=6).

Compound	Analysis type	Spiked concentration (ng/g)	Measured concentration (media±SD)	RSD%[a]	Accuracy (relative error %)[b]
DTX A	Intra-day	0.1	0.11±0.01	9.09	+8.20
		10	10.52±1.12	10.65	+5.20
		100	101.03±3.40	3.40	+1.00
	Inter-day	0.1	0.12±0.01	11.67	+14.00
		10	10.54±0.84	7.97	+5.40
		100	103.06±2.35	2.28	+3.06
BEA	Intra-day	0.1	0.09±0.01	14.44	-12.00
		10	9.90±1.24	12.53	-1.00
		100	101.20±4.23	4.18	+1.20
	Inter-day	0.1	0.09±0.01	13.33	-9.40
		10	9.95±0.90	9.35	-0.50
		100	109.20±3.68	3.37	+9.20

[a]Relative standard deviation.
[b]Accuracy = relative error % = (measured-spiked)/spiked x 100.

3.3. Analysis of DTX A and BEA content in cereals

The validated method (extraction accomplished with dichloromethane: ethyl acetate, 1:1 v/v) was successfully applied to quantify DTX A and BEA levels in 44 commercial products collected in the years 2015-2016 (Table 6).
Results showed that BEA contaminated 59 % of the sample even if in only 15 samples (34%) quantities were higher than LOQ (included in the range 0.11 and 7.51 ng/g). Contamination data are in accord with BEA occurrence reported by various authors and collected in the EFSA CONTAM Panel report (2014).
The high number of positive samples is due to the good LOD values obtained with our method (0.03 ng/g for both compounds). Either no significant differences were detected between percentages of contaminated samples based on the year of production or the agronomic technology used (organic or conventional farming).
Only one sample (red rice presenting the highest BEA levels) resulted contaminated by DTX A (0.28 ng/g).
Although the number of analyzed samples is limited, the low levels of BEA and the substantial absence of DTX A confirm that acute exposure to these toxins could do not indicate concern for human health. However, careful monitoring in foods is essential in order to provide a correct estimate of chronic exposure to these toxins.

Table 6. Occurrence and content of DTX A and BEA in commercial products (44 cereals samples) collected in the years 2015-2016 (each sample was analysed in triplicate).

Year	BEA content (ng/g±SD)									
	Amaranth	Barley	Oat	Oat flakes	Maize	**Millet**	**Red rice**	**Rice**	**Spelt**	**Wheat**
2015	---- [a]	---- [a]	0.18±0.01	0.17±0.02[a]	0.19±0.02[a]	0.58±0.05[a]	7.51±1.16[b]	1.28±0.16	0.53±0.07[a]	3.92±0.13
		0.27±0.03[a]	---- [a]	----	2.48±0.48		----	0.98±0.11[a]	----	----
				>LOD	----		>LOD	----		
2016	---- [a]	>LOD	>LOD	---- [a]	0.21±0.03	>LOD [a]	----	>LOD [a]	>LOD [a]	>LOD
			----	>LOD	1.54±0.18		>LOD	---- [a]	----	----
				----				0.11±0.01		5.12±0.59
								>LOD		
								----		

[a]Organic product.

[b]Red rice sample containing also DTX A (0.28±0.04 ng/g).

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