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Development and validation of a liquid chromatography/atmospheric pressure photoionization-tandem mass spectrometric method for the analysis of mycotoxins subjected to commission regulation (EC) No. 1881/2006 In cereals

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ABSTRACT

A sensitive and reliable liquid chromatography/photoionization (APPI) tandem mass spectrometry method has been developed for determining nine selected mycotoxins in wheat and maize samples. The analytes were chosen on the basis of the mycotoxins under EU Commission Regulation (EC) No. 1881/2006, i.e., deoxynivalenol (DON), zearalenone (ZON), aflatoxins (AFs), and ochratoxin A (OTA), and considering the possibility of a near future regulation for T-2 and HT-2 toxins. Mycotoxins were extracted from samples by means of an one-step solvent extraction without any cleanup. The developed multi-mycotoxin method permits simultaneous, simple, and rapid determination of several co-existing toxins separated in a single chromatographic run, in which AFs, T-2 and HT-2 toxin are acquired in positive, while OTA, DON and ZON in negative mode. Although a moderate signal suppression was noticeable, matrix effect did not give significant differences at $p = 0.05$. Then, calibration in standard solution were used for quantitation. Based on the EU Commission Decision 2002/657/EC, the method was in-house validated in terms of ruggedness, specificity, linearity, trueness, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ). For all the analytes, the regression coefficient r ranged between 0.8752 (DON in wheat) and 0.9465 (ZON in maize), biases related to mean concentrations were from −13% to +12% of the nominal spiking level, and the overall within-laboratory reproducibility ranged 3–16%; finally, CCα values did not differ more than 20% and CCβ not more than 42% from their respective maximum limit. Method quantification limits ranged from 1/20 (AFG1) to 1/4 (AFG2 and OTA) the maximum limit established by European Union in the Commission Regulation (EC) No. 1881/2006 and its subsequent amendments.

1. Introduction

Many cereals and other crops are susceptible to fungal attack either in the field or during drying, and subsequent storage. These fungi may produce as secondary metabolites diverse groups of naturally occurring, toxic chemical substances known as mycotoxins. The natural fungal flora associated with foods is dominated by three genera, i.e., *Aspergillus*, *Fusarium*, and *Penicillium* [1]. Contamination from *Fusarium* genus, e.g., deoxynivalenol (DON), T-2 and HT-2 toxin, can occur mainly pre-harvest, while contamination from *Aspergillus* and *Penicillium* genera, e.g., ochratoxin A (OTA) and aflatoxins (AFs), mainly post-harvest [2].

In terms of structural complexity, mycotoxins vary from simple C4-compounds, to complex substances [3,4]. When present in food in sufficiently high amounts, these fungal metabolites can have toxic effects that range from acute to chronic symptoms. Some mycotoxins were shown to be mutagenic, teratogenic, or/and carcinogenic. Mycotoxins may also cause developmental effects including birth defects, or affect the reproductive system, the immune system and specific target organs, or exhibit hormonal activity [1]. In addition to these diverse organ or site-specific actions, mycotoxins may affect the gastrointestinal system, cause skin irritation, have hematological effects and reduce growth [5]. Due to toxic effects on human and animals, the risk assessment of mycotoxins is of high relevance [5]. Over a hundred mycotoxins have been identified; however, only a few present significant food safety challenges. The International Agency for Research in Cancer (IARC) has classified AFs as carcinogenic to humans, while OTA and fumonisins B (FB) were classified as possibly carcinogenic. Trichothecenes (TRs) and zearalenone (ZON) were classified as non-carcinogenic but cause other adverse effects [6].

The frequent incidence of these toxins in agricultural commodities has a potentially negative impact on the health and economies of the affected regions. Generally, mycotoxins are stable chemical

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compounds and can neither be completely removed from the food supply nor destroyed during processing and heat treatment, thus, monitoring of this contaminants in food and in feed are important issues associated with public health, agricultural production, food processing, and trade. Especially in internationalization of food and feed trades, restrict regulation strategies have to be set to protect consumers from mycotoxin exposure. European Food Safety Authorities (EFSA) and US Food and Drug Administration (FDA) have set maximum levels (MLs) and guidelines for AFs, OTA, ZON, DON, FB1 and FB2 down to the ppb to ppt level in different food and feed products [7–9]. More recently, in Regulation (EC) No. 1881/2006 and Regulation (EC) No. 1126/2007, mycotoxin levels were set restrictively to: 4 $\mu g\,kg^{-1}$ for total AFs, and 2 $\mu g\,kg^{-1}$ for AFB1; 5 $\mu g\,kg^{-1}$ for OTA, 750 $\mu g\,kg^{-1}$ for DON, and 75 $\mu g\,kg^{-1}$ for ZON. Statutory regulations do not exist for T-2 and HT-2, yet. However, a selection of advisory and tolerance limits is available in the literature and from them we considered as possible maximum residue limit for T-2 and HT-2 the value of 50 $\mu g\,kg^{-1}$. Monitoring and control programs for mycotoxins in food and feed have been implemented in many countries, especially in the European Union (EU).

The analysis of mycotoxins is challenging as they are usually present in minute concentrations in complex sample matrices, and they may occur in various combinations produced by a single or by several fungal species. The fact that most mycotoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection. The analytical methods for the identification and determination of mycotoxins in food, feed and biological samples should be accurate and should provide reliable data.

Although Enzyme-linked immunosorbent assays (ELISA) have been used for screening purposes as well as for sensitive quantification of mycotoxins in various samples, modern analysis of mycotoxins relies heavily on high-performance liquid chromatography (HPLC) with UV or fluorescence detection [10–15] and, more recently, mass spectrometry (MS). An important and critical step in the mycotoxin analysis is sample preparation and sample cleanup. Different strategies have been performed, including solid phase extraction (SPE), liquid–liquid extraction, supercritical fluid extraction, and accelerated solvent extraction (ASE) [16]. Multifunctional columns (MycoSep) [17] and selective or specific antibodies (immunoaffinity columns: IAC) for isolation and purification from the matrix compounds have also been extensively used. HPLC–MS and HPLC–MS/MS has, in many cases, revolutionized the analysis of mycotoxins and it has become an important analytical tool for routine analysis in complex matrices because of unambiguous analyte identification, accurate quantification and sensitivity. Since some mycotoxins are inserted in legislation there is the need to determine mycotoxins by routine analysis in different types of matrices in one single extract and, if possible, in a single LC–MS run. Thus, to enable reliable and fast risk estimation of mycotoxin intake and poisoning, the development of multi-mycotoxin methods with a common sample preparation and final analysis procedure is highly desirable. The number of such multitoxin LC–MS methods is still relatively limited due to the complexity of the biological matrices as well as the wide range of physical and chemical properties of mycotoxins, challenging both sample preparation and LC–MS detection. For the most complex food matrices some of the published multitoxin LC–MS methods rely basically on multiple but parallel or sequential sample preparation strategies of one sample followed by separate analysis of each isolated class of mycotoxins in separate LC–MS runs [18–26]. This procedure is required either due to insufficient chromatographic separation of mutually interfering analytes [27,28] or by mycotoxin specific MS sensitivity differences in the positive and negative ion mode [22,23,29].

Positive/negative polarity switching has been shown to be a proper tool to solve this latter problem within one LC–MS run whenever modern MS instrumentation with sufficiently rapid polarity switching capability is available or, alternatively, analytes are sufficiently separated from each other by LC that a limited number of positive and negative mode windows can be set up within one LC–MS run [22,29]. Recent assays are focused on typical *Fusarium* toxins as trichothecenes, ZON and its metabolites [19,22–24,29], sometimes including fumonisins [22,23,30,31], OTA and AFs [22,32].

Aside the problematic sample preparation of complex matrices, LC–MS analyses of heterogeneous mixtures of mycotoxins suffer in principal from dramatic differences of analyte ionization efficiencies [23,29,33]. These are influenced by various parameters, as physical and chemical properties of the analytes, the employed ionization source, the preferred ionization polarity, the LC elution solvent and the presence of disturbing matrix components. For this reason, MS sensitivity can hardly be kept stable over a wide LC elution zone and polarity range.

The most recent trend in sample preparation with LC–MS/MS analysis has been the injection of a diluted crude sample extract with no further cleanup. This approach was first applied in multi-mycotoxin analysis by Sulyok et al. [34,35], who analyzed 39 different mycotoxins in a variety of grains. Later, the authors expanded the method for semi-quantitative analysis of 87 fungal metabolites [36]. Several different types of LC–MS multi-mycotoxin methods for food and feed matrices have been published recently [20,27,29,34–42].

In this respect Nielsen, Smedsgaard and coworkers [43] monitored simultaneously up to 474 mycotoxins in fungal cultures in order to compile a data base for pharmaceutical high throughput screening or to identify individual *Penicillium* species by their mycotoxin patterns [44]. The majority of multitoxin LC–MS methods has been done in fungal cultures and grain and to less extent in cheese, milk and other foodstuff. However, due to matrix effect, appropriate sample preparation and chromatographic separation of analyte from matrix compounds seem to be necessary to insure accurate quantification as well as unambiguous identification.

Modern LC–MS instrumentation is mostly based on atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), which exhibits the common problem of matrix effect. Atmospheric pressure photoionization (APPI) is the latest interface introduced in the field of soft ionization techniques for coupling MS to liquid-phase separation systems [45]. The additional analytical capabilities offered by APPI-MS, with respect to ESI and APCI-MS, have been optimized to improve the detection limits of some classes of compounds. In particular some studies have shown that APPI can provide higher signal-to-noise ratios with respect to APCI [46,47] and that it is less prone to matrix effect than ESI [48]. The use of LC/MS with an APPI source for analyzing AFs in some foodstuffs has been reported in precedent papers [45,49]. Using APPI, detection limits for the investigated compounds were lower than by using ESI, due to a much lower noise and matrix effect.

The aim of this work was to develop and validate a simple method for several mycotoxins based on one-step solvent extraction followed by LC/APPI-MS/MS. The analytes were chosen on the basis of the mycotoxins under EU Commission Regulation (EC) No. 1881/2006 and considering the possibility of a near future regulation for T-2 and HT-2. By taking advantage of the high specificity of LC–MS/MS, a simple sample treatment not involving any cleanup step was developed.

We decided to investigate the behaviour of APPI source considering that: (1) some of these compounds were not included in the cited works; (2) the easy and fast sample preparation may require a kind of source less sensible to matrix effect than ESI one; (3) none of the proposed method employing APPI has been validated following the guidelines given in the Decision 2002/657/EC. Preliminary experiments showed that the APPI source did not give a response

suitable for FB1 and FB2 determination at law regulated levels in maize. To our best knowledge, this is the first publication on the coupling of LC with APPI-MS for determining simultaneously mycotoxins produced by *Aspergillus*, *Fusarium*, and *Penicillium* genera in cereals.

2. Experimental

2.1. Reagents and chemicals

Standards of aflatoxins (AFs), namely aflatoxin M1 (AFM1), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2); ochratoxin A (OTA); trichothecenes A, namely T-2 toxin, and HT-2 toxin; trichothecene B, namely deoxynivalenol (DON); and macrocyclic lactone, namely zearalenone (ZON) were purchased from Sigma–Aldrich (Milan, Italy). AFM1, not present in vegetables since it is an AFB1 hepatic metabolite, was used as internal standard (IS).

Individual stock solutions were prepared in acetonitrile at 0.5 mg mL^{-1} level for AFM1, and at 1 mg mL^{-1} for the other mycotoxins. All the solution were stored at −20 °C in amber glass vials and kept in the dark at room temperature (20–25 °C) before use.

Working standard solutions were prepared by suitable dilution of stocks. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting with a suitable solvent obtaining the following concentrations: AFB1, AFB2, AFG1, and AFG2: 25 pg μL^{-1}, OTA: 125 pg μL^{-1}; T-2 and HT-2: 1 ng μL^{-1}; ZON: 1.5 ng μL^{-1}; DON: 15 ng μL^{-1}. The IS working standard solution was prepared at 50 pg μL^{-1}. These solutions were kept at 4 °C and renewed weekly. All organic solvents were HPLC grade from Carlo Erba (Milan, Italy) and were used as received. Concentrated ammonia, formic and acetic acids were RPE grade from Carlo Erba. Ultrapure water was produced from distilled water by a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Polypropylene tubes (6 mL) and polyethylene frit (20 μm) were purchased from Supelco (Bellefonte, PA, USA) while PTFE syringe filters (0.45 μm; 13 mm diameter) were from Alltech (Deerfield, IL, USA).

2.2. Samples

Wheat and maize samples, and reference materials were kindly provided by "Experimental Institute of Cereal Research" (Rome, Italy). Wheat and maize samples (50 g) used for method development were grounded by a laboratory mill (IKA, model A11, from Carlo Erba), sieved and the fraction <60 mesh sealed in plastic bags and maintained in a refrigerator at 4 °C until further use. Subsamples of 1 g were taken for analysis. A sub-sample of 1 g could be selected for extraction provided that the laboratory sample was finely grinded and thoroughly mixed [30].

Certified reference materials, namely maize flour, DON blank <50 μg kg^{-1}; wheat flour, DON blank <50 μg kg^{-1}; maize flour, DON 474 μg kg^{-1} were from Sigma–Aldrich and were stored following the supplier instructions.

2.3. Sample extraction

An aliquot of 1 g of ground sample was accurately weighed into a 50 mL screw cap 115 mm × 30 mm polycarbonate centrifuge tube, spiked with 50 μL of 50 pg μL^{-1} IS solution and homogenized by manually shaking for 15 s with 6 mL of acetone/water/acetic acid (80:19:1, v/v/v) mixture.

The extraction was carried out in an ultrasonic bath for 20 min. A model ST ultrasonic bath at a frequency of 50 ± 3 Hz from Stimin (Milan, Italy) was used. Extracted samples were then centrifuged for 10 min at 20 °C and 8000 rpm by a model PK131R multispeed refrigerated centrifuge (ALC, Milan, Italy). The supernatant was transferred to a clean 10 mL glass tube and evaporated to dryness at 40 °C under a gentle nitrogen stream. The residue was reconstituted with 500 μL of methanol/water/acetone 10:80:10 (v/v/v) mixture, and the obtained solution was forced through a PTFE syringe filter and 10 μL of the final solution was injected into the chromatographic system without further purification, and analyzed by APPI-MS/MS.

The extraction efficiency was evaluated for wheat and maize in terms of recovery and signal suppression due to matrix effect by spiking with 40 μL of the composite working standard solution analyte-free samples before and after the extraction step and triplicate experiments were done. In this way, the extraction effect on total recovery can be isolated from matrix effect and evaluated by comparing the peak areas for the same compound in samples spiked ante- and post-extraction step.

2.4. LC/APPI-MS/MS analysis

Liquid chromatography was performed by a series 200 Perkin-Elmer (Norwalk, CT, USA) apparatus consisting of a binary LC micropump, a vacuum degasser, and coupled with an autosampler equipped with a 15 μL loop. The chromatographic column was a 100 mm × 2.1 mm i.d. Kinetex (Phenomenex, Torrance, CA, USA) filled with C18 reversed-phase packing (2.6 μm average particle size), equipped with a SecurityGuard ODS 4.0 mm × 2.1 mm i.d. precolumn supplied by Phenomenex. An oven from Timberline Instruments Inc. (Boulder, CO, USA) was used for thermostatting the chromatographic column.

Analysis was performed using gradient elution with water/acetone (90:10, v/v) as mobile phase A and methanol/acetone (90:10, v/v) as mobile phase B; the flow rate was 200 μL/min. After an isocratic step at 15% B for 2 min, B was increased by a linear gradient from 15 to 25% in 10 min, then brought to 80% in 8 min, and to 95% in 1 min and held for 4 min to rinse the column. Finally, the B content was lowered to 15% and the column re-equilibrated for 10 min. The chromatographic column was kept in an oven at 40 °C to increase the retention time reproducibility. In this way, it was possible to subdivide the MS acquisition in nine periods, thus increasing the signal-to-noise ratio.

APPI-MS/MS was carried out on a Q TRAP 3200 mass spectrometer equipped with a PhotoSpray sources (Applied Biosystems/MDS Sciex, Concord, ON, Canada) and operating in both positive and negative ionization mode. The LC–MS system, data acquisition and processing were managed by Analyst software (1.4.1 version, Applied Biosystems/MDS Sciex).

The APPI source was equipped with a krypton discharge lamp, having a magnesium fluoride window that enabled transmission of the 10.0 and 10.6 eV photons emitted. Depending on the positive or negative ionization mode, capillary voltage was set at ±1400 V. High purity nitrogen (from liquid nitrogen) was used as curtain gas (set to 15 in arbitrary units), lamp gas and collision gas (CAD), while air was used as nebulizer (GS1) and auxiliary turbo spray (GS2) set, respectively, to 70 and 20 (arbitrary values). Probe temperature was set at 375 °C.

Mass calibrations and resolution adjustments on the resolving quadrupoles were automatically performed by using a 10^{-5} mol L^{-1} solution of poly(propylene glycol) introduced via a model 11 Harvard infusion pump (Harvard apparatus, Hollison, MA, USA). Source and compound-dependent parameters were optimized by Flow Injection Analysis (FIA). Each analyte was prepared at a concentration of 5 ng mL^{-1} (OTA, AFs), 50 ng mL^{-1} (T-2, HT-2, ZON) and 200 ng mL^{-1} (DON) in methanol/water 50:50 (v/v) and 10 μL were injected. Mobile phase was methanol:water 50:50 (v/v) at flow rate of 100 μL min^{-1} and a flow rate of 10 μL min^{-1} acetone was used as dopant.

Table 1
LC/MS/MS conditions and precursor ion/product ion pairs of the compounds studied for the acquisition in APPI (MRM) mode.

Analyte	Retention window (min)	Ionization polarity	Retention time (min)	Precursor ion	Selected transitions (*m*/*z*)	Relative collision energy (%)[a]	Declustering potential (V)	External potential (V)	Collision cell exit potential (V)
DON	0–4	–	1.8	$[M+HCOO]^-$	340.9 → 265.0	−15	−30	−5	−5
					340.9 → 295.0	−15			
AFG2	4–6.5	+	5.4	$[M+H]^+$	331.1 → 245.2	40	50	10	5
					331.1 → 313.1	30			
AFM1	4–6.5	+	5.6	$[M+H]^+$	329.1 → 229.1	50	−50	10	−5
					329.1 → 200.2	30			
AFG1	6.5–7.5	+	7.2	$[M+H]^+$	329.1 → 243.1	35	50	10	5
					329.1 → 200.2	50			
AFB2	7.5–9.0	+	7.9	$[M+H]^+$	315.2 → 287.1	30	50	10	5
					315.2 → 259.1	35			
AFB1	9.0–11.0	+	10.2	$[M+H]^+$	313.1 → 285.1	30	50	10	5
					313.1 → 241.1	45			
OTA	11.0–14.0	–	13.1	$[M+H]^-$	402.2 → 358.3	−30	−40	−10	−5
					402.2 → 211.1	−40			
HT-2	14.0–17.5	+	16.7	$[M+41+H]^+$ [b]	465.3 → 245.2	20	25	5	5
					465.3 → 227.1	20			
T-2	17.5–18.6	+	18.2	$[M+NH4]^+$	484.4 → 215.4	25	25	5	5
					484.4 → 305.4	20			
ZON	18.6–22.0	–	19	$[M-H]^-$	317.0 → 175.0	−35	−50	−5	−5
					317.0 → 130.8	−40			

DON, deoxynivalenol; AFG1, aflatoxin G1; AFM1, aflatoxin M1; AFG2, aflatoxin G2; AFB1, aflatoxin B1; OTA, ochratoxin A; HT-2, HT-2 toxin; T-2, T-2 toxin; ZON, zearalenone.
[a] Relative collision energy expressed as % respect to the maximum voltage difference value between the high pressure entrance quadrupole (Q0) and collisional cell quadrupole (RO2) (±130 V) permitted by the instrument.
[b] Probable composition of target ion: $[ROH + CH_3COCH_3 + H - H_2O]^+$.

The positive or negative ions were selected by the first quadrupole and fragmented in the collision cell (Q2) operating at medium pressure (7, in arbitrary scale). From the MS/MS full-scan spectra, two suitable transitions were selected for acquisition in multiple reaction monitoring (MRM) mode, and nine MRM periods were included in the MS method.

Periods, MRM transitions, and mass-spectrometric parameters (declustering potential, collision energy, external potential, collision cell Exit Potential) are reported in Table 1.

2.5. Linear dynamic range and matrix effect

The linear dynamic range was evaluated by constructing a six point calibration graph. For each analyte the combined ion current profile for both transitions was extracted from the LC–MRM dataset, and the peak area plot versus injected amount or concentration was obtained by measuring the resulting peak area and relating this area to that for the IS. Signal suppression on APPI-MS/MS response due to matrix (matrix effect) was assessed by comparing the slope of the calibration curve obtained from the standard solutions with the slope of calibration curve obtained by injecting the matrix-matched solutions spiked at the same concentration levels. Matrix-matched solutions were prepared by spiking 6 different analyte-free samples, after extraction and before evaporation, with known and appropriate volumes of the working standard and IS solutions, and following the remaining procedure.

2.6. Method validation

For method validation we followed a protocol developed in a previous work [50] following the guidelines suggested by the Commission Decision 2002/657/EC. Six sample of wheat and maize from different cultivars (cvs.) were used for validation. Previous experiments were done to check that the samples were analyte-free. Analyses of six blank samples, 5 samples fortified at 0.3 ML, 10 samples at 0.5 ML, 18 samples at the ML, 10 samples at 1.5 ML and 5 samples at 2 ML were performed for evaluating the linearity of the method around the critical value. Samples were placed in a flat amber glass vessel and soaked in 1 mL of acetone solution containing different volumes of the composite working standard solution, taking care to uniformly spread it on the sample. The samples were allowed to air drying at 25 °C in a ventilate oven, to eliminate the organic solvent. Then, spiked samples were treated following the procedure described above, and analyzed. The 18 samples spiked at the MLs were split in three groups of six and analyzed in different days by different operators.

For each analyte the combined ion current profile for both transitions was extracted from the LC–MRM dataset, and the peak area plot versus injected amount or concentration was obtained by measuring the resulting peak area and relating this area to that for the IS.

Instrumental limits of detection (LODs) were estimated by the MRM LC–MS/MS chromatogram resulting from the analysis of 10 μL injection of a mycotoxin standard solution containing 50 pg μL^{-1} DON; 1 pg μL^{-1} AFB1, B2, G1, G2; 3 pg μL^{-1} OTA; 20 pg μL^{-1} T-2, HT-2, and ZON. After extracting the selected transitions for each compound from data set, the resulting traces were smoothed twice by applying the smoothing method (Analyst software). Thereafter, the peak height-to-averaged background noise ratio (S/N) was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak.

Statistical evaluations were performed by ANOVA ($p = 0.05$).

3. Results and discussion

3.1. Extraction optimization

One of our goals in this study was to obtain, from cereal samples, extracts amenable for LC–MS/MS analysis with a simple, and time effective procedure, without cleanup step. Taking advantage to use APPI source, we tried to drastically simplify the analytical protocol and substantially reduce solvent consumption and analysis time.

After the examination of the very large literature, three different extraction mixtures were tested: acetonitrile/water/acetic acid 85:14:1 (v/v/v), Sol 1; methanol/water/acetic acid 80:19:1 (v/v/v), Sol 2, and acetone/water/acetic acid 80:19:1 (v/v/v), Sol 3; a volume of 6 mL per g of sample was used in every case. Two 15 s homogenizations (using a Polytron homogenizer, Kinematica, Lucerne, Switzerland) followed by centrifugation, solvent evaporation, and residue reconstitution were initially performed.

Table 2
The effect of dopant agent addition on signal-to-noise ratio (S/N) of mycotoxin target by atmospheric pressure photoionization.

Analytes	Toluene			Acetone		
	20% (v/v) added by a syringe pump	15% (v/v) added in the mobile phases	10% (v/v) added in the mobile phases	20% (v/v) added by a syringe pump	15% (v/v) added in the mobile phases	10% (v/v) added in the mobile phases
DON	57	82	80	93	104	98
AFG2	35	38	36	40	44	41
AFG1	60	68	61	78	90	82
AFB2	55	64	57	73	86	76
AFB1	102	118	105	140	160	146
OTA	45	47	47	56	85	58
HT-2	65	72	67	86	97	85
T-2	62	70	62	81	93	83
ZON	110	120	107	151	175	148

Sol 3 allowed the highest recovery for the analytes, ranging from 86% (DON and HT-2) to 104% (AFG1), while for Sol 1 recoveries were from 88% to 113%, with the exception of DON that was recovered only 62%. Sol 2 extracted no more than 79% of mycotoxin (99% for DON). Reported values are average recoveries for wheat samples, but similar values have been noticed for maize samples. In all cases relative standard deviation (RSD, %) was never higher than 10%. As previously experimented in our laboratory [19,24,26,30,51], maize does not need acidified solvents for efficient extraction, on the other hand, the presence of the acetic acid in the solvent mixtures did not interfere with the recovery of the selected analytes (data not shown).

Besides negatively affecting the extraction yield of the target compounds, Sol 2 also yielded a 30% decrease of DON response. This was the only important matrix effect measured. With Sol 2 a 20% decrease was found for ZON, whereas Sol 1 did not affect analyte response significantly. Extracting with more than 6 mL of hydroalcoholic mixture did not increase analyte recovery, while extracting with 5 mL volumes gave a loss of ca. 10%. Then, 6 mL of extracting volume were chosen.

Another widely used extraction method such as ultrasound assisted extraction was evaluated as an alternative to homogenization. The duration of ultrasound application was studied on the base of recoveries and a sonication time of 20 min was chosen. Results showed that recovery did not improve significantly neither using a longer time nor a larger solvent volume and did not differ significantly from those obtained by homogenization. For the sample preparation procedure we preferred sonication because the employment of ultrasounds is easy to handle, many samples can be treated at the same time, and the contamination from a sample to the successive is unlike to occur. Further purification of the extract was avoided in order to reduce analysis time; a defatting step was not necessary for our samples.

3.2. Optimization of analytical conditions: LC/APPI-MS/MS

By using the APPI source, solvents must be selected carefully because they can heavily affect the response of analytes [52,53]. As seen in previous works, the water/methanol mixture offered much better ionization conditions than the water/acetonitrile one, probably due to the proton affinity of some of the isomeric forms of acetonitrile generated by photoionization [45,54]. For the APPI source employment, the dopant addition is mandatory, and efficient doping agents must have ionization energies lower than UV Krypton lamp ionization. Toluene and acetone, the two most frequently used doping agents for APPI [52,53], were tested, and tests were conducted in FIA mode by using a 1:1 (v/v) mixture of methanol/water. In a recent work, the advantages of directly mixing the dopant with LC mobile phase has been reported; thus, also some mixtures of water/methanol/toluene or acetone, as reported in Table 2 were tested. In the literature, it is generally reported that the optimum dopant addition ranges 10–20%. As can be seen, the differences in terms of signal-to-noise ratio (S/N) were not large in the reported conditions. However, it should be taken into account the fact that those conditions are unrealistic when the column is used for separation, because the analytes, but ZON, are eluted with mobile phase containing less than 50% methanol. In these conditions, toluene must be added by a syringe pump to an immiscible nebulized liquid. The resulting effect was a decrease of the S/N value (both signal decrease and noise increase) that for DON was about 50% the value obtained in FIA. On the contrary, the S/N values did not decrease so dramatically when acetone was used as dopant, thus we decided to use 10% acetone in the mobile phase.

Both positive and negative mode were tested for ion acquisition. DON did not give any signal in positive mode, whereas AFs gave only a weak signal in negative mode. OTA and ZON may be detected in both positive and negative ion mode, with a slight sensitivity increase and a better stability in negative ion mode respect to positive. As reported in Table 1, DON molecule gave a formate adduct, although no formate was added to the mobile phase. Formic acid may be formed from methanol during photoionization. T-2 toxin ionizes giving ammonium adduct; also in this case, no ammonium was added to the mobile phase. A weak signal for protonated molecule was also present, whereas the signal of sodium adduct was less than 5% the signal of the ammonium one. The presence of some cation impurity in the HPLC solvents cannot explain this phenomenon considering that, in methanol, sodium impurities are much more abundant than ammonium ones. On the other hand the formation of ammonia from N_2 in the APPI source should not be possible. HT-2 toxin behaves in a very characteristic fashion: the protonated molecule at m/z = 425.4 and the ion $[M-H_2O+H]^+$ at m/z = 407.4 were present in the MS spectrum, but the most intense signal was at m/z = 465.4. This uncommon ion may be formed by addition of an acetone molecule after losing a water molecule, giving the ion $[M-H_2O+CH_3COCH_3+H]^+$. Small amount of some modifiers, such as formic acid or ammonium formate were added to the mobile phase in order to verify if their addition could promote the formation of ions amenable for fragmentation and MS/MS. Modifier addiction did not cause any signal increase or improvement, but only a noise increase with the exception of OTA, which peak shape improved whereas its intensity decreased dramatically in both negative and positive mode. For this reasons neutral phases were used.

Kinetex columns take advantage of the relatively new “fused core” packing technology. Operating with a short Kinetex C18 column (100 mm) it was possible to achieve, in a relatively short time, a very good resolution among analytes. This fact permitted to have an acquisition window for each compound, including three polarity switching, as reported in Table 1.

The aliquot of extracted sample submitted to analysis is an important parameter in determining the method quantification limit (MQL). A drawback of the fused core column is their reduced

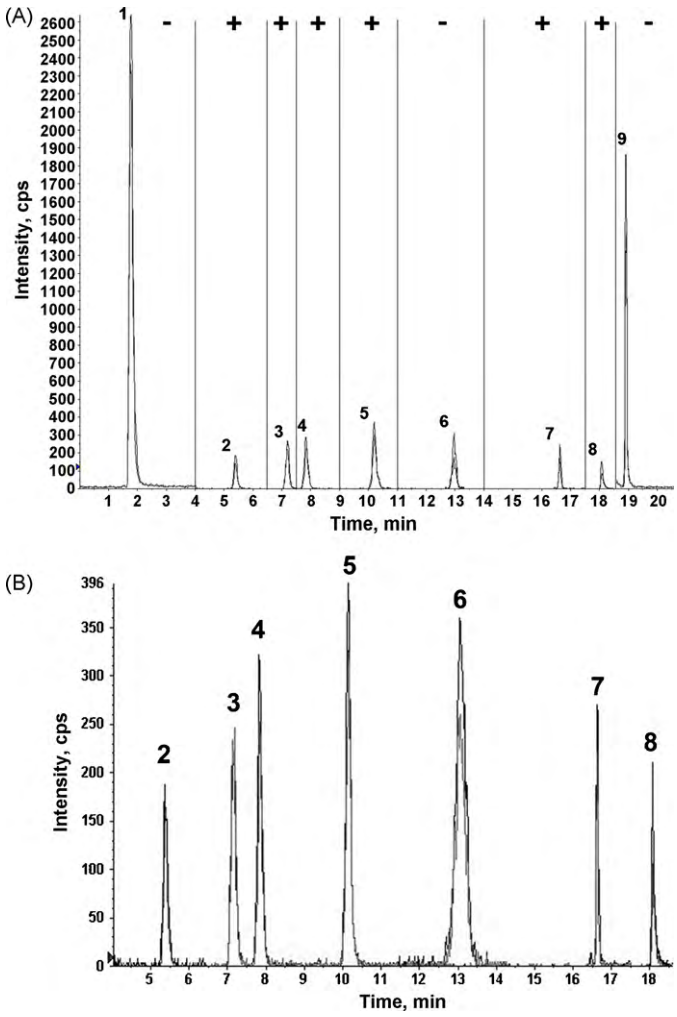

Fig. 1. (A) LC/APPI-MS/MS chromatogram relative to the selected transitions for each mycotoxin and obtained in MRM mode, resulting from 1 g of an analyte-free wheat sample spiked with the analytes at Maximum Limit (ML). Column: Kinetex C18; injected 10/500 of the extract (see text for chromatographic conditions) and (B) is a zoom of (A). 1-DON; 2-AFG2; 3-AFG1; 4-AFB2; 5-AFB1; 6-OTA; 7-HT-2; 8-T-2; 9-ZON.

loadability respect the fully porous materials (supplier advices to inject 2 μL sample volume). We found that the injection of 10/500 of the extract was an acceptable compromise. In these conditions, the peak of DON was only slightly broadened.

In Fig. 1, the mass-chromatogram relative to the selected transitions for each mycotoxin for an analyte-free wheat sample spiked with the analytes at MLs is reported (two smoothing by applying the mean smoothing method were done).

3.3. Method validation

When available, certified reference materials represent a widely accepted choice for method validation. For mycotoxins in cereals only three reference material for DON were available; in addition, preparing blanks and spiked samples in house has the advantage that also the effect of biological variability and linearity around the critical values can be tested.

3.3.1. Specificity and ruggedness

Working with contaminants largely diffused, it is not easy to found actual blank samples. Thus our blank samples, apart for DON in reference materials for which the levels were found under the MDL (<80 μg kg^{-1}), should be considered as apparently devoid of contamination, and specificity may only be supposed from the existence of some of this kind of sample. We had access to the collection of the Experimental Institute of Cereal Research (Rome, Italy) and tested only samples found apparently free from mycotoxin contamination. Nonetheless, small amount, especially of DON and ZON, were systematically found in the samples, so we decided to take into account these small amount as actual contamination.

For all the analytes, the weakest ion transitions ranged between 40% and 85% of the most intense one. Over 4 months, the tolerance of the relative ion abundances varied no more than 20%, and retention time no more than 2%. This meets requirements reported in the 2002/657/EC Decision, Sections 2.3.3.1 and 2.3.3.2 [9].

3.3.2. Calibration curves, matrix effect and linearity

Linear dynamic range was estimated for all the analytes from a six points standard calibration curve. The lowest concentration used for all the compounds was about the method detection limit (MDL) and the highest was MDL × 10^3. The coefficients of correlation r were always >0.9953; only for DON it was = 0.9830. Although a moderate signal suppression was noticeable for all the analytes and the IS, matrix effect, checked with the ANOVA test, did not give significant differences at $p = 0.05$. Then, calibration in standard solution were used for quantitation.

Linearity of the method was assessed by calibration curves obtained by analyzing spiked samples according to the protocol of validation previous described in Section 2.6. The regression coefficient r ranged between 0.8752 (DON in wheat) and 0.9465 (ZON in maize). This should be considered a satisfactory results, considering that the experiment for assessing linearity was conducted over a working week.

3.3.3. Trueness and within-laboratory reproducibility

Reference maize at certified concentration for DON = 474 μg kg^{-1} (about 2/3 ML) was analyzed six time. The mean concentration was 423 μg kg^{-1} (RSD 12%). Six wheat and six maize samples from different cvs. were spiked with the analytes at ML concentration, and analyzed. This series of analyses was repeated on two other days by different operators. Results are reported in Table 3. As already reported, some samples contained small amount of one or several analytes. As for linearity, these amount was considered and subtracted from all these measured in spiked samples. This procedure is arbitrary but can be assumed as a reasonable one. To verify that the method performance was not dependent on the particular operator or day of analysis, mean accuracy data were compared by using the ANOVA (analysis of variance) test at the $p = 0.05$ significance level. In any case, the calculated values were lower than the critical ones.

For all the analytes, bias related to mean concentrations measured by the three operators ($n = 18$) were from −13% to +12% of the nominal spiking level, and the overall within-laboratory reproducibility ranged 3–16%, satisfying the criteria suggested by the EU Decision for the concentration level considered.

3.3.4. Limits of identification and quantification, decision Limit (CCα), detection capability (CCβ)

When using a MS detector, the first condition to be satisfied for ascertaining the targeted compound presence is that the precursor ion and at least two product ions (i.e., two MRM transitions) produce signals distinguishable from the background ion current. According to it, for each analyte a definition of LOD as the amount giving S/N = 3 for the second most intense transition (qualifier ion) was adopted, while instrumental limits of quantification (LOQs), were defined as the amount giving S/N = 10 for the most intense transition ion currents (quantifier ion). Method identification limits (MILs) and method quantification limits (MQLs) were estimated in the same way of LODs and LOQs, respectively, by analyzing sam-

Table 3
Trueness and within-laboratory reproducibility resulting from analyzing on three different occasions and by different operators six wheat and six maize samples from different cultivar samples were spiked with the analytes at MR concentration level.

	Wheat				Maize			
	Day 1 Bias % (RSD%)	Day 2 Bias % (RSD%)	Day 3 Bias % (RSD%)	Overall Bias % (RSD%)	Day 1 Bias % (RSD%)	Day 2 Bias % (RSD%)	Day 3 Bias % (RSD%)	Overall Bias % (RSD%)
DON	−10 (13)	−8 (9)	−12 (7)	−10 (10)	−15 (6)	−14 (10)	−9 (11)	−13 (9)
AFG2	+4 (8)	−6 (8)	+5 (11)	+1 (9)	+3 (6)	+7 (9)	+8 (12)	+6 (9)
AFG1	+9 (11)	+1 (12)	+9 (6)	+6 (10)	+7 (22)	+11 (8)	+8 (15)	+9 (15)
AFB2	+6 (7)	−3 (10)	−7 (15)	−1 (11)	+2 (16)	+7 (14)	0 (18)	+3 (16)
AFB1	−8 (5)	+2 (8)	+6 (9)	0 (7)	−1 (11)	−7 (4)	−3 (7)	−4 (7)
OTA	−12 (12)	−4 (15)	+2 (17)	−5 (15)	+5 (18)	−6 (15)	−8 (11)	−3 (15)
HT-2	+11 (5)	+7 (8)	+2 (9)	+7 (7)	+4 (3)	+8 (12)	+2 (7)	+5 (7)
T2	−3 (15)	+7 (16)	+4 (7)	+3 (13)	+12 (13)	+8 (15)	−2 (17)	+6 (15)
ZON	−11 (4)	−8 (10)	−6 (11)	−8 (8)	+2 (9)	−6 (5)	−9 (11)	−4 (8)

Table 4
Method performances.

Analyte	LOD[a] (pg)	LOQ[b] (pg)	MIL[c] (μg/kg)	MQL[d] (μg/kg)
DON	470	670	80	120
AFG2	8	15	0.2	0.5
AFG1	1.5	4	0.07	0.1
AFB2	6	9	0.2	0.3
AFB1	3	5	0.2	0.2
OTA	16	30	1.0	1.2
HT-2	220	270	7	9
T-2	200	300	5	11
ZON	70	180	5	6

[a] Instrumental limit of detection.
[b] Instrumental limit of quantification.
[c] Method identification limit (S/N = 3 for the second most intense transition in MRM).
[d] Method quantification limit (S/N = 10).

ples fortified at 0.3 ML. Data are shown in Table 4 and are the mean of the values obtained from six wheat and six maize samples which did not differ each other more than about 30%. As can be seen, MQLs ranged from 1/20 (AFG1) to 1/4 (AFG2 and OTA) of the MLs established by European Union (EU) in Regulation (EC) No. 1881/2006 and subsequent amendments (i.e., Regulation (EC) No. 1126/2007) for mycotoxin limits.

In the 2002/657/EC European Decision, for substances for which a ML has been established, the CCα was defined as the limit above which it can be concluded with an error probability of α (α = 5%) that a sample is non-compliant, and CCβ as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (β = 5%). In our case the rigorous calculation by the formula reported in a previous work [50] cannot be performed because of the difficulty to find actual blanks from which a blank mean value μ_N and its RSD should be calculated. To overcome this impasse we used the MDL (calculated for the most intense transition) and its RSD calculated over a week instead of the rigorous parameter. CCα values did not differ more than 20% and CCβ not more than 42% from their respective ML. This approach, probably leads to a slightly underestimation of both CCα and CCβ. It is noteworthy that these values did not depend on the ML of the compounds. This is probably due to the fact that compounds with the lowest ML have the highest sensitivity.

4. Conclusions

A new LC/APPI-MS/MS method was developed to identify and quantify simultaneously the major mycotoxins included in EU 1881/2006 Regulation. The advantages of using an APPI source are the sensitivity for AFs, higher than by using ESI, and the reduction of matrix effect that allow the use of an external calibration without the use of isotopic internal standard.

The LC–MS/MS method developed in this work, specifically studied for confirmatory analysis purpose, fulfilled Commission Decision 2002/657/EC. The method has several advantages over the previously reported methods; it is rapid, accurate and selective. Finally, the same extraction and LC–MS/MS conditions can be used for a wide cereal matrix variety. Then, it could be applied for the establishment of monitoring programs for mycotoxins in different types of cereals.

It is noteworthy that a great variability in mycotoxin analyses may occur since usually contamination is not uniformly distributed in a load. Heterogeneity of mycotoxin distribution [55] is a technical difficulty in experiments and even under laboratory conditions, complete homogeneity was not attained. This caused substantial variations in results that often depend on how many contaminated samples were present in the sample, and on how high the level of contamination was in those samples. This source of variation could be much more important than both intra- and inter-laboratory reproducibility, thus the assessment of CCα and CCβ for a method for determining mycotoxins may be of very limited usefulness.

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