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Determination of trichothecenes in cereals and derived products by liquid chromatography tandem mass spectrometry

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Determination of trichothecenes in cereals and derived products by liquid chromatography tandem mass spectrometry

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Abstract

A sensitive, accurate and precise method for the simultaneous determination of nivalenol (NIV), deoxynivalenol (DON), T-2 toxin (T-2) and HT-2 toxin (HT-2) in different food matrices, including wheat, maize, barley, cereal-based infant foods, snacks, biscuits and wafers has been developed. The method, using liquid chromatography coupled with atmospheric pressure chemical ionization triple quadrupole mass spectrometry (LC-APCI-MS/MS), allowed unambiguous identification of the selected trichothecenes at low µg/kg levels in such complex food matrices. A clean up procedure, based on reversed phase SPE Oasis® HLB columns was used, allowing good recoveries for all studied trichothecenes. In particular, NIV recoveries resulted significantly improved as compared with those obtained by using Mycosep® #227 columns for the clean up of the extracts. Limits of detection in the various investigated matrices, ranged from 2.5 to 4.0 µg/kg for NIV, from 2.8 to 5.3 µg/kg for DON, from 0.4 to 1.7 µg/kg for HT-2, from 0.4 to 1.0 µg/kg for T-2. Mean recovery values, obtained from cereals and cereal products spiked with NIV, DON, HT-2 and T-2 toxins at levels from 10 to 1000 µg/kg, ranged from 72% to 110% with mean relative standard deviation lower than 10%. A systematic investigation of matrix effects in different cereals and cereal products was also carried out by statistically comparing the slopes of standard calibration curve with matrix-matched calibration curve for each of the 4 toxins and the 8 matrices tested herein. For seven out of the 8 matrices tested statistically significant matrix effects were observed, indicating that for accurate

quantitative analysis matrix-matched calibration was necessary. The method was applied to the analysis of 57 samples of ground wheat originated from South Italy, and of 9 cereal food samples collected from retail market.

Keywords: LC-MS/MS, nivalenol, deoxynivalenol, T-2 toxin, HT-2 toxin, cereals, cereal products, mycotoxins

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Introduction

Trichothecenes causing most concerns for human health are T-2 toxin (T-2) and HT-2 toxin (HT-2), as representative of type-A trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), as representative of type-B trichothecenes. These toxins occurr in cereal grains and are produced by different *Fusarium* species that may develop in field mainly in wheat, maize, barley and oats (Bottalico et al. 1998, WHO/FAO 2001). Trichothecenes are responsible for a wide range of disorders in animals including feed refusal, weight loss and vomiting and have been found to inhibit protein, DNA and RNA synthesis, and to have immunosuppressive and cytotoxic effects (Visconti et al. 1991; Prelusky et al. 1994; Visconti 2001, WHO/FAO 2001). The European Scientific Committee on Food (SCF) evaluated in a set of opinions the *Fusarium* toxins, including DON, NIV, T-2 and HT-2 (SCF 2002) and established a fully tolerable daily intake (TDI) for DON at 1 µg/kg body weight/day, a temporary TDI (t-TDI) for NIV at 0.7µg/kg body weight/day, and a combined t-TDI for T-2 and HT-2 at 0.06 µg/kg body weight/day. Health risks associated with human exposure to *Fusarium* toxins are worldwide recognized and depend on the extend to which they are assumed in a diversified diet. The major source of dietary intake of trichothecenes are products made from cereals, in particular wheat and maize. Processing of cereal crops into foods and commercial or home preparation of cereal based foods may decrease the level of mycotoxins but does not completely eliminate them (Visconti et al. 2004). Therefore, the presence of trichothecenes in the human diet, and especially in the diet of vulnerable populations such as infants, is of great concern. However, limited information are available on the distribution of these mycotoxins in cereal based foods (Lombaert et al. 2003; Jestoi et al. 2004), especially in infant foods, also due to the lack of reliable methods for trichothecene determination at low levels (µg/kg).

A recent data collection on the occurrence of *Fusarium* toxins in food in the European Union (EU), performed within an ad hoc SCOOP project (SCOOP 2003), showed that the dietary intakes of *Fusarium* toxins for the entire population and adults are often lesser than the TDIs for the respective toxin. Nevertheless for risk groups like infants and young children, the dietary intake of DON was found close to the TDI, and T-2 and HT-2 intakes were found to be higher than the t-TDI. As underlined in the same SCOOP report, intake data were strongly influenced

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by the limit of detection (LOD) of the analytical method used since negative samples (80% for T-2 and 86% for HT-2) were assumed to contain toxins at level of LOD/2. Therefore an overestimation of the intake was reported since LODs were up to 100 µg/kg.

Sensitive, accurate and reliable methods of analysis for these toxins in cereals and cereal-based foods are needed to better estimate human exposure and to assess compliance of food with European Union regulations, which are already in place for DON and programmed for T-2 and HT-2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is becoming the technique of choice for the simultaneous determination of type-A and type-B trichothecenes (Berger et al. 1999, Berthiller et al. 2005, Biselli & Hummert 2005, Cavaliere et al. 2005). Besides the high sensitivity, tandem mass spectrometry provides also the highest degree of certainty in analyte identification and may be employed in accordance with recent European Union guidelines to obtain data with relevant unambiguity (European Commission 2002).

Although the high selectivity of LC-MS/MS instruments should allow to reduce and simplify sample preparation, the presence of co-extracted matrix compounds can affect the analyte ionization, leading to signal suppression or enhancement that affect the accuracy of the measurement.

Methods using MycoSep® #227 multifunctional clean-up column (containing charcoal, celite and alumina) are simple and used in several laboratories for simultaneous analysis of type-A and type-B trichothecenes, although low NIV recoveries have been reported (Langseth & Rundberget 1998, Berthiller et al. 2005, Biselli & Hummert 2005).

In this paper we describe a new sample preparation procedure for determination of NIV, DON, HT-2 and T-2 in cereals and cereal-based products by LC-MS/MS. The developed method, using Oasis® HLB SPE columns to cleanup sample extracts, allows high recoveries for both polar (NIV, DON) and non polar (HT-2, T-2) toxins. A detailed investigation of matrix effects in different cereal and cereal-based foods has also been carried out.

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The method was used to analyze 57 samples of durum ground wheat obtained from wheat produced in Apulia region (South Italy) and 9 cereal-based food samples collected from retail market.

Materials and Methods

Chemicals and Reagents

Acetonitrile and methanol (both HPLC grade) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (for mass spectrometry), NIV, DON, T-2, and HT-2, were purchased from Sigma-Aldrich (Milan, Italy). Oasis® HLB columns were purchased from Waters (Milan, Italy), MycoSep® #227 columns from Romer Labs® Diagnostic GmbH (Herzogenburg, Austria). Filter papers (Whathman no. 4) were obtained from Whathman International Ltd., Maidstone, UK. HPLC syringe filters (0.45 μm) were from Alltech (Deerfield, IL, USA).

For method development three raw cereals, namely wheat, maize and barley, and five cereal-based foods, namely infant semolina, infant biscuits, bacon flavored biscuits, wafers and coconut snacks containing wheat, barley, oats, rice and maize were used. All these samples were collected from the Italian retail market, between 2005 and 2006. Durum ground wheat samples were obtained from grains cultivated in Apulia region (South of Italy) in 2005 and 2006.

Instrumentation

LC-MS/MS analyses were performed on a Q TRAP® MS/MS system, from Applied Biosystems (Foster City, CA, USA) equipped with either an APCI (atmospheric pressure chemical ionization) or an ESI (electrospray ionization) interface and a 1100 Series micro-LC system comprising a binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany).

Sample preparation and clean up with Oasis® HLB columns

Ground samples (10 g) were extracted with 50 ml acetonitrile/water (84:16, v/v) for 60 min on an orbital shaker (model 711 VDRL, Asal, Milan, Italy). The extract was filtered through filter

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paper (Whatman no. 4), and 5 ml of filtrate (equivalent to 1 g sample) were evaporated to dryness at 50 ºC under a stream of air. The residue was reconstituted with 1 ml of methanol/water 10/90 (v/v) and cleaned up through an Oasis® HLB column. The column was attached to a vacuum manifold (Supelco, Bellefonte, PA, USA), conditioned with 2 ml methanol, and equilibrated with 2 ml methanol/water 10/90 (v/v) at flow rate of about one drop per second prior to use. The reconstituted sample extract was passed through the column that was then dried by passing air for 10-20 seconds. Then NIV and DON were eluted with 1 ml methanol/water 45/55 (v/v) (first eluate), at flow rate of about one drop per second until air passed through the column. The column was then washed with 1 ml methanol/water, 55/45 (v/v) and dried again. Afterwards T-2 and HT-2 were eluted with 1 ml methanol (second eluate). The second eluate was dried, at 50 ºC under a stream of air, and redissolved with the first eluate containing NIV and DON by vortexing for 1 min. Twenty microliters (equivalent to 20 mg sample) were analyzed by LC-MS/MS.

Sample preparation and clean up with MycoSep® #227

For comparison purposes the filtered sample extracts were cleaned up by Mycosep® #227 columns according to Berthiller et al. (2005). In particular, ground samples (10 g) were extracted with 50 ml acetonitrile/water (84:16, v/v) by shaking for 60 min. The extract was filtered through filter paper (Whathman no. 4), and 9 ml of filtrate was slowly pressed through the MycoSep® #227 column. Five milliliters of purified extract (equivalent to 1 g sample) was evaporated to dryness at 50ºC, under a stream of air, and reconstituted with 1 ml of starting LC mobile phase (methanol/water, 20/80 v/v, containing 5 mM ammonium acetate). Twenty microliters were injected into the LC-MS/MS apparatus.

Standards and matrix-matched calibration

Stock solutions of each trichothecene were prepared by dissolving the solid commercial toxin in acetonitrile at concentration of 1 mg/ml. Mixed diluted standard solutions of NIV, DON, T-2 and HT-2 toxins were prepared in acetonitrile at concentrations of 100 µg/ml, 10 µg/ml, 1 µg/ml and 0.1 µg/ml. These solutions were used to prepare calibrant solutions and for spiking experiments.

Calibrant solutions for standard calibration curves were prepared in the starting LC mobile phase (methanol/water 20/80, v/v, containing 5 mM ammonium acetate) by dissolving adequate

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amounts of trichothecenes standard solutions, previously evaporated to dryness under nitrogen stream. Standard calibration curves were performed in the range 0.2 – 20 ng injected toxin (6 points).

Calibrant solutions for matrix-matched calibration curves were prepared in blank sample extract solutions passed through Oasis® HLB or Mycosep® #227 columns. In particular, for the blank extracts purified by Oasis® HLB, each mixed standard solution was added to the second eluate then dried and redissolved with the first eluate. For the blank extracts purified by Mycosep® #227, each mixed standard solution was added to the purified extract then dried and reconstituted with 1 ml of the starting LC mobile phase (methanol/water 20/80, v/v, containing 5 mM ammonium acetate). Matrix-matched calibrations (6 points) were performed in the range 0.2 – 20 ng toxin injected (corresponding to 10 – 1000 µg/kg when referred to the initial sample). For LC-MS/MS infusion experiments two solutions of each trichothecene, at concentration of 1 µg/ml, were prepared in methanol/water (40/60, v/v) with or without ammonium acetate (5mM).

Recovery experiments

For recovery experiments from the Oasis® HLB columns, dried blank crude sample extracts (equivalent to 1 g matrix) of infant semolina were spiked with 100 µg/kg of each toxin, dissolved in 1 ml methanol/water (10/90, v/v) and passed throughout different Oasis® HLB columns. Then each column was eluted with 1 ml of a methanol/water mixtures having different methanol content (10, 20, 30, 40, 45, 50, 55, 60, 70, 100%). Twenty microliters of each eluate were analyzed by LC-MS/MS.

Recovery experiments for the whole method were carried out in quadruplicate by spiking cereal-based food samples with each toxin at levels of 10, 100, 500, and 1000 µg/kg, and raw cereals at 100 µg/kg. Spiked samples were left overnight to allow solvent evaporation prior to extraction. After breaking lumps, samples were extracted and the filtered extracts were purified by Oasis® HLB or Mycosep® #227 as described in *Sample preparation* sections.

In all experiments, the amounts of recovered toxins were calculated by using matrix-matched calibration.

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Matrix effect study

Matrix effects were evaluated by comparing standard and matrix-matched calibration curves. The *t*-test was applied to statistically quantify the slope difference between standard and matrix-matched calibration curves using a significance alpha level of 0.05 (degrees of freedom = 8, critical *t* value = 2.3). *t* values were calculated by using the following formula

$$t_{(n-4)} = (b_1-b_2)/es_{(b1-b2)}$$

were:

N-4 are the degree of freedom;

b_1 and b_2 are the slopes of the two calibration curves to be compared;

$es_{(b1-b2)}$ is the standard error of the difference between the two slopes calculated as described by Soliani (2005).

LC parameters

The analytical column was a Synergi Hydro® (150 mm × 3 mm, 4 µm particles) (Phenomenex, USA), preceded by an Aqua C18 guard column (4 mm x 2 mm, 10 µm particles) (Phenomenex, USA). The flow rate of the mobile phase was 400 µl/min, while the injection volume was 20 µl. The column effluent was directly transferred into the APCI interface, without splitting. Two consecutive isocratic mixtures of methanol/water containing 5 mM of ammonium acetate were used. In particular, 20% methanol was pumped for 9 min followed by 80% methanol for 8 min. The column was re-equilibrated with 20% methanol for 5 min. Before LC-MS/MS analysis all samples were filtered through 0.45 µm membrane filter.

MS parameters

For LC-MS/MS analyses, the APCI interface was used with the following settings: temperature (TEM) 450 ºC and 380 ºC, for negative and positive ion mode, respectively. Curtain gas (CUR), nitrogen, 30 psi; nebulizer gas (GS1), air, 50 psi; auxiliary gas (GS2), air, 30 psi; corona discharge needle current (NC), -3 or +3 µA, respectively. The mass spectrometer operated in MRM (multiple reaction monitoring) mode, with a dwell time of 100 ms. Quantification of trichothecenes was performed by measuring peak areas in the TIC chromatogram (total ion chromatogram, sum of all monitored transitions) and by comparing them with the relevant calibration curve.

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For tuning experiments with the APCI interface, standard solutions of each mycotoxin (10 μg/ml) were infused, using a T-piece, at 10 μl/min into a flow of 400 μl/min of methanol/water (40/60 v/v), with or without ammonium acetate (5mM), delivered by the LC pump. The infusion was performed by using an Harvard infusion pump (model 11, South Natick, MA, USA). The ESI interface was solely used for tuning experiments performed by direct infusion at flow rate of 20 μl/min of the 1 μg/ml standard solutions of individual trichothecenes in methanol/water (40/60, v/v) with or without ammonium acetate (5mM). Interface parameters were as follows: TEM, 300 ºC, CUR, 20 psi, GS1, 15 psi, GS2, air, 30 psi, ionspray voltage + 4500 V or – 4500V, respectively.

Results and discussion

LC-MS/MS optimization

Preliminary studies were conducted to select, for each analyzed trichothecene, the most suitable precursor ion, and to identify the specific transitions to be used in the MRM (multiple reaction monitoring) acquisition mode. Moreover, since in LC – MS detection the response is strongly dependent on the interface conditions (Razzazi-Fazeli et al. 1999, 2002, Pallaroni et al. 2002), the interface parameters were optimized in order to obtain maximum sensitivity for all tested mycotoxins. For this purpose, trichothecene solutions were directly infused into the mass spectrometer and analyzed with two different interfacing systems, ESI and APCI. In agreement with previously published studies (Berthiller et al. 2005, Biselli & Hummert 2005, Razzazi-Fazeli et al. 2002), NIV and DON showed higher signal intensities in negative ionization mode, while T-2 and HT-2 toxins were more easily ionized in positive mode. Since the formation of sodiate adducts was observed using both ESI and APCI interfaces, 5 mM ammonium acetate was added to the solvent mixture, leading to mass spectra containing almost exclusively a single trichothecene-adduct ion species: the $[M+CH_3COO]^-$ in the negative ionization, or the $[M+NH_4]^+$ adduct in the positive ionization. The following precursor ions were therefore chosen to develop the MRM acquisition method: $[M+CH_3COO]^-$ for NIV (m/z 371.1) and DON (m/z 355.0), and $[M+NH_4]^+$ for T-2 (m/z 484.0) and HT-2 (m/z 442.0).

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After tuning of the interface parameters, the best results in terms of signal intensities were observed with APCI interface, giving for the precursor ions of all studied trichothecenes signals about five fold more intense as compared to that obtained with the ESI interface in agreement with the results obtained by Berthiller et al. (2005). Other authors found that for type-B trichothecenes ESI interface performed better than APCI (Laganà et al. 2003, Biselli & Hummert 2005). However they selected $[M-H]^-$ as precursor ion and the possibility to work with acetate adducts was not investigated. No comparison between ESI and APCI for T-2 and HT-2 have been reported by Biselli et al. (2005), working with sodiate adducts $[M+Na]^+$ for these toxins. Based on these results we decided to use the APCI interface in the successive steps of method development.

MS/MS experiments were performed to find the most intense and specific transitions for each trichothecene. For selective detection three transitions were chosen for DON and NIV, and five transitions for T-2 and HT-2, that exhibited a more complex fragmentation pattern. Collision energy (CE) and collision cell exit potential (CXP) were optimized for each selected transition. The selected transitions for the four toxins and the resulting MRM method are reported in Table 1.

A good separation of NIV, DON, T-2 and HT-2 was obtained by using a Synergi Hydro® column and two isocratic compositions of methanol/water containing 5 mM ammonium acetate, i.e. 20/80 (v/v) for the separation of NIV and DON, and 80/20 (v/v) for the separation of HT-2 and T-2. Retention times were: 4.1 min for NIV, 7.8 min for DON, 14.8 min for HT-2, and 15.5 min for T-2. As reported in Table 1, the selected precursor ions required the ionization polarity to be switched from negative (for the detection of NIV and DON) to positive (for the detection of HT-2 and T-2). The time windows defined from the two isocratic steps corresponded to the two periods of polarity switching of the APCI interface.

By operating under isocratic conditions it was also possible to define two distinct optimal temperatures of the heated nebulizer in the LC-MS/MS run. It is well documented that the temperature of the nebulizer (TEM) could significantly affect the APCI performances in terms of ionization efficiency (Razzazi-Fazeli et al. 1999, 2002, Pallaroni et al. 2002). The optimal temperature is affected not only by the type of sample, but also by the flow rate and the mobile

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phase composition. Low temperature produces incomplete vaporization of the sample, whereas too high temperature could induce thermal degradation of analytes. Moreover, the probe temperature should be inversely correlated to the percentage of organic solvent in the mobile phase. Under our operating conditions the optimal temperature was 450 ºC for the detection of NIV and DON (20% methanol in the mobile phase), and 380 ºC for the detection of HT-2 and T-2 (80% methanol in the mobile phase).

Development of the cleanup procedure

One of the aims of this work was to find a suitable SPE column allowing the simultaneous purification of type-A and -B trichothecenes, that vary considerably in polarity, from NIV (the most polar trichothecene) to T-2 toxin. For this reason we chose to test the performances of the Oasis® HLB column, filled with a patented hydrophilic/lipophilic balanced polymer [poly(divinylbenzene-co-N-vinylpyrrolidone)], which retains both polar and non-polar compounds.

The reconstitution in 10% methanol of dried extracts of infant semolina, spiked at 100 µg/kg of each toxin, allowed complete dissolution of the four toxins. When passed through the Oasis® HLB column the four toxins were quantitatively retained without any loss of loaded toxins. Mixtures with different methanol/water ratio were then tested for the washing and elution steps in order to achieve quantitative recoveries for all trichothecenes together with an efficient removal of matrix interfering compounds. Quantitative elution of NIV and DON was obtained by passing 1 ml methanol/water 45/55 (v/v). After washing with 1 ml methanol/water 55/45, quantitative elution of HT-2 and T-2 was obtained by passing 1 ml methanol. A washing step before the elution of NIV and DON was not possible since mixtures of methanol/water with methanol percentages between 10 and 45% resulted in significant loss of NIV. On the other hand a washing step with 55% methanol was possible before the elution of HT-2 and T-2 that occurred with methanol percentage >60%.

Matrix effect study

Despite the high sensitivity and selectivity, the influence of matrix components on the analyte ionization could represent a limit to the accuracy of the LC-MS/MS methods. The co-elution of

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matrix compounds could results in the enhancement or suppression of the analyte chromatographic signal. A detailed investigation of possible matrix effects was thus performed for the cereals and cereal-based foods considered in this study. Six points standard calibration curves and six points matrix-matched calibration curves were performed for each of the 4 toxins and the 8 matrices tested herein. The *t*-test was applied to statistically evaluate the slope differences between standard and matrix-matched calibration curves, giving a significance alpha level of 0.05, with 8 degrees of freedom. Under these conditions, the critical *t* value is 2.3. For *t* values higher than 2.3, the slope difference is considered statistically significant, i.e. considerable matrix effects occurs.

The obtained *t* values, are reported in Table 5. This study revealed that the presence of co-eluting matrix molecules resulted in a general enhancement (20/32 cases) of APCI response of all analyzed mycotoxins, even though in some cases, such as semolina, no significant matrix effects were observed. As expected, the strongest matrix effects were observed for NIV, having this compound the shortest retention time. Moreover, the slope of the regression varied considerably from matrix to matrix also revealing changing influences of co-eluting matrix compounds on the analyte signal. It was evident, for example, that no general regression curve could be used for different raw cereals, but calibration for each individual cereal grain was necessary. On the basis of these findings we conclude that, due to the observed matrix effects, the matrix-matched calibration is necessary for reliable quantitative analyses.

Our results are in agreement with Biselli & Hummert (2005) that recently performed a similar evaluation of matrix effect by comparison of slope differences between standard and matrix calibration for soybean and oats. The authors reported a strong signal suppression, up to 40%, in soybean extracts purified with Mycosep® #227 and "nearly no matrix effects" for oats, ground wheat and rye extract, although statistical evaluation of slope differences was not performed.
Matrix effects in trichothecene determination by LC/MS are well known since the first reports, and different approaches have been proposed to overcome this problem. For example, Razzazi-Fazeli et al. developed a double clean up procedure, using Mycosep #227® and 226® columns, to eliminate matrix effects in the determination of T-2 and HT-2 in oats (Razzazi-Fazeli et al. 2002).

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The use of isotope labeled internal standard is another mode to correct matrix effects in LC-MS/MS analyses. However few isotope labeled standards are commercially available. In particular, they are available for DON and T-2 but not for NIV and HT-2 (Häubl et al. 2006, Bretz et al. 2006). To date the most frequently used approach is the matrix-matched calibration (Laganà et al. 2003, Cavaliere et al. 2005, Dall'Asta et al. 2004, Razzazi-Fazeli et al. 2003). An alternative approach has been recently suggested to minimize or eliminate matrix effect by a drastic dilution of the sample extract combined with the use of high sensitive MS/MS instruments (Sulyok et al. 2006). However, signal enhancement/suppression was still observed for wheat and maize confirming that matrix effect have to be always carefully evaluated.

Performances of the analytical method

Method validation was performed in three different cereals (wheat, barley, and maize) and five cereal based foods (infant semolina, infant biscuits, bacon biscuits, cocoa wafers and coconut snacks). Matrix-matched calibration curves for all analytes were linear over the working range of 10 µg/kg – 1000 µg/kg, for all tested matrices, with r values between 0.9997 and 0.9999. Limits of detection for all investigated matrices are reported in Table 2. LOD values (S/N = 3) ranged from 2.5 to 4.0 µg/kg for NIV, from 2.8 to 5.3 µg/kg for DON, from 0.4 to 1.7 µg/kg for HT-2, and from 0.4 to 1.0 µg/kg for T-2. These results confirm that the use of the MRM detection mode allows not only a high degree of selectivity, but also high sensitivity. Similar LODs have been obtained by other authors (Berthiller et al. 2005, Biselli & Hummert 2005, Lagana' et al. 2003) that developed MRM methods for trichothecene detection, while previous methods based on Single Ion Monitoring (SIM) generally achieved higher detection limits (Dall'Asta et al. 2004, Razzazi-Fazeli et al. 1999, 2002, 2003).

Results of recovery experiments from infant semolina, infant biscuits, bacon biscuits, cocoa wafers and coconut snacks are reported in Table 3. Recoveries for type-B trichothecenes for the tested matrices ranged from 72 to 100% and from 73 to 110% for type-A trichothecenes, with relative standard deviation generally lower than 7%.

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Recovery and repeatability values of the method fulfill the criteria established by CEN (European Committee for Standardization) for acceptance of an analytical method also at concentrations about ten times lower to the lowest concentrations reported for these toxins in the Commission Regulation (EC) No 401/2006 (European Commission 2006). Moreover, the method fulfills the requirements established by the European Commission for the confirmation of substances listed in group B of Annex I of Directive 96/23/EC i.e. at least three identification points: retention time, molecular mass, one characteristic product ion (European Commission 2002).

Performances of the present method were compared with those of a previously reported method using Mycosep® #227 columns for clean up of the extract (Berthiller et al. 2005). For this purpose recovery experiments were carried out in both cereals and cereal-based foods spiked at 100 µg/kg of each toxin and the results are reported in Table 4. Good and comparable recovery values were obtained for DON, T-2 and HT-2 with the two methods, whereas significantly higher NIV recoveries were obtained for all tested matrices with the present method as compared with the method using the Mycosep columns. For cereal-based products the different recoveries of NIV were more pronounced between the two types of column as shown in Figure 2. In particular, for infant semolina, infant biscuits, cocoa wafers and coconut snacks mean NIV recoveries were 35% and 80% for Mycosep® #227 and Oasis® HLB columns, respectively. The low NIV recoveries (31-37%, see Table 4) obtained from cereal-based products (infant semolina, infant biscuits, cocoa wafers, coconut snacks) purified with Mycosep® #227columns could be correlated to their sugar content considering that recoveries from raw cereals and bacon biscuits were higher and in the range of 56-66%. By using Mycosep® 226 column clean up, higher recoveries for NIV (89%), but low recoveries for the less polar HT-2 and T-2 toxins (54% and 71%, respectively) have been reported in wheat (Biselli & Hummert 2005). The use of Oasis® HLB columns for clean up provided good recoveries for all toxins and matrices tested herein showing a wide range of applicability of the present method. Acceptable recoveries have been reported in corn and corn meal for the four toxins considered in this study after a time consuming clean up procedure by carbograph-4 cartridge applied to 1 l of diluted extract (Laganà et al. 2003, Cavaliere et al. 2005).

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Analysis of naturally contaminated samples

The total ion chromatogram (sum of all monitored MRM transitions) of a purified extract from a sample of bacon flavored biscuits naturally contaminated with NIV, near to its LOD, DON at 191 µg/kg, HT-2 at 8 µg/kg, and T-2 at 5 µg/kg is shown in Figure 1. The developed method has been applied to the analysis of 57 samples of ground wheat produced by durum wheat cultivated in Apulia, a region of Southern Italy, and of 9 cereal-based food samples collected from the Italian retail market in 2005-2006. Results are summarized in Tables 6 for ground wheat and Table 7 for cereal-based foods. Analyses of ground wheat samples revealed HT-2 to be the most frequently occurring trichothecene, with a percentage of positive samples of 44%, followed by DON and T-2 found in 18% and 11% of samples respectively. Nivalenol was found only in one out of 57 samples. The highest concentration values were 63.5, 17.3, 14.1, and 1.9 µg/kg for NIV, DON, HT-2 and T-2 respectively. In particular, DON contamination levels were quite below the maximum limit established by the European Commission (2005) in wheat flour, i.e. 750 µg/Kg. These data indicated the Apulia region as a low risk area for NIV, DON, HT-2 and T-2 contamination in agreement with Pascale et al. (2002) who reported that the northern Italian regions seem to be more susceptible to DON contamination than southern regions.

A widespread contamination of DON (8 positives out of 9 tested samples) was found in cereal-based food samples, and DON contamination levels were significantly higher as compared to T-2 and HT-2. Three samples were found contaminated with the four mycotoxins whereas the other samples contained different combinations of toxins. Interestingly, in most cereal-based food samples, the co-occurrence of two or more trichothecenes was found, confirming the need and the relevance of analytical methods for simultaneous monitoring of these trichothecenes in cereal-based foods. The percentage of positive samples for T-2 and HT-2 was higher than that reported for similar products in the SCOOP (2003) report. This finding can be attributed to the higher sensitivity of the method used herein. The levels of T-2 and HT-2 found in this study were lower than the mean levels reported in the SCOOP report supporting the hypothesis that the SCOOP data were overestimated due to the high LOD of the analytical methods reported therein.

Conclusions

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A sensitive, accurate and precise method for the determination of DON, NIV, T-2 and HT-2 was developed and applied to several cereals and cereal-based foods. The use of triple quadrupole analyzer in the MRM detection mode allowed unambiguous identification of the selected trichothecenes in complexes matrices at µg/kg levels. The method revealed its general applicability and robustness among a wide range of cereals and cereal-based foods, with detection limits generally lower than 5 µg/kg for DON and NIV and 1 µg/kg for T-2 and HT-2. The use of reversed phase SPE columns (Oasis® HLB) provided good sample clean up and high recoveries for all studied trichothecenes although matrix-matched calibration was necessary for accurate quantitative LC-MS/MS analyses. In particular, NIV recoveries resulted significantly higher than those obtained when Mycosep® #227 columns were used.

The performance of this method meet the CEN criteria at concentrations much lower than the lowest concentrations reported in the Commission Regulation (EC) No 401/2006 (European Commission 2006) for DON, HT-2 and T-2.

The co-occurrence of NIV, DON, HT-2 and T-2 was found in cereals or cereal-based products produced or commercialized in Italy. This method can be used for multi-mycotoxin analysis of food commodities for both control purposes and surveys aimed to accurately measure human exposure to this group of trichothecenes.

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Table 1: MS/MS parameters for detection of trichothecenes in the multiple reaction monitoring (MRM) method.

Analyte	Precursor ion	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
			311.1			-19	-2.0
NIV	$[NIV+CH_3COO]^-$	371.1	281.0	-23	-6.0	-19	-2.8
			59.1			-38	-6.2
			295.2			-17	-2.4
DON	$[DON+CH_3COO]^-$	355.0	265.1	-23	-6.0	-20	-2.1
			59.1			-36	-6.8
			323.2			13	3.0
			263.3			16	3.6
HT-2	$[HT-2+NH_4]^+$	442.0	245.2	16	4.5	15	3.8
			215.1			16	3.4
			185.3			25	3.4
			365.1			19	4.0
			305.2			19	4.0
T-2	$[T-2+NH_4]^+$	484.0	245.2	16	6.0	19	3.6
			215.1			26	3.3
			185.3			26	2.6

DP: declustering potential
EP: entrance potential
CE: collision energy
CXP: collision cell exit potential

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Table 2: Limits of detections (S/N = 3) of NIV, DON, HT-2 and T-2 in the tested cereals and cereal-based foods.

	LOD (µg/kg)			
	NIV	DON	HT-2	T-2
Infant Semolina	2.5	5.3	0.7	0.4
Infant Biscuits	4.0	4.6	0.8	0.7
Coconut Snacks	2.6	3.8	0.4	0.4
Bacon Biscuits	3.4	3.0	1.7	1.0
Cocoa Wafers	3.0	4.0	0.8	0.5
Wheat	2.6	2.8	0.6	0.4
Maize	2.7	5.1	0.8	0.4
Barley	3.6	5.1	0.9	0.5

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Table 3: Recoveries of NIV, DON, HT-2 and T-2 from cereal-based foods, spiked at different toxin levels, after clean up with Oasis® HLB column. Quantification was performed by using matrix-matched calibration curves.

	Spiking level (µg/kg)	Recovery, % (RSD_r, %)			
		NIV	**DON**	**HT-2**	**T-2**
Infant Semolina	1000	79.2 (4.0)	91.6 (2.3)	105.5 (5.7)	105.7 (3.4)
	500	78.5 (3.0)	94.0 (2.9)	108.6 (2.1)	107.5 (2.8)
	100	75.0 (3.6)	83.2 (5.2)	100.9 (6.9)	99.8 (6.1)
	10	77.8 (3.8)	97.0 (1.0)	99.2 (3.0)	98.8 (4.1)
Infant Biscuits	1000	76.2 (3.4)	85.0 (2.3)	104.5 (4.2)	90.0 (4.0)
	500	83.2 (3.1)	94.8 (3.8)	109.6 (1.0)	100.2 (0.9)
	100	79.4 (2.3)	87.2 (1.6)	109.3 (1.2)	108.4 (4.4)
	10	99.1 (1.3)	84.2 (1.9)	98.3 (1.1)	101.5 (3.9)
Bacon Biscuits	1000	76.5 (4.4)	84.3 (2.3)	89.1 (4.9)	88.2 (6.3)
	500	83.8 (1.8)	98.0 (4.0)	93.0 (1.0)	100.7 (4.0)
	100	75.8 (1.1)	86.3 (4.1)	83.2 (4.4)	88.2 (5.1)
	10	77.7 (1.6)	80.4 (4.5)	85.3 (3.6)	98.6 (2.0)
Cocoa Wafers	1000	79.7 (3.4)	94.7 (4.0)	99.5 (1.4)	98.2 (3.2)
	500	82.6 (2.4)	98.1 (5.7)	101.3 (5.6)	101.5 (3.1)
	100	72.3 (6.8)	80.2 (1.1)	89.6 (3.9)	85.2 (0.6)
	10	78.7 (3.4)	100.3 (4.6)	86.3 (5.6)	93.8 (3.8)
Coconut Snacks	1000	75.0 (7.6)	97.9 (4.2)	80.6 (2.1)	101.1 (6.7)
	500	73.1 (2.1)	81.0 (1.0)	88.8 (1.5)	91.0 (2.2)
	100	73.4 (5.4)	94.3 (1.4)	73.2 (7.9)	91.6 (9.7)
	10	71.8 (5.9)	80.0 (4.2)	73.6 (4.1)	88.1 (2.3)

RSD_r, relative standard deviation (n = 4).

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Table 4: Comparison of recovery values of NIV, DON, HT-2, and T-2 from cereal-based foods and raw cereals, spiked at 100 µg/kg, obtained after clean up with Mycosep® #227, and Oasis® HLB columns. Quantification was performed by using matrix-matched calibration curves.

Sample		Recovery, % (RSD_r, %)			
		NIV	**DON**	**HT-2**	**T-2**
Infant Semolina	Mycosep #227	36.1 (6.3)	83.3 (6.4)	97.8 (2.5)	100.9 (3.2)
	Oasis	75.0 (3.6)	83.2 (5.2)	100.9 (6.9)	99.8 (6.1)
Infant Biscuits	Mycosep #227	36.9 (2.7)	87.2 (3.7)	101.5 (4.2)	124.4 (2.1)
	Oasis	79.4 (2.3)	87.2 (1.6)	109.3 (1.2)	108.4 (4.4)
Bacon Biscuits	Mycosep #227	66.1 (2.2)	95.5 (4.7)	105.0 (2.4)	110.7 (5.6)
	Oasis	75.8 (1.1)	86.3 (4.1)	83.2 (4.4)	88.2 (5.1)
Cocoa Wafers	Mycosep #227	35.0 (6.6)	87.5 (5.2)	96.4 (1.7)	96.1 (3.5)
	Oasis	72.3 (6.8)	80.2 (1.1)	89.6 (3.9)	85.2 (0.6)
Coconut Snacks	Mycosep #227	30.8 (7.2)	90.5 (3.5)	81.8 (3.8)	94.5 (1.9)
	Oasis	73.4 (5.4)	94.3 (1.4)	73.2 (7.9)	91.6 (9.7)
Wheat	Mycosep #227	55.8 (2.9)	91.0 (1.8)	93.7 (8.4)	101.2 (4.4)
	Oasis	93.7 (1.6)	96.4 (2.1)	99.6 (1.5)	92.0 (1.3)
Barley	Mycosep #227	62.9 (7.6)	91.3 (2.9)	104.9 (2.1)	100.8 (0.5)
	Oasis	90.2 (3.1)	89.9 (3.4)	97.1 (8.7)	93.3 (9.3)
Maize	Mycosep #227	56.9 (1.0)	84.1 (0.8)	94.3 (3.9)	83.3 (3.8)
	Oasis	84.1 (0.9)	87.7 (2.6)	96.9 (3.0)	91.7 (2.5)

RSD_r, relative standard deviation (n = 4)

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Table 5: *t* values (α = 0.05) relevant to the slope differences between standard (5 points) and matrix-assisted (5 points) calibration graphs in different cereal and cereal-based foods. Calibration range: [0.2 - 20] ng injected. Degrees of freedom = 8; critical *t* value = 2.3.

Sample	**NIV**	**DON**	**HT-2**	**T-2**
Infant Semolina	0.52	-0.42	0.64	0.88
Infant Biscuits	8.80	2.20	0.81	0.43
Bacon Biscuits	5.57	4.66	4.69	6.45
Cocoa Wafers	10.98	3.50	3.24	0.83
Coconut Snacks	9.66	0.54	0.06	-0.06
Wheat	15.72	2.97	4.19	5.36
Barley	12.90	0.35	2.49	5.36
Maize	2.93	8.65	4.29	4.29

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Table 6. NIV, DON, HT-2 and T-2 content in naturally contaminated samples of ground wheat produced from grain cultivated in Apulia region (South Italy) in 2005 - 2006.

Toxin	Positives/total	% Positives	Mean of positives (µg/kg)	Range of contamination (µg/kg)
NIV	1/57	1.7	63.5	-
DON	10/57	17.5	7.7	3.3 – 17.3
HT-2	25/57	43.8	3.4	0.8 – 14.1
T-2	6/57	10.5	1.3	0.5 – 1.9

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Table 7. NIV, DON, HT-2 and T-2 content in naturally contaminated samples of cereal-based food samples collected from the Italian retail market.

Sample	Main Ingredients	µg/kg			
		NIV	DON	HT-2	T-2
Bacon Snacks	Wheat, eggs, bacon flavour, vegetal fats	traces	191.0	8.0	5.0
Rice Gallettes	Wheat, rice	traces	38.0	nd	nd
Rusks	Wheat, barley, oats, rice, rye	nd	29.7	0.6	nd
Cereal Biscuits	Wheat, oats, rice, spelt, butter, eggs, maltitol	nd	8.2	0.5	0.4
Breakfast Cereals	Wheat, rice, sugar	nd	23.4	nd	nd
Infant Semolina	Durum wheat flour, honey, sugar	nd	7.1	nd	nd
Infant Biscuits	Wheat, barley, oats, rice, rye flour, milk powder, brown sugar	nd	nd	1.8	1.5
Cocoa Wafers	Wheat, oats, milk powder, cocoa, sugar	6.3	38.4	7.5	10.0
Coconut Snacks	Wheat, barley, oats, rice, maize, dried coconut, chocolate, brown sugar	traces	9.5	2.8	3.7
Mean of positives		3.3	43.2	3.5	4.1
Percentage of positives		44%	89%	67%	56%

traces: near to its LOD

nd: not detected

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Caption to figures

Figure 1: Total ion chromatogram (sum of all monitored MRM transitions) of an extract of bacon flavored biscuits naturally contaminated with NIV, near to its LOD, 191 µg/kg DON, 8 µg/kg HT-2, and 5 µg/kg T-2. The mobile phase was methanol/water (20/80) containing 5 mM of ammonium acetate (0-9 min, APCI negative ion mode) followed by methanol/water (80/20) containing 5 mM of ammonium acetate (9-17 min, APCI positive ion mode) at flow rate of 400 µl/min.

Figure 2: Recovery values of NIV from raw cereals and cereal-based foods, spiked at 100 µg/kg, after clean up with Mycosep® 227 or Oasis® HLB columns.

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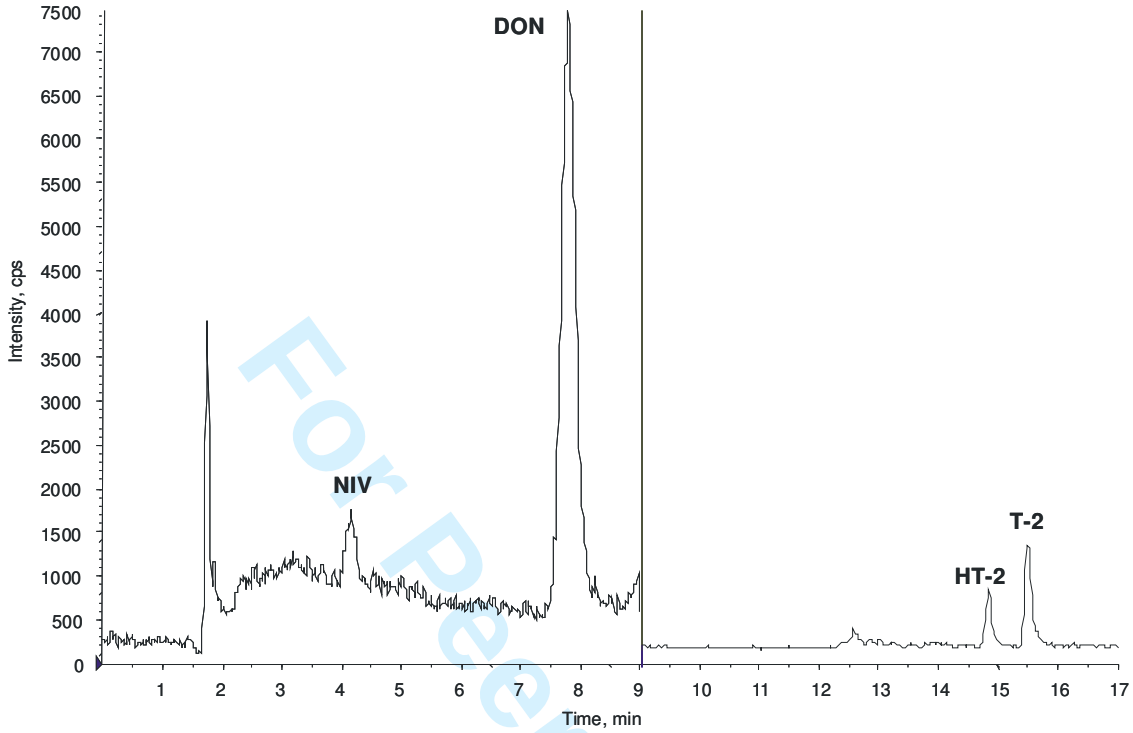

Fig. 1

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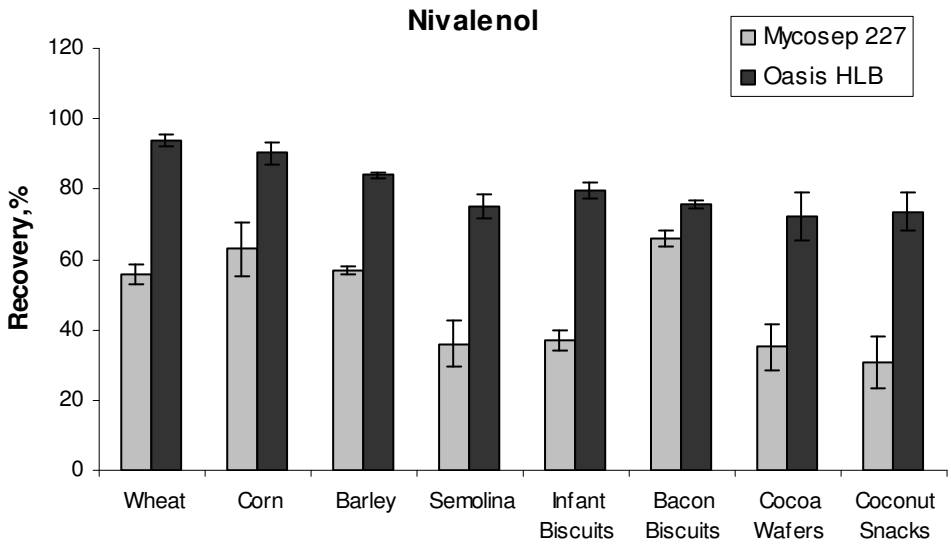

Fig. 2

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