Talanta 84 (2011) 1112–1117

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of type A and type B trichothecenes in paprika and chili pepper using LC-triple quadrupole–MS and GC–ECD

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ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 23 February 2011
Accepted 8 March 2011
Available online 16 March 2011

Keywords:
Chili pepper
Paprika
Mycotoxin
Mass spectrometry
Capsicum
Trichothecene
Liquid chromatography
Gas chromatography
Electron capture detection

ABSTRACT

There is a need to develop sensitive and accurate analytical methods for determining deoxynivalenol (DON), HT-2 toxin and T-2 toxin in paprika to properly assess the relevant risk of human exposure. An optimized analytical method for determination of HT-2 toxin and T-2 toxin using capillary gas chromatography with electron capture detection and another method for determination of DON by liquid chromatography–mass spectrometry in paprika was developed. The method for determination of HT-2 toxin and T-2 toxin that gave the best recoveries involved extraction of the sample with acetonitrile–water (84:16, v/v), clean-up by solid-phase extraction on a cartridge made of different sorbent materials followed by a further clean-up in immunoaffinity column that was specific for the two toxins. The solvent was changed and the eluate was derivatized with pentafluoropropionic anhydride and injected into the GC system. The limits of detection (LOD) for T-2 and HT-2 toxins were 7 and 3 μg/kg, respectively, and the recovery rates for paprika spiked with 1000 μg toxin/kg were 71.1% and 80.1% for HT-2 and T-2 toxins, respectively. For DON determination, the optimized method consisted of extraction with acetonitrile–water (84:16, v/v) solution followed by a solid-phase extraction clean-up process in a cartridge made of different sorbent compounds. After solvent evaporation in N_2 stream, the residue was dissolved and DON was separated and determined by LC–MS/MS. The LOD for this method was 14 μg DON/kg paprika sample and the DON recovery rate was 86.8%.

1. Introduction

Trichothecenes are secondary metabolites produced by several fungal genera, including certain species of *Myrothecium*, *Trichoderma*, *Cephalosporium*, and *Stachybotrys*, but mainly by *Fusarium* species [1–4]. *Fusarium* spp. are worldwide-found in plant debris and crop plants. Growth of *Fusarium* species and toxin production can occur at relatively low temperatures on agricultural commodities in the field or during storage [5].

Trichothecenes have a varying degree of cytotoxic potency and a sesquiterpenoid ring structure, which can be classified according to the presence or absence of characteristic functional groups [6–9]. All trichothecenes contain an epoxide at the C12,13 position, which is responsible for their toxicological activity. The trichothecenes have been classified into four groups: type A has an oxygen functional group other than ketone at C8; type B has a ketone at C8; type C has two epoxide groups; and type D has a macrocyclic ring. Type-A and type-B trichothecenes are the major *Fusarium* mycotoxins [10].

Deoxynivalenol (DON), also known as vomitoxin, is the most commonly found type-B trichothecene all over the world and is associated with various adverse health effects in animals and humans. The most prominent toxic effects of DON are growth retardation and immunotoxicity. It also causes nausea, vomiting and diarrhea and exhibits weight loss and food refusal in animals [11]. It is produced mainly by *Fusarium graminearum* and *Fusarium culmorum*. DON appears predominantly in wheat, corn, rye, rice and barley and also in some spices [12–16].

T-2 toxin (T2) and HT-2 toxin (HT2) are type-A trichothecene mycotoxins produced by various *Fusarium* species, mainly *F. sporotrichioides*, *F. poae*, *F. equiseti* and *F. acuminatum*. T2 is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunosuppressive and cytotoxic effects. The acute toxicity of T2 and HT2 is quite similar. T2 is rapidly metabolized *in vivo* by deacetylation to HT2 and, consequently, the toxicity of T2 *in vivo* might partly be attributed to HT2 [17–19]. These mycotoxins may contaminate a variety of cereal grains, such as wheat, maize, oats, barley, rice and some spices, such as pepper, especially in cold climate regions or during wet storage conditions. T2 and HT2 can also be found in by-products intended for direct human consumption [20].

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doi:10.1016/j.talanta.2011.03.017

The European Scientific Committee on Food has expressed its opinion on *Fusarium* toxins. It set tolerable daily intake (TDI) for DON (1 mg/kg body weight day) and provisional tolerable daily intakes for the sum of T2 and HT2 (0.06 mg/kg body weight day) [21]. A recent European survey in food showed that the TDI for the sum of HT-2 and T-2 toxin is often exceeded. Higher intakes than the recommended TDI were recognized especially for infants and children and indicated clearly that the presence of T2 and HT2 should be of concern regarding public health [22].

Paprika and chili pepper are spices made from the grinding of dried fruits of *Capsicum annuum*. They are used worldwide to add color and flavor to dishes in many cuisines. In Europe, Hungary and Spain are the two main countries for growing paprika peppers. The Spanish grades of paprika are sweet, semi-sweet and hot. Commercial food manufacturers use paprika in cheeses, processed meats, tomato sauces, chili powders and soups. Its main purpose is to add color. The worldwide importance of peppers and chillies (green and dry) could be inferred from the size of its market. According to the FAO [23] the production area for peppers and chillies is over 3,656,538 ha, with a production of 3.097×10^{10} kg of harvested product per year in 2008. China has the highest production of peppers and chillies in the world. Spain is one of the main producer countries of peppers and chillies in the world, with 20,548 ha, or 9.97×10^{7} kg per year. The consumption per person in Europe is lower than that in Latin America, Asia or Africa where peppers consumption per person per year could be higher than that of rice and potato [24]. According to these data, products derived from *Capsicum* are susceptible to fungal contamination owing to harvest conditions and poor storage conditions [25–30].

It is well known that paprika powder and peppers can be contaminated with ochratoxin A and aflatoxins [31–34] and it is very probable that these commodities are contaminated with other mycotoxins, such as the trichothecenes. However, few authors have carried out studies about the occurrence of trichothecene mycotoxins in paprika or chili pepper [35–37]. It is necessary to develop sensitive and accurate analytical methods for determining these mycotoxins in these products to properly assess the relevant risk of human exposure. Different methods, including gas chromatographic and liquid chromatographic methods for the determination of trichothecenes in cereals have been developed [17,38–44] but there are few methods for the determination of HT2, T2 and DON in paprika and chili pepper.

The aim of this study was to develop suitable chromatographic methodologies in terms of accuracy, precision and sensitivity for the determination of DON, HT2 and T2 in paprika and chili pepper and to apply them to the analysis of samples of these products taken from different supplier companies in Spain.

2. Materials and methods

2.1. Samples

A total of 32 samples (17 of paprika, 11 of chili pepper and 4 of smoked paprika) were collected between 2008 and 2010 from different suppliers in Spain. For the sampling plan, the European Regulation No. 401/2006, which stipulates the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs for small lots, was followed [45].

The lot weights of the bulk samples were between 200 and 10,000 kg. All aggregate samples were stored at −20 °C. To facilitate handling of samples, they were reduced to 500 g and these working samples were kept at −20 °C until analysis.

2.2. Chemicals and reagents

Trichothecene standards, including DON, HT2 and T2 were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Toluene and formic acid were purchased from Panreac (Castellar del Vallés, Spain). Acetonitrile and methanol were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were HPLC grade. Standardized 70–230 mesh aluminium oxide 90 (0.063–0.2 mm particle size) was purchased from Merck (Darmstadt, Germany). C18 and silica were purchased from Waters (Milford, MA, USA). Activated charcoal (Norit), heptafluorobutyric anhydride (HFBA), Celite 545 and ammonium acetate were purchased from Fluka (Sigma–Aldrich). Glass microfibre filters (GF/C) and filter papers (Whatman No. 4) were from Whatman (Maidstone, UK). Pentafluoropropionic anhydride (PFPA), sodium hydrogen carbonate and 4-dimethylaminopyridine (DMAP) were also purchased from Sigma. T-2/HT2™ HPLC and DONTEST™ HPLC affinity columns were from Vicam (Waters Business, Milford, MA, USA). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required. Phosphate buffer saline (PBS) was prepared by dissolving potassium chloride (0.2 g) (Panreac), potassium dihydrogen phosphate (0.2 g) (Sigma), anhydrous disodium phosphate (1.16 g) (Panreac) and sodium chloride (8.0 g) (J.T. Baker) in 1 l of pure water; the pH was brought to 7.4.

2.3. Preparation of standard solutions

Each standard of DON, HT2 and T2 was dissolved in acetonitrile at a concentration of 1.0 mg/ml and stored at −20 °C in a sealed vial until use. Working standards (10000, 2000, 1000, 500, 250, 100, 50, 20 μg) were prepared by appropriate dilution of known volumes of the stock solution with acetonitrile and used to obtain calibration curves.

2.4. Equipment

The GC system was composed of a HP-6890 plus gas chromatograph, equipped with a ^{63}Ni ECD (Hewlett-Packard, Avondale, PA, USA) and an Agilent 7683 Series injector (Agilent Technologies, Waldbronn, Germany). Signals were processed by HP GC ChemStation software version A.10:02(1757) (Agilent).

The LC system was an Acquity Ultra-Performance Liquid Chromatograph (UPLC®) coupled to an Acquity TQD® mass spectrometer (Waters Corporation, Manchester, UK). Empower 2 and MassLynx 4.0 software (Waters) were used to control the UPLC–MS system and for signal processing.

2.5. Determination of HT2, T2 and DON

2.5.1. Extraction and clean-up

For trichothecene extraction, three different solvent mixtures (acetonitrile–water 80:20, v/v; acetonitrile–water 84:16, v/v; methanol–water 90:10, v/v) were tested to optimize the extraction step. Two g of sample was placed into a 50-ml screw-capped tube. Then, 15 ml of solvent mixture was added and the tube was shaken in an orbital shaker (Infors-HT aerotron, Bottminghen, Switzerland) for 90 min. The extract of each tested solvent mixture was filtered through Whatman No. 4 filter paper. The filtrate extraction mixture was kept at −20 °C until used.

Solid-phase extraction (SPE) cartridges were prepared in the laboratory using 5-ml polypropylene syringes. Three types of solid-phases were assayed. A glass microfiber filter was placed at the bottom of the syringe. Then, a packed bed containing homogenized mixtures of various adsorbents was added: (i) 1.16 g alumina–charcoal–C18 (a) (75:1:40, w/w/w); (ii) 1.18 g alumina–charcoal–C18 (b) (75:3:40, w/w/w); (iii)

1.55 g alumina–charcoal–silica (90:1.5:50, w/w/w); (iv) 1.5 g alumina–charcoal–celite 545 (5:7:3, w/w/w) [44]. Three ml of filtrate sample extract was passed through the cartridge and collected into a vial. The cartridge was washed with 2 ml of the same extraction solvent. The eluate was collected in the same vial and the extract was dried at 45 °C, under a gentle stream of nitrogen.

A further clean-up step was made for HT2 and T2. Four ml of PBS was added into the tube with the dried extract and stirred vigorously. The extract was passed through a specific immunoaffinity column. The column was washed with 5 ml water and, finally, HT2 and T2 were eluted with 2 ml methanol. The extract was dried at 45 °C, under a gentle stream of nitrogen.

2.5.2. Derivatization and determination of HT2 and T2 by GC–ECD

HT2 and T2 were derivatized by adding 100 μl of a solution of DMAP (2mg/ml in toluene-acetonitrile 80:20, v/v) and 50 μl PFPA or HFBA and heating at 60 °C for 1 h. One ml of 3% $NaHCO_3$ aqueous solution and 400 μl of toluene were added to the derivatized extract. Then, the vial was vortexed for 15 s. When the phases were separated, the organic layer was transferred to a vial and analyzed by GC–ECD using a HP-5 column (5% phenyl-methylpolysiloxane; 30 m × 0.32 mm I.D., 0.25 μm film thickness, Agilent Technologies). The injector was operated in the splitless mode. The injection volume was 1 μl. The injector and detector temperatures were 250 °C and 300 °C, respectively. The oven temperature was programmed as follows: 90 °C (hold for 1 min), 40 °C/min to 160 °C, 1.5 °C/min to 182 °C, 5 °C/min to 240 °C, and 40 °C/min up to 275 °C (hold for 8 min). Helium at a constant pressure of 42.1 kPa was used as a carrier gas.

2.5.3. Determination of DON by LC–MS/MS

DON determination was done using UPLC® coupled to triple-quadrupole tandem mass spectrometer. Separation of DON was performed in an Acquity UPLC™ BEH C18 column 2.1 mm × 50 mm, 1.7 μm (Waters). The column oven was operated at room temperature. The injection volume was 20 μl. The mobile phase used was a mixture of two solvents (A: water with 0.5 mM NH_4Ac, 0.01% formic acid; B: methanol with 0.5 mM NH_4Ac, 0.01% formic acid) that was changed over time. The following gradient elution program was followed: the starting mobile phase composition (10% B) was linearly modified to reach 90% B in 5 min, and then returned to the initial composition in 0.2 min. Flow rate was 0.3 ml/min. The positive electrospray ionization (ESI+) was operated as follows: capillary voltage (3.5 kV), source temperature (120 °C), desolvation temperature (400 °C), gas desolvation flow rate (13 ml/min). Acquisitions of spectra were carried out operating in positive multiple reaction monitoring (MRM) mode. The precursor ion was $[M+H]^+$ 297. MRM transitions were 297.0 > 249.0 (collision energy 15 eV; cone voltage 30 V) and 297.0 > 203.1 (collision energy 20 eV; cone voltage 30 V).

2.5.4. Validation of analytical methods

The analytical methods used for DON, HT2 and T2 were assessed for linearity, precision and selectivity.

Linearity was assessed by performing triplicate injections of standard solutions whose concentrations were 20, 50, 100, 250, 500 and 1000 μg of each mycotoxin/l. Standard curves were generated by linear regression of peak areas against concentrations.

Precision and recovery rates were established for each method by determination of DON, HT2 and T2 in samples of paprika and chili pepper, covering the range of the method (between 50 and 1000 μg/kg). Recovery was determined by comparing the absolute responses of trichothecenes obtained from the spiked samples with the absolute responses of calibration standards. The number of replicates for recovery studies was five. Recovery rates between 70% and 110% and relative standard deviations (RSD) < 20% for DON and between 60% and 130% and RSD < 30% for HT2 and T2, were considered as acceptable according to the legislation [45].

Table 1
Recovery rates and relative standard deviations obtained in a blank paprika sample spiked with 100 μg/kg of HT2, T2 and DON and extracted with different solvent mixtures. $N = 5$.

Extraction solvent	Mycotoxin	Recovery (%)	RSD (%)
Acetonitrile–water (84:16)	HT2	78.2	6.4
	T2	106.9	25.7
	DON	118.7	14.6
Acetonitrile–water (80:20)	HT2	62.0	19.4
	T2	74.3	21.4
	DON	74.5	16.9
Methanol–water (90:10)	HT2	68.8	12.6
	T2	121.7	27.9
	DON	132.5	14.2

The limit of detection (LOD) was considered as the mycotoxin concentration that provides a signal equal to $b + 3S_b$, where b is the intercept of the calibration curve and S_b is the standard error of the estimate assuming to be the standard error of the blank. The limit of quantification (LOQ) was considered equal to 3× LOD [46].

Selectivity was checked by analysis of blank samples of the matrix (chili pepper or paprika) from six independent sources. Each blank sample was tested for interference by comparison of the retention times of peaks appearing in the vicinity of peaks from the toxins with those obtained from standard solutions run under the same conditions. Areas from interference peaks were always lower than the areas of toxin standards at concentrations near the LOQ. In addition, selectivity of the LC–MS/MS method was assessed by comparison of the MS spectra of the interference peaks from blanks with the MS spectrum from DON standard.

3. Results and discussion

3.1. Development and optimization of a method for determination of HT2 and T2

The extraction of the studied trichothecenes was optimized by comparison of the results obtained when the remaining parts of the method were the same through the process. The best results were obtained with the mixture acetonitrile–water (84:16, v/v) (Table 1). The SPE procedure that gave the best results in terms of reliability, low cost and less analysis time was a cartridge made with alumina–charcoal–C18 (a) (Table 2).

With regard of the derivatization procedure, PFPA was chosen as derivatization reagent because the coefficient of determination

Table 2
Recovery rates and relative standard deviations obtained in a blank paprika sample spiked with 100 μg/kg of HT2, T2 and DON in the different solid phase extractions studied. $N = 5$.

SPE	Mycotoxin	Recovery (%)	RSD (%)
Alumina/charcoal/C18 (a)	HT2	78.2	6.4
	T2	106.9	25.7
	DON	118.7	14.6
Alumina/charcoal/C18 (b)	HT2	68.6	16.6
	T2	64.9	22.7
	DON	73.3	18.0
Alumina/charcoal/silica	HT2	123.0	17.0
	T2	115.6	31.7
	DON	123.2	21.3
Alumina/charcoal/Celite 545	HT2	55.9	13.4
	T2	69.1	16.2
	DON	51.6	10.3

Table 3
Recovery rates and relative standard deviations obtained in a blank paprika sample spiked with different levels of HT2, T2 and DON. $N = 5$.

Mycotoxin	Spiking level (μg/kg)	Recovery (%)	RSD (%)
HT2	50	90.3	12.2
	100	78.2	6.4
	1000	80.1	5.9
T2	50	92.0	18.6
	100	106.9	25.7
	1000	71.1	5.0
DON	50	114.5	17.4
	100	118.7	14.6
	1000	86.8	4.5

(r^2) of the calibration curves (0.9778 for HT2 and 0.943 for T2) were higher than those obtained using HFBA (0.9498 for HT2 and 0.9209 for T2). HFBA is also a more expensive reagent, has less stability against moisture and trichothecene recovery rates were not significantly better than those obtained using PFPA.

The selected analytical method for detection and quantification of HT2 and T2 toxins showed good linearity in the studied range with coefficients of determination of 0.9778 and 0.943, respectively. The LODs were 7 μg/kg for HT2, and 3 μg/kg for T2. The LOQs were considered three times these limits, as previously stated.

As shown in Table 3, the recovery rates obtained by fortification of the samples with different amounts of T2 using the optimised method varied between 71.1% and 106.9% for spiking levels of 1000 μg/kg and 100 μg/kg, respectively. For HT2, recovery rates were 78.2% and 90.3% for spiking levels of 100 μg/kg and 50 μg/kg, respectively. Precision (repeatability) was assessed by the RSD, which was in every case <30%. Therefore, the method is considered appropriate for determination of these toxins in paprika and was subsequently applied to this purpose.

3.2. Development and optimization of the method for determination of DON

The best results concerning extraction and clean-up of samples for DON determination were obtained with acetonitrile–water (84:16, v/v) as in the case of T2 and HT2. Alumina–charcoal–C18 (a) (75:1:40, w/w/w) was the mixture of sorbents that provided the best results during the clean-up process of liquid extracts (Tables 1 and 2).

A LC–MS/MS method was developed providing good separation and sensitivity with respect to detection of DON using an electrospray interface (ESI) in positive ionization mode.

The parent ion $[M+H]^+$ 297 was chosen because it is the predominant ion in positive ionization mode. The daughter ions, once optimized the working conditions, were 249.0 and 203.1, and the relationship m/z (249.0) to m/z (203.1) was approximately 1.5. They were chosen for the MRM method because both fragments combined low interference and high detector response. The precursor ion using negative ionization mode $[M-H]^-$ was not selected as its breakdown produced a high number of low-sensitivity daughter ions.

The coefficient of determination and the LOD obtained by application of this LC–MS/MS method to DON determination were 0.9948 and 14 μg/kg, respectively. Thus, it seems suitable for detection and quantification of this toxin in paprika and chili pepper samples.

Table 3 shows the recovery rate obtained in a paprika sample spiked with 1000 μg DON/kg. It was 86.8%, and the RSD was 4.5%.

An alternative method similar to that developed for determination of HT2 and T2 was assayed. It consisted of the extraction of the sample with acetonitrile–water (84:16, v/v) followed by two clean-up procedures. First, the extract was passed through a SPE cartridge with different sorbents (see Section 2) and then the purified eluate was passed through an immunoaffinity column. Then, DON was derivatized by addition of PFPA and determined by GC–ECD. However, the results from these assays were not satisfactory since some interfering peaks prevented DON from being suitably detected and quantified.

Therefore, the optimized LC–MS method for DON determination in paprika and chili pepper is considered as appropriate on the basis of the values obtained for linearity, recovery and repeatability.

Table 4
Number of positive samples for HT2, T2 and DON, and their average and maximum levels found in the analysis of paprika and chili pepper samples. $N = 3$.

Mycotoxin	Positive samples	Average (μg/kg)	Maximum level (μg/kg)
HT2	3/32	82	199
T2	2/32	10	11
DON	4/32	125	269

3.3. Occurrence of DON, HT2 and T2 in paprika and chili pepper samples

Table 4 shows the range of trichothecene contamination found in paprika and chili pepper samples. Twelve percent of the samples were naturally contaminated with DON (27% of chili pepper and 6% of paprika samples), 6% were contaminated with T2 (9% of chili pepper and 6% of paprika samples) and 12% with HT2 (18% of chili pepper and 6% of paprika samples). One sample of chili pepper was also contaminated with the three studied mycotoxins. However, in all of the smoked paprika samples HT2, T2 and DON were not detected.

The highest concentrations of the studied mycotoxins found in samples were 269 μg/kg, 199 μg/kg and 11 μg/kg, for DON, HT2 and T2, respectively. The respective mean levels found (taking into account only positive samples) were 125 μg/kg, 82 μg/kg and 10 μg/kg. Fig. 1 shows a GC–ECD chromatogram of a chili pepper sample containing 37 μg HT2/kg and 11 μg T2/kg and Fig. 2 shows a LC–MS/MS chromatogram of a chili pepper sample naturally contaminated with 269 μg DON/kg.

The DON levels found were not very high in comparison with the maximum level permitted for this mycotoxin in the European regulation for cereals intended for direct human consumption, which is 750 μg/kg [47].

Trichothecene contamination of *Capsicum* products has been reported only in few occasions. Lincy et al. [31] studied the content of T2 in three paprika samples but none of them showed contamination with that toxin. Boonzaaijer et al. [32] analyzed five paprika samples and did not detect HT2, T2 and DON. However, Patel et al.

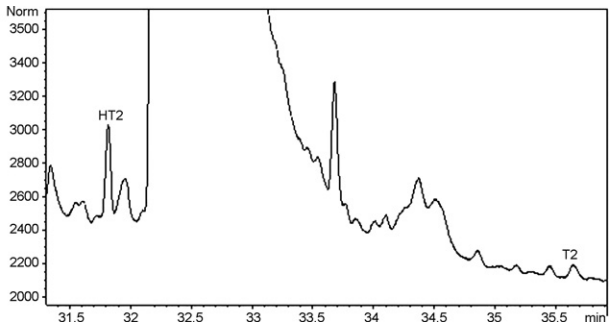

Fig. 1. GC–ECD chromatogram of a chili pepper sample containing 37 μg HT2/kg and 11 μg T2/kg. Retention times were 31.8 and 35.6 min for HT2 and T2, respectively. Extraction: acetonitrile–water (84:16, v/v); clean-up: alumina–charcoal–C18 (a) cartridge; derivatization: PFPA; separation: HP-5 column.

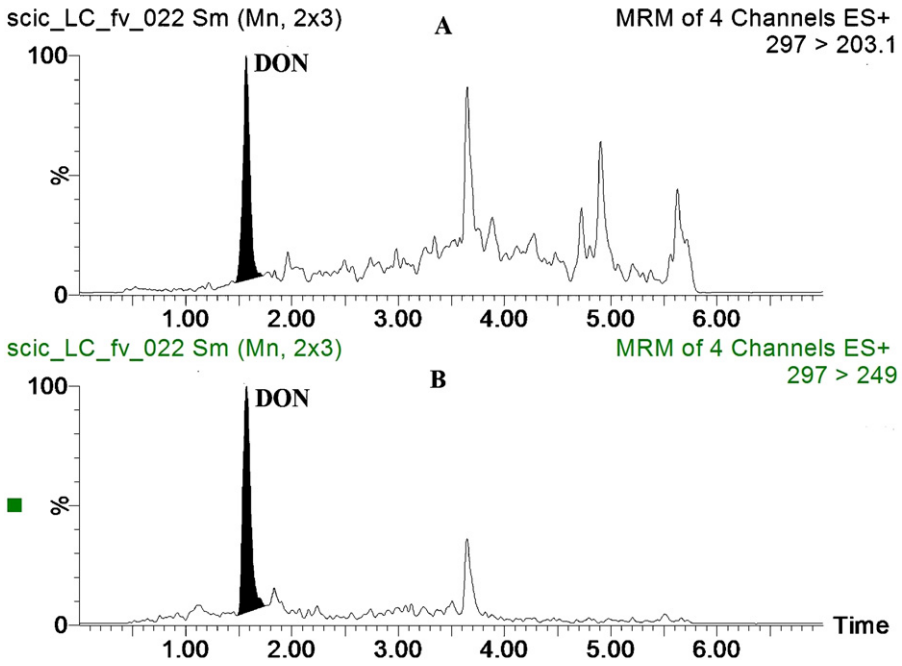

Fig. 2. LC–ESI-MS/MS chromatogram in MRM mode of a chili pepper sample containing 269 μg DON/kg. Transition-ion mass selection (A) *m/z* 297.0 > 203.1 and (B) *m/z* 297.0 > 249.0. Retention time: 1.57 min. Extraction: acetonitrile–water (84:16, v/v); clean-up: alumina–charcoal–C18 (a) cartridge; separation: Acquity UPLC™ BEH C18 column.

[33] found that one out of four analyzed *Capsicum* powder samples contained DON (8 μg/kg) and HT2 (24 μg/kg) while T2 was not detected.

4. Conclusions

The chromatographic methods for the determination of HT2, T2 and DON have been successfully optimized and validated. The concentration of HT2 and T2 was determined by GC–ECD and that of DON was determined by LC-triple quadrupole–MS.

These methods were applied to different samples of paprika and chili pepper, where it was found that some samples contained DON, HT2 and T2. Moreover, one of the samples was found to contain the three mycotoxins. Even when the levels found are not very high it is possible that higher concentrations may occur in other samples. Due to the toxicity of these metabolites and the high consumption of paprika and chili pepper in some countries, it is necessary to carry out a more exhaustive control of these trichothecenes in samples of these commodities to avoid health problems in consumers.

Acknowledgements

The authors wish to thank financial support from FEDER and Spanish Government "Ministerio de Ciencia e Innovación" (Projects AGL2007-66416-C05-01/ALI and AGL2010-22182-C04-03/ALI, and two research grants).

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