LC-MS/LC-UV analysis of type-A and -B trichothecenes after multifunctional MycoSep[®] clean-up

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Summary

A quick and efficient method for the determination of type-A and -B trichothecenes is described employing high performance liquid chromatography with UV (ultra violet) and MS (mass spectrometry) detection. Using MycoSep[®] multifunctional clean-up columns multi-toxin clean-up was done within one procedure and one solvent system, thus saving considerable time in comparison with other clean-up methods. The method was tested and validated in corn, wheat and barley with MS detection for the most common A-Trichothecenes (T-2 toxin, HT-2 toxin and DAS), using T-2 toxin-*d3* as internal standard. For most prevalent B-Trichothecenes, i.e. deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-deoxynivalenol, and fusarenon X (FUS-X), the method was validated with UV absorption and calibrated for MS detection.

Key words : mycotoxins, trichothecenes, MycoSep, liquid chromatography, LC-MS

Introduction

Trichothecenes are a large group of mycotoxins produced by various species of moulds, in particular by those belonging to the genus of *Fusarium*. Approximately 170 trichothecene mycotoxins have been identified up to date, with a sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system in common¹⁾. The most important structural features that contribute to the biological activities of trichothecenes are the presence of the 12,13-epoxy ring, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus and the structure and position of a side-chain.

Epidemiological surveys have revealed that the predominant type-A and -B trichothecenes are widely distributed in cereals as natural pollutants, whereas the macrocyclic trichothecenes occur rarely in food or feed. *F. sporotrichoides*, *F. poae*, and

F. equiseti produce type-A trichothecenes, while *F. graminearum* and *F. culmorum* are the most important type-B producing moulds¹⁾. The most prevalently occurring mycotoxins of these groups are deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-deoxynivalenol (3-AcDON), 15-acetyl-deoxynivalenol (15-AcDON), Fusarenon X (FUS-X) in case of B-trichothecenes, and T-2 toxin and HT-2 toxin of the type-A toxins. As some of these closely related compounds occur frequently simultaneously, analytical methods detecting more than one toxin of this group within an single analytical procedure are required.

For the determination of trichothecenes chromatographic methods have been used widely, including thin-layer chromatography (TLC), gas chromatography (GC) with electron capture detection (ECD) or mass selective detection (MS) as well as high-performance liquid chromatography (HPLC) with UV, fluorescence detection, and, described in more recent publications, also with mass spectrometry^{2,3)}. In contrary to this, also a variety of immunological methods, like immuno sorbent assays (ELISAs) or radio immune assays (RIAs) are available, which require usually no further sample purification but have the major disadvantage, that only one toxin can be determined by each test, referring to the specificity of the antibodies.

The results of the most sophisticated chromatographic procedures are determined by the efficiency of the prior sample preparation, in particular by sampling, extraction and the further clean-up of the extract. As a large number of interfering compounds present in samples contaminate the primary sample extract, these components must be removed as completely as possible⁴⁾. Commonly used clean-up methods employ column chromatography, liquid-liquid extraction, commercially available solid phase extraction columns (SPE), as well as immuno-affinity columns (IAC).

Extract purification with multifunctional clean-up (MFC) columns was first reported for aflatoxins. Described advantages comprise speed, simplicity, solvent efficiency, and, in some cases increased recovery and lower cost⁵⁾. The Mycosep[®] multifunctional column contains various adsorbents, such as charcoal, Celite, ion-exchange resins, and others. The packing material is housed in a plastic filter tube with a rubber flange on the lower end containing a porous filter and a one-way valve in the centre of the flange. Clean-up is done by pushing the filter tube into a culture tube, which contains the extract. The flange seals tight, thus forcing the sample through the porous frit, the valve, and through the packing material. The purified extract can be taken from the reservoir on top of the column. Without any rinsing steps required sample purification takes only 10 to 30 seconds.

This one-step purification represents a very rapid and efficient alternative to conventional solid phase extraction (SPE) or immunoaffinity (IAC) methods as both require usually three to four steps: precondition columns, retain extracted substances on packing material of the column, wash undesirable compounds, and elute analyte of

interest. This paper describes methods for the determination of the most important type-A and B trichothecenes by HPLC after a very simple and rapid clean up with multifunctional columns.

Materials and Methods

Reagents Mycotoxin standards were purchased from Biopure® Referenzsubstanzen GmbH, Austria (DON, 3- and 15-Ac-DON, NIV, FUS-X, DOM-1), and Sigma Chemical Co., MO, USA (T-2, HT-2, DAS, T-2 d3). Organic solvents, HPLC-grade water, ammonium formate, and formic acid were purchased from Merck AG, Germany.

Equipment and clean-up procedures MycoSep® #227 trichothecene cartridges and MultiSep® #216 columns were obtained from Romer® Labs. Inc., MO, USA. A Romer® Series II Mill (Romer Labs. Inc., MO, USA) was used for grinding and subsampling the grain. A total of 25 g of the milled and well-mixed sample was extracted with 100 ml of acetonitrile/water (84:16, v/v) for 3min using an Osterizer blender (USA) with 250 ml blender jars. The extract was filtered through a folded filter (# 595 ½, Schleicher & Schull, Germany) and 4 ml of the filtrate were slowly pressed through a Mycosep® #227. In case of further LC-MS analysis this extract was evaporated at 60 °C with a Romer® Evap system and then redissolved in 400 µl of HPLC starting solvent, containing 200 µg/l of the internal standard T2 toxin-d3. Samples were subsequently centrifuged at 10.000 rpm in an Eppendorf centrifuge and then transferred into an HPLC vial.

HPLC Analysis was performed with an LC-ESI-MS series 1100 from Agilent® technologies, comprising a micro vacuum degasser, a binary capillary pump, a micro autosampler, columns oven as well as an API-ES interface.

Chromatographic separation of A-trichothecenes was achieved with a Zorbax® Bonus-RP, (2.1 x 150 mm; 5 µm) using a Zorbax® Bonus-RP pre-column (2.1 x 12.5 mm, 5 µm) at a temperature of 30 °C (columns from Agilent® technologies, Germany). The HPLC was operated with a gradient mobile phase system consisting of ammonium formate buffer (10 mmol, formic acid and ammonium formate, pH 3.6) and acetonitrile at a flow rate of 0.3 ml/min. The pump was programmed as follows: start buffer: acetonitrile 70:30 (v/v) for 3 min, increase to 60 % acetonitrile over the next 7min, held for 2min and decreased to again 30 % within the next 2 min, which was finally held for 10 min (total gradient time: 25 min). Injection volume was 10 µl.

Chromatographic separation of B-trichothecenes prior to MS analysis was performed with a Zorbax® SB-Aq Stablebond Analytical column (4.6 x 150 mm, 5 µm; Agilent® Technologies, Germany). The gradient mobile phase system consisted of ammonium formate buffer (10 mmol, formic acid and ammonium formate, pH 3.6) and acetonitrile at a flow rate of 0.5 ml/min. The gradient was programmed as follows: start 10 % acetonitrile for 5 min, increase of acetonitrile 25 % in 1 min, held at 25 %

acetonitrile for 11 min, decrease to 10 % acetonitrile within the next 3 min and finally held at this 10 % for 6min (total gradient time: 25 min). Injection volume was 30 μ l.

The mass detector parameters were: electro spray (ES) interface with positive ionisation mode. The optimisation processes were carried out in scan mode, sample quantification in single ion monitoring (SIM) mode. The capillary voltage was 3000 V and a nebulizer pressure of 35 psi was applied. The drying gas stream (N_2) was at 9 L/min and a gas temperature at 350 $^{\circ}C$. The fragmentation voltage of each m/z ratio was optimised. Two characteristic m/z ratios of each analyte molecule were defined and programmed: For T-2 toxin the ratios m/z 484 $[M+NH_4]^+$ and the fragment m/z 305 were determined. The two most intensive m/z ratios of HT-2 toxin were 447 $[M+Na]^+$ and 442 $[M+NH_4]^+$. In case of DAS m/z 384 $[M+NH_4]^+$ and the fragment m/z 307 gave the best signal. The two signals of the internal standard T2 toxin-*d3* giving the most intensive signal were: m/z 487 and m/z 492. In case of B-trichothecenes the most intensive m/z ratios were given by the $[M+Na]^+$ ions, thus m/z of 319 for DON as well as $[M+H]^+$ 297; m/z 339 $[M+H]^+$ and 361 $[M+Na]^+$ for 15-AcDON and 3-AcDON; m/z of 355 $[M+H]^+$ and 377 $[M+Na]^+$ for FUS-X; m/z $[M+Na]^+$ 335 and $[M+H]^+$ 313 for NIV; and m/z 303 $[M+Na]^+$ and 281 $[M+H]^+$ for DOM-1. An internal calibration, using DOM-1, the de-epoxymetabolite of DON first described by Yoshizawa *et al.*⁶⁾, as internal standard, was calculated in the concentration range 10 - 1000 μ g/kg.

For validation of methods uncontaminated maize, barley, and oat were spiked with seven different concentrations in the range of 10 to 750 μ g/kg. Three replicates of each concentration level were analysed. Additionally blank samples were analysed. For spiking a mixture of all toxins dissolved in acetonitrile were used.

In case of type-B trichothecenes determination with HPLC and UV detection the 4ml eluate of the MycoSep # 227 column were transferred to the final clean-up column #216 (Romer Labs. Inc., MO, USA), which was prior conditioned with 5 ml of acetonitrile/water (84/16,v/v), that were discarded. The eluate of the MycoSep 227 column was transferred to the #216 column and eluted with 9 ml of acetonitrile/water (84/16, v/v), evaporated to dryness and then redissolved in 400 μ l of HPLC running solvent. Chromatographic separation of B-trichothecenes was performed with a HP-Hypersil ODS column (2.0 x 125 mm, 5 μ m) and HP-Hypersil ODS column (2.0 x 4 mm, 5 μ m) pre-column (Agilent[®] technologies, Germany). The gradient mobile phase system consisted of (A) water:acetonitrile:methanol (96:2:2, v/v/v) and (B) acetonitrile at a flow rate of 0.5 ml/min. The gradient was programmed as follows: start 100 % A for 2 min, increase of B to 2 % within 2 min, further increase to 7 % B in the next 4 min, held at 7 % B for the following 4 min, increase to 15 %B in 3 min, which is held for another 3 min, and finally decrease to 0 % B within 3 min, which is held for 5 min (total gradient time: 26 min). Injection volume was 50 μ l. Detection occurred with a Series 1100 diode array detector (DAD) at a wave length of 220 nm.

Quality control of routine analytical processes was performed with a naturally contaminated corn sample in case of DON, 3-AcDON and NIV, and spiked samples for T2-toxin, HT-toxin and DAS. The homogenised grain was analysed on five different days, four times each day, resulting in a control chart ($n=20$), which was blotted using a CCPro Plus computer program from Chem SW, USA. Control samples were analysed with each series of routine samples, results blotted in the computer system and tested for alignment.

Results and Discussion

Quantification of type-A trichothecenes was done by the use of an internal calibration with T-2 toxin-d3 as internal standard (ISTD). Losses during clean-up were corrected by means of a control sample, which was analysed in each analytical sample

Table 1. Validation data of type-A and -B trichothecenes.

Limits of detection (LOD) and quantification (LOQ), recovery, and relative standard deviation (RSD) for type-A trichothecenes (DAS, T2-toxin, HT2-toxin) from spiked sample matrices (8 spiking levels, 10-750 $\mu\text{g/kg}$) after mass spectrometric detection, and type-B trichothecenes (DON, NIV, 3-AcDON) from corn (8 spiking levels, 20-2000 $\mu\text{g/kg}$) as by UV detection.

	LOD [$\mu\text{g/kg}$]	LOQ [$\mu\text{g/kg}$]	Recovery [%]	RSD, n= 24 [%]
corn				
DAS	26	92	77	10.6
T2-toxin	9	32	103	11.1
HT2-toxin	25	90	70	8.9
oat				
DAS	32	88	93	5.0
T2-toxin	19	51	96	5.9
HT2-toxin	27	86	91	11.4
wheat				
DAS	30	90	84	9.5
T2-toxin	22	67	96	4.6
HT2-toxin	28	87	89	5.0
corn				
NIV	8	30	74	15.0
DON	16	60	72	7.0
3-AcDON	29	103	73	12.0
FUS-X	14	50	70	9.3

Table 2. SIM parameters of LC-MS determination of type-A trichothecenes with regard to time window of recording, fragmentor voltage and gain.

A-Trichothecenes				
Time [min] start-end	Toxin	SIM Ion m/z	Fragmentor [V]	Gain
Signal 1				
3.00-6.00	DAS	384	100	1.0
6.10- 0.00	HT-2	447	140	1.0
10.01-25.00	T-2d3	487	100	1.0
Signal 2				
3.00-6.10	DAS	307	160	1.0
10.00-25.00	T-2	484	120	1.0
Signal 3				
6.00-10.00	HT-2	442	100	1.0
10.10-25.00	T-2	305	200	1.0
Signal 4				
10.00-25.00	T-2d3	492.2	250	1.0

Fig. 1. Chromatogram of type-A trichothecenes: DAS, HT-2 toxin, T-2 toxin ($500 \mu\text{g/l}$) and T2d3 toxin as internal standard ($200 \mu\text{g/l}$).

series and results blotted in a control chart, that was established with spiked matrix samples, while fluctuations of MS performance were corrected by quantifying the trichothecenes relative to the ISTD.

The applied clean-up procedure allowed an almost complete removal of the sample matrix. Validation data for analytical procedures from corn, oat, and wheat, i.e. limit of detection (LOD), limit of quantification (LOQ), recoveries and relative standard deviation (RSD) can be seen in Table 1. Recoveries from 70 to 103 % are similar to reported recoveries from other working groups^{2,7,8}. The short chromatographic separation time of 25 min in total, including flushing and reconditioning, allowed a high sample throughput. Four signals were detected in parallel, thus allowing the detection of two mass signals of each of the three mycotoxins as well as the internal standard, at different fragmentor voltage in SIM mode (see Table 2, SIM parameters and Figure 1, chromatogram of type-A trichothecenes).

Calibration of B-type trichothecenes gave good data for 15-AcDON, FUS-X and NIV, with limits of detection and quantification of 8.3 and 28.2 $\mu\text{g/kg}$ for 15-AcDON, 4.4 and 15.2 $\mu\text{g/kg}$ for FUS-X, and 14.8 and 48.2 $\mu\text{g/kg}$ for NIV, respectively. DON resolution was unsatisfactory, as a broad peak tailing over 2 min was observed with this separation procedure, so that further optimisation is necessary.

Due to the B-trichothecene structure containing a carbonyl function at C8 conjugated with a double bond at C9-10 good UV absorption of this toxin group can be observed, so that analysis by LC-UV detection is still the method of choice for determination of these toxins from grain, food or feed. Application of an additional purification step with MultiSep[®] columns improved sensitivity of the method for example to limits of detection as low as 8.2 $\mu\text{g/kg}$ nivalenol in corn. Other method specific data can be seen in Table 1.

Samples of Austrian maize and oat, harvested in 2002, that were routinely tested for B-trichothecene contamination by LC-UV were additionally analysed for type-A trichothecenes in order to see whether any co-occurrence can be observed or not. As expected no correlation was observed between the occurrence of type-B trichothecenes and type-A trichothecenes. Diacetoxyscirpenol (DAS) was not found in any sample analysed, T-2 toxin and HT-2 toxin were found at approximately the same extent. Out of 23 corn samples 4 % showed T-2 concentrations of 100 to 150 $\mu\text{g/kg}$, while 30 % of samples detected positive for HT-2 toxin. Occurrence in oat (n=18) was somewhat higher, with 55 % of samples containing T-2 or HT-2 at levels of 90 to 150 $\mu\text{g/kg}$. Only three samples were highly contaminated with T-2 and HT-2 toxin levels of around 1000 $\mu\text{g/kg}$.

Multitoxin clean-up with MycoSep[®] columns in combination with liquid chromatographic separation allows simultaneous determination of trichothecenes in quick, reliable and rugged procedures. Detection methods reported range from UV

absorption⁴⁾ and tandem mass spectrometry (MS/MS)¹⁰⁾ which were preferably applied for B-trichothecenes, over APCI-MS methods, which were used either for type-A or type-B trichothecenes^{8,9)}, or multi-toxin detection comprising both groups, which was done by fluorescence detection after derivatisation with 4-dimethylaminopyridine¹¹⁾, APCI-MS¹²⁾ or ion trap multiple mass spectrometry³⁾.

In our studies best performance was achieved by subsequent mass spectrometrical detection of A-trichothecenes by using atmospheric pressure electrospray ionisation (AP-ESI) and employing a diode array detector (DAD) for ultra violet light absorption in case of B-trichothecenes.

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