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# A new solid phase extraction clean-up method for the determination of 12 type A and B trichothecenes in cereals and cereal-based food by LC-MS/MS

**Marianna Klötzel[1,2], Uwe Lauber[1] and Hans-Ulrich Humpf[2]**

[1]Chemisches und Veterinäruntersuchungsamt Stuttgart, Fellbach, Germany
[2]Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Münster, Germany

A new reliable and cost-efficient solid phase extraction-based clean-up method for the determination of 12 type A and B trichothecenes [deoxynivalenol (DON), nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, neosolaniol, monoacetoxy-scirpenol, diacetoxyscirpenol, T-2 triol and T-2 tetraol] in cereals and cereal-based food is presented. Furthermore, the suitability for the simultaneous determination of zearalenone is examined. Toxins were extracted from cereal samples using ACN/water (80/20, v/v), purified by means of a new Bond Elut Mycotoxin® column and analyzed via liquid chromatography-electrospray ionization tandem mass spectrometry. Limits of detection were calculated for the matrix wheat and ranged from 0.3 to 5 ng/g, depending on the toxin. Average recovery rates for the tested compounds in seven cereal-based matrices have been determined ranging from 65 to 104%. The relative standard deviations of the complete method ranged from 2.67 (DON, wheat) to 20.0% (T-2 toxin, oats).

**Keywords:** Cereals / Extraction / LC-MS/MS / SPE clean-up / Trichothecenes / Zearalenone

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## 1 Introduction

The occurrence of mycotoxins in agricultural commodities has long been recognized as a potential hazard for human and animal health and *Fusarium* fungi have been reported among the most prominent pathogens of various cereals.

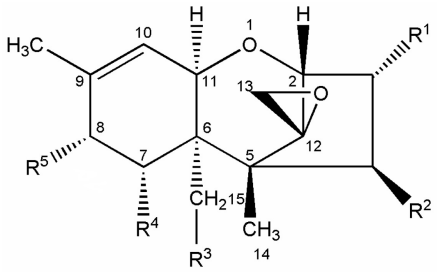
Trichothecenes are a large group of tetracyclic sesquiterpenoid mycotoxins produced by various *Fusarium* species. They are a group of over 150 different toxins that are classified into four groups: types A, B, C and D. Type A trichothecenes include, *e.g.* T-2 toxin (T-2), HT-2 toxin (HT-2), neosolaniol (NEO), monoacetoxyscirpenol (MAS), diacetoxyscirpenol (DAS), T-2 triol and T-2 tetraol and differ from type B trichothecenes deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON) and fusarenon-X (FUS) by the absence of a carbonyl group at position C-8 [1]. Chemical structures of the trichothecenes investigated in this report are shown in Fig. 1. DON and NIV are the most commonly found trichothecenes, followed by T-2, HT-2 and the DON-acetyl-derivates [1].

Trichothecenes are known to cause toxic effects on skin and mucous membranes, feed refusal, weight loss, vomiting, immunosuppressive and hemorrhage effects and furthermore inhibit protein biosynthesis as well as RNA and DNA synthesis [2, 3]. In national German food law, limits for DON, as low as 100 µg/kg in grains and grain products for infants, were established in February 2004. Further regulations for DON, T-2 and HT-2 in the µg/kg range will be introduced by the European Union authorities in 2006 and 2007. To comply with European regulations concerning mycotoxins in food and feed, suitable methods for the analysis of trichothecenes and other *Fusarium* toxins [*e.g.* zearalenone (ZEA), Fig. 1] are required, in particular sensitive and reliable multitoxin approaches. As trichothecenes differ strongly in polarity and solubility, such methods have to overcome problems arising from sample extraction, extract clean-up and instrumental analysis. In the following, a summary of the published data concerning the determination of trichothecenes is given.

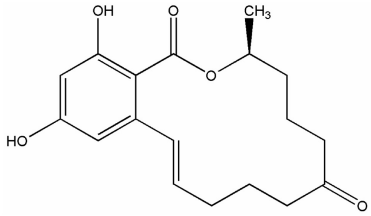
Extraction: In the first described methods for the analysis of trichothecenes the extraction was carried out using methanol/water [4]. Later, ACN/water in variable ratios was applied. Best recovery rates for DON and NIV were

**Correspondence:** Dr. Uwe Lauber, Chemisches und Veterinäruntersuchungsamt Stuttgart, Schaflandstr. 3/2, 70736 Fellbach, Germany
**E-mail**: uwe.lauber@cvuas.bwl.de
**Fax**: +49-711-588-176

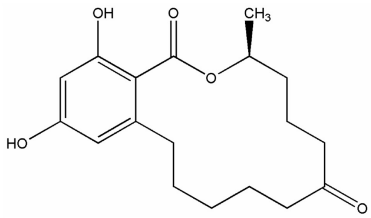
**Abbreviations: DOM-1**, deepoxy-deoxynivalenol; **DON**, deoxynivalenol; **IAC**, immunoaffinity columns; **ISTD**, internal standards; **NIV**, nivalenol; **ZAN**, zearalanone; **ZEA**, zearalenone




| Trichothecene | MW | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ |
|---|---|---|---|---|---|---|
| ***Type A*** | | | | | | |
| Neosolaniol (NEO) | 382 | OH | OAc | OAc | H | OH |
| HT-2 toxin (HT-2) | 424 | OH | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| T-2 toxin (T-2) | 466 | OH | OAc | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| T-2 triol | 382 | OH | OH | OH | H | $OCOCH_2CH(CH_3)_2$ |
| T-2 tetraol | 298 | OH | OH | OH | H | OH |
| Monoacetoxyscirpenol (MAS) | 324 | OH | OH | OAc | H | H |
| Diacetoxyscirpenol (DAS) | 366 | OH | OAc | OAc | H | H |
| ***Type B*** | | | | | | |
| Deoxynivalenol (DON) | 296 | OH | H | OH | OH | =O |
| 3-Acetyl-DON (3ADON) | 338 | OAc | H | OH | OH | =O |
| 15-Acetyl-DON (15ADON) | 338 | OH | H | OAc | OH | =O |
| Nivalenol (NIV) | 312 | OH | OH | OH | OH | =O |
| Fusarenon-X (FUS) | 354 | OH | OAc | OH | OH | =O |




Zearalenone




Zearalanone

**Figure 1.** Chemical structures of type A and B trichothecenes, zearalenone (ZEA) and zearalanone (ZAN).

observed with ACN/water (3 : 1; v/v) [5]. Nowadays, ACN/water (84/16; v/v), a ratio which was introduced by Chang *et al*. [6] is a common extraction solvent in combination with a clean-up step using charcoal-alumina columns. Trenholm *et al*. [7] showed that this mixture resulted in a lesser co-extraction of interfering matrix compounds than methanol/water solvents. In contrast, when applying immunoaffinity clean-up for the polar toxin DON, water is used for sample extraction [8].

Clean-up: Due to the different polarity of type A and B trichothecenes, compromises in the extraction and clean-up procedures have to be made. Interfering co-extracted matrix compounds with similar polarities as the trichothecenes have to be removed from the extract without substantial loss of toxins. The most often applied clean-up is SPE with different materials. Purification of the extracts with silica and Florisil (magnesium silicate) materials was reported in earlier published methods [4, 9 – 11], whereas nowadays, most laboratories use charcoal-alumina or Mycosep® (modified charcoal-alumina) columns [12 – 14]. The stationary phase of the Mycosep® column consists of charcoal, Celite and alumina. Recovery rates of approximately 90% are achieved with this clean-up procedure for type A and B trichothecenes, except for the polar toxin NIV, for which recovery rates of approximately 65% are reported. Nevertheless, Mycosep® columns are at the moment the best compromise for a rapid and effective clean-up of crude extracts in the analysis of trichothecenes. In the last few years, the application of immunoaffinity columns (IAC) has been a great success for several mycotoxins [8, 15]. This purification is highly specific and yields very good recovery rates, but is only applicable to one toxin at a time. Up to now, only IAC columns for the purification of DON and T-2/HT-2 are available.

Instrumental analysis: The first methods for the determination of trichothecenes were based on TLC [16, 17] or GC with electron-capture-detection [18 – 20], flame ionization detection [21] or MS detection [22 – 24]. However, due to matrix effects and the time-consuming derivatization procedure, GC methods are used more rarely [25]. Alternatively, HPLC methods with UV, fluorescence or MS detection are used. HPLC methods with UV detection are only applicable to B-trichothecenes and require very effective clean-up procedures such as IAC [15]. For fluorescence detection, different pre- and post-column strategies have been established after derivatization with anthracene- or cumarine-carbonyl chlorides for A-trichothecenes and methylacetoacetate for type B trichothecenes [26 – 29]. Nevertheless, the derivatization process requires a further step in the analysis, is time-consuming and results in higher limits of detection. The coupling of LC and MS is becoming more and more the method of choice, as it eliminates the need for sample derivatization. In addition, the selectivity and sensitivity of the analysis can be improved using MS/MS. The analysis of A-trichothecenes by LC-APCI-MS has been reported by Razzazi-Fazeli *et al*. [30] and by LC-ESI-MS by Thimm *et al*. [31]. Several methods for type B trichothecenes using ESI or APCI are described, *e. g*. by Lagana *et al*., Plattner *et al*. and Razzazi-Fazeli *et al*. [32 – 34]. However, only a few LC-MS methods for the simultaneous determination of both type A and B trichothecenes are described in the literature [13, 14, 35 – 37]. In these methods, ESI or APCI are applied and only a range of different trichothecenes is deter-

mined. Recently, a new LC-MS/MS method for the simultaneous analysis of the 12 above mentioned trichothecenes was presented by Klötzel *et al*. [38].

As we have described earlier [38], the recovery rates of this method (based on Mycosep® clean-up) concerning polar toxins such as DON, NIV and T-2 tetraol were low. In addition, we purified naturally contaminated samples with both IAC and Mycosep® columns. When applying the IAC-based method (sample extraction with water), we observed up to 57% more DON in comparison to the Mycosep® method (sample extraction with ACN/water). Therefore, the aim of this study was to develop a reliable, selective, fast and economic extraction and clean-up method for the simultaneous determination of 12 type A and B trichothecenes (T-2, HT-2, NEO, DAS, MAS, T-2 triol, T-2 tetraol, DON, NIV, 3ADON, 15ADON, FUS). For this purpose, different SPE materials were tested for their suitability for the LC-MS/MS analysis. Validation details including recoveries, LOD, accuracy and repeatability are given. The accuracy of the method was confirmed by analyzing a certified reference material for DON and the method was compared with two common methods. Moreover, the suitability of the simultaneous determination of ZEA was examined.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Trichothecene, ZEA, and zearalanone (ZAN) standards were obtained from Sigma (Taufkirchen, Germany) and deepoxy-deoxynivalenol (DOM-1) from Biopure (Tulln, Austria). ACN (HPLC grade), ammonium acetate (puriss. p. a.), ammonia solution 25% (analytical grade) were purchased from Merck (Darmstadt, Germany). For the filtration of the extracts, filter papers MN 619$^1/_4$ from Macherey-Nagel (Dueren, Germany) were used. Mycosep® 227 columns (Romer Labs, Union, MO, USA) were from Coring System Diagnostix GmbH (Gernsheim, Germany) and DONPrep® columns from R-Biopharm Rhone LTD (Darmstadt, Germany). Bond Elut® JR C18, Florisil, alumina, a new Bond Elut material (later defined as Mycotoxin®) each 1000 mg and Focus® columns were obtained from Varian Deutschland GmbH (Darmstadt, Germany). Oasis® columns were purchased from Waters (Eschborn, Germany) and Strata® X columns from Phenomenex (Aschaffenburg, Germany). Certified reference material T2210 was obtained from the Central Science Laboratory (Sand Hutton, UK). Deionized water was used for all procedures.

### 2.2 Sample preparation

#### 2.2.1 Using Mycosep® 227 columns

The finely ground sample (25 g) was extracted with 100 mL of a mixture of ACN/water (84/16; v/v) by blending at high speed for 3 min using an Ultra Turrax. The extract was filtered (MN 619$^1/_4$) and 5 mL of the filtrate was slowly pressed through a Mycosep 227 column. The eluate (2 mL) was evaporated to dryness on a heated aluminum block at 50°C using a gentle stream of nitrogen and the residue was reconstituted in 0.5 mL of ACN/water (1/4; v/v). DOM-1 solution [50 µL; 5 µg/mL in ACN/water (1/4; v/v)] was added, the solution was mixed and a 10-µL aliquot was used for LC-MS/MS analysis.

#### 2.2.2 Using Bond Elut Mycotoxin® columns

The finely ground sample (25 g) was extracted with 100 mL of a mixture of ACN/water (80/20; v/v) by blending at high speed for 3 min using an Ultra Turrax blender. The extract was filtered (MN 619$^1/_4$) and 4 mL of the filtrate was passed through a Bond Elut Mycotoxin® column. For simultaneous determination of ZEA the extract was spiked (prior to the filtration step) at a level of 50 ng/g sample with ZAN solution in ACN as internal standard. The rest of the procedure was identical to description given in Section 2.2.1.

#### 2.2.3 Using DONPrep® columns

The finely ground sample (25 g) was extracted with 200 mL water by blending at high speed for 3 min using an Ultra Turrax. The extract was centrifuged at 10 000 rpm and filtered through an MN 619$^1/_4$ filter paper. The filtrate (2 mL) was passed through a DONPrep® column at a flow rate of 1 drop/second. The column was washed with 5 mL of water at the same flow rate. Using a syringe, the column was "dried" by flushing it with air. DON was finally eluted with $2 \times 250$ µL and $2 \times 500$ µL of methanol and waiting 3 min after each elution step. The eluate was evaporated to dryness under nitrogen at 50°C and the residue reconstituted in 250 µL of ACN/water (1/4; v/v). DOM-1 solution (25 µL; 5 µg/mL in ACN/water (1/4; v/v)) was added, the solution was mixed and a 10-µL aliquot was used for LC-MS/MS analysis.

### 2.3 LC parameters

LC analysis was performed using an Agilent 1100 series system consisting of a binary pump, a degasser, a column oven and an autosampler (Agilent Technologies, Waldbronn, Germany). The analytes were chromatographed on a Synergi Fusion RP 80A column (4 µm, $250 \times 2$ mm id; Phenomenex, Aschaffenburg, Germany). The column was maintained at a temperature of 25°C. The flow rate was set to 0.2 mL/min, the injection volume was 10 µL. Solvent A was 0.00184 mM ammonia, 0.13 mM ammonium acetate in water (pH 7.4) and solvent B was ACN. A linear binary gradient was applied changing from 20 to 70% solvent B

within 25 min. Then, the content of solvent B was lowered to 20% within 1 min and the column was re-equilibrated for 9 min (for further details see [38]).

### 2.4 MS parameters

A Quattro Ultima MS/MS spectrometer (Micromass UK Limited, UK) was used with positive and negative ESI. The following settings were applied: source temperature: 120°C; desolvation temperature: 400°C; desolvation and cone gas flows: 600 and 250 L/h; capillary voltage: 3 kV; cone voltage: 30 V; collision energies: between 8 and 24 V, depending on the analyte. System control was carried out using Masslynx 4.0 software. Quantitative determination of all compounds was applied in the multiple reaction monitoring (MRM) mode with a dwell time of 0.2 s and a span of 0.1 u. The molecular ions and fragments used are given in Table 1.

**Table 1.** MS/MS parameters for the determination of 12 trichothecenes, ZEA, ZAN and DOM-1 in ESI negative and positive mode

| Toxin | Q1 *m/z* | Q3 (1) *m/z* | CE (eV) | Q3 (2) *m/z* | CE (eV) |
|---|---|---|---|---|---|
| DON | 295 $[M-H]^-$ | 265 | 12 | 247 | 12 |
| NIV | 311 $[M-H]^-$ | 281 | 11 | 191 | 19 |
| 3ADON | 337 $[M-H]^-$ | 307 | 11 | 173 | 11 |
| 15ADON | 337 $[M-H]^-$ | 219 | 11 | 150 | 18 |
| FUS | 353 $[M-H]^-$ | 263 | 12 | 187 | 23 |
| T-2 | 484 $[M+NH_4]^+$ | 245 | 14 | 215 | 17 |
| HT-2 | 442 $[M+NH_4]^+$ | 263 | 13 | 215 | 12 |
| NEO | 400 $[M+NH_4]^+$ | 305 | 13 | 215 | 15 |
| DAS | 384 $[M+NH_4]^+$ | 307 | 12 | 247 | 15 |
| MAS | 367 $[M+43]^-$ | 201 | 9 | 59 | 13 |
| T-2 triol | 381 $[M-H]^-$ | 101 | 20 | | |
| T-2 tetraol | 297 $[M-H]^-$ | 219 | 14 | 201 | 18 |
| ZEA | 317 $[M-H]^-$ | 175 | 24 | 273 | 20 |
| ZAN | 319 $[M-H]^-$ | 205 | 23 | 275 | 21 |
| DOM-1 | 279 $[M-H]^-$ | 231 | 17 | 249 | 10 |

### 2.5 Validation of the method

For external calibration, mixed standard solutions containing DON, NIV, 3ADON, 15ADON, FUS, T-2, HT-2, NEO, DAS, MAS, T-2 triol, T-2 tetraol, ZEA and ZAN with concentrations between 20 and 1500 ng/mL were prepared in ACN/water (1/4; v/v). To 500 µL of each solution (different concentration levels) 50 µL DOM-1 solution (5 µg/mL) was added. The peak areas of each toxin were plotted against the concentrations and the calibration curves were calculated by linear regression. The quantification of samples was carried out with external calibration (standard solutions in mobile phase; not matrix assisted) and correction of each value was performed using the respective internal standards (ISTD; DOM-1 for trichothecenes, ZAN for ZEA).

For the determination of the LOD, the matrix-assisted calibration was used. These calibration curves were performed by spiking standard solutions of different concentrations (20 – 1500 ng/mL) to cleaned extracts of a blank wheat sample. The S/N was calculated (peak to peak calculation with Masslynx 4.0 software) for each toxin by choosing the less intensive of the two mass transitions and the LOD determined at an S/N > 3 : 1.

For testing the recovery, extracts of blank wheat, corn, durum, oat, bread (rye and wheat), muesli (consisting of whole oats, wheat and barley) and cereal infant food (consisting of milk powder and whole wheat) were spiked with a trichothecene standard solution at levels of 50, 200 and 500 ng/g (DON was spiked at levels of 100, 400 and 1000 ng/g) and a ZEA/ZAN standard solution at a level of 50 ng/g, respectively, cleaned up with Bond Elut Mycotoxin® columns and analyzed. Three replicates were analyzed for each spiked sample extract.

To determine the repeatability of the complete method, two naturally contaminated samples (wheat, containing DON, NIV, 3ADON and 15ADON; oat, containing DON, NIV, T-2, HT-2 and T-2 tetraol) were analyzed each with ten replicates.

## 3 Results and discussion

### 3.1 Comparison of the immunoaffinity and Mycosep® method

To compare the IAC (DONPrep®, R-Biopharm) and the Mycosep® method, we analyzed ten naturally contaminated samples with both methods [15, 38] and determined the DON content by LC-MS/MS (Table 2). In all cases, we obtained significantly higher values ($f = 2$, $p = 0.05\%$) using the IAC method. The differences ranged from 23 to 57%. There are two possible reasons for these findings: the polar

**Table 2.** DON content of 10 naturally contaminated samples analyzed with DONPrep® IAC and Mycosep® 227 columns ($n = 3$)

| Sample | IAC (DONPrep®) DON (ng/g) | Mycosep® 227 DON (ng/g) |
|---|---|---|
| Corn | 1012 ± 40 | 819 ± 29 |
| Corn flour | 741 ± 22 | 460 ± 33 |
| Wheat flour | 1184 ± 15 | 788 ± 1.4 |
| Wheat bran | 1544 ± 8.1 | 1169 ± 4.7 |
| Wheat flour | 527 ± 2.3 | 283 ± 9.1 |
| Wheat bran | 580 ± 21 | 449 ± 15 |
| Durum | 1258 ± 5.7 | 539 ± 5.2 |
| Bread | 743 ± 2.8 | 349 ± 11 |
| Wheat flour | 3167 ± 10 | 2092 ± 54 |
| Wheat flour | 5876 ± 200 | 3930 ± 196 |

toxin DON is better extracted with water than with ACN/water and the IAC show usually higher recoveries [15]. In earlier studies we found recovery rates of about 80% with the Mycosep® clean-up of spiked samples [38]. As spiked samples do not represent the conditions in naturally contaminated samples, the efficiency of the extraction step is not included in this recovery. Thus, relatively "high" recovery rates are pretended when samples are spiked.

Therefore, we examined the extraction of naturally contaminated samples with different solvents as well as new SPE clean-up procedures to obtain higher values.

### 3.2 Extraction and spiking experiments

For the investigation of the best extraction efficiency, naturally contaminated wheat and oat samples were extracted with the following solvent mixtures: ACN/$H_2O$ 75/25 v/v, ACN/$H_2O$ 80/20 v/v and ACN/$H_2O$ 84/16 v/v ($n$ = 3). The extracts were cleaned up and analyzed using Mycosep® 227 columns (see Section 2). The highest concentrations for DON, NIV and T-2 tetraol were obtained with the ACN/$H_2O$ 80/20 v/v solvent, although we expected ACN/$H_2O$ 75/25 v/v to be the better solvent for polar toxins, due to the higher water content. The lower response for the internal standard DOM-1 indicated strong matrix effects in the MS when extracting the sample with ACN/$H_2O$ 75/25 v/v. Moreover, we suspect that the matrix effect is even more pronounced for the polar toxins in comparison to the ISTD. This might be the reason for the lower concentrations for DON, NIV and T-2 tetraol using this solvent. In comparison to the 84/16 v/v solvent mixture, the values for the polar toxins obtained with the ACN/$H_2O$ 80/20 v/v extraction were up to 33% higher, whereas the contents of the minor polar HT-2 was comparable for both solvents. Given that the extraction with ACN/$H_2O$ 80/20 v/v showed the best efficiency especially for polar trichothecenes and we observed less matrix effects in LC-MS/MS, we have chosen this solvent mixture for our method.

To compare two different ways of spiking, we spiked a blank wheat material directly into the sample and spiked the blank wheat extract at the same level. All extracts were analyzed with Mycosep® 227 columns and the recovery rates were calculated (Fig. 2). The differences between the two spiking procedures ranged from 1 to 21%, with a slight trend to higher recovery rates for the extract spiking. For NIV, FUS and T-2 tetraol, significant higher (f = 2, $p$ = 0.05%) recoveries were obtained using extract spiking, whereas for HT-2, significant higher values with direct spiking were observed. For all other trichothecenes no differences were perceived. Since our focus was on the performance of the clean-up step, we chose the spiking into the sample extract for further studies.

Additionally, we compared the extraction of the samples via high speed blending (3 min) and shaking (90 min). Only slight differences were found between these extraction methods and therefore, we used high speed blending in our experiments.

### 3.3 Development of a new clean-up procedure

Four SPE materials (Bond Elut® JR C18, Florisil, alumina and a new Bond Elut material) with different polarities and three polymer-based SPE columns (Focus®, Oasis® and Strata® X) were tested for their applicability for the clean-up of sample extracts. First, we evaluated the recovery performance of all materials by passing a trichothecene standard solution through the columns. Six of the seven tested materials showed satisfactory recoveries of 77 to 109%. In the next step, the purification efficiency was tested. For

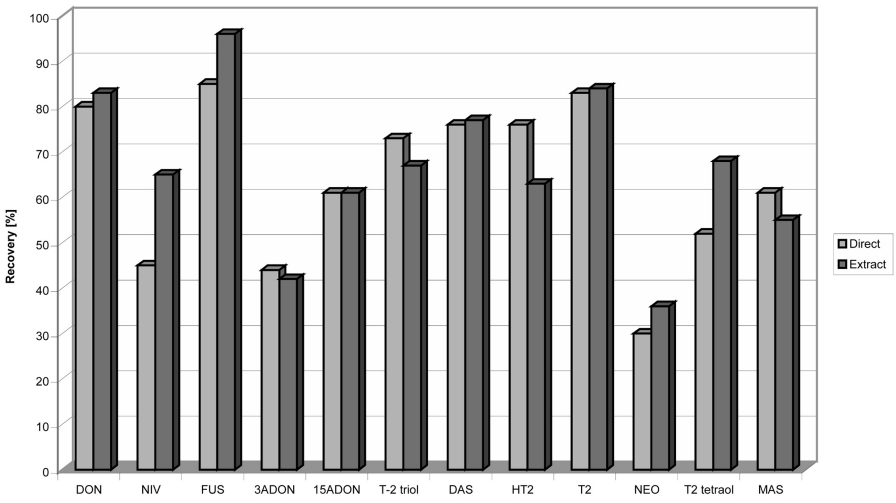



**Figure 2.** Recoveries for 12 trichothecenes obtained after direct or extract spiking, cleaned up with Mycosep® 227 columns.

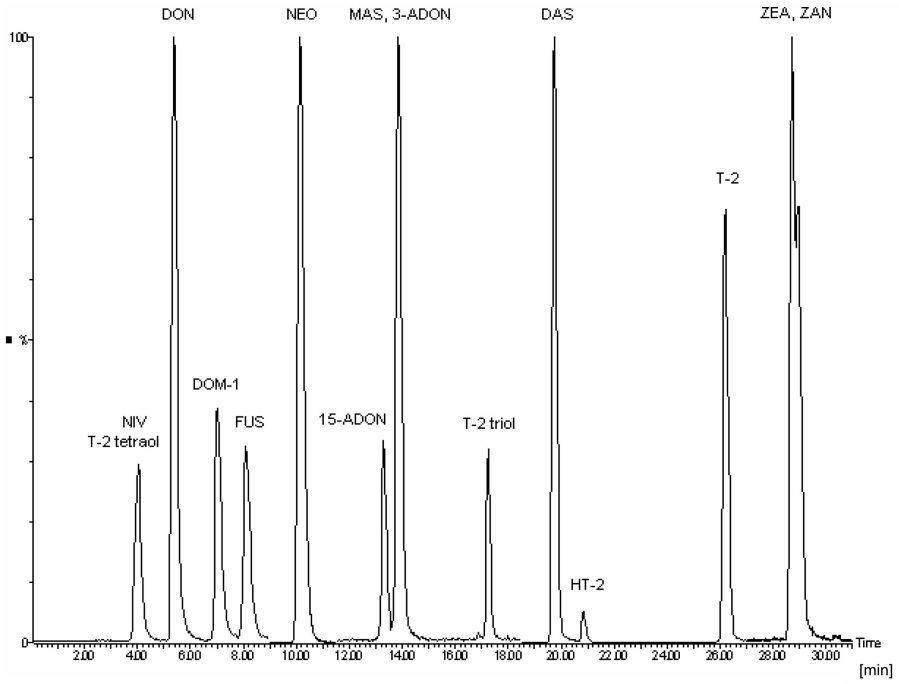



**Figure 3.** LC-ESI-MS/MS TIC of a wheat sample spiked with 12 trichothecenes at a concentration level of 500 ng/g (DON spiked at a level of 1000 ng/g), ZEA and ZAN (spiked at a level of 50 ng/g), cleaned up with Bond Elut Mycotoxin® columns (internal standard DOM-1, spiked immediately prior LC-MS-MS analysis at a concentration level of 400 ng/mL).

this, a spiked wheat extract was cleaned up with the columns and the recovery rates were determined and, additionally, an aliquot of the same extract was cleaned up with Mycosep® 227 columns. The clean-up performance of Bond Elut® JR Florisil, Focus®, Oasis® and Strata® X columns was too poor, so that the purified extracts were cloudy even after filtration and therefore could not be analyzed. The new Bond Elut® material showed the best recovery rates ranging from 62 to 108%. In comparison to the Mycosep® method, the recovery rates obtained with this material were at least similar and for six toxins even significantly higher (two-sided F-test and *t*-test; $f = 2$, $p = 0.05\%$). As the clean-up with the new Bond Elut® columns (later defined as Bond Elut Mycotoxin®) showed satisfactory purification of the crude extracts and good recoveries, this method was optimized and validated with different cereal-based matrices.

### 3.4 Performance validation

An LC-ESI-MS/MS total ion chromatogram (TIC) of a blank wheat sample spiked with 12 trichothecenes at a level of 500 ng/g (DON spiked at a level of 1000 ng/g), ZEA and ZAN (spiked at a level of 50 ng/g) and cleaned with Bond Elut Mycotoxin® columns is shown in Fig. 3.

Matrix-assisted calibration graphs were linear in the range of 20 – 1200 ng/g. The correlation coefficients ($R^2$) were between 0.995 and 0.9998 for five-point calibration curves, depending on the toxin. Limits of detection ranged from 0.3 ng/g for T-2 triol to 5 ng/g for HT-2. For the standards in mobile phase used for external calibration (not matrix assisted) the correlation coefficients were even higher and the LOD still lower.

For the determination of the recovery, trichothecene and ZEA/ZAN standard solutions were added to extracts of blank samples and the extracts were cleaned up with Bond Elut Mycotoxin® columns. The reasons for extract spiking are given in Section 3.2. In Table 3, the obtained recovery rates, corrected by the respective ISTD, are summarized. The recovery rates ranged from 65 to 104% (trichothecenes) and 95 to 116% (ZEA), showing the lowest values for the polar toxins NIV and T-2 tetraol. Biselli & Hummert [13] and Berthiller *et al*. [14] recently reported similar results using the Mycosep® method for the determination of trichothecenes and ZEA.

In the Guideline 2005/38/EG the recovery limits are given as follows: 60 to 120% for DON/ZEA and 60 – 130% for T-2/HT-2. The recovery rates of the new developed method are in these ranges and thus, the method satisfies the EU requirements.

As described in Sections 2.2.2 and 2.5 the Bond Elut Mycotoxin® method was also tested for its applicability for the simultaneous determination of zearalenone (ZEA) in cereals. The recovery rates for ZEA given in Table 3 were obtained for spiked extracts corrected with the ISTD ZAN (added before the clean-up step). The recovery for ZEA without the correction via the ISTD ZAN was about 50%. These results also match with those of Berthiller *et al*. [14] and Biselli & Hummert [13] who used Mycosep® 226 col-

**Table 3.** Average recoveries and RSD in% obtained for 12 trichothecenes (corrected by the ISTD DOM-1) and ZEA (corrected by the ISTD ZAN) from spiked wheat, corn, durum, oats, bread, muesli and cereal infant food samples (spiking levels of 50/100, 200/400 and 500/1000 ng/g for trichothecenes/DON and 50 ng/g for ZEA and ZAN), after clean-up with Bond Elut Mycotoxin® columns ($n = 3$)

| Toxin | Recovery [%] ± RSD [%], 3 levels, $n = 3$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | Wheat | Corn | Durum | Oats | Bread | Muesli | Infant food |
| DON | 90 ± 5.2 | 93 ± 2.8 | 98 ± 3.8 | 96 ± 5.1 | 87 ± 1.7 | 87 ± 3.7 | 88 ± 12 |
| NIV | 67 ± 5.9 | 74 ± 2.5 | 67 ± 6.3 | 73 ± 10 | 65 ± 5.7 | 71 ± 13 | 66 ± 10 |
| 3ADON | 89 ± 9.3 | 88 ± 7.6 | 97 ± 6.6 | 93 ± 11 | 100 ± 5.5 | 101 ± 7.1 | 91 ± 9.4 |
| 15ADON | 92 ± 13 | 87 ± 15 | 89 ± 11 | 89 ± 16 | 96 ± 9.5 | 98 ± 8.3 | 96 ± 6.6 |
| FUS | 91 ± 10 | 94 ± 4.2 | 91 ± 7.8 | 92 ± 5.2 | 98 ± 8.5 | 97 ± 6.4 | 6 ± 4.3 |
| T-2 | 87 ± 7.6 | 88 ± 8.8 | 84 ± 2.2 | 75 ± 1.9 | 83 ± 8.2 | 75 ± 11 | 70 ± 7.3 |
| HT-2 | 82 ± 7.3 | 91 ± 3.3 | 85 ± 5.0 | 76 ± 8.2 | 79 ± 3.3 | 70 ± 7.7 | 74 ± 0 |
| NEO | 91 ± 2.6 | 78 ± 11 | 68 ± 18 | 63 ± 17 | 80 ± 2.0 | 104 ± 10 | 71 ± 6.3 |
| DAS | 82 ± 8.3 | 89 ± 3.6 | 85 ± 5.2 | 82 ± 0.9 | 75 ± 3.7 | 82 ± 6.8 | 68 ± 4.6 |
| MAS | 86 ± 13 | 85 ± 12 | 93 ± 4.2 | 92 ± 5.5 | 86 ± 11 | 88 ± 16 | 91 ± 14 |
| T-2 triol | 69 ± 9.1 | 66 ± 1.2 | 83 ± 2.8 | 74 ± 1.9 | 76 ± 9.3 | 82 ± 3.3 | 71 ± 7.9 |
| T-2 tetraol | 69 ± 12 | 75 ± 6.8 | 73 ± 10 | 73 ± 12 | 65 ± 11 | 67 ± 17 | 70 ± 16 |
| ZEA | 110 ± 5.9 | 113 ± 5.0 | 108 ± 4.8 | 95 ± 7.5 | 111 ± 6.0 | 102 ± 2.7 | 116 ± 6.7 |

umns and the correction by the ISTD ZAN. Thus, the new Bond Elut Mycotoxin® method offers a simultaneous determination of trichothecenes and ZEA in cereals, too.

The $RSD_r$ of the complete method (analyzing two samples of wheat and oat, naturally contaminated with trichothecenes; ten replicates each) ranged between 2.67 for DON in wheat and 20.0% for T-2 in oats. Considering the above mentioned problems concerning simultaneous determination of toxins of different polarity, especially for the polar toxins (*e.g.* DON) these deviations are very low. In this regard, it should be considered that all analysis was performed under repeatability conditions (*e.g.* the same laboratory and person, defined series, replicates simultaneously analyzed). Under these conditions, comparatively low deviations were also observed for the IAS and Mycosep clean-up (see below). However, these values are satisfactory, when comparing them with the EU requirements in Guideline 2005/38/EG, where the $RSD_r$ are limited to 20% for DON and 40% for T-2.

Food Analysis Performance Assessment Scheme (FAPAS) certified reference material for DON is available from the Central Science Laboratory (CSL). For the confirmation of the accuracy and applicability of the Bond Elut Mycotoxin® method, we analyzed this certified reference material ($n = 4$) and compared our results with the certified concentrations. The results of our method matched very well, as we found 495 ± 5 ng/g DON compared to the assigned value of 463 ± 167 ng/g.

### 3.5 Method comparison

To characterize and classify the new method, we compared the recovery rates of the Bond Elut Mycotoxin® method with those of the Mycosep® method reported in our earlier studies [38]. All values were determined with the same LC-MS/MS method. The recoveries especially of the polar toxins such as DON, NIV, 3ADON and T-2 tetraol were increased by up to 31% when applying the new developed extraction and clean-up. As the performance of the Bond Elut Mycotoxin® columns is similar or even better and the columns are much cheaper, this new method is a very good alternative to the Mycosep® 227 columns.

Additionally, we analyzed six naturally contaminated samples with DONPrep® IAC, the Mycosep® method and the Bond Elut Mycotoxin® method. The obtained trichothecene contents are given in Table 4. Up to 43% higher values for the polar toxins DON, NIV, 3ADON, 15ADON and T-2 tetraol were achieved with the developed method in comparison to the Mycosep® clean-up. In the case of DON, the highest contents in all samples were obtained using the IAC. These results indicate that the solvent with the best extraction efficiency for DON is pure water. However, since our aim was the determination of 12 trichothecenes with different polarities, the new method is a very good compromise, as it shows similar values compared to the IAC for the most important trichothecene, DON. Furthermore, as was shown recently for Mycosep® 226 columns [13, 14] the simultaneous determination of ZEA is also possible with the new Bond Elut Mycotoxin® columns.

However, the new method yielded 20 and 16% lower concentrations for the minor polar T-2 and HT-2. This is probably due to the lower content of ACN in the extraction solvent (ACN/water, 80/20; v/v) in comparison to the solvent (ACN/water, 84/16; v/v) used for Mycosep® clean-up. Potentially, this problem could be reduced by using a combination of high speed blending (3 min) and shaking (30 min) for sample extraction.

**Table 4.** Trichothecene contents of six naturally contaminated samples analyzed with DONPrep® IAC, Mycosep® 227 and Bond Elut Mycotoxin® columns ($n = 3$)

| Sample | Clean-up | DON [ng/g] | NIV [ng/g] | 15ADON [ng/g] | HT-2 [ng/g] | T-2 [ng/g] | T2 tetraol [ng/g] |
|---|---|---|---|---|---|---|---|
| Bread | IAC | 690 ± 18 | | | | | |
| Bread | Mycosep® | 557 ± 19 | | | | | |
| Bread | Bond Elut Mycotoxin® | 648 ± 21 | | | | | |
| Corn | IAC | 368 ± 8.4 | | | | | |
| Corn | Mycosep® | 333 ± 14 | 12 ± 0 | 69 ± 2.0 | | | |
| Corn | Bond Elut Mycotoxin® | 356 ± 3.8 | 14 ± 0 | 99 ± 2.5 | | | |
| Wheat | IAC | 488 ± 5.5 | | | | | |
| Wheat | Mycosep® | 421 ± 16 | | | | | |
| Wheat | Bond Elut Mycotoxin® | 468 ± 19 | | | | | |
| Oats | IAC | 299 ± 11 | | | | | |
| Oats | Mycosep® | 220 ± 5.3 | 22 ± 3.3 | 7.0 ± 0.4 | 93 ± 12 | 15 ± 4.1 | 91 ± 6.2 |
| Oats | Bond Elut Mycotoxin® | 264 ± 13 | 19 ± 1.2 | 7.7 ± 0.1 | 78 ± 4.9 | 12 ± 4.3 | 106 ± 3.2 |
| Wheat | IAC | 1680 ± 32 | | | | | |
| Wheat | Mycosep® | 1590 ± 40 | 39 ± 3.7 | 24 ± 2.0 | | | |
| Wheat | Bond Elut Mycotoxin® | 1750 ± 120 | 64 ± 1.4 | 42 ± 2.2 | | | |
| Durum | IAC | 512 ± 15 | | | | | |
| Durum | Mycosep® | 407 ± 25 | 25 ± 4.1 | | | | |
| Durum | Bond Elut Mycotoxin® | 456 ± 42 | 22 ± 1.3 | | | | |

Although there is a small loss of the minor polar toxins, the new Bond Elut Mycotoxin® method is a good alternative to the commonly used Mycosep® method concerning the polar toxins (especially DON).

## 4 Concluding remarks

We developed a new rapid, reliable and cost-efficient method for the simultaneous determination of 12 trichothecenes and ZEA in cereals and cereal based food using LC-ESI-MS/MS detection. For the first time, Bond Elut Mycotoxin® columns were used for sample clean-up. In combination with a higher polarity of the extraction solvent these columns showed equal or, especially for the polar toxins, higher recoveries in comparison to different Mycosep® columns. Particularly in naturally contaminated samples significant higher amounts of the most important trichothecen DON were observed. As the performance of the Bond Elut Mycotoxin® columns is similar or even better and the columns are cheaper, the new clean-up procedure is a very good alternative to the standardized Mycosep® method commonly used.

Furthermore, using the internal standards DOM-1 (for the trichothecenes) and ZAN (for ZEA) application of the new sample preparation procedure prior to LC-MS/MS detection allows reliable and correct quantification without the need of a matrix assisted calibration.

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## 5 References

[1] Langseth, W., Rundberget, T., *J. Chromatogr. A* 1998, *815*, 103 – 121.

[2] European Commission, Scientific Commitee on Food, *Opinion on Fusarium Toxins Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol,* http://www.europa.eu.int/comm/food/food/chemicalsafety/contaminants/fusarium_en.htm (adopted: 02/27/2002).

[3] European Commission, Scientific Committee on Food, *Opinion on Fusarium Toxins Part 5: T-2 toxin, HT-2 toxin,* http://www.europa.eu.int/comm/food/food/chemicalsafety/contaminants/fusarium_en.htm (adopted: 05/30/2001).

[4] Scott, P. M., Lau, P. Y., Kanhere, S. R., *J. Assoc. Off. Anal. Chem.* 1981, *64*, 1364 – 1371.

[5] Tanaka, T., Hasegawa, A., Matsuki, Y., Ishii, K., Ueno, Y., *Food Addit. Contam.* 1985, *2*, 125 – 137.

[6] Chang, H. L., DeVries, J. W., Larson, P. A., Patel, H. H., *J. Assoc. Off. Anal. Chem.* 1984, *67*, 52 – 54.

[7] Trenholm, H. L., Warner, R. M., Prelusky, D. B., *J. Assoc. Off. Anal. Chem.* 1985, *68*, 645 – 649.

[8] Cahill, L. M., Kruger, S. C., McAlice, B. T., Ramsey, C. S. *et al*., *J. Chromatogr. A* 1999, *859*, 23 – 28.

[9] Cohen, H., Lapointe, M., *J. Assoc. Off. Anal. Chem.* 1982, *65*, 1429 – 1434.

[10] Kamimura, H., *Mycotoxins* 1981, *13*, 27 – 33.

[11] Tanaka, T., Hasegawa, A., Matsuki, Y., Ueno, Y., *Food Addit. Contam.* 1985, *2*, 259 – 265.

[12] Jimenez, M., Mateo, R., *J. Chromatogr. A* 1997, *778*, 363 – 372.

[13] Biselli, S., Hummert, C., *Food Addit. Contam.* 2004, *22*, 752 – 760.

[14] Berthiller, F., Schuhmacher, R., Buttinger, G., Krska, R., *J. Chromatogr. A* 2005, *1062*, 209 – 216.

[15] Klötzel, M., Schmidt, S., Lauber, U., Thielert, G., Humpf, H.-U., *Chromatographia* 2005, *62*, 41 – 48.

 

[16] Trucksess, M. W., Nesheim, S., Eppley, R. M., *J. Assoc. Off. Anal. Chem.* 1984, *67*, 40 – 43.

[17] Eppley, R. M., Trucksess, M. W., Nesheim, S., Thorpe, C. W. *et al*., *J. Assoc. Off. Anal. Chem.* 1984, *67*, 43 – 45.

[18] Walker, F., Meier, B., *J. AOAC Int.* 1998, *81*, 741 – 748.

[19] Kotal, F., Holadova, K., Hajslova, J., Poustka, J., Radova, Z., *J. Chromatogr. A* 1999, *830*, 219 – 225.

[20] Scott, P. M., Kanhere, S. R., Tarter, E. J., *J. Assoc. Off. Anal. Chem.* 1986, *69*, 889 – 893.

[21] Schothorst, R. C., Jekel, A. A., *Food Chemistry* 2001, *73*, 111 – 117.

[22] Tanaka, T., Yoneda, A., Inoue, S., Sugiura, Y., Ueno, Y., *J. Chromatogr. A* 2000, *882*, 23 – 28.

[23] Nielsen, K. F., Thrane, U., *J. Chromatogr. A* 2001, *929*, 75 – 87.

[24] Onji, Y., Aoki, Y., Tani, N., Umebayashi, K., *et al*., *J. Chromatogr. A* 1998, *815*, 59 – 65.

[25] Pettersson, H., Langseth, W., *Intercomparison of Trichothecene Analysis and Feasibility to Produce Certified Calibrants and Reference Material – Method Studies*; SMT4 – CT96 – 2047; European Commission: Brussels, Belgium, 2002.

[26] Bayliss, M. A. J., Homer, R. B., Shepherd, M. J., *J. Chromatogr. A* 1988, *445*, 393 – 402.

[27] Jimenez, M., Mateo, J. J., Mateo, R., *J. Chromatogr. A* 2000, *870*, 473 – 481.

[28] Sano, A., Matsutani, S., Suzuki, M., Takitani, S., *J. Chromatogr. A* 1987, *410*, 427 – 436.

[29] Dall'Asta, C., Galaverna, G., Biancardi, A., Gasparini, M., *et al*., *J. Chromatogr. A* 2004, *1047*, 241 – 247.

[30] Razzazi-Fazeli, E., Rabus, B., Cecon, B., Boehm, J., *J. Chromatogr. A* 2002, *968*, 129 – 142.

[31] Thimm, N., Handl, J., Binder, M., *HPLC-MS-Method for A-Trichothecenes*, Proc. 23, Mykotoxin-Workshop, Wien, Austria, 2000, 206 – 209.

[32] Lagana, A., Curini, R., D'Ascenzo, G., De Leva, I. *et al*., *Rapid Commun. Mass Spectrom.* 2003, *17*, 1037 – 1043.

[33] Plattner, R. D., Maragos, C. M., *J. AOAC Int.* 2003, *86*, 61 – 65.

[34] Razzazi-Fazeli, E., Böhm, J., Jarukamjorn, K., Zentek, J., *J. Chromatogr. B* 2003, *796*, 21 – 33.

[35] Huopalahti, R. P., Ebel, J., Henion, J. D., *J. Liq. Chromatogr. Relat. Technol.* 1997, *20*, 537 – 551.

[36] Berger, U., Oehme, M., Kuhn, F., *J. Agric. Food Chem.* 1999, *47*, 4240 – 4245.

[37] Dall'Asta, C., Sforza, S., Galaverna, G., Dossena, A., Marchelli, R., *J. Chromatogr. A* 2004, *1054*, 389 – 395.

[38] Klötzel, M., Gutsche, B., Lauber, U., Humpf, H.-U., *J. Agric. Food Chem.* 2005, *53*, 8904 – 8910.

 