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A Method Using Gas Chromatography – Mass Spectrometry for the Detection of Mycotoxins from Trichothecene Groups A and B in Grains

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1. Introduction

Mycotoxins are toxic secondary metabolites produced by several fungi species growing on many agricultural commodities and processed foods (Bennett & Klich, 2003). The term secondary metabolite designates compounds that are not indispensable for the growth and metabolism of the fungi as such, and in contrast to primary metabolites, such as amino acids, fatty acids, saccharides, nucleic acids and proteins, are not essential for living. These metabolites are generally believed to be produced in response to stress or altered conditions of fungal growth related to a variety of plant and environmental factors. Fungal growth requires a readily available carbohydrate source, adequate moisture, oxygen, and appropriate temperatures (often 15-25°C). Plant or fungal stressors such as drought, high ambient temperatures, insect damage, mechanical harvest damage and reduced plant vigour predispose crop plants to infestation by fungi with subsequent production of secondary metabolites (Osweiler, 1999).

Even if there is no generally accepted theory about the biological benefits of mycotoxin production, mycotoxins have been proposed to provide the following functions to fungal development: (1) ecological role in nature, such as substrate competition with bacteria; (2) regulatory role in metabolism; and (3) regulatory role in, or at least coincident with, differentiation (Miller, 2002).

Since toxigenic fungi are cosmopolitan, mycotoxins are environmental pollutants present in virtually all parts of the world. More than 300 chemically different mycotoxins formed by more than 350 fungal species and causing various diseases (mycotoxicoses) to living organisms are described (Stein, 1998). Moreover, it can be realistically assumed that further mycotoxins are likely to be discovered. Since only a few mycotoxins have been related to important food and feed borne diseases, the potential impact on human and animal health of many of them remains to be elucidated.

The presence of a toxigenic fungus in a food or feed commodity gives indication of the potential hazard, but definitive conclusions can be made only by the actual identification of the specific toxin, since the presence of a fungus itself provides no evidence that it is a...
producer of the toxin. Moreover, a given toxin may persist in a substrate when the fungus that has produced it is no longer present; or a given fungus may be capable of producing more than one toxin and finally, a given toxin may be produced by different genera of fungi (Fink-Gremmels, 1999).

Mycotoxins exhibit variable resistance to food processing such as cleaning and milling of cereals, bread- or pasta-making, roasting of nuts, production of vegetable oils, alcoholic fermentation, cooking and storage of meat, processing of milk to cheese, butter or yogurt. However, they are in general stable and capable of persisting in the final products (Bennett and Klich, 2003).

The mycotoxins that more frequently pose health risks to human and livestock are aflatoxins, ochratoxin A, trichothecenes, fumonisins, and zearalenone. Focusing on human and animals, biological effects, which may vary greatly among species, comprise hepatotoxicity, nephrotoxicity, neurotoxicity, immunosuppressive activity, teratogenicity, mutagenicity, genotoxicity, carcinogenicity, oestrogenicity, and diabetogenicity (Betina, 1989). Moreover, co-occurrence of different mycotoxins may lead to their interactions. Depending on the concentration and time of exposure to a mycotoxin and on the individual factors such as age, sex, diet or overall condition, clinical signs of mycotoxicoses may vary greatly among species (Grenier & Oswald, 2011).

Trichothecenes constitute a mycotoxin family produced by fungal species from several genera, notably *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichothecium*, *Trichoderma*, *Cylindrocarpon*, *Verticimonosporium*, *Acremonium*, and *Phomopsis*. Most of the trichothecenes that have been isolated and characterized chemically are from *Fusarium* species that grow in the field and are distributed worldwide, representing the important pathogens of grains and other food and feed plants. Trichothecenes appear as natural contaminants in cereal grains such as wheat, barley, oat, maize, rice, and derived products, such as bread, malt and beer (Scott, 1989).

There are more than 170 known trichothecenes which can be classified in four categories (A, B, C, and D) according to their chemical structure (Krska et al., 2001). All trichothecene mycotoxins have a basic tetracyclic sesquiterpene structure with a six-membered oxygen containing ring, an epoxide group on the 12,13 position, and an olefinic bond on the 9,10 position (Figure 1).

![Chemical structure of trichothecenes](www.intechopen.com)
The trichothecenes may also have side groups as hydroxyl, esterified hydroxyl, keto, or epoxide groups in various combinations. Type-A trichothecenes have a functional group other than a carbonyl group at C-8 and are differentiated by various combinations of hydroxyl or acyloxyl (OAc) groups at C-3, 4, 7, 8, and 15, similarly to the type-B trichothecenes, which have a typical carbonyl bond (keto group) at C-8 as well (Table 1).

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type-A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>HH</td>
<td></td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>COCH2CH(CH3)2</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>H</td>
<td>COCH2CH(CH3)2</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td><strong>Type-B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>O=</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>OH</td>
<td>O=</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>O=</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>OH</td>
<td>H</td>
<td>OAc</td>
<td>OH</td>
<td>O=</td>
</tr>
<tr>
<td>Fusarenon X</td>
<td>OH</td>
<td>OAc</td>
<td>OH</td>
<td>OH</td>
<td>O=</td>
</tr>
</tbody>
</table>

Table 1. The most important trichothecene mycotoxins (DAS: dyacetoxyscirpenol, DON: deoxynivalenol, 3-AcDON: 3-acetyl-DON, 15-AcDON: 15-acetyl- DON).

Group C trichothecenes are characterised by a second epoxide group at C-7, 8 or C-9, 10 (crotocin and baccharin), whereas type-D group includes toxins containing a macrocyclic ring between C-4 and C-15 with two ester linkages (satratoxin G and roridin). While type-A and B trichothecenes are commonly known as contaminants of food and feed, it is assumed that macrocyclic trichothecenes rarely occur in these matrices (Krska et al., 2001). However, these toxins have occasionally been determined in hay or straw. Over the past few years, they have attracted more attention as indoor pollutants (Gottschalk et al., 2009).

Several surveys suggest that the most prevalent trichothecenes are deoxynivalenol (DON), nivalenol, 3-acetyl-DON (3-AcDON) and 15-acetyl-DON (15-AcDON), as type-B trichothecenes, and HT-2 toxin and T-2 toxin, as type-A trichothecenes. They are mainly found on maize, oats, barley, and wheat. The latter, especially durum wheat, which is used nearly exclusively for the production of pasta, is susceptible to Fusarium infection and is often highly contaminated with DON. In European agricultural commodities type-A trichothecenes usually occur less frequently and at lower concentrations than DON. The simultaneous occurrence of DON with other Fusarium mycotoxins mainly type-B trichothecenes and zearalenone, has been reported for a variety of agricultural commodities (Gareis et al., 1989; Pettersson, 1992, as cited in Krska et al., 2001).

*Fusarium graminearum* (teleomorph Gibberella zeae) is the main species responsible for the natural contamination by DON and nivalenol; *F. sporotrichioides* is the major producer of T-2 and HT-2, while *F. poe* is a minor T-2 producer but, together with *F. sulphureum, F. roseum, F. equiseti,* and other species, forms diacetoxyscirpenol (DAS) (Krska et al., 2001).
All trichothecenes appear to inhibit peptidyl transferase. Peptidyl transferase is an integral part of the 60S ribosomal subunit and is involved in elongation and termination. All trichothecenes bind to the same ribosomal binding site, but produce different effects of protein synthesis. First, the 12,13 epoxide group is essential for inhibition of protein synthesis, and reduction of the 9,10 double bond reduces the toxic activity, both in vivo and in vitro. Second, substitution at R₂ enhances inhibition of peptidyl transferase. Those trichothecenes with substitutions at R₂ only are inhibitors of elongation or termination.

Third, substitution at R₁ and R₃ on the same side of the molecule produces initiation-like inhibitors. Substitution at R₂, which project away from the other side of the molecule, produces an inhibitor of elongation or termination. The more toxic trichothecenes are initiation-like inhibitors (Feinberg & McLaughlin, 1989).

Several surveys have shown toxicological and immunological effects on farm animals, produced by trichothecenes after ingestion of mould-contaminated cereal grains. The main symptoms observed, are skin and gastrointestinal irritation or necrosis, haematological disorders, diarrhoea, vomiting and feed refusal, decreased body weight gain, damage to the haematopoietic systems in bone marrow, spleen, thymus and lymph nodes and immunological alteration (Ibáñez-Vea et al., 2011).

From the point of view of known incidence, the most important trichothecene is DON. It may also co-occur in grains and feeds with other trichothecenes and zearalenone and other combinations of toxins are possible. The carryover of DON and other trichothecenes into human foods is considerable and a cause of concern for food safety agencies. The carryover of trichothecenes or their metabolites into farm animal products would not appear to be a major problem from experimental studies and no cases of their natural occurrence in meat, milk or eggs have been reported (Scott, 1989).

With regard to human diseases, these compounds have been related to several poison outbreaks such as alimentary toxic aleukia (ATA) and Akakaby-bio or red mould disease (Scott, 1989). During World War II, there was a devastating outbreak of ATA in Russia, and over 100,000 people died from this disease. Clinical signs included severe dermal necrosis, haemorrhaging, leukopenia and bone marrow degeneration. Bread prepared from over wintered grain contaminated by various toxigenic fungi was believed to be the cause of the illness (Trenholm et al., 1989).

Although perhaps outside the scope of this chapter, the controversial topic of the possible role of trichothecenes as biological warfare agents (“yellow rain”) should be mentioned as a footnote. T-2, DAS, DON, and nivalenol have been identified by gas chromatography - mass spectrometry (GC-MS) in environmental samples such as leaves, water, and yellow powder from Southeast Asia in 1981 (Scott, 1989).

Trichothecene levels in different matrices vary from µg/kg up to mg/kg depending on a toxin, matrix, climatic condition, as well as other factors. Since toxins can never be completely removed from the food supply, and since they are potential health risks for humans and animals, the European Union has implemented regulations for some of them. The co-occurrence of different trichothecenes (type-A and type-B) in one same foodstuff, could provoke additive or even synergistic effects on human or animal health; however, the knowledge regarding this aspects is still scarce. In order to be able to monitor several toxins,
it is necessary to develop analytical methods for their simultaneous determination that meets the regulatory requirements (Ibáñez-Vea et al., 2011).

According to reports on the incidence of mycotoxins, as mentioned above, one of the most frequently occurring mycotoxin in feed originating from Europe and Asia is DON (Binder et al., 2007). In addition, also the occurrence of T-2 toxin is reported to be significant. However, the indication that the presence of T-2 and HT-2 toxin in products intended for animal feeding could be a reason for concern and the statement that data on the presence of T-2 and HT-2 toxins in products intended for animal feeding are at present very limited are expressed in Commission Recommendation 2006/576/EC (European Commission, 2006b). Thus, the need for the development of a sensitive method and the need for collecting more occurrence data was announced and the guidance values for DON, zearalenone, ochratoxin A, and the sum of fumonisins B₁ and B₂ for judging the acceptability of compound feed, cereal and cereal products for animal feeding were given (European Commission, 2006b).

Reports on analytical methods for the determination of trichothecenes in various matrices using gas chromatography with either electron capture detection (Krska, 1998; Mateo et al., 2001; Radová et al., 1998; Valle-Algarra et al., 2005) or mass spectrometric detection (Ibáñez-Vea et al., 2011; Langseth and Rundberget 1998; Melchert and Pabel, 2004; Schollenberger et al., 1998; Schollenberger et al., 2005; Schothorst et al., 2005; Tanaka et al., 2000), liquid chromatography-mass spectrometry (Berthiller et al., 2005; Binder et al., 2007) and liquid chromatography with fluorescence detection (Dall’Asta et al., 2004; Pascale et al., 2003; Visconti et al., 2005) are available. Because the 12,13 epoxy trichothecenes are a group of closely related compounds, physicochemical methods of analysis are usually intended to determine more than one trichothecene at the same time (Krska et al., 2001).

In the present work, the validation of the procedure for the determination of eight trichothecenes – DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 toxin in animal feed with performance characteristics complying with requirements of Commission Recommendation 2006/576/EC (European Commission, 2006b), Commission Decision 2002/657/EC (European Commission, 2002), and Commission Regulation (EC) No 401/2006 (European Commission, 2006a) is described. However, Decision (European Commission, 2002) and Regulation (European Commission, 2006a) concern the residues in products of animal origin and mycotoxins in foodstuffs, but in view of the absence of suitable regulations, they seem to be a reasonable starting point. The procedure based on analytical methods described elsewhere (Binder et al., 2007; Langseth & Rundberget, 1998; Melchert & Pabel, 2004; Radová et al., 1998; Tanaka et al., 2000; Schothorst et al. 2005) consists of the extraction of trichothecenes with acetonitrile-water mixture, sample clean-up using MycoSep 227 Trich+ columns, derivatisation of trichothecenes with a mixture of bis(trimethylsilyl)acetamide, trimethylchlorosilane, and trimethylsilylimidazole (BSA-TMCS-TMSI) and determination using GC-MS. The problem with unusually high recoveries encountered was obviated with the use of matrix matched calibration suggested elsewhere (Pettersson & Langseth, 2002a, 2002b; Schollenberger et al., 2005). The validation procedure was performed according to Decision 2002/657/EC (European Commission, 2002).

In the years 2007, 2008, and 2009, altogether 175 samples of cereals produced in Slovenia were collected at farms, among them 79 samples of maize, 39 samples of barley, and 34 samples of wheat. In the samples, among analysed mycotoxins, trichothecenes were determined using the analytical procedure mentioned above.
2. Experimental

2.1 Apparatus

The linear shaker IKA HS 501 digital (IKA Labortechnik, Staufen, Germany) was used for the extraction and the system Syncore Polyvap (Büchi, Flawil, Switzerland) was used for the evaporation under vacuum. Gas chromatograph with mass selective detector 6890/5975B (Agilent Technologies) was combined with the column HP-5MS, 30 m, 0.25 mm I.D., 0.25 μm (Agilent Technologies) and a computer with a program ChemStation (Agilent Technologies) for the system control and data processing. The carrier gas was helium with the column flow rate of 1 mL/min. The injection volume was 1 μL and the splitless injection mode was used. The inlet temperature was 270°C, MSD ion source temperature 170°C, mass filter temperature 150°C and GC-MSD interface temperature 280°C. The column temperature program was: 60°C held for 2 minutes, 25°C/min to 240°C and 5°C/min to 300°C. Electron ionisation (EI) was carried out at 70 eV and spectra were monitored in selected ion monitoring (SIM) mode. Individual trichothecenes were identified by identification points given in Table 2.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Retention Time (min)</th>
<th>Target Ion</th>
<th>Qualifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>12.06</td>
<td>422.20</td>
<td>512.20</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>12.90</td>
<td>377.10</td>
<td>392.10</td>
</tr>
<tr>
<td>DAS</td>
<td>13.00</td>
<td>378.10</td>
<td>350.20</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>13.04</td>
<td>392.10</td>
<td>350.20</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>13.26</td>
<td>379.20</td>
<td>510.30</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>14.27</td>
<td>350.20</td>
<td>436.20</td>
</tr>
<tr>
<td>HT-2</td>
<td>16.58</td>
<td>466.20</td>
<td></td>
</tr>
<tr>
<td>T-2</td>
<td>16.62</td>
<td>350.20</td>
<td>436.20</td>
</tr>
</tbody>
</table>

Table 2. Retention time and ions monitored for trichothecenes.

2.2 Reagents

A certified combined crystalline trichothecenes standard of DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 was purchased from R-Biopharm Rhône (Glasgow, Scotland). After reconstitution in acetonitrile, the concentration of each trichothecene in the solution was 100 μg/mL. Working standard solutions with the concentrations of each trichothecene of 0.2 and 2.0 μg/mL were prepared diluting the stock standard solution with acetonitrile. Dichlorodimethylsilane, hexane, acetonitrile and methanol (analytical or chromatography grade purity) were purchased from Merck (Darmstadt, Germany) and MycoSep 227 Trich+ columns from Romer (MO, USA). Phosphate buffer saline (PBS) tablets (Oxoid, Basingstoke, Hampshire, UK) were used for a solution with pH = 7.4 prepared according manufacturer’s instructions. Sylon BTZ (BSA:TMCS:TMSI = 3:2:3) was purchased from Supelco (Bellefonte, PA, USA). As the extraction solvent, the mixture of acetonitrile and deionised water (84:16) was used. Prior to use, glass vials for the derivatisation were deactivated with 5% dichlorodimethylsilane solution (25 mL of dichlorodimethylsilane diluted to 500 mL with hexane).
2.3 Samples

The samples of raw material were taken directly at the farms. Several incremental samples were taken randomly from the whole lot and combined to the aggregate sample. After homogenisation and grinding of the aggregate sample in the lab, a laboratory sample of 1.5 kg was taken for the examination and stored at 8°C for mycotoxicological analyses.

The validation of the procedure was performed with different types of feed (maize, barley, wheat, oat, soya meal, and compound feeds for pigs, lactating sows, pregnant sows, cattle, laying hens, and chicken reared for fattening) spiked with trichothecenes using the working standard solution with the concentration of 0.2 and 2 µg/mL. The selected volume of the standard solution was added to the weighed portion of a ground feed sample and the spiked sample was kept for half an hour prior to the addition of the extraction solvent.

To follow the contamination of grains with mycotoxins in primary production in Slovenia, altogether 175 samples of cereals produced in Slovenia were collected at farms in the years 2007, 2008, and 2009. Among them, there were 79 samples of maize, 39 samples of barley, and 34 samples of wheat. In the collected samples, mycotoxins were determined. For the determination of trichothecenes, the analytical procedure mentioned above was used. As positive, samples with the concentration of mycotoxins higher than limit of detection (LOD) were designated.

2.4 Analytical procedure

The procedure based on analytical methods described elsewhere (Binder et al., 2007; Langseth & Rundberget, 1998; Melchert & Pabel, 2004; Radová et al., 1998; Schothorst et al., 2005; Tanaka et al., 2000) was used. 10.0 g of a ground sample spiked with trichothecenes was extracted with 100 mL of the acetonitrile-water mixture (84+16) by shaking for one hour at the ambient temperature using linear shaker. 10.0 mL of the sample extract was slowly pressed through a MycoSep 227 Trich+ column. 2.0 mL of the purified sample extract was pipetted into a deactivated vial for the derivatisation and the solvent was evaporated at around 60°C under vacuum to dryness. To the dry residue in the vial, 100 µL of Sylon BTZ reagent for derivatisation was added and the vial was capped promptly. It was mixed well and the mixture was let react for 15 minutes at ambient temperature. After 15 minutes, 0.5 mL of hexane and 1 mL of PBS solution were added to the mixture and shaken for 1 minute. The layers were let separate and the upper layer was transferred into GC vial. Trichothecenes were determined by GC-MS at the conditions described above. Matrix matched calibration curves were prepared separately for each matrix type by adding a suitable amount of trichothecenes standard solution into aliquots of the purified matrix extract. Into each of five vials for the derivatisation, 2.0 mL of the purified matrix extract and a suitable amount of a trichothecenes standard solution was pipetted (e.g. 0.01 µg, 0.02 µg, 0.1 µg, 0.2 µg, and 0.3 µg which corresponded to the concentrations of 0.050, 0.100, 0.500, 1.0, and 1.5 mg/kg, respectively) and the solvent was evaporated at around 60°C under vacuum to dryness. The derivatisation and the extraction into hexane were carried out in the same way as with a spiked sample described above.

2.5 Validation procedure

For the linearity test as well as determination of LOD and limit of quantification (LOQ), the matrix matched calibration standards corresponding to 0.050 mg/kg, 0.100 mg/kg, 0.500
mg/kg, 1.0 mg/kg, and 1.5 mg/kg were prepared as described above. In the present study, LOD was defined as a concentration giving a signal \[ b_0 + 3 \times s(b_0) \] and LOQ was defined as a concentration giving a signal \[ b_0 + 10 \times s(b_0) \], where \( b_0 \) and \( s(b_0) \) were the intercept of the calibration curve and the standard deviation of the intercept, respectively. For the within-laboratory reproducibility test, ten samples of feed were spiked with the trichothecenes standard solution at the concentration level of 0.3 mg/kg, ten samples at the concentration level of 0.5 mg/kg, and ten samples at the concentration level of 1 mg/kg. They were prepared according to the procedure described above. After the extraction, each sample was prepared in duplicates. Along samples, matrix matched calibration standards were prepared as described above. The results of these experiments were used also for testing the repeatability and for the determination of the recovery and measurement uncertainty. The listed parameters for each trichothecene at the concentration of LOQ were tested by measurements performed with six different feed samples spiked at 0.1 mg/kg.

2.6 Statistical procedure

To demonstrate the repeatability of the procedure, differences between results of duplicate measurements performed within the within-laboratory reproducibility test were calculated. The within-laboratory reproducibility was expressed with the standard deviation \( s_W \), the relative standard deviation \( RSD_W \), and the reproducibility limit \( R_W \) (\( R_W = 2.8 \times s_W \)). \( RSD_W \) values were compared to the values derived from the Horwitz equation, given as the reference value in Decision 2002/657/EC (European Commission, 2002) and to the performance criteria given in Regulation (EC) No 401/2006 (European Commission, 2006a). Prior to the calculation of the listed parameters, results of experiments were examined by the Cochran test and the Grubbs test to eliminate results with too high difference between duplicate measurements and outliers (Miller & Miller, 2000). The expanded measurement uncertainties (European Co-operation for Accreditation (EA), 2003) at the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1 mg/kg were calculated from the within-laboratory reproducibility standard deviation \( s_W \) using coverage factor 2 (\( U = \pm 2 \times s_W \)). Validation parameters, obtained in the validation procedure were compared to parameters given in Recommendation 2006/576/EC (European Commission, 2006b), to parameters given in Decision 2002/657/EC (European Commission, 2002), and to parameters given in Regulation (EC) No 401/2006 (European Commission, 2006a).

3. Results and discussion

A procedure for the simultaneous quantification of DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 in feed including different grain samples using the same analysis procedure for all of them along with GC-MS technique has been successfully validated. Most often, samples contained DON; it was present in 121 samples (69.1% of all samples).

Matrix matched calibration curves for DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 were tested in the range from 0.050 mg/kg to 1.5 mg/kg. The correlation coefficients were higher than 0.99, thus the linearity was considered appropriate. The procedure using pure standards calibration tested previously did not give reasonable results. The problem was unreasonably high recoveries (often higher than 110%, 120% or
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130% given in Regulation (European Commission, 2006a). The use of available internal standard(s) seemed to be very expensive and was not expected to solve the problem for all determined trichothecenes satisfactorily, thus the matrix matched calibration was chosen. Concerning compound feedingsstuffs, experiments showed that the slope of calibration curves prepared in different types of feedingsstuffs were different, but were similar in similar matrices, so the possibility of the calibration using combined matrices (mixture of similar types of feed) was examined. The slope of calibration curves prepared in combined matrices was usually similar to slopes of calibration curves prepared in individual matrices included in the combined matrix, but often outliers (calibration curves with completely different slopes) were detected. Additionally, the concern was raised that in routine analysis it is difficult to judge whether samples are similar or not and whether the combined matrix is justified or not. Therefore, concerning compound feedingsstuffs, the decision to prepare the calibration curve on each individual sample was made (standard addition method rather than matrix match calibration). A chromatogram of derivatised barley sample containing trichothecenes at the contamination level of 0.5 mg/kg is shown in Figure 2.

![Chromatogram of derivate barley sample](image)

**Fig. 2.** Total ion chromatogram of barley sample containing trichothecenes at the contamination level of 0.5 mg/kg.

The determined LOD of each trichothecene (DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2) in feed was around 0.03 mg/kg. For practical purposes, LOD of 0.05 mg/kg, tested with all examined samples was accepted. LOQ of each trichothecene in feed was 0.1 mg/kg. The ability to determine each of trichothecenes at this concentration level was proven by measurements performed with six different feed samples spiked at 0.1 mg/kg. The obtained parameters are given later on. The determined LOQ is satisfactory in regard to Recommendation 2006/576/EC (European Commission, 2006b) where the lowest guidance value given for DON is 0.9 mg/kg. It means that the determination of such a concentration is possible with the procedure in question.

The repeatability, within-laboratory reproducibility, recovery and measurement uncertainty were tested at the concentration levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1 mg/kg. The levels were chosen according to the lowest guidance value given in Recommendation
2006/576/EC (European Commission, 2006b) for DON and according to the concentrations of DON expected in samples. In Table 3, the average differences between results of duplicate measurements with DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2 and T-2 are given.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Average differences (mg/kg)</th>
<th>0.1 mg/kg</th>
<th>0.3 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.0 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.04 (13%)</td>
<td>0.11 (22%)</td>
<td>0.20 (20%)</td>
</tr>
<tr>
<td>3-AcDON</td>
<td></td>
<td>0.02 (20%)</td>
<td>0.03 (10%)</td>
<td>0.10 (20%)</td>
<td>0.18 (18%)</td>
</tr>
<tr>
<td>DAS</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.02 (7%)</td>
<td>0.07 (14%)</td>
<td>0.10 (10%)</td>
</tr>
<tr>
<td>15-AcDON</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.02 (7%)</td>
<td>0.09 (18%)</td>
<td>0.13 (13%)</td>
</tr>
<tr>
<td>Nivalenol</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.03 (10%)</td>
<td>0.10 (20%)</td>
<td>0.18 (18%)</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.03 (10%)</td>
<td>0.06 (12%)</td>
<td>0.10 (10%)</td>
</tr>
<tr>
<td>HT-2</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.02 (7%)</td>
<td>0.08 (16%)</td>
<td>0.14 (14%)</td>
</tr>
<tr>
<td>T-2</td>
<td></td>
<td>0.01 (10%)</td>
<td>0.02 (7%)</td>
<td>0.08 (16%)</td>
<td>0.12 (12%)</td>
</tr>
</tbody>
</table>

Table 3. Repeatability of measurements, expressed with the average differences between results of duplicate measurements.

In Tables 4-7, the within-laboratory reproducibility standard deviation ($s_W$), the reproducibility limit ($R_W$), and the relative standard deviation ($RSD_W$), as well as CV derived from Horwitz equation are given for the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg. The results for $s_W$, $R_W$ and $RSD_W$ for each individual trichothecene were calculated from six experiments done in duplicates at the contamination level of 0.1 mg/kg and from ten experiments done in duplicates at the other three contamination levels except those for DON and nivalenol at the concentration levels of 0.3 mg/kg and 1.0 mg/kg which were calculated from nine experiments done in duplicates since one result at each of the two contamination levels was eliminated by the Cochran test.

The experimental $RSD_W$ values were compared to the CV values derived from Horwitz equation. Majority of experimental $RSD_W$ values were lower than reference values, only a few exceeded it. However, they were much lower than upper limits for $RSD_W$ given in Regulation (EC) No 401/2006 (European Commission, 2006a) which were 40% for DON and 60% for T-2 and HT-2, thus the determined $RSD_W$ are considered acceptable.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of experiments</th>
<th>$s_W$ (mg/kg)</th>
<th>$R_W$ (mg/kg)</th>
<th>$RSD_W$ (%)</th>
<th>Horwitz value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>6</td>
<td>0.01</td>
<td>0.04</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>6</td>
<td>0.02</td>
<td>0.04</td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>DAS</td>
<td>6</td>
<td>0.01</td>
<td>0.04</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>6</td>
<td>0.01</td>
<td>0.04</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>6</td>
<td>0.02</td>
<td>0.05</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>6</td>
<td>0.02</td>
<td>0.05</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>HT-2</td>
<td>6</td>
<td>0.02</td>
<td>0.05</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>T-2</td>
<td>6</td>
<td>0.02</td>
<td>0.05</td>
<td>19</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 4. Within-laboratory reproducibility of measurements at the contamination level of 0.1 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation ($RSD_W$).
A Method Using Gas Chromatography – Mass Spectrometry for the Detection of Mycotoxins from Trichothecene Groups A and B in Grains

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of experiments</th>
<th>$s_W$ (mg/kg)</th>
<th>$R_W$ (mg/kg)</th>
<th>RSDN (%)</th>
<th>Horwitz value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>9</td>
<td>0.04</td>
<td>0.12</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>10</td>
<td>0.04</td>
<td>0.11</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>DAS</td>
<td>10</td>
<td>0.03</td>
<td>0.09</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>10</td>
<td>0.03</td>
<td>0.09</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>9</td>
<td>0.04</td>
<td>0.12</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>10</td>
<td>0.04</td>
<td>0.10</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>HT-2</td>
<td>10</td>
<td>0.04</td>
<td>0.10</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>T-2</td>
<td>10</td>
<td>0.04</td>
<td>0.10</td>
<td>14</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 5. Within-laboratory reproducibility of measurements at the contamination level of 0.3 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation (RSDN).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of experiments</th>
<th>$s_W$ (mg/kg)</th>
<th>$R_W$ (mg/kg)</th>
<th>RSDN (%)</th>
<th>Horwitz value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>10</td>
<td>0.09</td>
<td>0.24</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>10</td>
<td>0.08</td>
<td>0.22</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>DAS</td>
<td>10</td>
<td>0.06</td>
<td>0.17</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>10</td>
<td>0.07</td>
<td>0.21</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>10</td>
<td>0.09</td>
<td>0.25</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>10</td>
<td>0.07</td>
<td>0.19</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>HT-2</td>
<td>10</td>
<td>0.09</td>
<td>0.25</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>T-2</td>
<td>10</td>
<td>0.07</td>
<td>0.20</td>
<td>15</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 6. Within-laboratory reproducibility of measurements at the contamination level of 0.5 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation (RSDN).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of experiments</th>
<th>$s_W$ (mg/kg)</th>
<th>$R_W$ (mg/kg)</th>
<th>RSDN (%)</th>
<th>Horwitz value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>9</td>
<td>0.17</td>
<td>0.47</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>10</td>
<td>0.17</td>
<td>0.49</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>DAS</td>
<td>10</td>
<td>0.11</td>
<td>0.32</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>10</td>
<td>0.12</td>
<td>0.35</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>9</td>
<td>0.13</td>
<td>0.37</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>10</td>
<td>0.12</td>
<td>0.34</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>HT-2</td>
<td>10</td>
<td>0.13</td>
<td>0.37</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>T-2</td>
<td>10</td>
<td>0.12</td>
<td>0.35</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 7. Within-laboratory reproducibility of measurements at the contamination level of 1.0 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation (RSDN).

The mean recoveries determined at the concentration levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg using results obtained in the within-laboratory test are given in Table 8. The number of experiments taken into account is stated above.
The determined recoveries were between 78% and 103% and the mean recovery for individual trichothecenes was between 87% and 97%. This is in accordance to the requirements of Regulation (EC) No 401/2006 (European Commission, 2006b), which demands the recovery of DON from 60 to 110% and from 70 to 120% for different concentration levels and the recovery of T-2 and HT-2 from 60 to 130%.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean recovery of the level (%)</th>
<th>Mean recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mg/kg</td>
<td>0.3 mg/kg</td>
</tr>
<tr>
<td>DON</td>
<td>99</td>
<td>84</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>DAS</td>
<td>99</td>
<td>93</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>101</td>
<td>91</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>89</td>
<td>84</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>99</td>
<td>92</td>
</tr>
<tr>
<td>HT-2</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>T-2</td>
<td>94</td>
<td>86</td>
</tr>
</tbody>
</table>

Table 8. Recoveries of trichothecenes.

The measurement uncertainties determined at the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg are given in Table 9.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Measurement uncertainty (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mg/kg</td>
</tr>
<tr>
<td>DON</td>
<td>0.03</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>0.03</td>
</tr>
<tr>
<td>DAS</td>
<td>0.03</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>0.03</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>0.04</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>0.04</td>
</tr>
<tr>
<td>HT-2</td>
<td>0.04</td>
</tr>
<tr>
<td>T-2</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 9. Measurement uncertainties of results of trichothecenes determination.

From the 175 analysed samples of different cereals, 139 (74.3%) samples contained at least one of the trichothecenes listed above. Most often, samples contained DON; it was present in 121 samples (69.1% of all samples). 15-AcDON and nivalenol were found in 16.0% and 5.1% of investigated samples, respectively. In Table 10, the concentrations of trichothecenes determined in samples of maize, wheat, barley, oats, and triticale are given. Further, 7 samples of fodder pea, one sample of rye and one sample of millet were analysed, but none of them contained any trichothecene. It is obvious, that most often DON was present in wheat, maize, triticale, oats, and barley, which represent the largest part of the meal in the intensive production of poultry and pigs in Slovenia and worldwide (Binder et al., 2007).
A Method Using Gas Chromatography – Mass Spectrometry for the Detection of Mycotoxins from Trichothecene Groups A and B in Grains

Type | Maize | Wheat | Barley | Oats | Triticale |
--- | --- | --- | --- | --- | --- |
DON | 79/68 | 34/30 | 39/13 | 7/4 | 7/6 |
| 2 | 0.1-14.4 | 0.1-2.86 | 0.05-0.72 | 0.15-0.52 | 0.13-1.56 |
3-AcDON | 47/2 | 29/0 | 30/0 | 3/0 | 4/0 |
| 2 | 0.08-0.14 | - | - | - | - |
DAS | 47/0 | 29/0 | 30/0 | 3/0 | 4/0 |
| 2 | - | - | - | - | - |
15-AcDON | 47/28 | 29/0 | 30/0 | 3/0 | 4/0 |
| 2 | 0.05-0.83 | - | - | - | - |
Nivalenol | 47/5 | 29/26 | 30/2 | 3/0 | 4/0 |
| 2 | 0.05-1.14 | 0.06-0.07 | 0.34-0.51 | - | - |
Neosolaniol | 47/1 | 29/0 | 30/0 | 3/0 | 4/0 |
| 2 | 0.08 | - | - | - | - |
HT-2 | 79/3 | 34/0 | 39/1 | 7/0 | 7/0 |
| 2 | 0.06-2.3 | - | 0.05 | - | - |
T-2 | 79/1 | 34/0 | 39/0 | 7/0 | 7/0 |
| 2 | 0.290 | - | - | - | - |

Table 10. The presence of trichothecenes regarding the cereal type in the period 2007-2009 in Slovenia (1- number of investigated samples/number of positive samples, 2- concentration ranges in mg/kg).

However, besides recommendations on the presence of DON, zearalenone, ochratoxin A, T-2 and HT-2 toxins, and fumonisins in feed (European Commission, 2006b), the legislative limits have been set only for aflatoxin B1 in feed for different species and categories of livestock. For DON the recommended highest concentration per kg cereals and cereal products is 8 mg. Recommended maximum levels for T-2 and HT-2 toxins are not set yet in Europe. T-2 toxin is the most toxic of the naturally occurring trichothecenes (LD50 4.0 mg/kg body weight for rats and swine, oral; 7 mg/kg body weight for mice, oral). Comparison of the LD50 of T-2 toxin with those of other toxins, e.g. DON (46 mg/kg body weight for mice, oral), reveals its acute toxicity (Roth et al., 1990, as cited in Krška et al., 2001). But, as it was mentioned, despite the high toxicity of the type-A trichothecenes, only few countries have established legal regulation of recommendations for T-2 toxin (FAO, 1997, as cited in Krška et al., 2001). In our study, T-2 toxin was present in only one sample of maize in the concentration of 0.290 mg/kg.

From the available data (Schuhmacher-Wolz et al., 2010), it can be concluded that raw oats and barley can be highly contaminated with T-2 and HT-2 including high incidence and concentrations. Maize can be contaminated occasionally at moderate concentrations. T-2 and HT-2 contamination of wheat seems to be very infrequent and at low concentrations. Feed products that are of major concern include by-products from oat processing (pellets). Food products generally show low incidence and concentration of T-2 and HT-2, however, oat products may contain some T-2 and HT-2. Field factors that influence T-2 and HT-2 include region-year (climate), variety, sowing date, pre-crop, and organic production. Fungicides seem to have no or only a weak effect. Processing cereals will substantially reduce T-2 and HT-2 contamination in most food products due to redistribution over the various fractions. As a consequence, the levels in the by-products, often used for animal feeding are increased (Schuhmacher-Wolz et al., 2010).
In general, the levels of mycotoxins in our research did not reach the maximum levels set by Slovenian or European legislation. The results are similar to those reported from around the world. DON is the most widely spread mycotoxin, which is confirmed also by the investigation including samples from eleven European countries examined on trichothecenes contents. 57% samples were DON positive and 20% contained T-2 toxin and HT-2 toxin (SCOOP, 2003). Most contaminated were maize samples, with average concentrations of DON 300-3700 \( \mu \text{g/kg} \). Similar results were gained by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2001). Maize is known to be a good substrate for mould infection and production of mycotoxins harmful to both humans and animals (Kumar et al., 2008). Under natural conditions, two mono-acetylated derivatives 3- and 15-AcDON accompany DON, albeit they are produced at lower concentrations (EFSA Journal, 2004). Also in our study, the second most frequent toxin was 15-AcDON. It was found in 59.6% of maize samples in concentration 0.05-0.83 mg/kg. Acetylated toxins are rapidly deacetylated in vivo. DON is therefore discussed together with acetylated forms (Eriksen et al., 2004).

A world-wide survey of DON, nivalenol, and zearalenone, on 500 agricultural samples from 19 countries and districts, reported that approximately 40-50% of the samples were positive for these mycotoxins. Average concentrations of 0.292 mg/kg for DON, 0.267 mg/kg for nivalenol, and 0.045 mg/kg for zearalenone were reported. DON was often found in maize (69%), followed by mixed feed (65%) and wheat (60%) (Tanaka et al., 1988, as cited in Krska et al., 2001).

In Croatia, the neighbouring country of Slovenia, the seven years long investigation of grains (1998-2004) revealed 9.5-66.7% of T-2 toxin positive samples (average 150-410 \( \mu \text{g/kg} \) and 9.1-50% positive for DAS (average 300-130 \( \mu \text{g/kg} \) (Sokolović & Šimpraga, 2006). In 2008 a total of 139 samples of various grains were investigated for the presence and concentration of DON (Jajić et al., 2008). The average incidence rate of DON in maize was 44.7%, in wheat 37.5 %, and in barley 25%. In the positive samples, DON was found in concentration range between 0.04 and 2.46 \( \mu \text{g/kg} \). Recently, Ibáñez-Vea et al. (2011) investigated the type-A and type-B trichothecenes in 44 barley samples. The higher occurrence was found for DON (89% of the samples), although at concentrations below the maximum permitted level. Two or more thichothecenes were present in 41% of the samples.

However, also in our study, quite a number of investigated samples contained more than one trichothecene and other mycotoxins; some of them even up to seven (Jakovac-Strajn et al., 2010). It is stated elsewhere (Tanaka et al., 2000), that subclinical and chronic concentrations as well as synergistic effects of mycotoxins have even higher impact on the health of animals and human than acute concentrations. The response of affected animals to exposure to more than one mycotoxin can be the same as the response predicted from the summation of the response to each mycotoxin individually (additive), less than the predicted response from each toxin individually (antagonistic), or more than the predicted summation of the responses from each individual mycotoxin (synergistic) (Wyatt, 2005). In addition, very little is known about the effects of long-term, low-level exposure, especially with regard to the co-contamination with multiple mycotoxins (Kumar et al., 2008). But, interactions between different mycotoxins are still under investigation (Grenier & Oswald, 2011). Combination of \textit{Fusarium} mycotoxins, in general, result in additive effects, but synergistic and/or potentiating interactions have been observed and are of greater concern.
in livestock health and productivity. Synergistic effects between DON and fusaric acid, DON and fumonisin B₁, and DAS and aflatoxins (D’Mello et al., 1999, as cited in Kolosova & Stroka, 2011) have been reported. Additive and synergistic effects between known and unidentified mycotoxins may account for enhanced adverse effects observed on feeding mycotoxin-contaminated diet. Furthermore, the toxic effect of any single mycotoxin may be amplified due to synergistic interactions with other substances (Sergent et al., 2008, as cited in Kolosova & Stroka, 2011). The issue of masked mycotoxins should also be taken into consideration. They attracted attention in the eighties already, because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. The unexpected high toxicity was thought to be attributed to undetected, conjugated forms of mycotoxins that hydrolyse to the precursor toxins in the digestive tract of animals. As reported, combining HPLC with tandem MS results in a powerful tool for characterisation and identification of masked mycotoxins (Berthiller et al., 2005; Binder, 2007).

Except in the case of DON, the concentrations of mycotoxins in our study were mainly not high enough to cause clinically detectable health or production problems. In a range of species, reduced feed intake seems to be one of the most sensitive indicators of dietary exposure to trichothecenes. At higher doses or with repeated exposure, this may be followed by actual feed refusal, emesis in capable species, and possibly the development of perijoral, oral, pharyngeal, oesophageal or gastroenteric lesions or combination of these. Perijoral and oral lesions have been most thoroughly documented in birds, swine, and horses (Trenholm et al., 1989). Of special interest are trichothecenes immunosuppressive effects and subsequent increased susceptibility to infections. It is clear that the trichothecene mycotoxins suppress the immune network, but the specific function of various cell types affected by trichothecene mycotoxins have yet to be definitively ascertained. As would be expected, newborn animals without fully developed detoxifying mechanisms, and with short-lived energy stores, are even more sensitive to trichothecene toxicoses (Taylor et al., 1989).

In the discussion about results, the problem of representative sampling must be also taken into account. The problem of sampling is less pronounced with *Fusarium* toxins which are regarded as less heterogeneously distributed than the aflatoxins (Larsen et al., as cited in Krska & Molinelli, 2007). Anyway, Hallier et al. (2011) showed recently that within the procedure of DON analysis, the most critical step is grain sampling. Nevertheless, little scientific evidence of mycotoxin distribution and variability have limited the development of alternative sampling plans to be used depending on the mycotoxins and the type and size of commodities, which would reduce the total variability and enable the estimation of uncertainty in the evaluation of mycotoxin concentrations. Nowadays, with relatively few sampling plans available for different commodities, the sampling step contributes to the largest variability in mycotoxin determination (Krska & Molinelli, 2007).

To conclude, DON occurrence is almost exclusively associated with cereals, and the levels of occurrence are in the order of hundreds of μg/kg upwards. DON occurs as a field (pre-harvest) rather than a storage contaminant, and almost always co-occurs with other *Fusarium* toxins. Preventive measures are difficult to implement, and even the effect of fungicide treatment on DON levels is controversial (Edwards et al., 2001). As seasonal variations significantly influence the extent of *Fusarium* infections, levels of DON tend to
vary from year-to-year making it difficult to generalise the typical levels of occurrence (EFSA Journal, 2004).

Prevention of fungal infections during plant growth, harvest, storage and distribution would seem the most rational and efficient way to avoid mycotoxins in agricultural commodities (Huwing et al., 2001; Ramos and Hernandez, 1997, as cited in Kolosova & Stroka, 2011). Recently, European regulation (EC) No 1831/2003 (European Commission, 2003) on additives for use in animal nutrition has been amended. A new functional group defined as “substances for reduction of the contamination of feed by mycotoxins” has been added in the category of technological feed additives (European Commission, 2009). It contains substances most often termed as mycotoxin binders. It should be pointed out that the use of such products does not mean that animal feed exceeding the established maximum limits may be used (Kolosova & Stroka, 2011).

For both, A- and B- trichothecenes there is still a lack of simple and reliable screening methods enabling the rapid detection of these mycotoxins at low cost. Besides the increasing demand for rapid screening methods for both A- and B-trichothecens, the use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) enabling both quantification and identification of several trichothecenes simultaneously can be considered a major future trend in the analysis of these Fusarium mycotoxins in cereals (Krska et al., 2001).

4. Conclusions

Mycotoxins are secondary metabolites of fungi. It is not possible to predict their presence or to prevent their occurrence during preharvest, storage, and processing operations by current agronomic practices. Therefore, their presence in food and feed represents a constant health risk for animals and humans. To protect the consumer from the harmful effects of these compounds, regulations have been established in many countries, resulting in the development of monitoring methods. The performance characteristics for all grains, obtained from validation in this study, confirmed that GC-MS, a very common technique for the quantification of type-A and B trichothecenes in grains, food, and feedstuff, is well suited for their determination. Among DON, 3-AcDON, 15-AcDON, nivalenol, neosolaniol, T-2, HT-2 and DAS, two mycotoxins DON and 15-AcDON were detected most often. In general, the levels of mycotoxins in the tested samples did not reach the maximum levels set by legislation. Anyway, the long term of chronic exposure to low concentrations of mycotoxins, the co-occurrence of several mycotoxins in the same sample, masked mycotoxins, and the problems connected to sampling must be taken into account when determining permitted levels or carrying out risk assessment.

Experience gained during intercomparison studies clearly shows the need for further improvements in the determination of trichothecenes – more accurate and comparable results are required. Regular maintenance of the GC instrument seems to be of great importance to the achievement of long term reproducibility.

Besides the increasing demand for rapid screening methods for both A- and B-trichothecenes, the use of LC-MS/MS enabling both quantification and identification of several trichothecenes simultaneously can be considered a major future trend in the analysis of these Fusarium mycotoxins in cereals.
5. References


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The aim of this book is to describe the fundamental aspects and details of certain gas chromatography applications in Plant Science, Wine technology, Toxicology and the other specific disciplines that are currently being researched. The very best gas chromatography experts have been chosen as authors in each area. The individual chapter has been written to be self-contained so that readers may peruse particular topics but can pursue the other chapters in the each section to gain more insight about different gas chromatography applications in the same research field. This book will surely be useful to gas chromatography users who are desirous of perfecting themselves in one of the important branch of analytical chemistry.

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