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**Study on Trichothecenes, Zearalenone
and Ochratoxin A in Finnish Cereals:
OCCURRENCE AND ANALYTICAL TECHNIQUES**

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Helsingin yliopisto
Soveltavan kemian ja mikrobiologian laitos
University of Helsinki
Department of Applied Chemistry and Microbiology



Eläinlääkintä- ja elintarviketutkimuslaitos
Kemian tutkimusyksikkö
National Veterinary and Food Research Institute
Department of Chemistry



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AND OCHRATOXIN A IN FINNISH CEREALS:
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Mari Eskola

Academic Dissertation

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.....

ON THE TURNING AWAY
FROM THE PALE AND DOWNTRODDEN
AND THE WORDS THEY SAY
WHICH WE WON'T UNDERSTAND
"DON'T ACCEPT THAT WHAT'S HAPPENING
IS JUST A CASE OF OTHERS' SUFFERING
OR YOU'LL FIND THAT YOU'RE JOINING IN
THE TURNING AWAY"

IT'S A SIN THAT SOMEHOW
LIGHT IS CHANGING TO SHADOW
AND CASTING IT'S SHROUD
OVER ALL WE HAVE KNOWN
UNAWARE HOW THE RANKS HAVE GROWN
DRIVEN ON BY A HEART OF STONE
WE COULD FIND THAT WE'RE ALL ALONE
IN THE DREAM OF THE PROUD

ON THE WINGS OF THE NIGHT
AS THE DAYTIME IS STIRRING
WHERE THE SPEECHLESS UNITE
IN A SILENT ACCORD
USING WORDS YOU WILL FIND ARE STRANGE
AND MESMERISED AS THEY LIGHT THE FLAME
FEEL THE NEW WIND OF CHANGE
ON THE WINGS OF THE NIGHT

NO MORE TURNING AWAY
FROM THE WEAK AND THE WEARY
NO MORE TURNING AWAY
FROM THE COLDNESS INSIDE
JUST A WORLD THAT WE ALL MUST SHARE
IT'S NOT ENOUGH JUST TO STAND AND STARE
IS IT ONLY A DREAM THAT THERE'LL BE.
NO MORE TURNING AWAY ?

ON THE TURNING AWAY
-PINK FLOYD 1987-



.....

Eskola, M. 2002.
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ABSTRACT

Aims of this study were to improve and develop analytical methods for trichothecenes, ochratoxin A (OA) and zearalenone (ZEN) analysis in cereals, and to survey the presence and amounts of trichothecenes, ZEN and OA in Finnish cereals after a growing season with severe mould infection of cereals. Cereal samples, mainly rye and wheat, harvested in 1998 were analysed for the mycotoxins of interest and although heavy *Fusarium* mould infection was detected on the kernels, especially in the case of rye, the amounts of trichothecenes and ZEN were very low. Deoxynivalenol (DON) was the trichothecene most often detected, but the most toxic T-2 and HT-2 toxins were also occasionally observed. ZEN was found only in a few samples and in low amounts and OA was not detected in any of the samples analysed. *Fusarium avenaceum* was the mould strain most frequently isolated.

The analytical methods used (gas chromatography with mass spectrometric detection or electron capture detection; GC-MS, GC-ECD, and high performance liquid chromatography with fluorescence detection; HPLC) were reliable and had relatively low detection limits. However, during the course of this investigation and an intercomparison study of trichothecenes analysis (EU-project, SMT-4-CT96-2047), relatively high variation in the trichothecenes results was observed. The main sources of variation in the trichothecenes analysis were studied and were identified as between-day changes in performance of GC-MS, matrix effects and incomplete derivatisation reaction. The GC-ECD method had less day-to-day variation, but the matrix effect and the problems in derivatisation were similar. By using cereal matrix-assisted calibration, internal standard, Constant Standard with GC-MS and GC standard with GC-ECD to control the functioning of the instruments and effective derivatisation reagent, some of the variation could be eliminated, which further assisted in the evaluation of the trichothecenes results.

The use of immunoaffinity column purification prior to the HPLC analysis of OA and ZEN in cereals increased the reliability and specificity over the conventional methods and automation of the clean-up step by applying ASPEC (automated solid phase extraction) further facilitated the methods.

This study enhanced the status of information on the occurrence and the concentration levels of trichothecenes, ZEN and OA in Finnish cereals in relation to *Fusarium* moulds. Due to the low concentration levels the adverse health effects caused by the mycotoxins of interest were almost negligible in 1998. Valuable knowledge was generated concerning in the method performance of trichothecenes analysis. In comparison to the analytical techniques previously applied, this investigation resulted in significant improvement of the methods, updating them to the international level currently prevailing in leading mycotoxin laboratories.



PREFACE

This study was conducted at the Department of Chemistry, National Veterinary and Food Research Institute (EELA) and at the Department of Applied Chemistry and Microbiology, University of Helsinki during the years 1998-2001. The academic dissertation was performed under the Applied Bioscience – Bioengineering, Food & Nutrition, Environment (ABS) program of the Finnish Graduate School and was financially supported by ABS Graduate School and EELA.

I thank Docent, Dr. Aldo Rizzo for introducing me to the fascinating world of mycotoxins and for his enthusiasm towards this work, including many interesting discussions on mycotoxins and useful comments on my manuscripts. I thank Aldo for supervising me on behalf of EELA, and Professor Vieno Piironen for supervision on behalf of the University of Helsinki. I thank Aldo and Professor Timo Hirvi for their frequent encouragement. I also thank Timo and the Department of Chemistry at EELA for providing the facilities and instrumentation for carrying out the work.

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Helsinki, December 2001

Mari Eskola



LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications,
referred to in the text by Roman numerals **I-V**.

- I** Eskola, M., Parikka, P. and Rizzo, A. 2001.
Trichothecenes, ochratoxin A and zearalenone contamination and *Fusarium* infection in Finnish cereal samples in 1998. *Food Addit. Contam.* 18: 707-718.
- II** Eskola, M., Rizzo, A. and Soupas, L. 2001.
Occurrence and amounts of some *Fusarium* toxins in Finnish cereal samples in 1998. *Acta Agric. Scand., Sect. B, Soil and Plants Sci.* 50: 183-186. (In the published paper incorrect year 2000)
- III** Eskola, M., Boonzaaijer, G., van Osenbruggen, W.A., Rizzo, A. and Tijmensen, G. 2001.
A study of the suitability of gas chromatography-electron capture detection for the analysis of deoxynivalenol in cereals. *Mycotoxin Research* 16: 73-90. (In the published paper incorrect year 2000)
- IV** Eskola, M., Kokkonen, M. and Rizzo, A. 2002.
Application of manual and automated systems for purification of ochratoxin A and zearalenone in cereals with immunoaffinity columns. *J. Agric. Food Chem.* 50: 41-47.
- V** Eskola, M. and Rizzo, A. 2002.
Sources of variation in the analysis of trichothecenes in cereals by gas chromatography-mass spectrometry. *Mycotoxin Research*. In press.

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Contribution of the author to papers I to V:

The author participated in planning the studies, performed most part of the mycotoxin analysis, was responsible for analytical data and was the main author of the papers.



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LIST OF ABBREVIATIONS

3-AcDON	3-acetyldeoxynivalenol
15-AcDON	15-acetyldeoxynivalenol
ACN	acetonitrile
ASPEC	automated solid phase extraction
BSA	bis(trimethylsilyl)acetamide
CAST	Council for Agricultural Science and Technology
CRM	certified reference material
CS	Constant Standard
DAS	diacetoxyscirpenol
DDE	di(chlorophenyl)dichloroethylene
DMAP	dimethylaminopyridine
DON	deoxynivalenol
EC	European Community
ECD	electron capture detector
EELA	National Veterinary and Food Research Institute
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAPAS	food analysis performance assessment
FID	flame ionization detector
Fus-X	fusarenon-X
GC	gas chromatography
GC-ECD	gas chromatography with electron capture detection
GC-MS	gas chromatography with mass selective detection
HFB	heptafluorobutyryl
HFBA	heptafluorobutyryl anhydride
HFBI	heptafluorobutyryl imidazole
HPLC	high performance liquid chromatography
HT-2	HT-2 toxin
IA	immunoaffinity
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantification



LIST OF ABBREVIATIONS

MeOH	methanol
IMS	mass spectrometry
MS/MS	tandem mass spectrometry
n	number of samples
NEO	neosolaniol
Ni	nickel
NICI	negative ion chemical ionisation
NIV	nivalenol
OA	ochratoxin A
PBS	phosphate buffered saline
PCI	positive chemical ionisation
PFP	pentafluoropropionyl
PFPA	pentafluoropropionyl anhydride
PFPI	pentafluoropropionyl imidazole
RIA	radioimmunoassay
RSD	relative standard deviation
SIM	selected ion monitoring
SMT	Standard, Measurement and Testing project
SPE	solid phase extraction
T-2	T-2 toxin
TFA	trifluoroacetyl
TFAA	trifluoroacetyl anhydride
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TMSI	trimethylsilyl imidazole
TNO	Netherlands Organization for Applied Scientific Research
TRI	trichothecolone
UNEP	United Nations Environment Programme
UV	ultra violet
WHO	World Health Organization
ZEN	zearalenone



1

INTRODUCTION

.....

Mycotoxins are toxic secondary metabolites produced by moulds, and they are harmful to both humans and animals. One definition is that "mycotoxins are natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route" (Bennet, 1987). Some moulds are able to produce more than one mycotoxin and some mycotoxins are produced by more than one mould species, and thus several mycotoxins are often simultaneously found in a single commodity.

Mycotoxins with the greatest public health and agro-economic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins and ergot alkaloids, and among these aflatoxin is the most intensively studied (Hussein and Brasel, 2001). Recently, however, several other toxins have attracted increasing attention (Galvano et al., 2001). A total of 300 mycotoxins have been isolated and characterised (Betina, 1984; Fink-Gremmels, 1999) and it is probable that new mycotoxins will be found in future.

The history of mycotoxins is related to several outbreaks of the associated diseases (Malloy and Marr, 1997). One of the first known diseases in humans caused by mycotoxins was ergotism in the Middle Ages (Varga and Téren, 1999) and e.g. in 944, as many as 40 000 inhabitants died in France (Fink-Gremmels and Georgiou, 1996). The outbreak of this mycotoxicosis was due to consumption of rye contaminated with ergot mycotoxins. The last major mycotoxicosis caused by ergot was in France in 1935 and since then several sporadic cases of mycotoxicosis in humans and animals have been reported (Beardall and Miller, 1994a; Fink-Gremmels and Georgiou, 1996; Malloy and Marr, 1997). Probably the most recent outbreak affecting humans occurred in China in 1984-85 (Coker, 2000). Hundreds of thousands of turkeys and ducklings died due to feed contaminated with aflatoxin in Great Britain in 1960, launching rapid expansion in mycotoxin research (Malloy and Marr, 1997; D'Mello and Macdonald, 1997). Nowadays chronic effects caused by mycotoxins are a more important public health issue than acute toxicity (Fink-Gremmels and Georgiou, 1996).

Mycotoxins cause economical losses in widespread areas of living: human and animal lives, increased health care and veterinary care costs, reduced cereal and livestock production, extra handling and processing requirements, reduced nutritional value of food and feed, disposal of contaminated food and feed, and investment in research and applications to reduce the mycotoxin problem (Shane, 1994; Charmley et al., 1995; Boutrif and Canet, 1998; Hussein and Brasel, 2001).

Cereals are the most important source of human food. The annual world crop of cereals exceeds 2000 million tonnes meaning over 160 kg per inhabitant and the production of cereals is still growing (Chelkowski, 1991; FAO, 1993a, 2001). It is estimated that 10-30% of the harvested grains is lost due to mould infection (Chelkowski, 1991) and the Food and Agriculture Organization of the United Nations (FAO) estimates that about 25% of the world's food crops are affected by mycotoxins (Mannon and Johnson, 1985; CAST, 1989). Usually, and especially in developing countries, the best quality cereals are exported while the crop with poorer quality is consumed in the homeland (Dawson, 1991). Hence the citizens in developing countries, mainly living in rural areas, are especially sensitive to adverse health effects of mycotoxins due to the malnutrition and low standard of living (Shane, 1994).

The expanding trade of cereals has created a need for the authorised directives for sampling methods, analytical methods and tolerance limits of mycotoxins. These directives are especially needed in order to avoid trade barriers (Dawson, 1991; Boutrif, 1995; Vasanthi and Bhat, 1998), but most of them are still not achieved and more knowledge on mycotoxins in cereals is certainly needed.

A great number of analytical techniques have been used to analyse mycotoxins in cereals (Chu, 1991, 1995; Scott, 1991, 1995; Betina, 1993a; Gilbert, 1993, 1996a; Sydenham and Shephard, 1996; Wilson et al., 1998). However, recently several authors have expressed the need to improve the analytical quality of these methods in order to obtain more reliable results (Larsson and Möller, 1996; Wood et al., 1996; Schuhmacher et al., 1997; Pettersson, 1998; Pettersson and Langseth, 2000; Josephs et al., 2001; Krska et al., 2001). It is a well known problem that variation in the results of mycotoxins between laboratories and even within laboratory is high.

In this thesis trichothecenes (deoxynivalenol; DON, nivalenol; NIV, 3-acetyldeoxynivalenol; 3-AcDON, fusarenon-X; Fus-X, diacetoxyscirpenol; DAS, HT-2 toxin; HT-2 and T-2 toxin; T-2), ochratoxin A (OA) and zearalenone (ZEN) were studied. The literature review includes a summary on the production, occurrence and toxicity of trichothecenes, OA and ZEN in cereals. In addition, sampling and analytical methods mainly used for the analysis of these toxins are reviewed. Due to the need of harmonisation of the analytical methods, existing among the laboratories involved in mycotoxin research, the analytical techniques for mycotoxins were developed and improved. The GC-MS and GC-ECD methods were investigated in order to decrease the variation associated with trichothecenes analysis and to use them in routine analysis. In order to increase the reliability of the OA and ZEN analysis in cereals and to simplify these methods, the use of immunoaffinity column clean-up and automation of the clean-up step prior to the HPLC analysis were investigated. Moreover, due to the lack of recent information on the presence and amounts of mycotoxins in Finnish cereals, and due to the heavy *Fusarium* mould contamination on kernels in 1998, the survey study on mycotoxins of interest in cereals was performed. In principle, the experimental part contains two sections: a survey study section, and a method improvement and development section. The first section reports general improvements of the analytical methods prior to the survey study, and the occurrence and amounts of trichothecenes, ZEN and OA in Finnish cereals. The next section describes the investigation of the sources of variation in trichothecenes analytical methods and development of the analytical methods for ZEN and OA. This study is based on the five original papers that are presented at the end of this thesis.



2 LITERATURE REVIEW

2.1 Chemistry of trichothecenes, OA and ZEN

Mycotoxins are relatively stable compounds, mostly with low molecular weight. Several of them are generally lipophilic and accumulate in the fat fractions of plants and animals (Hussein and Brasel, 2001). Trichothecenes are tetracyclic, with a sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system and they are arbitrarily divided into four groups A, B, C and D according to their chemical structure (Ueno, 1977). In type A trichothecenes the carbonyl group in position C-8 is missing, whereas in type B the carbonyl group appears in position C-8 (Figure 1, Table 1). Type C trichothecenes have a second epoxide group and type D trichothecenes are macrocyclic compounds. The chemical name of OA is 7-carboxy-5-chloro-8-hydroxy- 3,4-dihydro- 3R-methylisocoumarin-7-L- β -phenylalanine (Cole and Cox, 1981) and its chemical structure is given in Figure 2. Several OA-related metabolites are also detected especially in fungal cultures (Kuiper-Goodman and Scott, 1989; Moss, 1996; Höhler, 1998). The chemical name of ZEN is [6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic acid lactone] (Cole and Cox, 1981) (Figure 3). A number of metabolites related to ZEN have also been isolated from various fungi (Kuiper-Goodman et al., 1987; Betina, 1989).

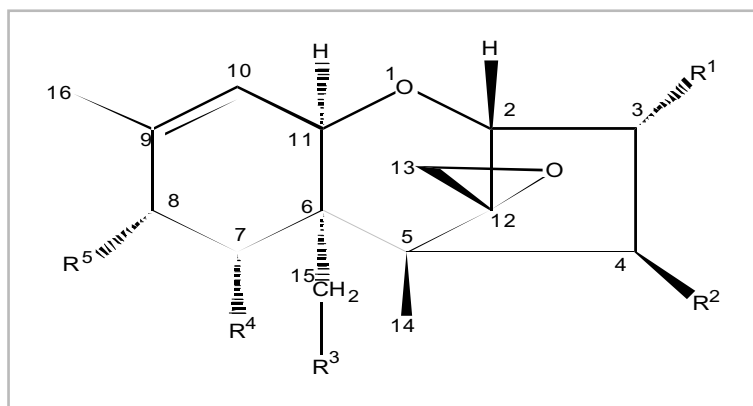


Figure 1. Basic structure of 12,13-epoxytrichothecenes. See also Table 1.

Table 1. Structures of the trichothecenes investigated.

Trichothecene	MW ^a	R ¹	R ²	R ³	R ⁴	R ⁵
Type A						
Diacetoxyscirpenol (DAS)	366	OH	OAc	OAc	H	H
HT-2 toxin (HT-2)	424	OH	OH	OAc	H	OCOCH ₂ CH(CH ₃) ₂
T-2 toxin (T-2)	466	OH	OAc	OAc	H	OCOCH ₂ CH(CH ₃) ₂
Neosolaniol (NEO)	382	OH	OAc	OAc	H	OH
Type B						
Deoxynivalenol (DON)	296	OH	H	OH	OH	O
3-acetyldeoxynivalenol (3-AcDON)	338	OAc	H	OH	OH	O
Nivalenol (NIV)	312	OH	OH	OH	OH	O
Fusarenon-X (Fus-X)	354	OH	OAc	OH	OH	O
Trichothecolone (TRI)	264	H	OH	H	H	O

^a Molecular weight (g/mol), R¹-R⁵ are expressed in Figure 1.

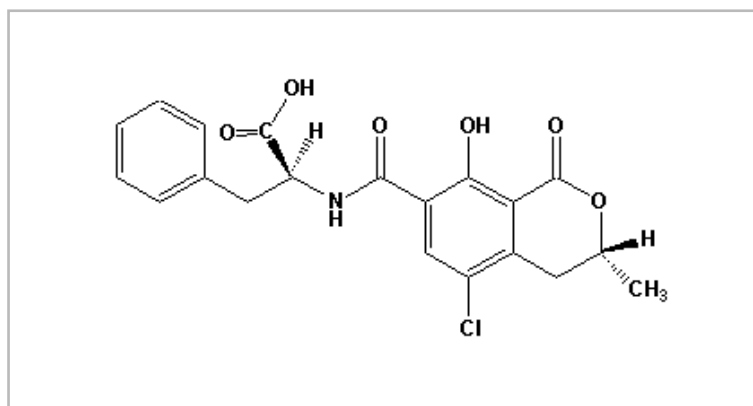


Figure 2. Structure of ochratoxin A (OA).

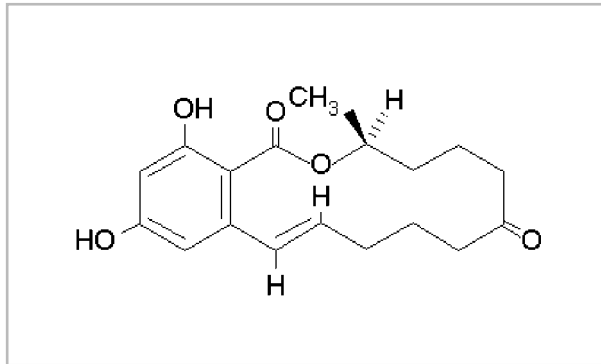


Figure 3. Structure of zearalenone (ZEN).

2.2 Production of mycotoxins in cereals

Contamination of foods and feeds with mycotoxins is very difficult to control. Cereals, including wheat, barley, oats, rye, maize and rice, are susceptible to contamination by mycotoxins since many of the toxigenic species of fungi are plant pathogens. Mycotoxin production due to the growth of fungi may occur on crops in the field during harvesting and drying and continue in storage, or it may begin in storage (Abramson, 1991; Wilson and Abramson, 1992). Various environmental factors affect the growth of fungi and further the production of mycotoxins, but optimal conditions for mould growth are not necessarily optimum for mycotoxin production (Hussein and Brasel, 2001).

2.2.1 Factors affecting mould growth and mycotoxin production

Moisture content and temperature are the most critical factors affecting mould growth, but several other factors are also important, such as spore inoculation, mechanical injury, insect damage, storm and rainfall damage to crops, plant stress, time, mineral nutrition of the plant, chemical treatment, rapidity of drying, rewetting from condensation in storage, leakage in storage and hot spots (Hesseltine, 1976; Lacey, 1986; Wilson and Abramson, 1992; Langseth et al., 1993a; Scudamore et al., 1999; Birzele et al., 2000). The hot spot is a small area with only a few kernels which are moist and may start the mould growth in a lot (Hesseltine, 1976). However, interactions of several factors operating simultaneously is usually more important than any single factor in mycotoxin production (Moss, 1991). It is essential to realise that mould growth on the grains does not necessarily mean that they are contaminated with mycotoxins, and vice versa (Fink-Gremmels, 1999). Mould growth may not be visible on the kernels due to drying or the use of fungicides, although even high amounts of mycotoxins may be found. Social conditions and behaviour, such as methods of preservation of foods, traditional feeding and environmental pollution may play a significant role in the growth of moulds and mycotoxin production (Maaroufi et al., 1996), and factors such as stubble and weeds may also serve as a source of fungi infection (Trenholm et al., 1989). Minimising the presence of mycotoxins in foods and feeds has been summarised by Trenholm et al. (1989), Harris (1997), Placinta et al. (1999) and Hussein and Brasel (2001).

2.2.2 Important moulds producing trichothecenes, OA and ZEN

The fungi which are known to produce mycotoxins and which are involved with the human and animal food production chain belong mainly to three genera: *Aspergillus*, *Fusarium* and *Penicillium*. *Fusarium* species produce mycotoxins before or soon after harvesting, whereas *Penicillium* and *Aspergillus* species are more often detected in food and feed during drying and storage (Sweeney and Dobson, 1998). A typical generalisation is that *Penicillium* is associated with temperate regions of the world, whereas *Aspergillus* is often detected in the tropics (Moss, 1991; Smith et al., 1994; Sweeney and Dobson, 1998). *Fusarium* is common in the area of temperate climates and is thus probably the most important class of moulds infecting cereals in northern regions (Ueno, 1980; Chelkowski, 1989a; Moss, 1991).

The mycotoxins of greatest significance worldwide are aflatoxins produced by *Aspergillus* species, but another very important mycotoxin is OA (Sweeney and Dobson, 1998). OA is mainly produced by *A. ochraceus* and related species, but it is also widely produced by *Penicillium* species, particularly *P. verrucosum* (Sweeney and Dobson, 1998). Both *Penicillium* and *Aspergillus* are capable of growing in relatively dry conditions (Hocking and Pitt, 1979; Moss, 1991) and therefore they have often been called storage fungi (Höhler, 1998).

A variety of different *Fusarium* species produce trichothecenes, but other fungal genera are also known to produce these toxins (Smith et al., 1994). However, *Fusarium* species are the most important, because they are distributed worldwide, they are important mycotoxin producers infecting cereals, and they produce the greatest amounts of trichothecenes (Ueno, 1980; Chelkowski, 1989; Smith et al., 1994). Some common *Fusarium* species are *F. acuminatum*, *F. avenaceum*, *F. chlamydosporium*, *F. crookwellense*, *F. culmorum*, *F. equesti*, *F. graminearum*, *F. moniliforme*, *F. nivale*, *F. proliferatum*, *F. sambunium*, *F. scirpi*, *F. semitectum*, *F. solani*, *F. sporotrichioides*, *F. oxysporium* and *F. tricinctum* (Smith et al., 1994), and of these the species most often found in association with cereals are *F. graminearum* and *F. culmorum* (Abramson, 1998). Trichothecenes are a group of mycotoxins and are often produced in mixtures (Sweeney and Dobson, 1998). A total of 172 trichothecenes, of which 67 are macrocyclic, have been isolated from natural sources, and the number is increasing (Grove, 1993; Langseth and Rundberget, 1998). However, only a few of them have been found to contaminate food and feed, including DON, NIV, DAS, 3-AcDON, 15-AcDON, Fus-X, HT-2 and T-2, and of these the most commonly found is DON (WHO, 1990).

ZEN is also produced by *Fusarium* genera. The most important producers are *F. graminearum* and *F. semitectum*, but other species can also produce this toxin (Smith et al., 1994). Several other mycotoxins produced by *Fusarium* genera have also been reported (Marasas et al., 1984, 1986; Blais et al., 1992; Savard and Blackwell, 1994; Bottalico et al., 1995; Doko and Visconti, 1993; Logrieco et al., 1996; Munkvold et al., 1998).

2.3 Food and feed as sources of trichothecenes, OA and ZEN

The main sources of trichothecenes, ZEN and OA worldwide are cereals and cereal products (Miller and Trenholm, 1994; Sinha and Bhatnagar, 1998), but they have also been found in other plant species (Kuiper-Goodman, 1987). OA has been detected in many food items such as coffee, wine, beer and spices (Studer-Rohr et al., 1995; Zimmerli and Dick, 1996; Stegen et al., 1997; Jørgensen, 1998; Trucksess et al., 1999; Bresch et al., 2000; Leoni et al., 2000; Pietri et al., 2001; Thirumala-Devi et al., 2001). OA has also been detected in food products of animal origin, e.g. in pig's kidney and cow's milk (Büchmann and Hald, 1985; Jonker et al., 1999; Breitholtz-Emanuelsson et al., 1993; Jørgensen, 1998; Skaug, 1999) and has also been found in various foodstuffs in Finland (Tyllinen and Hintikka, 1982; Rintala et al., 1995; Nuotio, 1997, 2000; Hieta-niemi, 1999; EELA 2001). Moreover, OA has been found in human plasma and milk in many countries (Breitholtz et al., 1991; Hald, 1991; Breitholtz-Emanuelsson et al., 1993, 1994; Zimmerli and Dick, 1995; Radic et al., 1997; Olsen et al., 1998; Scott et al., 1998; Rosner et al., 2000; Skaug et al., 2001).

Not much is known of the possible carry-over of trichothecenes and ZEN from feed to animal products, but according to Jonker et al. (1999) it is not significant. FAO (1998) considers contaminated cereals as the main source of mycotoxins for humans, rather than animal products. Cereals are also the main source of trichothecenes and ZEN in the Nordic countries (Eriksen and Alexander, 1998), and the major sources of OA in the human diet are cereals and cereal products in the EU states (European Commission, 1996).

2.4 Occurrence of trichothecenes, OA and ZEN in cereals

2.4.1 Worldwide

Many studies on the occurrence and amounts of OA, ZEN and trichothecenes, especially DON, have been carried out worldwide (Tanaka et al., 1988; Scott, 1990; Beardall and Miller, 1994b; Doko et al., 1996; European Commission, 1996; Dalcerio et al., 1997; D'Mello et al., 1997; Ali et al., 1998; Desjardins et al., 2000). Scott (1990) reviewed the incidence and levels of trichothecenes in North America, Asia, Australia, New Zealand, South America and several European countries and concluded that the most often detected trichothecene was DON. Recently Placinta et al. (1999) presented data on the occurrence of *Fusarium* mycotoxins worldwide in the 1990s and summarised that potentially harmful levels of DON were reported in cereals in Norway, Germany, Poland, Japan, New Zealand, USA, Canada and Argentina. The amounts of NIV and ZEN in cereals were generally low. It is also known that 3-AcDON is prevalent in Europe and 15-AcDON in North America (European Commission, 1999). Pittet (1998) reviewed the incidence and concentration of DON, OA and ZEN in cereals in Germany, Denmark, Switzerland, the United Kingdom, Canada, Norway, Bulgaria, Argentina, USA, Korea and Egypt in 1995-1997. The incidence and concentration of OA varied from 4% to 54% and from 0.05 µg/kg to up to 121 µg/kg, respectively. For samples contaminated with DON, the incidence varied between 33 and 100% and concentration between 5 and 62050 µg/kg. ZEN was found in 1-69% of the samples, with a concentration range of 4-8036 µg/kg. In general, lower levels of OA have been found in the USA than in Europe (Sydenham and Shephard, 1996).

2.4.2 In Europe and the Nordic Countries

A summary of the incidence and amounts of OA in Europe was prepared in cooperation between different countries (European Commission, 1996). Especially in Denmark, high occurrences and concentrations of OA in cereals have been reported (Nordic Council of Ministers, 1991; Jørgensen et al., 1996). In 1997 in the United Kingdom, 21% of cereal samples were contaminated with OA, barley being more frequently contaminated than wheat, with a mean concentration below 0.7 µg/kg (Scudamore et al., 1999). In Germany, cereals and related products were analysed for OA. In over 2300 samples approximately 70% were positive for OA, but only 1.4% of the samples contained more than 3 µg/kg (Wolff, 2000).

The occurrence of NIV in Swedish cereals in 1987-90 varied between 0 and 63%, generally in the range of 50-300 µg/kg (Pettersson et al., 1995). Oats was the most frequently and heavily contaminated. In Norway 70% of the 5000 cereal samples collected in 1988-96 were contaminated with >30 µg/kg DON, oats being more frequently contaminated (Langseth and Elen, 1997). DON, ZEN, 3-AcDON, NIV, T-2 and HT-2 were detected from 7% of HT-2 to 96% of DON in wheat samples, with an average amount from 7 µg/kg of 3-AcDON to 1632 µg/kg of DON in Germany in 1987 (Müller and Schwadorf, 1993). DON was also the main toxin found in oats in 1987-92 in Germany, but other *Fusarium* toxins were also detected (Müller et al., 1998). Langseth and Rundberget (1999) investigated a total of 449 cereal samples collected in 1996-98 in Norway and concluded that DON and HT-2 were the most often detected, followed by T-2 and NIV. Incidences were from 10% to 70% and mean concentrations of positive samples from 56 µg/kg to 115 µg/kg in oats. Incidence and average concentration varied between 5 and 22% and between 30 and 155 µg/kg, respectively, in barley and between 0 and 14% and 0 and 53 µg/kg, respectively, in wheat. Even though a number of studies have been carried out, the Scientific Committee on Food of the European Commission concluded that there is still need for more accurate information on the occurrence of trichothecenes in Europe (European Commission, 2000).

2.4.3 In Finland

In general the data of occurrence and amounts of mycotoxins in Finnish cereals is rather limited. However, mycotoxicosis in animals has been reported, indicating the presence of mycotoxins in feeds (Rainio, 1932; Ylimäki et al., 1979). Ylimäki et al. (1979) reported that ZEN was detected in approximately 5% of samples in 1976-77. In 1982-84 a number of animal toxicoses caused by trichothecenes were observed (Karppanen et al., 1985; Hintikka et al., 1988). In 1984, 94% of cereal samples were contaminated with trichothecenes, including DON in 91% of the samples (Rizzo et al., 1991). Hietaniemi and Kumpulainen (1991) analysed about 180 Finnish cereal samples in 1987-88. They detected DON in over 90% of samples in the range of 7-2600 µg/kg, lower amounts of 3-AcDON in over 30%, and NIV, HT-2, T-2 and ZEN in 1-11% of the samples. In the investigation of Rintala et al. (1995), low but detectable concentrations of ZEN were found in 1% of Finnish cereals and over 150 cereal samples gave negative results for the presence of OA.

2.5 Legislation of trichothecenes, OA and ZEN in cereals and cereal products

There are no harmonised regulations for trichothecenes, OA and ZEN in cereals and cereal products between countries. Over 90 countries worldwide have their own regulations or guideline levels for some mycotoxins depending on the type of commodity (de Koe, 1999). Van Egmond and Dekker (1995), D’Mello and MacDonald (1997) and Placinta et al. (1999) pointed out that OA and *Fusarium* toxins have been less stringently regulated than aflatoxins. However, in recent years harmonisation of the regulations, also for mycotoxins other than aflatoxins, has been in progress in the European Union. The Third Joint FAO/WHO/UNEP International Conference on Mycotoxins (1999a) concluded that worldwide harmonised limits for mycotoxins will probably not be achieved in the near future due to several factors: lack of information on the distribution of mycotoxins in foods, lack of reliable analytical methods and the possible distortion of the food market especially in developing countries. However, there is general consensus that exposure to mycotoxins should be limited to the lowest possible level, although they cannot be totally avoided (Rosner, 1998).

FAO (1997) has published the regulations for mycotoxins existing in different countries. The limits of tolerance for DON are 1000 µg/kg in finished wheat products in USA, 2000 µg/kg in uncleaned soft wheat in Canada and in Austria 500 µg/kg in wheat and rye and 750 µg/kg in durum wheat. Recently, considering children to be the most vulnerable to DON exposure, the limits of tolerance for DON in wheat and bread were estimated to be 120 and 60 µg/kg, respectively, in the Netherlands (Pieters et al., 1999). The introduction of limits for DON in cereal products and cereals to 500 µg/kg and 750 µg/kg, respectively, has recently been under consideration in the EU (Minutes of the Second meeting of the C.A. Mycotochain, 2000; Personal communication, W.A. van Osenbruggen, 2000). In Austria, Denmark and France the limit of tolerance is 5 µg/kg of OA in cereals and cereal products (FAO, 1997), and the same limit has been under consideration in the EU (Personal communication, Ministry of Trade and Industry, 2002). In Austria the limit of tolerance for ZEN is 60 µg/kg in wheat and rye, and in France 200 µg/kg (FAO, 1997). Some countries even have zero tolerance for mycotoxins in cereals (FAO, 1997). In Sweden, there are no maximum tolerated levels for trichothecenes, but for OA the national maximum level is 5 µg/kg (Olsen et al., 1998; Thuvander et al., 2001). In Finland, there are no maximum tolerance levels for trichothecenes, OA and ZEN in cereals and related products.

2.6 Toxicity of mycotoxins to humans and animals

Exposure to mycotoxins has been associated with health problems in both humans and animals. The nature of toxic effects varies depending on the chemical structure and concentration of the mycotoxins, time of exposure, animal species and health status (Charmley et al., 1995; Fink-Gremmels, 1999). It has been shown that the biological effects of mycotoxins are manifold (Betina, 1984), but occasional production of mycotoxins and unclear symptoms of mycotoxicosis make it difficult to estimate the health problems (Prelusky et al., 1994). Recently Hussein and Brasel (2001) reviewed the toxicity and mode of action of mycotoxins on humans and animals and concluded that swine are probably the most sensitive animals and that ruminants are the least sensitive, probably due to degradation of mycotoxins in the rumen.

2.6.1 Toxicity of trichothecenes, ZEN and OA

Trichothecenes are known to be immunosuppressive compounds and to inhibit protein synthesis (Prelusky et al., 1994; Cheeke, 1998). They may cause neural disturbance, haemorrhages, skin irritation, vomiting, diarrhoea and reduced feed intake (Kuiper-Goodman, 1994; Cheeke, 1998). It has been reported that T-2 was the cause of alimentary toxic aleukia in humans in Russia and that DON was the cause of human toxicosis in Japan, China and India (Beardall and Miller, 1994a). In general the trichothecenes of type A are more toxic than those of type B and DON is the least toxic and T-2 the most toxic of the commonly detected trichothecenes (Rotter et al., 1996; Scott, 1990; Eriksen and Alexander, 1998).

ZEN may cause reproductive and infertility problems in animals, as well as immunotoxic effects (Pestka and Bondy, 1994; Prelusky et al., 1994). The presence of ZEN in feed causes hyperestrogenism, especially in swine, and has for long been a problem (Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, 1999b; Hussein and Brasel, 2001). ZEN has also been considered as a possible agent that caused pubertal changes among young children in Puerto Rico (Sáenz de Rodríguez, 1984). Estrogenic activity of ZEN-related metabolites has also been reported (Hagler et al., 1979; Kuiper-Goodman et al., 1987; Kennedy et al., 1998).

OA is a nephrotoxic, teratogenic and carcinogenic compound and may also cause immunotoxic effects (Kuiper-Goodman, 1991; Pestka and Bondy, 1994; Prelusky et al., 1994). Due to its nephrotoxicity, OA has been regarded as an important factor for human endemic nephropathy in the Balkan area (Petkova-Bocharova and Castegnaro, 1991; Fuchs et al., 1991; Beardall and Miller 1994a), although the evidence is not unambiguous (Plestina, 1996; Joint FAO/WHO Expert Committee on Food Additives, 2001). One serious disease is porcine nephropathy, associated with pigs consuming feed contaminated with OA (Hussein and Brasel, 2001). OA is also known to segregate slowly from the organs of animals due to its strong ability to bind to serum albumin (Krogh, 1991; Hussein and Brasel, 2001), and Hald (1991) concluded that OA may also remain in human blood for a long period.

2.6.2 Toxicity due to co-occurrence of mycotoxins

Food and feed may contain various mycotoxins simultaneously, but synergism between different mycotoxins has not been well studied (Petkova-Bocharova and Castegnaro, 1985; Osweiler, 1990; Lemmens and Krska, 1996). According to Thuvander et al. (2001), trichothecenes of types A and B act by the same mechanism in the body and therefore their effects were assumed to be additive. It is suspected that mycotoxins have an additive or synergistic effect with other fungal metabolites or metabolites originating from host plants (Foster et al., 1986; Scudamore and Hetmanski, 1995; Thrane 2000). According to Foster et al. (1986), Eriksen and Alexander (1998) and Thrane (2000), other non-toxic compounds may enhance the toxicity of trichothecenes. Kuiper-Goodman et al. (1987) concluded that pure ZEN is less toxic than naturally contaminated cereals, indicating the presence of additional toxic substances in matrix. Other metabolites of moulds than known mycotoxins may also cause toxic effects (Fuchs et al., 1991; Scudamore and Hetmanski, 1995), and other components occurring in food and feed, e.g. pesticide residues, food additives and contaminants, may enhance the toxicity (Kuiper-Goodman, 1998).

Höhler (1998) proposed that the long-term uptake of small amounts of mycotoxins simultaneously may increase the risk of cancer and suppression of the immunological system. Miller (1991) stated that the co-occurrence of different mycotoxins in grains has a major effect on public health, especially in developing countries. Due to the versatile human diet, exposure to low amounts of a number of mycotoxins simultaneously over a long period is possible (Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, 1999c). It is also important to be aware that guidelines regulating the amount of mycotoxins in food and feed are usually based on toxicological investigations performed only with pure compounds (D'Mello et al., 1997).

2.7 Mycotoxins are stable compounds in food and feed processes

Mycotoxins are relatively heat-stable within the range of conventional food processing (European Commission, 1994). Major losses of DON and other *Fusarium* toxins were not observed during cleaning, milling and baking processes (Scott et al., 1984; Seitz et al., 1986; Kamimura, 1997). On the contrary, the concentrations of DON, 3-AcDON and ZEN even increased during malt germination and DON was found to migrate to beer (Schwarz et al., 1995). Scott (1990) summarised the effects of food processing on trichothecenes and concluded that carry-over of DON into processed cereal foods may be considerable. Trigo-Stockli et al. (1996) found that the levels of DON and ZEN were usually highest in the bran and lowest in the flour, indicating that they exist on the surface of grains. In contradiction to this, Jørgensen et al. (1996) found that fungi penetrated rather deep into the kernel and produced OA. Patey and Gilbert (1989) expressed that it is also possible to reduce the toxicity or the levels of mycotoxins physically or chemically, and many of these approaches have been presented in the literature (Charmley et al., 1995, 1996; Cole et al., 1998; Sinha, 1998; Galvano et al., 2001).

2.8 Determination of trichothecenes, OA and ZEN in cereals

Several authors have summarised the analytical techniques used for analysis of trichothecenes, OA and ZEN in cereals (Chu, 1991, 1995; Scott, 1991, 1995; Betina, 1993a; Gilbert, 1993, 1996a; Sydenham and Shephard, 1996; Wilson et al., 1998). The number and types of techniques employed are very high and versatile. Many authors have also expressed the need to improve the analytical quality of trichothecenes, OA and ZEN analysis in cereals, because many of the recent intercomparison studies have shown very high variability in the analytical methods, especially in the case of trichothecenes (Larsson and Möller, 1996; Wood et al., 1996; Schuhmacher et al., 1997; Pettersson, 1998; Pettersson and Langseth, 2000; Josephs et al., 2001; Krska et al., 2001). In this review only the main techniques presently used for trichothecenes, OA and ZEN analysis in cereals are presented in detail. Furthermore, the sampling of cereals for mycotoxin analysis is presented, because it is probably the most critical step in the analysis of mycotoxins, and one of the most difficult steps to perform in an appropriate way.

2.8.1 Sampling of cereals for mycotoxin analysis

Risks associated with sampling

Chu (1991) proposed that sampling is one of the most serious problems associated with mycotoxin determination. The primary objective of the sampling is to obtain a sample that accurately represents the concentration of mycotoxins in the entire lot. Theoretically, a lot in which mycotoxin concentration is higher than a defined guideline should be rejected and a lot in which the concentration is less than or equal to the guideline should be accepted (Johansson et al., 2000a). However, the mycotoxin concentration in a lot is estimated by analysing a sample taken from the lot and due to variation in sampling, sample preparation and analytical procedures, the estimated concentration differs from the true concentration (Johansson et al., 2000a). Only a small part of the kernels is analysed for the presence of mycotoxins. The difference between the estimated and the true concentration leads to an incorrect decision in some lots, meaning that some lots contaminated with mycotoxins higher than the guideline are accepted and enter the food chain, and some lots with concentrations lower/or than the guideline are rejected (Johansson et al., 2000a). These two types of risks are known as buyer's and seller's risks. Several statistical studies have been published to evaluate methods which decrease these risks (Whitaker et al., 1979, 1994, 1995, 2000; Sharman et al., 1994; Coker, 1998; Johansson et al., 2000a, 2000b, 2000c; Freese et al., 2000). Most of these studies are for commodities contaminated with aflatoxin.

A heterogeneous cereal lot

Mycotoxin contamination in cereals is heterogeneous (Davis et al., 1980; Campbell et al., 1986; Dickens and Whitaker, 1986; Park and Pohland, 1989; Hult, 1991). Since mycotoxins are produced by moulds they are found at isolated locations in the bulk product, where humidity and temperature have been favourable for mould growth (Crosby, 1996). Only a small portion of kernels in a lot may be contaminated, but with even very high mycotoxin levels (Campbell et al., 1986). Moreover, the distribution of kernels of different size, density and shape varies throughout the lot, and during loading fine particles tend to concentrate near the centre.

Sampling methods for mycotoxins in cereals

According to Campbell et al. (1986) most sampling methods have been developed for aflatoxin, but there is no remarkable evidence that the nature of contamination for other mycotoxins is different, and therefore the sampling methods of aflatoxin should be adequate for the other mycotoxins (Dickens and Whitaker, 1986). FAO (1993b) and the European Community (1998) have released sampling instructions for aflatoxin analysis in certain foods. However, the distribution of mycotoxins other than aflatoxin in the bulk product is not well studied. Only little is known concerning the distribution of OA and *Fusarium* toxins in contaminated foodstuffs and therefore less attention has been paid to the sampling of these toxins (Gilbert, 1996b; Joint FAO/WHO Expert Committee on Food Additives, 2001). Scudamore et al. (1999) suggested that in the absence of information about the heterogeneity of OA in cereals, a less stringent sampling procedure than for aflatoxin may be acceptable. Recent studies on the variation associated with the measurement of DON in cereals concluded that the main source of variation was sample preparation and analysis when the sample was properly taken (Freese et al., 2000; Whitaker et al., 2000). In contrast to the

analysis of aflatoxin in large kernels, a higher amount of small grains was contaminated with DON and the number of grains was higher in the collected sample, which caused smaller variation in sampling (Freese et al., 2000; Whitaker et al., 2000). A more heterogeneous sample with larger particles generally requires a larger sample size (Betina, 1993b).

It is particularly difficult to obtain representative samples from a large amount of a bulk product with irregularly shaped components, at very low contaminant levels and with heterogeneous contamination throughout the lot. An increase of the sample volume usually results in increased reliability of the final results (Park and Pohland, 1989) and therefore sampling is a very important step. A sufficient number of equal sample portions should be taken at random points throughout the lot and the number of particles in the sample is more important than its size for obtaining a representative sample (Smith and Moss, 1985; Richard, 2000). The size of the lot, the number and size of subsamples taken from the lot and the size of the combined sample for the laboratory analysis are recommended in several publications (Smith and Moss, 1985; Campbell et al., 1986; Commission Directive 98/53/EC, 1998; Richard, 2000). In practice, costs of the sampling are usually so high that it is not always possible to carry out an ideal sampling strategy (Crosby, 1996) and the acceptable sampling procedure for quality control may be very different from that needed for enforcement purposes (Gilbert, 1991; Davis et al., 1980).

Recommended routine sampling techniques

The most effective sampling method is to use an automatic stream sampler and to take small portions from a moving cereal stream at certain intervals, and finally combining the portions to the sample (Davis et al., 1980; Campbell et al., 1986; Dickens and Whitaker, 1986; Park and Pohland, 1989; Crosby, 1996). If this is impossible the sampling can be performed manually, e.g. with a probe or a scoop as described by Campbell et al. (1986) and Richard (2000). The combined sample must then be mixed carefully and subdivided by a mechanical sample divider or by manually (Dickens and Whitaker, 1986; Park and Pohland, 1989). After subdividing, the sample will be ground and divided for the laboratory sample (Park and Pohland, 1989).

2.8.2 Immunochemical and biological methods

Recently, the development of immunochemical and biological methods as screening techniques for mycotoxins in foods and feeds has been of great concern due to the need for rapid techniques to detect mycotoxins with minimal sample preparation and inexpensive equipment. In the past these techniques had relatively poor reliability, but at present and especially in future they will probably be important techniques.

Research on the application of immunochemical assays to detection of mycotoxins began in the late 1970s and was originally based on radioimmunoassay (RIA) (Pestka et al., 1995). Later research has concentrated on studying the feasibility of using enzyme-linked immunosorbent assay (ELISA), but often the preparation of a suitable mycotoxin immunogen has been a limiting step (Pestka et al., 1995). Nevertheless, immunochemical methods for the analysis of trichothecenes, OA and ZEN in cereals have been used in a number of studies (Barna-Vetró et al., 1994, 1996;

Trucksess et al., 1995; de Saeger and van Peteghem, 1996, 1999; Park and Chu, 1996; Usleber et al., 1996; Hart et al., 1998). Recently, Krska et al., (2001) summarised ELISA methods as a screening test for trichothecenes detection in cereals.

Biological methods are not specific and their sensitivity is generally low, and thus they can only be used for screening purposes of general toxicity (Chu, 1995). Recently, Widestrand (2001) concluded that cell culture-based bioassay as a screening test for trichothecenes in cereals was effective and suitable method. Other applications of biological methods for trichothecenes analysis have also been published (Rotter et al., 1993; Langseth et al., 1997, 1999).

2.8.3 Thin-layer chromatographic methods

Thin-layer chromatography (TLC) is one of the earliest analytical techniques used for mycotoxin analysis. TLC is simple and inexpensive, and is thus one of the most commonly used techniques (Chu, 1995). However, higher variance has been associated with TLC methods than with high performance liquid chromatographic (HPLC) and gas chromatographic (GC) techniques (Wilson et al., 1998), and due to their precision these techniques are preferred over TLC methods. Nevertheless, TLC methods remain still important in many laboratories (Wilson et al., 1998) and high performance TLC may be a potential technique as a screening test (Krska et al., 2001). Recently several authors have reviewed the use of TLC techniques for trichothecenes, OA and ZEN analysis (Chu, 1995; Sydenham and Shephard, 1996; Lin et al., 1998; Wilson et al., 1998).

2.8.4 Gas and liquid chromatographic methods for trichothecenes analysis

Recently Langseth and Rundberget (1998) and Krska et al. (2001) reviewed the most important methods used for the determination of type A and B trichothecenes in cereals. The authors reviewed different extraction, clean-up and derivatisation procedures, and discussed the possibilities and limitations of GC, HPLC and supercritical fluid chromatography including various detection techniques. The authors concluded that GC methods are the most often used, and that mass spectrometric (MS) detection is essential for reliable detection of small concentrations of trichothecenes. The one advantage of the GC method is the simultaneous analysis of several trichothecenes (Langseth and Rundberget, 1998; Krska et al., 2001). It has also been reported that the trichothecenes results obtained are method-dependent (FAPAS, 2000, 2001; Josephs et al., 2001).

Sample preparation

Langseth and Rundberget (1998) and Krska et al. (2001) pointed out that no single published method is optimal for the analysis of all trichothecenes at the same time due to the considerable variation in polarity of various trichothecenes, from the most polar NIV to the less polar T-2. Usually, however, the same purification procedure is applied, leading to different recoveries for various trichothecenes.

Most common is to use a mixture of acetonitrile-water for extracting trichothecenes, but methanol-water and other solvents have also been applied (Langseth and Rundberget, 1998; Krska et al., 2001). Charcoal-alumina column, MycoSep column, silica column, Florisil column and combinations of different columns have been used to purify the extracts, but in recent years the MycoSep column has been applied in many laboratories (Weingaertner et al., 1997; Berger et al., 1999; Krska, 1998; Malone et al., 1998; Radová et al., 1998; Razzazi-Fazeli et al., 1999; Schuhmacher et al., 1997; Trucksess et al., 1996; Langseth and Rundberget, 1998; Walker and Meier, 1998; Krska et al., 2001) (Table 2). The MycoSep column contains different adsorbents including charcoal, celite and ion-exchange resin (Langseth and Rundberget, 1998). Recently an immunoaffinity (IA) column clean-up was applied for purification of DON in cereal extract (Scott and Trucksess, 1997; Cahil et al., 1999; Krska et al., 2001) and the use of supercritical fluid extraction showed promise for screening purposes of trichothecenes (Järvenpää et al., 1997; Josephs et al., 1998; Krska, 1998; Krska et al., 2001).

GC methods

Trichothecenes are normally derivatised in order to attain the required volatility and sensitivity prior to GC analysis. The derivatisation step is also one of the most critical steps in the analysis of trichothecenes (Gilbert et al., 1985; Kientz and Verweij, 1986; Kanhere and Scott, 1990; Scott, 1993; Langseth and Rundberget, 1998; Krska et al., 2001). Derivatisation of trichothecenes is usually based on the derivatisation of hydroxyl groups to form trimethylsilyl (TMS), trifluoroacetyl (TFA), pentafluoropropionyl (PFP) or heptafluorobutyryl (HFB) derivatives (Langseth and Rundberget, 1998). For TMS derivatives, reagents such as bis(trimethylsilyl)acetamide (BSA), trimethylsilyl imidazole (TMSI), a mixture of TMSI and trimethylchlorosilane (TMCS) and commercial mixtures such as bis(trimethylsilyl)trifluoroacetamide-TMSI-TMCS (3:3:2) and more commonly BSA-TMSI-TMCS (3:3:2) have been used. For fluoroacetyl derivatives, reagents such as heptafluorobutyryl imidazole (HFBI), heptafluorobutyryl anhydride (HFBA), pentafluoropropionyl imidazole (PFPI), pentafluoropropionyl anhydride (PFPA) and trifluoroacetic anhydride (TFAA) have been employed. Krska et al. (2001) reported that less interfering peaks are obtained by using silylated than fluorinated derivatives.

Langseth and Rundberget (1998) reported that either 100% dimethyl siloxane, 5% phenyl or 7% phenyl 7% cyanopropyl dimethyl siloxane are the most often used column types for separating derivatised trichothecenes. Either an electron capture detector (ECD) or an MS detector is generally used for detecting trichothecenes (Langseth and Rundberget, 1998). The use of an MS detector reduces the need for extensive clean-up, is very selective for types A and B trichothecenes and allows simultaneous analysis of the toxins. Electron impact (EI), positive chemical (PCI) and negative ion chemical (NICI) ionisation have been used (Langseth and Rundberget, 1998), and tandem mass spectrometry (MS/MS) has also been applied (Kostiainen and Rizzo, 1988; Kostiainen et al., 1989). With ECD, rather selective detection is obtained for type B trichothecenes derivatised with trimethylsilyl reagents due to the conjugated carbonyl group in their molecules. To increase the sensitivity of the ECD detector for type A trichothecenes, fluorinated derivatives must be used (Langseth and Rundberget, 1998). Langseth and Rundberget (1998) concluded that GC-MS using the EI mode and applying selected ion monitoring (SIM) is sufficiently simple while giving reliable results. Furthermore, Tanaka et al. (2000) recently concluded that GC-MS is a powerful and practical tool for simultaneous detection and confirmation of trichothecenes and

also ZEN. Although GC-MS appears to be the most suitable technique for trichothecenes analysis in cereals, GC-ECD is still used in many laboratories due to its simplicity and lower cost of the instrument. To a lesser extent, the GC method with flame ionisation detection (FID) has also been applied (Furlong and Valente Soares, 1995). Due to the two recent reviews of the GC methods, only some techniques are presented in Table 2 in order to express the present situation in trichothecenes analysis.

Table 2. A description of the GC methods currently used for trichothecenes analysis in cereals.

Toxins analysed	Extraction	Clean-up	Derivatisation	Separation, detection/internal standard	Reference
DON, 3-AcDON, NIV, Fus-X, T-2, NEO, DAS, (ZEN)	ACN-water	Defatting with hexane, Florisil-column	TMSI-TMCS-ethyl acetate	GC-MS	Tanaka et al. (2000)
DON, NIV, HT-2, T-2	ACN-water	Mycosep 225	PFFPA	GC-MS/ α -chloralose	Langseth and Rundberget (1999)
DON, NIV, DAS, Fus-X, T-2 tetraol, HT-2, T-2	ACN-water or gel permeation chromatography	Florisil and C18 columns or MycoSep 225	TFAA	GC-ECD	Radová et al. (1998)
DON, NIV, Fus-X, 15-AcDON, 3-AcDON, HT-2, T-2	ACN-water	Florisil-column, cation-exchange column	TFAA	GC-MS	Schollenberger et al. (1998)
DON, NIV, 3-AcDON, 15-AcDON, Fus-X	ACN-water	Mycosep 225	Sylon BTZ	GC-ECD/Mirex	Weingaertner et al. (1997)
DON	ACN-water	C18-alumina-column	TMSI-TMCS	GC-ECD/Mirex	Tacke and Casper (1996)
DON, NIV, 3-AcDON, DAS, T-2, HT-2, T-2 triol	MeOH-water	Florisil-column, C18-column	HFBI, HFBA+ DMAP/poly-styrene, TMSI	GC-ECD/nandrolone	Seidel et al. (1993)
DON, DAS, Fus-X, NIV, T-2, 3-AcDON, trichothecin, NEO, HT-2	Ethyl acetate-ACN-water	Florisil-column	Tri-Sil-TBT	GC-ECD/DDE	Möller and Gustavsson (1992)

ACN = acetonitrile, MeOH = methanol, DDE = di(chlorophenyl)dichloroethylene

HPLC methods

HPLC techniques have also been applied for trichothecenes, but interferences from other compounds are usually more problematic with HPLC than with GC methods (Langseth and Rundberget, 1998). In addition to Langseth and Rundberget (1998), Lawrence and Scott (1993) and Frisvad and Thrane (1993) have also reviewed the use of HPLC methods. Due to the very poor ultra violet (UV) absorption of type A trichothecenes, HPLC with UV detection has mainly been used for the analysis of type B trichothecenes (Frisvad and Thrane, 1993). Reversed phase HPLC technique has also been applied in a number of studies using detection other than by UV, such as electrochemical detection and fluorescence detection together with post-column derivatisation (Visconti and Bottalico, 1983; Sylvia et al., 1986; Sano et al., 1987; Cohen and Boutin-Muma, 1992; Trucksess et al., 1996; Josephs et al., 1998; Walker and Meier, 1998; Cahill et al., 1999; Jiménez et al., 2000; Josephs et al., 2001). Liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS have also been used for trichothecenes analysis (Tiebach et al., 1985; Rajakylä et al., 1987; Berger et al., 1999; Razzazi-Fazeli et al., 1999) and they appear to be potential methods (Krska et al., 2001).

2.8.5 Liquid and gas chromatographic methods for OA analysis

Sample preparation

To extract OA from cereals, a mixture of organic solvent (e.g. chloroform, dichloromethane, acetonitrile) and water, with or without a small amount of acid, has generally been used (Wood et al., 1996, 1997). Solid phase extraction (SPE) has frequently been used for purification of the cereal extract (Langseth et al., 1989; Hald et al., 1993; Wood et al., 1996, 1997), but recently purification with a specific IA column has become popular (Sharman et al., 1992; Marley et al., 1995; Bisson et al., 1994; Scott and Trucksess, 1997; Scudamore and MacDonald, 1998; Solfrizzo et al., 1998; Trucksess et al., 1999; Vrabcheva et al., 2000). Furthermore, automation has been employed in some of the studies (Sharman et al., 1992; MacDonald et al., 1993; Bisson et al., 1994).

Separation and detection techniques

Due to the native fluorescence of OA, mainly reversed phase HPLC technique with fluorescence detection has been applied (Larsson and Möller, 1996; Sydenham and Shephard, 1996; Scudamore and MacDonald, 1998; Wilson et al., 1998), but other techniques such as GC-MS and especially recently LC-MS and LC-MS/MS methods have also been used (Abramson, 1987; Rajakylä, 1987; Trucksess et al., 1999; Sydenham and Shephard, 1996; Becker et al., 1998; Jørgensen and Vahl, 1999). The most common types of HPLC methods for OA analysis in cereals currently applied are presented in Table 3.

Table 3. A description of the methods currently used for OA analysis in cereals.

Toxins analysed	Extraction	Clean-up	Mobile phase	Separation, detection	Reference
OA	ACN-water	IA column	MeOH-water-phosphoric acid	HPLC-Flu	Vrabcheva et al. (2000)
OA	Chloroform-water-phosphoric acid	Silica-column	ACN-water-phosphoric acid	HPLC-Flu	Scudamore et al. (1999)
OA	MeOH-water-sodium bicarbonate	IA column	ACN-water-acetic acid	HPLC-Flu	Trucksess et al. (1999)
OA	ACN-water	IA column	ACN-water-phosphoric acid	HPLC-Flu	Scudamore and MacDonald (1998)
OA	Dichloromethane-ethanol-water-phosphoric acid	Liquid-liquid extraction with sodium bicarbonate in water-EtOH and with dichloromethane	ACN-water-acetic acid	HPLC-Flu	Jørgensen et al. (1996)
OA	PBS buffer-MeOH	IA column	ACN-water-acetic acid	HPLC-Flu	Sharman et al. (1992)
OA, (ZEN)	Chloroform-water-phosphoric acid	Silica-column	MeOH-water-phosphoric acid	HPLC-Flu	Langseth et al. (1989)

MeOH = methanol, ACN = acetonitrile, EtOH = ethanol, HPLC-Flu = high performance liquid chromatography with fluorescence detector

2.8.6 Liquid and gas chromatographic methods for ZEN analysis

Sample preparation

A mixture of organic solvent (acetonitrile, ethyl acetate, methanol, chloroform, acetone) with water or aqueous acidic solutions has commonly used for extracting ZEN from cereals (Schuhmacher et al., 1997). For purification of the extract, SPE cartridges (Tanaka et al., 1985; Langseth et al., 1989; Onji et al., 1998; Zöllner et al., 1999; Scudamore and Patel, 2000), liquid-liquid partition (Schwadorf and Müller, 1992; Schuhmacher et al., 1997; Scott, 1999; Josephs et al., 2001), MycoSep column (Silva and Vargas, 2001) and recently specific IA columns (Schuhmacher et al., 1997, 1998; Scott and Trucksess, 1997; Krska, 1998; Rosenberg et al., 1998; Visconti and Pascale, 1998; Kruger et al., 1999; Zöllner et al., 1999; Josephs et al., 2001) have been used.

Separation and detection techniques

HPLC has been the most often used technique for ZEN analysis in cereals (Sydenham and Shephard, 1996; Schuhmacher et al., 1997; Josephs et al., 2001). Reversed phase separation with fluorescence detection, due to native fluorescence of the ZEN molecule, has frequently been applied. In addition to fluorescence detection, electrochemical, UV and diode array detection have been used (Sydenham and Shephard, 1996; Schuhmacher et al., 1997). GC techniques, mainly with MS detection, have also been utilised, with reports of simultaneous detection of different *Fusarium* toxins, and LC-MS and LC-MS/MS also appear to be a promising techniques (Rajakylä et al., 1987; Schwadorf and Müller, 1992; Ryu et al., 1996; Sydenham and Shephard, 1996; Müller et al., 1998; Onji et al., 1998; Rosenberg et al., 1998; Zöllner et al., 1999; Tanaka et al., 2000). A description of the most common HPLC methods currently used for ZEN analysis in cereals is presented in Table 4.

Table 4. A description of the methods currently used for ZEN analysis in cereals.

Toxins analysed	Extraction	Clean-up	Mobile phase	Separation, detection	Reference
ZEN	ACN-water	MycoSep 224	MeOH-water	HPLC-Flu	Silva and Vargas (2001)
ZEN	ACN	IA column	ACN-water	HPLC-Flu	Schuhmacher et al. (1998)
ZEN	ACN-water	IA column	ACN-water-MeOH	HPLC-Flu	Visconti and Pascale (1998)
ZEN, (OA)	Chloroform-water-phosphoric acid	Silica-column	MeOH-water-phosphoric acid	HPLC-Flu	Langseth et al. (1989)

ACN = acetonitrile, MeOH = methanol, HPLC-Flu = high performance liquid chromatography with fluorescence detector



3

OBJECTIVES OF THE PRESENT STUDY

The main objectives of the present study were:

1. To improve and develop the analytical methods for trichothecenes, OA and ZEN analysis in cereals in order to use them in routine analysis.
2. To survey the presence and amounts of trichothecenes, ZEN and OA in the Finnish cereals wheat, rye, barley and oats after a growing season with heavy mould infection of cereals.
3. To identify the main sources of variation in the trichothecenes analytical method due to the problems observed during the survey study and in the intercomparison study of "Trichothecene analysis and feasibility to produce certified calibrants and reference material" (EU-project, SMT-4-CT96-2047).



4

MATERIALS AND METHODS

4.1 Sampling of cereals (I, II)

The cereal samples were taken after the rainy and cool growing season of 1998, when heavy *Fusarium* mould infection was observed on the cereal fields, particularly on rye fields, throughout Finland. Samples were collected from different regions of Finland, but mainly they were from the south due to the importance of cereal production in this area. In the autumn of 1998 the sampling was performed for cereals that were suspected to contain mycotoxins due to visible mould growth on the grains and therefore most of the samples were rye (I). In the study II the sampling was performed from the stored cereals during the winter and spring of 1999 and now the main cereal sampled was wheat. In the study I, millers and farmers took and delivered the samples to the laboratory, but in the study II health inspectors co-operated with the National Food Agency in the sampling. A total of 115 cereal samples including 49 of rye, 31 of wheat, 22 of barley and 13 oats samples were analysed. 7 barley and 2 oats samples were collected from cereal lots intended for animal feed, but all the other samples were from lots intended for human consumption.

Sampling was performed mainly using stream sampling. At the industrial mills the automatic stream samplers were applied and at the local mills and farms, millers and farmers were asked to take samples manually from the cereal stream. However, this was not always possible to realise and was especially difficult in the study II because the cereal was stored. Samples were then taken from cereal silos using a probe or manually with a scoop. Several portions were taken, which were combined and carefully mixed. A total of 0.5-1 kg subsample was separated and delivered to the laboratory (I) or the whole combined sample (1-10 kg) was delivered to the laboratory (II). The whole subsample was ground (I) or the whole combined sample or at least 3 kg was ground and divided by a sampler divider (II).

After the grain sample arrived at the laboratory, approximately 60 g was divided from it and sent to Agrifood Research Finland for mould detection (I). The cereal samples were stored in a refrigerator (+4 °C) before grinding and analysis, which were performed within 1-5 weeks. The samples were finely ground in a laboratory mill with a sieve size of 0.8 mm at the Plant Production Inspection Centre.

4.2 Reagents and apparatus

All the standards for mycotoxins, including internal standards, were obtained from Sigma St. Louis, MO, USA. TMS and HFB derivatisation reagents for trichothecenes analysis were purchased from Pierce, Rockford, IL, USA or Supelco, Bellefonte, PA, USA.

GC-MS with electron impact ionisation mode was used for measurements (**I-III, V**). The GC was a Hewlett Packard model 5890 series II, the MS a Hewlett Packard Mass Selective Detector model 5971 A and the autosampler a Hewlett Packard model 7673 (Hewlett Packard, Palo Alto, CA, USA). The capillary column was a DB-5MS (30 m x 0.25 mm, 0.25 µm film thickness), purchased from J&W Scientific, Folsom, CA, USA. In the study **III** the GC-ECD applied was from Carlo Erba Instruments, Milano, Italy. The GC was a model 6000 Vega Series 2 equipped with a ⁶³Ni electron capture detector model ECD 850 and an autosampler model A200S. The capillary column was an HP-5MS (30 m x 0.25 mm, 0.25 µm film thickness) and was purchased from Hewlett Packard, Palo Alto, CA, USA.

In the study **I**, HPLC (Hewlett Packard model 1090) with a fluorescence detector (Hewlett Packard model 1046 A, Hewlett Packard, Palo Alto, CA, USA) was used. In the study **II** the HPLC contained a Waters 510 pump and an autosampler Waters 717 (Waters, Milford, MA, USA). In the investigation **IV** the HPLC was a Waters model 2690 Alliance Separation module (Waters, Milford, MA, USA). A scanning fluorescence detector Waters 474 was used in both studies **II** and **IV**. The analytical column was a Waters Symmetry C18 column, 3.9 x 150 mm, 5 µm (**I, II, IV**). The automatic sample preparation system applied in the study **IV** was a model ASPEC XL coupled with a model 401 C diluter from Gilson Villiers Le Bel, France.

4.3 Sample preparation

4.3.1 Principles of sample preparation for trichothecenes analysis (**I-III, V**)

The analytical method used for simultaneous analysis of trichothecenes was a modification of the methods presented by Rizzo et al. (1986), Möller and Gustavsson (1992) and Weingaertner et al. (1997). Ground cereal sample was extracted with a mixture of acetonitrile-water in a flask shaker. To purify the cereal extracts, MycoSep column types 227 (**I, II, V**) and 225 (**III**) (Romer Labs INC, Union, MO, USA) were used. They were used manually by pushing the MycoSep column through the test tube filled with the cereal extract in 10-15 s. Thereafter the purified cereal extract was collected from the top of the column. After the purification step neosolaniol (NEO) or trichothecolone (TRI) was added as internal standard (Figure 1, Table 1) and the sample was then evaporated to dryness under a nitrogen stream. Thereafter the sample was derivatised with TMS-reagent with temperature control. TMS-derivatives were then diluted with hexane (**I, II**) or with a solution of Constant Standard (CS) (**V**) or GC standard (**III**) in hexane and then washed with water (**I-III, V**) or phosphate buffer (**III, V**). The CS was behenic acid methyl ester or pentacosane and the GC standard was 1,1-bis-(4-chlorophenyl)-2,2-dichloroethylene (DDE). The samples were

then analysed with GC-MS (**I-III, V**) or with GC-ECD (**III**). When the performance of the trichothecenes method was investigated, a mixture of cereals uncontaminated with trichothecenes (wheat : rye : barley, 3:2:1, w/w/w) was often used for sample preparation (**III, V**).

4.3.2 Principles of sample preparation for OA and ZEN analysis (**I, II, IV**)

The analytical method used for OA and ZEN determinations was a modification of the method described by Langseth et al. (1989) (**I**). Ground cereal sample was mixed with phosphoric acid solution and both of the toxins were extracted with dichloromethane. After evaporation the dry residue was dissolved in dichloromethane and purified with a silica Bond Elut column (Varian, Harbor City, CA, USA). The samples were then evaporated to dryness under a nitrogen stream and dissolved in the mobile phase used for OA and ZEN analysis by HPLC.

In the studies **II** and **IV**, the IA columns from two different manufacturers (Vicom, Watertown, MA, USA and Rhône-Diagnostics Technologies, Glasgow, Scotland) were used for purification of cereal extracts. A method used for ZEN analysis was a modification of the analytical procedure described by the Vicam (1997a) (**II**). In the study **IV** the method including a vacuum manifold was a modification of the method presented in the Vicam OchraTest (Vicom, 1997b) and Zearalatest Instruction Manuals (Vicom, 1997a) for OA and ZEN analysis, respectively. When ASPEC was used, the procedures presented for the Ochraprep immunoaffinity column (Rhône Diagnostics, 1999a) and the Easi-Extract Zearalenone immunoaffinity column (Rhône Diagnostics, 1999b) were slightly modified, as were the procedures described by Sharman and Gilbert (1991) and Sharman et al. (1992). First the ground cereal sample was extracted with a mixture of acetonitrile-water in a flask shaker. The extract obtained was then diluted with water or phosphate-buffered saline (PBS) and purified on IA columns using a vacuum manifold system (**II, IV**) or ASPEC (**IV**). Thereafter the samples were evaporated to dryness under the nitrogen stream, dissolved in a convenient mobile phase or methanol and analysed by HPLC. When the performance of the OA and ZEN methods was investigated, wheat, rye, barley and/or oats uncontaminated with OA and ZEN were used for sample preparation (**I, II, IV**).

4.3.3 Analytical techniques for trichothecenes, OA and ZEN determinations

Separation and quantification of trichothecenes (**I-III, V**)

Trichothecenes were separated and quantified simultaneously by GC-MS in SIM mode using NEO (**I, II, V**) or TRI (**V**) as an internal standard. Only a few samples were quantified using the method with an external standard (**I**). When GC-ECD was used for determination of DON the method with an internal or external standard was used (**III**). Usually trichothecenes were quantified using a cereal matrix-assisted calibration curve (**I-III, V**), but a calibration curve without cereal matrix was also applied (**III**). Rye (**I**) or a cereal mixture of wheat, rye and barley (wheat : rye : barley, 3:2:1, w/w/w) (**II, III, V**) was used for preparation of the matrix-assisted calibration curve.

Separation and quantification of OA and ZEN (I, II, IV)

OA and ZEN were determined by HPLC with fluorescence detection using the method with an external standard (I, II, IV). In the study I the mobile phase was acetonitrile-water-acetic acid (99:99:2, v/v/v) and to detect OA an excitation wavelength of 333 nm and an emission wavelength of 450 nm were used. For detection of ZEN the excitation wavelength was 270 nm and the emission wavelength 465 nm (I). In the investigation II the mobile phase was prepared with acetonitrile, water and methanol (40:52:8, v/v/v) and excitation and emission wavelengths of 274 and 440 nm, respectively, were used for ZEN detection. The chromatographic conditions used in the studies I and II were applied in the study IV for analysis of OA and ZEN, respectively.

4.4 Method improvement for trichothecenes, OA and ZEN analysis in cereals

The analytical methods were improved in two steps, first for the survey study (I, II), and subsequently on the basis of observations made during the survey study and taking into account international developments in mycotoxin analysis (III, IV, V).

4.4.1 Method improvement for trichothecenes analysis (I-III, V)

Prior to the survey study (I, II)

Before the study I, to purify cereal extract, MycoSep 227 columns were tested and compared with the MycoSep 215 columns earlier used in the laboratory. The end volume of the sample and the injection volume were optimised and the type of calibration curve and the quantification were tested. The extraction, silylation with TMSI and the washing step of excess reagent with water were left unchanged. The method was then validated (I, II).

After the survey study (III, V)

Due to problems observed during the validation studies, the survey studies I and II, and in the intercomparison study of trichothecene analysis (EU-project, SMT-4-CT96-2047), the performance of the trichothecenes method was investigated in order to improve it (III, V). Injection parameters such as the hold time of the injector and the injection volume for GC analysis were tested. The use of various silylation reagents was studied and the behaviour of internal standards relative to trichothecenes was investigated. The GC standard and Constant Standard for monitoring the performance of GC-ECD and GC-MS, respectively, within day and between days were introduced and their suitability was studied. Furthermore, the matrix effect was investigated (III), and finally, the methods were validated (III, V).

4.4.2 Method improvement for OA and ZEN analysis (I, II, IV)

To improve the reliability of the analysis, the sample clean-up step based on the use of SPE column in the study I was replaced with specific IA column purification in the study II. At first, IA column purification was performed by employing a vacuum manifold (II, IV), but later in order to automate the sample purification step, the ASPEC (automated solid phase extraction) system was introduced and studied (IV). Finally, the methods were validated.

4.5 Method validation

4.5.1 Validation of the trichothecenes method (I-III, V)

Validation of the trichothecenes method was performed using a spiked mixture of ground wheat, rye and barley. Repeatability (same operator and day), variation between days (different operators and days) and recovery were tested. The limit of detection (LOD) (signal to noise ratio 3:1) and the limit of quantification (LOQ) (signal to noise ratio 6:1) were estimated. To test the performance of the trichothecenes analytical method, the concentration of DON was measured in the certified wheat reference materials CRM 396 and CRM 379 (Community Bureau of Reference-BCR, Commission of the European Communities, Brussels, Belgium).

4.5.2 Validation of the OA and ZEN methods (I, II, IV)

Recovery and repeatability (same operator and day) of the OA and ZEN methods were tested by using spiked cereal samples (I, II). After the method improvement and automation, the methods applying both sample clean-up procedures were validated (IV). Recovery and repeatability of the methods were studied in various cereals species. The LOD (signal to noise ratio 3:1) and LOQ (signal to noise ratio 6:1) values were estimated. Moreover, samples of certified reference material for OA (CRM 472) purchased from the Community Bureau of Reference-BCR, Commission of the European Communities, Brussels, Belgium, and samples of wheat test material for ZEN (FAPAS) obtained from FAPAS, Central Science Laboratory, York, UK, were prepared and analysed (IV).

4.6 Confirmatory methods for samples positive for trichothecenes, ZEN and OA (I, II, IV)

Samples positive for HT-2, T-2, DAS, Fus-X and 3-AcDON were confirmed in a second GC-MS run (I, II). HT-2 and T-2 were confirmed by preparing HFB-derivatives. To confirm DAS, Fus-X and 3-AcDON, silylated samples were reanalysed using the SIM mode with extra ions. Confirmatory methods for ZEN and OA were introduced and tested (II, IV). To confirm the cereal samples positive for ZEN the method described by Visconti and Pascale (1998) was applied. The confirmatory method for OA was the method presented by Langseth et al. (1993b) with some modifications in the post-column pH shift system.



5 RESULTS

5.1 Method improvement for trichothecenes, OA and ZEN analysis prior to the survey study

In the analytical method of trichothecenes (**I, II**) the purification step with MycoSep 215 columns was replaced by using MycoSep 227 columns which were fast and easy to use. Due to the method optimisation, the end volume of the sample was four times smaller and the injection volume three times higher than in the earlier method. Furthermore, the matrix-assisted calibration curve was introduced into the method, the internal standard (NEO) was applied for controlling the derivatisation reaction and the samples were quantified with the method using an internal standard.

The methods for OA and ZEN analysis were not improved for the survey study **I**. In the second survey study (**II**) the sample clean-up step, previously based on the use of an SPE column (**I**), was replaced with IA column purification in ZEN analysis. The new method was fast and easy to perform and clean chromatograms were observed.

5.2 Method reliability in the survey study

The LODs were below 15 µg/kg for all of the trichothecenes investigated (**I, II**). For the repeatability the relative standard deviations (RSD) of trichothecenes generally varied from 2 to 12% (**I**). Poor RSD (24%) was obtained for DON and the same was also occasionally observed when the variation between days was investigated. Variation (RSD) between days was from 8% to 25% (**II**). Generally the recoveries were at an acceptable level (83-103%), but for NIV the recovery was low (57%) (**II**). The concentration of DON measured in the reference material was approximately 20% lower than the assigned value (**I, II**).

When SPE column purification was applied the LOD for OA was 0.5 µg/kg and for ZEN 3.5 µg/kg (**I**), but when IA column clean-up was used the LOD for ZEN was slightly lower (2 µg/kg) (**II**). The recovery for OA was 79% and the RSD was 20%, and the recovery for ZEN was 94% and the RSD 4% (**I**). The recovery percentages obtained for ZEN varied between cereal species from 55% for barley to 84% for rye and the related RSDs for repeatability from 4% for rye to 13% for wheat when the sample was purified with IA column (**II**).

5.3 Trichothecenes, ZEN and OA in Finnish cereal samples

Low amounts of trichothecenes and ZEN were found, but OA was not detected in any of the cereal samples. These results are summarised in Table 5 (I, II). Most of the samples were contaminated with DON at levels below 100 µg/kg and only four samples had concentrations exceeding 100 µg/kg, with the highest amount of 955 µg/kg being found in oats. However, the occurrence of DON was high, and over 72% of samples were contaminated with DON (I, II). HT-2 and T-2 were detected in some of the samples at low concentration levels, as were 3-AcDON and NIV, but Fus-X and DAS were not detected in any samples (Table 5). Contrary to the study I, low amounts of ZEN were detected in four cereal samples in study II. Simultaneous contamination of the samples with different *Fusarium* toxins was also detected.

5.4 Occurrence of *Fusarium* moulds and their simultaneous occurrence with trichothecenes

A total of 63 grain samples were analysed for the presence of *Fusarium* moulds (I). Most of the samples were heavily infected by *Fusarium* species, rye having the most severe contamination. Although every cereal sample was infected with *Fusarium* species, not all of them contained *Fusarium* toxins and although most of the samples were heavily infected, the detected concentrations of DON, NIV and HT-2 were low. *Fusarium avenaceum* was the most frequently found species and almost all the DON-contaminated samples were infected with this mould. About half of the DON-positive samples were infected with *F. arthrosporioides* and/or *F. tricinctum* together with *F. avenaceum*.

Table 5. The occurrence and concentration ranges of *Fusarium* toxins in the cereal samples harvested in 1998.

		Number of samples in range (µg/kg)							
		DON			3-AcDON	NIV	HT-2	T-2	ZEN
Cereal	n	5-100	100-500	500-1000	5-100	15-100	10-100	10-50	2-8
Wheat	31	24	2	0	1	0	0	0	2
Rye	49	40	1	0	0	0	0	0	0
Barley	22	13	0	0	0	3	5	0	1
Oats	13	12	0	1	2	2	4	1	1

n = number of samples

5.5 Main sources of variation in the trichothecenes analytical method

5.5.1 Derivatisation (III, V)

A double peak of DON appeared subsequent to silylation with reagents based only on TMSI (TMSI, Tri-Sil-Z), contrary to the case with a mixture of silylation reagents (Tri-Sil-TBT) (III). This was confirmed by GC-MS (III). When the cereal matrix was present together with the standard, the derivatisation was incomplete, but with pure standard this problem did not occur. A similar reagent to Tri-Sil-TBT, named Sylon BTZ, was used in the study V and complete reaction was again obtained with relatively low variation in the response of trichothecenes.

5.5.2 Functioning of the instrument (III, V)

Low between-day variation was observed in the functioning of GC-ECD, since the variation of the GC standard was below 5% (III). However, high variation within day and between days was occasionally observed in the functioning of GC-MS (V). The RSD of CS was sometimes as high as 34% during a single test including a sequence of standards and samples (Figure 4). When the instrument was working properly the RSD of the response for CS was below 10%. The injection volume and the hold time of the injector had only a small effect on the variation (III, V).

The terms "GC standard" and "Constant Standard (CS)" were introduced by the author and they are not official analytical terms.

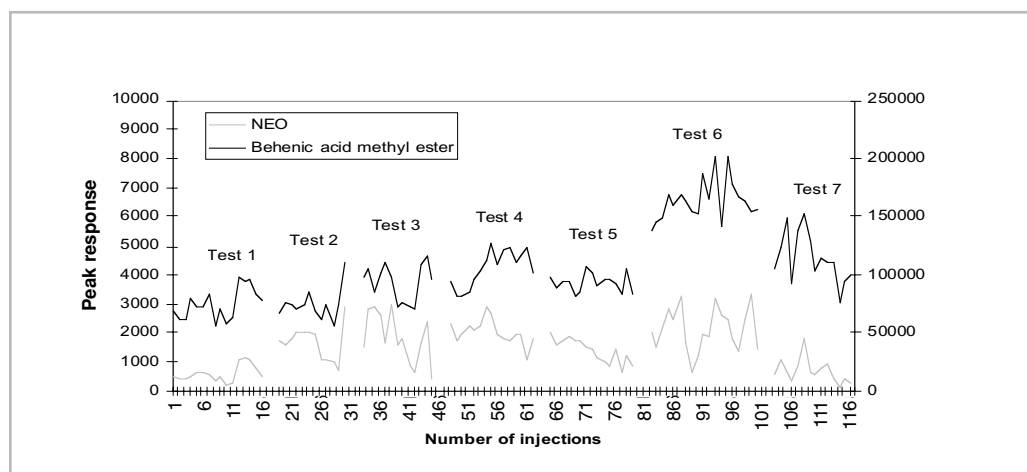


Figure 4. The variance of response for NEO and behenic acid methyl ester as a CS in sequential analytical tests during three weeks.

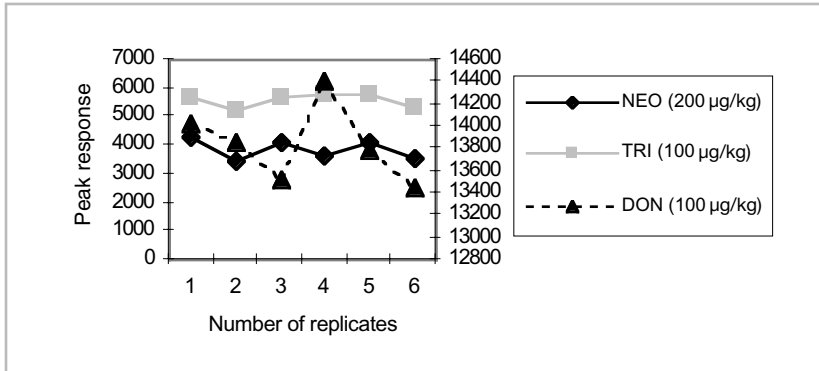
5.5.3 Use of internal standard (III, V)

NEO and TRI were used as internal standards in trichothecenes analysis in order to follow the effectiveness of the derivatisation reaction. The response of TRI correlated the response of trichothecenes relatively well in the cereal samples when the GC-ECD was applied (III), but not when GC-MS was used (V) or when the samples without matrix were analysed (III). However, the response of NEO followed the response of trichothecenes relatively well and NEO showed to be a rather suitable internal standard when GC-MS was used, especially after the change of derivatisation reagent from TMSI to Sylon BTZ (Figure 5).

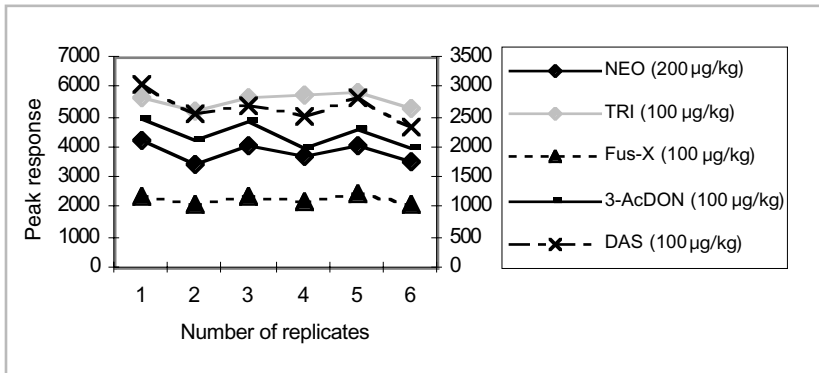
5.5.4 Matrix effect (III)

Matrix effect was present in each study (I-III, V), but especially it was observed in the study III when the spiked and reference material samples were quantified with the matrix-assisted calibration curve and the calibration curve without matrix. Much higher concentrations and recoveries were observed when the samples were quantified with the calibration curve without matrix. The matrix effect is expressed in Figure 6. The use of internal standard decreased the variation between the results quantified using different types of calibration curves. Moreover, lower variation between days for the calibration curves prepared with matrix was observed, in contrast to calibration curves prepared without cereal matrix (III). Subsequent to silylation, the use of an aqueous instead of a non-polar environment in the sample decreased the matrix effect between the matrix-assisted calibrants and calibrants without cereal matrix (III).

A



B



C

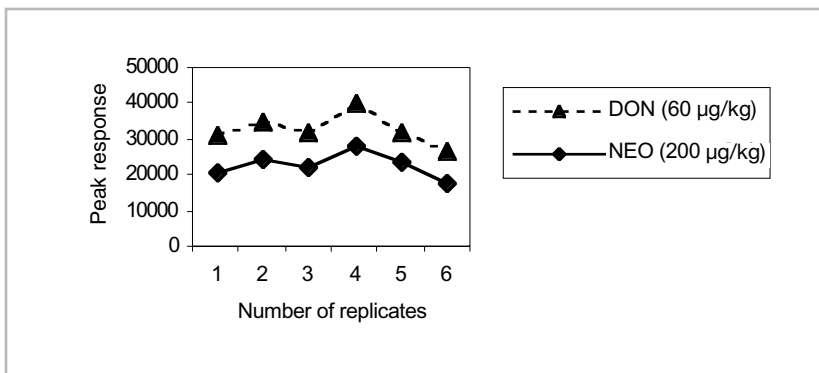


Figure 5. The peak responses of some trichothecenes together with internal standards (NEO and TRI). In the Figures 5A and 5B TMSI derivatisation reagent was used and in the Figure 5C Sylon BTZ reagent was used.

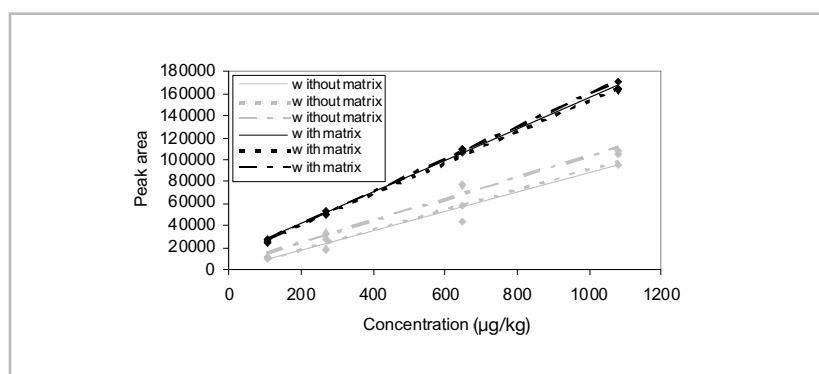


Figure 6. The matrix effect between six parallel calibration curves of DON prepared without and with cereal matrix and plotted using external standard.

5.5.5 Method reliability after subtracting the variation observed (III, V)

The validation tests of the trichothecenes analytical methods showed that the repeatabilities of the GC-ECD and GC-MS methods were below 10%. The recoveries varied from 46% of NIV to 96% of HT-2 in the GC-MS method and from 95% up to 146% for DON in spiked samples and in reference material with the GC-ECD method.

5.6 Method improvement and automation of OA and ZEN analysis (IV)

After method improvement of OA and ZEN analysis, enhanced specificity and cleaner chromatograms were observed by applying the IA column in comparison with earlier methods based on an SPE column clean-up (I). For OA, fivefold lower LOD (0.1 µg/kg) was achieved when IA columns were employed, but no major improvement was observed in the LOD of ZEN analysis (2 µg/kg). The RSD for repeatability was below 12% for both methods and the recoveries were above 82%. The concentration of OA measured in the reference material was 11% lower than the assigned value. More samples (from 2 to 5) without hazardous organic solvents and considerable manual work, were prepared in a working day than with the earlier method.

ASPEC enhanced the sample throughput and was easy to use. The RSD for the repeatability of the method including ASPEC was in agreement with the method comprising a vacuum manifold. However, almost all the recoveries were significantly lower when ASPEC was used (> 68%) instead of a vacuum manifold (Two-Sample T test, $p < 0.05$, Statistix for Windows, version 2.0). The concentration of OA measured in the reference material was in agreement (2% lower) with the assigned value, but the concentration of ZEN in the test material was 25% lower than the given value. It was found that the use of ASPEC changed the solubility of the ZEN sample residue after the purification and methanol was then used instead of mobile phase as a dissolving agent. The chromatograms with a baseline peak separation were observed after changing the dissolving solution.



6

DISCUSSION

6.1 Sampling and representativeness of the cereal samples

Because most of the samples were collected from southern Finland, although some samples were from other parts of the country, and due to the relatively small number of samples analysed, a definite conclusion on the mycotoxin contamination over the whole Finland in 1998 cannot be drawn. Only an approximate evaluation of the mycotoxin contamination in cereals can be presented. Most of the samples were from lots intended for human consumption and only a few were for feed. Hence the representativeness of the sample number for estimating the mycotoxin contamination in feeds is clearly too low.

Sampling of cereals is one of the most difficult problems in mycotoxin analysis and is usually the cause of the highest variance and error. Therefore in the present study, the requirements proposed by Davis et al. (1980), Campbell et al. (1986), Park and Pohland (1989) and Crosby (1996) for cereal sampling for mycotoxin analysis were fulfilled as well as possible. The sampling methods here applied were not originally planned for the purposes of mycotoxin investigations, but for the quality control of the cereals at the mills. The samplings at the farms were mainly performed as requested by the cereal inspection. In most cases the automatic or manual sampling was performed by taking several subsamples at intervals from a moving cereal stream and finally combining the subsamples. This type of sampling is generally considered to be the most effective for mycotoxin analyses (Davis et al., 1980; Campbell et al., 1986; Dickens and Whitaker, 1986; Park and Pohland, 1989; Crosby, 1996). However, sampling from a cereal stream was not always possible, especially in the study **II** when cereal was stored. The subsamples were then taken at different points from cereal silos by using a probe or manually with a scoop as described by Cambell et al. (1986) and Richard (2000). After subsampling the portions were combined and mixed. For practical reasons, the sampling procedure at the private farms and the local mills was not as sophisticated as in the industry, but it was performed as well as possible.

According to one study of sampling variability for aflatoxin in peanuts, the highest variance was due to the first sampling from the lot (Whitaker et al., 1994). The situation in cereal sampling in the present study was probably similar. However, a recent investigation of the sampling of DON in cereals showed that the main source of variation was sample preparation and the analytical step when the sample was properly taken (Freese et al., 2000; Whitaker et al., 2000). The variation in the size of the grain lots sampled was very high, as was the sample size delivered to the laboratory (0.5-10 kg but usually approximately 0.5-1 kg). According to Commission Directive 98/53/EC

(1998) for sampling cereals for aflatoxin analysis, the laboratory sample should be 10 kg. Richard (2000) expressed a general guideline for sample size of 1.4 kg in mycotoxin analyses of cereals. The representativeness of the laboratory samples of 0.5 kg might then be too small. However, the whole sample or at least 3 kg were ground to reduce the particle size before the analysis and the sieve size was small in order to achieve reliable results. It can be concluded that the sampling and the representativeness of the samples were sufficient in order to obtain the approximate evaluation of the mycotoxin contamination in Finnish cereals in 1998.

6.2 Trichothecenes, ZEN and OA in Finnish cereal samples

In 1998 the growing season was rainy, as it was in most areas of the Northern Europe, and also relatively cold, and heavy *Fusarium* mould growth on kernels was detected in Finland. The wet weather also caused high *Fusarium* mould infection in Central Europe in 1998 (The 21st German Mycotoxin-Workshop, 1999; Marketing Board for Cereals, Seeds and Pulses, 1999; Birzele et al., 2000). Although even high mycotoxin contamination was expected due to the heavy mould infection on the Finnish grains, especially in rye, the amounts of *Fusarium* toxins were very low. DON, NIV, 3-AcDON, HT-2, T-2 and ZEN were found, but OA, DAS and Fus-X were not detected in any of the samples (Table 5).

Low concentrations of trichothecenes in cereals cultivated during the wet and cool growing season in 1998 were also reported in a Norwegian study and it was suggested that the cool weather seemed to favour other *Fusarium* species than DON-producing species (Langseth and Rundberget, 1999). This conclusion was well in agreement with the results obtained in this study. In contrast to the present investigation, clearly higher concentrations of trichothecenes were found in Central Europe in the same year (Birzele et al., 2000; Personal communication, W.A van Osenbruggen, 2000). As in the present study, the occurrence of DON in cereal samples was also high in Europe in 1998 (Birzele et al., 2000). Comparing the results obtained in this study to an earlier Finnish study of Hietaniemi and Kumpulainen (1991), the concentrations of trichothecenes and ZEN were lower, but the toxins identified were similar. The occurrence of samples contaminated with DON was high, as in the previous Finnish investigations (Karppanen et al., 1985; Hietaniemi and Kumpulainen, 1991; Rizzo et al., 1991; Rizzo, 1993).

In general OA and ZEN have only occasionally been detected in Finnish cereals, usually in low amounts. Even though the number of samples was low in the present study, the results indicate that the levels of OA and ZEN were also low in 1998, as in the previous Finnish investigations (Hietaniemi and Kumpulainen, 1991; Rintala et al., 1995). In 1998 low concentrations of OA were detected in wheat in Germany (Birzele et al., 2000), at the same level as the LOD of the method applied in this work; hence the sensitivity of the method might be the reason for the negative finding of OA. This is probably confirmed by the fact that low amounts of ZEN were detected in the cereals after the method with specific IA column purification was introduced in the study II.

Co-occurrence of the *Fusarium* toxins was observed in the samples investigated, and this finding was in agreement with other Nordic studies (Hietaniemi and Kumpulainen, 1991; Pettersson et al., 1995; Langseth and Rundberget, 1999; Thuvander et al., 2001).

Health effects caused by the mycotoxins detected

Due to the importance of rye in the Finnish diet, and to the observed heaviest contamination of rye with *Fusarium* moulds, the cereal industry was concerned about the quality of this cereal. Several rye lots were rejected due to low quality and mouldiness in the autumn of 1998, but several suspected lots were also examined for the presence of trichothecenes, ZEN and OA. Due to the mouldiness of rye, other cereals, mainly wheat, were also investigated for the presence of *Fusarium* toxins. However, because of the low quality of the cereal crop, only 20-30% of wheat of the total wheat crop was accepted for food production in Finland in 1998 (Personal communication, Fazer, 2001). Most of the wheat samples here investigated were included in this accepted wheat proportion.

Although DON was frequently detected, its concentration in the cereals was so low that the health risk for consumers was almost negligible, but this did not necessarily represent the true situation in Finland due to the rather low number of samples analysed. However, only the samples highly infected with moulds were analysed and it may be expected that mycotoxin contamination in other cereals did not greatly exceed the amounts detected. This was later confirmed by analysing stored cereals. Nevertheless, it is important to realise that mould growth on the kernels does not necessarily mean that they are contaminated with mycotoxins and vice versa (Fink-Gremmels, 1999). The amount of DON in one oat sample was very near the limit of tolerance applied for wheat in the USA (FAO, 1997), and the amount of HT-2 in the same sample was also relatively high. This might indicate that high concentrations of *Fusarium* toxins in cereals also occurred. It might be that the most contaminated lots were left on the farms and fed to domestic animals or destroyed. However, symptoms caused by *Fusarium* toxins in animals were not observed during the end of 1998 or in 1999, which further indicates that the amounts of *Fusarium* toxins were low. Only a few samples obtained from the individual farms were analysed and thus producers who used the cereals themselves might have been exposed to these toxins if the cereals were contaminated.

The co-occurrence of different *Fusarium* toxins was observed in several samples and thus the adverse health effect caused by these toxins simultaneously, even though they were found at low levels, might be moderately harmful for some consumers. The possible synergistic effects of different toxins on health have not been well studied (Petkova-Bocharova and Castegnaro, 1985; Osweiler, 1990; Lemmens and Krska, 1996; Thuvander, 2001), but it is considered that long-term uptake of small amounts of mycotoxins simultaneously may harmfully affect human health (Miller, 1991; Höhler, 1998; Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, 1999c).

Fusarium infection related to amounts and occurrence of trichothecenes

The weather conditions during the growing season of 1998 were wet and also relatively cold, which had the strongest effect on heavy *Fusarium* infection, especially in rye. *Fusarium avenaceum* was the most common species and other *Fusarium* species were only rarely found, as also previously reported by Uoti and Ylimäki (1974) and Ylimäki et al. (1979). Contrary to the present investigation,

Hietaniemi and Kumpulainen (1991) most frequently detected *F. graminearum* in Finland. It appears that the occurrence of *Fusarium* species varies between years due to different growing and weather conditions, as concluded by Chelkowski (1989b).

F. avenaceum, *F. arthrosporioides* and *F. tricinctum* found in many DON-contaminated samples analysed are reported as being important mycotoxin producers in northern Europe (Eriksen and Alexander, 1998). The low trichothecene concentrations observed in this study may be explained by the finding that fungal species found have not been proved to produce trichothecenes (Thrane, 2000; Nielsen and Thrane, 2001). In one Norwegian study, *F. avenaceum* did not produce any trichothecenes but it produced rather high amounts of moniliformin toxin (Langseth et al., 1999).

Old data on the occurrence and the amounts of trichothecenes, OA and ZEN in Finnish cereals were updated and new information on the relation of *Fusarium* infection and occurrence of trichothecenes in Finland was generated.

6.3 Factors sensitive to variation in trichothecenes analytical methods

Already before the beginning of the survey investigations, the intercomparison study of trichothecenes analysis (EU-project, SMT4-CT96-2047, Pettersson, 1998) concluded that the reliability of trichothecenes analytical methods was poor, which prompted activities for improving the methods. The new modern MycoSep columns were fast and easy to use and enabled high sample throughput. One cereal sample extract was purified without the need of a vacuum manifold system, performing the washing and elution step in a few seconds. The MycoSep columns also enabled relatively easy preparation of the calibrants for a matrix-assisted calibration curve. The application of a matrix-assisted calibration curve complicated the method, but was essential for decreasing the matrix effect reported as being one of the sources of high variation (Pettersson, 1998). The use of cereal matrix in the preparation of the calibration curve decreased the between-day variation of the curves and improved the linearity and quantification (III), as was also reported by Tacke and Casper (1996). Subsequent to silylation, the use of an aqueous environment instead of a non-polar environment in the sample appeared to reduce the matrix effect between different types of calibrants (III). This, however, should be confirmed by further studies.

The matrix effect is probably partly due to adsorption onto the glass liner in the injector of the GC and/or the GC-column. It is likely that active sites on the glass liner and in the column adsorb silylated components of cereals as described by Pareles et al. (1976) for silylated milk samples. According to Pettersson (1998), the adsorption of derivatised trichothecenes in the injector and in the first part of the chromatographic column is a problem in GC analysis of trichothecenes. In another study on mycotoxins, Rosen and Pareles (1974) reported that silylated carbohydrates saturated the active sites on the column wall, resulting in higher sensitivity for silylated patulin in apple juice samples.

The derivatisation step is one of the most critical steps in the analysis of trichothecenes (Gilbert et al., 1985; Kientz and Verweij, 1986; Kanhere and Scott, 1990; Scott, 1993; Langseth and Rundberget, 1998; Krska et al., 2001), and therefore the silylation reagent used must be effective

for achieving complete silylation. However, the appearance of a double peak for DON showed that TMSI and Tri-Sil-Z reagents were not fully effective (III). Difficulties in silylation of type B trichothecenes have also previously been reported. Langseth and Rundberget (1998) observed the appearance of double peaks for type B trichothecenes derivatised with pure TMSI, and Gilbert et al. (1985) suggested that in type B trichothecenes the double peaks were due to difficulties in derivatisation of the C-7 hydroxyl group, due to hydrogen bonding with the adjacent carbonyl group (Figure 1, Table 1). Derivatisation performed with pure standards often gives rise to different products than those formed in the presence of matrices (Little, 1999), which is probably why the double peaks were not detected in the samples prepared without matrix (III). The mixture of silylation reagents (Tri-Sil-TBT, Sylon BTZ) proved to be effective, as also reported by Möller and Gustavsson (1992), Scott et al. (1986), Bennet et al. (1984) and Hastings and Stenroos (1995), and lowered the variation due to complete silylation.

A variety of components have been reported to have been used as internal standards in trichothecenes analysis (Rood Jr et al., 1988; Sydenham and Thiel, 1987; Möller and Gustavsson, 1992; Seidel et al., 1993; Furlong and Valente Soares, 1995; Tacke and Casper, 1996; Weingaertner et al., 1997; Abramson et al., 1998; Kotal et al., 1999), but none of them have achieved great success, and therefore NEO (Figure 1, Table 1) was tested as a new potential internal standard. NEO is a type A trichothecene and its derivatisation reaction is similar to those of other trichothecenes. It was chosen as the internal standard because it is one of the trichothecenes very rarely detected. It has been found in fungal extracts (Schmidt, 1986; Park and Chu, 1993). The disadvantage was that NEO is a type A trichothecene and not a type B, because type B trichothecenes may have incomplete derivatisation reactions (Gilbert et al., 1985; Langseth and Rundberget, 1998). Originally, NEO was included in the method for controlling the derivatisation reaction and therefore it was added to the sample after the extraction step. Previously Schollenberger et al. (1998) used other trichothecenes (DAS) to control the efficiency of derivatisation. The low probability of detecting NEO naturally in the samples was compensated by controlling the response of NEO and by reanalysing the samples without internal standard if its presence was suspected.

The use of NEO decreased the variation caused by the problems in derivatisation, but still some variation, particularly in the response of DON, was sometimes observed (Figure 5). The next approach was that NEO and TRI were both used as internal standards, NEO for type A trichothecenes and TRI for type B (Figure 1, Table 1). However, it appeared that the effectiveness of the silylation of TRI differed from that of the other trichothecenes, but NEO appeared to be a rather suitable internal standard for the GC-MS method, especially after the change of silylation reagent from a less effective to a more powerful reagent (Figure 5). Currently NEO appears to be a rather convenient internal standard. Some of the matrix effect may also be reduced due to similar behaviour of NEO with other silylated trichothecenes in GC-MS analysis.

As a new approach in the analysis of trichothecenes, GC standard and CS were used for controlling the operation of the GC-ECD and GC-MS instruments. Using DDE as a GC standard it is possible to control the operation of the GC-ECD instrument throughout the analytical runs. Contrary to GC-ECD very high variation was occasionally observed in the functioning of GC-MS by monitoring the response of behenic acid methyl ester and pentacosane. They are stable components and therefore rather appropriate as a CS for controlling malfunctioning or contamination of the GC-MS and for indicating derivatisation problems occurring for the internal standard. The CS and GC standard

were added to the sample immediately before the GC-MS or GC-ECD analysis in order to avoid any effect caused by the sample preparation. Simultaneously recording the responses of both internal standard and CS was used in a control chart and it was very useful for evaluating the results for trichothecenes (Figure 4). It appears that higher variation is caused by GC-MS than by GC-ECD, which is also in accordance with the results of Pettersson (1998). This variation is probably due to the functioning of the MS detector rather than GC.

The validation results obtained before the survey investigation showed that the GC-MS method was relatively reliable, specific and low LODs were obtained, and it was practicable for survey studies. Later the high variation observed in trichothecenes analytical methods was minimised before accomplishing the final validation, which showed that the GC-ECD and GC-MS methods were both adequately reliable. However, the recovery of NIV was still low, as also reported by Weingaertner et al. (1997) and Krska (1998), suggesting that this is due to adsorption of NIV on polar sites in the packing material of the MycoSep column. The validation results generally demonstrate that the variation observed in trichothecenes analysis may be decreased by using matrix-assisted calibration, internal standard, control chart and effective derivatisation reagent.

Valuable information on the use of matrix-assisted calibration curve and different silylation reagents in the trichothecenes analytical method was obtained. A new internal standard (NEO) and a control chart were introduced in the method. These new factors in the trichothecenes analytical method resulted in better reliability of the method and considerably assisted in the evaluation of the results determined.

6.4 Performance of IA columns and automation in OA and ZEN analysis

The methods for OA and ZEN analysis were not improved for the first survey study, because the validation results showed that the methods were relatively reliable and rather low LODs were achieved. However, for the next study a more specific method including IA column clean-up for ZEN analysis, applied in many routine laboratories (Schuhmacher et al., 1997, 1998; Scott and Trucksess, 1997; Krska, 1998; Visconti and Pascale, 1998; Josephs et al., 2001), was tested.

The use of IA columns simplified the analyses, sample throughput increased and less hazardous solvents were required, as also reported by Visconti et al. (1999). Due to the increased specificity, low levels of ZEN and OA were detected with confidence. The validation results showed that the methods were adequately reliable and low detection limits were achieved, and they were in agreement with the earlier studies of Bisson et al. (1994), Krska (1998), Scudamore and MacDonald (1998), Schuhmacher et al. (1998), Visconti and Pascale (1998), Trucksess et al. (1999) and Vrabcheva et al. (2000).

Only in a few studies had ASPEC been used to purify food and feed samples contaminated with aflatoxin and OA by applying IA columns (Sharman and Gilbert, 1991; Sharman et al., 1992; Gilbert, 1993; Bisson et al., 1994; MacDonald et al., 1999), but no studies were found on the use of ASPEC for ZEN analysis. Sharman and Gilbert (1991) proposed that due to their simplicity of use, IA columns are especially adaptable to automation. The validation results showed that ASPEC

worked adequately well for both analyses and the results were in agreement with the investigations of Sharman et al. (1992), Krska (1998), Schuhmacher et al. (1998) and Visconti and Pascale (1998). The methods were relatively reliable and low LODs were achieved. The present study is the first one published on the use of ASPEC for the analysis of ZEN by applying IA columns (**IV**). The notable difference in validation results between the method including ASPEC and a vacuum manifold was the lower recoveries obtained with ASPEC. The other major finding was that the use of ASPEC changed the solubility of ZEN sample residue after the purification compared to the manual method. This was presumed to be due to an interfering substance originating from packing material of the IA column. Rossi and Zhang (2000) pointed out that the improvement of the automated method in comparison to the manual method is not always obvious. However, in order to facilitate the IA column purification step the use of ASPEC overnight remarkably enhanced the efficiency of the sample preparation and further enabled avoiding some of the health risks caused by mycotoxins and solvents.



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CONCLUSIONS

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Cereal samples, mainly rye and wheat, collected after harvest in 1998 and during the winter of 1999, were analysed for the presence of trichothecenes, ZEN and OA. Although heavy *Fusarium* mould infection was detected on the kernels, the amounts of the mycotoxins determined were very low. DON was clearly the trichothecene most often detected, whereas other toxins were only occasionally found. *Fusarium avenaceum* was the strain causing the heaviest mould infection, but was not the source of trichothecenes. These results were also in accordance with other Scandinavian and European studies. The low amounts of mycotoxins found in cereals indicate that the mycotoxins in Finnish cereals were not posing health risk for the consumers in 1998-99.

During this work and in the intercomparison study (EU-project, SMT-4-CT96-2047), relatively high variation in the results of trichothecenes was observed. This gave rise to an investigation of the main sources of variation in the trichothecenes analysis, which turned out to be the between-day changes in performance of GC-MS, the matrix effect and incomplete derivatisation reaction. Some of these findings have been reported in earlier studies, in works aiming at solving one specific problem, but in this investigation they are presented simultaneously for the first time. By using cereal matrix-assisted calibration, internal standard, Constant Standard with GC-MS, GC standard with GC-ECD and effective derivatisation reagent, some variation could be identified and eliminated, which further improved the reliability of the trichothecenes results. The GC-MS and GC-ECD methods including these factors are clearly well suitable for the analytical methods of trichothecenes analysis in cereals. Further improvements may be possible in the use of two suitable internal standards not existing among the natural mycotoxins, one for the trichothecenes of type A and one for the type B, decreasing the variation still appearing in the analytical methods, and improving the recovery of NIV. The use of IA column purification in the analytical methods for OA and ZEN in cereals increased the reliability and specificity over the conventional methods, and automation of the clean-up step by applying ASPEC further facilitated the use of the methods. This was well in agreement with previous studies, but the use of ASPEC for the analysis of ZEN by applying IA columns has not been reported earlier. The application of ASPEC for IA column purification in OA and ZEN analysis is recommended, although it needs further optimisation.

This study enhanced the status of information on the occurrence and the concentration levels of trichothecenes, ZEN and OA in Finnish cereals in relation to *Fusarium* moulds. Valuable information was obtained in the method performance of trichothecenes analysis. With respect to the analytical methods previously applied, the investigation resulted in significant method improvement, enabling their use in routine analysis and updating them to the international level currently existing in leading mycotoxin laboratories.



8

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