

# **TECHNISCHE UNIVERSITÄT MÜNCHEN**

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt  
Lehrstuhl für Tierhygiene

## **NEW APPROACHES FOR ZEARALENONE ANALYSIS**

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All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy.

*Paracelsus* (1493-1541)

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**LIST OF ABBREVIATIONS**

APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Interface
ASE	Accelerated Solvent Extraction
BLE	Blending
bw	Body Weight
°C	Celsius degree
Conc	Concentration
DAD	Diode Array Detector
DON	Deoxynivalenol
E2	17 $\beta$ -estradiol
ELISA	Enzyme Linked ImmunoSorbent Assay
ER	Estrogen Receptor
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization of the United Nations
FAPAS <sup>®</sup>	Food Analysis Performance Assessment Scheme
FLD	Fluorimetry Detector
GC	Gas Chromatography
hER	Human Estrogen Receptor
HPLC	High Performance Liquid Chromatography
IA	Immunoaffinity
IARC	International Agency for Research on Cancer
Ig	Immuno globuline
ISTD	Internal Standard
LC-MS	Liquid Chromatography - Mass Spectrometry
LD <sub>50</sub>	Lethal Dose 50
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
min	minute
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometer
MW	Molecular Weight
NIV	Nivalenol
NOEL	No Observed Effect Level
PCA	Principal Component Analysis
PLE	Pressurized Liquid Extraction
PMTDI	Provisional Maximum Tolerable Daily Intake
R <sup>2</sup>	Correlation coefficient
RP	Reversed Phase
S/N	Signal-to-Noise
SHA	Shaking
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
TAL	Taleranol ( $\beta$ -Zearalanol)
TEA	Triethylamine
TIC	Total Ion Chromatogram

TDI	Tolerable Daily Intake
TLC	Thin Layer Chromatography
UE	Ultrasonic Extraction
UV	Ultraviolet
v/v	volume / volume
ZAL	Zeranol ( $\alpha$ -Zearalanol)
ZAN	Zearalanone
ZOL	Zearalenol
ZOLs	$\alpha$ -Zearalenol and $\beta$ -Zearalenol
ZON	Zearalenone



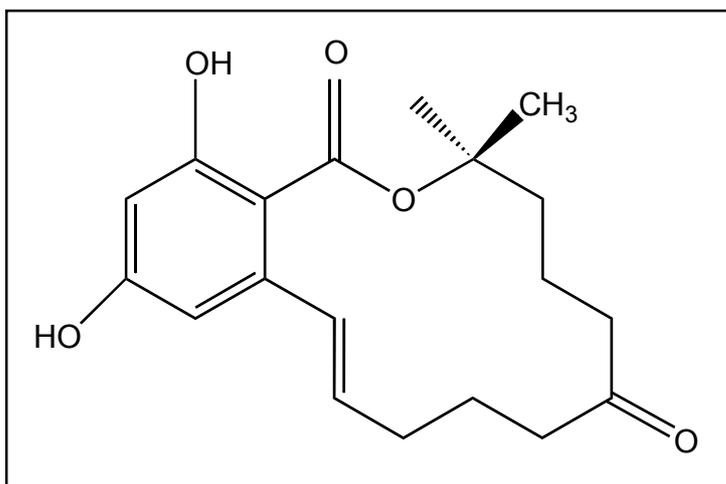
## 1 INTRODUCTION

### 1.1 Mycotoxins

Mycotoxins are low molecular weight compounds produced by many fungal species. Mycotoxins are “secondary metabolites”; this definition refers to the distinction that these metabolites are not essential for the survival and the growth of the fungi (primary metabolism). Generally speaking, secondary metabolites play an important economical role, being either useful as antibiotics, or harmful as mycotoxins. Over 300 mycotoxins synthesized by about 350 different fungi species are known (Cole and Cox, 1981). Among the mycotoxin producers the most important ones belong to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Betina, 1989).

### 1.2 Zearalenone

Zearalenone (ZON) was isolated in 1962 by Stob (1962) and derives its name from the fungus *Giberella zeae* (perfect state of *Fusarium graminearum*) from whose mycelia contaminating maize (*Zea mays*) was first isolated. Its structure (Figure 1-1) was elucidated in 1966 by Urry (1966), who named the discovered compound F-2, later on re-named ZON.



**Figure 1-1:** Chemical structure of zearalenone.

#### 1.2.1 Chemical and physical properties

ZON can be identified with the systematic name 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione or 6-(10-hydroxy-6-

oxo-*trans*-1-un-decenyl)- $\beta$ -resorcyclic-acid-lactone or by the CAS number 17924-92-4. Together with other compounds it belongs to the group of resorcyclic acid lactone (RALs) (Budavari, 1989). ZON, C<sub>18</sub>H<sub>22</sub>O<sub>5</sub>, has a molecular weight (MW) of 318.36, and is a white crystalline compound with a melting point of 164-165 °C. ZON is naturally fluorescent under ultraviolet (UV) light, its spectrum is characterised by three maxima at UV<sub>max</sub> 236, 274 and 316 nm with an extinction coefficient in methanol of 29,700, 13,900 and 6,020, respectively. This mycotoxin is practically insoluble in water, while it is soluble in aqueous alkaline solutions, ethyl acetate, acetonitrile, alcohols, diethyl ether, benzene, chloroform and methylene chloride (Betina, 1989; Budavari, 1989).

### **1.2.2 Producing organism**

ZON has been isolated from strains of several species belonging to the genus *Fusarium* such as: *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. nivale*, *F. roseum*, *F. tricinctum*, *F. sporotrichioides*, *F. oxysporum*, *F. verticilloides (moniliforme)*, *F. gibbosum*, *F. avenaceum*, *F. semitectum* and the teleomorphic states (sexual state) *Giberella zeae* and *G. fujikuroi* (Betina, 1989, Logrieco et al, 2002). *Fusarium* genus is characterised by the production of macroconidia, which are septate and fusiform; this genus produces fast growing colonies with felty aerial pale or brightly coloured mycelium (Pitt and Hocking, 1985).

*Fusarium* species are widely spread in soil, especially if cultivated, and are able to decompose cellulosed plant materials leading to plant diseases of major interest such as *Fusarium* head blight (FHB) in wheat and ear rot in maize (Bottalico and Perrone, 2002; Logrieco et al, 2002).

*Fusarium* colonise cereals in the field (flowering is the optimal phenological phase), but are able to continue their toxic activity during the storage period. The production of mycotoxins during this period is influenced by the interaction of several factors, such as moisture level of the seeds, air temperature and humidity, fungal strains, extent of infection (Moss, 1984). In a study testing 36 different *Fusarium* species isolated from corn, it was shown that only a few of them were able to produce ZON at different water activities, incubation times and temperatures (Jeménez et al, 1996). Although several ZON producing *Fusarium* strains have been isolated, the most important ones are *F. graminearum* and *F. culmorum* followed by *F. equiseti*, *F. cerealis* and *F. semitectum*

(Bottalico et al, 1983; Logrieco et al, 2002). ZON is often associated with  $\alpha$ -zearalenol (ZOL) and  $\beta$ -ZOL (Logrieco et al, 2002).

### 1.2.3 Natural occurrence of zearalenone

ZON is among the most widespread *Fusarium* mycotoxins in agricultural commodities (Logrieco et al, 2002). Unexpectedly high concentration of mycotoxins can be found in relation to the severity of the *Fusarium* infection. Surveys on ZON occurrence in several commodities are routinely published, ZON varying within a wide concentration range, which is mainly influenced by the considered matrix, the harvest season (year), the climate and the countries. Data from the most recent published surveys on ZON occurrence in corn and wheat (both matrices were further investigated in this study) are presented in Table 1-1.

**Table 1-1:** Recent data on the occurrence of ZON in corn and wheat in several countries.

Commodity	Positive Samples range $\mu\text{g/Kg}$	Country	Reference
Corn, corn flakes	2-20	Switzerland	Rhyn, 2003
Wheat	2-10	Switzerland	Rhyn, 2003
Wheat flour	1-24	Germany	Schollenberger, 2002
Wheat	Not detected	Finland	Eskola, 2001
Corn	10-100	Switzerland	Noser, 2001
Corn	36.8-719	Brazil	Vargas, 2001
Corn	2-7300	Korea	Sohn, 1999
Corn	60-1350	Hungary	Rafai, 2000
Wheat	50-890	Hungary	Rafai, 2000

### 1.2.4 Fate of zearalenone

ZON is a very stable compound, which is not affected by high temperatures. In alkaline conditions the ZON lactone ring can be hydrolysed and cleavage of the ester ring leads to loss of ZON (Krska and Josephs, 2001; Schuhmacher et al, 1998). Experiments carried out during the development of this project have shown that ZON is extremely

unstable in sodium hydroxide and potassium hydroxide, while it is stable in bicarbonate and triethylamine (TEA) aqueous solution.

During grain processing, the water insolubility of ZON plays an important role in the redistribution of this mycotoxin in different fractions such as in gluten (49-56%), solubles (17-26%), fibre (15-19%), and germ (9-11%), while it was not found in starch (Bennet et al, 1978; Lauren and Ringrose, 1997). No appreciable effects were registered on spiked corn having undergone typical cooking conditions such as inoculation at different humidity levels (10-50%) and at temperatures ranging from ambient temperature up to 110 °C for a period from 2 up to 12 days (Lauren and Smith, 2001); a great reduction of ZON levels has been reported during the extrusion process (Ryu et al, 1999).

### ***1.2.5 Toxicokinetics***

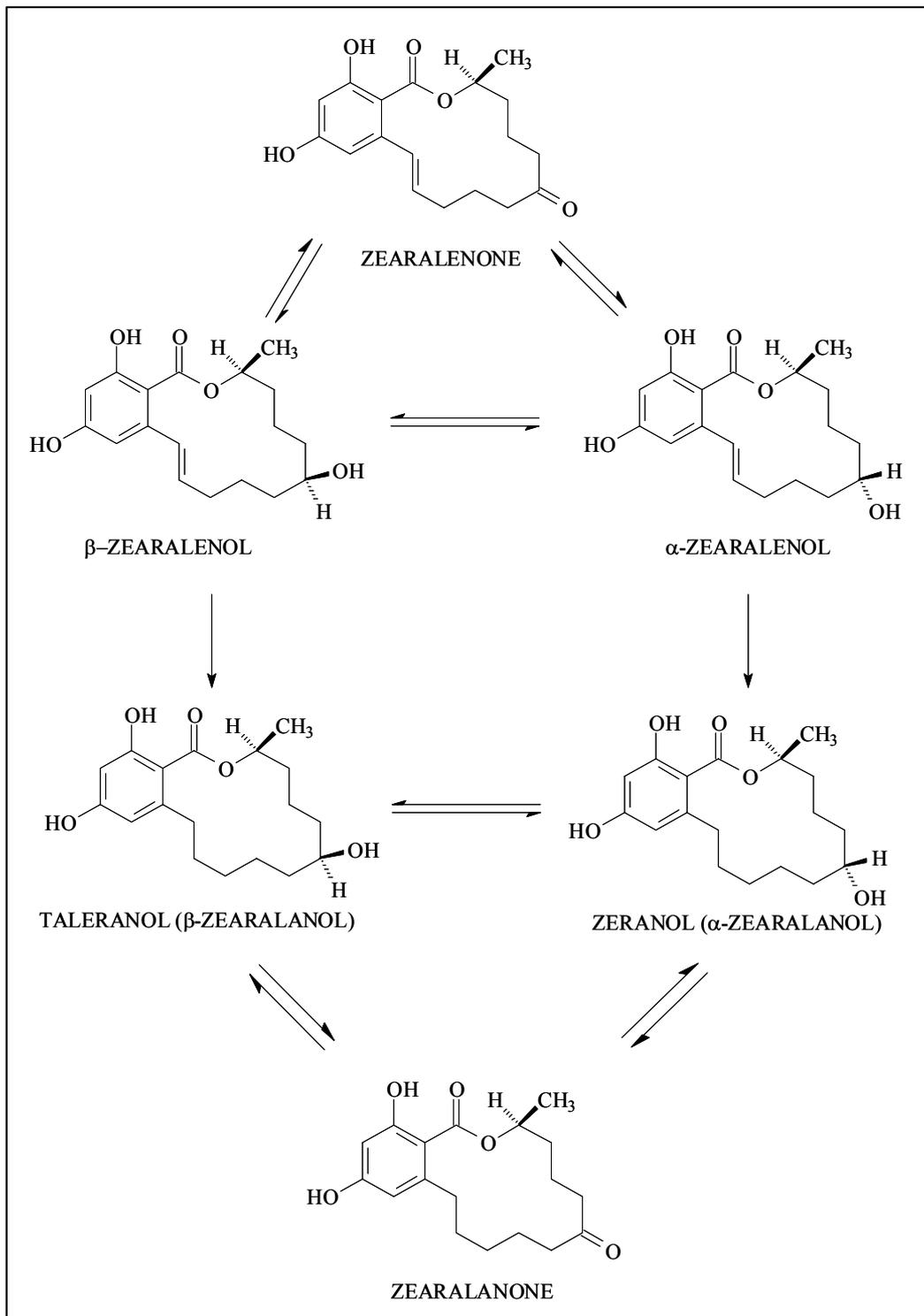
ZON is fairly rapidly absorbed following oral administration. In mice and rats ZON seems to be excreted through the bile duct with entero-hepatic circulation, while in rabbits, pigs and humans (as far as the very limited data available allow to reach a conclusion) excretion is through the urinary tract (Kuiper-Goodman et al, 1987). The in vivo metabolism of ZON is presented in Figure 1-2, showing that ZON can be transformed into  $\alpha$ -ZOL and  $\beta$ -ZOL, and both can undergo a further reduction leading to zeranol (ZAL) and taleranol (TAL), respectively. In vivo ZON and its metabolites are conjugated with glucuronic acid (Kuiper-Goodman, 1987).

The concentration of each metabolite is strictly dependent on the species considered. In swine ZON is mainly converted in  $\alpha$ -ZOL (Bauer et al, 1987; Biehl et al, 1993); a recent study has evidenced that in pigs ZON is metabolised mostly in  $\alpha$ -ZOL, but  $\beta$ -ZOL does occur in different ratios depending on the investigated tissue or biological fluid ( $\alpha$ -ZOL /  $\beta$ -ZOL liver = 2.5; urine = 3) (Zöllner et al, 2002).

Ruminants metabolise ZON predominantly to  $\beta$ -ZOL (Jodlbauer et al, 2000) or equally to  $\alpha$ -ZOL and  $\beta$ -ZOL (Kennedy et al, 1998). ZOLs may undergo a further reduction resulting in partly significant concentration of ZAL and TAL (Zöllner et al, 2002). In slight disagreement Kennedy et al (1998) reported that while  $\alpha$ -ZOL could be converted to ZAL,  $\beta$ -ZOL reduction to TAL does not occur. This complex metabolism in ruminants leads to legal problems since ZAL falls into the existing European Union ban on administration to domestic livestock of all hormonal substances used as growth

promoters (Council Directive 88/146). In fact ZAL can be detected in bile following in vivo metabolism of naturally occurring ZON.

In humans exposed to ZON contaminated foods, the mycotoxins have mostly been found in urine as glucoronide conjugate of ZON and  $\alpha$ -ZOL (Kuiper-Goodman et al, 1987).



**Figure 1-2:** Metabolism pathway of ZON.

### ***1.2.6 Toxicity***

ZON was evaluated by the International Agency for Research on Cancer (IARC) in 1993, based on evidence judged to be inadequate in humans and limited on experimental animals ZON was placed into Group 3, containing all the compounds which are not classifiable as carcinogenic to humans (IARC, 1993; IARC, 1999). Nevertheless, a provisional maximum tolerable daily intake (PMTDI) of 0.5 µg/Kg body weight (bw) has been established on the basis of the no observed effect level (NOEL) of 40 µg/Kg bw in a 15 days study on pigs (Kuiper-Goodman et al, 1987). A safety factor of 100 was utilised for determining the PMTDI. On the basis of the same data and a safety factor of 200, the European Commission obtained a tolerable daily intake (TDI) of 0.2 µg/Kg bw (European Commission, 2000).

The different toxic effects of ZON are reported below.

#### *Acute toxicity*

ZON has shown low acute toxicity in mice and rats after either oral or intraperitoneal administration; Marasas (Marasas et al, 1979) reported that a single dose of 20,000 mg/Kg does not cause death in these species. The lethal dose (LD<sub>50</sub>) has been estimated between 4,000 and 20,000 mg/Kg bw. A dietary level of 500 µg/Kg of ZON is considered to be a biological active dose, which should not be consciously given to breed animals (Marasas et al, 1979).

#### *Sub-chronic and chronic toxicity*

In long term experiments the effect in experimental as well as in domestic animals appeared to be dependent on the interaction between ZON and the estrogen receptor (ER). Pigs are definitely the most sensitive species. Discrepancies can be found in the reported NOEL for pigs set either at 40 µg/Kg bw per day (Edwards et al, 1987; Kuiper-Goodman et al, 1987) or at 60 µg/Kg bw per day (Prelusky et al, 1990); whereas the NOEL for rats is 100 µg/Kg bw per day (Kuiper-Goodman et al, 1987).

Long-term studies (over 100 weeks) carried out on mice fed with 0.5 or 100 mg/Kg ZON, did not show any difference in survival and body weight between treated- and control-groups. No neoplastic-lesions were evidenced in males, while in females estrogen related effect were evidenced in several tissues (NTP, 1982).

#### *Genotoxicity*

ZON has been tested using several genotoxicity tests; it does not induce mutations in

the Ames test or mitotic crossover, it was also negative in the eukaryotic cell mutation assay with *Saccharomyces cerevisiae* (Wang and Groopman, 1999); while it shows a positive DNA damaging effect in the recombination test performed with *Bacillus subtilis* (Ueno and Kubota, 1976), sister chromatid exchange (SCE), chromosomal aberration in hamster and weak effects in human lymphocytes. There is no evidence of DNA-adduct formation in rats and only limited on mice (Li et al, 1992).

### Immunotoxicity

*In vitro* experiments have shown several alterations of immunological parameters (increase interleukine (IL)-2 and IL-5 production) (Eriksen and Alexander, 1998) when testing high concentration of ZON. Administration of ZON (Pestka et al, 1987; Forsell et al, 1986) and of ZOLs (Pung et al, 1985) did not evince differences between control and treated groups in the serum concentration of immunoglobulin (Ig) M, IgG, IgA and in white blood cell count, which are typical indicators of immunotoxic activity.

### Reproductive and developmental toxicity

The most important toxic effect of ZON is, without any doubt, the estrogenic effect, which has recently led to identify ZON and ZOLs as endocrine disrupters.

The chemical structure of ZON and of its derivatives resembles 17 $\beta$ -estradiol (E2), enabling them to competitively bind to the ER as has been demonstrated in several *in vitro* experiments carried out using various cell lines of different species. For example, the affinity of ZON to uterine and oviduct ER followed this order: pig, rat and chicken (Coulombe, 1993). Based on a proliferation assay performed on MCF7 human breast cancer cell, the following relative estrogenicity compared to E2 has been found:  $\alpha$ -ZOL=92%, ZAL=18%, TAL=3.5, ZON=1% and  $\beta$ -ZOL=0.44% (Shier et al, 2001).

Evaluation of the estrogenic potency using an *in vivo* fish model reported values of about 50% compared to E2 with the following decreasing order  $\alpha$ -ZOL>ZON> $\beta$ -ZOL, emphasising a remarkable discrepancy between binding affinities, obtained *in vitro*, and the estrogen-regulated end product observed *in vivo*, where smaller differences between  $\alpha$ -ZOL, ZON and E2 have been observed (Arukwe et al, 1999).

Experiments performed on human cell lines, transfected using both  $\alpha$ -human estrogen receptor ( $\alpha$ -hER) and  $\beta$ -hER, have shown that ZON activates both regions, behaving as a full antagonist for  $\alpha$ -hER and as a mixed agonist-antagonist for  $\beta$ -hER (Kuiper et al, 1998).

In vivo ZON causes alterations of the reproductive tract on laboratory animals (mice, rat, hamster), decreased fertility, increased embryo-lethal re-absorption, changes in weight of adrenal, thyroid and pituitary glands, variation in serum levels of hormones (estradiol and progesterone) (Williams et al, 1989).

Swine are the most sensitive species. The most evident symptoms of ZON poisoning, enlargement, swelling and reddening of the vulva, are evident when ZON feed contamination exceeds 1 mg/Kg, but they can also occur at lower concentrations (Mirocha et al, 1977). Indeed, Bauer et al (1987) reported estrogenic effects at low ZON doses (50 µg/Kg in the diet) in prepubertal female pigs. In this study the typical ZON symptoms, such as redness and swelling of the vulva, swelling of the mammary glands and many vesicular follicles and cystic follicles on the ovaries, were observed when higher doses of ZON were administered. Although external changes were not appreciable in the low dose treatment group, the autopsy revealed vesicular follicles on the ovaries. The high female susceptibility to ZON has not been observed in male pigs, although some studies have reported that ZON can cause inflammation of the prepuce, reduced testes, epididymides, vesicular glands weight and cessation of spermatogenesis in boars (Diekman and Green, 1992), but no significant effects were reported when boars received contaminated feed at level of 1 mg/Kg (Stolla et al, 1987), 40 mg/Kg (Berger et al, 1981) and even 200 mg/Kg of complete ration (Ruhr et al, 1983). According to these studies ZON does not have adverse effect on the reproductive potentiality of mature boars, so the differences in results could be due to the synergistic effect of other mycotoxins when naturally contaminated feed has been used during the study.

Ruminants are definitely more tolerant to ZON than pigs. ZON exposure in ewes determined decreased ovulation rate and an increase of estrus length, but did not affect the pregnancy rate or embryonic loss (Smith et al, 1990). When pure ZON has been given to dairy heifers at a dose of 250 mg/day, no significant effects were noticed, despite a slightly lower conception rate, which in any case remained very high in the treated group (Weaver et al, 1986 a). Similar results were obtained giving pure ZON (31.25 mg/day up to 500 mg/day) to dairy cows, where the only appreciable effect of ZON intake was an apparently smaller corpora lutea (Weaver et al, 1986 b). The results of other studies, in which ZON exposure was associated to hyperestrogenism (Diekman

and Green, 1992), infertility and decreased milk yields (Bloomquist et al, 1982), could again be due to the simultaneous presence of other mycotoxins in the feed used to carry out the experiments. Poultry are quite resistant (Prelusky et al, 1990).

There are few epidemiological studies suggesting the estrogenic potency of ZON in humans. ZON and ZOLs were suspected to be the cause of epidemic precocious pubertal changes in young children in Puerto Rico (Saenz de Rodriguez, 1984) and of increased incidence of early telarche in the south-east region of Hungary, where high levels of ZON have been found in serum (Szuetz et al, 1997).

### ***1.2.7 Zearalenone legal limit***

The increasing awareness of mycotoxin risks are leading to setting guidelines and/or regulatory limits for mycotoxins. Indeed, an increase of 35% in countries known to have mycotoxin regulations has been registered from 1987 to 1997 (Food and Agriculture Organization of the United Nations (FAO), 1997). Most of these regulations concern aflatoxins. At European level legislation has only been harmonised for aflatoxin and ochratoxin, while some countries have already set limits for other mycotoxins. Few countries have applied the “zero tolerance level” approach for one or more matrices, that has been judged as impractical by FAO (1997) for a number of reasons. First of all, as natural toxicants, mycotoxins cannot be completely avoided in the food/feed chain; enforcement of such a strict regulation requires reliable analytical methods and so far there are no methods with a limit of detection (LOD) equal to zero. Table 1-2 shows ZON legal or recommended limits set by some countries on different commodities. Few discrepancies on the limit or the regulated commodities have been found in the literature.

**Table 1-2:** Maximum limit for ZON in foods and feeds in various countries.

Country	$\mu\text{g}/\text{Kg}$ $\mu\text{g}/\text{L}$	Matrix	Remarks	Reference
Austria	60	Cereals (Food)		Creppy, 2002
	50	Feed for pubertal female pig	Rec	Razzazi Fazeli, 2003 personal communication
	150	Feed for sow		
Germany	50	Feed for pubertal female pig	Rec	Razzazi Fazeli, 2003 personal communication
	250	Feed for sow		
	100	Feed for calf		
	500	Feed for dairy cow		
France	200	Cereals, vegetable oils		Conseil Supérieur d'Hygiène Publique de France
Hungary	500	Feed	AL	Rafai, 2000
	80	Feed for reproducing animal		
Italy	20	Baby food		Ministero della Sanità della Repubblica Italiana
	100	Cereals		
Romania	30	Cereals, vegetable oils		Creppy, 2002
		All food		FAO, 1997
Russia	1000	Cereals; vegetable oils, nuts		FAO, 1997
Brasil	200	Maize		Legislação sobre micotoxina
Cyprus *	0.5	Milk, dairy products		FAO, 1997
Canada *	0	Feedstuff for reproducing animal	Rec	FAO, 1997
The Netherland *	0	Cereals, pulse, legume		FAO, 1997

\* Limit set for all mycotoxins  
 Rec = Recommendation Limit  
 AL = Advisory Limit

### **1.3 Analytical methods for zearalenone**

Analytical methods can be divided into the following steps: extraction, clean-up, separation, detection and quantification. The characteristics of each step contribute to the final performance of the analytical method.

#### **1.3.1 Extraction**

In the analytical method sample extraction is the first step whose efficiency influences all the following steps. Indeed, only a complete extraction of the target analyte from the matrix justifies the development of sophisticated techniques in the following steps.

ZON is generally extracted either by conventional liquid shaking (SHA) for a period varying from 30 min up to 1 h (Tanaka et al, 2000; Fazekas and Tar, 2001) or by blending (BLE) for few minutes (ISO, 2001; Krska and Joseph, 2001). Beside various extraction mixtures have been used to extract ZON from cereals (Joseph et al, 2001), the most commonly used are acetonitrile-water (Visconti and Pascale, 1998 a; Kruger et al, 1999; Eskola et al, 2002), and methanol-water (Fazekas and Tar, 2001; Llorens et al, 2002) mixed at different ratios.

Recently, several studies have been carried out to compare the efficiency of different extraction procedures for mycotoxin analysis. The most investigated factors were the solvent extraction mixture and the solvent/matrix ratio. Although most of these studies have been performed on aflatoxins and fumonisins, it is worthwhile to consider the results obtained for other mycotoxin analyses too. The most relevant discovery is that, when dry material is extracted, the use of an aqueous solution based on acetonitrile could result in phase separation and water absorption leading to artificially higher recoveries (Stroka et al, 1999). After this discovery other authors have also taken this misleading phenomenon into consideration before drawing conclusions (De Girolamo et al, 2001; Solfrizzo et al, 2001). In the extraction of fumonisin, the extraction solvent mixture acetonitrile-water was replaced with a modified mixture acetonitrile-methanol-water (Solfrizzo et al, 2001; Visconti et al, 2001), where the presence of methanol avoids phase separation. In the same extensive study no statistical differences were observed when comparing shaking (SHA) versus blending (BLE) and different solvent/matrix ratios. It was reported that for difficult matrices (corn flakes) the extraction efficiency greatly improved when multiple versus single extraction was

performed (Solfrizzo et al, 2001; Visconti et al, 2001).

In a work focused on ZON, the performance of five different extraction solvent mixtures have been compared and the mixture methanol-1% aqueous sodium chloride (80:20 or 60:40 v/v) and acetonitrile-water (86:14 v/v) provided similar recoveries (Llorens et al, 2002).

Although several studies have been published on the influence of different factors on extraction efficiency in mycotoxin analysis, sample extraction is still nowadays the least developed part of almost all analytical methods, and it remains a critical time-consuming step. Over the last decade the demand for modern extraction techniques has driven to the development of alternative extraction techniques supported by external sources of energy such as Microwave Assisted Extraction (MAE) and Pressurized Liquid Extraction (PLE) [known with the trade name of Accelerated Solvent Extraction (ASE)]. MAE and PLE techniques enable the process to be automated, shorten the required extraction time and reduce the organic solvent consumption (Sparr-Eskilsson et al, 2000). Although alternative extraction methods have already found their way into environmental analysis and are now starting to be applied in pharmaceutical analysis, their use is still very limited in food analysis. Mycotoxin analysis does not constitute an exception; only a few papers applying alternative extraction techniques have been published. So far MAE has been used solely for the determination of ergosterol, a marker of fungi contamination, allowing the extraction and the saponification step to be performed simultaneously (Young, 1985). PLE has been applied for the determination of fumonisin (Lawrence et al, 2000; Klaffke et al, 2000) and recently for ZON (Rhin and Zoller, 2003). The other published papers on MAE and PLE are part of the present thesis (Pallaroni et al, 2002; Pallaroni and von Holst, 2003 a; 2003 b). Interesting conclusions have been drawn when the effect of temperature on fumonisin extraction by PLE was examined (Lawrence et al, 2000); a clear influence of temperature and solvent composition on the target analyte recovery was shown; in some corn-products the level of extracted fumonisin tripled, passing from ambient temperature to 80 °C. Moreover the temperature enabled the use of new, more environmentally friendly and less toxic extraction solvent mixtures such as ethanol-water 3:7 v/v and water 100% (Lawrence et al, 2000).

### *1.3.2 Clean-up*

Traditionally, the clean-up of ZON was based on liquid-liquid partitioning with chloroform-aqueous sodium hydroxide (Mirocha et al, 1974, Schuhmacher et al, 1998). The exposure of ZON to sodium hydroxide should be minimized to avoid losses of ZON, therefore this technique requires an experienced analyst to achieve reproducible and reliable results. To avoid the drawbacks of liquid-liquid partitioning, the trend is to substitute it by a solid phase extraction (SPE), which can be performed using different solid phases such as Florisil, aluminium oxide, C-8, C-18 and most recently Mycosep<sup>®</sup> (Silva and Vargas, 2001). SPE cartridges represent an advantage compare to liquid-liquid partitioning because they are easier to use and provide good recoveries and reproducible results, especially if commercial products are used. As a main disadvantage, the lack of specificity should be mentioned and, when columns are self packed, the low reproducibility.

In a recent study, three SPE cartridges for ZON clean-up were compared; Florisil provided comparable results to C-18, though the chromatograms obtained using the C-18 extracts showed more peaks, therefore Florisil should be preferred (Llorens et al. 2002; Mateo et al, 2002). The third stationary phase considered was silica, which has been shown to be totally unsuitable for ZON purification, providing a recovery lower than 20%; however, these data disagree with a previously reported recovery of 94% (Eskola et al, 2001); in cases like this, the different recoveries could also depend on existing differences within the column, using the same stationary phase but from different producers.

Over the last years, the production of specific antibodies for mycotoxins led to the development of immunoaffinity (IA) based columns, which gave a strong impulse to improving mycotoxin clean-up. IA columns are easy to use and provide good recoveries, repeatability and reproducibility. The main advantages of IA columns are high selectivity and specificity, which allow particularly clean chromatograms to be obtained compared to the one registered for samples purified by SPE, with two resulting aspects: on one side, it facilitates the analysis of the most complex matrices starting from cereals-based foods and feeds to biological fluid and animal tissues (Visconti and Pascale, 1998 a; b); on the other, allows the use of low-tech detection methods lacking in separation power such as thin layer chromatography (TLC) while retaining good

method performances (Stroka et al, 2000; Gilbert and Anklam, 2002).

Commercially available IA columns for ZON are based on monoclonal antibodies and present good cross reactivity with  $\alpha$ -ZOL,  $\beta$ -ZOL and zearalanone (ZAN), also enabling their use for the determination of some growth promoters (Visconti and Pascale, 1998 b).

Compared to SPE cartridges, IA have a lower sample capacity (4 $\mu$ g), so methods should be adapted by switching from SPE to IA columns. Although similar recoveries were obtained when comparing the performance of the two technologies SPE (Florisil and C18) and IA, the latter had a cleaner extract demonstrated by less interferences in the chromatogram (Zöllner et al, 1999; Mateo et al, 2002). The recoveries accomplished on corn using different clean-up procedures are reported in Table 1-3; it should be noted that the data presented by Mateo (Mateo et al, 2002) and Llorens (Llorens et al, 2002) differ only for the target contamination level, which in the second case has been standardized with respect to the highest value obtained (100%).

**Table 1-3:** Influence of the different clean-up steps on the recovery of ZON.

Clean-up method	Recovery %	ZON target concentration	Reference
Silica	94	25 ng/g	Eskola, 2001
Liquid/liquid	80	10;30;50;100;200 ng/g	Schuhmacher, 1998
IA	94		
RP-18	104.2	126 ng/g	Zöllner, 1999
IA	96.0		
C-18	91.5	40 $\mu$ g/g	Mateo, 2002*
Florisil	95.3		
Silica	16.9		
IA	91.8		
C-18	95.9	38.1 $\mu$ g/g	Llorens, 2002*
Florisil	100		
Silica	17.8		
IA	96.1		

To summarise, the use of IA columns boasts several advantages compared to the other clean-up methods, including: high selectivity leading to extremely clean extracts, high precision and accuracy in a wide range of concentration, ease of use, environmentally friendly due to the reduced use of organic/dangerous solvents. Despite the optimal performance of IA column for ZON (Visconti and Pascale, 1998 a; Kruger et al, 1999), its use might be limited due to the higher cost of these columns compared to most SPE cartridges (De Saeger et al, 2003).

Whichever clean-up techniques are used, this step experiences analyte losses. Liquid/liquid partitioning has almost been abandoned due to high sample handling, low reproducibility and the most probable analyte losses. Both SPE and IA provide good recovery. Overall IA reports better performances. However, the choice between SPE and IA techniques is mainly determined by the cost of IA together with the characteristics of each single study.

To underline the importance of IA, it must be mentioned that the analytical methods for mycotoxin determination most recently validated by the AOAC apply this technique.

### ***1.3.3 Detection and determination***

Analytical methods for the detection of ZON include both chromatographic and immunological methods.

#### *Chromatographic method*

Although there are methods for the determination of ZON by TLC and gas chromatography (GC), liquid chromatography (LC) is definitely considered the method of choice. ZON can be detected either by diode array detector (DAD) or by fluorimetry detector (FLD), the former allows the display of the whole UV spectrum, providing additional information and confirming the identity of the compound, but the latter is without any doubt the most sensitive. According to a recent study, the FLD reaches a LOD three times lower than DAD (Mateo et al, 2002). Several wavelengths can be used when working in fluorimetry. An interesting ZON determination was presented using an electrochemical detector (Hurst et al, 1987).

When high performance liquid chromatography (HPLC) was not available, ZON was detected by TLC, as confirmed by the first AOAC validated method published in 1976 (Shotwell et al, 1976). Nowadays the use of TLC is limited to confirmation purposes (Abbas et al, 1988); nevertheless, whenever HPLC is not available (too high investment

or lack of experienced staff) the application of TLC coupled to IA purification is foreseen. A simplified method for the determination of ZON without performing a chromatographic separation has been presented, in which the IA purified extracts were directly determined using a fluorometer allowing for a LOD of 100 ng/g (Kruger et al, 1999).

Determination of ZON by GC requires a tedious derivatization step. The development of existing GC methods for ZON was mainly driven by the aim of developing simultaneous methods for several mycotoxins, since ZON often co-occurs with trichothecenes (Tanaka et al, 2000).

#### *Liquid Chromatography – Mass Spectrometry (LC-MS)*

The state-of-the-art in chromatography is LC-MS. From its development almost 20 years ago, the use of LC-MS was restricted to a group of innovators, who mainly focused on mass spectrometry science. In the last 5 years the availability of commercial integrated LC-MS systems made this technology accessible to a broader analytical community of new users with a different background. Thus resulting in an increasing number of applications. Before 1997, which could be considered a milestone in LC-MS history, applications in food analysis, including mycotoxins, were limited. In fact, the few published papers dealt principally with the most critical mycotoxins, which normally needed a derivatization step to be determined, such as trichothecenes (Voyksner et al, 1987; Krishnamurthy et al, 1989; Kostianen, 1991; Kostianen and Kuronen, 1991; Kostianen et al, 1991) and fumonisin (Young and Lafontaine, 1993; Thakur and Smith, 1994; Doerge et al, 1994). With the introduction of the Atmospheric Pressure Interfaces (API) LC-MS became easier to be used, moreover the commercially available instruments became quite affordable. Nowadays LC-MS is becoming a routine tool for several scientific disciplines. From 1997 up to now, the number of publications applying LC-MS as a detection method for mycotoxins analysis has greatly increased.

Most likely, determination of ZON by LC-MS has been limited due to the good performance of the conventional methods (Visconti and Pascale, 1998 a; b).

A new interface design has been presented in the first published paper on ZON determination by LC-MS. In this new Atmospheric Pressure Chemical Ionization (APCI), the HPLC inlet is perpendicular to the ion optics, preventing uncharged molecules from entering the mass spectrometer (MS), thus resulting in a lower

background (Rosenberg et al, 1998). Most of this work dealt with interface optimization, finally preferring positive ionisation mode due to a higher stability of the signal and resulting in a more favourable signal-to-noise. The second part of this work attempted to reduce the overall time required for analysis, either by injecting the raw extract directly or by shortening the chromatographic separation. Both approaches can be considered to exclude samples not containing the target analyte, re-routing the others to a more precise quantification method. It is worthwhile underlining that no internal standard (ISTD) has been used. Although an overestimation of the actual ZON concentration has been observed when raw extracts were injected, it should be noted that this overestimation (30%) was comparable with the one obtained injecting extracts purified by IA column (24%) (Rosenberg et al, 1998).

In disagreement with the above mentioned study, Zöllner et al (1999) selected to work in the negative ionisation mode, where a better signal-to-noise was recorded.

The widespread perception that the high selectivity obtained working in MS-MS mode allows the matrix effect to be eliminated, has been thoroughly investigated. Data clearly shows that the use of ZAN as ISTD greatly improves the accuracy of the obtained data. Indeed ZAN takes into account both variations due to the MS detector, which are estimated at about 10% due to the matrix effects deriving from the presence of compounds co-eluting with the target analytes. Although the authors foresaw the use of a deuterated-ISTD as a best choice, ZAN remains an ideal ISTD for compensating the matrix effect. Comparison of the use of SPE versus IA clean-up provided similar performances, however, SPE is preferable, as it is less selective and allows for the development of a multi-mycotoxins method. Moreover IA results in a loss of 20% of ZAN.

Further investigations performed on beer have confirmed the importance of the use of ISTD, although the authors underlined that elimination of the matrix effect is possible only within each single beer brand (Zöllner et al, 2000). However, a deviation within the range  $\pm 4.4\%$  has been noted from evaluation of the raw data presented in the paper, meaning that when an internal calibration is used, the deviation obtained for the different brand falls within the feasible variation given by the MS detector. In a recent publication (Zöllner et al, 2002) presenting a LC-MS-MS method for the determination of ZON and its metabolites ( $\alpha$ -ZOL,  $\beta$ -ZOL; ZAL, TAL and ZAN) in difficult matrices

such as urine, muscle tissue and liver, the authors took into consideration the conclusions of previous studies, developing a multi-method purification by SPE. Moreover, since ZAN can occur naturally in biological matrices, a deuterated-ZON was used as ISTD.

Table 1-4 presents the operation mode, MS type and performance of the LC-MS method for the determination of ZON, for a complete overview on existing methods, including the work developed during this thesis. It should be remembered that the limit of quantification (LOQ) is influenced by the concentration step performed during the analysis.

## INTRODUCTION

**Table 1-4:** Characteristics of LC-MS methods for the determination of ZON in various matrices.

Matrix	Clean-up	Interface	Ionization mode	MS	Operation mode	ISTD	Method LOD	Method LOQ	Reference
Food	None	APCI	Pos	Single-Quad	SIM	None	2.5 ng/mL	0.12 ng/g	Rosenberg, 1998
Feed	IA								
Cereals	RP-18 IA	APCI	Neg	Triple-Quad	MRM	ZAN	0.5 ng/g	1.0 ng/g	Zöllner, 1999
Beer	RP-18	APCI	Neg	Triple-Quad	MRM	ZAN	0.03 ng/mL	0.06 ng/mL	Zöllner, 2000
Urine	RP-18								
Muscle tissue	RP-18	APCI	Neg	Triple-Quad	MRM	D <sub>2</sub> -ZON	0.1-0.5 ng/g	0.5-1.0 ng/g	Zöllner, 2002
Liver	SPE								
Wheat	Florisil	ESI	Pos	Single-Quad	SIM	None	/	10 ng/g	Schneweis, 2002
Corn	None	APCI	Neg	Ion-Trap	Full	ZAN	2 ng/g	20 ng/g	Pallaroni, 2002
Wheat					scan		3 ng/g	30 ng/g	
Urine	RP-18 NH <sub>2</sub>	ESI	Neg	Triple-Quad	MRM	D <sub>4</sub> -ZAL D <sub>4</sub> -TAL	0.03 ng/mL	< 1.0 ng/mL	van Bennekom, 2002
Corn	None	ESI	Neg	Ion-Trap	Full	ZAN	4 ng/g	12 ng/g	Pallaroni, 2003 a
Wheat					scan		5 ng/g	15 ng/g	

*Immunological methods*

Simple and rapid immunoassay methods have been developed since the availability of antibodies specific for several mycotoxins, including ZON. Several Enzyme Linked ImmunoSorbent Assay (ELISA) kits, based either on polyclonal or monoclonal antibodies, are nowadays commercially available. After extracting the sample, the extract is applied directly onto the kit; the main advantage of the ELISA kit is rapidity, as the fastest ELISA kit only needs 15 min to provide results, assuring a LOD of 50 ng/g, while longer kits allow for a LOD of 0.2 ng/mL (Barna-Vetró et al, 1994). In principle, ELISA test kits are able to provide rapid results ensuring good accuracy (Krska and Joseph, 2001). It has been shown that ELISA kits are also affected by the matrix effect, since the extract is applied onto the kit without performing any clean-up step (Hock et al, 1992; Barna-Vetró et al, 1994).

To conclude, several methods for the determination of ZON can be applied. A realistic picture of those most frequently used and preferred by monitoring and control laboratories can be obtained by evaluating the two most recent proficiency test reports provided by Food Analysis Performance Assessment Scheme (FAPAS<sup>®</sup> 2000; 2002).

The extraction step is generally performed using the extraction solvent mixture acetonitrile-water (59%) followed by methanol-water (14%). Only conventional extraction techniques are used, with a preference for SHA (67%) versus BLE (33%); single extraction is by far the most applied (82.5%), multiple extraction only rarely (8.5%). The clean-up is normally based on the most reliable and selective IA columns (74%), SPE almost one quarter (21%). For the detection TLC, GC and fluorometry are seldom applied, 3%, 4.5% and 3%, respectively. ELISA covers 10%, while HPLC is definitely the method of choice (74%). Different detectors can be coupled to HPLC, the favoured one being FLD (72%), followed by DAD (20%) while MS is still rarely applied in routine analysis (8%).

## 2 OBJECTIVE OF THE STUDY

Today's awareness of food safety has led to an increasing interest in mycotoxins, resulting in a constantly growing number of analyses required to be performed routinely. For this reason, fast, reliable, efficient, automated and economic analytical methods are required.

This thesis discusses the possibility to develop a fast automated method for the determination of ZON in cereals, which are the most frequently contaminated commodities. Two different approaches were considered in this attempt; on one hand the development of a fast detection method based on direct injection of raw extracts into LC-MS without performing the clean-up step; on the other the application of modern extraction techniques allowing to reach fast and automated sample extraction.

Firstly, the influence of several MS interface parameters on the target analyte signal was investigated (Appendix I). The strong role played by the matrix effect has to be carefully considered when developing an LC-MS method injecting raw extracts. A wide investigation on how to overcome or at least minimize the matrix effect has been performed (Results and Discussion, 4.1). Finally, a fast method for the determination of ZON without performing the clean-up step was developed.

The possibility to analyse raw extract without performing the purification step allowed losses of the target analyte occurring during the clean-up to be completely avoided, therefore it was possible to clearly evaluate the efficiency of alternative extraction techniques with emphasis on MAE (Appendix II) and PLE (Appendix III). A factorial design approach was utilised to choose the most suitable extraction conditions. As a logical conclusion, the extraction efficiencies of conventional and alternative extraction techniques, for a total of five different methods, were compared on a batch of 8 corn samples (Appendix IV). Lastly, an attempt to reduce the use of organic solvent in favour of aqueous solutions was performed by coupling PLE, environmentally friendly solvents and LC-MS (Appendix V).

### 3 MATERIALS AND METHODS

#### 3.1 Samples

Spiking experiments were performed on uncontaminated wheat and corn samples grown during an *in field* trial under controlled conditions and tested for ZON presence. Naturally contaminated corn samples belonging to different harvest seasons were collected from various countries (Austria, France, Germany, Argentina, Italy). When naturally contaminated samples were not available, method performance was determined by analysing wheat and corn which had been previously used in proficiency tests organized by FAPAS<sup>®</sup> (DEFRA, UK). The characteristics of these materials are described in the respective reports for wheat (FAPAS<sup>®</sup>, 2000) and for corn (FAPAS<sup>®</sup>, 2002).

All samples were ground in a laboratory ultra centrifuge mill ZM100 (Retsch, Haan, Germany) using a 0.5 mm sieve. Samples were mixed in a head over heels mixer (Turbula Type T2 F, WAB, Basel, Switzerland) to assure sufficient homogeneity of each sample and stored at  $-20^{\circ}\text{C}$ .

#### 3.2 Equipment

##### 3.2.1 Extraction Equipment

Several extraction techniques have been applied during the development of the project. The following equipment was used:

<u>Blending:</u>	Stick homogenizer Ultra-Turrax 125 (IKA-Labortechnik, Staufen, Germany)
<u>Shaking:</u>	Horizontal shaker Compact Shaker SK 15 A (Edmund Bühler, Tübingen, Germany)
<u>Ultrasonication:</u>	Stick sonicator U 200 S control (IKA-Labortechnik, Staufen, Germany), which allows varying amplitude and cycle
<u>Microwave Assisted Extraction:</u>	A closed-vessel microwave assisted extraction unit, MSP 1000 (CEM Corp., Matthews, NC, USA)

Pressurized Liquid Extraction: ASE<sup>TM</sup> 200 System (Dionex, Sunnyvale, CA, USA)

### 3.2.2 HPLC-Equipment

All analyses were performed in reversed phase (RP)-HPLC using a LC-MS-ion trap for method development and an HPLC-FLD when comparing the developed method with the most commonly used one. The following instruments were used:

**LC-MS** - SpectraSystem (Finnigan Mat, San Jose, CA, USA) consisting of a SCM degasser, a P4000 (low flow) quaternary pump and an AS3000 autosampler. The HPLC system was coupled to an MS ion trap, LCQ-Deca (Finnigan Mat, San Jose, CA, USA) equipped with an APCI interface.

- HP 1100 Series HPLC equipped with a degasser, a binary or quaternary pump (depending on the setting), an auto-injector and thermostat coupled to an ion-trap mass spectrometer (LC-MSD) equipped with an Electrospray Ionization (ESI) or APCI interfaces (Hewlett-Packard, Palo Alto, CA, USA). In addition, instruments with slightly modified performances such as the Esquire-LC ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) and the ion-trap mass spectrometer HP (MSD-SL), which is an improved version of the LC-MSD, were used.

**HPLC-FLD** - HP 1100 Series HPLC equipped with a degasser, a binary pump, an auto-injector, thermostat and FLD ( $\lambda_{\text{exc}}=274\text{nm}$ ;  $\lambda_{\text{em}}=440\text{nm}$ ) (Hewlett-Packard, Palo Alto, CA, USA).

## 3.3 Development of the LC-MS method

### 3.3.1 Optimisation of the interface parameters

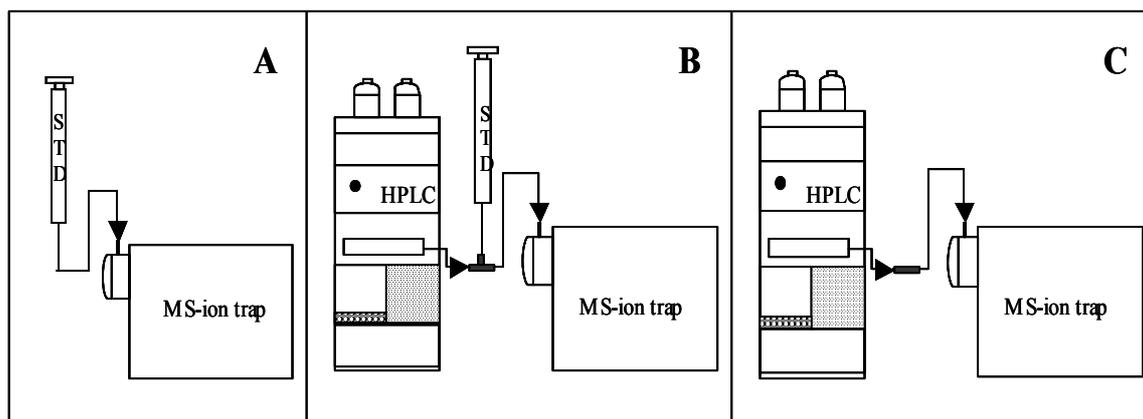
The role played by several interface parameters on the target analyte signal has been investigated. Three different set-ups were used:

*Infusion* (Figure 3-1 A): the standard solution was infused directly into the instrument interface; it was used to choose interface (ESI versus APCI) and operation mode (positive versus negative.);

*In-flow infusion* (Figure 3-1 B): the standard solution was infused after the column

directly into the LC flow, allowing the effect of the LC flow to be taken into account during optimization. This set-up was used for these interface parameters, which could be optimised automatically by the instrument. For the LCQ-Deca (Finnigan) they were capillary voltage, tube lens offset, first and second octapole offset, inter-octapole lens and entrance lens, while for the HP-LC-MSD capillary, skimmer, cap exit offset, octapole, trap drive and lenses.

*In-flow injection* (Figure 3-1 C): the interface parameters, not automatically optimised by the instrument, were optimised using this set-up, where the standard solution was injected into the flow, by-passing the chromatographic column and by manually increasing in steps the investigated parameters, which were, for the LCQ-Deca Finnigan, nitrogen sheath gas flow, auxiliary gas flow (only for APCI), vaporizer temperature, capillary temperature, and nebulizer, dry gas, dry temperature, APCI temperature (only for APCI) for the LC-MSD.



**Figure 3-1:** Scheme of the different settings utilized for interface parameters optimization. A) Infusion; B) In-flow infusion; C) In-flow injection.

Effects of mobile phase modifiers [formic acid (0.05%; 0.1%; 0.2%), acetic acid (0.05%; 0.1%; 0.2%; 0.5%) and ammonium acetate (10 mM; 50 mM)] were investigated in in-flow injection.

### 3.3.2 Matrix effect evaluation

An extensive investigation on the matrix effect has been performed for ZON analysis, both by applying a linear gradient and working in isocratic. The matrix effect has been

evaluated by comparing mobile phase-matched standard versus matrix-matched standard calibration curves.

The initial experiments were carried out on the LCQ-Deca instrument coupled to an APCI using a linear gradient; the matrix effect on ZON was investigated on several matrices (corn, wheat, barley, oat) within the range 62.5-1000 ng/mL on the extract, which corresponds to 250-4000 ng/g on the matrix.

More extensive and complete investigations on the matrix effect were performed when working in isocratic using the second instrument (LC-MSD). Calibration curves were prepared by dissolving aliquots of ZON standard solutions and a fixed amount of ISTD into mobile phase or different matrix extracts. Experiments were performed within the range 40-4000 ng/g of ZON (corresponding to 10-1000 ng/mL of ZON on the extract) on six types of corn (3 corn kernels, polenta, pop corn, corn-flakes), wheat, barley and two types of feedstuffs. Differences in working with or without the use of ISTD and full scan versus MS/MS mode were investigated. Evaluation of the matrix effect was performed by considering the linearity and the equation of each calibration curve, which was plotted in figures for a easier visual interpretation. Finally, in order to provide a clear and convenient estimation of the matrix effect, three hypothetical peaks having a peak area falling within the investigated concentrations range were quantified based on the different calibration curves. Based on the hypothetical area obtained by injecting crude extract, it is possible to calculate the concentration based on the matrix-matched calibration curve and the concentration which would have been obtained by performing the quantification on the mobile phase calibration curve. Comparison of the two concentrations allows to establish if injection of the crude extract performing the quantification on the mobile phase results in an over- or under-estimation.

### ***3.3.3 Zearalenone analysis***

Zearalenone analysis has been performed by injecting the filtered raw extract directly into LC-MS. The chromatographic separation was achieved using a Discovery C8 column (100 x 2.1 mm, 5 µm particle size, 180Å pore size; SUPELCO, Milano, Italy) kept at 35 °C and working in isocratic 45% H<sub>2</sub>O (0.2% acetic acid) and 55% methanol at a flow rate of 200 or 250 µl/min for a 12 min run. The injection volume was 5 µl.

Extracts from low contaminated samples were reduced in volume by a factor of 3 prior

to analysis.

Both interfaces, APCI and ESI, were operated in negative ionisation mode. The mass spectra were recorded in full scan mode (250-400  $m/z$ ); the quantification was based on matrix-matched calibration curves using the extracted ion chromatograms of the deprotonated ion  $[M-H]^-$   $m/z$  317.4 for ZON and  $[M-H]^-$   $m/z$  319.4 for (ISTD) ZAN. When MS/MS mode was applied, fragmentation was performed on the deprotonated ion  $[M-H]^-$   $m/z$  317.4 for ZON, resulting in the product ions  $m/z$  299 and  $m/z$  273 and  $[M-H]^-$   $m/z$  319.4 for ZAN, product ions  $m/z$  301 and  $m/z$  275.

### **3.4 Extraction Techniques**

#### ***3.4.1 Development of the extraction method***

All extraction experiments were developed on spiked materials both for ensuring a systematic approach and for overcoming the lack of accessible contaminated materials for most of the period covered by this project.

Briefly, 5 g of matrix were spiked; subsequently different extraction procedures were followed according to the adopted extraction techniques (conventional or alternative). After the extraction was performed, 1.0 mL of ISTD (ZAN 2  $\mu\text{g/mL}$ ) was added and, after thoroughly mixing, a sub-sample was filtered into vials using a TITAN PTFE 0.45  $\mu\text{m}$ . Once the extraction parameters were selected, the method performance was tested on material previously used for a proficiency assessment scheme and on naturally contaminated samples.

#### ***3.4.2 Microwave Assisted Extraction (MAE)***

The most important extraction parameters such as time (5 min; 10 min), temperature (40°C; 80°C) and extraction solvent mixture (acetonitrile; methanol; acetonitrile-methanol 1:1 v/v) were tested to find the most suitable extraction conditions.

Further information is provided in APPENDIX II.

#### ***3.4.3 Pressurized Liquid Extraction (PLE)***

The following parameters were investigated: flush volume (60%; 75%), temperature (40°C; 80°C; 120°C), static time (5 min; 10 min), extraction solvent mixture (acetonitrile-water 9:1 v/v; methanol-water 8:2 v/v; acetonitrile-methanol 1:1 v/v) and

number of static cycles (2; 3).

The method development is presented in detail in APPENDIX III, while applications are reported in APPENDIX IV and APPENDIX V.

### ***3.4.4 Comparison of conventional versus alternative extraction methods***

The extraction efficiency of conventional (SHA and BLE) and alternative (ultrasonic extraction (UE), MAE; PLE) extraction techniques was compared by extracting eight naturally contaminated corn samples.

Further details are described in APPENDIX IV.

The basic principles of MAE and PLE are presented in APPENDIX B–EXTRACTION TECHNIQUES (10.2).

### ***3.4.5 Reduction of organic solvents for zearalenone extraction***

By applying the optimised setting of the PLE, the possibility to reduce the use of organic solvent for ZON analysis was investigated, substituting acetonitrile with more environmentally friendly solvents (methanol; ethanol; isopropanol) and increasing the percentage of water up to 50%. The possibility to use an alkaline solution to increase extraction efficiency was considered. Method development was performed following a simplex strategy. Suggested trials were performed in duplicate and the average was inserted as a response in the Simplex program.

Further details are provided in APPENDIX V.

## **3.5 Data evaluation**

Statistical analysis has represented a fundamental tool for the development of the whole project. The effects and the interactions of the investigated parameters on extraction efficiency were evaluated using a factorial design approach (Bayne and Rubin, 1986), which is able to estimate the variation due to a single parameter as well as to interaction among factors. The STATISTICA™ software (Stat Soft Inc., Tulsa, OK, USA) was used (APPENIDX II and APPENIDX III).

Conclusions drawn when comparing different extraction techniques were strengthened by post-hoc comparison with Scheffé's test and by principal component analysis (PCA) performed using THE UNSCRAMBLER® (Camo ASA, Oslo, Norway) (APPENDIX

IV).

During the development of an environmentally friendly extraction method, a sequential simplex optimization supported by the use of the software Multisimplex® (Grabitech Solutions AB, Sweden) was applied (APPENDIX V).

Further details on the background of the statistical tools applied in this research are presented in APPENDIX B–STATISTIC (10.3).

## 4 RESULT AND DISCUSSION

### 4.1 Development of the LC-MS method

#### 4.1.1 *Optimisation of the interface parameters*

Interface parameters can have a strong influence on the signal acquired on MS. Once the API to be used and the operation mode have been decided, the analyst can either rely on the interface parameters suggested by the instrument manufacturer or proceed with the interface optimization. LC-MS neophytes are particularly easily misled by a wrong set-up resulting in low or even absent signal, as occurred at the beginning of this study for the deoxynivalenol (DON) signal. For this specific reason the influence of several APCI parameters has been extensively investigated (APPENDIX I). The latter was performed on a Finnigan LCQ Deca instrument, which was replaced by an instrument from Hewlett-Packard. Although both ion trap mass spectrometers use ESI and APCI atmospheric pressure interfaces, they are built in a different way. Consequently, different interface parameters had to be investigated in order to achieve a good signal response.

#### 4.1.2 *Matrix effect evaluation*

The matrix effect, which can lead to enhancement or quenching of the MS signal, constitutes a crucial aspect in MS analysis. In this section several approaches to avoid or at least to minimize the matrix effect have been investigated. The following were considered: linear gradient versus isocratic, full scan versus MS/MS mode and the use or not of ISTD.

##### 4.1.2.1 *Evaluation of the matrix effect using a linear gradient elution*

The strong role played by the matrix effect on quantification had been noticed when developing a simultaneous method for the determination of nivalenol (NIV), DON and ZON. The possibility to inject the raw extracts directly without performing any clean-up step was evaluated on several matrix-matched standard curves. Promising results were obtained for NIV, which showed comparable calibration curves for mobile phase, corn, wheat and oat, but not for barley, whose calibration curve had a different slope; quantification performed on the mobile-phase calibration curves would have resulted in a significant underestimation of NIV concentration. This situation worsened slightly

when considering DON, as the different matrix-matched calibration curves presented a good linearity but had a very different slope.

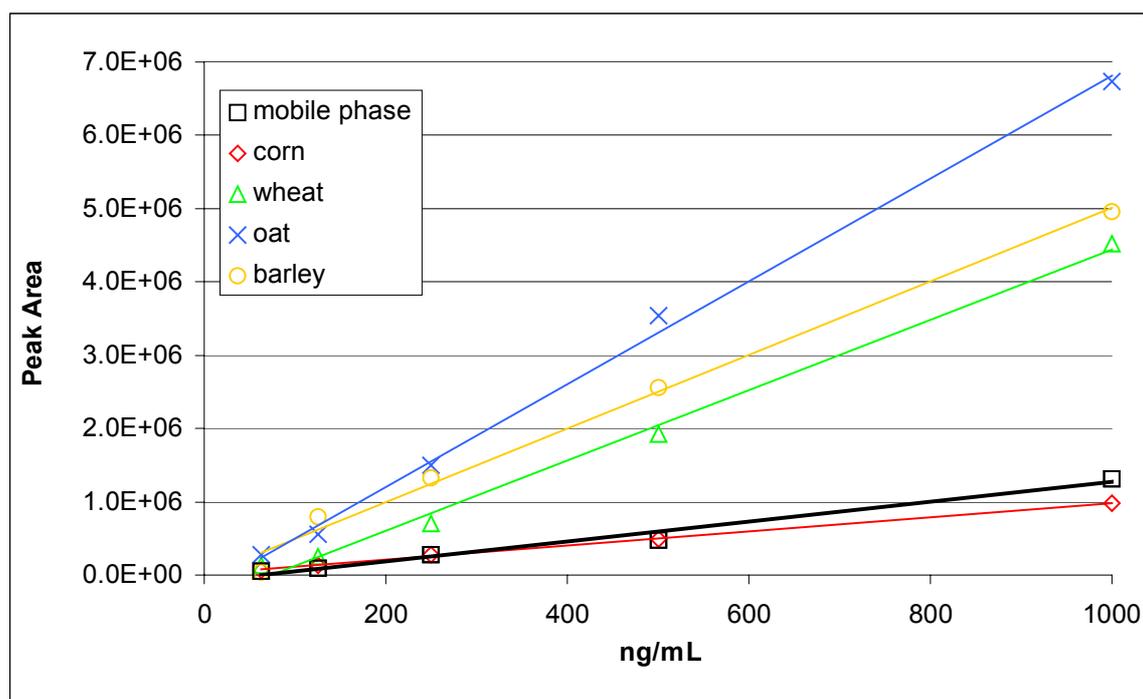
Finally, ZON calibration curves were evaluated; once more all of them showed good linearity, but the quantification of samples based on the mobile phase-matched calibration curve was highly unreliable and led to high overestimation of the real sample concentration (Table 4-1).

As clearly shown in Figure 4-1, calibration curves had different slopes. When the mobile phase acquired an elution power strong enough to allow ZON to elute, several other co-extracted matrix compounds also contemporarily co-eluted. Evaluation of the Total Ion Chromatogram (TIC) chromatogram clearly showed that ZON was eluting at the beginning of a huge elution front, which led to a strong matrix effect.

Although all matrices reported linear responses for the analytes of interest, the response factor varied within matrices and mobile phase. Quantification on the mobile-phase calibration curves would result in a dramatic over or under-estimation of the real concentration of the target analyte in the sample, therefore it was foreseen to quantify each sample on a matrix-matched calibration curve.

**Table 4-1:** Zearalenone calibration curves equations and regression coefficients obtained for different matrices and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in full scan mode without using ISTD.

Matrix	Equation	R <sup>2</sup>	ZON Peak Area		
			1.0E+05	5.0E+05	1.0E+06
	x=Peak Area		ng/mL	ng/mL	ng/mL
Mobile phase	1347.3x-79390	0.983	133	430	801
Corn	963.55x+17444	0.999	86	501	1020
Wheat	4784.4x-346446	0.995	93	177	281
Oat	7014.2x-195432	0.997	45	99	170
Barley	5027x-12978	0.992	22	102	202



**Figure 4-1:** Zearalenone: matrix effect evaluated by mobile phase and matrix-matched standard curves. Calibration obtained in full scan mode on the peak area without using ISTD.

#### 4.1.2.2 Evaluation of the matrix effect when working in isocratic mode

Following the change of the LC-MS instrument, the investigations using a linear gradient were suspended and interest moved towards finding suitable conditions for analysing ZON by injecting raw extracts. A reduction of the matrix effect was foreseen working in isocratic. An extensive investigation on the matrix effect within the range 10-1000 ng/mL was performed working in isocratic.

##### Full scan mode

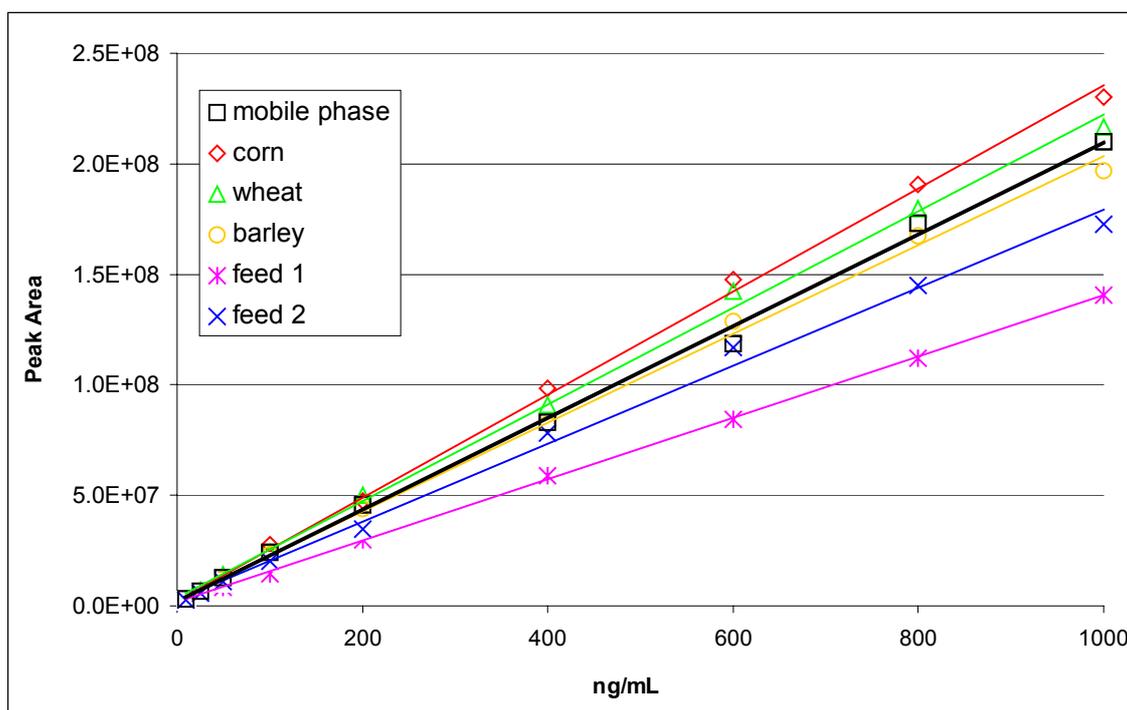
During the initial investigation, the matrix effect was evaluated by operating the MS in full scan mode. In order to determine if the use of an ISTD resulted in better performances, the calibration curves were calculated both on the target analyte peak area and on the ratio between target analyte (ZON) and ISTD (ZAN). Calibration curve equations and correlation coefficients ( $R^2$ ) based on the target analyte peak area, without taking into account the ISTD, are presented in Table 4-2 and the calibration curves in Figure 4-2.

First of all it was noticed that the matrix effect occurring during the determination of ZON was drastically reduced when working in isocratic (Figure 4-2). This observation is supported by the analysis of the TIC chromatogram, where it was evident that most of the co-extracted compounds were eluting within the first 3-4 min (Figure 1, APPENDIX II). ZON is eluted at a retention time where the TIC is constant, meaning that even in the presence of co-eluting ions the system is more stable.

It could be noticed at a first glance that all calibration curves are linear within the considered range and, except for the two feed matrices, presented a similar slope (slope RSD = 6.6%). The calibration curve slopes for the two feeds are lower, and this can be explained by the fact that, as feedstuff is a more complex matrix, a higher amount of substances are co-extracted, as confirmed by the analysis of the absolute intensity of the TIC at the ZON retention time (mobile phase =  $3.0E+05$ ; corn, wheat, barley =  $1.0E+07$ ; feed 1 =  $3.0E+07$ , feed 2 =  $2.0E+07$ ).

**Table 4-2:** Zearalenone calibration curves equations and regression coefficients obtained for different matrices and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in full scan mode without using ISTD.

	Equation	R <sup>2</sup>	ZON Peak Area		
			2.0E+07	5.0E+07	2.0E+08
	x=Peak Area		ng/mL	ng/mL	ng/mL
Mobile phase	207829x+1667942	0.998	88	401	904
Corn	233458x+2234191	0.999	76	355	847
Wheat	218992x+3408856	0.998	76	373	898
Barley	200906x+2601157	0.998	87	410	983
Feed 1	138662x+1718645	0.9998	132	601	1430
Feed 2	176755x+2715253	0.996	98	466	1116

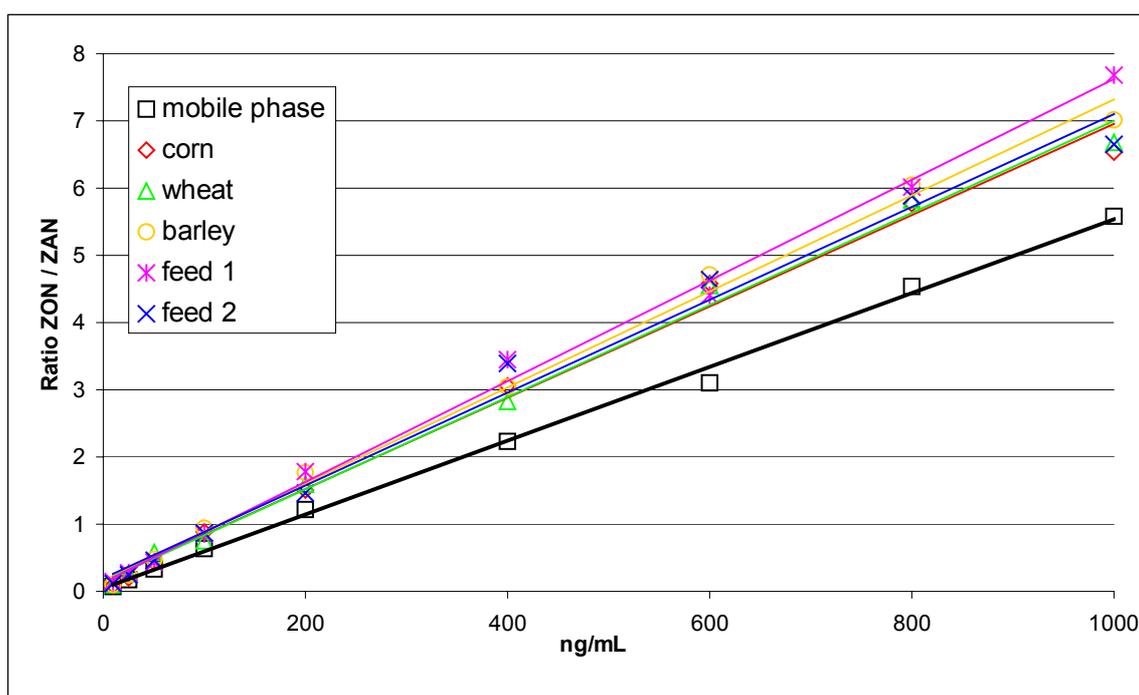


**Figure 4-2:** Zearalenone: matrix effect evaluated by mobile phase and matrix-matched standard curves. Calibration curves obtained in full scan mode on the peak area without using ISTD.

A slow decrease in MS efficiency was noticed when a large number of samples was injected in a row. A feasible possibility to overcome the different behaviour shown by different matrices due to the presence of co-eluting compounds could be the use of an ISTD. The same calibration curves have been recalculated taking into account the ISTD. The new obtained results for the same matrices are presented in Table 4-3 and Figure 4-3. From the presented data and the graph, a drastic change can be seen when using the ISTD. As presented in Figure 4-3, the mobile phase calibration curve is different from all the matrix-matched calibration curves (matrices slope, RSD = 4.4%; matrix slope including MP, RSD = 13.0%). This fully agrees with what has been previously presented for the TIC. When the ISTD is used, quenching or enhancement of the target analyte signal due to the matrix effect is minimised, since the ISTD is exposed to the same ionisation process as the target analyte. Moreover, the use of ISTD allowed to take into account the decreasing performances of the MS when numerous samples are injected. Therefore the use of ISTD should be preferred to take into account the presence of co-eluting compounds. In any case, quantification of raw extracts on the mobile phase-matched calibration curves results in an overestimation of the real concentration if it is not based on matrix-matched calibration curve.

**Table 4-3:** Zearalenone calibration curves equations and regression coefficients obtained for different matrices and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in full scan mode using ISTD.

	Equation	R <sup>2</sup>	ZON Peak Area / ZAN Peak Area		
			0.5	2	5
	x=Peak Area		ng/mL	ng/mL	ng/mL
Mobile phase	0.005x+0.0447	0.998	91	391	991
Corn	0.0068x+0.1589	0.992	50	271	712
Wheat	0.0068x+0.1534	0.995	51	272	713
Barley	0.0071x+0.1726	0.996	46	257	680
Feed 1	0.0075x+0.135.	0.997	49	249	649
Feed 2	0.0069x+0.1904	0.989	45	262	697



**Figure 4-3:** Zearalenone: matrix effect evaluated by mobile phase and matrix-matched standard curves. Calibration curves obtained in full scan mode on the ratio ZON/ISTD.

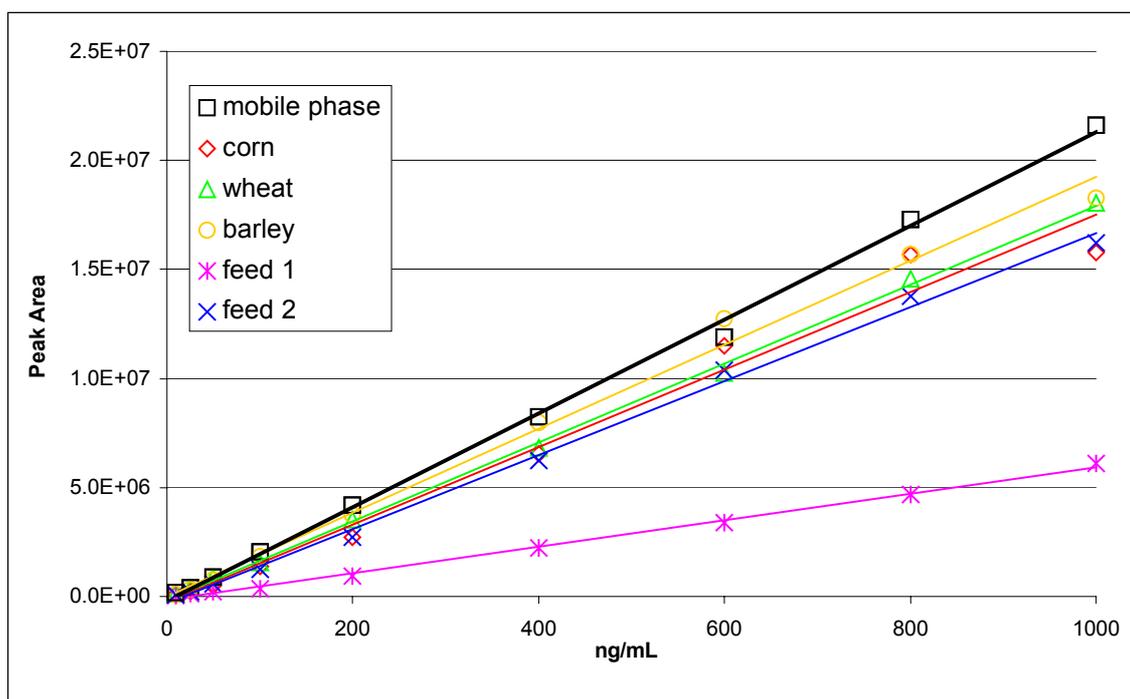
*Evaluation of MS/MS acquisition mode*

The common perception is that high selectivity and reduced matrix interferences are guaranteed when working in MS/MS mode, despite recently reported cases in which co-eluting compounds determined a matrix effect also in MS/MS mode (Zöllner et al, 2000). As a follow up of the matrix effect investigation, the calibration curves and equations obtained in MS/MS mode without ISTD (Table 4-4; Figure 4-4) and with ISTD (Table 4-5; Figure 4-5) are reported below.

If the quantification is made without ISDT, the matrix effect plays an important role also in MS/MS mode. Calibration curves (Figure 4-4) are similar to the one obtained in full scan mode (Figure 4-2). In both cases feed 1 presented the lowest slope of the calibration curves, which would have resulted in an underestimation of the ZON concentration if quantification were performed on the mobile phase calibration curve. Over- and under-estimation due to the matrix effect were reduced but still appreciable in MS/MS using ISTD (Figure 4-5).

**Table 4-4:** Zearalenone calibration curves equations and regression coefficients obtained for different matrices and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in MS/MS mode without using ISTD.

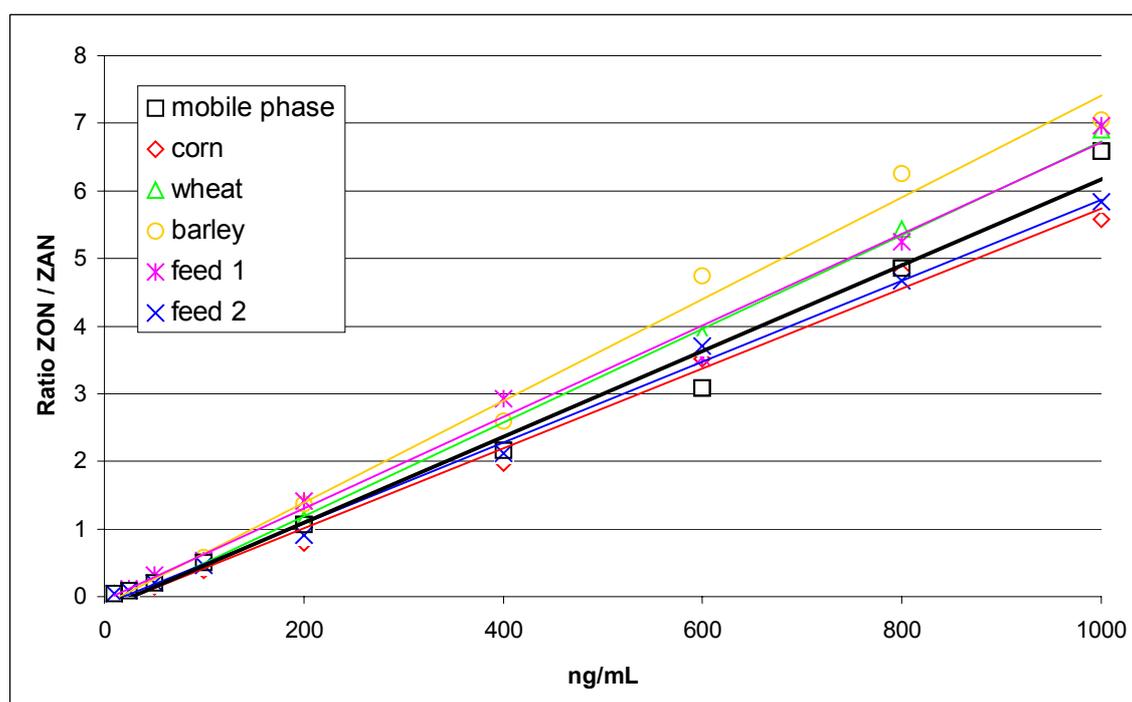
	Equation	R <sup>2</sup>	ZON Peak Area		
			2.0E+06	5.0E+06	2.0E+07
	x=Peak Area		ng/mL	ng/mL	ng/mL
Mobile phase	21509x-200855	0.998	102	242	939
Corn	17757x-246379	0.978	127	295	1140
Wheat	18086x-160120	0.999	119	285	1115
Barley	19288x+5732	0.994	103	259	1034
Feed 1	6085.5x-145922	0.997	353	846	3310
Feed 2	16961x-284868	0.997	135	312	1196



**Figure 4-4:** Zearalenone: matrix effect evaluated by mobile phase and matrix-matched standard curves. Calibration curves obtained in MS/MS mode on the peak area without using ISTD.

**Table 4-5:** Zearalenone calibration curves equations and regression coefficients obtained for different matrices and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in MS/MS mode using ISTD

	Y Equation	R <sup>2</sup>	ZON Peak Area / ZAN Peak Area		
			0.5	2	5
	x=Peak Area		ng/mL	ng/mL	ng/mL
Mobile phase	0.0063x-0.1761	0.988	107	345	822
Corn	0.0059x-0.1678	0.994	113	367	876
Wheat	0.0069x-0.1924	0.994	100	318	753
Barley	0.0075x-0.115	0.992	82	282	682
Feed 1	0.0068x+0.0529	0.992	66	286	728
Feed 2	0.0060x-0.1180	0.997	103	353	853



**Figure 4-5:** Zearalenone: matrix effect evaluated by mobile phase and matrix-matched standard curves. Calibration curves obtained in MS/MS mode on the ratio ZON/ISTD.

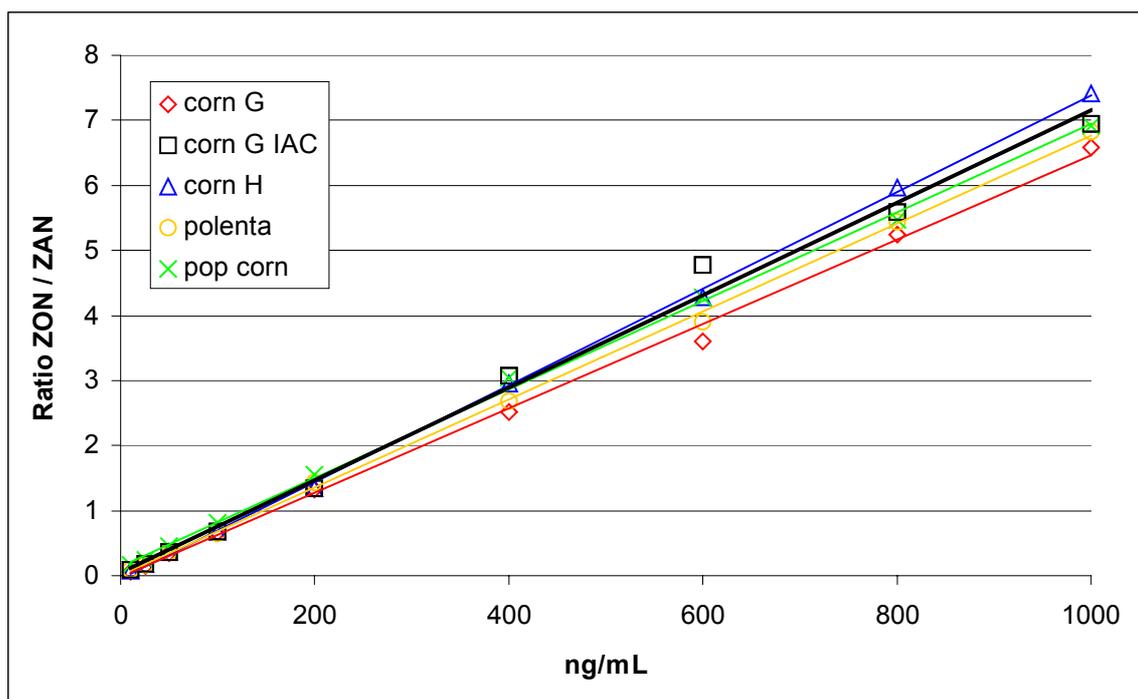
### *Evaluation of different type of the same matrix*

For the final method the MS was operated in full scan. This decision was mainly driven by the characteristic of the ion-trap, which performs the fragmentation for the MS/MS mode in a time-depending way instead of in a space-depending way as occurs in the triple quadrupole. This way of operating results in a lower performance of the ion-trap versus the triple quadrupole. In this particular case ZON and ZAN have a similar retention time, the fragmentation should be performed simultaneously, meaning that while ZON is fragmented no signal for ZAN is acquired and vice versa. Moreover, improvements in the LOD were not noticed.

Most of the method development was performed on spiked matrices, therefore it was easy and convenient to work on matrix-matched calibration curves. Nevertheless once the experiments on naturally contaminated matrix started, the doubt that different batches of the same matrix could behave differently (Zöllner et al, 2000) gave rise to concern. For this reason the matrix effect for corn from different origins and variety was investigated. Concentration of the theoretical samples calculated based on the obtained calibration curves, varied in a small range and presented a RSD = 4.5% in the whole investigate range (10-1000 ng/mL).

**Table 4-6:** Zearalenone calibration curves equations and regression coefficients obtained for different types of corn and theoretical concentrations (ng/mL) estimated using the different calibration curves obtained in full scan mode using ISTD.

	Y Equation	R <sup>2</sup>	ZON Peak Area / ZAN Peak Area		
			0.5	2	5
	x=Peak Area		ng/mL	ng/mL	ng/mL
Corn G	0.0065x-0.0179	0.998	80	310	772
Corn G IAC	0.0071x+0.0428	0.994	76	288	710
Corn H	0.0074x-0.0414	0.999	73	276	681
Polenta	0.0068x+0.0053	0.999	73	293	735
Pop corn	0.0068x+0.01415	0.999	71	292	733



**Figure 4-6:** Zearalenone: matrix effect evaluated on different corn matrices. Calibration curves obtained in full scan mode on the ratio ZON / ISTD.

### *Conclusion on matrix-effect*

The entire work performed on the matrix effect can be summarized as follow.

The matrix effect, as quenching or enhancing of the MS signal response, is a consequence of the presence of substances co-eluting with the target analyte. The matrix effect has a dramatic impact when analysing ZON using a linear gradient, but better results are achieved in isocratic, where most of the co-extracted compounds elute before the target peak.

The use of ISTD is strongly recommended; although it does not overcome the matrix effect, it compensates for changes in the instrument performance and variations in the response factor when different matrices are analysed. ZAN showed to have all the prerequisites of a reliable ISTD, having almost the same chemical structure as ZON (a double bond is missing) it behaves as ZON, is stable in the sample and has very similar response and retention time (peaks are not fully separated resembling isotopic standard). The only drawbacks is that ZAN can only be used when ZON is determined in vegetable matrices (as in this case), since it can be present as a natural metabolite when animal tissues have to be analysed.

In this study, which has been performed in an ion-trap, no significant improvements were noticed when working in MS/MS compared to full scan mode. Quantification should be always performed on matrix-matched instead of mobile phase calibration curves, allowing the impact of the matrix effect to be reduced or at least minimized.

### **4.1.3 Zearalenone analysis**

Both APCI and ESI have been used, although the most frequently used for the determination of ZON is APCI. In this study ESI was preferred, as this could be a good option when developing simultaneous methods for mycotoxin determination, since fumonisin can only be detected with this interface.

During the whole study the LODs (signal to noise (S/N) = 3) and the LOQs (S/N = 10) were determined on the matrices. The LODs and LOQs reported in the different part of this study are slightly different for two main reasons: instruments with different performances were employed and the increased skill in handling the instrument resulted in a more confidence in optimising the interfaces and evaluating the spectra. As an indication, the LOD on the matrix, calculated injecting fortified raw extracts, was 15 pg in wheat and 10 pg in corn, while the method LOQ obviously depended on the method

procedure (remember that PLE provides a double extraction volume compared to the other techniques). The LOQs were in the range of 15-30 ng/g and 10-20 ng/g for wheat and corn, respectively.

## **4.2 Extraction techniques**

Statistical evaluation of the influence of each investigated parameters on the extraction efficiency, both for MAE and for PLE, was performed using an experimental design, which has the advantage of simultaneously evaluating main effects and interaction effects. The results of the evaluation can be represented using a histogram, called pareto chart, which shows the estimated effects sorted by their absolute size. The vertical line indicates the minimum magnitude of statistically significant effects; all the interactions represented by bars not reaching the significant line are not to be considered significant. The pareto charts for MAE and PLE are discussed below.

### **4.2.1 Microwave Assisted Extraction (MAE)**

The main drawbacks of using MAE for extracting cereals is associated with the percentage of starch, which does not allow the use of aqueous extraction solvent mixtures since the presence of a percentage of water tends to cook the starch, resulting in a bulky porridge

Parameters influencing the extraction efficiency of ZON in corn can be visibly estimated from Figure 4-7. ZON recovery is significantly ( $\alpha=0.05$ ) influenced by temperature and time. The most important factor is the temperature, the positive value (2.63) means that the higher level of temperature (80 °C) allows for a better extraction efficiency, in contrast the negative value for time (-2.62) means that better extraction efficiency is achieved when a lower level of the extraction time is applied (5 min).

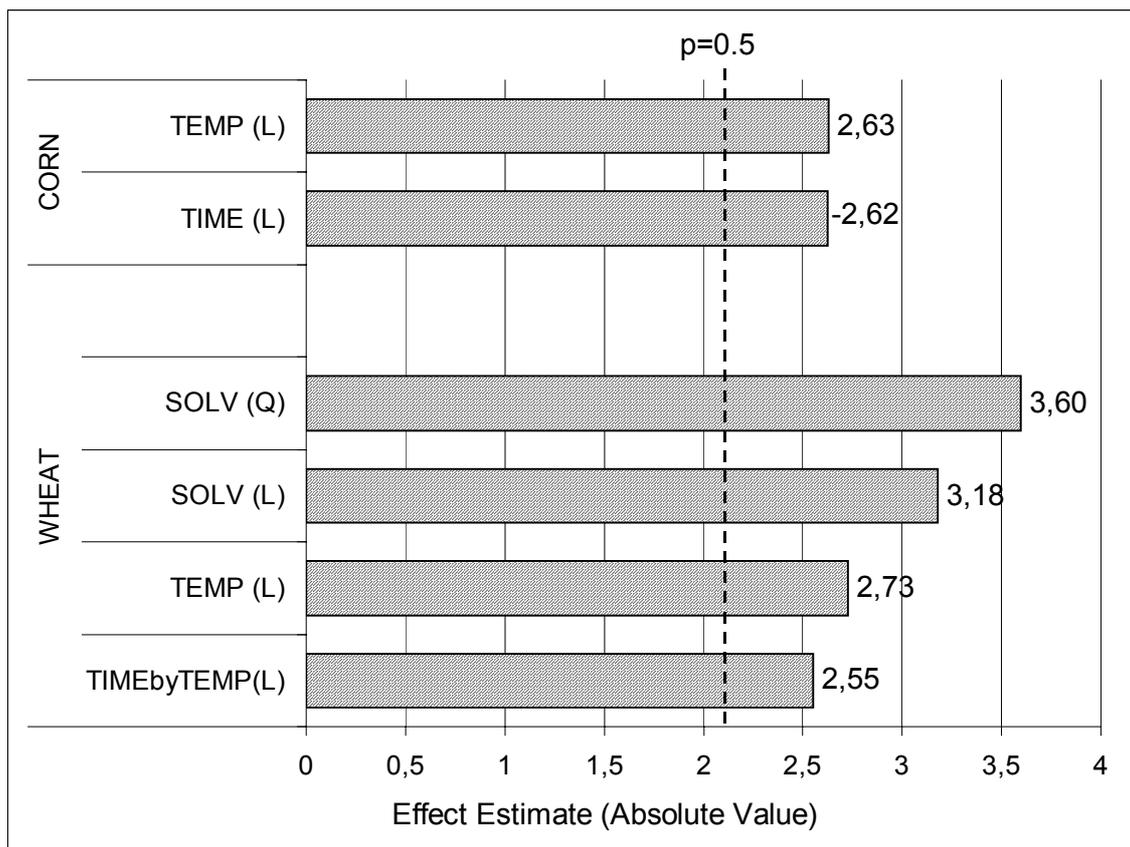
When analysing the data for wheat, which has shown to be a more complicated matrix, more parameters influenced ZON extraction efficiency (Figure 4-7).

The solvent shows the main effect on the extraction efficiency. In the case of solvent which is a qualitative factor, from the graph it is possible to extrapolate only that it did play an important role, but in theory it is not correct to assign a value to this main effect. As has been previously shown a higher temperature level (80 °C) allows for better results. Finally, the interaction between time and temperature is statistically significant, indicating that there is an interaction between time and temperature levels. Although

time does not have a significant effect, it can be noticed that the higher level of time resulted in lower extraction efficiency for wheat as well (-1.99).

The final chosen MAE extraction conditions for both corn and wheat were: time 5 min, temperature 80 °C and extraction solvent mixture acetonitrile-methanol 1:1 v/v.

For further details please refer to APPENDIX II.



L = linear

Q = quadratic

**Figure 4-7** Pareto chart of the standardised effect evaluated on MAE extraction of corn and wheat.

Factorial design is a unique powerful tool for evaluating the interaction of effects, however, it can also be used to simply evaluate main effects (as in corn). Despite the advantages of factorial design for data evaluation, the analyst should always supervise and assess the results provided by the statistical program. Indeed, quadratic effects do not have any meaning when referred to qualitative factors, for which is not possible to

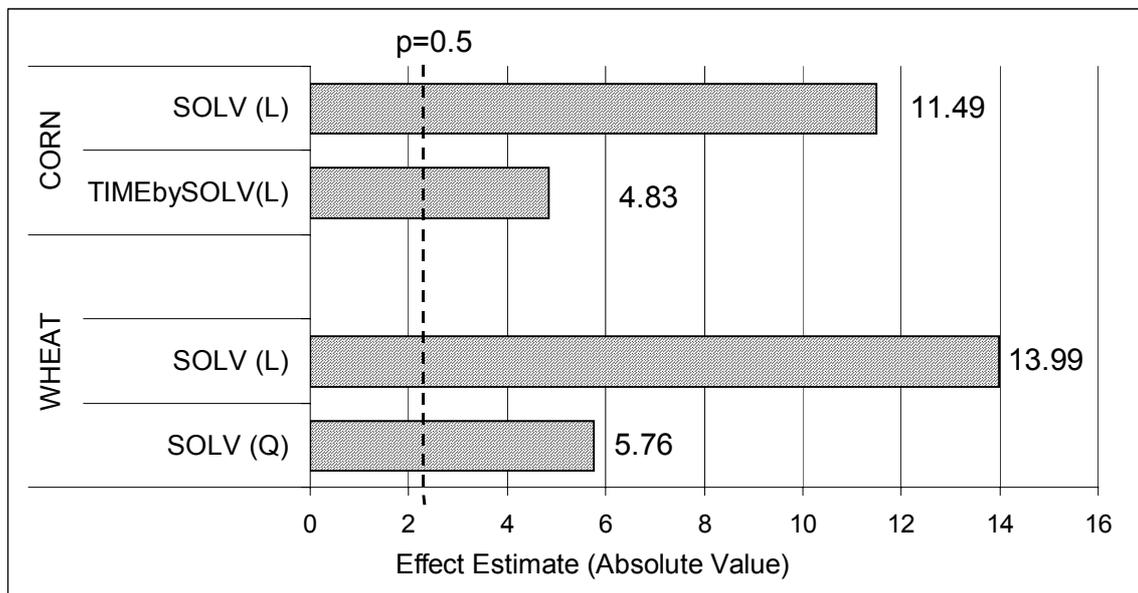
establish an order among levels.

#### 4.2.2 Pressurized Liquid Extraction (PLE)

Experimental design was also applied when evaluating the effect of several parameters on extraction efficiency of ZON by using PLE. This extraction technique allows the use of aqueous extraction solvent mixtures, therefore the three solvent mixtures that were tested have been chosen taking into account the most frequently used methods in literature and the 100% organic mixture, which has provided satisfactory performances when developing the MAE extraction method.

Contrary to what has been seen during the MAE method development, ZON was not always as easily extracted. This could be due to the use of aqueous organic solvent mixture compared to the 100% organic solvent previously used. For both matrices a reduced extraction efficiency of ZON was noted at the highest temperature (120 °C) and when methanol was used.

Evaluation of the pareto chart obtained for corn and wheat showed more similarity compared to what has been shown for MAE.



L = linear  
Q = quadratic

**Figure 4-8** Pareto chart of the standardised effect evaluated on PLE extraction of corn and wheat.

For corn, the solvent used had a major impact on the extraction of the target analyte, an interaction effect between time and temperature was also reported (Figure 4-8). A similar situation was presented for wheat (Figure 4-8), where once more the solvent was the most important factor.

The final chosen extraction conditions providing satisfactory performances were: time 5 min, temperature 80 °C and extraction solvent mixture acetonitrile-methanol 1:1 v/v.

As previously explained, the significant quadratic interaction evidenced by the statistical analysis does not have any meaning when referred to a qualitative factor.

For further details please referred to APPENDIX III.

### ***4.2.3 Comparison of conventional versus alternative extraction methods***

In principle the use of FAPAS<sup>®</sup> material to test method performances allowed a comparison among tested methods and those most frequently used. Actually, the target value provided by FAPAS<sup>®</sup> is determined from data submitted by different laboratories, obtained using different extraction solvent mixtures, extraction techniques as well as different clean-up procedures and detection methods. In this respect, the methods developed for MAE and PLE provided performances comparable to the most frequently used method, since the obtained values fall into the accepted range. However, it was of main interest to perform a direct in-house comparison between conventional (BLE; SHA) and alternative (UE, MAE, PLE) extraction techniques. The experimental setting was carefully evaluated with the aim to protect against unsuspected sources of bias to be able to draw truthful and unbiased conclusions. PLE has been claimed to be able to break possible existing bonds between the target analyte and the matrix (as shown for fumonisin, Lawrence et al, 2000), therefore naturally contaminated corn samples were employed for this investigation.

#### Experimental settings

Samples were handled “one at a time”; each sample was extracted 25 times (5 methods; 5 replicates) in the same day and quantified on LC-ESI-MS within the same sequence on the same matrix-matched standard curve. Performing the extraction during the same day allows the same reproducible conditions within a sample batch, avoiding the influence of external factors on the reliability of the differences detected between the extraction methods. The condition causing most concern was the detection by LC-MS, which is responsible for the major part of the whole method variability (LC-MS around

10%). The best option to reduce experimental error is to quantify the 25 extracts on a freshly prepared calibration curve, using the same ISTD used during the extraction procedure.

The last most critical point raising concern, was to decide whether to use the same extraction solvent mixture for all the different extraction techniques or to use the optimised solvent mixture for MAE and PLE and one of the most frequently world wide used aqueous solvent mixtures (acetonitrile-water 90:10 v/v; acetonitrile-water 86:14 v/v; methanol-water 80:20 v/v) for conventional extraction methods. After weighing the pros and the cons of the two alternatives, it was chosen to perform the comparison using the same extraction solvent mixture for all techniques. The decision was mainly driven by the fact that if different extraction solvent mixtures were used for the comparison of different extraction techniques, the main criticism would have been that an extraction technique could not be selected as the most efficient since differences in the extraction efficiency could be ascribed to the different solvent mixtures utilised. Moreover, ZON is practically insoluble in water, therefore using a 100% organic solvent mixture in principle could not result in a lower extraction efficiency compared to the extraction efficiency achieved using a solvent mixture containing a percentage of water. In favour of this statement are the results obtained during the PLE method development, where 100% organic solvent mixture was compared to two of the most frequently used aqueous solutions and by a short additional experiment in which the mixture (acetonitrile-methanol 1:1 v/v) was compared to two well established extraction solvent mixtures (acetonitrile-water 86:14 v/v; methanol-water 8:2 v/v) for conventional extraction techniques. Although the mixture acetonitrile-water provided satisfactory results both during PLE method development and during the SHA comparison, the use of 100% organic extraction solvent circumvented the phenomena of phase separation and water absorption by the matrix (Stroka et al, 1999). These phenomena could easily lead to apparently higher recovery when using acetonitrile-water as extraction solvent mixture compared to solvent mixtures where phase separation did not occur.

#### Statistical evaluation

A data set of 200 results was obtained from all the extraction performed. Since the concentration of ZON in the eight samples covered a wide range, in order to elide the effect that different ZON concentration levels would have had during the statistical

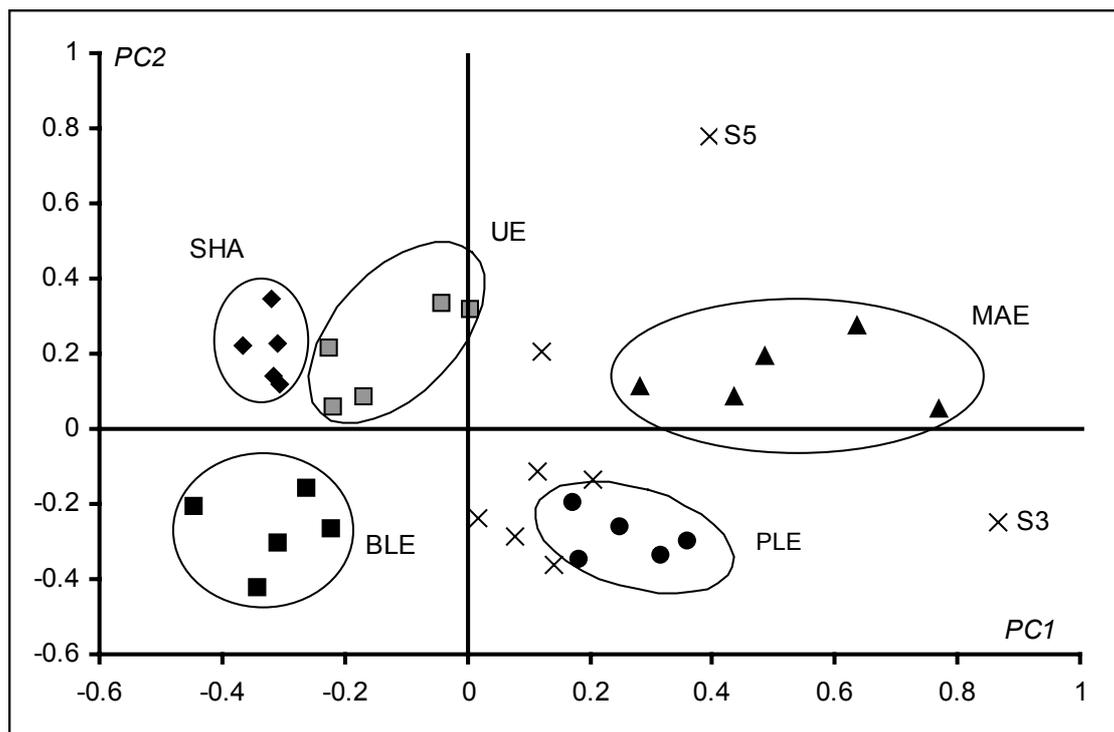
evaluation, the obtained results (Table 2, APPENDIX IV) were normalised (Table 4-7). This table clearly shows that the higher extraction efficiency averages are always obtained using MAE (5 times) or PLE (3 times), indicating a superiority of these methods over the others, which have the lowest value BLE (2 times), SHA (2 times) and UE (4 times). Therefore, a first division of the extraction techniques into two groups can already be perceived; however, it is necessary to base any conclusion on unequivocal evidence.

After subjecting the results to analysis of variance and subsequently to Scheffe's post-hoc comparison test, extraction techniques can be divided into two groups divided as follows: UE, BLE and SHA versus MAE and PLE (Table 3, APPENDIX IV).

**Table 4-7:** Normalized concentration of zearalenone in 8 different corn samples obtained by applying different extraction techniques. Normalised values (centred around 1).

	CONVENTIONAL						ALTERNATIVE								
	BLE			SHA			UE			PLE			MAE		
	Conc±SD	RSD (%)	RSD (%)	Conc±SD	RSD (%)	RSD (%)	Conc±SD	RSD (%)	RSD (%)	Conc±SD	RSD (%)	RSD (%)	Conc±SD	RSD (%)	RSD (%)
<b>1</b>	0.940±0.092	9.8	10.3	0.969±0.100	10.3	8.3	0.895±0.074	8.3	8.3	1.090±0.138	12.7	12.7	1.106±0.095	8.6	8.6
<b>2</b>	0.907±0.077	8.5	11.6	1.016±0.118	11.6	14.9	0.999±0.149	14.9	14.9	1.002±0.097	9.6	9.6	1.076±0.077	7.1	7.1
<b>3</b>	0.783±0.060	7.6	7.4	0.647±0.048	7.4	9.7	0.872±0.084	9.7	9.7	1.298±0.088	6.7	6.7	1.400±0.189	13.5	13.5
<b>4</b>	1.046±0.058	5.5	4.7	0.997±0.046	4.7	6.2	0.888±0.055	6.2	6.2	1.113±0.041	3.7	3.7	0.956±0.092	9.7	9.7
<b>5</b>	0.687±0.071	10.4	8.6	1.050±0.090	8.6	12.6	1.066±0.134	12.6	12.6	0.858±0.047	5.5	5.5	1.340±0.140	10.5	10.5
<b>6</b>	1.051±0.064	6.1	6.2	0.889±0.055	6.2	7.5	0.868±0.065	7.5	7.5	1.164±0.104	8.9	8.9	1.028±0.118	11.5	11.5
<b>7</b>	1.153±0.190	16.5	2.3	0.910±0.021	2.3	5.3	0.880±0.047	5.3	5.3	0.979±0.029	2.9	2.9	1.078±0.107	9.9	9.9
<b>8</b>	0.980±0.051	5.2	4.1	0.902±0.037	4.1	2.2	1.017±0.022	2.2	2.2	1.094±0.060	5.5	5.5	1.008±0.056	5.6	5.6

Data were also subjected to PCA in order to elaborate on the relationship between the clustering of the results according to the various extraction techniques and the different samples used in this study. By applying PCA the results from the replicate analyses of the five methods are projected onto a smaller number of variables called principal components (Ebensen, 2000) in order to facilitate the visual interpretation of the relationship between the results from the different methods and the various samples used. Principal components (PCs) are linear combinations of the relative concentrations of the extraction methods calculated in such a way that the first PC represents the major part of the overall variance of the analytical results (Ebensen, 2000). The outcome of PCA, presented in Figure 4-9, shows the coordinates (scores) of the results from the five extraction methods in the coordinate system established by PC 1 and PC 2 along with the contribution (loading) of each sample to PC 1 and PC 2, respectively. Large positive scores correspond to high values of the relative concentration. In principle, the results from PCA coincided with those obtained with the Scheffe's test, since the scores of PC 1 for MAE and PLE were positive, whereas the corresponding scores for the BLE, SHA and UE were negative. PC 2 mainly allowed a separation of the methods that belonged to group 1 as attributed by Scheffe's test. Additionally, evaluating the loadings of the samples showed that the samples do not behave uniformly across all extraction methods. For example, sample 5 (S5) has a large loading for PC 2 indicating that the obtained relative concentrations of this specific sample were higher for UE and lower for BLE. In contrast, sample 3 had a lower loading for PC 2, since the corresponding relative concentrations did not vary very much between the UE, SHA and BLE. The loading for PC 1 of this sample was very high thereby showing elevated relative concentrations when using MAE. In conclusion, the results from PCA were comparable to those from the post-hoc comparison. In addition, the outcome of the PCA analysis demonstrates that comparing the efficiency of different extraction methods requires the use of real world samples from *various* origins to draw unbiased information, which is anyway confined to the batch of studied samples.



**Figure 4-9:** Two-dimensional scatter plot of PC 1 and PC 2 showing the clustering of the results according to the various extraction methods applied and the loadings for the two principal components illustrating the impact of the different samples on PC 1 and PC 2. Samples are represented by crosses. S3 and S5 are the sample influencing most the PCs.

From these observations it may be concluded that although UE is often considered an alternative extraction method either because it employs an additional source of energy (ultrasounds) during extraction, or because it is not frequently used, it should be more correctly considered a conventional extraction method with performances similar to BLE and SHA.

The significantly higher extraction efficiency achieved for sample 3 and 5 when using MAE and PLE can be explained by the positive effect of temperature in providing energy to break the interaction occurring between target analyte and matrix in a naturally contaminated sample. Although all the analysed samples were naturally contaminated, no information was available concerning the stage at which the mycotoxin had been produced. A feasible explanation for the different behaviour of samples 5 and 3 could be based on the period in which the mycotoxin had been

produced. In fact, if the mycotoxin was produced in the field, could be hypothesised an interaction between the infected plant and the mycotoxin leading to a bind between target analyte and matrix; on the other hand, if the mycotoxin had been produced directly on the kernel during the storage phase, an interaction between mycotoxin and matrix is not foreseen.

Drawing a conclusion on which is the best extraction technique is quite straightforward; since all the methods applied for evaluating the extraction efficiencies agreed in addressing alternative extraction methods, MAE and PLE as the best performing. MAE provided the highest results in the evaluation of the data set and comparison of the relative concentrations of the various extraction methods. It is more controversial to establish which is the worst method. In fact, if the comparison of the relative concentration suggest SHA as the worst method, evaluation of the data set fairly disagreed, indicating UE as the worst one. In principle, the extraction efficiency obtained by using the extraction techniques falling in the first group hardly showed any significant difference.

For further information please refer to APPENDIX IV.

To conclude the use of MAE or PLE is to be preferred when the instruments are available, otherwise if only conventional extraction techniques are on hand, BLE and SHA should be preferred over UE. However, since BLE and SHA results were comparable, each single lab should choose between conventional methods taking their needs into account. The method characteristics are presented in Table 4-8.

**Table 4-8:** Characteristics of the different extraction techniques.

ADVANTAGES	DRAWBACKS
<b>BLENDING</b>	
Satisfactory recoveries Short time of extraction (2-5 min) Inexpensive system Easy to use	High manpower Single extraction Handling of the sample Very noisy
<b>SHAKING</b>	
Satisfactory recoveries Inexpensive system Easy to use Simultaneous extractions	Long extraction (30min-2h) Handling of the sample
<b>ULTRASONIC EXTRACTION</b>	
Satisfactory recoveries Short time of extraction (2-5 min) Inexpensive system Easy to use	High manpower Single extraction Lowest recovery Very noisy
<b>PRESSURIZED LIQUID EXTRACTION</b>	
Good recoveries High efficiency Automated system Dynamic extraction technique Temperature/pressure controlled Reduced handling of the sample	High instrument investment Packing thimble
<b>MICROWAVE ASSISTED EXTRACTION</b>	
Good recoveries High efficiency Short time of extraction (5-10min) Temperature/pressure controlled Simultaneous extractions (12) Reduced handling of the sample	Instrument investment Need of experienced operator Time required to reach room temperature Solvent limitation (able to absorb microwave)

#### 4.2.4 Reduction of organic solvent for zearalenone extraction

Considering the rising concern for environmental issues, the use of organic solvent in the laboratories has also become a hot topic. After the Montreal protocol established to discontinue the use of chloroform, other solvents (especially acetonitrile) are foreseen to be slowly substituted. In this optic, the use of organic solvent employed for ZON

extraction was reduced. The extraction solvent mixture so far suggested by this study was a 100% organic solvent; this percentage does not differ much from the world-wide most frequently used extraction solvent mixture acetonitrile-water 90:10 v/v and methanol-water 80:20 v/v.

The extractions were performed by using PLE, which combined higher extraction efficiency with the possibility to use a percentage of water, which is not feasible for MAE.

The simplex strategy was applied for the method development. The advantages of operating using the simplex were partially hidden since quite good recoveries were obtained starting from the first trials. Nevertheless, this was the result of the initial investigation aiming to find a suitable alkaline solution able to improve the extraction efficiency of water.

A final method with performances comparable to the one using 100% organic solvent was developed. The percentage of organic solvent was reduced and a less toxic solvent than those commonly applied was employed. In addition, the amount of water was increased up to 50%, which corresponds to halving the organic solvent compared to the previously developed method and to reducing 1.8 times compared to the method proposed by ISO. The final extraction was based on isopropanol-water (1% TEA) 1:1 v/v at the following conditions: time 5 min, temperature 80 °C, 2 cycles.

For further information please refer to APPENDIX V.

## 5 ABSTRACT

Nowadays more than 75 countries have regulations on mycotoxins and, due to increasing concern, the existing limits are likely to be extended to additional commodities and other mycotoxins. Monitoring is foreseen in this context not only for consumer protection but for the world trade market as well, resulting in an increase in the number of analyses to be performed, which should be taken into account when developing new detection methods.

The aim of the present study was to develop an analytical method for zearalenone taking into account today's requirements for analytical methods such as high throughput, speed, automation, costs, reliability and reduced use of hazardous solvents. This purpose was achieved by coupling recent extraction techniques and improving the extraction efficiency while reducing the time required for analysis and enabling automation, with highly sophisticated detection systems as LC-MS.

The first part of this study was devoted to the development of a fast LC-MS method based on the direct analysis of raw extracts. Initially, the influence of several interface parameters on the signal acquired for the target analyte was investigated in order to achieve the highest instrument sensitivity. Afterwards the role played by the matrix effect in different working conditions was evaluated. A reliable method for the determination of ZON in crude extracts was developed and the matrix effect was overcome by performing the quantification on matrix-matched calibration curves and using the ISTD (ZAN).

The second part of this research was dedicated to finding suitable operating conditions for ZON extraction by alternative extraction techniques and the subsequent comparison of the extraction efficiency of conventional versus alternative extraction techniques. The elimination of the clean-up step abolishes losses of the target analyte during this step, thus allowing to truly evaluate the extraction efficiency of the different techniques without being hampered by variations due to handling the sample during purification.

The influence of time, temperature and extraction solvent mixture on the extraction

efficiency of ZON using two alternative extraction techniques, MAE and PLE, were evaluated using a factorial design approach. This statistical tool allows to simultaneously evaluate the effects of single factor and the interactions among them.

Suitable extraction conditions were chosen for both MAE and PLE, allowing good recoveries which were confirmed by analysing materials previously used in a proficiency test scheme. As a consequent follow up, conventional and alternative extraction techniques were compared on a batch of naturally contaminated corn. Following Sheffe's test, the investigated methods were divided into two groups; on one side BLE, SHA, UE and on the other MAE and PLE. Alternative extraction techniques showed a statistically significant higher extraction efficiency compared to the conventional. MAE proved to be slightly superior to all of them. However, if the instrumentation for alternative extraction techniques is not available, conventional methods provide satisfactory recoveries in most cases.

Lastly, the extraction solvent mixture used for PLE was modified using the simplex strategy, allowing, the move to a less toxic solvent while maintaining satisfactory extraction efficiency and to increase the percentage of water up to 50%, almost halving the amount of organic solvent commonly used for ZON analysis.

Summarizing, in this study alternative extraction techniques were coupled to a highly sophisticated analytical technique (MAE-LC-MS or PLE-LC-MS). A part from providing higher recoveries, the use of alternative extraction techniques permits either to reduce the time required for the extraction or to automate the process with the possibility to reduce the use of organic solvent. By taking adequate precautions, it is possible to inject raw extracts directly into LC-MS, with the main advantages of avoiding losses of the target analyte during the clean-up step, reducing both time required for the whole analysis and costs per analysis, to which clean-up column contributes most.

## 5.1 Zusammenfassung

Weltweit existieren zur Zeit in mehr als 75 Ländern Gesetze über Mykotoxine in Lebensmitteln und es kann erwartet werden, daß diese Gesetze auf weitere Lebensmittel und andere Mykotoxine ausgeweitet werden. Im Sinne des Verbraucherschutzes und des freien Warenverkehrs werden deshalb gehäuft Monitoring Programme durchgeführt, woraus sich bestimmte Kriterien für die anzuwendende analytische Methodik ableiten.

Das Ziel der vorliegenden Arbeit war es, eine analytische Methode für die Bestimmung des Mykotoxins Zearalenon (ZON) zu entwickeln, die einen großen Probendurchsatz bei gleichzeitiger Verwendung von weniger organischen Lösemitteln erlaubt. Dieses Ziel wurde im wesentlichen durch die Anwendung moderner und automatisierbarer Extraktionsverfahren in Kombination mit LC-MS als hochspezifisches Detektionsverfahren erreicht.

Der erste Teil der Arbeit konzentrierte sich auf die Entwicklung einer LC-MS Methode, wobei unaufgereinigte Extrakte von ZON direkt in das System eingespritzt wurden. Zusätzlich wurde der Einfluß der Matrix auf die Quantifizierung untersucht. Diese Arbeiten führten zur Entwicklung einer Methode, bei der als interner Standard (Zearalanon) verwendet wurde. Zusätzlich wurde ermittelt, dass die Quantifizierung mittels Matrix angepaßter Standardlösungen erfolgen muss.

Im zweiten Teil der Arbeit wurden optimierte Verfahrensbedingungen für moderne Extraktionsverfahren ermittelt und die erzielten Konzentrationen von ZON in Getreide mit den Ergebnissen verglichen, die mittels herkömmlicher Verfahren erzielt wurden. Indem auf die Verwendung von Aufreinigungsschritten verzichtet werden konnte, war es möglich, die gefundenen Unterschiede bezüglich der Wiederfindungsrate auf die verschiedenen Extraktionsverfahren zurückzuführen.

Die Entwicklung der Extraktionsbedingungen wie zum Beispiel Temperature, Zeit oder Zusammensetzung der Lösemittel der verwendeten Verfahren (PLE und MAE) wurde mittels faktorieller Versuchsplanung durchgeführt. Dieses statistische Verfahren erlaubt

die Bestimmung von signifikanten Einflüssen der verschiedenen Extraktionsbedingungen und ihrer Wechselwirkung auf die Wiederfindungsrate von ZON.

Die optimierten Extraktionsbedingungen wurden durch die Anwendung auf realistische Proben überprüft, deren Gehalt zuvor in einem internationalen Ringversuch ermittelt wurde. Der Vergleich von verschiedenen Methoden zeigte, dass BLE, SHA und UE eine Gruppe von herkömmlichen Verfahren bilden, die signifikant niedrigere Wiederfindungsraten liefern als eine zweite Gruppe (PLE und MAE) von modernen Verfahren. Der Vergleich wurde mittels post-hoc Vergleich durchgeführt. Insgesamt hat MAE zu den besten Ergebnissen geführt. Schließlich zeigen die Ergebnisse auch, dass herkömmliche Methoden ebenfalls zu zufriedenstellenden Resultaten führen können.

In dem letzten Teil der Arbeit wurde eine Methode für die PLE Extraktion entwickelt, bei der der Wasseranteil auf 50% erhöht werden konnte. Zusätzlich wurde ein weniger toxisches organisches Lösemittel verwendet. Bei dieser Arbeit wurde ein anderes Optimierungsverfahren (Simplexoptimierung) angewendet.

Zusammenfassend wurde in der vorliegenden Arbeit eine Analyenmethode entwickelt, bei der moderne Extraktionsverfahren mit hochspezifischen Detektoren gekoppelt wurden. Dies führte insgesamt zu einer höheren Automatisierung, ausgezeichneten Wiederfindungsraten bei gleichzeitiger Minimierung von organischen Lösemitteln. Zusätzlich wurde gezeigt, dass unter Berücksichtigung bestimmter Rahmenbedingungen die Aufreinigung gänzlich vermieden werden kann, was zu einer weiteren Beschleunigung der Verfahrensabläufe, verminderten Kosten und Ausschluss einer Fehlerquelle führte.

## 5.2 Riassunto

Attualmente, in oltre 75 stati vige una legislazione inerente le micotossine. Dato il crescente interesse per le micotossine è probabile che i limiti esistenti vengano estesi ad altre derrate alimentari e ad altre micotossine. In quest'ottica è prevedibile l'attuazione di un piano di monitoraggio al duplice scopo di proteggere, la salute del consumatore e il commercio. Durante lo sviluppo di nuovi metodi analitici sarebbe, quindi, opportuno considerare l'elevato numero di analisi che dovranno essere effettuate.

Lo scopo del presente studio è quello di sviluppare un metodo analitico ad alta produttività, velocità, automazione, affidabilità e che consenta di ridurre i costi e l'uso di solventi pericolosi. Tale scopo è stato raggiunto accoppiando sistemi di determinazione altamente sofisticati, quali LC-MS, con tecniche di estrazione moderne, che permettono di migliorare l'efficienza di estrazione riducendo contemporaneamente il tempo necessario per l'analisi e permettendone l'automazione.

Durante la prima fase di questo studio è stato sviluppato un metodo rapido per la determinazione dello zearalenone, basato sull'iniezione diretta dell'estratto crudo in LC-MS. Inizialmente, al fine di raggiungere la migliore sensibilità dello strumento, sono stati valutati gli effetti di diversi parametri dell'interfaccia sul segnale acquisito. Successivamente è stato valutato, in diverse condizioni di lavoro, il ruolo dell'effetto matrice, che è stato superato effettuando la quantificazione su curve di calibrazione prodotte sulla matrice ed utilizzando uno standard interno (ZAN).

Nella seconda parte della ricerca sono state selezionate le condizioni operative migliori per l'estrazione dello zearalenone utilizzando tecniche alternative, la cui efficienza di estrazione è stata paragonata a quella delle tecniche convenzionali. Grazie all'eliminazione del passaggio di purificazione del campione, è stato possibile eliminare le perdite dell'analita che si verificano durante questa fase, permettendo in tal modo di valutare realmente l'efficienza di estrazione delle diverse tecniche senza che queste fossero influenzate dalle perdite derivanti dalla manipolazione del campione.

L'influenza del tempo, della temperatura e della miscela di estrazione sull'efficienza di

estrazione dello ZON, estratto con due tecniche di estrazione alternativa, MAE e PLE, sono state valutate tramite factorial design. Questo metodo statistico permette di valutare simultaneamente gli effetti di ogni singolo fattore e l'interazione esistente tra i vari fattori considerati.

Adeguate condizioni di estrazione sono state determinate per entrambe le tecniche di estrazione (MAE, PLE), permettendo di raggiungere buoni recuperi, confermati analizzando campioni precedentemente utilizzati per uno schema di valutazione. Quale ovvio evolversi del lavoro, sin qui svolto, è stata effettuata una comparazione tra le tecniche di estrazione convenzionali e quelle alternative. Secondo il test di Sheffe's, i metodi indagati si possono dividere in due gruppi, da una parte BLE, SHA e UE e dall'altra MAE e PLE. I metodi alternativi di estrazione hanno una efficienza di estrazione statisticamente significative rispetto a quelli convenzionali. In assoluto MAE è risultato leggermente superiore a tutti gli altri metodi. Tuttavia, nel caso in cui lo strumento per effettuare l'estrazione alternativa non sia disponibile, i metodi di estrazione convenzionale hanno comunque dimostrato una buona efficienza di estrazione.

Infine la strategia del simplex è stata usata per modificare la miscela di estrazione utilizzata per la PLE, pur senza rinunciare all'efficienza di estrazione, con l'intento di utilizzare solventi meno tossici e di aumentare la percentuale dell'acqua impiegata sino al 50%, dimezzando la quantità di solvente organico normalmente usata per la determinazione dello ZON.

Riassumendo, in questo studio tecniche di estrazione alternative sono state accoppiate a sistemi analitici altamente sofisticati (MAE-LC-MS o PLE-LC-MS). L'utilizzo di tecniche di estrazione alternative fornisce oltre a migliori recuperi, la possibilità sia di ridurre il tempo richiesto per l'estrazione sia di automatizzare il processo con l'opzione di ridurre il consumo di solvente organico. Con le dovute precauzioni è possibile iniettare in LC-MS direttamente gli estratti crudi, con il vantaggio di evitare perdite dell'analita ricercato durante la fase di purificazione del campione e di ridurre i tempi di analisi. Inoltre, l'eliminazione delle colonne di purificazione ad immunoaffinità riduce i costi d'analisi.

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## 7 CURRICULUM VITAE

### Personal Data

Name: Lea Pallaroni  
Date of birth: 25-08-1971  
Place of birth: Piacenza, Italy

### Education

1985- 1990: Scientific high school “L. Respighi”  
1990-1996: University of Agricultural science, Faculty of Agriculture  
July 1996: Degree in Agriculture, Faculty of Agriculture, Institute of Food and Nutrition Science, Piacenza, Italy  
Thesis: “Mycotoxins and markers of fungal growth in feeds”  
1996: Specialization course: “Quality system management in food industry”  
2001-2003: External doctorand, chair of Animal Hygiene Faculty of Agriculture Technische Universität München, Germany in collaboration with the European Commission, Directorate General-Joint Research Centre, Ispra, Italy.

### Work Experience

Sept 1996 Dec 1998: Institute of Food and Nutrition Science, Faculty of Agriculture, **Catholic University of Sacred Heart**, Italy  
Jul-Oct 1998: Department of Veterinary Anatomy and Public Health, Faculty of Toxicology, College of Veterinary Medicine **Texas A&M University**, Texas, USA  
Jan-Nov 1999: Department of Veterinary Physiology and Pharmacology, Faculty of Toxicology, College of Veterinary Medicine **Texas A&M University**, Texas, USA  
Dec 1999-Mar 2000: Research Institute of Toxicology (RITOX), Veterinary Faculty, **Utrecht University**, The Netherlands  
Mar 2000-Feb 2003: Food Products Unit, Institute for Health and Consumer Protection, Food and Safety Quality Unit Institute for Reference Materials and Measurements European Commission Directorate General-**Joint Research Centre**, Ispra, Italy  
April-July 2003: Inland and Marine Water Unit, Environmental Institute and sustainability, European Commission Directorate General-**Joint Research Centre**, Ispra, Italy  
Since August 2003 Assalzo, National Association of Feeding Industry Rome, Italy

## Publications

Pallaroni L, von Holst C

Comparison of alternative and conventional extraction techniques for the determination of zearalenone in corn

Anal Bioanal Chem, 2003; 376: 908-912

Pallaroni L, von Holst C

Determination of zearalenone from wheat and corn by pressurized solvent extraction and liquid chromatography – electrospray-mass spectrometry

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Stroka J, Capelletti C, Papadopoulou-Bouraoui A, Pallaroni L, Anklam E

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Pallaroni L, Björklund E, von Holst C

Optimization of Atmospheric Pressure Chemical Ionization Interface Parameters for a Simultaneous Determination of Deoxynivalenol and Zearalenone using High-Performance Liquid Chromatography-Mass Spectrometry

J Liq Chrom & Rel Techn, 2002; 25 (6): 913-926

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Analysis of procyanidins in chocolate by reversed-phase high-performance liquid chromatography with electrospray ionisation mass spectrometry and tandem mass spectrometry detection

J Chromatogr A, 2001; 926: 211-220

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Huebener HJ, Mayura K, Pallaroni L, Ake CL, Lemke SL, Herrera P, Phillips TD  
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J Food Prot, 2000; 63 (1): 106-110

## 8 LIST OF PUBLICATIONS

### 8.1 Refereed publication

- [I] Pallaroni L, Björklund E, von Holst C  
Optimization of Atmospheric Pressure Chemical Ionization Interface Parameters for a Simultaneous Determination of Deoxynivalenol and Zearalenone using High-Performance Liquid Chromatography-Mass Spectrometry  
J Liq Chrom & Rel Techn, 2002; 25 (6): 913-926
- [II] Pallaroni L, von Holst C, Sparr-Eskilsson C, Björklund E  
Microwave assisted extraction of zearalenone in wheat and corn  
Anal Bioanal Chem, 2002; 374: 161-166
- [III] Pallaroni L, von Holst C  
Determination of zearalenone from wheat and corn by pressurized solvent extraction and liquid chromatography – electrospray-mass spectrometry  
J Chromatogr A, 2003; 993 (1-2): 39-45
- [IV] Pallaroni L, von Holst C  
Comparison of alternative and conventional extraction techniques for the determination of zearalenone in corn  
Anal Bioanal Chem, 2003; 376: 908-912
- [V] Pallaroni L, von Holst, C  
Development of a Zearalenone extraction method using reduced organic solvents supported by Simplex optimisation  
Anal Bioanal Chem, Submitted

**By the author of this dissertation the following work was performed:**

**Analysis**

- Collection, handling and testing of the cereals sample to be used during the research.
- All laboratories works from the extraction to the analytical part
- Data preparation for the evaluation
- Statistical evaluation in APPENDIX III and simplex strategy in APPENDIX V

**Paper Writing**

- Introduction
- Materials and Methods
- Results and Discussion together with von Holst

**Table 8-1:** Contribution of the various authors

APPENDIX	EXPERIMENTAL PART				PAPER WRITING			Contribution
	Setting	Extraction	LC-MS Analysis	Data evaluation	INTRO	M&M	R&D	
I	EB LP	LP	LP	LP EB	LP	LP	LP EB	CvH
II	EB LP	LP	LP	LP EB CvH	EB LP	EB LP	CvH LP EB	
III	LP	LP	LP	LP	LP	LP	LP	CvH EB
IV	LP	LP	LP	CvH	LP	LP	CvH LP	
V	LP	LP	LP	LP	LP	LP	LP	CvH
Matrix effect	LP	LP	LP	LP	/	/	/	/

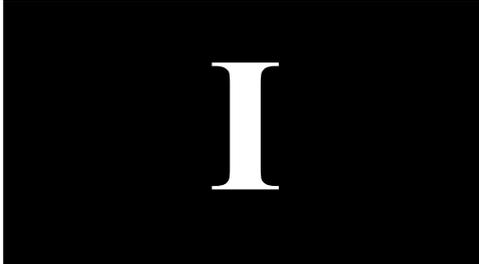
LP: Lea Pallaroni  
 CvH: Christoph von Holst  
 EB: Erland Björklund

INTRO: Introduction  
 M&M: Materials and Methods  
 R&D: Results and Discussion



**9 APPENDIX A - PUBLICATIONS**

**9.1 Appendix I**



**I**

**OPTIMIZATION OF ATMOSPHERIC  
PRESSURE CHEMICAL IONIZATION  
INTERFACE PARAMETERS FOR THE  
SIMULTANEOUS DETERMINATION OF  
DEOXYNIVALENOL AND ZEARELENONE  
USING HPLC/MS**

**Lea Pallaroni, Erland Björklund, and  
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**ABSTRACT**

In this paper, the influence of several atmospheric pressure chemical ionization (APCI) parameters were investigated in flow-injection for the simultaneous determination of deoxynivalenol (DON) and zearalenone (ZON) using liquid chromatography (LC) coupled to a mass spectrometer (MS). During the optimization procedure of the APCI interface, it was revealed that vaporiser temperature and capillary temperature had a strong influence on the MS signal of DON and ZON in the positive and in the negative ionization mode. In the positive mode a higher absolute signal of both compounds was obtained,

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while in the negative mode a higher selectivity was achieved. Thereafter, tube lens offset, corona discharged current, and capillary voltage were investigated in the negative mode; it was shown that only the tube lens offset had a large influence on the MS signal of the analytes. Finally, the optimized conditions were confirmed injecting and separating a standard mixture on a LC column prior to MS detection.

## INTRODUCTION

The presence of mycotoxins in agricultural commodities can present a major health concern for animal and humans due to their biological activity (1). Deoxynivalenol (DON) and zearalenone (ZON) are two commonly analyzed mycotoxins, which often co-occur in cereals (2–5). The most common approach to determining DON is gas chromatography (GC) (6–8), providing good sensitivity. However, the main disadvantage is the requirement of derivatization of the sample. An alternative approach to avoid the derivatisation step has been developed by Tiebach (9) using LC-MS, applying direct liquid introduction (DLI). Since then, several additional methods have been published, using different interfaces, such as fast atom bombardment (FBA) (10–11), thermospray (11–13), and plasmaspray (11), coupled to MS detector. The most recent approaches are based on HPLC coupled to MS using APCI interface (14,15). The generally applied method for ZON is based on HPLC with fluorescence detection since ZON has good fluorescence properties (16–18). However, the recent introduction of APCI has enabled LC-MS to become a more diverse tool and, as a result, new LC-MS methods have been published, including the analysis of ZON (19–21).

Since DON and ZON normally are analyzed with two completely different approaches (GC versus HPLC), two different analyses have to be carried out if both analytes are to be determined. Only recently, a simultaneous method for determination of trichothecenes and zearalenone has been developed (22). However, this method is based on GC-MS and requires a time-consuming derivatisation step and also has the disadvantage of a long GC run.

In this paper, the basis for a simultaneous determination of DON and ZON using LC coupled to an MS ion trap equipped with an APCI interface is presented. It is well known that interface parameters play a main role in enhancing the transmission response of the MS and, hence, these parameters were carefully optimized, since no data presently are available for a simultaneous analysis of the two mycotoxins. The absolute responses and spectra were studied in the positive and negative mode by ramping vaporizer and capillary temperatures in different combinations. Further investigations on tube lens

offset, corona discharged current, and capillary voltage were carried out only in the negative ionization mode due to its better selectivity. The optimized APCI conditions were tested by separating a standard mixture of DON and ZON using liquid chromatography prior to MS detection.

## EXPERIMENTAL

### Chemical and Reagents

DON and ZON standards were purchased from Sigma (Milano, Italy). Acetonitrile, ethanol, and methanol were of HPLC grade (Aldrich, Milano, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Milano, Italy).

### Preparation of Standards Solutions

Standard solution of DON was prepared in ethanol, while ZON was dissolved in methanol obtaining two stock solutions at a concentration of 100  $\mu\text{g}/\text{mL}$  and 1000  $\mu\text{g}/\text{mL}$ , respectively. The stock solutions were tightly sealed and stored at  $+4^\circ\text{C}$ . The concentrations were checked regularly using a spectrophotometer (Shimadzu, Duisburg, Germany) according to the UV-max and the extinction coefficient ( $\epsilon$ ) reported in The Merck Index (23). The DON absorbance was measured in ethanol at 218 nm ( $\epsilon$  4500), while ZON was determined in methanol at 274 nm ( $\epsilon$  13,909) and at 316 nm ( $\epsilon$  6020). Working standard solutions were prepared by taking an exact volume of the stock solution and evaporating it under a gentle stream of nitrogen and re-dissolving the residue in acetonitrile/water (1 : 1, v/v).

### HPLC-MS Equipment

Chromatographic separation was performed using a SpectraSystem (Finnigan Mat, San Jose, CA, USA) consisting of a SCM degasser, a P4000 (low flow) quaternary pump, and an AS3000 autosampler. The HPLC system was coupled to an MS ion trap, LCQ-Deca (Finnigan Mat, San Jose, CA, USA) equipped with an APCI interface. The system was controlled with Xcalibur software, version 1.2 (Finnigan Mat, San Jose, CA, USA).

### Optimized APCI Parameters

During the optimization of the APCI the following parameters were investigated: ionization mode (positive and negative); vaporizer temperature (ramped from 150°C to 350°C in steps of 50°C), and capillary temperature (increased from 150°C to 250°C in steps of 25°C). In order to determine the interactions of the vaporizer and the capillary temperatures, all capillary temperatures were tested at all vaporizer temperatures in both ionization modes. The other parameters were kept as follows: nitrogen carrier gas and helium sheath gas flows were set at 70 and 9 arbitrary units, respectively. The capillary voltage was held at 3 V in the positive mode and -3 V in the negative mode. All other settings were pre-optimized for DON by the autotune program in the continuous infusion mode, to achieve maximum transmission for DON protonated molecular ions  $[M + H]^+$  and deprotonated molecular ions  $[M - H]^-$ .

The investigation was performed at a flow rate of 0.2  $\mu\text{L}/\text{min}$  in flow-injection mode by bypassing the column. Optimization was carried out in acetonitrile : water 15 : 85 for DON and acetonitrile : water 50 : 50 for ZON, as estimated from the retention time of the chromatographic method. A short experiment was done shooting DON and ZON in acetonitrile : water 50 : 50 and 90 : 10, respectively, and no major changes were realized. Injections of 5  $\mu\text{L}$  of 5  $\mu\text{g}/\text{mL}$  standard solutions were done in triplicate for each mycotoxin, both in positive and negative mode, while ramping the respective parameters. The MS response was acquired in full scan mode ( $m/z$  150–350) allowing evaluation of the peak area, the signal to noise ratio, and the spectrum. The evaluation of the parameters was based on the average of the peak area, calculated on the protonated molecular ions  $[M + H]^+$  and deprotonated molecular ions  $[M - H]^-$ , in positive and negative ionization mode, respectively.

Once the vaporizer and the capillary temperature were set for the negative ionization mode, the interactions between the following parameters were investigated: tube lens offset (increased from -100 V to 100 V in steps of 50 V), corona discharged current (from 3  $\mu\text{A}$  to 9  $\mu\text{A}$  in steps of 2  $\mu\text{A}$ ), and capillary voltage (from -120 V to 0 V in steps of 20 V).

### LC-MS Analysis at Optimized Conditions

For the chromatographic separation of the two analytes a liquid chromatography column XTerra RP-18 (150 mm  $\times$  2.1 mm, 3.5  $\mu\text{m}$ , C18 end-capped, pore size 120 Å, Waters, Milano, Italy) was used. A linear binary gradient, at a flow rate of 200  $\mu\text{L}/\text{min}$  was applied, starting with 1 min 100% water, increasing to 90% acetonitrile in 4 min, remaining at 90% acetonitrile for 7 min, then lowering to 0% acetonitrile in 1 min, followed by 100% water

for 2 min. After the run, the column was re-equilibrated in 100% water for 2 min. The APCI was used in the negative ionization mode and mass spectra were registered in full scan mode ( $m/z$  150–350).

## RESULTS AND DISCUSSION

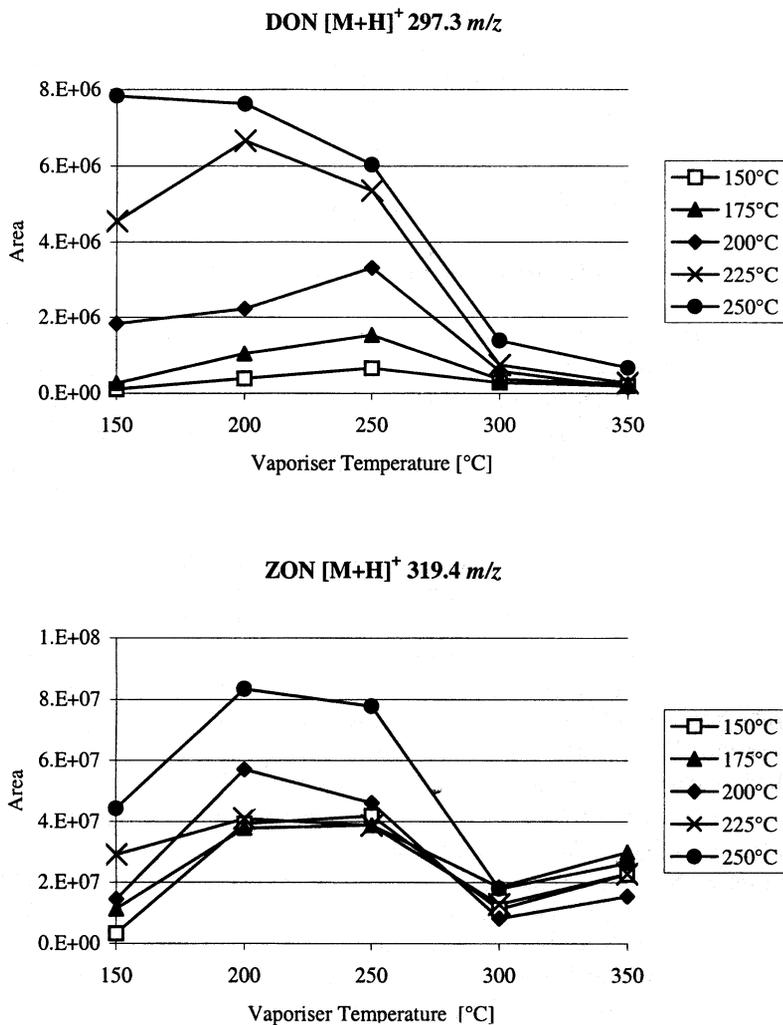
### Optimization of APCI Parameters

The LC-MS interface parameters were optimized in order to improve sensitivity and selectivity. This was done in both the positive and in the negative ionization mode by taking into account the interaction of the vaporizer and the capillary temperature. The influence of the studied parameters on the response of the target analytes and the interaction of these parameters were evaluated based on the protonated molecular ions  $[M + H]^+$   $m/z$  297.3 for DON and  $[M + H]^+$   $m/z$  319.4 for ZON in the positive mode.

The results (Figure 1) show that the DON and the ZON signals are enhanced at higher capillary temperatures and the absolute signal is higher when a vaporizer temperature below 300°C is applied. The best results for DON were obtained with a low vaporiser temperature. However, a vaporizer temperature that is too low does not allow for a satisfactory evaporation of the liquid solvent flowing from the LC system. In the positive ionization mode, a high capillary temperature (250°C) and a medium vaporizer temperature (250°C) have to be applied to work at optimized conditions using a flow rate of 0.2  $\mu$ L min. A careful evaluation of the signal to noise (S/N) ratio demonstrated a low selectivity in the positive ionization mode. Indeed, despite the high absolute signals (based on peak area), the S/N ratio for DON never exceeded 60 when injecting as much as 25 ng of the compounds. For this reason the positive ionization mode was not further considered for DON analysis in this study.

The deprotonated molecular ions for DON ( $[M - H]^-$   $m/z$  295.3) and for ZON ( $[M - H]^-$   $m/z$  317.4) were evaluated for the optimization of the APCI parameters in the negative ionization mode and the results can be seen in Figure 2.

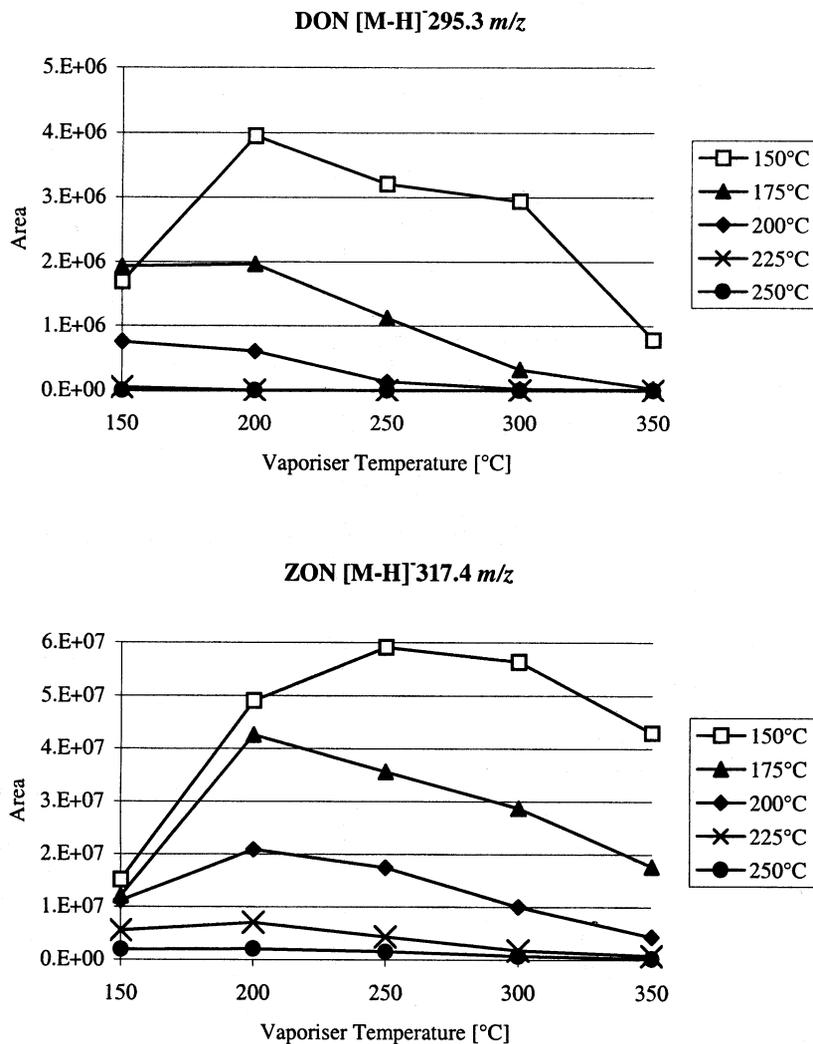
In the negative ionization mode, a higher signal is achieved when applying a lower capillary temperature, while the vaporiser temperature shows an optimum between 200°C and 300°C. In Figure 2 it can also be seen that DON has a 10 fold lower signal than ZON and, consequently, optimised parameters must mainly be based on DON detection. From Figure 2 it can be concluded that a vaporiser temperature of 200°C and a capillary temperature of 150°C would be preferable. However, the chosen parameters for APCI were 250°C for the vaporizer and 150°C for the capillary, due to the fact that 200°C in some cases might be insufficient for a complete evaporation of the solvent. The optimum of the



**Figure 1.** Influence of the vaporizer temperature and the capillary temperature (see legend) on DON and ZON responses in the positive ionization mode.

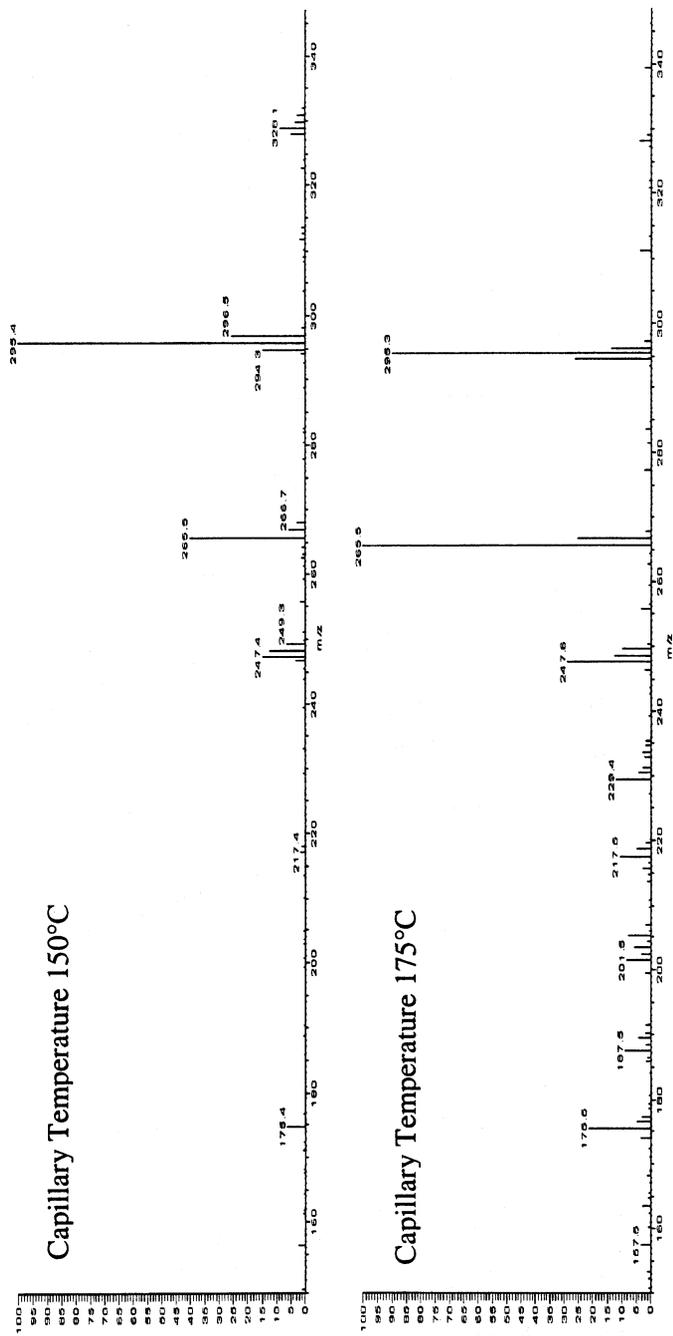
vaporizer temperature is linked to the LC flow rate and higher vaporizer temperatures are required when higher flow rates are utilized (data not shown).

After evaporating the solvent in the ionization chamber the analytes reach the first vacuum stage via the capillary. While the analytes never reach the set vaporizer temperature (due to the evaporation process), the analytes are fully



**Figure 2.** Influence of the vaporizer temperature and the capillary temperature (see legend) on DON and ZON responses in the negative ionization mode.

exposed to the capillary temperature. Consequently, thermally labile species will be more affected by the capillary temperature than by the vaporiser temperature. In Figure 3 the fragmentation pattern of DON in the negative ionization mode applying different capillary temperatures (150°C to 250°C) at a constant vaporiser temperature of 250°C, is shown.



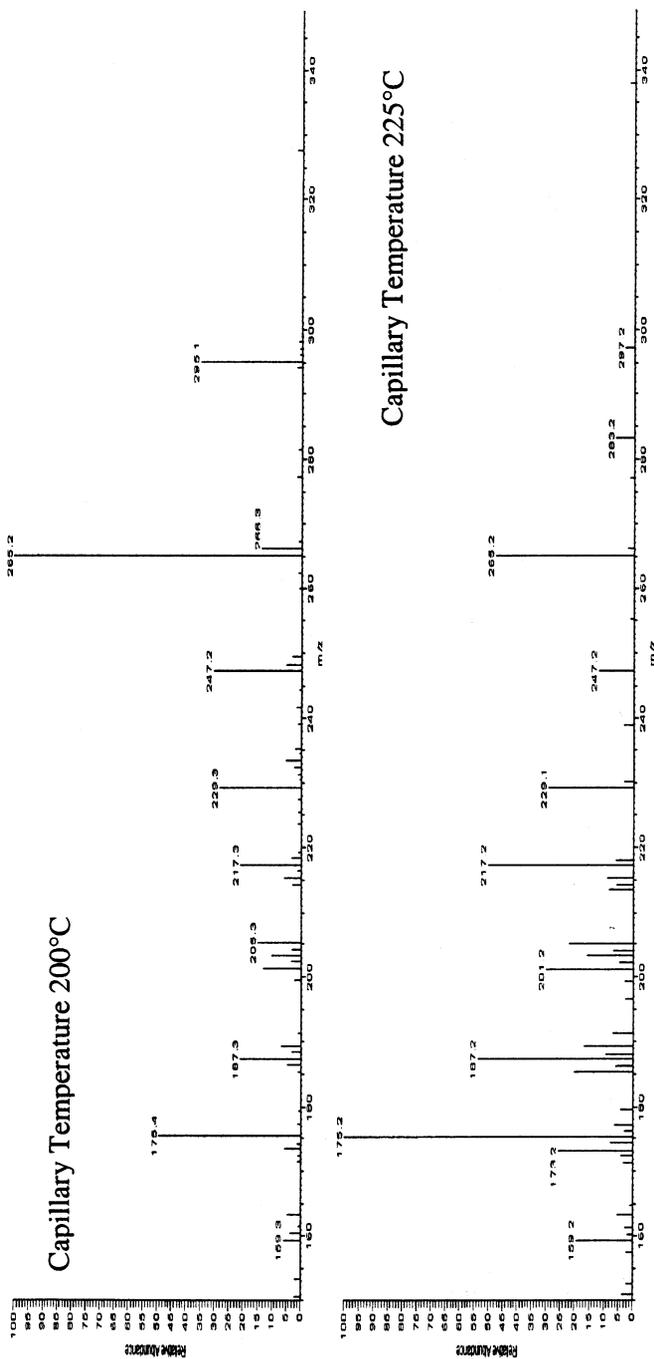
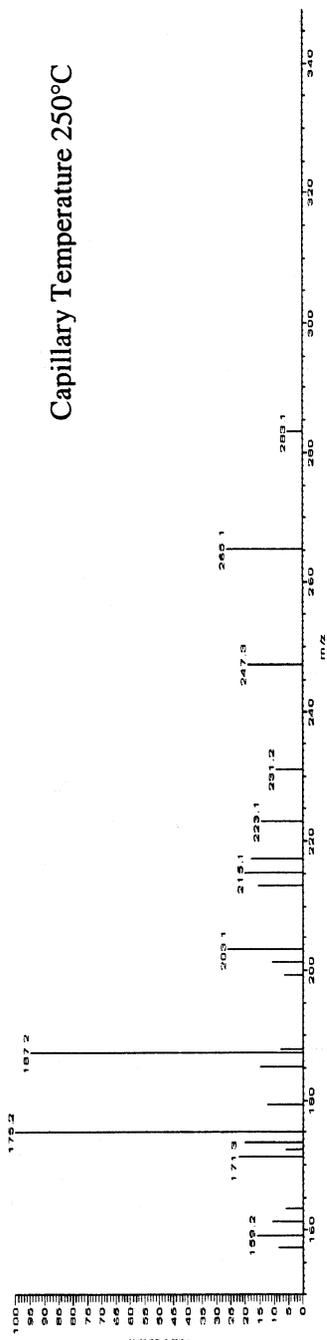


Figure 3. Spectra of DON acquired in the negative ionization mode setting the vaporizer temperature at 250°C, ramping the capillary temperature from 150°C to 250°C.

(continued)



At high capillary temperatures (225°C–250°C) the deprotonated molecular ion ( $[M - H]^-$   $m/z$  295.3) has disappeared. At the chosen optimized conditions (vaporizer temperature 250°C, capillary temperature 150°C),  $m/z$  295.3 ion gives the highest absolute signal in comparison to all other investigated combinations (except for 200°C vaporizer temperature, as discussed above). Despite the fact that a thermal degradation of DON lowers the absolute signal, it has the advantage of providing an identification fingerprint by the formation of two additional ions ( $m/z$  265, 247), which help in excluding matrix interferences when not running in the MS/MS mode. The fact that these ions are derived from the degradation of the DON ion was confirmed by acquiring the same spectrum as the DON fragmentation pattern when applying direct infusion in MS/MS scan mode.

In order to further improve DON signal in negative ionization mode, the interaction between the tube lens offset and the corona discharged current was evaluated.

From Figure 4, it is evident that the corona discharged current is responsible only for minor changes, therefore, it was set at 5  $\mu$ A, since values between 3  $\mu$ A and 5  $\mu$ A are generally recommended in order to avoid arcing in the APCI due to too high value. Figure 4 clearly shows that setting of the tube lens offset has a strong influence on DON signal, hence, it was more extensively investigated between -100 V to 0 V confirming the value of -50 V as the one providing the highest signal (data not shown). Experiments carried out ramping

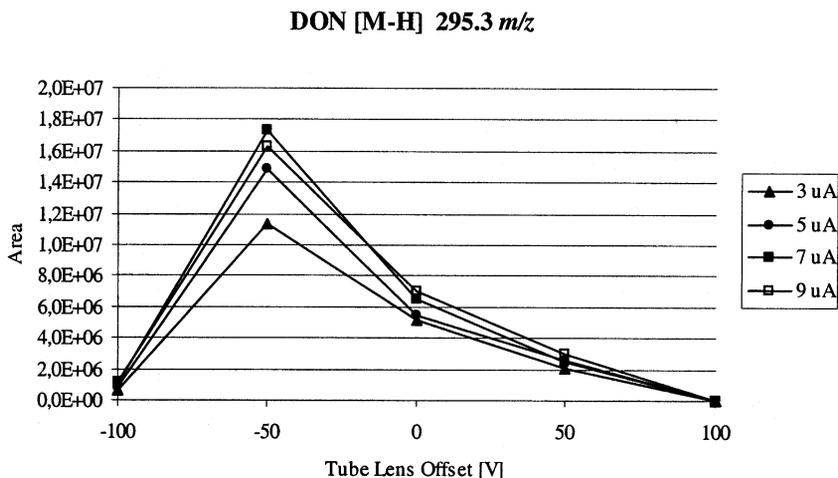
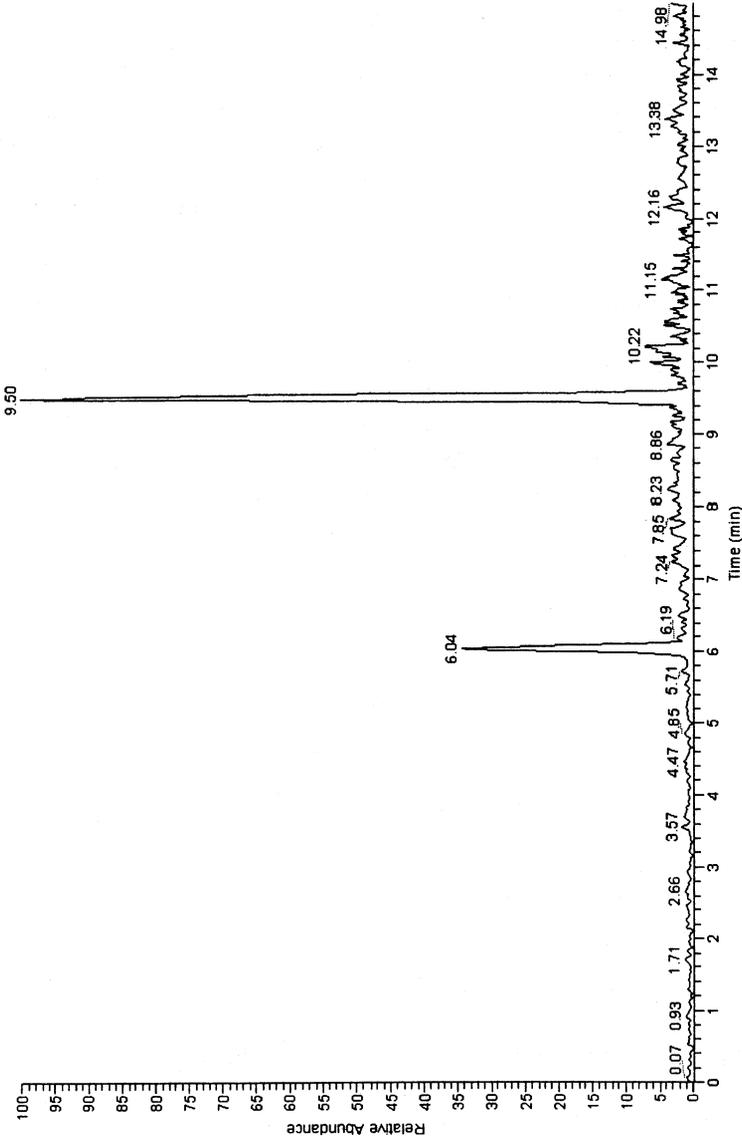


Figure 3. Continued.



**Figure 4.** Influence of the tube lens offset and the corona discharged current (see legend) on DON response in the negative ionization mode.

the capillary voltage at the optimized conditions only showed slight changes in the DON signal, reaching an optimum response between  $-40$  V and  $-20$  V.

The achieved optimised instrument APCI parameters were: vaporizer temperature  $250^{\circ}\text{C}$ ; capillary temperature  $150^{\circ}\text{C}$ ; tube lens offset  $-50$  V; corona discharged current  $5\ \mu\text{A}$ , and capillary voltage  $-20$  V.

### HPLC-MS Analysis at Optimized Conditions

The above optimised parameters, obtained by flow injection by-passing the column in the negative ionization mode, were further confirmed by analyzing a  $5\ \mu\text{L}$  mixed standard solution ( $2.5\ \mu\text{g}/\text{mL}$  of each mycotoxin) through the LC-column. In this case, using a capillary temperature of  $150^{\circ}\text{C}$  the optimal vaporizer temperature was observed at  $250^{\circ}\text{C}$  for both DON and ZON, ramping from  $150^{\circ}\text{C}$  to  $250^{\circ}\text{C}$ .

Applying the optimized APCI parameters, a standard chromatogram acquired in full scan mode, injecting  $5\ \mu\text{L}$  of a standard solution containing  $500\ \text{ng}/\text{mL}$  and  $60\ \text{ng}/\text{mL}$  of DON and ZON, respectively, was easily obtained as seen in Figure 5. Such concentrations are presently under discussion within the European Union as legal limit for food.

### CONCLUSION

During the optimization of APCI parameters it has been shown that the ionization mode combined with the vaporizer temperature and the capillary temperature had a strong influence on the signal acquired in MS.

The optimized APCI parameters allows for a simultaneous detection of DON and ZON using LC-MS. Future investigation will be devoted to quantitative analysis, as well as the development and evaluation of a simultaneous extraction method for DON and ZON.

### ACKNOWLEDGMENT

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**9.2 Appendix II**



II

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## Microwave-assisted extraction of zearalenone from wheat and corn

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**Abstract** A microwave-assisted extraction (MAE) method has been developed for determination of zearalenone in wheat and corn by LC–MS with an atmospheric pressure chemical ionization interface (APCI). Matrix effects were minimized by use of matrix-matched standard curves for quantification of the analyte. The limit of quantification (LOQ) of the method was 30 ng g<sup>-1</sup> in wheat and 20 ng g<sup>-1</sup> in corn. The rapid LC–MS method enabled analysis of the extracts without clean-up, thereby reducing analyte losses, the time required for the analytical procedure, and costs. A factorial design approach was used to examine the effect on extraction efficiency of the main extraction conditions – time, temperature, and solvent. On the basis of results from statistical assessment extraction was performed with 1:1 (v/v) methanol–acetonitrile at 80 °C for 5 min. When these extraction conditions were applied to a wheat sample from a recently conducted international proficiency test, 92% (103 ng g<sup>-1</sup>) of the assigned zearalenone concentration (112 ng g<sup>-1</sup>) in the test material was obtained. This result was within the uncertainty (u) range of the assigned value of the test material (u=±15.8 ng g<sup>-1</sup>, α=0.05) thereby demonstrating the accuracy of the method was sufficient. The precision of the whole method was also confirmed to be adequate, because the observed relative standard deviation (RSD) of 12% (n=10) also fulfils the quality criteria recommended by European guidelines for in-house method validation.

**Keywords** Zearalenone · Microwave-assisted extraction · Mycotoxin · Liquid chromatography–mass spectrometry · Food analysis

### Introduction

Microwave-assisted extraction (MAE) has started to replace conventional liquid extraction techniques; its main advantages are reduced solvent consumption and increased sample throughput [1]. Although most investigations have been devoted to determination of organic contaminants such as PAH [2] and PCB [3] in environmental samples, MAE has also started to find its way into pharmacy [4] and agriculture [5]. An important research area within the agricultural/food sector is the determination of mycotoxins in cereals. No reports of the use of MAE for extraction of mycotoxins have yet been published, apart from an interesting work on ergosterol (primary fungal metabolite) in fungal cultures [6]. A frequently investigated *Fusarium* toxin is zearalenone. Besides its low acute toxicity concentrations [7], zearalenone is a health concern in animal husbandry (especially in pigs) [8] and for humans, because of its estrogenic properties [9]. No legal limits have yet been set within the European Union, although they are under discussion. Several countries have already set their own limits, e.g. Austria 50 ng g<sup>-1</sup> in cereals, Italy 100 ng g<sup>-1</sup> in cereals and cereal products, France 200 ng g<sup>-1</sup> in cereals and vegetable oils, and Russia 1000 ng g<sup>-1</sup> [10]. Traditionally this mycotoxin is extracted by conventional liquid shaking for between 30 min up to 1 h [11, 12] or by blending for a few minutes [13, 14]. The method most commonly used for analysis of zearalenone is HPLC with fluorescence detection. To perform this analysis a clean-up step comprising liquid–liquid partitioning, solid-phase extraction, or immunoaffinity (IAC) is required [15]. Since the introduction on the market of bench-top LC–MS equipped with the atmospheric pressure chemical ionization (APCI) interface, LC–MS methods have been published for the analysis of zearalenone [16, 17, 18]. The possibility of injecting raw extract was briefly investigated in one paper, but resulted in overestimation of the level of zearalenone contamination [16].

The aim of the work discussed in this report was to develop a simple method for determination of zearalenone

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in wheat and corn based on MAE and final LC–MS analysis without a clean-up step. This would combine modern sample preparation with sophisticated analysis, reducing the overall analysis time. This paper reports the application of LC–MS to analysis of raw extracts. Quantification of zearalenone was performed by use of matrix-matched standard curves to compensate for matrix-related adverse effects on the accuracy of the determination. Zearalanone was, moreover, used as internal standard to take into account changes of the extraction solvent when conducting MAE.

## Experimental

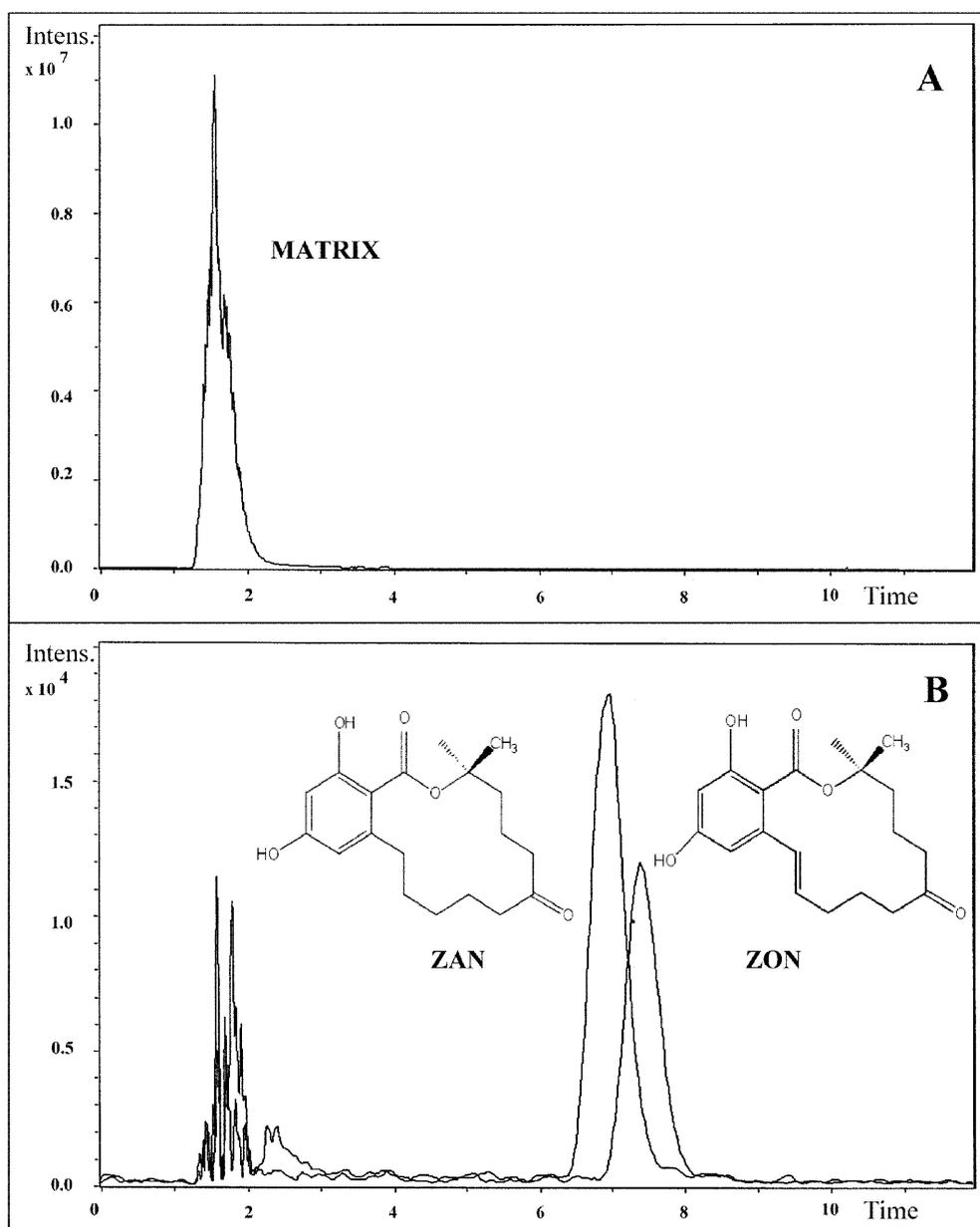
### Chemical and reagents

Zearalenone and zearalanone standards (Fig. 1 B) were purchased from Sigma (Milano, Italy). Acetonitrile and methanol were of HPLC grade (Aldrich, Milano, Italy).

Zearalenone and zearalanone standard solutions, 1000 and 200  $\mu\text{g mL}^{-1}$  respectively, were prepared in methanol, tightly sealed, and stored at  $-20^\circ\text{C}$ . Zearalenone spiking standard solution was prepared by diluting an exact volume of the stock solution in acetonitrile at a concentration of 10  $\mu\text{g mL}^{-1}$ . Two spiking levels were used, 400  $\text{ng g}^{-1}$  and 100  $\text{ng g}^{-1}$  in cereal. Zearalanone working solution was prepared at a concentration of 2  $\mu\text{g mL}^{-1}$  (1  $\mu\text{g mL}^{-1}$  for the lower investigated level) by diluting the stock solution with the extraction solvent mixture used in the respective microwave extraction.

Uncontaminated wheat and corn samples were produced during a field trial under controlled conditions and were tested for zear-

**Fig. 1** Chromatogram obtained from a wheat sample spiked with 100  $\text{ng g}^{-1}$  zearalenone and 200  $\text{ng g}^{-1}$  zearalanone. (A) TIC Chromatogram acquired in full-scan mode (m/z 250–400). (B) Extracted ion chromatogram of zearalenone (m/z 317.4) and of zearalanone (m/z 319.4)



alene contamination. Samples were ground in a Retsch (Haan, Germany) ZM100 laboratory ultra centrifuge mill, using a 0.5 mm sieve. The performance of the method was tested by analysis of contaminated wheat [19] which has been used in a proficiency test organized by the Food Analysis Performance Assessment Scheme (FAPAS) of the Central Science Laboratory of DEFRA (Department for Environmental Food and Rural Affairs) UK. As described in the FAPAS report [19] of this proficiency test, naturally contaminated wheat was fortified with an additional amount of zearalenone and blended with blank material. The homogeneity of subsamples containing 60 g material was established as sufficient by the organizer of the proficiency test; the target zearalenone concentration ( $112 \text{ ng zearalenone g}^{-1}$ ) in the test material and the corresponding uncertainty ( $u=\pm 15.8 \text{ ng g}^{-1}$ ,  $\alpha=0.05$ ) were derived from the results submitted by the participants in the study.

### LC-MS Analysis

Analysis was performed by use of an HP 1100 series liquid chromatograph equipped with a degasser, a binary pump, an auto-injector, and a thermostat (Hewlett-Packard, Palo Alto, CA, USA) coupled to an Esquire-LC ion-trap mass spectrometer equipped with an APCI interface (Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed on a  $100 \text{ mm} \times 2.1 \text{ mm}$  i.d.,  $5 \mu\text{m}$  particle size,  $180\text{\AA}$  pore size, Discovery C8 column (Supelco, Milano, Italy). The column temperature was  $35^\circ\text{C}$  and isocratic elution was performed with 45:55 (% v/v)  $\text{H}_2\text{O}$ -methanol (both containing 0.2% acetic acid) at a flow rate of  $250 \mu\text{L min}^{-1}$  for a 12 min run. The injection volume was  $5 \mu\text{L}$ .

The APCI interface was used in the negative ionization mode because of its better selectivity. Optimization of the APCI settings was performed by in-flow injection of  $5 \mu\text{L}$  of a  $1 \mu\text{g mL}^{-1}$  zearalenone standard solution. The APCI settings used were: nebulizer 50 psi, dry gas  $3 \text{ L min}^{-1}$ , dry temp  $300^\circ\text{C}$ , APCI temp  $300^\circ\text{C}$ , HV capillary 680 V, and HV corona 4900 V. Mass spectra were recorded in full scan mode ( $m/z$  250–400), by averaging five spectra, while rolling averaging tool was disabled. The retention times for zearalanone and zearalenone were 7.1 and 7.6 min, respectively.

### Procedures

All extractions were performed with a CEM (Matthews, NC, USA) MSP 1000 microwave-assisted extraction unit. Method development to find the most suitable extraction conditions was performed on wheat and corn spiked at a concentration of  $400 \text{ ng g}^{-1}$ . By applying the optimized extraction conditions the effect on zearalenone recovery of the power applied was investigated at two different spiking levels, 400 and  $100 \text{ ng g}^{-1}$ . All trials were performed on 5-g samples spiked with 200 or  $50 \mu\text{L}$  of the  $10 \mu\text{g mL}^{-1}$  zearalenone spiking solution directly into the extraction vessels to ensure complete and reproducible introduction of the spiked compound. The spiking solvent was left to evaporate for approximately 1 h and the spiked material was then mixed with a metal spoon, to crush aggregates formed as a result of adding the spiking solvent, before addition of 20 mL solvent. After installation of a new rupture membrane the extraction vessel was closed and the operating steps started. Different power settings (150–500 W) were chosen to give similar heating times when a different number of vessels was present in the oven. The heating time was always a minor proportion of the total extraction time. It was important that heating was not too crude, because this would result in a fluctuating temperature profile during the extraction. At the end of the extraction time the extracts were left to cool below  $35^\circ\text{C}$  before the vessels were opened. Internal standard solution (zearalanone concentration  $2 \mu\text{g mL}^{-1}$ , 1.0 mL) was added and the extraction solvent that had condensed on the wall of the vessel was collected by addition of ca 3 mL extraction solvent. After thorough mixing a 2-mL subsample was transferred to a 2.5-mL disposable plastic syringe and filtered into vials by use of a Titan PTFE  $0.45\text{-}\mu\text{m}$  filter, which has been tested to ensure it did not interact with the target analytes. After filtration the raw extract was injected directly into the LC-MS system without further clean-up.

Quantification was based on extracted ion chromatograms of the deprotonated molecular ions  $[\text{M}-\text{H}]^-$ ,  $m/z$  317.4 for zearalenone and  $m/z$  319.4, for (ISTD) zearalanone. To compensate for matrix interference effects the calibration solutions containing zearalenone and zearalanone were diluted with blank matrix solution. Three matrix solutions were prepared by extracting blank wheat and corn at  $40^\circ\text{C}$  for 10 min with the different solvents investigated in this study, thereby ensuring that the solvent used in the method development trials and the solvent used in the matrix standard solution were identical.

Statistical evaluation of the results was performed by use of Statistica software (Stat Soft, USA). A factorial design approach [20] was used to determine the effects of the main factors time, extraction temperature, and solvent on the concentration of zearalenone measured in the test materials. It was of particular interest to establish which of these factors had a significant influence on the recovery of zearalenone from both matrices. The time (5 min, 10 min) and temperature ( $40^\circ\text{C}$ ,  $80^\circ\text{C}$ ) were varied at two levels using three different solvents (ACN, MeOH, 1:1 v/v ACN-MeOH) thereby resulting in a total of 12 different extraction conditions to be evaluated. Because all experiments were performed in triplicate, the final number of trials was 36 for each matrix.

## Results and discussion

### LC-MS analysis

With the LC-MS method set up the target analytes were separated from most of the matrix compounds, which elute mainly within the first 3–3.5 min (wheat and corn, respectively) as shown in Fig. 1A. The target analyte peaks were not completely separated by liquid chromatography (Fig. 1B), but this did not hamper determination of zearalenone and zearalanone, because of the different masses used for quantification.

Matrix interferences, which might have led to signal suppression or enhancement in LC-MS analysis, resulting in under or over-estimation of the analytes, were reduced by chromatographic separation of matrix interferences and target analytes. Quantification was, moreover, based on matrix-matched standard curves, enabling precise quantification; results were confirmed to be within 6% of the amount present in quality-control samples, which were standard, diluted into matrix at a known concentration level, set within the sequence. The need to use matrix-matched curves was demonstrated by evaluating calibration curves based on standards diluted in the mobile phase or in the matrix extracts. Although high values for  $R^2$  were obtained for all standard curves, the equations of the curves were different for the different matrices (wheat and corn), and also when using different extraction solvents (ACN, MeOH, and MeOH-ACN) for the same matrix. These effects resulted in over-estimation of the zearalenone content when quantification was based on a standard calibration plot prepared on mobile phase (data not shown). Quantification was based on a five-point calibration plot in the concentration range  $25\text{--}250 \text{ ng mL}^{-1}$  for the higher contamination level and  $5\text{--}50 \text{ ng mL}^{-1}$  for the lower level of zearalenone; the internal standard zearalanone was added at 100 and  $50 \text{ ng mL}^{-1}$ , respectively. Calibration curves were linear with values for  $R^2$  varying from 0.9829 to 0.9981 for wheat and from 0.9980 to 0.9999 for corn. To compensate for variations in solvent

**Table 1** Recovery of zearalenone (spiking level=400 ng g<sup>-1</sup> sample) from ground wheat and corn using microwave-assisted extraction with different solvents, times, and temperatures

Time (min)	Temperature (°C)	Solvent <sup>a</sup>	Recovery from wheat (%)			Recovery from corn (%)		
5	40	MeOH	65	80	73	91	102	99
5	80	MeOH	93	86	77	97	109	102
10	40	MeOH	65	73	73	93	76	66
10	80	MeOH	72	84	90	85	84	100
5	40	ACN	78	98	87	81	96	97
5	80	ACN	74	70	65	92	97	95
10	40	ACN	51	51	49	86	86	90
10	80	ACN	79	84	79	93	108	96
5	40	MeOH-ACN	72	101	84	88	86	96
5	80	MeOH-ACN	81	103	93	102	95	93
10	40	MeOH-ACN	83	94	83	86	80	100
10	80	MeOH-ACN	96	96	76	96	82	92

<sup>a</sup>MeOH=methanol, ACN=acetonitrile

volume, the concentration of zearalenone calculated from the calibration plot was multiplied by the ratio of the mean zearalanone response in the five calibration solutions and the zearalanone response in the respective sample extract.

The limits of detection (LOD) (signal-to-noise ratio (S/N)=3:1) and of quantification (LOQ) (S/N=10:1) for zearalenone were determined for the matrixes. The instrumental LOD was 15 pg and 10 pg on column for wheat and corn, respectively. The method LOQ was 30 ng g<sup>-1</sup> for wheat and 20 ng g<sup>-1</sup> for corn. Although detection limits are somewhat lower for other methods than for the approach presented in this paper, this method has the main advantages of drastically reducing analyte losses during sample handling, the time required for the analytical procedure, and the costs of material and manpower, which are desirable characteristics during method development.

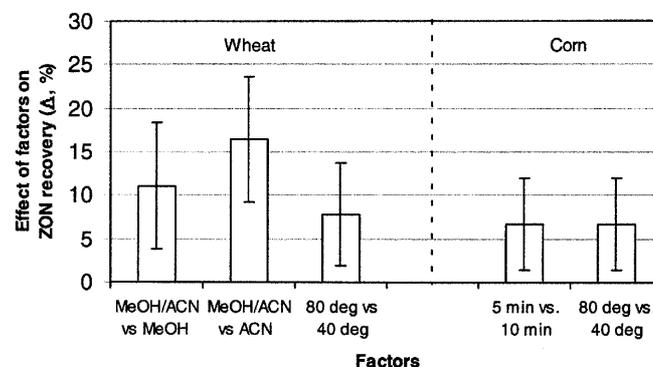
#### Evaluation of different extraction conditions – solvent, temperature, and time

The first part of the study was devoted to finding a suitable extraction solvent, temperature, and extraction time. Conventional shaking methods normally utilize a variety of mixtures of acetonitrile (ACN) or methanol (MeOH) and water. In this study water was excluded, because it formed a thick “porridge” when cooked together with wheat and corn flour. Instead, ACN and MeOH were investigated separately and as a 1:1 (v/v) mixture. The temperature was set to 40 or 80 °C with 5 and 10 min extraction time. Higher extraction temperatures were not investigated because preliminary trials at 120 °C resulted in slight thermal degradation of the target analyte, as has previously been reported [21], and at high temperatures more matrix material was extracted, resulting in a very dirty raw extract. Combination of 80 °C with a shorter extraction time was not considered, because the instrument took 50 s to reach the temperature set and reducing the length of the treatment did not enable significant time saving. The experiment design and results showing the recovery of zearalenone from wheat and corn are presented

in Table 1. The recovery values were calculated from the zearalenone concentration obtained in the respective experiments and the spiking level of the matrixes.

Subjecting the results shown in Table 1 to statistical analysis enables estimation of the analytical error on the basis of the replicates of all factor combinations and the effect of the main factors on the recovery of zearalenone in the test materials. Calculation of the overall mean value of the recovery of zearalenone obtained in all experiments revealed that zearalenone can be more easily extracted from corn than from wheat, irrespective of the experimental conditions used, because the average recovery of zearalenone was 79% for wheat and 92% for corn. Comparable values for the standard deviation (SD) of the zearalenone recovery were obtained for wheat (SD=8.5) and for corn (SD=7.6). The standard deviation representing the analytical error of the method was used to check for the significance of the factor effects calculated from the results from the experimental design.

Significant effects of factors ( $\alpha=0.05$ ) influencing zearalenone recovery from wheat and corn are shown in Fig. 2.



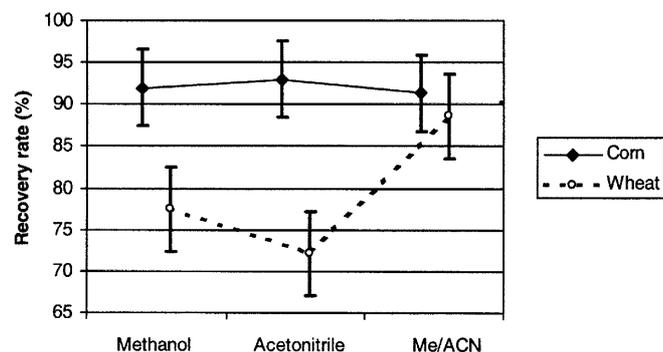
**Fig. 2** Significant effects ( $\alpha=0.05$ ) of factors on the recovery of zearalenone, with 95% confidence intervals. The bars represent the difference ( $\Delta$ , %) between the average values obtained under two different sets of experimental conditions. The first conditions always yielded the higher zearalenone recovery (i.e. by using MeOH-ACN zearalenone recovery was increased by 11% compared with use of MeOH); deg=°C

Effects of factors that did not exceed the 5% significance level were omitted from this figure. The effect of a factor is the difference between the mean response from all trials performed at different factor levels; this indicates the impact on zearalenone recovery when the level of the factor is changed. For example, the average zearalenone recovery from wheat for all trials performed at 80 °C was 83% and the average zearalenone recovery at 40 °C was 76%, resulting in a temperature effect of 7.8%.

Error bars at the 95% confidence level are shown to indicate the uncertainty of the estimated effects. All other effects were calculated accordingly. Study of the results from wheat and corn revealed that the effect of temperature was significant for both, and that extracting the samples at 80 °C resulted in increased zearalenone recovery compared with 40 °C. The optimized temperature was therefore set at 80 °C. The recovery of zearalenone from wheat depended significantly on the extraction solvent whereas for corn the extraction time, not the solvents, was important.

Evaluation of the effect of extraction time on the recovery of zearalenone from corn revealed, surprisingly, that recovery of zearalenone was significantly higher when samples were extracted for 5 min (average zearalenone recovery 95%) rather than 10 min (average zearalenone recovery 89%). The same tendency was observed for wheat but the corresponding effect was not significant and is, therefore, not presented in Fig. 2. Statistical analysis of the results from wheat showed, however, that because of interaction between temperature and time this effect was present only when the extraction temperature was 40 °C, because the average zearalenone recovery was 70% for 5 min and 82% for 10 min. In contrast with this result, extraction time had no effect when the extraction was performed at 80 °C – the corresponding zearalenone recovery was 88% for 5 min extraction and 90% for 10 min. For these reasons conditions chosen for extraction of both matrixes were 5 min at 80 °C.

The impact of the solvents on zearalenone recovery was quite different for the different matrixes, as shown in Fig. 3. For corn the average zearalenone recovery exceeded 90% irrespective of the solvent used, whereas for



**Fig. 3** Comparison of mean values for the recovery of zearalenone from corn and wheat, depending on the solvent used. The error bars represent the 95% confidence interval

wheat only the MeOH–ACN mixture gave acceptable results – the corresponding zearalenone recovery was 89%. In contrast with this result the average zearalenone recovery was 72% when using ACN and 77% when using MeOH. As shown in Fig. 2 MeOH–ACN yielded significantly higher results than those obtained by use of pure acetonitrile or methanol. From these results we concluded that the preferred solvent, suitable for both matrixes, was the mixture of methanol and acetonitrile.

#### Evaluation of different power settings

Although satisfactory recoveries had already been obtained at 500 W by applying the optimized conditions (5 min, 80 °C, MeOH–ACN), experiments were performed to establish whether further improvement could be achieved at higher power. These experiments were performed on wheat, which had been shown to be a more difficult matrix than corn. Two different spiking levels – 400 and 100 ng g<sup>-1</sup> – chosen to match more closely the levels found in nature – were investigated. In these trials the microwave oven was completely filled (12 vessels) with five replicates (n=5) for each spiking level and two blanks. At a power setting of 600 W recovery for the five replicates spiked at 400 and 100 ng g<sup>-1</sup> were 96% (RSD=10%) and 95% (RSD=14%), respectively. The same experiment performed at 900 W gave, for the same spiking levels, average recoveries of 90% (RSD=7%) and 94% (RSD=7%). This means the power setting is less important in the range 600–900 W, because quantitative recoveries were always obtained for reasonable contamination levels.

#### Verification of the final extraction method

To confirm the results obtained with the test material prepared in our laboratory we applied the optimized method to a wheat sample from an international intercomparison study [19]. Although this material was a mixture of naturally contaminated and fortified wheat, we used it because a commercially available reference material for zearalenone was not available. The target concentration was 112 ng g<sup>-1</sup>, which is close to the lower spiked level investigated during method development. The wheat sample was also the material that seemed to interact most strongly with the analyte and was, therefore, the best choice for evaluation of the method. It should also be stressed that spiked samples are still considered valid for evaluation of extraction methods, and only recently have certified materials for certain mycotoxins started to become available.

The first evaluation was performed in triplicate with a total of six cells in the oven (three blanks). The power setting was 500 W, in accordance with the discussion above. The average recovery in this experiment was 93% (RSD=7%, n=3) corresponding to 104 ng g<sup>-1</sup> compared to 112 ng g<sup>-1</sup>, the assigned value of the material. A second trial was also tested in which 10 samples of certified material were extracted simultaneously. In total 12 cells were

present in the oven (two blanks) meaning that it was completely filled. The power setting was, in this case, 600 W, because no increase in recovery had been observed for the spiked samples at a higher power setting. At these settings the average recovery was 92% corresponding to 103 ng g<sup>-1</sup> (RSD=12%, n=10), identical with the result from triplicate analysis performed on the same material. Both results can be considered satisfactory, because they are within the uncertainty range ( $u=\pm 15.8$  ng g<sup>-1</sup>,  $\alpha=0.05$ ) of the target zearalenone concentration (112 ng g<sup>-1</sup>). The RSD obtained use of this material was also considered adequate, because the values are below acceptable limits of the RSD (15%) set by a proposed guideline of the European Commission on in-house validation [22].

The data clearly demonstrate that MAE can be used for simultaneous extraction of multiple samples with good recovery of the analyte of interest. One disadvantage of using 80 °C is that the cooling time is increased to approximately 20 min compared to the treatment at 40 °C. According to a previous paper [4] the handling time can be reduced by cooling the sample vessel on ice for 2 min immediately after extraction, without reducing extraction efficiency.

## Conclusions

The LC–MS method presented in this paper enables accurate quantification of zearalenone in wheat and corn without requiring any clean-up step before analysis. The limit of quantification was somewhat higher than that of IAC–LC–FLD. By eliminating possible loss of the target analyte in sample-preparation steps, this LC–MS method enabled unbiased examination of the main factors affecting the zearalenone extraction efficiency; this was required to establish optimized extraction conditions.

The MAE method developed is capable of simultaneous extraction of 12 samples in less time than extraction by shaking. MAE extraction is also superior to the mixing procedure, which requires only a few minutes for extraction, but the total work load is much higher for the technician conducting the mixing procedure. Combination of MAE with LC–MS is, therefore, a rapid and competitive method for determination of zearalenone in food, especially when analyzing a large number of samples; this compensates for the higher cost of the instrumentation re-

quired. Another feasible application of this LC–MS method could be the identification of zearalenone and other estrogenic compounds in complex matrixes such as animal tissue.

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**9.3 Appendix III**



III

# Determination of zearalenone from wheat and corn by pressurized liquid extraction and liquid chromatography–electrospray mass spectrometry

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## Abstract

Zearalenone (ZON) was extracted from wheat and corn by using pressurized liquid extraction (PLE) and the PLE extracts were analyzed using liquid-chromatography–mass spectrometry (LC–MS) without further clean-up procedures. A statistical design approach was applied to evaluate the influence of several extraction parameters such as temperature (40 °C; 80 °C; 120 °C), time (5 min; 10 min) and solvent extraction mixture [acetonitrile–water (9:1, v/v); methanol–water (8:2, v/v); methanol–acetonitrile (1:1, v/v)] on fortified cereals. The results showed a strong influence of the solvent composition on recovery of ZON. Quantification of the analytes was performed by LC–MS analysis of the raw extract using matrix-matched standard curves. The method performance was tested in the selected conditions (80 °C; 5 min; two cycles; methanol–acetonitrile) on samples which had been previously used for an international proficiency test. Compared to the assigned value, the recovered ZON was 118% [relative standard deviation (RSD)=5.2%,  $n=3$ ] and 107% (RSD=2.2%,  $n=3$ ) in wheat and corn, respectively. Therefore, PLE can be used for ZON extraction, achieving good performances and allowing for an automated handling of the sample extraction step. Successively, the influence of temperature and number of cycles was investigated on naturally contaminated corn. From these results it could be concluded that fortified experiments perfectly mimicked naturally contaminated samples.

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*Keywords:* Cereals; Food analysis; Zearalenone

## 1. Introduction

The extraction step has often proved to be the bottleneck of most analytical procedures, as it is one of the least evolved parts of the whole method. One

of the most promising and recent sample preparation techniques is the pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), which offers the advantages of reducing solvent consumption and allowing for automated sample handling [1]. Although PLE has started to replace conventional extraction in environmental [2,3] and food analysis [4–6], its application in the mycotoxin field has up to now been limited to fumonisin [7,8]. It has been reported that increasing

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the temperature in PLE allows for a higher extraction efficiency of fumonisin from corn products [7].

Zearalenone (ZON) is a *Fusarium* toxin of great importance due to its negative health effect on animal husbandry, e.g. pigs [9], and on humans [10]. Traditionally, this mycotoxin is extracted by conventional liquid shaking for about 30–60 min [11,12] or by blending for a few minutes [13,14]. Various extraction mixtures have been used to extract ZON from cereals, the most commonly used are acetonitrile–water [13–16], and methanol–water [12,16] mixed at different ratios. Extraction performances of conventional methods for mycotoxins analysis are constantly put under discussion, evaluating the effect of shaking versus blending [17]; additionally, different extraction solvent mixtures [16] as well as single versus multiple extractions are also discussed [18]. Analysis of ZON is commonly performed by applying a clean-up step (liquid–liquid partitioning, solid-phase extraction or immunoaffinity) after the extraction and using HPLC with fluorescence detection [19]. Since the availability of bench-top LC–MS systems equipped with atmospheric pressure interfaces, several LC–MS methods have been published using atmospheric pressure chemical ionisation (APCI) [20–23]. The possibility of injecting raw extract was briefly investigated in one paper, re-

sulting in an overestimation of the ZON level [20], whilst it has been already used for developing a microwave assisted extraction (MAE) method [23].

The aim of this work was to develop a simple method for the determination of ZON in wheat and corn based on PLE and detection in LC–MS equipped with an electrospray ionisation (ESI) interface without performing any clean-up step. This will merge automated and modern sample preparation with sophisticated analysis, resulting in an overall reduction of analysis time, manpower and an increase in throughput. To evaluate the feasibility to use PLE instead of a conventional extraction technique, several extraction parameters such as temperature, static time and solvent composition have been investigated using fortified cereals. Quantification of ZON was carried out by using matrix-matched standard curves to compensate for matrix-related adverse effects, and zearalanone (ZAN) was used as internal standard.

## 2. Experimental

### 2.1. Chemicals

ZON and ZAN (Fig. 1) standards were purchased

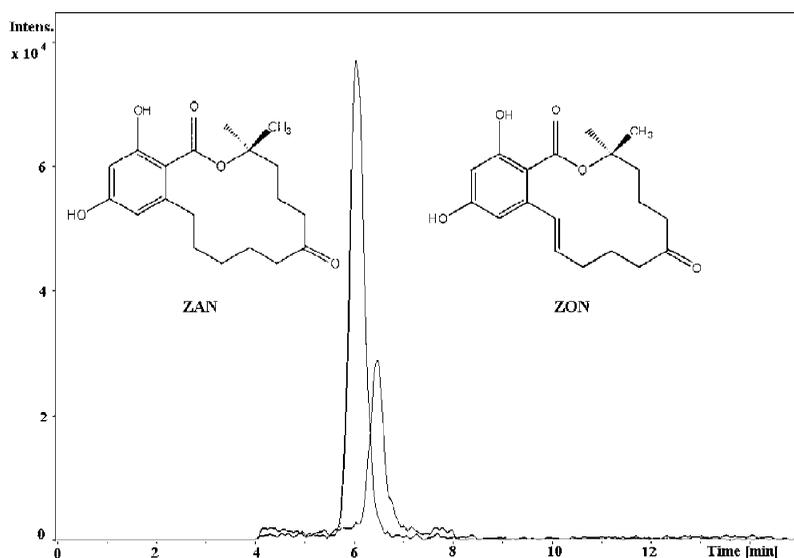


Fig. 1. Extracted ion chromatogram obtained from a corn sample spiked with 80 ng/g of ZON ( $m/z$  317.4) and 200 ng/g of ZAN ( $m/z$  319.4) as internal standard.

from Sigma (Milan, Italy) and diatomaceous earth sorbent Hydromatrix from Varian (Dionex, Milan, Italy). Solvents were of HPLC grade (Aldrich, Milan, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Milan, Italy).

## 2.2. Samples

Method development was performed using fortified samples of wheat and corn and a naturally contaminated corn. Samples were ground in a laboratory ultra centrifuge mill ZM100 (Retsch, Haan, Germany) using a 0.5-mm sieve.

The method performance was determined by analysis of wheat [24] and corn [25], which had been previously used in proficiency tests organized by the Food Analysis Performance Assessment Scheme (FAPAS) of the Central Science Laboratory of DEFRA (Department for Environmental Food and Rural Affairs) UK. Characteristics of these materials are described in the respective reports [24,25]. The target ZON concentration (112 ng/g wheat; 285 ng/g corn) and the corresponding uncertainty ( $u = \pm 15.8$  ng/g,  $\alpha = 0.05$  in wheat;  $u = \pm 19.2$  ng/g,  $\alpha = 0.05$  in corn) of the test materials derived from the results submitted by the participants of the study.

Naturally contaminated corn sample was provided by a German laboratory. Its ZON target value was established by LC–MS at 5051 ng/g ZON (RSD = 7.4%;  $n = 10$ ) according to a modified International Standard Organisation (ISO) method [14] as reported in the procedure.

## 2.3. LC–MS analysis

Analysis of the PLE extracts was performed using an HP 1100 Series HPLC system equipped with a degasser, a binary pump, an autoinjector and thermostat coupled to an ion-trap mass spectrometer equipped with an ESI interface (Hewlett-Packard, Palo Alto, CA, USA). The chromatographic separation was achieved working in isocratic [methanol–water (0.2% acetic acid) 55:45 (v/v)] at a flow-rate of 0.2 ml/min on a Discovery C<sub>8</sub> column (100 × 2.1 mm, 5 μm particle size, 180 Å pore size; Supelco, Milan, Italy) kept at 35 °C. The injection volume was 5 μl.

The ESI interface was used in the negative

ionization mode due to its better selectivity. The following ESI parameters were applied: nebulizer 50 p.s.i. (1 p.s.i. = 6894.76 Pa), dry gas 10 l/min, dry temperature 350 °C and high voltage capillary 4000 V. The mass spectra were recorded in full scan mode (200–550  $m/z$ ). The quantification was based on extracted ion chromatograms of the deprotonated molecular ion  $[M-H]^-$   $m/z$  317.4 for ZON and  $[M-H]^-$   $m/z$  319.4 for ZAN, which was used as the internal standard for the LC–MS analysis.

## 2.4. Procedures

All the extractions were performed on an ASE 200 System (Dionex, Sunnyvale, CA, USA). An amount of 5 g of sample was weighed in a small beaker and mixed thoroughly with 3 g of Hydromatrix to obtain a porous mixture to enable the extraction solvent to flow through the sample during the extraction. The mixture was poured into a 22 ml thimble, which was packed by adding a layer of Hydromatrix at the base and at the top in order to fill the thimble completely according to the instrument's manufacturer recommendations. The final volume of the solvent after extraction was always close to 35 ml. When the extraction solution reached room temperature, 1.0 ml of internal standard solution (ZAN 2 μg/ml) was added and the volume was filled up to 40 ml. After thoroughly mixing, an aliquot was filtered into vials using a Titan PTFE 0.45-μm filter, which has been tested for not interacting with the target analytes. After filtration, the raw extract was injected directly into the LC–MS system without any further clean-up step. Extracts from low contaminated samples were reduced by a factor of 3 prior to analysis.

Preliminary experiments have been carried out either to evaluate a likely interaction between the Hydromatrix and the target analyte or to guarantee the most appropriate settings of the parameters, which were not further investigated in the study. ZON was spiked directly into the Hydromatrix in the absence of sample matrix and extracted with two sequential static extractions of 5 min each, at two temperatures (40 and 80 °C) applying two different flush volumes of 60 and 75%.

A statistical design approach [26] was applied to evaluate the influence of PLE parameters on extraction efficiency on samples of wheat and corn

fortified at a concentration level of 400 ng/g. This level was chosen in order to work in a range where changes in the response were not given by instrument variation but were imputable to changes in the extraction efficiency. The investigated parameters were temperature (40 °C; 80 °C; 120 °C), static time (5 min; 10 min) and extraction solvent [acetonitrile (ACN)–water (9:1, v/v); methanol (MeOH)–water (8:2, v/v); methanol (MeOH)–acetonitrile (1:1, v/v)]. These three extraction solvent mixtures were selected because ACN–water and MeOH–water have been reported to be the most frequently used for ZON analysis [19], while the third solvent has previously shown satisfactory results by using MAE [23]. The PLE was operated applying the instrument settings reported in Table 1.

Presuming that in a naturally contaminated sample ZON could be bound to the matrix, parameters which did not significantly affect the recovery of ZON from fortified samples could play a role when extracting naturally contaminated sample. In order to evaluate the influence of PLE parameters on naturally contaminated matrices, a naturally contaminated corn was extracted by PLE using MeOH–ACN by applying a 5-min static cycle, varying the temperature (40 °C; 80 °C; 120 °C) and the number of static cycles (2; 3). The target ZON concentration of this naturally contaminated corn sample was determined using a modified ISO [14] method by substituting the suggested solvent mixtures (ACN–water) by MeOH–ACN in order to avoid problems due to phase separation or water absorption by the matrix as reported by Stroka et al. [27]. Finally the chosen extraction conditions (5 min; 80 °C; MeOH–

ACN) were tested on the FAPAS wheat and corn samples [24,25].

All experiments were performed in duplicates; the statistical evaluation of the results was performed using STATISTICA™ software (Stat Soft Inc., USA).

### 3. Results and discussion

#### 3.1. LC–MS analysis

The matrix compounds, which predominantly elute in the first 3.5 min, were directed to the waste by using the divert valve, allowing the target analytes to be separated from the main matrix interferences often leading to under or overestimation of the target analytes. The target analyte peaks were not completely separated by liquid chromatography (Fig. 1); selectivity was achieved by extracted ion chromatogram tool.

Quantification of the extracts was based on a six points matrix match standard curves covering the range of 10–100 ng/ml to achieve better precision. Matrix-matched standard curves were prepared by extracting blank wheat and corn at 40 °C for 5 min using the different solvents investigated in this study, thus assuring a perfect match between samples analysed and standard curves. The need to use matrix-matched standard curves was demonstrated by evaluating calibration curves based on standard diluted into the mobile phase or into matrix extracts. These calibration curves presented good linearity ( $R^2$  value of all curves were  $>0.995$ ), but different slopes. For example, in the case of ACN–MeOH, a

Table 1  
Extraction parameters applied during the PLE extraction

Fixed parameters			
Cell volume	22		
Pre heat time	1 min		
Heat time	5 min (6 min when 120 °C applied)		
Flush volume	75%		
Purge time	100 s		
Cycle	2		
Pressure	1500 p.s.i.		
Investigated parameters			
Temperature	40 °C	80 °C	120 °C
Static time		5 min	10 min
Solvent	ACN–water (9:1)	MeOH–water (8:2)	MeOH–ACN (1:1)

50 ng/g standard diluted in corn or wheat matrix would have resulted in an overestimation of 34 and 23%, respectively, if quantification was based on standard-mobile phase calibration curve (mobile phase  $R^2=0.9998$ ;  $y=0.0062x+0.0194$ ; corn  $R^2=0.9999$ ;  $y=0.009x-0.0127$ ; wheat  $R^2=0.9954$ ;  $y=0.0071x+0.0523$ ). The limits of detection (LODs) [signal-to-noise ( $S/N$ ) 3:1 and of quantification (LOQs)  $S/N$  10:1] for ZON were determined on the matrices. The method LOD was 5 ng/g in wheat and 4 ng/g in corn, while the LOQ was 15 ng/g (RSD=8.6%,  $n=5$ ) and 12 ng/g (RSD=10.2%,  $n=5$ ), respectively. Though other methods show lower detection limits compared to the approach presented in this paper, this method has the main advantage of drastically reducing analyte losses during sample handling, time required for the analytical procedure and costs for material and manpower, which are desirable characteristics during method development.

### 3.2. Pressurized liquid extraction

Preliminary experiments have been carried out to evaluate a likely interaction between the Hydromatrix and the target analyte and to establish the

most appropriate settings of the flush volume. All the pre-trials produced recoveries above 92%, ensuring that no losses of the analytes occurred due to adsorption of ZON into the Hydromatrix. A flush volume of 75% was chosen for the following experiments since it provided a statistically significant higher recovery (flush effect of 6.0%).

Subsequently, experiments were devoted to evaluating the influence on extraction efficiency of (1) temperature, (2) time and (3) solvent, with the aim to find the most suitable conditions to be used for ZON extraction. The experimental design and the results of the recovery of ZON in wheat and corn are shown in Table 2.

The effect of each single parameter investigated and their interaction was determined by submitting the presented results to statistical evaluation. The standard deviation (SD) reflecting the analytical error of the method was derived from the duplicate analyses of the factorial design experiments (SD=7.0% for wheat; SD=4.0% for corn) and was used to check for the significance of the factor effects calculated from the results of the experimental design. Only the solvent had a significant effect on ZON recovery from wheat and corn. The extraction temperature did not show a significant influence on

Table 2

Recovery of zearalenone (spiking level=400 ng/g sample) from ground wheat and corn using PLE with different solvent mixtures, times and temperatures

Static time (min)	Temperature (°C)	Solvent	Recovery (%)			
			ZON wheat		ZON Corn	
5	40	ACN–water	100.2	102.5	92.0	94.2
5	80	ACN–water	86.5	105.6	86.6	91.6
5	120	ACN–water	98.4	107.4	97.9	100.9
10	40	ACN–water	86.1	105.3	92.7	90.6
10	80	ACN–water	104.2	96.7	92.9	91.1
10	120	ACN–water	100.0	104.5	86.4	89.1
5	40	MeOH–water	70.7	63.4	80.8	81.4
5	80	MeOH–water	68.8	57.1	83.8	95.5
5	120	MeOH–water	69.0	62.7	83.9	85.9
10	40	MeOH–water	68.8	73.0	66.6	79.9
10	80	MeOH–water	56.4	63.2	94.8	98.8
10	120	MeOH–water	67.9	64.2	84.0	78.5
5	40	MeOH–ACN	93.2	112.7	113.8	112.7
5	80	MeOH–ACN	112.0	116.5	108.4	110.7
5	120	MeOH–ACN	114.1	104.2	104.3	105.0
10	40	MeOH–ACN	115.6	111.3	107.2	107.4
10	80	MeOH–ACN	109.3	104.9	113.5	111.0
10	120	MeOH–ACN	110.8	107.4	105.4	109.2

the recovery of ZON in either matrix irrespective of the extraction solvent used. The results from the experiments in which MeOH–ACN was used showed that the recovery was above 100% at all temperatures, thereby indicating that there was no thermal degradation of ZON at elevated temperatures. On the other hand, the extraction efficiency when using MeOH–water as extraction solvent could not be improved by changing the temperature from 40 to 120 °C since the recovery of ZON was constant at about 65% from wheat and 85% from corn at all applied temperatures. For the subsequent trials, a medium temperature of 80 °C was applied. Since the length of the static cycle did not influence the extraction efficiency, the extraction time was set to 5 min to assure a rapid extraction. The solvent MeOH–water provided low recoveries compared to the other mixtures; ACN–water showed better recoveries for wheat (99.8%) than for corn (92.1%). MeOH–ACN gave significantly better recoveries for both matrices. Therefore, this latter solvent mixture was selected for subsequent extractions.

An increase in colour and in cloudy suspension could be visibly noticed when increasing the temperature (40 °C < 80 °C < 120 °C) and varying the extraction solvent mixture from MeOH–ACN to ACN–water and finally MeOH–water. Extremely dirty extracts were obtained when MeOH–water was used, and also when the extraction was performed at 120 °C. Storing the extracts in the refrigerator overnight facilitated precipitation of the co-extracted matrix component. Consistent results were obtained by analyzing the extract after filtration, after centrifugation and after overnight precipitation.

Presuming that ZON could be bound to the matrix in a naturally contaminated sample, a small statistical design was set up in order to evaluate if parameters which did not significantly affect the recovery of ZON from a fortified sample could play a role when extracting a naturally contaminated sample. This design applied the selected condition, solvent (MeOH–ACN), time (5 min) and varied the temperature (from 40 to 120 °C) and number of static cycles (2; 3). The recoveries of all PLE extractions were between 93 and 103% (RSD=8.4%), thereby indicating that the experimental conditions established by using fortified material also apply to naturally contaminated corn samples.

### 3.3. Verification of the final extraction method

Since ZON reference material is still not available on the market, two samples used in an international proficiency study were analysed to evaluate the parameters chosen for the PLE extraction instrument. In this way the extraction method, including the LC–MS analysis, was compared to the most frequently applied methods used by the participants of the proficiency test, which are normally based on purification of the samples using either solid-phase extraction or immunoaffinity clean-up followed by LC–fluorimetry detection. The samples were extracted in triplicate for both matrices. ZON was detected in wheat at a level of 132 ng/g (RSD=5.2%;  $n=3$ ), which is 118% of the target value assigned (112 ng/g). In corn the target ZON was at a concentration of 305 ng/g (RSD=2.2%;  $n=3$ ), which is equal to a recovery of 107%, the target value being 285 ng/g.

## 4. Conclusion

Pressurized liquid extraction of ZON in wheat and corn provided results comparable to the most commonly used extraction method. Therefore, PLE could be an optimal choice in the attempt to automate sample handling. In particular, sample extraction could be run overnight. Acceptable recoveries (>90%) were obtained using the solvent mixture ACN–water. However, the alternative extraction mixture MeOH–ACN provided better performances for both matrices (cleaner extracts and higher recoveries). Temperature and time did not play an important role in the extraction efficiency irrespective of whether fortified or naturally contaminated material was used.

The limits of quantification were 15 ng/g in wheat and 12 ng/g in corn, while the RSD values were 8.6% and 10.2%, respectively, showing that this method is suitable for food and feed analysis.

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**9.4 Appendix IV**



**IV**

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## Comparison of alternative and conventional extraction techniques for the determination of zearalenone in corn

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**Abstract** Naturally contaminated corn samples of different origin were extracted using two conventional techniques (blending and shaking) and three alternative approaches (ultrasonic extraction, accelerated solvent extraction, and microwave-assisted extraction). Use of the same extraction mixture for all trials enabled the efficiency of the various extraction techniques to be compared. Extracts were filtered and directly analyzed by LC–ESI–MS, without further clean-up. The yield from the alternative extraction techniques showed efficiency to be higher than for conventional techniques. In particular, microwave-assisted extraction was slightly superior to other techniques.

**Keywords** Zearalenone · Extraction · Microwave-assisted extraction · Accelerated solvent extraction · Ultrasonic extraction

### Introduction

Sample extraction is a critical and time-consuming step in quantitative analysis; nevertheless it is still the least developed part of most analytical methods. Over the last ten years, the demand for new extraction techniques has encouraged the development of alternative extraction techniques, such as ultrasonic extraction (UE), microwave-assisted extraction (MAE) and pressurized-liquid extraction (PLE) (known by the trade name ASE, for accelerated solvent extraction), enabling automation, shortened extraction times, and reduction in organic solvent consumption [1]. Comparisons of extraction techniques have been published for the determination of PCB, showing either the similarity of the results obtained by Soxhlet (SOX),

shaking (SHA) and MAE [2] or better extraction efficiency of dynamic extraction techniques (ASE, fluidized bed extraction and SOX) compared with batch extraction (Blending (BLE), SHA, MAE and UE) [3]. Alternative extraction methods have already found their way into environmental analysis, and have just started to be applied in pharmaceutical [4, 5] and food analysis [6]. Application of alternative extraction methods in the mycotoxin field has been limited to a few published papers based on MAE [7, 8] and ASE [9, 10, 11, 12].

The extraction performance of conventional methods for mycotoxin analysis is constantly under discussion. The effects of different extraction techniques [13] and of different extraction solvent mixtures [14] have been evaluated, and single and multiple extractions have been compared [15].

In this research we have considered zearalenone (ZON), which is a *Fusarium* toxin of interest due to its health concern for animal husbandry (especially in pigs) [16] and for humans [17], as a case study. Traditionally, this mycotoxin is extracted from corn by blending for a few minutes [18, 19] or by shaking for about 30–60 min [20, 21]. The objective of this study was to compare the extraction efficiency of conventional versus alternative extraction techniques in order to establish whether the extraction can be performed whatever extraction technique is applied or whether one or more of these techniques gives higher extraction efficiency.

### Experimental

#### Chemical and reagents

ZON and zearalanone (ZAN) standards were purchased from Sigma (Milano, Italy) and diatomaceous earth sorbent Hydromatrix from Varian (Dionex, Milano, Italy). Solvents were of HPLC grade (Aldrich, Milano, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Milano, Italy).

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**Table 1** Comparison of the extraction efficiency obtained by shaking with the 100% organic solvent mixture and with two of the most frequently used aqueous extraction solvent mixtures

Sample	Acetonitrile–methanol 1:1		Acetonitrile–water 86:14		Methanol–water 80:20	
	Concn±SD	RSD (%)	Concn±SD	RSD (%)	Concn±SD	RSD (%)
4	114.0± 1.6	1.9	120.8±23.3	17.9	101.6± 8.2	8.1
6	274.3±10.3	4.2	216.5±18.0	8.3	245.6± 6.8	2.8
7	145.3± 5.3	3.6	141.4± 6.3	4.4	144.7±11.3	7.8
8	949.6±29.6	3.1	949.0±57.4	6.0	930.2±54.5	5.9

Concentration is in  $\text{ng g}^{-1}$   
RSD=relative standard deviation,  $n = 3$

#### Test material

Eight different corn samples naturally contaminated with ZON and taken from various origins (Austria, France, Germany, Argentina) were ground in a laboratory ultra centrifuge mill ZM100 (Retsch, Haan, Germany) using a 0.5 mm sieve. The samples were mixed in a head over heels mixer (Turbula Type T2 F, WAB, Basel, Swiss) to ensure sufficient homogeneity of the test material.

#### Procedure

All extractions were performed on 5 g corn, with addition of 20 mL extraction mixture, except for ASE, where the final volume was about 35 mL. The extraction mixture was MeOH–ACN, 1:1 ( $v/v$ ), which had previously given satisfactory results for MAE [8] and ASE [11]. MAE represented the limiting factor in choosing the extraction mixture, since the presence of water tends to form a thick “porridge” when cooked together with corn flour. Comparable extraction efficiency has been observed when corn was extracted by a conventional extraction technique (SHA) using MeOH–ACN or the most frequently used extraction solvent mixtures such as ACN–water, 86:14, ( $v/v$ ) and MeOH–water 80:20 ( $v/v$ ) (Table 1).

Using the MeOH–ACN mixture as solvent was also justified by the result from another study showing that an aqueous solvent mixture could result in phase separation or water absorption, leading to false analytical results [22]. The MeOH–ACN mixture was therefore used in all the extraction procedures in order to evaluate only the difference in efficiency due to the extraction techniques, extrapolating the value from the influence of the extraction mixture composition. After the extraction 1.0 mL internal standard (ZAN)  $2 \mu\text{g mL}^{-1}$  was added to all samples in order to take into account changes in extraction volume and variation in the electrospray (ESI) ionization process. After thorough mixing, a sub-sample was filtered into vials using a Titan PTFE  $0.45 \mu\text{m}$  filter, which had been tested for not interacting with the target analytes. After filtration the raw extract was injected directly into the LC–MS system without performing any further clean-up step.

Extract analysis was performed using an HP 1100 Series HPLC (Hewlett–Packard, Palo Alto, CA, USA) coupled to an ion-trap mass spectrometer (MSD-SL) equipped with an ESI interface (Hewlett–Packard) according to a previously published method [11]. Briefly, chromatographic separation was performed on a  $\text{C}_8$  column (Discovery 100×2.1 mm,  $5 \mu\text{m}$  particle size,  $180 \text{ \AA}$  pore size; Supelco, Milano, Italy) working in isocratic  $\text{H}_2\text{O}$  (0.2% acetic acid)–MeOH 45:55 ( $v/v$ ) at a flow rate of  $250 \mu\text{L min}^{-1}$ . The interface parameters were optimised by standard in-flow infusion. MS detector was operated in full scan mode ( $250\text{--}400 m/z$ ) and quantification was based on extracted ion chromatograms. The instrumental limit of detection (LOD) (signal to noise ratio ( $S/N$ ) = 3:1) was 8 pg on corn. If the ASE extracts were reduced to 20 mL prior to analysis (as for the other extracts) the limit of quantification of the method ( $\text{LOQ } S/N = 10:1$ ) was  $15 \text{ ng g}^{-1}$ . Quantification was based on matrix-matched calibration curves to enable more precise quantification, because different equations are obtained using mobile-phase versus matrix-matched calibration curves [8].

#### Blending

Sample was blended using an Ultra-Turrax 125 (IKA-Labortechnik, Staufen, Germany) for 3 min [19]. Starting from room-temperature, samples reached a temperature of  $43 \pm 1 \text{ }^\circ\text{C}$  after the blending process.

#### Shaking

Sample was shaken for 30 min with a Compact Shaker SK 15 A horizontal shaker (Edmund Bühler, Tübingen, Germany) [21].

#### Ultrasonication

Sonication was performed using a U 200 S control (IKA-Labortechnik), a stick sonicator that allows variation of amplitude and cycle. Suitable conditions were investigated by testing for recovery from spiked corn. Samples were stirred and extracted for 5 min setting the cycle at 0.4 and the amplitude at 90% (recovery  $103.1 \pm 3.1\%$ ). Samples reached a temperature of  $35 \pm 1 \text{ }^\circ\text{C}$  after the extraction procedure.

#### Accelerated solvent extraction

ASE was carried out using an ASE 200 System (Dionex, Sunnyvale, CA, USA). Extraction was performed according to a previously optimized method [11]. Briefly, the sample was thoroughly mixed with 3 g Hydromatrix to obtain a porous mixture to enable the extraction solvent to flow through the sample during the extraction and was poured into a 22-mL thimble. Extraction conditions were: temperature  $80 \text{ }^\circ\text{C}$ ; pressure 1500 psi; static time 5 min; 2 cycle; flush volume 75%, and purging time 100 s.

#### Microwave-assisted extraction

A closed-vessel microwave-assisted extraction unit (MSP 1000; CEM, Matthews, NC, USA) was used in all extractions. This unit is equipped with a pressure and temperature probe and a safety features solvent vapor detector. Six vessels (five replicate and a control) were simultaneously extracted using microwave power 900 W, temperature  $80 \text{ }^\circ\text{C}$ , pressure 20 psi, and extraction time 5 min [8].

#### Statistical analysis

Statistical evaluation of the results was performed using Statistica software (StatSoft, Tulsa, OK, USA).

## Results and discussion

The whole data set contained 200 analytical results since five extraction methods were applied to eight different test samples and each combination of the sample and the

**Table 2** Concentration (ng g<sup>-1</sup>) of zearalenone in eight different corn samples (*n* =5) obtained by applying different extraction techniques

	Conventional <sup>a</sup>				Alternative <sup>a</sup>					
	BLE		SHA		UE		ASE		MAE	
	Concn±SD	RSD (%)	Concn±SD	RSD (%)	Concn±SD	RSD (%)	Concn±SD	RSD (%)	Concn±SD	RSD (%)
1	5446±532.8	9.8	5613±577.7	10.3	5188±430.4	8.3	6317±801.6	12.7	6410±551.7	8.6
2	90.2±7.7	8.5	101.0±11.7	11.6	99.3±14.8	14.9	100±9.6	9.6	107.0±7.6	7.1
3	110.4±8.4	7.6	91.2±6.7	7.4	123.0±11.9	9.7	183±12.3	6.7	195.5±26.4	13.5
4	123.9±6.9	5.5	118.0±5.5	4.7	105.0±6.6	6.2	132±4.9	3.7	113.1±10.9	9.7
5	54.6±5.7	10.4	83.5±7.2	8.6	84.7±10.7	12.6	68±3.8	5.5	106.5±11.1	10.5
6	285.5±17.4	6.1	241.5±14.9	6.2	235.7±17.6	7.5	316±28.3	8.9	279.3±32.2	11.5
7	179.2±29.6	16.5	141.5±3.2	2.3	136.8±7.3	5.3	152.4±	2.9	167.6±16.6	9.9
8	1022.1±52.7	5.2	940.4±38.1	4.1	1060.5±22.9	2.2	1141±62.7	5.5	1051.6±58.7	5.6

<sup>a</sup>BLE=blending, SHA=shaking, UE=ultrasonic extraction, ASE=accelerated solvent extraction, MAE=microwave assisted extraction, Concn=concentration, SD=standard deviation, RSD=relative standard deviation

extraction method was performed five times. The mean value and the corresponding standard deviation are presented in Table 2.

#### Statistical evaluation

The concentrations obtained were subjected to statistical analysis in order to establish which of the extraction methods investigated in this study gave significantly higher results than the other methods. In addition it was of interest to examine whether the relative extraction efficiencies of the various extraction methods differ between the samples included in the study.

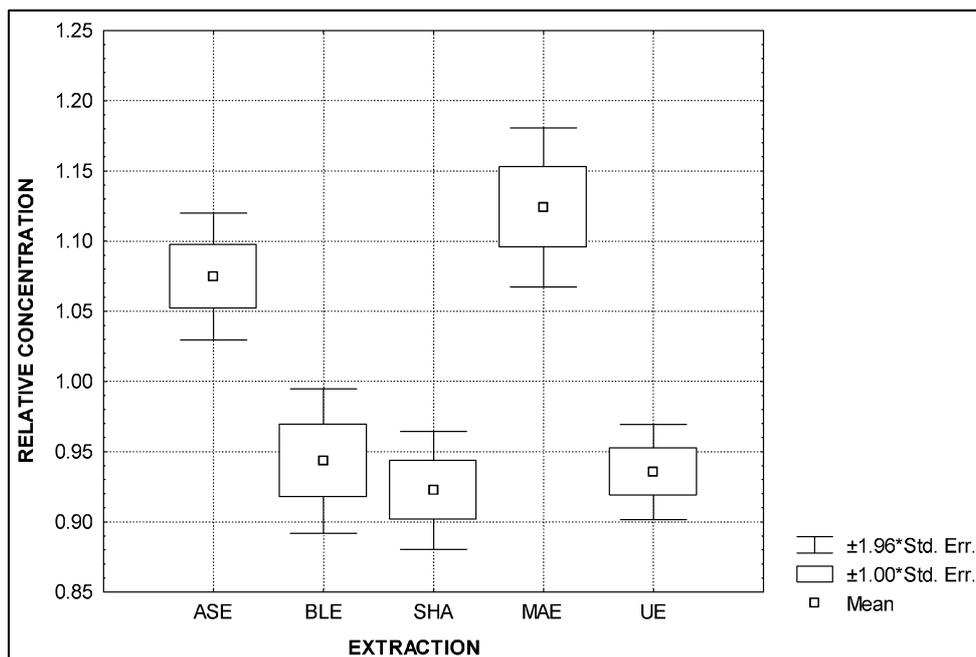
Prior to statistical analysis the concentrations obtained had to be transformed to achieve variance homogeneity.

This was required since the concentrations of ZON measured in the various samples covered a large range from 80 ng g<sup>-1</sup> to 5795 ng g<sup>-1</sup> thereby leading to a low standard deviation for the slightly contaminated samples and a large standard deviation for the samples containing ZON at high concentrations. Therefore relative concentrations were calculated by dividing the concentrations from the 25 extractions performed for each sample (five extraction methods performed on five replicates) by the corresponding mean of these 25 results. Applying this transformation led to a new data set in which all values centered around 1.

Comparing the relative concentrations of the various extraction methods showed that MAE gave the highest results, followed by ASE, as shown in Fig. 1.

Applying two-factor analysis of variance (ANOVA) to the transformed data revealed that the observed differ-

**Fig. 1** Comparison of the relative concentrations obtained from the different extraction methods



**Table 3** Results from Scheffe's test ( $\alpha=0.05$ )

Extraction method	Relative concentration	Group	
		1	2
Blending	0.94	xxxx	
Shaking	0.92	xxxx	
Ultrasonic	0.94	xxxx	
Accelerated solvent	1.07		xxxx
Microwave assisted	1.12		xxxx

ences in the results obtained by applying the different extraction methods were significant ( $\alpha=0.05$ ). However, ANOVA did not indicate which of the investigated methods gave significantly better results compared with the other methods. In principle, the *t*-test could be applied to establish whether the results from *two* methods are significantly different. However, repeatedly performing of the *t*-test to compare the various pairs of the mean values from the different extraction methods increases the risk of indicating a significant difference between methods which are actually equivalent. Therefore, we applied post-hoc comparisons ( $\alpha=0.05$ ) to establish which of the methods form a group of equally performing methods. Use of Scheffe's test, considered as the most conservative post-hoc test, demonstrated that the five extraction methods could be divided into two groups as shown in Table 3.

The best performing methods MAE and ASE were in group 2, whereas group 1 contained the methods BLE, SHA and UE, which gave significantly lower results compared to group 2. Additionally, the result showed that the average values of the methods in group 1 were *below* the average values of *all* methods, whereas the mean values of MAE and ASE (group 2) were *above* the overall mean of *all* methods. The general applicability of this conclusion is ensured by taking into account that we used different corn samples of different origin. The outcome of the statistical assessment also revealed that the relative standard deviations (RSD) of the investigated methods were very similar, since the RSD (%) was 8.5 for BLE, 6.8 for SHA, 8.3 for UE, 6.9 for ASE and 8.8 for MAE.

#### Conventional versus alternative extraction techniques

As shown by the statistical analysis MAE and ASE are similar, indeed both techniques have the possibility to work at elevated temperatures and pressures, vastly improving the speed of the extraction process. A preliminary experiment performed by spiking a blank matrix with ZON provided acceptable recovery for all the techniques (BLE=97.8%; SHA=93.8%; UE=93.6%; ASE=101.4%; MAE=90.4%). The increased extraction efficiency for naturally contaminated samples could be explained by the interaction between the target analyte and the matrix, which is weakened by the high temperature and microwave energy. Extraction efficiency varied depending on the sample extracted. For instance, sample 2 showed higher re-

covery rates for SHA compared to BLE whereas the other way around was observed when extracting sample 4, since in this case BLE gave higher results than SHA. These data demonstrated the importance of including a large number of different samples in the study in order to minimize the risk of drawing wrong conclusions, which might occur when analyzing only one or two different samples. Nevertheless, the results from the statistical assessment regarding the extraction efficiency of the various tests are valid within the frame of the analyzed materials.

#### Blending

This is one of the most widely used extraction techniques; it has the advantages of very fast extraction providing good recovery. BLE is convenient whenever few samples have to be extracted, as each sample has to be handled separately and the analyst should be fully devoted to the extraction step while performing it.

#### Shaking

This is the most frequently used extraction procedure. It is definitely very user-friendly, in fact several samples could be extracted simultaneously and the presence of the analyst is not required. Moreover the instrumentation required is not expensive and is normally available in any analytical laboratory.

#### Ultrasonic extraction

UE has to be considered as an alternative extraction technique, however, according to Scheffe's test it fell onto the same cluster as BLE and SHA. Despite the use of ultrasonic energy, which implies an increase in extraction power due to mechanistic aspects, better extraction efficiency was not noticed. It should be emphasized that the UE was performed using a professional stick sonicator instead of a sonicator bath as normally occurs.

#### Accelerated solvent extraction

ASE combined high temperature with pressure and dynamic extraction leading to a slightly better extraction efficiency than conventional techniques. It has previously been shown that ASE may greatly improve extraction efficiency of other analytes (e.g. fumonisin [9]). Although this technique required a little more time for packing the thimbles, it has the great advantages of being fully automatic, samples could be easily extracted overnight, extra handling is not needed to separate the matrix from the solvent, and reduced manpower is required.

## Microwave-assisted extraction

The use of microwave energy enables the solvent mixture to heat rapidly, accelerating the speed of the heating and consequently reducing the extraction time required. Extraction time is as short as in BLE, with the advantage of enabling the simultaneous extraction of 12 samples. MAE was the most efficient technique. The natural matrix moisture could play an important role, because the microwave energy tends to swell the matrix and/or to interfere with matrix–analyte interactions, as has already been shown [23].

## Conclusions

This study demonstrates that the five different extraction techniques are in principle interchangeable for the determination of ZON. Although UE has previously been considered as an alternative method [3], results clearly classified UE as a conventional method with a performance similar to that of BLE. Amalgamating the results from the various samples and applying post-hoc comparisons revealed that alternative extraction methods, ASE and MAE, were statistically more efficient than other conventional methods. Since *individual* samples behaved differently when the extraction efficiencies obtained with the techniques covered by this study were compared; future studies should focus on investigating the impact of samples from various sources on the extraction characteristics of these techniques.

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**9.5 Appedix V**



**DEVELOPMENTS OF ZEARALENONE EXTRACTION METHOD USING  
REDUCED ORGANIC SOLVENTS SUPPORTED BY SIMPLEX  
OPTIMIZATION**

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**Keywords:** Zearalenone; Accelerated Solvent Extraction; Environmentally friendly;  
Liquid chromatography–mass spectrometry

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**Abstract**

A method for the determination of zearalenone in corn has been developed applying automated sample handling and avoiding noxious and environmentally unacceptable organic solvents. Optimization of the extraction solvent mixture was performed using the sequential simplex strategy. The samples were extracted applying Accelerated Solvent Extraction and the extracts were analysed by using an LC-MS equipped with an ESI interface. The chosen extraction mixture was isopropanol-water (1% triethylamine) 50:50 (v/v), which allowed to halve the use of organic solvent compared to the method proposed by ISO, and thereby to reduce the cost per analysis. When applying the optimised method to five different naturally contaminated corn samples an average recovery rate of 109% was obtained and the relative standard deviation varied between 4.4% and 10% depending on the concentration level of the target analyte in the test material.

**INTRODUCTION**

Zearalenone (ZON) is a mycotoxin produced by several species of fungi belonging to the genus *Fusarium*, which is well known for colonizing cereals [1]. Although its incidence and concentration levels vary considerably between geographical areas and substrate, corn is surely the most frequently contaminated commodity [2]. ZON represents a health concern for animal husbandry (particularly for pigs) [1] and for humans, due to its estrogenic properties [3]. Traditionally this mycotoxin is extracted by conventional extraction methods such as liquid shaking [4,5] or blending [6,8], nevertheless alternative extraction methods have been investigated [9-11]. The most commonly used solvent extraction mixture for ZON is acetonitrile-water in various ratios; the mixture acetonitrile-water 90:10 v/v has been suggested by the method proposed by ISO [7].

Evaluation of the safety data for organic solvents clearly shows the higher toxicity of acetonitrile compared to the non-aromatic alcohols used in this study [12]. For example the United States Government under the Occupational Safety and Health Act (OSHA) has set the Permissible Exposure Limit (PEL) of 40 ppm for acetonitrile and

200, 1000 and 400 ppm for methanol, ethanol and isopropanol, respectively. According to the opinion expressed by the European Scientific Committee for Toxicity, Ecotoxicity and the Environment on the results of the risk assessment of acetonitrile, the estimated indirect exposures of humans via the environment is so low that no concern is perceptible. Nevertheless the users of acetonitrile for HPLC mobile phase has been identified between the highlighted risks categories [13]. Therefore a reduction in the use of acetonitrile moving towards less toxic and more environmentally- and user-friendly solvent is both, desirable and foreseeable.

Method development requiring the optimisation of various parameters can either be conducted in a non-systematic way, based on the experience of the analyst, or by applying a systematic approach, in which the parameters are varied according to a well defined plan. Most frequently, such a plan allows changing only one parameter whereas the other parameters are kept constant. By measuring the response of each experiment the optimal combination of the parameters can be achieved. However this approach leads to a large number of experiments, which amplifies drastically when increasing the number of variables taken into account, resulting in a very time-consuming and inefficient way of operate. Moreover the result could be influenced by the initial conditions chosen; during the development this approach does not take into account effects due to the interaction between investigated parameters. Alternatively other techniques are available that allow simultaneous variation of the parameters such as the experimental design being an efficient tool to establish which parameters are most important in respect to optimising the method; and the simplex method which use information from a previous set of experiments to establish a new combination of parameters expected to show an improvement of the response. In this study the *modified* simplex, that allows the step size and not only the direction of the optimisation process to be adjusted to the results from previous trials, was applied. Compared to the basic simplex method the modified one is based on other rules such as expanding the step size in a direction of more favourable conditions or contracting the step size if the Simplex algorithm proposed a set of parameters with less favourable conditions. The procedures for expansion and contraction enable the modified simplex to accelerate along a successful track of improvement. Therefore the modified simplex will usually reach the optimum quicker than the basic method [14].

In this study the simplex method [15] was applied to optimize the composition of the solvent extraction mixture and the extraction temperature, which could play a main role in increasing extraction efficiency using solvents of less toxic profile [14]. Extractions were performed on an automated extraction system (trade name Accelerated Solvent Extraction, ASE) and the extracts were analysed by using liquid chromatography (LC)-electrospray (ESI)- mass spectrometer (MS) [10].

## **EXPERIMENTAL**

### ***Chemical and Reagents***

ZON and ZAN standards were purchased from Sigma (Milano, Italy). Acetonitrile, methanol, ethanol and isopropanol were of HPLC grade (Aldrich, Milano, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Milano, Italy). Sodium hydroxyde (NaOH), potassium hydroxide (KOH) and ammonia 25% v/v (NH<sub>3</sub>) were purchased from Fluka (Milano, Italy), Triethylamine (TEA) from Aldrich (Milano, Italy). Diatomaceous earth sorbent Hydromatrix was from Varian (Dionex, Milano, Italy).

### ***Test material***

The simplex method to optimised the extraction conditions was performed using corn fortified at a concentration of 400 ng/g. The selected extraction conditions were tested on naturally contaminated corn samples from various origins (Austria, France, Germany), which were ground in a laboratory ultra centrifuge mill ZM100 (Retsch, Haan, Germany) using a 0.5 mm sieve. The samples were mixed in a head over heels mixer (Turbula Type T2 F, WAB, Basel, Swiss) to assure sufficient homogeneity of the test material.

### ***Equipment***

All the extractions were performed on an ASE<sup>TM</sup> 200 System (Dionex, Sunnyvale, CA, USA). Analyses were performed using an HP 1100 Series HPLC with degasser, quaternary pump, auto-injector and thermostat coupled to an MSD-SL ion-trap mass spectrometer equipped with an ESI interface (Agilent Technologies, Milano, Italy). The

chromatographic separation was achieved working in isocratic methanol-water (0.2% v/v acetic acid) 45:55 (v/v) at a flow rate of 0.2 mL/min and injecting 5  $\mu$ L on a Discovery C8 column (100 x 2.1 mm, 5  $\mu$ m particle size, 180 Å pore size, SUPELCO, Milano, Italy) kept at 35 °C.

Possibility to load on immunoaffinity column the extracts, obtained applying the optimised conditions, was investigated using EASI-EXTRACT™ ZEARALENONE (Rhône-diagnostic, Glasgow, Scotland) and quantifying the extract by using an HP 1100 with fluorescent detector ( $\lambda_{exc}$ = 274 nm;  $\lambda_{em}$ = 440 nm) (Agilent Technologies, Milano, Italy). Chromatographic separation was performed working in isocratic acetonitrile-water (2% (v/v) acetic acid) 40:60 (v/v) at a flow rate of 0.8 mL/min on a Discovery C8 column (100 x 4.0 mm, 5  $\mu$ m particle size, 180 Å pore size, SUPELCO, Milano, Italy) kept at 35 °C.

### ***Stability study***

ZON was dissolved into various alkaline aqueous solutions (NaOH 1M, KOH 1M, NH<sub>3</sub> 1M, TEA 1%, TEA 1%-MeOH 1:1 (v/v)) at two concentration levels (100; 500 ng/g) to investigate its stability. Standards solutions were injected every 2h over a period of 24h. The trend of ZON concentration was calculated comparing the obtained area with the area of ZON standard diluted in pure water.

### ***Procedure***

An amount of 5 g of sample was mixed thoroughly with 3 g of Hydromatrix to obtain a porous mixture to enable the extraction solvent to flow through the sample during the extraction. The mixture was poured into a 22 mL thimble, which was packed by adding a layer of Hydromatrix at the base and at the top (about 1g) in order to fill the thimble completely according to the instrument's manufacturer recommendations. Based on previous experience [10], the following ASE parameters were set: pressure 1500 psi; static time 5 min; 2 cycle; flush volume 75% and purging time 100 sec. When the extraction solution reached room temperature, 1.0 mL of internal standard solution (ZAN 2  $\mu$ g/mL) was added. After thoroughly mixing, an aliquot was filtered into vials using a TITAN PTFE 0.45  $\mu$ m filter, which has been tested for not interacting with the target analytes. Without performing any other clean-up the raw extract was injected directly into the LC-MS.

Naturally contaminated corn samples were extracted in triplicate applying the selected extraction conditions using isopropanol-TEA 1% 50:50 (v/v) as extraction solvent and adjusting the temperature at 80°C. Results from these experiments were compared with results obtained for the same samples extracted by ASE using methanol-acetonitrile 50:50 (v/v) [10].

### *Simplex*

Multisimplex® (Grabitech Solutions AB, Sweden) was applied for the sequential simplex optimization. The optimization algorithm used in the MultiSimplex® software contains some generally accepted modifications to the original version of the modified simplex method.

To start the simplex algorithm the following information were given to the program: for all control variables a step size of 20, for the solvents (TEA 1%, isopropanol, ethanol and methanol) a reference value of 25%, setting their sum at 100%; the starting value for the temperature was 80 °C. Each suggested trial was performed in duplicate and the average was inserted as a response in the program.

## **RESULTS AND DISCUSSION**

The challenge to develop an environmentally friendly method was tackled either by using solvents having a low toxicity or by increasing the percentage of water present in the extraction solvent mixture. The method proposed by ISO uses as extraction solvent mixture acetonitrile-water 90:10 v/v [7,8], while the method provided with the immunoaffinity column (Rhône-diagnostic) suggests acetonitrile-water 75:25 v/v. Methanol was considered as alternative and less toxic solvent compared to acetonitrile since it has been already used for zearalenone extraction showing acceptable performance characteristics [17]. Other non-aromatic alcohols having similar properties, but lower toxicity, were also considered in this study. For the simplex optimisation were finally selected methanol, ethanol and isopropanol. Increasing the water percentage in the extraction mixture led to technical inconveniences and reduced efficiency, due to the presence of starch, which tended to cook forming thick porridge and clogging the thimble. To facilitate the solvent flow through the matrix several ratio matrix/Hydromatrix were tried. Since it was established as a minimum requirement to

work on a minimum sample size of 5 g, the amount of Hydromatrix was limited by the size of the thimbles available with the ASE 200. Thus it was not possible to find a suitable ratio for using 100% water. In addition using solvent mixtures having a water percentage higher than 50% led to unsteady; in fact when several samples were extracted in a row the extracted volume slowly decreased till the instrument clogged. The increase of the water percentage in the final extraction mixture was also limited by the fact that ZON is practically insoluble in water. Consequently, increasing the percentage of pure water could result in a severe reduction in the extraction efficiency. Preliminary experiments were performed using ethanol-water at different ratios (ethanol-water 70:30; 50:50; 30:70). The most successful was ethanol-water 70:30 v/v, which allowed only for a recovery of 73.8% (RSD% 1.6).

### ***Stability study***

To overcome low extraction efficiency of water several trials were performed to find a suitable alkaline aqueous solution for substituting water, since the solubility of ZON is improved when increasing the pH value. On the other hand ZON is not stable in alkaline conditions [2]. ZON has shown to be completely unstable in NaOH and KOH, further investigations on the fate of ZON were not performed because extraction was impossible since these solutions in contact with corn formed a gel sticking to the glassware. When ZON was dissolved in NH<sub>3</sub> 1M solution only 40.0% (RSD 12.4%) for 100 ng/g and 44.3% (RSD 5.7%) for 500 ng/g was recovered. ZON was stable in TEA 1% (101.9% RSD 9.7% for 100 ng/g; 105.3% RSD 4.3% for 500 ng/g), nevertheless after 16h it was noticeable a change in the peak shape (fronting), if the whole peak was considered the peak area was constant. Finally, stability and peak shape were improved dissolving ZON in a mixture of methanol-TEA 1% 50:50 v/v (103.6% RSD 6.3% for 100ng/g; 106.0% RSD 4.2%). From the stability investigation it was concluded that ZON is stable when dissolved into organic solvent-TEA 1% 50:50 (v/v).

To evaluate the extraction efficiency of water containing TEA at various concentrations, additional experiments were performed by extracting spiked corn (200 ng/g) applying the conventional shaking method. The following values for the recovery were obtained: 3% (100% water), 10.5% (0.1% TEA), 59.0% (0.5% TEA) and 61.2% (1% TEA). These results demonstrate insufficient recovery for ZON when using 100% aqueous solutions. TEA 1% is a good choice for substituting pure water in the extraction solvent

mixture merging both stability and extraction efficiency for the target compound.

### ***Simplex optimisation***

The set-up of the trials and the results of the optimisation are shown in Table 1. Each combination of parameters is called in simplex terminology “vertex”. Based on initial values and the step size for the parameters the simplex algorithm calculated a first set of 5 experiments. The step size fixed for the first set, varied during the optimisation process decreasing when approaching the optimal range. However, in this specific case for technical reason (precision of the solvent mixer) a minimum step of 5 was kept. Thanks to the pre-trials performed to find suitable extraction solvent a satisfactory recovery was already achieved in the first set of experiments (1-5) (Table 1). Indeed the average recovery for the whole experiments was 95.2% with a RSD of 5.7%.

Table 1. Database and recovery obtained from the simplex optimization

Vertex number	Vertex	H2O %	IsopOH %	EtOH %	MeOH %	Degree °C	Recovery %
1	F	15	15	15	55	70	93.2
2	F	15	35	35	15	90	93.6
3	F	35	15	35	15	70	97.8
4	F	35	15	15	35	90	87.1
5	F	35	35	15	15	70	95.4
6	R	15	35	35	15	60	90.8
7	C+	20	30	30	20	68	97.9
8	C-	25	20	20	35	72	99.0
9	R	45	15	15	25	50	87.9
10	C-	20	30	30	20	80	92.4
11	R	15	15	40	30	75	100.5
12	E	5	0	60	35	78	97.1
13	R	50	25	25	0	75	94.0
14	C+	25	15	30	30	67	84.9
15	R	50	0	25	25	75	101.1
16	R	50	50	0	0	75	101.5
17	R	50	15	10	25	80	104.3
18	C+	50	50	0	0	80	101.5

- F = first vertex  
R = reflection vertex  
C+ = contraction vertex on the positive side  
C- = contraction vertex on the negative side  
E = expansion

The simplex objective, 100% recovery, was achieved in trial number 11, in which only 15% of aqueous solution was used. In order to establish whether extraction solutions containing a higher percentage for water would gain comparable high values for the recovery of ZON, the percentage of TEA 1% was set on purpose at 50% in trials 15 to 18. In these trials only the percentage of isopropanol, ethanol and methanol and the extraction temperature were varied by the simplex algorithm. Since the recovery of

ZON in these trials was always above 100%, the optimisation was stopped after trial 18 as shown in Fig 1.

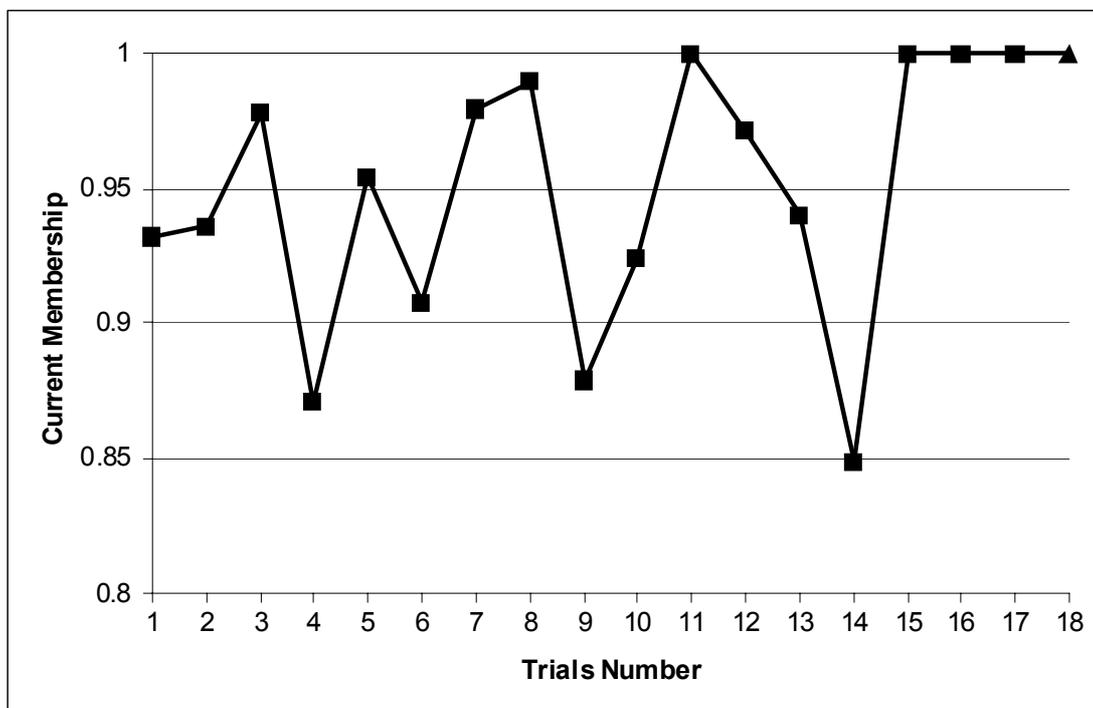


Figure 1. Plot of the Current membership against the trials number for the simplex optimization

The final solvent mixture was selected taking into account obtained recovery, handling aspects (facility in filtering the extract; number of solvent to be used), visual aspects (colour, limpidity, clearness) and cost of the solvents required. Trial 18 (repetition of trial 16), where only two solvents were used, was selected. These extracts were dark yellow, limpid and easy to be filtered, which was not the case for extracts of trial 15. Moreover isopropanol is far less toxic than acetonitrile and methanol. In addition, setting the price of methanol equal to  $x$  €/L, isopropanol is cheaper ( $2x$  €/L) than ethanol ( $5.2x$  €/L) and acetonitrile ( $3.5x$  €/L). Compared to the extraction mixture suggested by the ISO method the solvent mixture proposed in this study reduce the cost for the extraction solvent 3 times (acetonitrile-water 90:10 v/v  $3x$  €/L versus isopropanol-TEA 1% 50:50 v/v  $x$  €/L) and 2.5 times compared to the immunoaffinity

protocol.

When ZON is determined applying a traditional detection method the extraction step is followed by a clean-up step using an immunoaffinity column. The extract, obtained applying the selected conditions, was diluted with PBS (Phosphate Saline Buffer) and the pH value was adjusted to 7.4 with acetic acid before loading it onto the immunoaffinity column. Results showed that once the pH has been adjusted the extract obtained with the presented method could be purified using the immunoaffinity column without hampering their performances (recovery 97.9% n=5, RSD 2.2%).

Table 2 shows the comparison between the ZON concentrations in various naturally contaminated samples obtained with the presently proposed method and another ASE method using a 100% organic solvent mixture (acetonitrile-methanol 50:50 (v/v)) [10]. Since the results from the different methods are comparable, it was concluded that the selected solvent mixture containing 50% aqueous solution is suitable for the determination of ZON in corn.

Table 2. Comparison of ZON level of naturally contaminated corn sample determined using the selected extraction solvent mixture and an extraction solvent mixture used in a previous study.

Sample Cod.	Isopropanol-TEA (1%) 50:50 v/v		Acetonitrile-methanol 50:50v/v	
	Average ng/g	RSD%	Average ng/g	RSD%
2	114	10.1	100	9.6
3	202	10.8	183	6.7
6	307	4.4	316	8.9
7	164	6.8	152	2.9
8	1322	9.6	1141	5.5

## CONCLUSION

The preliminary investigation, which has been carried out to select an alkaline aqueous solution to substitute water in the extraction of ZON merging extraction efficiency and stability properties, led to high recovery. The sequential simplex method resulted in

successful optimisation allowing for halving the amount of organic solvent and switching to a less toxic solvent. Moreover the cost for the extraction solvent mixture has been reduced by a factor of three.

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## 10 APPENDIX B

### 10.1 LC-MS

Two decades of constant and rapid development of LC-MS technologies have lead them to become promising tools even for routine analysis.

#### *10.1.1 MS Interface*

Interfaces have the critical duty to couple the LC system to the MS detector. In order to fulfil these requirements three processes should be performed at this level: evaporation process necessary to convert the liquid phase into the gas phase; pressure reduction needed to transfer samples which have been separated at atmospheric pressure to the MS detector which operates at high vacuum and ionisation process to transform molecules into ions, which are visible to the detector. Coupling a system working at atmospheric pressure with one at high vacuum was a difficult task. Interface development can be divided into three phases, the first generation grouped direct liquid introduction, moving belt and particle beam; to the second belong the termospray, while to the third the API, representing an amazing improvement.

#### *Atmospheric pressure interface*

APCI and ESI have been used in this study. APCI and ESI are based on the same concept, therefore can be easily configured on the same instrument. Both interfaces create gas-phase ions at atmospheric pressure but at two different stages. The ions should already be present in solution when using ESI, while are formed in the gas phase using a discharged source in APCI.

Afterwards the ions are introduced to the vacuum system through a series of apertures into successive vacuum chambers up to the highest vacuum. In APCI the eluent is sprayed into a heat chamber where the heat rapidly evaporates the solvent.

At the beginning of this study the influence of several interface parameters was investigated.

#### *10.1.2 MS-analyser*

The whole study was developed on an ion-trap analyser.

This analyser, as its name implies, works by trapping the ions and detecting them on the base of the ration  $m/z$ . Basically, the ion-trap applies the same principles used in the single quadrupole. In the quadrupole the whole range of masses can be passed through the detector by stepping up the voltage of each set of pole, whereas in the trap the ions are contained in a three dimensional space. Ions are detected by placing them in an unstable orbit to allow them to leave the trap. It is possible to perform MS/MS by trapping the ions inside the trap. In this case, fragmentation is performed in a time-depending way, in contrast with the triple quadrupole were fragmentation occurs in a space-depending way.

The main advantage of the ion trap is the possibility to perform  $MS^n$ , therefore it is the detector of choice for structure elucidation, however it is also a good option to work in MS/MS mode at a cheaper price compared to the triple quadrupole.

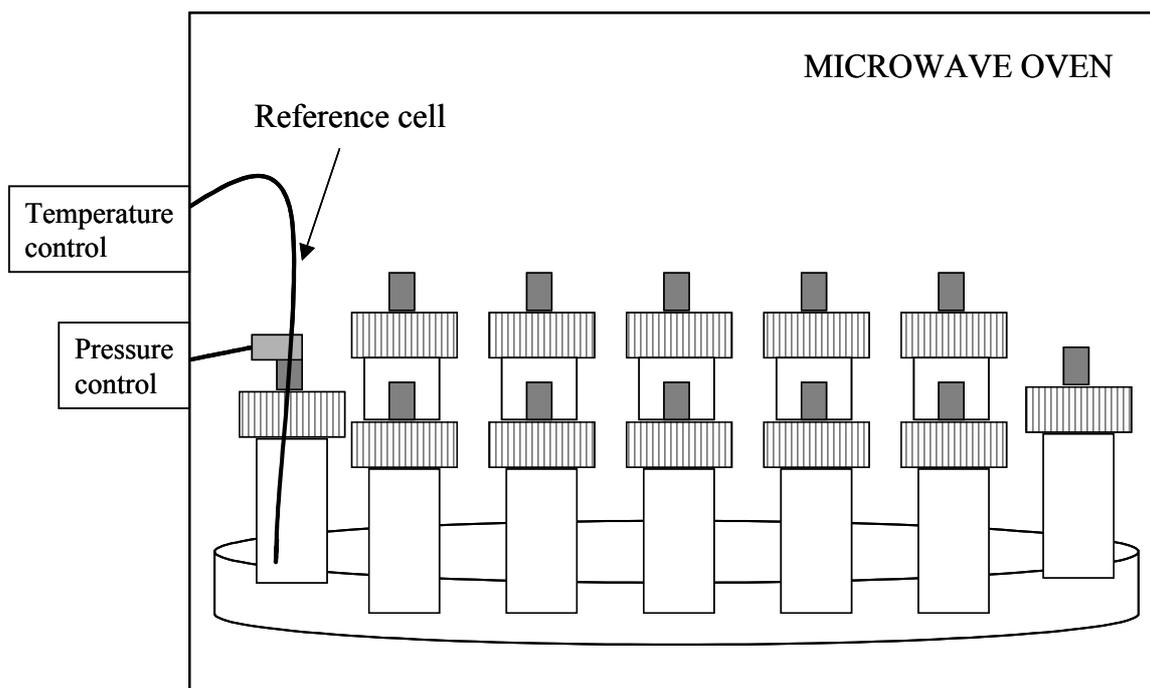
## **10.2 Alternative extraction techniques**

The simplest extraction is performed by mixing the sample with a suitable solvent and then shaking or blending at ambient temperature for a certain period. Sometimes it is advisable to repeat the extraction process several times to overcome limited extraction efficiency due to a low distribution coefficient of the analyte between the extraction solvent and the matrix. Although sample extraction is a critical and time-consuming step in quantitative analysis, it is still the least developed part of most analytical methods. In the last decade there has been a demand for new extraction techniques allowing automation, reduction of required time and reduction of organic solvent consumption. To round up these principles, new extraction techniques such as microwave-assisted extraction and pressurised solvent extraction have been developed. Both have the similar option to apply elevated temperature and pressure. These techniques have already found their way into environmental analysis, especially for PAH and PCBs; while they have only recently been applied to pharmaceutical and agricultural field.



carousel and able to collect vapour in case of solvent leakage; the rupture membranes, which resist up to 200 psi, an exhaust fan, which evacuates air from the instrument cavity; and the isolator allowing the reflected microwave energy diverts and reduce the overall load.

The MSP-1000 basic set-up is reported in Figure 10-2.



**Figure 10-2:** Schematic diagram of a closed-vessel microwave assisted extraction system.

### Basic Principle

Microwaves are high frequency electromagnetic waves; commercially available systems generally work at 2450 MHz frequency. The uses of microwave energy leads to two simultaneous occurring mechanisms: the *ionic conduction* consisting in the electrophoretic migration of ions within an electromagnetic field and the *dipole rotation* of the dipoles, which tends to realign with the field applied. Both mechanisms result in heating, the first due to friction occurring between the ion flow and the solvent resistance and the second to the forced molecular movement.

### Parameters influencing the extraction efficiency

#### Solvent

When performing extraction using a MAE, the choice of solvent is fundamental for achieving an optimal extraction process. First of all, the solvent should be able to absorb microwave energy in order to heat up when exposed to microwave energy. This ability partly depends on the dissipation factor ( $\tan \delta$ ) expressed as:

$$\tan \delta = \varepsilon''/\varepsilon'$$

where  $\varepsilon''$  is the dielectric loss (efficiency of converting microwave energy into heat) and  $\varepsilon'$  is the dielectric constant (capacity of a molecule to polarize in an electric field). Consequently polar molecules and ionic solutions with a permanent dipole moment will absorb microwave energy strongly, while non-polar solvents as hexane are not able to warm up when exposed to microwave energy.

The other solvent characteristics to be taken into account are the analyte solubility in the solvent and the interaction between matrix and solvent.

#### Temperature

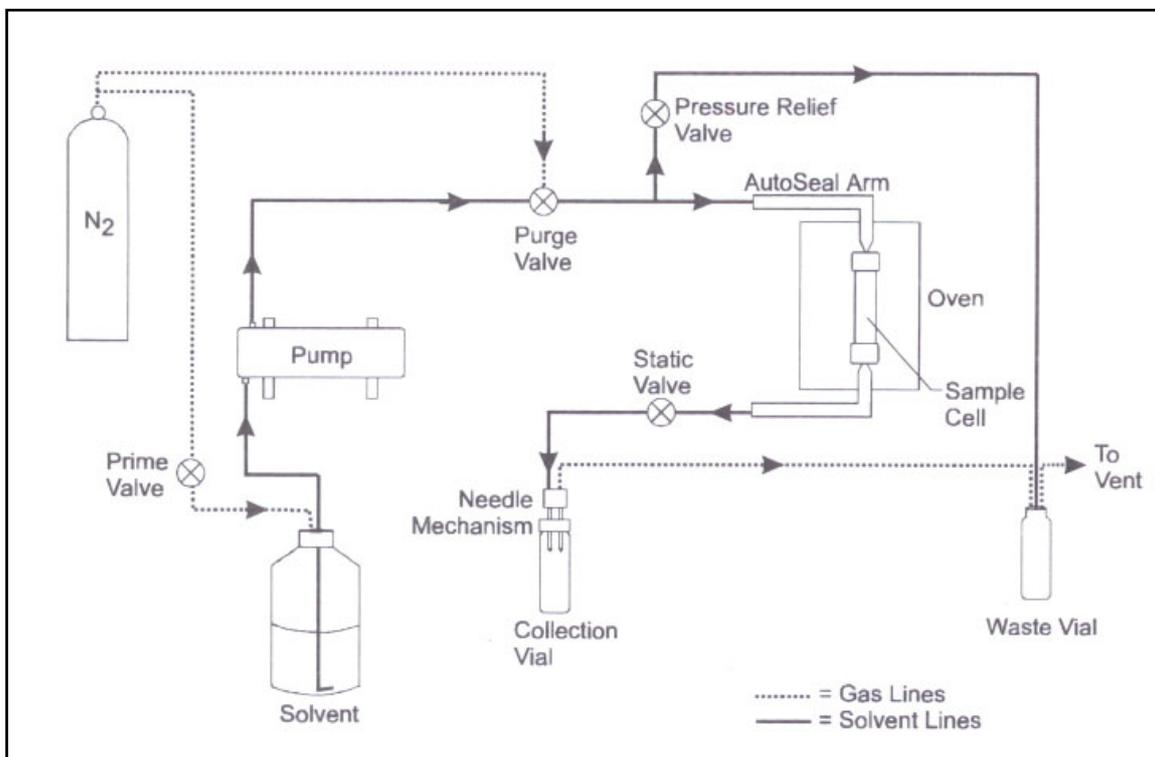
The effects of temperature on MAE extraction are not always easily explained. In principle, high temperature are to be avoided when working with thermolabile compounds, otherwise extraction efficiency is improved by using elevated temperatures due to the higher solvent capacity to solubilize analyte, decrease solvent viscosity and surface tension, and promote easier desorption of analytes from active sites. When MAE extraction is performed in closed-vessel the temperature can rise above the solvent boiling point.

### **10.2.2 Pressurised Liquid Extraction**

Pressurized Liquid Extraction (PLE) is most commonly known as Accelerated Solvent Extraction, which is the trade name of the only instrument available on the market nowadays (ASE<sup>TM</sup>, Dionex, Sunnyvale, CA, USA). The first experiments were performed using in-house built system (Richter et al, 1996), which resulted in the development of the commercialised system (ASE). Most published applications have been performed on the Dionex ASE<sup>TM</sup> or in a few cases on in-house built systems or adapting supercritical fluid extraction systems (Kenny and Olesik, 1998).

The basic instrument set-up presented in the first application resembles the scheme presented in Figure 10-3. The commercially available instrument ASE is an automated

system for extracting organic compounds from a variety of solid and semisolid samples. This system is equipped with safety tools such as a gas sensor to monitor solvent leaks.



**Figure 10-3:** Schematic diagram of a PLE system

Sample extraction requires the following steps:

- **Loading:** sample is loaded in a stainless steel extraction cell (possible dimension 11mL; 22mL; 33mL) When the thimble is tightened, it is set in the trial for performing the extraction.
- **Filling:** thimble is filled with the extraction solvent. The static valve will close when the cell is full and about 1 mL is collected in the collection vial.
- **Pre-heating:** this step is performed to ensure that the sample reaches thermal equilibrium. During the pre-heating the solvent expands; to prevent overpressure the static valve opens periodically.
- **Static-extraction:** during the static extraction all the valves are closed, the static valve opens to maintain pressure at the set point.
- **Flushing:** the static valve is opened, fresh solvent is pumped into the extraction cell and the flushed extract is collected into the collection vial.

- Purging: the remaining solvent is flushed out of the extraction cell by pressurised nitrogen onto the collection cell. During this step the pump valve is closed while the purge valve is opened.
- Unloading: pressure is vented and the cell is unloaded from the oven returning to the tray.

There are few hints to be followed when loading the sample. First of all, the use of a paper filter to cover the metal frit is a good practice to avoid obstruction. According to the manufacturers instructions, any empty volume should be filled using an inert matrix: sand, Hydromatrix, diatomaceous earth, anhydrous sodium sulphate or glass wool have been successfully used for this purpose. Often the inert matrix is mixed directly with the sample providing a porous mixture enabling the extraction solvent to flow through the sample. Moreover when using a flush volume of 60% no carry-over occurred between extraction (Richter et al, 1995).

#### *Parameters influencing the extraction efficiency*

##### Temperature

Temperature plays a key role in increasing extraction efficiency. This statement is justified by physico-chemical explanations reported by Richter (Richter et al, 1996). Indeed the use of a high temperature increases the solvent capacity to solubilize analytes and the diffusion rate. In detail, high temperatures can disrupt the existing matrix-analyte interaction, in addition the solvent viscosity decreases, allowing better contact between particle and solvent, thus resulting in an enhanced extraction.

As a drawback, the increase of temperature can cause a breakdown of the analytes when dealing with thermolabile compounds.

##### Pressure

The use of elevated pressure is the basic criteria to allow for working conditions at temperatures above the solvent boiling point. Pressure plays a minor role in increasing extraction efficiency. Nevertheless, pressure forces the solvent into areas where it would have not gone at atmospheric pressure, increasing the contact surface between solvent and matrix, facilitating the extraction procedure. In particular, the extraction efficiency is improved when analytes are trapped in water-blocked pores (Richter et al, 1996), because the solvent is pushed into the pores by the pressure.

##### Solvent

An optimal extraction process is strictly dependent on the choice of solvent. Conventional extraction methods are usually transferred to the PLE. Investigation on the use of solvents are limited and in any case restricted to the application investigated.

### **10.3 Methods optimization**

Method optimization can be performed following two approaches, the non-systematic based on the experience of the analyst, or “trial-and-error” manner, and the systematic approach based on vary-one-factor-at-a-time while holding the others fixed. The latter leads to a large number of experiments, which amplifies exponentially when increasing the number of variables taken into consideration, resulting in a very time-consuming and inefficient way of operating. To overcome this problem, two ways of proceeding have been considered in this study such as factorial design, which increases efficiency by studying several factors simultaneously and simplex based on sequential optimization.

#### ***10.3.1 Factorial design***

The factorial design set-up allows the effect of several factors to be investigated simultaneously. In an experiment the independent variables are named *factors* and the values within a factor are called *levels*. When designing the experiment it is necessary to decide how many factors and how many levels are to be investigated. If a full experiment design is carried out the number of experiments can be quite high. Basically the number of experiments required is the product of the number of levels for each factor and the number of replicate for each treatment.

$$\text{Experiments} = (\text{levels factor } 1) \times \dots \times (\text{levels factor } K) \times \text{number of replicates}$$

The main advantage of operating using a factorial design is evident during the data evaluation. Indeed factorial design is highly efficient since every observation provides information about all the factors investigated. The change of response due to switching a factor from one level to another is defined as *main effect*, while when a factor depends on the level of another factor then an *interaction of effects* occurs. The factorial design allows interactions among factors to be evaluated, avoiding misleading conclusions, moreover if no interactions exist, a factorial design is able to provide information concerning the main effects anyway.

In this study the influence of the investigated extraction parameters on extraction efficiency was evaluated with a factorial design approach (Bayne and Rubin, 1986), by using the STATISTICA™ software (Stat Soft Inc., Tulsa, OK, USA).

The most frequently used factorial design during the development of the project was the mixed two and three level design. When studying the two-level factor, the levels will be coded  $-1$  and  $+1$ , corresponding to the lowest and the highest level, respectively, if working with quantitative factors or representing just two different sets for nominal levels if working with qualitative factors. The code  $0$  will be added when introducing factors having three-levels.

Two-level factorial design allows to evaluate main effects as well as interaction effects. Nevertheless to estimate the quadratic curvature of a response it is necessary to apply a three-level factorial design. However, it should be borne in mind that interpretation of results must be cautious when using qualitative factors (e.g. solvent) since the lack of an ordered relationship among factors level should be taken into account.

Factorial design has the main advantage to be highly efficient and flexible; indeed each observation provides information about the factors considered in the experiment.

To avoid misleading and erroneous conclusions, factorial design should be used whenever an interaction among factors is suspected; in fact, basic statistical analysis is not able to investigate and take into account interaction among factors. For this specific reason factorial design is the only effective way to examine interaction effects.

The only drawback of factorial design is the large number of experimental units required when many factors and/or levels are investigated, which can be practically overcome by using a fractional factorial design.

Within the experimental design *randomization* is the method applied to take into account the experimental errors, performing the experiments so that every possible arrangement of treatments on experiments units has the equal probability of occurring. Its main purpose is to protect the results from unsuspected *bias* to have unbiased estimates of treatments effects and experimental error variance and to obtain independent results.

### **10.3.2 Simplex**

The simplex is based on the geometrical figure of a simplex. This means that the

simplex optimises the response depending on K factors starting with an initial design of K+1 experiments; successively a new simplex is created as a reflection in the opposite direction of the worst results obtained.

In this project the Multisimplex® (Grabitech Solutions AB, Sweden), whose optimization algorithm contains some generally accepted modifications to the original version of the modified simplex method, was applied for the sequential simplex optimization. In this respect the modified simplex can adjust its shape and size depending on the response achieved in each step becoming a powerful tool for optimization where all conditions can be varied in a small number of trials and multiple control variables can be handled simultaneously. Compared to the basic simplex method the modified one is based on other rules, such as expansion in a direction of more favourable conditions or contraction if a move was taken in a direction of less favourable conditions. The procedures for expansion and contraction enable the modified simplex to accelerate along a successful track of improvements. Therefore the modified simplex will usually reach the optimum faster than the basic one.

### ***10.3.3 Comparison of different extraction method***

#### Post-hoc comparison

To highlight statistically significant differences between sets of data it is possible to perform a series of simple t-test comparing all pairs of means. However, this way of proceeding capitalizes on the chance of overestimating the statistical significance of mean differences. To take into account that more than two samples are considered, it is recommended to apply the post-hoc comparison technique. In this particular case the Scheffe's test has been used, which can be applied to determine the significant differences between group means in an analysis of variance setting. Scheffe's test is considered to be one of the most conservative post hoc tests, meaning conservatively strict.

#### Principal component analysis

When large data tables containing a large amount of information have to be evaluated, the resulting interpretation can be too complex and most of the information can remain hidden in the table. In this respect PCA is a projection method that helps to visualize all the information contained in a data table. PCA is able to find out in what respect one

sample is different from another, which variables contribute most to this difference, and whether those variables are correlated or independent.

In this project the PCA was performed using THE UNSCRAMBLER<sup>®</sup> (Camo ASA, Oslo, Norway).

