Fresenius J Anal Chem (2001) 369 : 469–476

REVIEW ARTICLE

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The state-of-the-art in the analysis of estrogenic mycotoxins in cereals

Received: 25 September 2000 / Revised: 2 January 2001 / Accepted: 3 January 2001

Abstract The increasing public awareness of chemicals that mimic or otherwise interfere with the activity of natural hormones – so-called endocrine disrupters – has also led to greater study of mycotoxins with estrogenic potential. The purpose of this paper is to introduce the topic of estrogenic mycotoxins and to discuss the state-of-the-art in the analysis of these substances in cereals, with special emphasis on zearalenone (ZON) as its most relevant representative. Because the use of immunoaffinity columns (IAC) followed by high-performance liquid chromatography with fluorescence detection (HPLC–FLD) and immunoassays are currently the most frequently used methods for the determination of ZON and its metabolites, these techniques are discussed in more detail. Recent papers, which have revealed the great potential of HPLC–MS(MS) for the simultaneous detection and identification of several estrogenic mycotoxins, are discussed. The performances of the state-of-the-art methods are finally compared by study of the results obtained in recent international intercomparison studies. On the one hand, these studies revealed the good performance of both chromatographic and antibody-based methods. On the other hand, the need for better means of external quality assurance measures, especially the availability of certified reference materials and certified standards, has clearly been demonstrated.

Introduction

Estrogens are female sex hormones characterized chemically as steroids with an aromatic A-ring, a phenolic group on the A-ring, and no methyl group at the bridgehead between the A and B rings. There are three natural estrogens: 17 β-estradiol, estriol, and estrone. Estrogens have a variety of actions in the body. They control sexual differentiation both during prenatal development and at puberty. Estrogens act by binding to specific soluble receptor proteins in the target cell cytosol [1]. For unknown reasons the cytoplasmic receptors for estrogens enable a variety of chemicals or their metabolites to bind. The most important environmental estrogens derived from industrial processes are chemicals (e.g. DDT, PCBs) which are unusually persistent in the environment and tend to accumulate in food chains [2, 3]. Also, various plants, particularly soybeans, contain large amounts of flavonoids that have substantial estrogenic activity [4].

Mycotoxins are natural secondary metabolites, formed by the action of fungi on agricultural commodities in the field or during storage, which are toxic to man and farm animals. The only estrogenic mycotoxins yet established are the zearalenones, although there is no reason to believe that other structural classes will not be discovered in the future. The discovery of estrogenic mycotoxins developed from the study of estrogenism, a condition in swine first reported in 1927 by Buxton [5], who observed swelling and eversion of the vagina in young gilts. The major active toxin was given the name zearalenone by Urry et al. [6] who determined the structure in 1966. Zearalenone (ZON) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic-acid-lactone] derives its name from *Gibberella zeae*, the name of the first producing organism studied; *r*esorcylic *a*cid *l*actone, the generic name for this group of natural products; *ene*, the standard suffix indicating the presence of the C-1′ to C-2′ double bond; and *one*, the standard suffix indicating the presence of the C-6′ ketone (Fig. 1).

ZON is a secondary fungal metabolite produced by several species of Fusarium fungi, mainly by *F. graminearum* and *F. culmorum*. These species are known to colonize maize, barley, oats, wheat, and sorghum [7] and tend to develop during prolonged cool, wet growing and harvest seasons in the temperate and warm regions of the

This overview of mycotoxin analysis will be continued shortly with a critical review on the most important class of Fusarium-mycotoxins, the analysis of trichothecenes.

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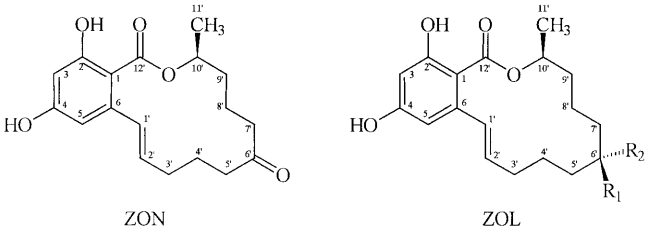

Fig. 1 Graphical illustration of the molecular structures of zearalenone and its metabolites α–zearalenol (R_2 = OH) and β-zearalenol (R_1 = OH)

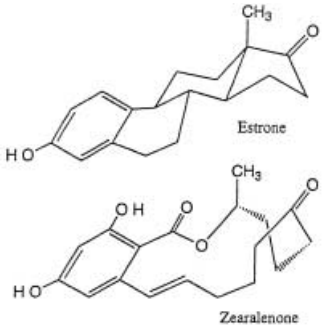

Fig. 2 Possible conformations of estrone and zearalenone

world [8]. In relation to foods and animal feedstuffs, besides aflatoxin B_1 and M_1, deoxynivalenol, ochratoxin A, and the fumonisins, ZON is one of the most important mycotoxins worldwide [9]. Although reported incidence rates and concentration levels in cereals and mixed feed vary considerably, maize is the most frequently contaminated commodity. Depending on climatic, harvest, and storage conditions in maize and maize products, the levels of ZON found are between 1 and 2900 μg kg^{-1} [10]. Of numerous ZON derivatives that can be produced by *Fusarium* spp., only *trans*-α-zearalenol (α–ZOL), has been found to occur naturally in cereal grain [11]. After consumption of ZON, the two stereoisomeric metabolites, α- and β-ZOL (Fig. 1), are produced in mammals by reduction of the keto-group at C-6′. Another structurally similar compound is zearalanol (zeranol, Ralgro), which is synthetically produced from ZON, is used as a growth promoter in animals [12, 13], and has been banned in the European Union since 1985. Zeranol is distinguished from ZON by the lack of a C-1′–C-2′ double bond. This substance can also be formed in vivo from ZON and α-ZOL, which can be carried over from contaminated feed stuff to animals.

ZON has been characterized as a compound that is not acutely toxic, because a single oral dose of 20 000 mg kg^{-1} (w/w) does not cause death in mice and rats [14]. ZON and ZOLs act as estrogens because they can adopt a conformation which sufficiently resembles 17β-estradiol and other natural estrogens to enable binding to the estrogen receptor (Fig. 2). Owing to their frequent occurrence, ZON and ZOLs are an important class of endocrine disrupter. Their estrogenic potential is comparable with that of the naturally occurring estrogens estrone and estriol and is several orders of magnitude higher than those of well-known environmental estrogens, e.g. organochlorine pesticides. In animal husbandry the presence of ZON in feed, causing hyperestrogenism, especially in swine, has long been a problem. Although the marked estrogenic and anabolic properties of ZON can cause severe reproductive and infertility problems in farm animals [15], the overall contribution of ZON and its metabolites to the total environmental estrogen load has not been determined.

Because of existing regulations worldwide, the risk of ZON contamination has been addressed by several food authorities in different countries [16] and will increasingly become a quality criterion in trade, especially for maize and maize products. Currently, nine countries have set guidelines or maximum tolerable levels for ZON in food (mainly cereals), ranging from 0 to 1000 μg kg^{-1} [17]. ZON is, therefore, a frequently analyzed mycotoxin, and analysis of ZON has been included in the internal quality control of maize production by many cereal handling companies. Besides sampling, the major problems for regulation and control of the ZON content have so far been the lack of fast analytical methods and a lack of comparability of measurement results and of appropriate reference materials.

Analytical methods

Because estrogenic mycotoxins usually occur at μg kg^{-1} levels there is special interest in analytical procedures for reliable detection of ZON and its metabolites between 10 and 100 μg kg^{-1}. In response to the risk of great economic loss in the industry and the threat to human health as a result of exposure to ZON, several methods have been developed for the quantitation of ZON and its metabolites in different foods, feeds, animal tissues, blood, and urine. Detailed reviews have been given by Steyn [18], Betina [19], Frisvad and Thrane [20], Scott [21], and Lawrence and Scott [22]. The determination of ZON in cereals can be divided into five steps–grinding and extraction of the sample, clean-up, separation, and detection.

Detection

Although TLC [23, 24], GC [25, 26], and GC–MS [26, 27] methods are available, HPLC is usually chosen for the determination of ZON and its metabolites. Most of the HPLC methods have been developed for maize and other cereals. Recent HPLC methods for ZON have employed reversed-phase chromatography with direct fluorescence detection (275/450 nm) [28, 29]. Fluorescence detection is also used in a recently described method based on immunoaffinity columns (IAC) and quantitation by reaction with aluminium chloride hexahydrate ($AlCl_3.6H_2O$) then measurement with a fluorimeter [30]. Increasingly, HPLC–MS with atmospheric-pressure chemical ionization (APCI)

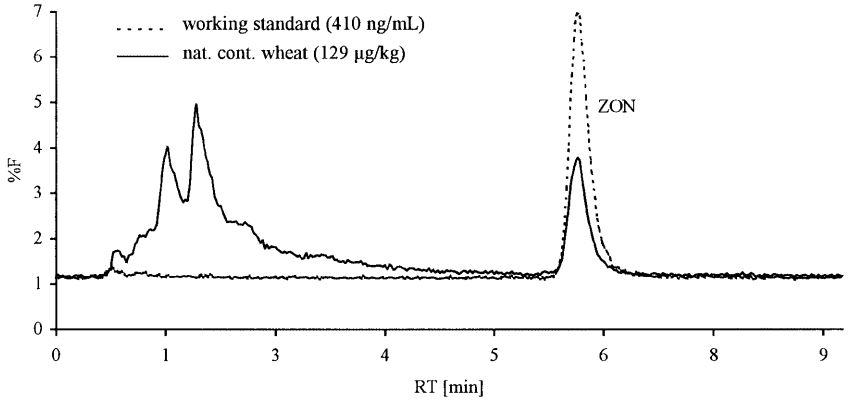

Fig. 3 RPHPLC–FLD chromatogram obtained from a 410 ng mL^{-1} ZON standard solution (*dotted line*) overlaid with that from a wheat sample (*full line*) spiked with 129 µg kg^{-1} ZON

and enzyme-linked immunosorbent assays (ELISAs) are used for the determination of ZON in foods and feeds. Because of the selectivity of MS detection, it was possible to quantitatively determine ZON both in raw extracts without clean-up by use of a normal size (100 mm) column, or a short (20 mm) chromatographic column, when the clean-up was performed to minimize possible interferences [31]. Recent work has shown that the sensitive simultaneous determination of ZON, α-/β-ZOL and zeranol in animal urine and tissue is feasible by means of LC–APCI–MS–MS after enzymatic digestion [32]. This might enable differentiation of zeranol abuse from natural contamination. Analysis of all four compounds seems to be advantageous in improving assessment of the risk of overall estrogenic and anabolic effect in food and feed. Similar work was conducted by Kennedy et al. [33], who developed a method for the simultaneous determination of ZON, α-/β-ZOL, zeranol (α-ZAL), and taleranol (β-ZAL). Because the use of LC–MS instruments is steadily becoming routine, and enables quantitation and identification of a variety compounds, this technique is applied by an increasing number of laboratories worldwide.

In contrast to LC–MS techniques, ELISA methods usually enable the determination of only a single mycotoxin at one time, although simply and very rapidly. An ELISA has been tested collaboratively and approved by the AOAC (Association of Official Analytical Chemists) as a first-action screening method for ZON concentrations higher than 800 µg kg^{-1} [34]. Several ELISAs for the detection of ZON are available commercially and will be discussed later as a major technique for the screening of ZON in cereals.

Extraction and clean-up

The extraction of ground samples is usually performed with a mixture of organic solvents (e.g. ethyl acetate, acetonitrile, methanol, or chloroform) and water or aqueous acidic solutions.

Conventional methods for the analysis of ZON and its metabolites generally use successive liquid–liquid partitioning with chloroform–aqueous NaOH [29, 35] or solid-phase extraction steps [36] during sample clean-up. These methods require sufficient practical experience to achieve precise and accurate analytical results. In particular, the time ZON is exposed to aqueous NaOH must be minimized because the ZON lactone ring can be hydrolyzed under alkaline conditions, and cleavage of the ester bond would result in loss of ZON during the clean-up procedure.

The most applicable and adaptable procedures for the purification of estrogenic mycotoxins are antibody-based IAC before separation and quantification by RPHPLC with FLD (fluorescence detection). IACs for purification of ZON are marketed by Rhône-diagnostics Technologies (Glasgow, Scotland) under the trade name Easi-Extract and by VICAM (Watertown, MA, USA) under the trade name Zearala Test columns. According to our experience both products perform equally well. Analysis of ZON and its metabolites by use of IAC is simple and robust, can be used as a semi-quantitative method for the screening of estrogenic mycotoxins, or it can be coupled with physicochemical equipment such as HPLC for the complete and accurate estimation of mycotoxin quantities. After preconditioning of the column with a buffer the sample is applied to the column. As the solution passes through the column the mycotoxin molecules bind specifically to the antibodies attached to the solid phase of the column. Other components in the solution are unaffected by the antibodies and, therefore, pass directly through the column. After washing with buffer solution or distilled water at neutral pH to remove unwanted substances, the mycotoxin is eluted (desorbed) by the use of an appropriate solution (e.g. methanol, acetonitrile) which causes antibody denaturation (e.g. methanol, acetonitrile). Generally, HPLC–FLD chromatograms with analyte peaks well separated from interferences can be obtained by the methods described by the IAC suppliers VICAM and Rhône-diagnostics Technologies. Figure 3 shows a chromatogram of a ZON standard overlaid with that from a spiked wheat sample. Studies on the cross-reactivity of ZON with the also naturally occurring α-ZOL when using IAC have shown that both toxins can be detected simultaneously by HPLC–FLD in combination with IAC, with no cross-reaction (Fig. 4). A typical limit of detection (LOD) of an IAC method is 3–6 µg kg^{-1}. In the concentration range 10–200 µg kg^{-1} ZON the mean recovery of the method is high–98–100% [29, 37]. A major environmental advantage of the IAC is that in

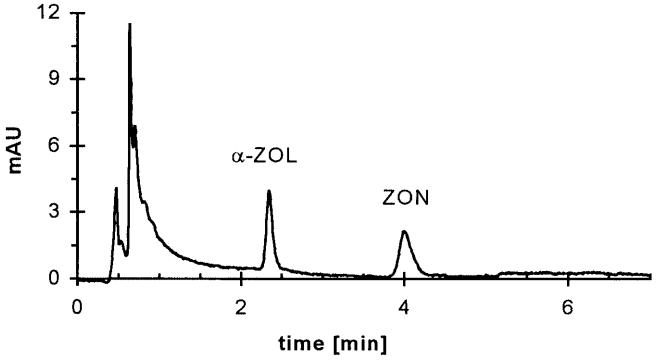

Fig. 4 HPLC–DAD chromatogram obtained from of a sample of maize spiked with α-ZOL and ZON at a level of 50 μg kg^{-1} (for each toxin)

contrast with conventional methods employing liquid–liquid extraction the use of a chlorinated solvent (chloroform), is no longer necessary. Furthermore, ZON analysis by means of IAC is easy to learn and to employ.

The analytical results from a method-comparison [29] revealed good agreement between the results obtained from methods using IAC and liquid–liquid extraction. In addition, a recent study did not demonstrate a significant difference between the results obtained with IAC and by use of AOAC Official Method 985.18 for ZON determination [38], which is also based on liquid–liquid extraction. As will be described later, the clean-up procedures employed in the interlaboratory comparison studies of 1996 and 2000 organized by the IFA–Tulln [39, 40] indicate that conventional liquid–liquid separation techniques will be rapidly replaced by use of IACs.

Another alternative clean-up technique, introduced only recently for ZON analysis after successful application in the analysis of trichothecenes [41], is the use of Mycosep columns #224 (Romer Labs Inc., Union, MT, USA) [37]. Mycosep multifunctional clean-up columns consist of packing material containing a variety of adsorbents, e.g. charcoal, celite, ion-exchange resins, and others [19]. The packing material is housed in a plastic tube between filter discs with a rubber flange on the lower end containing a porous frit and a 1-way valve. When the column is inserted into the culture tube the flange seals tightly, thus forcing the extract through the packing material of the column to the top of the plastic tube. The Mycosep column enables rapid sample purification within 10 to 30 s. A major advantage of this column is that there are no time-consuming rinsing steps required as in solid-phase extraction. Although promising results, with sensitivities and recoveries comparable with those of IAC–HPLC–FLD methods [39], have been achieved for maize, according to our experience the use of Mycosep column before HPLC–FLD results in less selectivity and can, therefore, not be recommended for the analysis of complex matrices such as mixed feed.

The second major technique for the determination of ZON, which is also based on antibodies, is the use of immunoassays.

Immunoassays

Since 1977, when the first immunoassays were described for aflatoxin B_1 [42], there has been rapid development of these assays for mycotoxin detection [43]. The initial stages involve the production of antibody – because mycotoxins are low-molecular-weight molecules they are not immunogenic. After conjugation to a protein carrier, however, the toxins can be used for immunization to induce antibody production. By use of this approach several antibodies have been developed for mycotoxins such as the aflatoxins, ochratoxin A, ZON, T-2 toxin, and many more. With the availability of these antibodies, simple and rapid immunoassay methods have been developed for the determination of these mycotoxins in food samples, animal feed samples, and in biological fluids such as milk. The spectrum of immunoassays for the determination of ZON range from radio-immunoassays (RIA) based on porcine antibodies to enzyme immunoassays based on polyclonal rabbit and monoclonal mice antibodies and immuno-chromatography. The production of polyclonal ZON antibodies in rabbits was reported for the first time by Liu et al. [44]. Warner et al. published a direct competitive ELISA based on rabbit antibodies for the determination of ZON in corn [45]. Investigations with naturally contaminated and spiked corn samples showed recoveries to be sufficient for concentration levels above 100 μg kg^{-1}.

Székács developed an indirect competitive ELISA by use of a ZON-conalbumin conjugate as immunogen [46]. The working range of the assay was described as 1 to 70 μg L^{-1}. Cross-reactivities with other resorcyclic acid lactone derivatives were found to vary between 1 and 22% compared with ZON. Remarkably good performances of IgY-based immunoassays for the determination of ZON down to the μg kg^{-1}-range have also been reported recently [47].

A multi-analyte dipstick immunoassay for AFB1, T-2 toxin, 3-AcDON, roridin A, and ZON has been developed by Schneider et al. [48]. Studies on the application of ELISAs for *Fusarium* mycotoxins such as ZON have also been conducted by Usleber et al. [49].

Immunoassays for the determination of ZON are now available from several suppliers, e.g. r-Biopharm (Darmstadt, Germany) with its product Ridascreen Fast with an LOD of 50 μg kg^{-1}, 5 min extraction with methanol, and a total test procedure taking 15 min [49], or Neogen (Lansing, MI, USA) with a ZON ELISA known under the trade name Veratox-Zearalenon, which enables the detection of ZON, although only down to 250 μg kg^{-1}. The latter ELISA is therefore not suitable for monitoring of ZON at the guideline level established in Austria (60 μg kg^{-1}). Cross-reaction with α-ZOL, zeranol, and β-ZOL is observed for both commercial test-kits.

As a result of the intercomparison study mentioned below has been demonstrated that ELISA methods are well suited for obtaining analytical results of good accuracy in ZON detection. Because completely equipped assay kits are commercially available, this rapid analytical method is accessible to any chemical laboratory equipped for, and familiar with, routine analysis. The high costs of such

Table 1 Overview of methods for the determination of ZON employed by the laboratories participating in the intercomparison study of 1998

Extraction	Clean-up	Detection
14 × Acetonitrile/water	10 × Immunoaffinity columns	18 × HPLC–FLD
5 × Methanol/water	7 × Liquid–liquid-partitioning	5 × ELISA
2 × Acetonitrile/aq. KCl	3 × Mycosep columns	1 × HPLC–DAD
2 × Ethyl acetate/water	2 × SPE C18	1 × HPLC–MS
2 × Chloroform	1 × SPE silica	1 × GC–MS
1 × Ethyl acetate/aq. acetic acid	1 × Extrelut	2 × TLC
1 × Methanol/dichloromethane	1 × Liquid–liquid+SPE silica	
1 × Ethyl acetate	3 × None	

commercial kits do, however, limit wide-spread application, in particular for monitoring programs, e.g. in resistance-breeding studies.

Intercomparison studies

The great interest of European laboratories in the two intercomparison studies carried out by us in 1996 [29] and 1998 [37] (with 28 participants) show the increasing awareness and the need for external means of quality assurance in the field of *Fusarium* mycotoxin analysis. The different extraction, clean-up, and detection methods employed during the 1998 study reflected the state-of-the-art in ZON-analysis 2 years ago (Table 1) with IAC (10 of 28 participants) and liquid–liquid-partitioning (7 of 28) as the major clean-up techniques and HPLC–FLD (18) and ELISA (5) as the most frequently used end determination steps. Our most recent intercomparison study, organized in 2000 within the scope of our SMT-project, described below, revealed that the number of laboratories using IAC had increased to 24 (of 28).

Different results were obtained from the two interlaboratory comparison studies of 1996 and 1998 on ZON in maize. In the first study satisfactory coefficients of variation (CV) between the laboratory means of 15.0 to 27.7% for both ZON-spiked maize materials and 16.6% for naturally ZON contaminated maize were obtained. The reasonable precision and accuracy were mainly because of the use of a common ZON calibrant provided by the IFA–Tulln. Within the scope of this study the participants were asked to perform the calibration with the common ZON calibrant. The participants were also asked to analyze a ZON calibrant containing a concentration of ZON unknown to the participants. The calibration for the determination of this unknown calibrant had to be performed by use of the in-house ZON calibrant of the participants. In this case only 9 of 17 labs submitted values for the unknown ZON calibrant. An interlaboratory CV of 16.6% (not outlier rejected) and a deviation of 10.8% from the target concentration calculated on the basis of the weighed-in amount of ZON was obtained for the unknown calibrant. The results indicate that a major problem in ZON analysis can be traced back to the application of in-house calibrants.

The second intercomparison study performed in 1998 was designed without providing a common ZON calibrant. Under realistic routine analysis conditions using in-house ZON calibrants, high interlaboratory CVs were obtained – 27.7% for ZON-spiked maize and from 40.5 to 41.2% for two naturally ZON contaminated maize materials (Fig. 5). These results confirmed our findings from the study of 1996 that a ZON calibrant is crucial to ensure the reliability and traceability of ZON determination. Moreover, method-dependence of the analytical results was

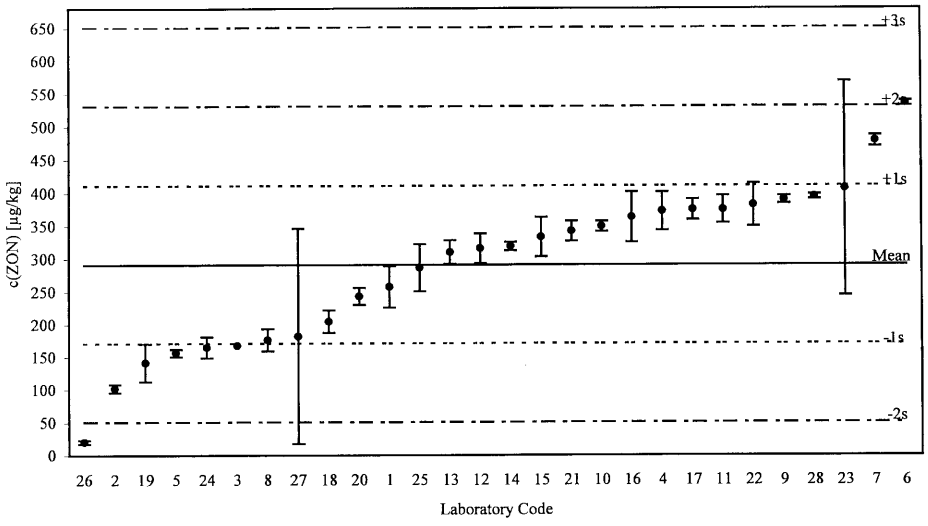

Fig. 5 1998 Results of the intercomparison study conducted in 1998 for maize naturally contaminated with zearalenone (mean = 291 µg kg^{-1}, CV = 40.5%, n = 28)

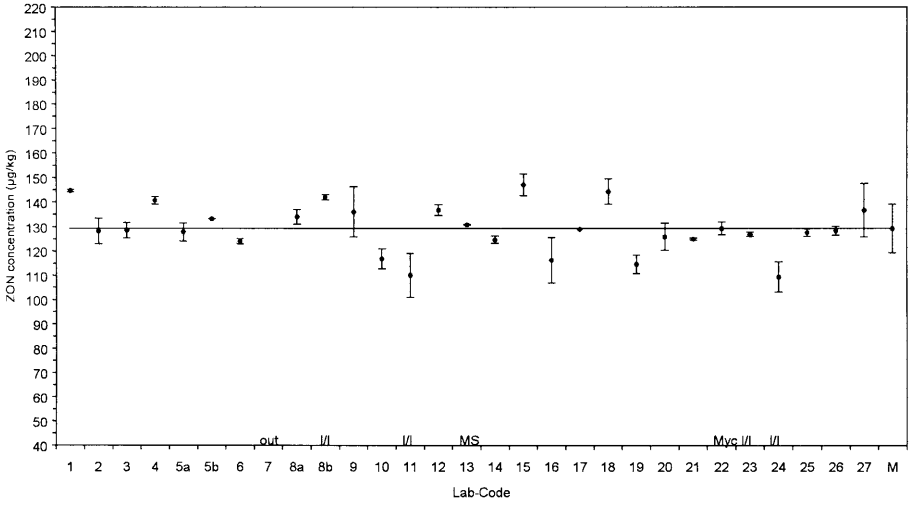

Fig. 6 Results of an intercomparison study conducted in 2000 for maize naturally contaminated with zearalenone (mean = 129.2 µg kg^{-1}, CV = 8%, n = 28). (Legend: **out** = outlier, **l/l** = liquid–liquid extraction, **MS** = mass spectrometer, **Myc** = Mycosep column)

demonstrated in this study. The analytical results obtained for ZON show that although the HPLC and ELISA methods cannot be statistically distinguished, all other methods, particularly TLC, furnished significantly lower results than the assigned mean value [50].

Because of the results obtained from the previous interlaboratory comparison studies an SMT-project, funded by the EC, dealing with the preparation and certification of reference materials for ZON has been launched [51]. The overall objective of this 4-year project, which began at the end of 1998, is the preparation and certification of both a blank maize material (< 5 µg kg^{-1} ZON in maize) and a maize naturally contaminated with ZON (c = 40–120 µg kg^{-1} ZON in maize) to be used as reference samples for the determination of ZON in maize. An additional goal of this project was the production of a common standard solution of ZON in acetonitrile to be available over the whole project duration which will finally be certified on the basis of the preparation procedure. Moreover, determination of a common extinction coefficient at a given reference wavelength in acetonitrile was a further objective, because most laboratories employ mixtures of acetonitrile and water as mobile phase and UV measurements can be performed more precisely in this solvent, because of lower background absorption. So far, a common extinction coefficient was only available for ZON in benzene and methanol [52]. Because the preliminary results of our measurements showed that from the three UV-absorption maxima of ZON the absorption band at 274 nm could be measured most precisely (CV = 1.2% compared with 2.3% at 314 nm and 5.6% at 236 nm), the common extinction coefficient of ZON in acetonitrile was determined at that wavelength by the three main partners in this EC project. An extinction coefficient of 12623 ± 111 (CI(95%)) was finally obtained for ZON in acetonitrile at 274 nm using temperature-controlled and newly calibrated UV-spectrophotometers [37].

In the course of this project the performances of the participants and the analytical methods employed were investigated and improved by means of two large-scale interlaboratory comparisons. The results of the first interlaboratory comparison with 28 participants and IAC (23 labs) before HPLC–FLD (27 labs) as the predominant method showed an improvement of the comparability of the laboratory mean results, because of the production and successful application of a common ZON calibrant certified on the basis of the preparation procedure. Another reason for the better improved agreement of results might also be the use of IAC by most participants.

Moreover, extensive recovery studies showed that the method recoveries generally fulfil the given criteria of 70–110% and the required between day variations of less than 15%, specified by the European Commission. Minor method-dependence does, however, seem to be present: recovery rates for methods employing liquid–liquid extraction for clean-up tend to be lower than those for IAC methods.

In general, no matrix effects could be observed, because all analyses of the ZON-spiked maize were performed by external and matrix calibration with a common ZON calibrant. The overall mean recovery was 93.7% with a CV of 10% for external calibration and 95.9% with a CV of 14% for matrix calibration.

As a main result of the second intercomparison study a good CV of 8% of the laboratory mean results was achieved, even for the naturally ZON-contaminated maize; this is an improvement of 32% compared with the 1998 study. The laboratory mean results and the assigned between-laboratory mean of 129.2 µg kg^{-1} for naturally ZON-contaminated maize are presented in Fig. 6.

Subsequent to these extensive interlaboratory comparison and preparatory studies certification of a „blank“ and a naturally contaminated maize material was performed by selected European mycotoxin laboratories in October 2000.

Conclusions and outlook

Increasing awareness of the presence of estrogenic mycotoxins has led to the development of improved methods

for the quantification and identification of ZON and its metabolites. In the last four years IAC, followed by HPLC–FLD, has become the predominant analytical method for ZON using a clean-up, whereas immunoassays without the need for further clean-up have become the most relevant rapid method (and the accuracy of this method is improving). As a future trend, which is already well known, e.g. from environmental analysis, on-line clean-up by IAC coupled with HPLC–FLD seems feasible and promising. The availability of commercial, relatively easy-to-use LC–MS–(MS) instruments for routine analysis, which enable both quantification and identification, has led to an increasing use of this technique, also for the analysis of estrogenic mycotoxins. LC–MS can currently be considered as the most important method for the simultaneous determination of ZON, α-, β-ZOL, and zeranol. Because LC–MS has also successfully been used for the detection of trichothecene mycotoxins [53], this technique could become a powerful tool for the simultaneous determination of trichothecenes and estrogenic mycotoxins in cereals in the routine analysis of *Fusarium* mycotoxins.

Several initiatives aimed at improving the comparability of measurement results between European laboratories have, moreover, been started in recent years. Results from several intercomparison studies demonstrate that the production of a common ZON calibrant (in acetonitrile) and the determination of a common extinction coefficient for ZON in acetonitrile are important steps in achieving this goal.

Acknowledgements The author would like to thank the European Commission for financial support and Mrs. Renate Hoermann for her assistance during the preparation of the manuscript.

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