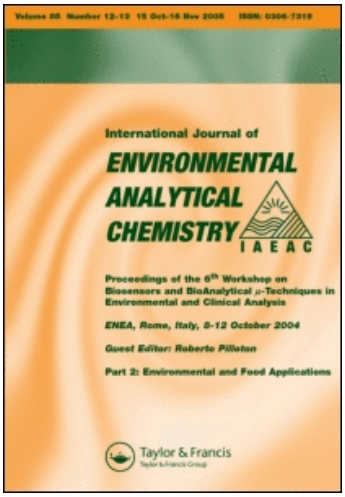

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Application of an innovative matrix solid-phase dispersion-solid-phase extraction-liquid chromatography-tandem mass spectrometry analytical methodology to the study of the metabolism of the estrogenic mycotoxin zearalenone in rainbow trout liver and muscular tissue

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APPLICATION OF AN INNOVATIVE MATRIX SOLID-PHASE DISPERSION–SOLID-PHASE EXTRACTION–LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY ANALYTICAL METHODOLOGY TO THE STUDY OF THE METABOLISM OF THE ESTROGENIC MYCOTOXIN ZEARALENONE IN RAINBOW TROUT LIVER AND MUSCULAR TISSUE

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The metabolic fate of the estrogenic mycotoxin zearalenone in rainbow trout is presently unknown. In this study, the tissue concentration of zearalenone and its principal metabolites (α-zearalenol and β-zearalenol) was determined. A known amount of zearalenone was administered as a single bolus to ten fish, and the biological tissue concentration was determined at various times following administration. The analytes were extracted from liver and muscular tissue using an on-line matrix solid-phase dispersion–solid-phase extraction sample preparation protocol, and their concentration determined by HPLC–Turboionspray–tandem mass spectrometry. The results showed that zearalenone is mainly metabolized into α-zearalenol in both liver and muscular tissues. The maximum concentrations of each analyte found in liver were 76.1, 211.2 and 63.7 ng/g respectively for zearalenone, α-zearalenol and β-zearalenol, while in muscular tissue they were 10.7, 8.2 and 6.5 ng/g. These values were reached after 2 h in liver tissue and 12 h in muscular tissue. Moreover the data obtained showed that the elimination rate in liver is quite fast since 48 h after the exposure less than 7% of the maximum concentration found is still present. In muscular tissue, however, about one-third of the maximum concentration found is still present after 48 h.

Keywords: Zearalenone; Rainbow trout; HPLC-MS/MS; MSPD

INTRODUCTION

Heightened public awareness concerning chemicals that are able to interfere with natural hormone activity has recently led to a greater study of natural substances with estrogenic potential.

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Many plant molds can affect grain quality by producing, in specific conditions of moisture content and temperature [1], secondary metabolites, namely mycotoxins, some of which can cause reproductive disorders in cattle, swine and other animals with cycling fertility. Zearalenone (ZON), whose structure resembles mammalian steroid estrogens, is a mycotoxin of major concern for human and animal health. ZON is produced by *Fusarium* spp that usually parasitize important crops such as corn, wheat, barely, oat and hay during growth, harvesting or storage [2,3].

Several studies, conducted in Europe and North America, reported the high incidence of ZON in cereals and animal feeding stuffs [4–7]. Such mycotoxins belong to a class of resorcylic acid lactones that bind to the estrogen receptor (ER) despite their non-steroidal structure. The estrogenic potential of ZON has usually been assessed by *in vivo* uterotropic assay in mice [8] and the proliferating activity of ZON has been assessed by estrogen-sensitive breast cancer cell lines such as MCF-7 [9]. The binding affinity of ZON to the estrogen receptor has also been explored [10,11]. The general conclusion drawn from these studies was that the estrogenic potential of ZON is approximately 1/100 of estradiol-17β. However, it has been reported that the biological effect *in vivo* of ZON may be greater than expected, because of its metabolites. Studies carried out on mammalian species have shown that ZON's metabolism leads to two biologically active isomers: α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). The ratio between these isomers varies in relation to the animal species, and the difference in sensitivity between species seems to be related to the proportion of α-ZOL formed. The most sensitive species for reproduction alteration caused by ZON are those in which α-ZOL is the major metabolite [12]. This can be explained considering that the estrogenic potency decreases in the order α-ZOL > ZON > β-ZOL, so the reduction of ZON to α-ZOL involves increased toxicity [13]. Arukwe *et al*. [14] compared the estrogenic potential of the above-mentioned compounds in terms of their binding affinities to the ER and their potencies to induce *in vivo* synthesis of two proteins (produced by the liver of female fish in response to estrogen): vitellogenin and zona radiata. It was shown that *in vivo* effects of α-ZOL and ZON are more pronounced than those of β-ZOL; it was also shown that α-ZOL possesses an estrogenic potency that is approximately 50% that of 17-β-estradiol (E2); additionally it was shown that ZON may not be an active estrogen in itself, but rather that its estrogenicity is a result of its metabolic product α-ZOL [14]. This demonstrates that *in vitro* tests lack the essential metabolic competence of a living fish, since they lack the endocrine effects caused by metabolism or endocrine modulation caused by interference with steroid metabolism. Fish can be easily subjected to contamination because of antinutritional factors often recoverable in fish feed produced using plant-derived materials.

Rainbow trout (*Oncorhynchus mykiss*) is one of the most abundant aquaculture species worldwide (in Italy there are over 1000 fish farms). Trout feed is composed mainly of animal proteins, fats, oils, oily seed by-products and cereals [15]. When contaminated, these components are the source of mycotoxins through ingestion.

It is important to get information concerning the metabolic fate of mycotoxins and their bioaccumulation in such species, as human exposure to those substances may occur from eating both contaminated cereal products and contaminated animal products.

Dealing with complex matrices, such as meat and vegetable tissues, the sample preparation task is often long, tedious and difficult to perform: it consists not simply in extraction of the analytes from the solid matrix but also in their clean-up from

many interfering substances of biological origin; matrix solid-phase dispersion (MSPD) extraction, however, is more efficient because it combines disruption of the gross architecture of the sample with the dissolution and dispersion of sample components into the bound organic phase on the surface of the particles. This is the reason why it has been recently applied to a variety of matrices for the extraction of analytes with a broad range of polarity.

In this study we have applied a newly developed sample preparation analytical methodology for the investigation of the metabolism of ZON in trout tissues. Sample preparation was performed using a matrix solid-phase dispersion (MSPD) procedure coupled on-line with a solid-phase extraction (SPE) clean-up process. The analytes were then quantified by means of liquid chromatography–tandem mass spectrometry (LC-MS/MS).

EXPERIMENTAL

Chemicals

Zearalenone (2,4-dihydroxy-6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]benzoic acid μ-lactone), α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-*trans*-1-undecenyl]benzoic acid μ-lactone), β-zearalenol (2,4-dihydroxy-6-[6β,10-dihydroxy-*trans*-1-undecenyl]benzoic acid μ-lactone), and the internal standard zearalanone (2,4-dihydroxy-6-[10-hydroxy-6-oxo-undecenyl]benzoic acid μ-lactone), were purchased from Sigma (St Louis, MO, USA). C18 (Lichroprep® RP 18 25–40 μm) was purchased from Merck (Darmstadt, Germany). Carbograph-4, a particular type of graphitized carbon black (GCB) was supplied by L.A.R.A. srl (Rome, Italy). The particle size range was 120–400 μm. All solvents employed were HPLC-grade, and were supplied by Carlo Erba (Milan, Italy).

Fish

Fourteen rainbow trout (*Oncorhyncus mykiss*) with a mean weight of 237 ± 29 g were obtained from the Agroittica Pontina srl (Latina, Italy). Animals were kept in an exposed 260-L ($1.8 \times 1.8 \times 0.8$ m) tank supplied with running ground water, passed through an activated charcoal cartridge. The water flow was adjusted in order to allow a complete water change every 2 h. Water temperature was continuously monitored and its mean value was 16 ± 2°C. Experimental animals were allowed to acclimatize to these conditions for seven days before the experiment started. During the first seven-day period trout were fed with commercial trout feed (1% of animal weight per day) ''Hendrix Truvit'' (Hendrix, Varese, Italy). 48 h before the beginning of the experiment feed administration was suspended. Two animals were killed at the end of this phase, serving as a control (control A), for the following experiment. Also as an initial control two trout were killed prior to acclimatization (control B).

Substance Administration and Sampling

Fish were anaesthetized by placing them in water at 0°C for 5 min. For oral administration, a 48% ethanol solution (500 μg/mL) of ZON was prepared and administered as a single bolus by means of a gastric tube. A standardized quantity of toxin was

administered by weighing each trout before the experiment and administering 1 μg of toxin for each gram of body weight (2 μL/g).

Two fish were killed by a blow to the head 2, 6, 12, 24 and 48 h after the mycotoxin administration. After the death of each animal the liver was removed and muscular tissue samples were taken from the trunk region. These samples were collected and stored at −18°C for further analysis.

Analytical Procedure

Analysis for ZON and metabolites was carried out applying a previously developed HPLC-MS/MS analytical procedure [16]. In short, liver or muscular tissue portions were taken from the animals and made homogeneous; 0.50 g of homogenate was placed in a glass mortar, 2.00 g of C18 was added and the mixture ground to obtain a homogeneous paste which was allowed to become dry and powdery. Then the sample was transferred to a PTFE column fitted with a Teflon frit at the bottom. The column thus obtained was stacked on-line with a similar cartridge containing 250 mg of carbograph-4. The stacked columns were then eluted with 15 mL of methanol–water 70 : 30, v/v; this solution was discarded. Subsequently the upper cartridge was removed and the cartridge containing GCB was sequentially washed with 10 mL of water, 10 mL of CH_3OH acidified with 10 mM HCOOH and 3 mL of CH_3OH, all of these fractions were discarded. The analytes were extracted with 15 mL of a dichloromethane–methanol 80 : 20, v/v solution. This solution was collected in a round-bottomed vial, dried in a water bath at 40°C under a gentle nitrogen stream and reconstituted with 250 μL of acetonitrile–methanol–water 37 : 16 : 47, v/v. HPLC was performed using a Series 200 Perkin-Elmer pump equipped with a Rheodyne Model 7125 injector with a 50-μL loop. Chromatographic separation was achieved under isocratic conditions on an Alltima Prevail C-18 column 250 × 4.6 mm i.d., average particle size 5 μm (Alltech, Deerfield, IL, USA) with a precolumn Supelguard 20 × 4.6 mm i.d. (Supelco, Bellefonte, PA, USA). The composition of the mobile phase was acetonitrile–methanol–water 37 : 16 : 47, v/v. Flow rate was set at 1 mL/min, but only one-fifth (200 μL/min) of the total column effluent was diverted to the mass spectrometer. Tandem mass spectrometry was performed using a PE Sciex (Concord, ON, Canada) API 365 triple quadrupole mass spectrometer equipped with a TurboIonSpray^{TM} (TISP) source, operating in the negative-ion mode. The tandem MS analysis was performed in the multiple reaction monitoring (MRM) mode. The deprotonated molecular species of ZON ($m/z = 317$) and of α-ZOL, β-ZOL and ZAN ($m/z = 319$) were selected as precursor ions and the following fragment ions were selected: $m/z = 175$, $m/z = 160$, $m/z = 131$ for ZON; $m/z = 174$, $m/z = 160$, $m/z = 130$ for α-ZOL; $m/z = 160$, $m/z = 144$, $m/z = 130$ for β-ZOL and $m/z = 205$, $m/z = 161$ for ZAN.

Data processing was performed with the software MassChrom 1.1.1 on a Power Macintosh G3.

Quantitation of each analyte was carried out by relating the areas of the analytes with the area of the internal standard (ZAN) and comparing to those obtained from a standard solution of each analyte. To be considered positive, an analyte's signal had to exceed the average of the blank signal by three times the standard deviation of background signal obtained from a procedural blank. Calculated analyte concentrations were corrected for recovery. Recovery and relative standard deviation

values for target analytes were the following: α-ZOL 85% (7.8), β-ZOL 88% (7.0), ZON 92% (9.3); limit of detection of the method were: α-ZOL 0.2 ng/g, β-ZOL 0.2 ng/g, ZON 0.1 ng/g. More information about how recovery experiments were carried out are given in reference [16].

RESULTS AND DISCUSSION

All of the fish survived the mycotoxin administration and none showed any visible behavioral alterations during the experiment. Before the beginning of the two experiments, as a control, two fishes were killed and analyzed (control A). The analyte concentrations found, reported in Table I, were below or near to the limit of detection of the analytical method employed. In contrast, the concentration values found for the other control group (control B), were close to the detection limits, but still detectable, confirming the real risk of animal contamination through ingestion of contaminated feed.

Table I reports the concentrations of ZON, α- and β-ZOL found by analyzing the ten remaining fish tissues 2, 6, 12, 24 and 48 h after the mycotoxin administration.

As can be easily seen in Fig. 1a, the ZON concentration in liver tissue reaches a maximum 2 h after administration. Moreover, after ZON is administered it undergoes a nearly immediate metabolization to α-ZOL. The kinetics of α-ZOL also show a maximum concentration after 2 h, when the free ZON concentration in liver corresponds to 22% of the sum of the determined analytes, compared to 60% of α-ZOL. Considering the concentration data found respectively 12 and 24 h after exposure there is evidence of a quick turnover, and therefore excretion, of all the three compounds, whose concentration is less than one-half of the maximum concentration found 48 h after the administration. Twelve hours after administration, the ratios between substance concentrations found in liver tissue were: 33% for ZON, 15% for β-ZOL and 52% for α-ZOL. Analysis performed on muscular tissue samples shows a different behavior. Two hours after administering the mycotoxin, only ZON (5.3 ng/g) could be found, while metabolite concentrations were not significant. Concentration values for all three analytes reach their maximum 12 h after administration. By juxtaposing the data shown in Fig. 1b with the data obtained from the trout killed after 2 h, the ZON quantity is still seen to be higher, as demonstrated by the isomer concentrations.

TABLE I Mean values of the analytes concentration found (ng/g)

Time after dose (h)	*Liver tissue*			*Muscular tissue*		
	α-ZOL	*β-ZOL*	*ZON*	*α-ZOL*	*β-ZOL*	*ZON*
Control (B)	2.2	0.4	1.0	0.1	0.3	0.6
Control (A)	0.4	n.d.	0.3	n.d.	n.d.	0.2
2	211.2	63.7	76.1	0.5	0.4	5.3
6	163.4	39.2	48.8	7.1	5.2	9.9
12	51.1	14.9	32.7	8.2	6.5	10.7
24	29.3	4.9	19.2	6.1	4.2	6.9
48	6.9	2.8	5.1	2.9	1.7	3.7

Each data point represents the analyte concentration obtained analyzing two samples taken from two different animals killed at the specified time.
n.d. = non-detectable.

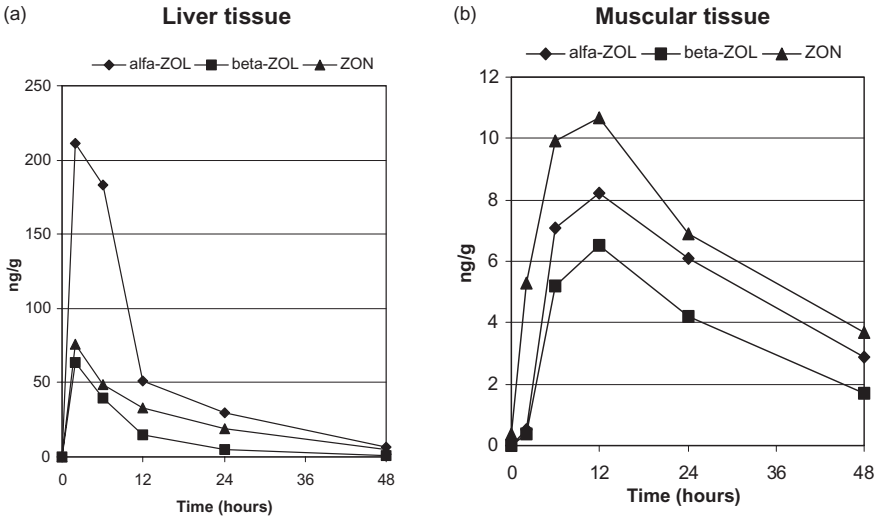

FIGURE 1 Plot of liver (a) and muscular tissue (b) mean analyte concentration. Each data point represents the concentration obtained by analyzing two samples taken from two different animals killed at the specified time.

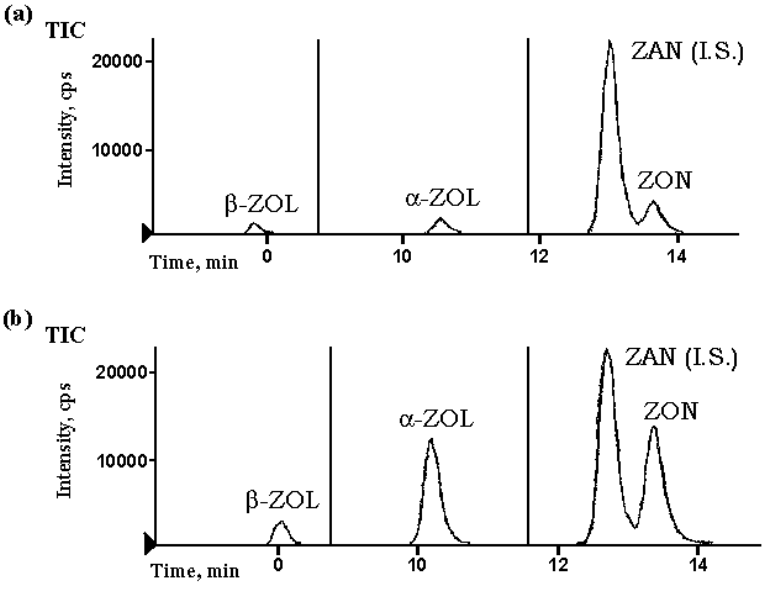

FIGURE 2 Chromatographic traces of ZON and metabolites in trout liver (a) and muscular tissue samples (b). The plot was obtained by injecting the extracts taken from an animal killed 12 h after ingestion of the mycotoxin.

The relative percentages of analytes were 43% ZON, 30% α-ZOL and 28% β-ZOL. In muscular tissue, just as in liver, α-ZOL is the major metabolite, even though it is less abundant.

Figure 2 shows the chromatograms obtained from a liver and muscular tissue sample taken from an animal killed 12 h after substance administration.

Various factors must be taken into consideration when evaluating the physiological response of an organism to a potentially estrogenic compound. Bioaccumulation and the metabolic fate of the compound are of primary importance. Other factors may be represented by interactions with other substances and their ability to bind plasmatic proteins [17]. Studying mycotoxin metabolism turns out to be useful for improving the understanding of why in rainbow trout the estrogenic potential of ZON is higher than that predicted by *in vitro* experimentation. This study, whose aim was to evaluate the metabolism and bioaccumulation of ZON in rainbow trout, turns out to be relevant since the literature is silent about experiments of this kind performed using ZON. It is well known that ZON metabolism leads to two isomers produced in different ratios. Both metabolites have estrogenic activity, but the potency of the α isomer is higher. Actually it has been observed that the estrogenic activity decreases in the following order: α-ZOL > ZON > β-ZOL [13].

Several studies carried out using different animal species have proved that for ZON there is a correlation between the endocrine disrupting potential and production of the α isomer. From the results obtained in this study, it can be observed that ZON metabolism in rainbow trout produces mainly α-ZOL, and its metabolization is primarily achieved in the liver. Therefore, it is appropriate to consider rainbow trout as species sensitive to the estrogenic effects of this mycotoxin. This conclusion is in good agreement with those of Arkuwe *et al*. [14], which assigned to ZON, administered *in vivo*, an estrogenic potential approximately 50% of that of 17-β-estradiol. This was done by measuring the plasmatic concentration of vitellogenin and zona radiata. Rainbow trout show the same behavior observed in animals sensitive to ZON, for example pig or rabbit.

As a further consideration it can also be observed that in muscular tissue the turnover of ZON and metabolites is slow, and therefore there is a risk that the continuous intake of small quantities of ZON can lead to tissue bioaccumulation. It is evident that a real risk of contamination exists when consuming fish farmed without safety procedures for both storage and animal feeding. Even if ZON is one of the most frequent mycotoxins found in crops, there is little concern about its presence in fish food; however, its presence in the harvested fish should be taken into serious consideration.

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