# HPLC assay of zearalenone and reduced metabolites in S9 fractions of duck liver

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**SUMMARY**

HPLC analysis of zearalenone (ZEA), zearalenols (α-ZOL and ß-ZOL) and zearalanols (α-ZAL and ß-ZAL) was developed, in order to obtain a sensitive and reproducible method to quantify ZEA and its reduced metabolites in subcellular fractions of animal livers (S9 samples).

Optimal *in vitro* metabolism was observed by incubating 5 mg S9 proteins with 0.016 $\mu$mol. ZEA. Acetonitrile and diethylether/chloroform mixture were compared for extraction, as well as different mobile phases and two detection modes in HPLC analysis. Extracted samples were eluted with water/acetonitrile (55:45, v/v) at a flow-rate of 1.0 ml/min$^{-1}$, resulting in well separated peaks between ZEA and the metabolites. The limits of detection ranged from 0.5 to 2 ng/mg S9 proteins using UV, and from 0.04 to 4 ng/mg S9 proteins, using fluorescence detection. Fluorescence showed a ten-fold higher sensitivity than UV detection for ZEA and α-ZOL. Repeatability (10 assays) was 2.7% to 6.99% for zearalenols. Day-by-day coefficients of variation for zearalenone and zeranols with UV detection were 3.3 to 8.5 %, and 2.5 to 4.3 %, respectively.

This analysis applied to S9 samples from ducks after 30 min of ZEA incubation allowed to demonstrate that α-ZOL is the main reduced metabolite in the duck. The present method is particularly adapted for studying *in vitro* metabolism of ZEA and inter-species variations.

**Keywords : Zearalenone, zearalenols, S9 liver fractions, duck, metabolism, HPLC.**

**RÉSUMÉ**

**Analyse HPLC de la zéaralénone et de ses métabolites réduits dans des fractions S9 de foie de canard**

L'analyse HPLC de la zéaralénone (ZEA), des zéaralénols (α-ZOL et ß-ZOL) et zéaralanols (α-ZAL et ß-ZAL) a été mise au point pour disposer d'une méthode sensible et reproductible pour quantifier la ZEA et ses métabolites réduits dans des fractions hépatiques subcellulaires.

Le métabolisme *in vitro* optimal a été observé après incubation de 5 mg de protéines de S9 avec 0.016 $\mu$mol de ZEA. L'acétonitrile et des mélanges diéthyléther/chlorofome ont été comparés pour l'extraction, ainsi que différentes phases mobiles et deux détecteurs pour l'analyse HPLC. Les échantillons extraits élués avec un mélange eau/acétonitrile (55:45, v/v) à un débit de 1.0 ml/min$^{-1}$, ont conduit à des pics bien séparés entre ZEA et ses métabolites réduits. Les limites de détection ont été comprises entre 0.5 et 2 ng/mg de protéines avec détection UV, et entre 0.04 et 4 ng/mg de protéines avec détection fluorimétrique. Cette dernière a montré une sensibilité dix fois supérieure à l'UV pour la ZEA et l'α-ZOL. La répétabilité (10 répétitions) a été de 2.7% à 6.99% pour les zéaralénols. Les coefficients de variation jour-à-jour mesurés en détection UV ont été de 3.3% à 8.5% et de 2.5% à 4.3% pour la zéaralénone et les zéranols, respectivement.

Cette méthode appliquée à des S9 de canard incubés 30 minutes avec de la ZEA a permis de montrer que l'α-ZOL constitue le principal métabolite réduit chez le canard. La méthode décrite est particulièrement adaptée à l'étude du métabolisme *in vitro* et aux variations interspécifiques.

**Mots-clés : *Zearalénone, zéaralénols, fraction S9 hépatique, canard, métabolisme, HPLC.***

## Introduction

Fusariotoxins which contaminate foods and feed worldwide, are metabolites from several species of Fusarium genus, soil fungi producing probably the most important quantity of toxin in temperate north countries [16]. Zearalenone (ZEA), one of the most widely distributed fusariotoxins, is common in maize and maize products but also in soybeans and various cereals grains, which represent a major part of human food and animal feed. Crop contamination is often inevitable and constitutes a potential risk for human and animal health [3]. Human and animal exposure comes mainly from chronic contaminated food ingestion, but human exposure can be direct via corn and cereals or indirect via animal products.

ZEA causes oestrogenic syndrome in animals, resulting in reproductive disorders and decreased fertility in a great number of species, with high variations of sensitivity. Female pigs are considered as the most sensitive animal species while poultry and ruminants show a lower responsiveness to ZEA [7]. Absorption and metabolism are implicated in these variations between species [6]. In particular, following oral exposure in mammals, ZEA is metabolized in various tissues, particularly in the liver, to the main metabolites identified *in vitro* and *in vivo*: alpha zearalenol, α-ZOL and beta zearalenol, ß-ZOL [13,14,25]. The rate of conversion and the ratio α-ZOL/ß-ZOL shows species variations, and may account for the species differences in the sensitivity to ZEA, as α-ZOL shows a several-fold higher oestrogenic activity compared to the parent compound ZEA whereas ß-ZOL has approximately the same activity as ZEA [5,24]. Both these ZEA derivatives have been described in naturally contaminated cereal grains [10]. Further reduced forms, zearananone and zearalanols (Figure 1) have also been identified in mammal urine [12,27]. In poultry species, only little information is available concerning ZEA metabolism whereas exposure is likely to occur via feedstuffs, specially during force-feeding, corresponding to a final average feed intake of approximately 10 kg of maize per duck [22].

Various methods of analysis exist for monitoring the presence of ZEA in food and feed commodities [8] or for investigating biological fluids. Most quantitative methods applied for determination of ZEA and derivatives include high-performance liquid chromatography (HPLC) followed by fluorescence detection [1,2,4,9,10,15,18,19,21,26], or UV detection [11, 23]. The aim of this work was to develop a simple, sensitive, and reproducible HPLC method analysis to identify and quantify zearalenone and its reduced metabolites in S9 fractions of duck-liver homogenates. First, optimal conditions of incubation of ZEA for producing metabolites were defined before extraction of ZEA and reduced metabolites, followed by their separation and quantification. In the present study, different extraction mixtures before HPLC, different mobile phases, and two detection modes following HPLC were tested.

# Materials and Methods

## CHEMICALS AND REAGENTS

Pure mycotoxin zearalenone (ZEA), cofactors NADH and NADPH, reduced derivatives: ß-zearalenol (α-ZOL), α-zearalenol (α-ZOL), zearalanone (ZAN), and α-zearalanol (ß-ZAL) and ß-zearalanol (ß-ZAL) were purchased from Sigma Chemical Co (Saint Quentin Fallavier, France). Stock solutions were prepared in acetonitrile (5 mg/ml: ZEA, α and ß-ZOL; 1 mg/ml: ZAN, α and ß-ZAL); after sonication (15 minutes) they were stored in the dark at -20°C. The following chemicals were used for the preparation of hepatic fractions: $KH_2PO_4$ and $K_2HPO_4$ from Prolabo, trisacetate and buthylhydroxytoluene (BHT) from Sigma, KCl, EDTA $2H_2O$, NaOH, HCL from Merck. Dye reagent concentrate (Biorad) and bovine serum albumin (BSA, 100 µg/ml) were used for the dosage of total proteins. Distilled deionized water and HPLC grade solvents were used.

## SAMPLES PREPARATION

### Subcellular fractions

Two mallard ducks were used for the preparation of liver fractions (Pygavi, Muret, France, 4-weeks old). They had free access to complete diet (Aliso, Auch, France). They were killed according to the ethical recommendations and in accordance with European Guidelines for the care and use of animals for research purposes. The livers were removed and all the subsequent operations were carried out at 0-4°C.

The subcellular fractions were prepared in duplicate for each animal. The livers were cut into small pieces, and 8 g samples were homogenized in 15 ml of ice-cold buffer : potassium phosphate pH 7.4 buffer (0.1M $KH_2PO_4$, 0.1M $K_2HPO_4$) and 0.1M Tris acetate, 0.1M KCl, 1 mM EDTA and 0.02 mM BHT (40 mg/10 ml ethanol) in a glass Potter homogenizer with a Teflon pestle. After filtration, the homogenate was centrifuged at 9000xg for 30 min in a TGA-65 Kontron ultracentrifuge. The supernatant, as 1-ml aliquots, was stored at -80°C until use.

## *IN VITRO* METABOLISM OF ZEA AND EXTRACTION

Protein concentration in the subcellular fraction was determined by the method of Bradford, using BSA as standard and the kit Biorad (Microassay procedure). For each animal sample, proteins were dosed in triplicate, and repeated for four days, to estimate reproducibility and repeatability of the colorimetric assay. The mean of these measurements was used for estimating S9 volume required for incubations. First, incubation conditions were tested, to ensure that activities were measured under linear conditions with respect to substrate concentrations.

Preliminary experiments were conducted in duck fractions to ensure linearity without saturation of enzyme activities: 2.5, 5 and 10 mg of duck S9 proteins on one part, in a 1ml-incubation volume. On another part, 50 µL from 25, 50, 100 or 200 µg/ml ZEA corresponding to 4, 8, 16 and 30 µM respectively of final ZEA, were incubated with 5 mg S9 proteins. After these preliminary assays, 5 mg of proteins from the subcellular fractions and 100 µg/ml ZEA (50 µL), were incubated in 1ml phosphate buffer with co-factors in excess: 2.4 µmol of NADH and NADPH. The temperature of incubation was adapted to poultry metabolism, and maintened at 40°C for 30 min. All samples were extracted twice with 10 ml of extraction solution (diethylether/chloroform, 3:1, v/v) for 30 min and centrifuged at 2000 g for 10 min. Two different solvents were tested as extraction solvents: acetonitrile and diethylether/chloroform mixtures. The organic phases were collected, evaporated to dryness under nitrogen (50°C, 30 min), and residues were dissolved in 100 µL of acetonitrile, and sonicated.

Extraction control samples were prepared for each animal and each analysis (ZEA without incubation) and serve to estimate global rate of ZEA extraction of the assays.

## HPLC ASSAY OF ZEA, ZOLS, AND ZALS

The HPLC apparatus include an isocratic pump (model 2200, ICS) and a Rheodyne injection loop of 20 µL (ICS). The injection followed immediately sonication of the reconstituted extract. Elution was through a Prontosil column 120-5 C18 H (250x4 mm, 5 µm particle size; Bischoff) with water/acetonitile (55:45, v/v) at a 1ml/mn flow-rate. Various solvents were tested to elute ZEA, ZOLS and ZALS mixtures, to obtain well separated peaks: MeOH:$H_2O$ (70:30 v/v and 65:35 v/v), ACN:$H_2O$ (60:40; 50:50 and 45:55), MeOH:ACN:$H_20$ (22:35:43 v/v/v), MeOH:ACH:$H_2O$:$CH_3COOH$ (22:35:43:0.5). The mobile phase was prepared and degased before each assay. The column was kept at 20°C, and was connected to a guard column (C18, 5µm, Prontosil). Two detectors were used: a UV detector (Spectra Focus), at 235 nm, and a fluorescence detector (Shimadzu, RF-10AXL) at excitation wavelength $\lambda_{exc}$ 274 nm/emission wavelength, $\lambda_{em}$ 440 nm. Chromatograms were integrated with ANAPIC3 software.

The dosage method of the following reduced metabolites was validated: ß-zearalenol (ß-ZOL), α-zearalenol (α-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and ß- zearalanol (ß-ZAL). Standard solutions of each compound in acetonitrile

(0.004 to 50 $\mu$g/ml: 6 concentrations for ZEA (0.097, 0.391, 1.563, 6.25, 25 and 50 $\mu$g $l^{-1}$), 9 concentrations for the metabolites: 0.097, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12,5 and 25 $\mu$g $l^{-1}$) were analysed in triplicate and calibration curves were obtained. Detection and quantification limits were defined as signal to noise ratio equal or superior to 3 and 10, respectively. A mixture of the standards were extracted and injected in parallel to the samples to serve as controls.

# Results

## DEVELOPMENT OF THE HPLC METHOD

The whole UV spectrum from 190 to 320 nm of each metabolite (6.25$\mu$g/ml in acetonitrile) showed maximum absorbance at 235 nm for zearalenone and zearalenols, and at 218 nm for zearalanone and zearalanols. The HPLC method was adapted from several studies [2,4,9,15,18,19,21,26].

We tested various solvent mixtures as mobile phase in order to obtain a retention time for zearalenone well after the reduced metabolites but not too long (less than 30 minutes) and to obtain a high peak. The higher peak resolution was observed in water/acetonitile (55:45, v/v) with a retention time of 16-18 minutes for zearalenone (table I). In our chromatographic conditions, zearalenone (ZEA), α-zearalenol (α-ZOL) and ß-zearalenol (ß-ZOL) showed well separated peaks at retention times of 16-18 minutes, 9-10 minutes, and 6-7 minutes respectively, using UV-detection at 235 nm or fluorescence detection. For this detection mode, we tested two excitation wavelengths with emission wavelength $\lambda_{em}$ 440 nm: $\lambda_{exc}$ 236 nm and $\lambda_{exc}$ 274 nm, and we choose this latter $\lambda_{exc}$., because of higher signals, in accordance with other studies [4,10,15,26]. Zearalanone, α-zearalanol and ß-zearalanol showed also well separated peaks using UV-detection at 218 nm or fluorescence detection. Elution times were of 17-18 minutes, 6-7 minutes and 9-10 minutes respectively (data not shown).

Calibration curves and limits of detection are summarized in table II. $R^2$ of 0.9992, 0.9995 and 0.9999 for ZEA, α-ZOL and ß-ZOL respectively were observed. The limits of detection using UV were 0.5 to 2 ng/mg proteins and ranged from 0.04 ng to 4 ng/mg with fluorescence detection for all the six metabolites tested. The highest sensitivity was observed for the zearalenols, which are described as activated metabolites, key factors in zearalenone toxicity. For ZEA and α-ZOL, fluorescence detection was ten fold more sensitive than UV one, in agreement with published results [2]. For ß-ZOL, fluorescence detection was less sensitive than UV detection, because of less fluorescent properties of this metabolite than ZEA and α-ZOL [1,15]. Our results demonstrate that both zearalenols metabolites can be quantified by the two studied detection modes. We preferred UV detection at two wavelength to differenciate ZOLs and ZALs showing similar retention times. Our HPLC assay is characterized by simplicity compared to other assays requiring more sophisticated materials to quantify all zearalenone reduced metabolites, for example mass spectrometric detection [26].

Repeatability, estimated by ten separate measurements of the same sample was less than 5% for reduced metabolites but 12.5% for ZEA. Reproducibility (day-by-day variation, 3 assays per day, 3 days) was measured for zearalenols and zearalanone with UV detection, showing variation coefficients of 2.5 to 4.3% and of 3.3 to 8.5% respectively. The HPLC method analysis is reliable and reproducible, as all the variation coefficients of reduced metabolites were less than 5%. For ZEA, we observed a day-by-day CV of 8.5 % and CV for response factor of 12.66 % (table II), which is consistent with previous published results for this toxin [2]. Considering the main objective of this work was quantifying reduced metabolites, we have considered this result acceptable.

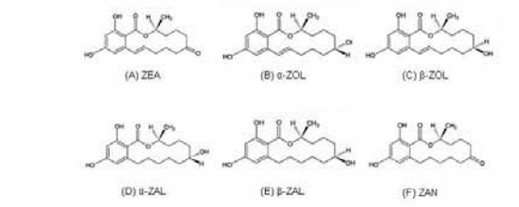


FIGURE 1: Structures of zearalenone (A), and its reduced metabolites (B to F): zearalenols, α-zearalenol (B) and ß-zearalenol (C), and zearalanone (F), α-zearalanol (D) and ß-zearalanol (E).

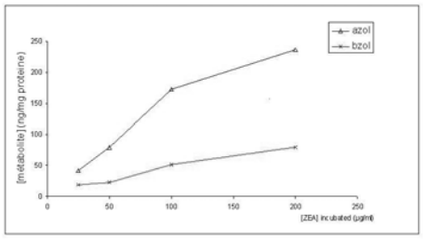


FIGURE 2: Incubations of 5 mg duck S9 proteins/ml with 50 $\mu$l of ZEA concentrations of 25, 50, 100 and 200 $\mu$g/ml, at 40°C, for 30 minutes. The reduced metabolites of zearalenone (ZEA) are represented according to the conditions of incubations: α-zearalenol (open triangles), and ß-zearalenol (squares).

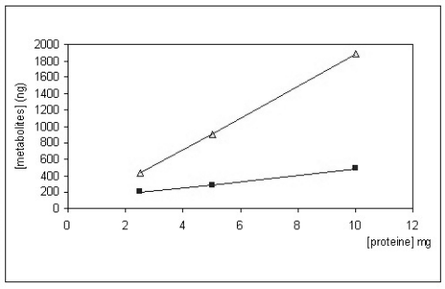


FIGURE 3: Incubations with 2.5 mg, 5 mg and 10 mg duck S9 proteins with 190 $\mu$g/ml of ZEA at 40°C, for 30 minutes. The reduced metabolites of zearalenone (ZEA) are represented according to the conditions of incubations: α-zearalenol (open triangles), and ß-zearalenol (squares).

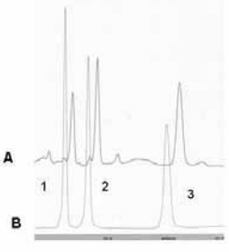


FIGURE 4: **A**: Chromatogram representative of duck samples (S9 fractions incubated with 16 $\mu$M zearalenone for 30 minutes); **B**: mixture of standards 1 = ß-zearalenol; 2 = α-zearalenol; 3=zearalenone; [1]=[2]=[3]= 30 $\mu$g/ml C18 250*4 mm, 5$\mu$m column, mobile phase acetonitrile/H20 45/55 v/v, injected volume: 20 $\mu$L; 1 ml/mn flow rate; range 0.1 AUFS; UV detection at 235nm.

| Mobile phase composition | Ratio v/v | RT (minutes) | H |
|---|---|---|---|
| $CH_3OH/H_2O$ | 70/30 | 10.437 | 14396 |
| | 65/35 | 23.187 | 7117 |
| $CH_3CN/H_2O$ | 60/40 | 6.566 | 17033 |
| | 50/50 | 12.075 | 9 142 |
| | 45/55 | 18.533 | 13170 |
| $CH_3OH/ CH_3CN/H_2O$ | 22-35-43 | 15.870 | 14883 |
| $CH_3OH/CH_3CN/H_2O/CH_3COOH$ | 22-35-43-0.5 | 15.25 | 12300 |
| $CH_3CN/H_2O/CH_3COOH$ | 22-35-43-0.5 | 15.770 | 14523 |

Zearalenone in acetonitrile, 6.25 $\mu$g/mL; UV detection ($\lambda$max 235 nm), injected volume: 20 $\mu$L; 1 ml/mn flow rate; range 0.1 AUFS.

TABLE 1: Influence of the mobile phase on the retention time of zearalenone (RT) and on the area of the peak (H).

| | Detection | Linearity range (ng) | CV* (%) | $R^2$ | Detection limit (ng/mg proteins) |
|---|---|---|---|---|---|
| Zearalenone | UV 235 nm | 25-1000 | 12.6 | 0.999 | 1 |
| (ZEA) | Fluo. | 0.3-150 | 2.7 | 1.000 | 0.15 |
| α-zeaealenol | UV 235 nm | 1-5000 | 4.3 | 0.999 | 0.5 |
| (α-ZOL) | Fluo. | 4-75 | 4.4 | 0.999 | 0.04 |
| ß-zeaealenol | UV 235 nm | 25-500 | 6.9 | 0.999 | 0.5 |
| (ß -ZOL) | Fluo. | 9-300 | 3.8 | 0.999 | 0.3 |
| Zearalanone | UV 218 nm | 1-500 | 4.1 | 0.999 | 0.5 |
| | Fluo. | 8-62 | 3.4 | 0.999 | 4 |
| α-zeaealanol | UV 218 nm | 62.5-500 | 1.9 | 1.000 | 2 |
| | Fluo. | 16-62 | 12.6 | 0.991 | 2 |
| ß-zeaealanol | UV 218 nm | 5-500 | 4.6 | 0.995 | 0.5 |
| | Fluo. | 8-126 | 4.3 | 0.999 | 4 |

* CV = coefficient of variation

Elution with acetonitrile/H20 45/55 v/v; 1ml/min flow-rate; injected volume: 20 $\mu$L; column C18 250*4mm, 5$\mu$m; UV detection (235 or 218 nm), or fluorescence detection ("fluo": $\lambda_{exc}$ 274 nm/ $\lambda_{em}$ 440 nm).

TABLE 2: Validation of HPLC assay

### EXTRACTION PROCEDURE AND HPLC ANALYSIS IN LIVER SUBCELLULAR FRACTIONS (S9) AFTER INCUBATION OF ZEA

We adapted the incubation and extraction procedure from Pompa et al. [17]. The results of experiments to test incubation conditions are shown in figures 2 and 3. A volume corresponding to 5mg S9 proteins and ZEA concentration of 100$\mu$g/ml (final concentration of 16 $\mu$M) were selected for incubations, in order to observe a linear production of both α-ZOL and ß-ZOL without saturation. The extraction mixture diethylether/chloroform 3:1, v/v was chosen because of higher extraction rate and higher reproducibility of signals compared to acetronitrile. The extraction with acetonitrile was rejected because of very slow evaporation and of lower solubilization of metabolites compared to diethylether/chloroform. Using diethylether/chloroform 3:1, the extraction rate was of 82 % ± 6 % for ZEA, consistent with the results of previous works [1,4,15,23].

Metabolites were identified by comparison of retention times with those of standards and quantified by their peak area ratio with standards. Using the UV-detection wavelengths of 218 nm and 235 nm, neither zearalanone nor zearalanols were found in any of the S9 samples. In the ducks S9 fractions (Figure 4), metabolized zearalenone accounted for about one half of the extracted fraction after 30 min of incubation (50±3%, n=4) , α-ZOL and ß-ZOL representing 39 % (39±5%) and 11% (11±3%) respectively. Non metabolized zearalenone represented the remainder of the fraction. These are the first published results concerning the metabolism of ZEA in the mallard duck. These *in vitro* results show that ducks produce mainly α-ZOL, as described in mammals.

## Conclusion

The method presented here is particularly adapted for studying zearalenone metabolism, showing sensitivity, reproducibility and repeatability for the main recognized reduced metabolites: zearalenols. The performance of UV detection appears to be sufficient and adapted for *in vitro* metabolism studies. For kinetic and residues studies, fluorescence detection,

showing ten-fold higher sensitivity for alpha-zearalenol and zearalenone, will be prefered. The present results tend to show that zearalanols are not produced in the duck, as it is the case in most of mammalian species, notably rat or pig. These results have to be confirmed in other poultry species.

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