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Development of a New HPLC Method Used for Determination of Zearalenone and Its Metabolites in Broiler Samples. Influence of Zearalenone on the Nutritional Properties of Broiler Meat

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This paper presents the development, optimization and validation of a new HPLC method used for the separation and determination of zearalenone, ZON, and its metabolites in biological samples of Leghorn broiler. ZON and its metabolites can be separated with good resolution in 11 min, using a Hypersil Gold C18 column, a mobile phase mixture of 50 mM aqueous ammonium acetate: acetonitrile:methanol, 45:8:47 (v/v/v), flow rate 1 mL/min and column temperature 40 °C. Based on the results obtained by this method applied on biological samples one can conclude that liver is the site for zearalenone localization and detoxification. Influence of zearalenone on the nutritional properties of broiler meat (weight variation, gross chemical composition, fatty acids profile of the meat) was studied, also. Results obtained during 4 days of treatment with ZON showed minimal or no effects of the dietary zearalenone on broiler meat nutritional quality.

KEYWORDS: Zearalenone; HPLC; broiler biological samples

INTRODUCTION

Mycotoxins are secondary toxic metabolites produces by over 200 fungi species such as *Fusarium*, *Aspergillus* and *Penicillium*, which grow in a wide range of climatic conditions and on different agricultural products (grains, straws, fruits, coffee and peanuts) in the field or during storage. Their presence in the foods, beverages and feedstuffs is acknowledged as a potential threat to human and animal health, which can occur by direct contamination of the vegetal materials and their products by "carrying" the mycotoxins and their metabolites in animal tissues, milk and eggs after the ingestion of contaminated feeds (*1*). A few hundred different species of mycotoxins have been discovered so far, with a wide structural diversity. They can be removed partially, or they cannot be removed at all, by food processing or by alternative methods of decontamination. Most have a strong thermal and chemical stability. Currently, the focus is on aflatoxins, trichotecenes, ochratoxin A, zearalenone, fumonisin, moniliformin and patulin, because of the high incidence and severe effects on animal and human health.

Zearalenone, ZON, is a secondary metabolite of several *Fusarium* species (*Fusarium graminearum*, *F. culmorum*, *F. equiseti* and *F. crookwellense*), which colonize the cereals particularly (corn, oats, barley, wheat and sorghum) under conditions of cold, wet weather, in the temperate and warm regions. Studies on the physical and chemical properties of zearalenone revealed traits ideal for an easy diffusion into the tissues. Zearalenone is low toxic, and there is no evidence on its carcinogenetic potential, all studies being conducted on animals. On the other hand, this mycotoxin has strong estrogenic and anabolic properties because of the agonistic effect on the estrogenic receptors, many animal species displaying severe disturbances of the reproductive system. In the USA, starting with 1969, α-zearalenol, α-ZOL, is used as growth stimulator in cattle. In the European Union, the use of this drug has been banned as of 1985 (*2*), this ban causing the interdiction of imports of cattle meat or meat products, if the respective animals had been treated with α-ZOL for other than veterinary purposes (*3*). The European Union policy relies on the fact that the effects on the human organism of long consumption of meat produced using growth promoters are not properly documented; very few long-term tests have been conducted on rats, dogs or monkeys (*4*). The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) proposed in 1987 an accepted maximum ingested amount of 0.5 μg/kg body mass, which corresponds to a residual level in the liver of 10 μg/kg or 2 μg/kg in the muscles, which are α-zearalenol levels which do not produce hormonal disturbances (*4*). The Food and Drug Administration (FDA) set the accepted highest levels of α-zearalenol at 150 μg/kg in muscles, 300 μg/kg in the liver, 450 μg/kg in kidneys and 600 μg/kg in fat from cattle (*5*). The European Union set, in 2006, the maximal admitted levels of zearalenone in feeds to 100 μg/kg in the feeds for growing pigs and 250 μg/kg in the feeds for sows and fattening pigs. The zearalenone level in the feeds for calves, dairy cows, sheep and goats must not exceed 500 μg/kg (*6*).

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Food contamination with zearalenone comes in two ways: direct contamination of the grains or indirect contamination by mycotoxins and their metabolites "carrying" into animal tissues, milk and eggs after the intake of contaminated forages.

The strong estrogenic effects of zearalenone, ZON, and its metabolites (α-zearalenol, α-ZOL; β-zearalenol, β-ZOL; zearalanone, ZAN; α-zearalanol, α-ZAL; β-zearalanol, β-ZAL) and the legislated maximal dietary levels prompted the necessity to determine amounts between 10 and 100 μg in foods and forages. Very sensitive methods (up to ppb) are compulsory for the determination in body fluids and human and animal tissues, for a good determination of the risk level and in the studies of zearalenone metabolism (*7*). Because of the strong native fluorescent activity of the zearalenone and its metabolites, HPLC methods using a fluorescence detector are used as sufficiently sensitive and efficient in terms of separation, but only after zearalenone extraction from the sample (*7*, *8*). The use of gas chromatography, GC, is limited by the need to derivate the phenol hydroxyl groups; in consequence, only GC/MS determinations have been used largely and deliver reliable data (*9*). Thin layer chromatography, TLC, and enzyme-linked immunosorbent assay, ELISA, classical or "open sandwich" (*10*) are frequently used to determine zearalenone and its metabolites. The facile and robust traits of TLC determinations and its costs are higher than those of the other methods, but the sensitivity and selectivity are inferior. ELISA determinations offer sensitivity comparable to that of fluorescent detection. Because of the potential of cross reaction of the antibodies with the matrix components, it is desirable to confirm the results using other techniques, just to avoid false positive erroneous results, or results with overestimation. Due to the same cause, the simultaneous determination of zearalenone and its metabolites is not possible by ELISA. In general and independent of the method of detection used, or of the sample matrix, difficult protocols are required for the extraction, cleaning and enrichment of the sample, in order to reach a minimal level necessary to determine zearalenone and its metabolites. This is why the very long and laborious liquid/liquid extraction has been replaced by solid phase extraction, SPE with inverse aminopropyl phase and absorbent immunoaffinity materials or with MycoSep columns containing a mixture of charcoal, ion exchange resins and other materials (*11*, *12*). During the past years methods have been developed for zearalenone determination by laser-induced fluorescent capillary electrophoresis, these methods being capable to analyze between 5 ng/g and 500 ng/g in corn samples, but they are very expensive (*13*).

The literature describes several chromatographic methods for zearalenone and its metabolites' separation, identification and determination from biological samples. Most times, zearalenone is determined alone in cereal samples (*14*). Zearalenone metabolites α-ZAL and β-ZAL were determined by several methods from fat and kidney samples, from cow muscle and liver and from chicken liver (*15*). Other methods are dedicated to the separation and determination of ZON, α-ZOL and β-ZOL from beer and fish samples (*16*). There are papers published which describe the separation and determination of ZON, α-ZOL, β-ZOL, α-ZAL and β-ZAL from river water, pig urine and tissue, cattle urine, eggs and milk samples (*17*). In the latter methods ZAN was used as internal standard, but it has two major inconveniences: it is a natural metabolite of zearalenone, and it has the same elution behavior as ZON, if C18 columns are used. Few are the liquid chromatography methods presented in the literature for zearalenone and its metabolites' separation from biological samples such as cattle urine, pig liver and cattle liver, plasma and urine (*18*).

This paper presents the development, optimization and validation of a HPLC-DAD method for the separation of zearalenone and its metabolites in biological samples from Leghorn broilers treated with zearalenone (25 mg/kg body weight, bw). The proposed method was used for the chicken food quality control and in the view to determine the biotransformations undergone by zearalenone within the animal organism. Because zearalenone interferes with several metabolic pathways, among which is lipid metabolism, the influence of zearalenone on the nutritional properties of broiler meat was monitored.

MATERIALS AND METHODS

Instrumentation. HPLC studies and analyses were performed on a HPLC Finnigan Surveyor complete system (Thermo-Electron Corporation, Waltham, MA). A Hypersil Gold (C18), 150 × 4.6 mm column was employed. Kjeltec 2300 and Soxtec 2055 (FOSS, Tecator, Hilleroed, Denmark) were used. A gas chromatograph with a Clarus 500 system FID detector from Perkin-Elmer (Waltham, MA) was also used. Chromabond C18 polypropylene columns were obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). The HPLC system used in LC−MS coupling consisted of a LC 1100 series system (Agilent, Santa Clara, CA), coupled with an ion trap mass spectrometer (Esquire HCT) using an ESI interface operating in the positive or negative mode (Bruker Daltonics, MA). The ZearaStar immunoaffinity columns (IAC) were purchased from Romer Laboratories Diagnostic GMBH (Tulin, Austria). Water was purified in a Milli-Q Ultrapure water purification system (Millipore, Billerica, MA). Thermo SpeedVac concentrator from Thermo-Electron Corporation (Waltham, MA) was used. A Perkin-Elmer GC-FID with N_2 carrier gas with a capillary chromatographic column (BPX 70, 60 m) was also used. A Grindomix GM 200 knife mill from Retsch (Haan, Germany) was used for dried sample grinding. A 2-16K Sigma centrifuge (Deisenhofer, Germany) was used to centrifuge the biological samples; a GFL 3015 vibration shaking system (Burgwedel, Germany) was used for sample stirring; an SW23 shaking water bath from Julabo Labortechnik GmbH (Sellback, Germany) was used to incubate the sample.

Chemicals and Solvents. Zearalenone, ZON, α-zearalenol, α-ZOL, β-zearalenol, β-ZOL, α-zearalanol, α-ZAL, β-zearalanol, β-ZAL, and zearalanone, ZAN, standards were purchased from Sigma (Deisenhofer, Germany); ammonium acetate (AmAc), glacial acetic acid, sulfuric acids (density of 1.840 g/mL), disodium phosphate and monosodium phosphate as well as sodium hydroxide (all analytical reagent grade) were supplied by Sigma (Deisenhofer, Germany). Both buffer solutions ammonium acetate−glacial acetic acid (50 mM; pH 4.8) and phosphate (50 mM, pH 7.4) were prepared in our laboratory. A standard mixture of fatty acids, PUFA No.2 (Animal Source) from Sigma (Deisenhofer, Germany), was used. Glucuronidase/arylsulfatase from *Helix pomatia* (30/60 U/mL), HPLC grade methanol and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). The standards were individually dissolved in acetonitrile to give 10^{-3} M stock solutions, which were stored at −20 °C until use. Standard working solutions of 50 μM ZON and its metabolites were prepared by diluting each stock solution with a mixture consisting of water:acetonitrile (50:50, v/v). Liquid nitrogen was purchased from Linde Gas (Saint Priest Cedex, France). Ultrapure Milli-Q water was used.

Broiler Treatments. Leghorn broilers aged 2 weeks, housed and treated in agreement with the acting European legislation (*19*) in facilities authorized by the Ministry of Agriculture within the French Food Safety Agency (AFSSA), Ploufragan, France, were used. The broilers were fed *ad libitum* with a standard diet. The birds (5 broilers per group) were treated intraperitoneally for 3 days as follows: group 1 named CONTROL, NaCl 9% solution 1 mL/day; group 2 named ZON IP, 25 mg of ZON/kg bw every day; group 3 named PB-ZON, 80 mg of phenobarbitone natrium salt/kg bw every day, followed by a single treatment with 25 mg of ZON/kg bw. The animals were slaughtered 24 h after the last treatment.

Sample Collection. The broiler meat, blood and liver samples were collected at AFSSA Ploufragan.

The broiler meat samples (breast and whole left leg) were collected according to ISO 3100 (Part 1):1996/ISO 3100-1:1991 (*20*). The samples were frozen in liquid nitrogen and stored at −80 °C.

The broiler blood samples were collected in sterile tubes with heparin, left for 2 h at room temperature and then centrifuged at 2500 rpm, and the

supernatants were collected. The plasma was frozen in liquid nitrogen and stored at −80 °C.

The liver samples consisted of the whole liver, frozen in liquid nitrogen and stored at −80 °C.

Determination of the Gross Chemical Composition of the Broiler Meat. The following determinations were performed: dry matter, according to SR ISO 6496:2001 (*21*), crude protein by Kjeldhal method using a Kjeltec 2300, in agreement with SR ISO 13325:1995 (*22*), ether extractives using Soxtec 2055, in agreement with SR ISO 6492:2001 (*23*), gross ash, residue insoluble in hydrochloric acid, according to STAS 10542/1:1986 (*24*) on meat broiler samples.

Determination of the Fatty Acids Profile. The fatty acids were determined by transforming the fatty acids of the sample into methyl esters, using an acidulated methanol solution (2% sulfuric acid in methanol); followed by their separation in the capillary chromatographic column (BPX 70, 60 m). The fatty acids were identified and quantified using a standard mixture of fatty acids on a Perkin-Elmer gas chromatograph with FID detector and nitrogen gas carrier according to SR ISO 5508:1990 (*25*).

Determination of Zearalenone and Its Metabolites. The samples were prepared for analysis according to P. Songsermsakul et al. (*18*).

Broiler Meat Sample. A quantity of 1 g of broiler meat sample, ground and dried at 65 °C, was extracted thereafter in 50 mL of water:methanol (50:50, v/v), while stirring at 150 rpm, for 60 min. The sample was centrifuged for 10 min at 4000 rpm; a volume of 20 mL of supernatant was collected and mixed with 40 mL of buffer solution acetic acid−ammonium acetate, pH 4.8. This solution was incubated for 15 h at 37 °C with 80 μL of a solution of glucuronidase/arylsulfatase and then brought to pH 4.0 with glacial acetic acid. The solution was loaded into a Chromabond C18 extraction column preconditioned with 10 mL of methanol and then rinsed with 10 mL of ultrapure water. Thereafter, the columns loaded with the sample were rinsed with 5 mL of ultrapure water and 5 mL of a methanol:water (30:70, v/v) mixture and dried for 3 min under vacuum. The analytes were eluted with 1.25 mL of methanol. The eluate was mixed with 15 mL of buffer phosphate solution, pH 7.4. This mixture was introduced into a ZearaStar immunoaffinity column preconditioned with 10 mL of buffer phosphate solution, pH 7.4. The immunoaffinity column loaded with the sample was rinsed with 15 mL of ultrapure water and dried for 3 min under vacuum. The analytes were eluted with 1.5 mL acetonitrile, the samples were dried using a Speed Vac concentration system and then 150 μL mixture of water:methanol (50:50, v/v) was added; 20 μL of this solution was injected into the HPLC system for separation and determination.

Plasma Sample. A volume of 1 mL of plasma was mixed with 6 mL of buffer ammonium acetate solution, pH 4.8. This solution was incubated for 15 h at 37 °C with 25 μL of a solution of glucuronidase/arylsulfatase before adding 6 mL of phosphate buffer adjusted to pH 7.4 using NaOH 1 M. The sample was centrifuged at 4000 rpm; the supernatant was introduced into a ZearaStar immunoaffinity column preconditioned with 10 mL of buffer phosphate solution, pH 7.4. The immunoaffinity column loaded with the sample was rinsed with 15 mL of ultrapure water and dried for 3 min under vacuum. The analytes were eluted with 1.5 mL acetonitrile, the samples were dried using a Speed Vac concentration system and then 150 μL mixture of water:methanol (50:50, v/v) was added; 20 μL of this solution was injected into the HPLC system for separation and determination.

Liver Sample. The thawed liver samples were used to prepare microsomal extracts by successive centrifugation according to the method described by Peyronneau (*26*). A quantity of 2.5 mg (10 mL of S9, the subcellular fraction obtained after the first centrifugation) of liver was used; this sample was extracted in 40 mL of a water:methanol (50:50 v/v) mixture, while stirring at 150 rpm, for 60 min and was prepared for analysis using the same methodology as for the broiler meat sample.

Chromatographic Separation. Chromatographic separation was performed by reverse phase chromatography using a Hypersil Gold column having dimensions 5 μm, 150 × 4.6 mm purchased from ThermoElectron Croporation, USA. During the separation the temperature of the column was 40 °C. The mobile phase was a mixture of 50 mM aqueous ammonium acetate:acetonitrile:methanol (45:8:47 v/v/v) and the flow rate 1 mL/min. The detection was at λ = 280 nm and the volume of injection 20 μL.

The data were acquired and processed by the ChromQuest Chromatography Data System. The EXCEL software was employed for the statistical analysis.

Validation of the HPLC Method for the Determination of Zearalenone and Its Metabolites. The HPLC method was validated according to the international rules (*27*, *28*): selectivity, linearity, limits of determination and quantification, precision, accuracy, robustness and stability of the solutions were determined. Selectivity was studied on a standard mixture of ZON, ZAN, α-ZOL, α-ZAL, β-ZOL and β-ZAL containing 50 μM of each compound. Linearity was checked for zearalenone and its metabolites using standard solutions having the concentrations between 0.32 μg/mL (1 μM) and 31.8 μg/mL (100 μM). The precision of the proposed method, expressed by repeatability (intraday assay), was determined by performing 6 repeated determinations on 3 samples containing 12.7 μg/mL (40 μM), 15.9 μg/mL (50 μM) and 19.1 μg/mL (60 μM) standard mixture of ZON, ZAN, α-ZOL, α-ZAL, β-ZOL and β-ZAL, respectively. In order to determine the precision expressed by reproducibility (interday assay) 15.9 μg/mL standard samples were analyzed within 5 consecutive days by 2 different analysts. Precision was reported as percentage of relative standard deviation, RSD, calculated with the formula [(standard deviation/mean) × 100] (*29*, *30*). The accuracy was determined using the results obtained for the precision determination. Accuracy was calculated according to the formula [(mean of n measurements/true mean) × 100] (*30*). The bias interval was calculated with the relation {[(measured concentration − true concentration)/true concentration] × 100}. The lower limit of detection, LOD, and the lower limit of quantification, LOQ, were determined on 6 consecutive determinations of a standard sample of 0.03 μg/mL. The LOD and LOQ were calculated as a sum of 3 times standard deviations and 10 times standard deviations, respectively, and the average concentration was determined for the used standard. The recovery was determined by performing 10 repeated determinations on a standard sample of 15.9 μg/mL and other 10 determinations on the same standard sample with spiked analyte up to a final concentration of 19.1 μg/mL. The stability of the solution was studied during two weeks. Robustness was explored during the development of the method.

RESULTS AND DISCUSSION

HPLC Method Development. The determination of zearalenone and its metabolites' content in biological samples is a current topic as supported by the wide contamination of the forages and implicitly of the animal products. In **Figure 1** the structures of zearalenone and its metabolites are presented. In developing a HPLC method for the simultaneous separation and determination of zearalenone and its metabolites in biological broiler samples, the method described by Songsermsakul et al. (*18*) for biological samples, horse urine, plasma and feces was used as model. This method required the use of two serial chromatographic columns: a Polar-RP column with stationary phase containing phenyl ether groups (150 mm × 4.6 mm) and a Hydro-RP column with stationary phase C18 (150 mm × 4.6 mm), and a mixture of water:ACN:MeOH (35:30:35, v/v/v) as mobile phase, with a flow rate of 1 mL/min. Working under these conditions, but using only a Hypersil Gold column, the separation of a standard mixture containing 50 μM of ZON, ZAN, α-ZOL, β-ZOL, α-ZAL and β-ZAL was performed. Three chromatographic peaks corresponding to the separation of mixtures ZON + ZAN from α-ZOL + α-ZAL and from β-ZOL + β-ZAL were obtained, but it was not possible to separate ZON from ZAN, α-ZOL from α-ZAL and β-ZOL from β-ZAL. In order to optimize the method, the following parameters were successively varied: mobile phase flow rate, ammonium acetate concentration in the solvent mixture, ratio of components water:acetonitrile:methanol in the mobile phase and the chromatographic column temperature. By modifying the mobile phase flow rate only an improved separation of ZON from ZAN at a flow rate of 0.4 mL/min was obtained, but not the separation of α-ZOL from α-ZAL

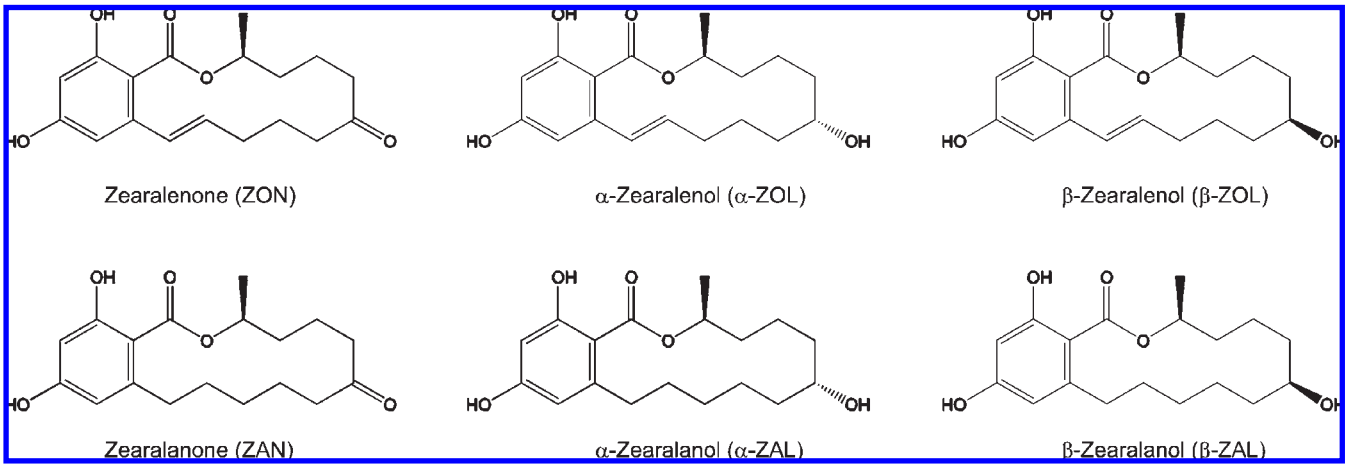

Figure 1. Structure of zearalenone and its metabolites.

and β-ZOL from β-ZAL. Knowing the influence of ammonium acetate, AmAc, in separation of ZON and its metabolites (*16*, *17*), the influence of the ammonium acetate concentration in the mobile phase was studied. Concentrations of ammonium acetate between 10 μM and 70 μM were studied. A concentration greater than 50 μM ensures a good separation of the entire mixture. For an optimal separation of the pairs of substances which lead to neighboring peaks—ZON from ZAN, α-ZOL from α-ZAL and β-ZOL from β-ZAL—the influence of the mobile phase composition was studied. Aqueous phase containing 50 mM AmAc was mixed with acetonitrile, ACN, and methanol, MeOH, in various proportions starting from 50 mM aqueous AmAc:ACN:MeOH, 35:30:35 (v/v/v) ratio to a 45:5:50 (v/v/v) ratio. The resolution for each of the three pairs of substances displays minimal values when a mobile phase with 50 mM aqueous AmAc:ACN:MeOH ratio of 35:20:45 is used, after which it increases continuously with the increase of the content of aqueous component and the decrease of the acetonitrile content in the solvent mixture (**Figure 2A**). Evaluating the separation resolution values as a function of the solvent mixture composition and the time of analysis, the mixture 50 mM aqueous AmAc:ACN:MeOH with 45:8:47 (v/v/v) ratio was chosen as optimal mobile phase composition. The correctness of this decision is also supported by the values of the equivalent theoretical plate height, *H* (**Figure 2B**).

In order to optimize the total analysis time the influence of the mobile phase flow rate and of the chromatographic column temperature on the retention time values were studied. As shown in **Figure 3**, varying the flow rate from 0.5 mL/min to 1 mL/min, the total analysis time decreased from 30 to 20 min. Subsequently, by increasing the column temperature from 25 to 40 °C, the total analysis time decreased from 20 min to just 11 min. Because the resolution of β-ZOL from β-ZAL separation decreases with the increase of the flow rate, the flow rate value of 1 mL/min was considered optimal.

In conclusion, ZON and its metabolites can be separated with a good resolution in 11 min, using a Hypersil Gold C18 column, the mobile phase a mixture of 50 mM aqueous ammonium acetate: ACN:MeOH, 45:8:47 (v/v/v) with a flow rate of 1 mL/min and a chromatographic column temperature of 40 °C. The resolutions between ZON and its metabolites are ZON/ZAN 1.19, α-ZOL/α-ZAL 1.81 and β-ZOL/β-ZAL 1.68 respectively. **Figure 4** shows a typical chromatogram obtained in these working conditions.

Validation of the HPLC Method for the Determination of Zearalenone and Its Metabolites. This new HPLC method for separation, identification and quantitative determination of zearalenone and its metabolites was validated according to the international rules (*27*−*30*). The performance criteria required are as follows: accuracy within the bias interval of (−2)−2% and the recovery values in the interval 80−120%. The obtained results were evaluated using the Student's *t* test: the value given in the table is $t = 2.26$ for $p \leq 0.05$ and 9 degrees of freedom (*31*), and it is in all cases greater than the calculated ones, showing that the data belong to the same population of values as the reference value. The correlation coefficients, *R*, of the linear regression equations exceeded the value 0.998, demonstrating a good correlation between the measured response (area of the peak) and the concentration of the analyte. The relative standard deviation for repeatability and reproducibility ranged from 0.27

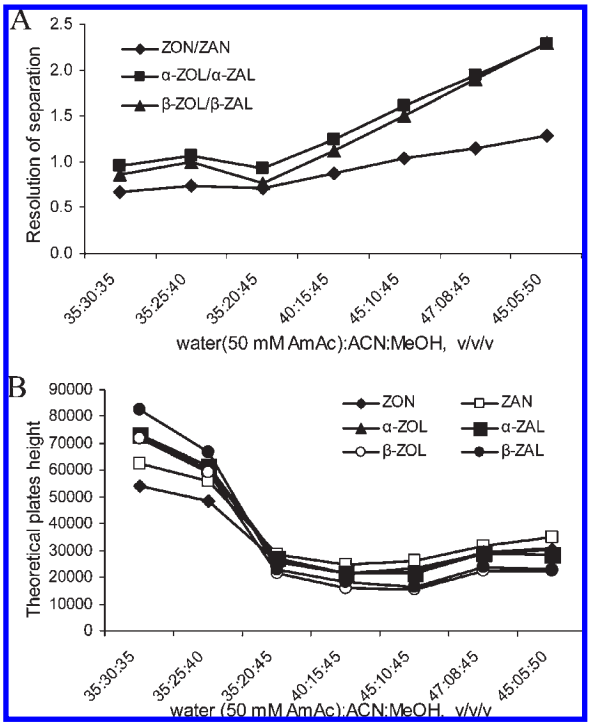

Figure 2. Variation of resolution for 3 pairs of neighboring peaks (**A**) and of the height of the equivalent theoretical plates (**B**) function of the ratio of the components from the mobile phase (Hypersil Gold (C18) column, 150 × 4.6 mm; elution isocratic gradient − mixture of water (containing 50 mM ammonium acetate):acetonitrile:methanol; total flow rate of the mobile phase 0.4 mL/min; temperature of the chromatographic column 25 °C; DAD detector, λ = 280 nm).

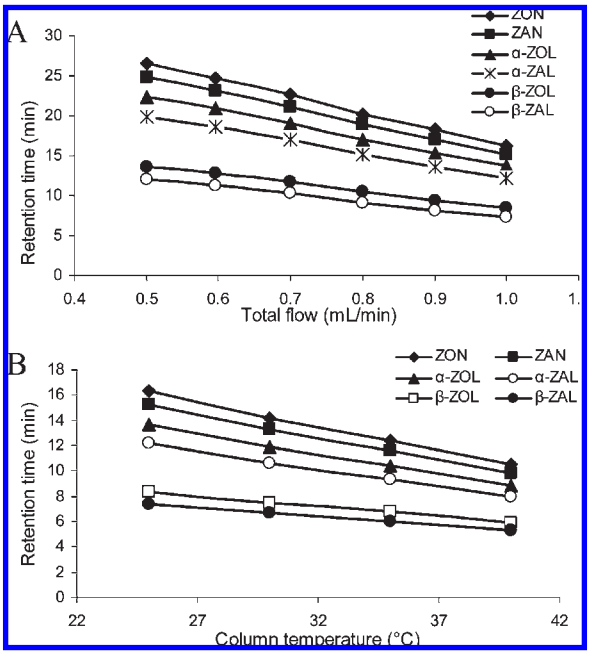

Figure 3. Variation of the retention time according to the total flow rate of the mobile phase (**A**) and to the temperature of the chromatographic column (**B**) for zearalenone and its metabolites (Hypersil Gold (C18) column, 150 × 4.6 mm; elution isocratic gradient − mixture of water (containing 50 mM ammonium acetate):acetonitrile:methanol (45:8:47); DAD detector, λ = 280 nm; temperature of the chromatographic column 25 °C (**A**); total flow rate of the mobile phase 1 mL/min (**B**)).

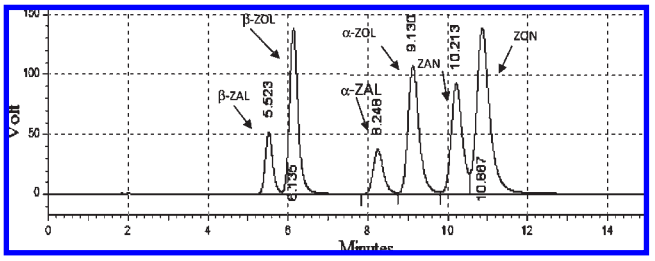

Figure 4. Example of chromatographic separation of a standard mixture 50 μM de ZON, ZAN, α-ZOL, α-ZAL, β-ZOL, β-ZAL (Hypersil Gold (C18) column, 150 × 4.6 mm; elution isocratic gradient − mixture of water (containing 50 mM ammonium acetate):acetonitrile:methanol (45:8:47); total flow rate of the mobile phase 1 mL/min; temperature of the chromatographic column 40 °C; DAD detector, λ = 280 nm).

to 2.21% for all analytes. The results of the validation are presented in **Table 1**.

Effects of Zearalenone on Broiler Meat Nutritive Quality. The studies of groups of broilers presented in this paper are part of a larger experiment. The final purpose of the study was the *in vivo* induction of cytochromes P450, which generally reflects in the increase of liver mass. The aim of the experiment is to detect the forms of cytochromes P450 induced by the presence of zearalenone in broilers (knowing that the broilers are the most "resistant" species to the presence of zearalenone) and to compare them with classical P450 inducers, like phenobarbital.

The first step for the nutritional characterization of the broiler meat was to observe the weight variation in the experimental broilers (**Table 2**). No noticeable differences were observed in terms of average daily weight gain. A trend of increased liver weight/final weight ratio was observed for the experimental groups: group 2, named ZON IP, and group 3, named PB-ZON (see Materials and Methods).

No significant differences were noticed between the control and experimental groups in terms of the gross chemical composition: dry matter, crude protein, ether extractives and ash as meat quality indicators (**Table 2**).

The fatty acids profile of the meat is one of the most important quality criteria, a higher level of unsaturated fatty acids being desirable. In view to observe the effects of the higher doses of zearalenone used in the experiment on the fatty acids profile of the samples, the fatty acids concentrations of 15 broiler meat samples were determined. As seen in **Table 3**, there is no difference between the experimental groups and the control group in terms of fatty acids profile.

The experimental results obtained during 4 days of treatment with ZON showed minimal or no effects of the dietary zearalenone on broiler meat nutritional quality.

Distribution of Zearalenone and Its Metabolites in Broiler's Organism. *Broiler Meat Sample Analysis.* Using the optimized and validated HPLC method, the concentrations of zearalenone and its metabolites in broiler meat, plasma and liver samples were determined. The samples were prepared as described in Materials and Methods. In parallel, part of the samples was not deglucuronized. The results obtained on the deglucuronized samples and on the samples that were not deglucuronized were comparable (**Table 4**). This shows that, in Leghorn broilers, the animal organism only contains ZON and its metabolites in the free form and not in the glucuronized form. A similar situation was described by Peter Zollner et al. in 2002, upon analyzing meat samples from pigs fed on contaminated barley (*32*).

ZON and its metabolites ZAN, α-ZOL, β-ZOL and β-ZAL have been determined and identified in the broiler meat samples analyzed; α-zearalanol, α-ZAL, was not detected. Previous research (*33*) revealed the presence of a new monohydroxylate metabolite of zearalenone, hydroxy-zearalenone (molar mass 334), which forms *in vitro* particularly upon incubation of microsomes from phenobarbital-treated rats. In the present paper in meat samples from broilers treated with ZON for 3 days (experimental group 2, named ZON IP), as well as for broilers treated with PB and thereafter with ZON (experimental group 3, named PB-ZON) an unknown compound having the retention time at 7.32 min (**Figure 5**) was identified by the proposed HPLC method. This compound may be a degradation product of zearalenone and its mass was determined by LC−MS method already described (*33*). The MS spectrum corresponding to the peak obtained for this compound indicates a molar mass of 217. One can conclude that this compound is not hydroxy-zearalenone as this one has the molar mass 334. In addition this compound is different from the product with the molar mass 274, that has been observed during the *in vitro* metabolic tests (*34*). Further work is in progress in order to characterize the unknown compound identified by the proposed HPLC method applied on meat samples from broilers treated with ZON.

Plasma Sample Analysis. The plasma samples of broilers from the groups 2, named ZON IP, and 3, named PB-ZON, respectively, slaughtered 24 h after the last treatment were prepared according to the method described previously in Materials and Methods and were analyzed using the optimized and validated HPLC method proposed in this paper. Only the zearalenone content was been determined. None of zearalenone metabolites have been detected. The plasma samples from group 2, named ZON IP, had an average zearalenone concentration of 6.62 ± 1.04 ng/mL, and those from group 3, named PB-ZON, had an average zearalenone concentration of 3.40 ± 0.54 ng/mL.

Liver Sample Analysis. ZON and important amounts of α-ZOL and β-ZOL were detected in the liver samples (**Table 4**).

Table 1. Results Obtained for the Validation of the HPLC Method for the Determination of Zearalenone and Its Metabolites

parameters	ZON	ZAN	α-ZOL	α-ZAL	β-ZOL	β-ZAL
			Precision			
repeatability, RSD (%)	1.36	0.61	2.20	0.48	0.50	0.27
reproducibility, RSD (%)	2.22	2.21	2.00	2.18	2.00	1.86
			Accuracy			
accuracy	100.76	100.78	101.31	101.35	100.65	100.35
bias	0.76	0.78	1.31	1.35	0.65	0.35
calcd *t*	1.65	2.09	2.12	2.16	1.92	1.12
			Lower Limits of Detection and Quantification			
LOD (μg/mL)	0.03	0.03	0.03	0.14	0.04	0.04
LOQ (μg/mL)	0.04	0.04	0.04	0.17	0.05	0.07
			Linearity			
domain (μg/mL)	0.04−31.8	0.04−31.8	0.04−31.8	0.17−31.8	0.05−31.8	0.07−31.8
R value	0.9996	0.9990	0.9990	0.9969	0.9984	0.9980
			Stability of the Solution			
$t_R \pm$ sd (min)	10.753 ± 0.185	10.100 ± 0.150	9.031 ± 0.132	8.161 ± 0.118	6.076 ± 0.080	5.470 ± 0.081
			Recovery			
value (%)	104.93	102.28	98.86	103.64	119.87	107.13

Table 2. Total Broiler Weight, Liver Weight and Gross Chemical Composition of Broiler Meat[a]

determinations	units	group 1: control	group 2: ZON IP	group 3: PB-ZON
		Total Weight and Liver Weight		
av initial wt	g	221.2 ± 21.40	222.7 ± 32.11	222.8 ± 35.54
av final wt	g	259.2 ± 22.03	259.8 ± 38.34	257.0 ± 41.83
av daily gain	g	38.0 ± 6.21	37.2 ± 10.1	34.2 ± 8.30
av liver wt	g	6.0 ± 0.5	6.8 ± 1.1	7.1 ± 0.8
% of liver wt from final wt	%	2.30 ± 0.14	2.60 ± 0.21	2.79 ± 0.19
		Gross Chemical Composition		
dry matter (DM)	g/100 g sample	26.91 ± 2.442	26.66 ± 1.120	25.62 ± 0.624
crude protein	g/100 g DM	75.76 ± 1.941	72.98 ± 3.784	74.69 ± 1.473
ether extractives	g/100 g DM	13.19 ± 2.391	15.78 ± 4.035	14.32 ± 1.890
ash	g/100 g DM	4.28 ± 0.26	4.21 ± 0.32	4.20 ± 0.42

[a] The results are the average of analysis of 5 samples, with double determinations. The statistical significance was evaluated with the *t* test; no significant differences were noticed for $p \leq 0.05$.

Table 3. Fatty Acids Concentration (g/100 g EE) in Broiler Meat[a]

fatty acids	group 1: control	group 2: ZON IP	group 3: PB-ZON
myristic acid	0.50 ± 0.08	0.46 ± 0.10	0.51 ± 0.05
palmitic acid	22.71 ± 1.085	22.32 ± 1.903	21.56 ± 0.981
palmitoleic acid	4.53 ± 0.44	4.82 ± 0.71	4.70 ± 0.56
stearic acid	7.51 ± 0.81	7.28 ± 1.04	6.98 ± 0.55
oleic acid	33.53 ± 1.532	35.15 ± 5.925	34.72 ± 2.965
linoleic acid	27.24 ± 0.992	25.46 ± 7.440	27.56 ± 1.590
linolenic acid	1.44 ± 0.15	1.39 ± 0.21	1.50 ± 0.11
arachidonic acid	2.54 ± 0.48	3.12 ± 0.73	2.45 ± 1.65
unsaturated to saturated fatty acids ratio	2.26 ± 0.18	2.33 ± 0.25	2.44 ± 0.15

[a] The results are the average of analysis of 5 distinct samples from each group, with double determinations. The statistical significance was evaluated with the *t* test; no significant differences were noticed for $p \leq 0.05$.

ZAN, α-ZAL and β-ZAL were not detected, either because they were below the limit of detection or because they were not present in the samples.

Similar results were reported in the broilers fed on a diet containing 100 mg/kg zearalenone for 8 days; the samples were analyzed by GC−MS, and only ZON, α-ZOL and β-ZOL were identified in the liver at 30 min after exposure (*35*). Comparable levels of zearalenone were also reported by other studies on poultry meat, liver and plasma, but no zearalenone metabolites have been identified (*35*, *36*).

Table 4. Concentration of Zearalenone and Its Metabolites in Samples of Broiler Liver and Meat[a]

	broiler meat		broiler liver	
compound	ZON IP (ng/g DM)	PB-ZON (ng/g DM)	ZON IP (μg/g)	PB-ZON (μg/g)
ZON	35.72 ± 6.123	22.98 ± 8.863	3.01 ± 0.32	3.69 ± 0.77
ZAN	1.72 ± 0.681	0.56 ± 0.30		
α-ZOL	13.42 ± 2.503	4.08 ± 1.11	26.46 ± 2.220	5.48 ± 0.22
unknown compound	21.4 ± 11.5	2.74 ± 1.15		
β-ZOL	11.47 ± 3.951	5.48 ± 1.29	23.69 ± 2.052	9.57 ± 0.95
β-ZAL	5.61 ± 0.94	7.10 ± 1.60		

[a] The results are the average ± standard deviation of 5 distinct samples from each group, with double determinations.

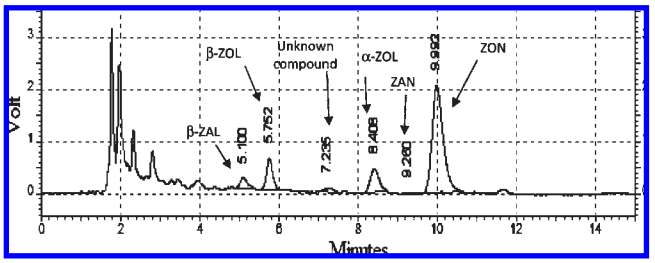

Figure 5. Example of ZON group chromatographic separation in a broiler meat sample (Hypersil Gold (C18) column, 150 × 4.6 mm; elution isocratic gradient; mobile phase mixture of water (containing 50 mM ammonium acetate):acetonitrile:methanol (45:8:47); total flow rate of the mobile phase 1 mL/min; temperature of the chromatographic column 40 °C; DAD detector, λ = 280 nm; total time of separation 12 min).

This paper presents the development and validation of a HPLC-DAD method for the separation and determination of zearalenone and its metabolites in broiler biological samples. The method responds to the requirements to determine zearalenone and its metabolites at the ng/g level in broiler biological samples, allowing the quality control of the samples; it also is a useful instrument in the studies of zearalenone metabolism. ZON and its metabolites can be separated with a proper resolution in 11 min using a Hypersil Gold C18 column (150 mm × 4.6 mm), the mobile phase a mixture of 50 mM aqueous ammonium acetate:ACN:MeOH with 45:8:47 (v/v/v) ratio, at a mobile phase flow rate of 1 mL/min and chromatographic column temperature of 40 °C.

Based on the results obtained using the proposed method for sample analysis of meat (35.72 ± 6.12 ng ZON/g dry matter (DM), 1.72 ± 0.68 ng ZAN/g DM, 13.42 ± 2.50 ng α-ZOL/g DM, 11.47 ± 3.95 ng β-ZOL/g DM, 5.61 ± 0.94 ng β-ZAL/g DM), of plasma (6.62 ± 1.04 ng ZON/mL) and of liver (3.01 ± 0.32 μg ZON/g, 26.46 ± 2.22 μg α-ZOL/g, 23.69 ± 2.05 μg β-ZOL/g) collected from broilers treated with zearalenone, one can conclude that liver is one of the centers for zearalenone localization and detoxification. Although the broiler meat was not altered nutritionally by the presence of zearalenone; it cannot be regarded as edible because of the presence and buildup of ZON, ZAN, α-ZOL, β-ZOL and β-ZAL in the broiler meat, products which disturb the estrogenic metabolism. Using the new HPLC method developed and validated in this paper, an unknown compound (21.45 ± 11.51 ng/g DM) with the retention time 7.32 and the molar mass of 217 was revealed between the zearalenone metabolites. Further research is needed to characterize them.

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