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Study on the transmission of deoxynivalenol and de-epoxy-deoxynivalenol into eggs of laying hens using a high-performance liquid chromatography-ultraviolet method with clean-up by immunoaffinity columns

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The transmission of deoxynivalenol (DON) and of its metabolite de-epoxy-DON into eggs has not been sufficiently elucidated until now. This question was addressed within the scope of a 16-week experiment with laying hens which were fed a maize-based diet with a DON concentration of 11.9 mg · kg^{-1} dry matter. Eggs were collected during weeks 2, 4, 8, and 16 of the experiment, and DON and its metabolite de-epoxy-DON were analyzed in freeze-dried yolk and albumen. In order to cover possible conjugates, all samples were incubated with β-glucuronidase prior to extraction. Yolk and albumen were extracted with acetonitrile-water, and the extracts were purified with immunoaffinity columns (IACs) after a precleaning step. The toxins were determined by high-performance liquid chromatography (HPLC) with UV detection. The detection limits of both toxins were 5 and 8 μg · kg^{-1} in freeze-dried yolk and albumen, respectively, corresponding to approximately 2.5 and 1 μg · kg^{-1} in fresh samples. The recovery of DON and de-epoxy-DON in yolk was 80% and 78%, respectively, and in albumen 77 and 72%. Neither DON nor de-epoxy-DON or glucuronide conjugates of both substances could be detected in any of the samples. These results indicate that eggs do not contribute significantly to the dietary DON intake of humans.

Keywords: Deoxynivalenol / Egg / Mycotoxin / Poultry

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1 Introduction

Trichothecene mycotoxins are secondary metabolites produced by *Fusarium* sp. The trichothecene deoxynivalenol (DON) is prevalent worldwide in crops used for food and feed production. Consumption of DON-contaminated feed can cause reduced feed intake or feed refusal, immune suppression and vomiting in the case of high dietary concentrations [1, 2]. Poultry and ruminants are more tolerant to DON than swine. Chickens and laying hens respond to increasing dietary DON concentrations with a reduction in productivity only at high levels above 5 mg · kg^{-1} but there is no evidence of a clear dose-response relationship [2].

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Abbreviations: DAD, diode array detection; **DON**, deoxynivalenol; **de-epoxy-DON**, de-epoxy-deoxynivalenol; **IAC**, immunoaffinity column

Outbreaks of acute human disease involving nausea, vomiting, gastrointestinal upset, and diarrhoea have been attributed to the presence of DON at concentrations of 3 – 93 mg · kg^{-1} in grain for human consumption in Asia [3]. The EU Scientific Committee on Food established a tolerable daily intake (TDI) at 1 μg · kg^{-1} body weight for DON [4]. Human exposure to DON occurs predominantly *via* ingestion of cereals and grains. On the basis of studies to date, it is estimated that food of animal origin does not significantly contribute to this exposure [5]. In swine, following extended (3 – 7 weeks) consumption of DON-contaminated feed (6.0 – 7.6 mg · kg^{-1}), only trace tissue residue levels below 10 μg · kg^{-1} were found [6]. In cow's milk, no DON but trace levels of the less toxic metabolite de-epoxy-DON were found after feeding a diet with a very high DON concentration (66 mg · kg^{-1}) over a period of 5 days [7].

The carry-over of DON to eggs was investigated in two feeding experiments with laying hens with a DON concentration in feed of 4 – 5 and 83 mg · kg^{-1}, respectively [8, 9]. No residues of DON could be detected in eggs above the detection limit of 10 μg · kg^{-1} in either study. Recently, using a very sensitive LC-MS/MS method, trace levels of

DON below 1 μg · kg^{-1} were detected in eggs of laying hens which were fed a diet containing DON at 5 – 10 mg/kg [10]. However, the metabolite de-epoxy-DON and conjugates of DON and of de-epoxy-DON were not analyzed in either study. Low levels of radioactive residues were transmitted to eggs of laying hens following a single oral dose of [^{14}C]DON (2.2 mg/bird) [11] or of [^{3}H]DON (0.1 mg · kg^{-1} body weight) [12] or during prolonged administration of a diet containing 5.5 mg · kg^{-1} [^{14}C]DON [13]. In the study by Prelusky *et al.* [11], only 10% of the radioactivity in yolk could be identified as the parent toxin DON.

In the present study, the carry-over of DON to eggs was investigated within the scope of a 16-week experiment with laying hens fed a maize-based diet with a DON concentration of 11.9 mg · kg^{-1} dry matter. Emphasis was laid on the detection not only of DON, but also of its metabolite de-epoxy-DON and the conjugates of both substances in yolk and albumen. For analysis, an HPLC-UV method including a clean-up with immunoaffinity columns (IACs) was used which was optimized for this type of samples. The study on carry-over of DON into eggs was undertaken with regard to possible impairment of human health. Another objective was to test whether DON or de-epoxy-DON concentration in eggs could be a specific parameter indicating the effect of so-called detoxifying agents for mycotoxins on DON absorption in laying hens.

2 Materials and methods

2.1 Feeding experiment and egg collection

The feeding experiment is described in detail by Dänicke *et al.* [14]. Maize-based diets (70% maize on an air-dry basis) with a DON concentration of 11.9 mg · kg^{-1} dry matter (DON group) and 0.5 mg · kg^{-1} dry matter (control group), respectively (analyzed by HPLC with diode array detection after IAC clean-up according to a slightly modified protocol of R-Biopharm, Darmstadt, Germany), were each tested on 25 Lohmann Brown laying hybrids in a 112-d experiment. The maize used in the diet of the DON group was not only highly contaminated with DON, but also with 15-acetyl-DON, nivalenol, and zearalenone (Table 1). Eighteen-week-old hens were placed into single cages and fed a commercial layer diet until the commencement of the experiment when the hens were 22 weeks old. The mean body weight of the flock at the beginning of the experiment was 1380 g ± 132 g. Feed was offered in a meal form for *ad libitum* consumption and water was supplied by nipple drinkers. Two eggs were collected from each hen during weeks 2, 4, 8, and 16 for the determination of mycotoxin residues. Yolk, albumen, and shell were separated and yolk and albumen were pooled in such a way that five pooled samples were available per group and period. The samples were freeze-dried and ground to pass through a 1-mm screen.

Table 1. Mycotoxin contamination of maize used in the diet of the DON group (DON maize) and of the control group (control maize), respectively (μg · kg^{-1})

Mycotoxin	DON maize	Control maize
DON[a)]	17 630	212
15-Acetyl-DON[b)]	5 500	n.a.
3-Acetyl-DON[b)]	90	n.a.
T-2 Toxin[b)]	< 4	n.a.
HT-2 Toxin[b)]	< 10	n.a.
Nivalenol[b)]	1 600	n.a.
Zearalenone[c)]	1 580	43
Fumonisins[d)]	< 100	n.a.
Beauvericin[e)]	< 46	n.a.
Moniliformin[e)]	< 89	n.a.

n.a., not analyzed

a) Analyzed by HPLC-DAD after clean-up with Mycosep™ Trichothecen according to a slightly modified procedure of Coring System Diagnostics, Gernsheim, Germany

b) Analyzed by GC-MS at the Institute of Animal Nutrition of the University of Hohenheim, Germany

c) Analyzed by HPLC with fluorescence detection after incubation with 2 U β-glucosidase and clean-up with IAC (Easi-Extract Zearalenone, Coring System Diagnostics, Gernsheim, Germany)

d) Analyzed by an enzyme-linked immunosorbent assay (ELISA, RIDASCREEN® FAST Fumonisin; R. Biopharm, Darmstadt, Germany)

e) Analyzed by HPLC at the Institute for Agrobiotechnology (IFA) Tulln, Austria

2.2 Reagents and standards

DON standard was obtained from Sigma-Aldrich (Taufkirchen, Germany). A 40 μg · mL^{-1} stock solution in acetonitrile was prepared and the concentration was checked by spectrophotometry according to Pettersson [15] using the molar absorption coefficient of 6400 at 219 nm. The de-epoxy-DON standard was a kindly gift from Dr. Meyer, Department of Animal Hygiene at the Technical University of Munich. Chemicals were of analytical-reagent grade, except for solvents which were of HPLC-grade. Water was purified and deionized using a PURELAB Option-E50 water purifier (ELGA Labwater, VSP, Ransbach-Baumbach, Germany). For enzymatic hydrolysis, β-glucuronidase from *Helix pomatia*, type H-2 (min. 100 000 units · mL^{-1}, sulfatase up to 7500 units · mL^{-1}) (Sigma-Aldrich), was employed. Polyvinylidene fluoride (PVDF) membrane syringe filters (0.45 μm) were obtained from Amchro (Hattersheim, Germany).

2.3 Incubation, extraction, and clean-up of yolk and albumen

A 3.0 g portion of freeze-dried yolk (2.5 g portion of freeze-dried albumen) was mixed with 10 mL (12 mL) phosphate buffer solution, pH 6.8. A volume of 0.12 mL (0.10 mL)

β-glucuronidase was added and the mixture was incubated overnight at 37°C. After the addition of 50 mL (63 mL) acetonitrile, the mixture was shaken for 1 h and filtered. A 40-mL aliquot was defatted with an equal volume of petroleum ether and 30 mL of the water-acetonitrile phase were further cleaned up by shaking with 3 g of a mixture of charcoal, alumina, and celite® (Mallinckrodt Baker, Griesheim, Germany) (7+5+3 w+w+w) for 10 min. After filtration, an aliquot of 15 mL was evaporated to dryness with the addition of 5 mL ethanol on a rotary evaporator. The last traces were blown out under a gentle stream of nitrogen. The residue was dissolved in 3 mL water by treatment in an ultrasonic bath and filtered through a membrane syringe filter. A 2-mL volume of the precleaned sample extract, equivalent to 0.5 g freeze-dried yolk and 0.333 g freeze-dried albumen, respectively, was passed through an immunoaffinity column (DONprep®, R-Biopharm) under gravity. The column was washed with 5 mL water by applying gentle vacuum and the toxins were eluted from the column by slowly passing four 1-mL portions of methanol. The solvent was evaporated to dryness on a rotary evaporator and the last traces were blown out under a gentle stream of nitrogen.

2.4 Analysis by HPLC

The HPLC equipment consisted of a pump, model LC-10ADvp, an auto-injector, model SIL-10ADVP, a column oven, model CTO-10ASVP, a spectrophotometric detector, model SPD-10AVVP, and Class VP 6.12 data system, all of Shimadzu (Duisburg, Germany). Separations were carried out on a Synergi Hydro-RP column (4 μm, 250 × 3 mm) in combination with a C18Aq guard column (4 × 2 mm), both of Phenomenex (Aschaffenburg, Germany). The column was maintained at 40°C. The detector wavelength setting was 218 nm. The purified sample extract was dissolved in 0.2 mL of mobile phase A (acetonitrile-water, 12 : 88 v/v) by treatment in an ultrasonic bath and filtered through a membrane syringe filter. A 50-μL aliquot was injected onto the column. Initially, 100% mobile phase A was pumped at a flow rate of 0.5 mL · min^{-1} for 15 min. Under these isocratic conditions, DON and de-epoxy-DON eluted at approximately 7 and 12 min, respectively. Subsequently, a linear gradient of mobile phase A and mobile phase B (100% acetonitrile) was run: 15 – 18 min: 100–5% A; 18 – 33 min: 5% A; 33 – 36 min: 5 – 100% A; 36 – 53 min: 100% A. Quantification of DON was performed using external five-point calibration based on peak area. Calibration standard solutions in the range of 0.05 – 5.0 μg · mL^{-1} were prepared in mobile phase A. The calibration curve was linear in the concentration range of the standard solutions with correlation coefficient of 0.999 or higher. De-epoxy-DON was quantified according to the calibration curve for DON.

2.5 Recovery of DON and de-epoxy-DON in yolk and albumin

Recovery of DON and de-epoxy-DON was carried out by spiking noncontaminated freeze-dried yolk and albumen samples with appropriate amounts of standard solutions according to final concentrations of 30 – 50 μg · kg^{-1} in the case of yolk and 30 – 100 μg · kg^{-1} in the case of albumen, one day before analysis. The spiking solvent was allowed to evaporate before addition of reagents for incubation. The spiked samples were analyzed using the methods described above. The ongoing performance of the HPLC equipment was checked by measuring at least two calibration standards within each batch of samples. The retention time, peak area, and peak height were compared with the results of the previous calibration run.

3 Results and discussion

3.1 Analytical method

A previously described method for the determination of DON and de-epoxy-DON in feces [16], which was based on a method of VDLUFA [17] for the determination of DON in feeds, was modified for the analysis of yolk and albumen. All samples were incubated with β-glucuronidase prior to extraction in order to detect possible glucuronide conjugates. Subsequently, the samples were extracted with an acetonitrile-water mixture and purified by IAC after a precleaning step. In contrast to the method described in [16], another type of IAC columns was used (DONprep® of R-Biopharm instead of DONtest HPLC® of VICAM). It was demonstrated for the columns DONtest HPLC® in the previous study [16] that not only DON but also de-epoxy-DON is bound to the antibody within the column. The same was shown for the columns DONprep® in the present study, as indicated by the recovery of DON and de-epoxy-DON in spiked yolk and albumen samples which was in the same order of magnitude for both toxins (Table 2). Therefore, both types of IAC columns are suitable for the analysis of physiological samples which may contain not only DON but also the metabolite de-epoxy-DON. However, the DON-

Table 2. Recovery of DON and de-epoxy-DON from freeze-dried yolk and albumen

	DON			De-epoxy-DON		
	Spiking level (μg · kg^{-1})	*N*	Recovery (mean ± SD) (%)	Spiking level (μg · kg^{-1})	*N*	Recovery (mean ± SD) (%)
Yolk	30 – 50	4	80 ± 12	30 – 50	4	78 ± 11
Albumen	30 – 100	6	77 ± 12	30 – 100	6	72 ± 7

N = number of samples; SD = standard deviation

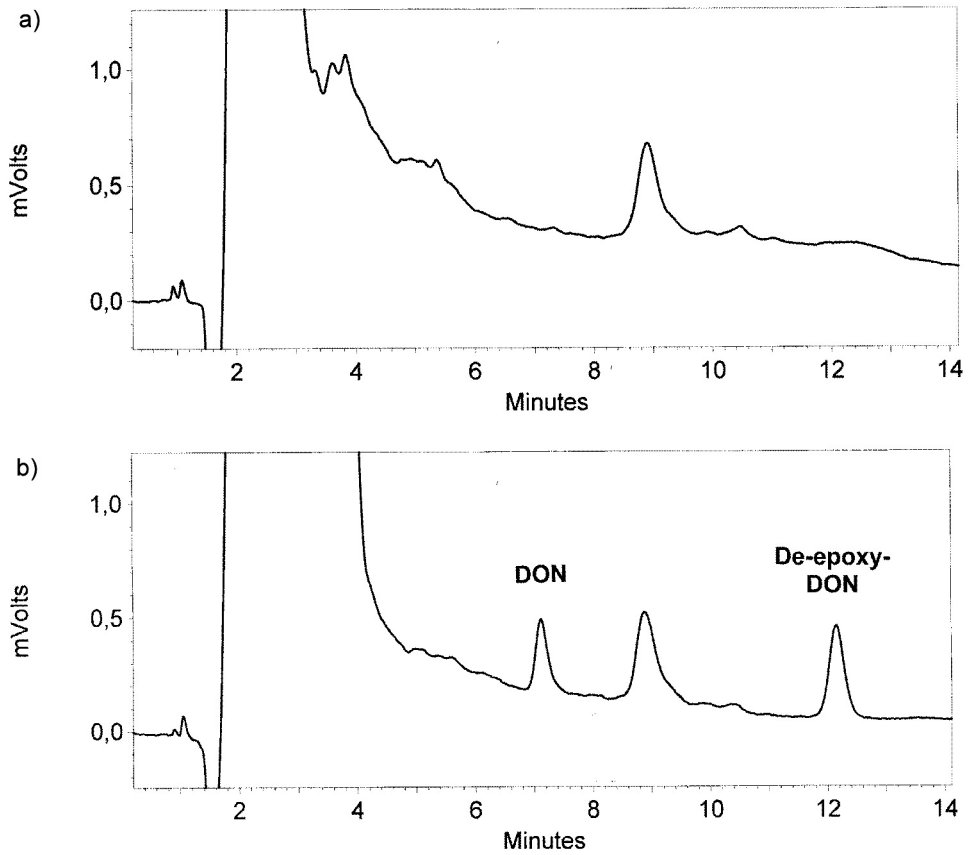

Figure 1. HPLC chromatograms of (a) a blank albumen sample, (b) an albumen sample spiked with DON and de-epoxy-DON at a level of 30 μg · kg^{-1} related to freeze-dried albumen.

prep® columns seem less sensitive to impurities in sample extracts and therefore more appropriate for difficult samples like yolk and albumen.

According to the HPLC conditions described by Valenta *et al.* [16], a gradient was run after the elution of de-epoxy-DON in order to clean the HPLC column. In contrast to the mentioned method, a more sensitive UV detector and a 4 μm HPLC column with a smaller diameter were used to improve the detection limits. The detection limits of both DON and de-epoxy-DON, at a signal-to-noise ratio of 3, were 5 and 8 μg · kg^{-1} in freeze-dried yolk and albumen, respectively, corresponding to approximately 2.5 and 1 μg · kg^{-1} in fresh samples (calculation based on a mean dry matter of 51% for yolk and 13% for albumen). Thus, the detection limits were improved by the factor of 4 (yolk) and 2 (albumen), respectively, compared to the nonoptimized method used in a preliminary study on transmission of DON into eggs [18]. An example of LC chromatograms of blank albumen and spiked albumen is shown in Fig. 1. No interfering compounds were noted at the elution time of either DON or de-epoxy-DON.

Gas chromatography (GC) with mass spectrometric (MS) detection was used for the determination of DON in whole eggs in a previous study [8]. Eggs were extracted with acetonitrile-water and cleaned up on an alumina-charcoal column. The detection limit of DON in this study was 10 μg · kg^{-1} related to fresh eggs and thus higher than the detection limit of the present study. Lun *et al.* [9] used GC with electron capture (EC) detection and the sample preparation method of El-Banna *et al.* [8] for the determination of DON in yolk and albumen.

The development of IAC columns for purification of sample extracts resulted in an increased use of HPLC-UV methods for the analysis of DON in cereals in the last several years. The reason for this is, that due to the IAC purification step, clean extracts and consequently HPLC chromatograms without interfering peaks can be obtained. Moreover, derivatization and common method problems in gas chromatographic analysis of trichothecenes [15] can be avoided. To date, only few studies used HPLC-UV and IAC columns for the determination of DON in physiological samples. Janes and Schuster [19] described an HPLC-UV method for the determination of DON in blood, bile, urine, and feces of swine with an IAC clean-up after a precleaning step by preparative HPLC. The detection limits ranged from 10 to 20 μg · kg^{-1}. The analysis did not include the metabolite de-epoxy-DON. The method of Valenta *et al.* [16] allows the determination of DON and de-epoxy-DON in serum, urine, bile, and feces of livestock using HPLC-diode array detec-

tion (DAD) after IAC clean-up and a precleaning step with a mixture of charcoal, alumina, and celite® in the case of bile and feces. The detection limits were in the range of 4 – 20 μg · kg^{-1}. Meky *et al.* [20] described an IAC-HPLC method for the determination of urinary metabolites of DON in rat and human urine. Recently, Sypecka *et al.* [10] used a very sensitive LC-MS/MS method with IAC clean-up for the determination of DON in whole eggs with a detection limit of 0.01 μg · kg^{-1}. The method did not include the analysis of de-epoxy-DON and conjugates.

In conclusion, the described HPLC-UV method with IAC clean-up allows the determination of DON, de-epoxy-DON and glucuronide conjugates in yolk and albumen down to concentrations of approximately 2.5 μg · kg^{-1} and 1 μg · kg^{-1}, respectively, related to fresh samples. Thus, this method is a useful alternative to more complicated and expensive GC-MS and HPLC-MS methods. These latter methods can be used for confirmation of positive findings [20 – 22].

3.2 Carry-over

Neither DON nor de-epoxy-DON or glucuronide conjugates of both were detected in any of the yolk and albumen samples. The nondetection of DON in the yolk and albumen samples is in accordance with the results of two feeding trials with laying hens fed diets containing lower (4 – 5 mg · kg^{-1}) [8] or higher (83 mg · kg^{-1}) [9] DON concentrations in the feed compared to the described experiment. Also, with exception of the gizzard, Lun *et al.* [9] and Kubena *et al.* [23] could not detect DON in edible tissues (liver, muscle, kidney, heart) of chickens, fed a diet with a DON concentration of 9, 18, or 83 mg · kg^{-1}. Recently, Sypecka *et al.* [10] measured trace levels of DON in the range of 0.13 – 0.79 μg · kg^{-1} in whole eggs of laying hens which were fed diets containing DON at 5, 7.5, and 10 mg · kg^{-1}, respectively, for three weeks. The levels of DON in eggs, reported by Sypecka *et al.* [10], were below the detection limit of the present study. No statistical difference between treatment groups for concentrations of DON in eggs was found by Sypecka *et al.* [10]. DON levels in the eggs rose rapidly in the first seven days of the feeding with contaminated rations and were reduced to undetectable levels within three to seven days of withdrawal of contaminated grain [10]. Transmission rates of 15 000 : 1 to 29 000 : 1 were calculated for the three treatment levels. However, no metabolites or conjugates of DON were analyzed in eggs and other tissues of poultry until now. *In vitro* inoculation of the contents of the large intestine of chickens with DON showed the ability of the microorganism of the large intestine to transform DON to the metabolite de-epoxy-DON [24].

Regarding other *Fusarium* mycotoxins, carry-over of nivalenol (NIV) and zearalenone (ZON) into eggs and tissues of laying hens was studied [10, 14, 25]. Trace concentrations of NIV and DON were detected in the liver of hens fed a diet containing 5 mg NIV per kg, but neither NIV nor any of the other investigated metabolites (DON, de-epoxy-NIV, fusarenon X) were found in yolk and albumen [25]. Low residues of ZON and α-zearalenol were detected in livers, but no ZON or metabolites were detected in yolk, albumen, abdominal fat, and breast meat of laying hens fed a diet containing ZON at 1.1 mg · kg^{-1} [14]. No detectable levels of ZON or metabolites were found in whole eggs at a ZON concentration in feed of 0.25 mg · kg^{-1}, as well [10].

The nondetection of DON, de-epoxy-DON, and conjugates in yolk and albumen in the present study and the nondetection or only trace-detection of DON in eggs in other studies [8 – 10] are supported by experiments on poultry with radioactive labelled DON [12, 26] which indicate poor bioavailability and rapid elimination of the toxin from the body. The systemic absorption was estimated to be < 1% of a single oral dose of [^{14}C]DON to laying hens [26]. A more extensive absorption from the gastrointestinal tract, but a highly efficient hepatic or renal first-pass effect which removes the toxin before it reaches the systemic (blood) circulation, is also discussed [1].

Detectable levels of radioactive residues were transmitted to eggs of laying hens during a 6-day period with the administration of 2.2 mg [^{14}C]DON per bird and day [11]. Radioactivity levels in eggs increased until the last exposure to the toxin; maximum levels accounted for 4.2 μg DON-equivalents per 60-g egg. Because the results indicated an accumulation of residues of DON and/or metabolites in eggs, transmission of residues to eggs during prolonged administration for a 65-day period of a diet containing 5.5 mg/kg [^{14}C]DON to laying hens was investigated in a subsequent study [13]. Total residues increased daily until the 8th day of [^{14}C]DON exposure when levels reached a plateau for several days, then decreased slowly thereafter. Maximum radioactivity measured was equivalent to 1.7 μg DON or metabolites per 60-g egg; the yolk, albumen and shell membrane contributed 70, 29, and 1% of the total amount, respectively. 10% of the radioactivity in yolk was identified as the parent toxin DON in the first study [11].

Applying the results of Prelusky *et al.* [13] to the present study, maximum residues of 124 μg DON equivalents (approximately 12 μg of it as DON) per kg yolk and 27 μg DON equivalents per kg albumen were to be expected in week 2. The reasons for the nondetection of DON, de-epoxy-DON and glucuronide conjugates in the present study could be that DON is transferred to eggs in the form of other not yet identified metabolites, or DON and/or metabolites are bound to some constituents of eggs and can-

not be released by the applied incubation and extraction procedure. It is also possible that DON is completely degraded.

4 Concluding remarks

Using an HPLC-UV method which was optimized for the analysis of yolk and albumen, neither DON nor de-epoxy-DON or glucuronide conjugates of both toxins were detected in yolk and albumen samples of a feeding study with laying hens fed a diet containing a relatively high DON level of 11.9 mg · kg^{-1}. Regarding DON, the results are in accordance with other feeding studies with laying hens which found no or only trace residues of DON in eggs. No other studies are known which analyzed residues of de-epoxy-DON and conjugates of both toxins in eggs. In contrast to the present study, detectable levels of radioactive residues were transmitted to eggs of laying hens during the administration of [^{14}C]DON. It is possible that DON is transferred to eggs in the form of other not yet identified metabolites, DON and/or metabolites are bound to some constituents of eggs, or DON is completely degraded. However, de-epoxy-DON is the only known metabolite of DON from other studies on livestock.

Considering the results of the present study together with the earlier results, it cannot be excluded that DON and/or metabolites are transmitted to eggs to a minor degree. However, compared to other dietary sources of DON, especially cereals and cereal-based products, it is assumed that eggs do not contribute significantly to the dietary DON intake of consumers.

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