Analysis of Deoxynivalenol and De-Epoxy- Deoxynivalenol in Horse Blood Through Liquid Chromatography After Clean-up with Immunoaffinifty Column

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ABSTRACT

The Fusarium toxin, deoxynivalenol (DON) has a great significance in human and animal nutrition. Five Haflinger mares were selected to study the concentration of Deoxynivalenol (DON) and De-Epoxy-Deoxynivalenol (deepoxy-DON) in plasma samples of horse fed a diet contaminated with DON. The experimental animals were feed contaminated oat (12mg /kg oat), total being 36 mg DON/day. A series of blood samples were carried out on the day of feeding (day 1) and day 10. The concentration of DON and deepoxy-DON were determined using liquid chromatographic (HPLC-UV) method coupled with the immunoaffinity columns. The method was validated with the range of 10-100 ng/mL DON and deepoxy-DON. The Relative Standard Deviation (RSD) intra-day and inter-day for DON were less than 9% and 7% and for deepoxy-DON were less than 11% and 9%, respectively. The average recoveries of both analytes were found to be approximately 81.9 ± 5.3 % and 80.5 ± 6.4%, respectively. Generally, free form of DON (without treatment with ß-glucuronidase) in the plasma samples collected 24 hour post-feeding on day ten were under the limit of detection in all animals except in two animals (11.7 and 7.6 ng/mL). The higher concentration of DON was found in the plasma one hour post-feeding. In plasma, it seems that majority of DON is present in the glucuronide-conjugated form. The levels detected are not very high, but even long-term exposure to low doses of these mycotoxins may represent a threat to horse health.

***Key Words*:** DON, Horse, HPLC, Mycotoxins, De-Epoxy-Deoxynivalenol

INTRODUCTION

Mycotoxins are secondary metabolites of phytopathogenic and food spoilage fungi that cause a variety of public health and agro-economic problems. Mycotoxins may enter in food chain through the growth and toxin production of fungi in food and feedstuffs. However, the presence of fungi in food does not always indicate toxin formation. On the other hand, the absence of fungi does not necessarily mean that food and feedstuffs are free from mycotoxins (Janssen et al., 1996; Smith et al., 1994). Mycotoxins can be produced by more than one species and different mycotoxins could simultaneously occur in one food product. Mycotoxin contaminations in food and feedstuffs are strongly dependant on environmental conditions during pre-harvest and storage steps. Water availability and temperature are the most conducive factors for fungi germination and growth and for production of secondary metabolites (Creppy, 2002).

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Contamination of foods and feeds by major mycotoxins, for instance Aflatoxins (AFs), Ochratoxin A (OTA), Fumonisins (FBs), Deoxynivalenol (DON), T-2 Toxin (T-2), HT-2 Toxin and Zearalenone (ZEA), has been recognized as significant source of food-borne illnesses.

DON is a 3*α*, 7*α*, 15-trihydroxy-12, 13-epoxytrichothec-9-en-8-one ($C_{15}H_{20}O_6$) with a molecular weight of 296.3 g/mol. Because of the higher relevance and reduced yield and performance in animal production, deoxynivalenol is considered as the major cause of economic loss (Josephs et al., 2001). Cumulative evidences showed that there is a lack of published information regarding the quantification of DON and deepoxy-DON in the biological samples of horses. Therefore, we used High Performance Liquid Chromatography-UV (HPLC-UV) coupled with immunoaffinity column for the detection of DON and deepoxy-DON in plasma of horses.

MATERIALS AND METHODS

Standard, Chemicals and Clean-up Cartridge

Deepoxy-DON standard (50µg/mL) was purchased from Biopure (Tulln, Austria) which were in crystalline and solution forms, respectively. DON (Biopure, Tulln, Austria) standard (5mg) was dissolved in acetonitrile to a final concentration of 1,000 µg/mL. The standard solutions (stock solutions) of DON (1,000 µg/mL) and deepoxy-DON (50 µg/mL) were stored at -20°C until further processing. The solutions of DON and deepoxy-DON standards were measured using an ultraviolet-spectrophotometry at 218 and 215 nm respectively (Biopure, 2005).

ß-Glucuronidase enzyme type HP-2 from *Helix pomatia* (100,000 unit/mL) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Steinheim, Germany). Polyethylenglycol (PEG)-8000 was purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile of HPLC-gradient grade were purchased from Fisher Scientific Co. (Leicestershire, UK) while water was purified in a UPW2 system (F & L, Austria).

DON-Prep immunoaffinity columns and DONTest™-immunoaffinity column were purchased from R-Biopharm Rhône (Glasgow, Scotland) and Vicam L.P. (Watertown, USA) respectively. The liquid-liquid partitioning was also performed in this study as additional step of clean-up method using ChemElut Varian cartridge (five mL) with hidromatrix diatomaceous earth (Walnut Creek, USA).

Experimental Animals and Feeding Trial

Five fertile Haflinger mares (Gaby, Lucy, Regina, Sylvia, and Twiggy) with a mean body weight of 450kg maintained at the Clinic for Obstetrics, Gynaecology, and Andrology, University of Veterinary Medicine, Vienna, were used in the study. At the start of experimental phase, the animals were fed a diet primarily composed of hay (07kg) and oats (03kg) per day. The DON was administered orally by feeding contaminated oat (12mg /kg oat); hence total being 36 mg DON/day. The ration was provided twice a day with an interval time of twelve hours throughout the experimental period (10 days).

Sample Collection and Preparation

Blood was collected on a day before the experimental phase was categorized as initial value (IV). In the experimental phase, collection of blood was done on day 1 and 10. Blood samples from each animal were collected after hour 1, 2, 3, 4, 8, 12 of first feeding and 12 of second post-feeding *i.e.* at hour 24 of first post-feeding.

Plasma (1.0 mL) was mixed with 2.0 and 3.0 mL of phosphate buffer (pH 6.8),

respectively. The solutions were mixed with 80 µl of ß-glucuronidase (~ 8,000 unit enzyme) and incubated overnight at room temperature. For the investigation of glucuronidation degree, three replicates of blood sample of horses were collected from 24 hour post-feeding on day 10 and prepared with and without ß-glucuronidase treatment. Samples without ß-glucuronidase treatment after mixing with phosphate buffer were poured directly into DON-Prep immunoaffinity column.

Clean-up Methods for Samples

Plasma samples were cleaned-up using DON-Prep immunoaffinity column with certain modification in the washing step. Plasma (3.0 mL) was loaded into an immunoaffinity column after incubation. An intensive washing procedure was carried out with 14.0 mL of phosphate buffered saline (PBS) and 6.0 mL of water, respectively. Later on, the analyte was eluted with 4.0 mL of methanol. Afterwards, eluate was evaporated using a stream of nitrogen and heating at 40°C, reconstituted in 300 µL of mobile phase and 100 µL was injected into HPLC-UV system.

Validation Analysis

The validation procedures were performed by spiking plasma into final concentrations of DON and deepoxy-DON ranging from 10 to 1,00 ng/mL. Evaluations of recoveries and repeatability were performed in three replicates within-day and between-days. Each of 950 µL of plasma was spiked with 50 µL of mixed working standard of DON and deepoxy-DON (0.2, 0.5, 1.0 and 2.0 µg/mL) and the final concentrations were 10, 25, 50 and 100 ng mixed of DON and deepoxy-DON/mL. Determination of detection limits (LOD's) and quantification limits (LOQ's) were based on response signal to noise of 3.0 to 1.0 and 10 to 1.0, respectively.

HPLC-UV

Mobile phase consisted of HPLC-water (82%), methanol (9%), and acetonitrile (9%). An amount of 100µl of reconstituted aliquot from food and biological samples were injected using an autosampler (Merck-Hitachi AS-2000, Tokyo, Japan). An Iso-Chrom LC pump (Spectra Physics, San Jose, USA) maintained at the flow rate of 1.00 mL/min was used. Analytes were separated on Synergy polar reversed-phase column (80Å, 250 mm length x 4.6 mm i.d., 4 µm) from Phenomenex (Cheshire, UK) and an analytical guard column C_{18} (4 x 3 mm; Phenomenex, Aschaffenburg, Germany) was installed to protect the HPLC-column. The wavelength, rise time and sensitivity range of ultraviolet detector was set at 220 nm, 0.3 seconds and 0.05 AUFS. The chromatograms were integrated based on a Stratos-V.4 Program (Polymer Laboratories, UK).

RESULTS AND DISCUSSION

Adaptation and Validation of Clean-up Method

In the current study, only immunoaffinity column was used as a clean-up method prior to HPLC-UV for determination of DON and deepoxy-DON. Figures 1 and 2 show that DON and deepoxy-DON have retention times of 8.5 and 11.5 minutes, respectively. The Relative Standard Deviation (RSD) within-day and between-day for DON were less than 9% and 7% and for deepoxy-DON were less than 11% and 9%, respectively (Tables 1 and 2). On the other hand, the average recoveries of both analytes were found to be approximately 81.9 ± 5.3 % and 80.5 ± 6.4%, respectively (Table 3).

Since immunoaffinity column was originally intended for clean-up of food samples, therefore, adaptation of this procedure was carried out in the current study for analysis of biological samples. The immunoaffinity

column was originally adapted for biological samples by additional washing step using a PBS. In addition, PBS has been commonly used as a washing solution in antibodies production and its application in immunochemical assay (Gathumbi et al., 2001). A prior study showed that immunoaffinity columns are able to remove impurities and pre-concentrate DON and deepoxy-DON in plasma samples. Furthermore, it has also been demonstrated that antibody of DON Prep-immunoaffinity column is also able to bind deepoxy-DON within the column (Valenta and Dänicke, 2005). Moreover, intensive washing steps consisting of 14 mL of PBS and 6.0 mL of water were able to exclude the additional application of diatomaceous-earth cartridge. However, Döll et al. (2003), Dänicke et al. (2004a, 2004b, 2004c, 2004d, 2004e, 2005a and 2005b) and Goyarts and Dänicke (2006) performed a clean-up using diatomaceous-earth cartridge coupled with immunoaffinity column when analysing the DON and deepoxy-DON in serum samples of pig and duck.

Various reports have mentioned higher recoveries of DON and deepoxy-DON using diatomaceous-earth cartridges coupled with immunoaffinity column in the pig's serum (Döll et al., 2003; Dänicke et al., 2004a; 2004b; 2004c; 2004e, 2005a, 2005b; Goyarts and Dänicke, 2006) and in Pekin duck (Dänicke et al., 2004d) ranging from 75 to 95 % and 64 to 104 %, respectively. The recoveries observed in the current study were also comparable to those of Valenta and Dänicke (2005) who employed this clean-up method to determine DON and deepoxy-DON in yolk (80 and 78 %) and albumen (77 and 72 %) of egg.

Similarly, the detection limits of both DON and deepoxy-DON in serum were reported to be 4.0 ng/mL in pigs (Döll et al., 2003; Dänicke et al., 2004b, 2004c; Goyarts and Dänicke, 2006), 5.0ng/mL in pig (Dänicke et al. 2005b) and 6.0 ng/mL in Pekin duck (Dänicke et al., 2004d). On the other hand, higher limit of detection (10µg/kg) was reported by Dänicke et al. (2004a, 2004e) compared to other studies. Our study showed the similar limit of detections in horse's plasma using a HPLC-UV method through immunoaffinity column clean-up without employing additional clean-up of diatomaceous-earth cartridges. However, the present study still could not reach lower detection limit (2.0ng/mL) as reported by Dänicke et al. (2005a). In contrast, Eriksen et al. (2003) reported a lower (1.0 ng/mL) detection limit for DON in pig's plasma using a GC-ECD. Although the GC-ECD is a more sensitive and selective method for the determination of DON and deepoxy-DON compared to HPLC-UV, but incorporation of immunoaffinity column coupled with HPLC-UV and employing a 4.0 µm HPLC column with a smaller diameter made it possible to improve limit of detection of analytes comparable to GC-ECD method (Valenta and Dänicke, 2005). Therefore, in the current study, more attention was given to enhance the effectiveness of the immunoaffinity column because of relatively high recoveries (80–82%). Furthermore, other validation studies mentioned the recoveries ranging from 70–110 % and RSD of repeatability less than 20 % were considered acceptable in validation methods (Gilbert and Anklam, 2002; Sugita-Konsihi, et al., 2006).

Application of Clean-up and Analytical Method for Analysis of DON and Deepoxy-DON

The concentrations of DON and deepoxy-DON in horse's plasma are shown in Figures 3 and 4 on day 1 and Figure 5 and 6 on day 10, respectively. Before the start of experiment, it was assumed that horses were fed only DON-free rations. Therefore, on day 1 at zero hour, DON could not be detected in plasma of Twiggy, Sylvia, and

Lucy except in the plasma of Regina (7.2 ng/mL) and Gaby (18.5 ng/mL). Deepoxy-DON could not be detected in Lucy and Gaby at zero hour on day one (Figure 4). In the remaining three animals (Regina, Twiggy, Sylvia), deepoxy-DON was detected at zero hour with concentrations ranging from 5.0 to 17.0 ng/mL.

Some studies (King et al., 1984; Swanson et al., 1987; He et al., 1992; Kollarczik et al., 1994) demonstrated successful metabolism of DON to deepoxy-DON *in vitro* by anaerobic intestinal microorganisms of ruminant and pig. The lack of appearance of deepoxy-DON in the serum samples of few horses might be due to individual variation and type of intestinal microorganisms present as it has been documented that each individual has own specific intestinal microbial population (Rehman et al., 2007). There is a dearth of report regarding the successful isolation of pure culture that has capability to metabolize DON to deepoxy-DON (Schatzmayr et al., 2006). Nevertheless, Binder et al. (1998) has described the capability of *Eubacterium sp.* to metabolize DON into deepoxy-DON.

Feeding of DON-contaminated feed to pigs have shown that the maximum concentration of DON in serum were reached after 4.1 hours (Dänicke et al., 2004b) and attained up to 6.0 hours after ingestion of contaminated feed. The authors reported that DON level in serum was approximately half of the maximum level attained one hour post-feeding. In the current study, the higher concentration of DON after one hour of feeding appeared in Regina and Gaby (Figure 3). In Regina, the magnitude of DON peak was 50 % after one hour of feeding compared to highest peak (six hours post-feeding) which is in accordance with the finding of Dänicke et al. (2004b). However, the highest concentration of DON appeared one hour post-feeding in Gaby on day 1. Goyarts and Dänicke (2006) also demonstrated that maximum concentration appeared in the pig's serum between 0.81 and 2.27 hours after chronically feeding DON-contaminated diet. The highest concentration of DON was observed in Sylvia after four hours of feeding on day ten (Figure 6) which is comparable to Eriksen et al. (2003) who mentioned maximum serum level of DON in pigs reached three hours after feeding of DON-contaminated feed.

Glucuronidation Degree of DON and Deepoxy-DON

The conjugation of DON and its metabolites with activated glucuronic acid (uridine diphospho-glucuronic acid) is a major Phase II reaction to increase the water solubility, and therefore facilitate the excretion of DON or its metabolites in urine and bile. It is well documented that glucuronide conjugation occurs particularly in hepatocytes (Prelusky et al., 1988).

Generally, free form of DON (without treatment with ß-glucuronidase) in the plasma samples collected 24 hour post-feeding on day ten were under the limit of detection in all animals except in Twiggy (11.7 ng/mL) and Lucy (7.6 ng/mL). Similarly, free deepoxy-DON was detected only in Regina (5.8 ng/mL) and Twiggy (5.1 ng/ml). Incubation of plasma samples with ß-glucuronidase increased the concentration of DON and deepoxy-DON. In case of Twiggy, the concentrations of DON and deepoxy-DON increased and were 49.2 and 10.9 ng/mL respectively. While concentrations of DON and deepoxy-DON in plasma treated with ß-glucuronidase were found to be 30.3 ng DON/mL (Lucy) and 19.8 ng deepoxy-DON/mL (Regina). The degree of conjugated-DON in the plasma was found to be 76.1 and 79.1 % for Twiggy and Lucy, respectively. In plasma, it seems that majority of DON is present in the glucuronide-conjugated form. The degree of conjugated deepoxy-DON was higher in Regina (70.6 %) compared to Twiggy (52.8 %).

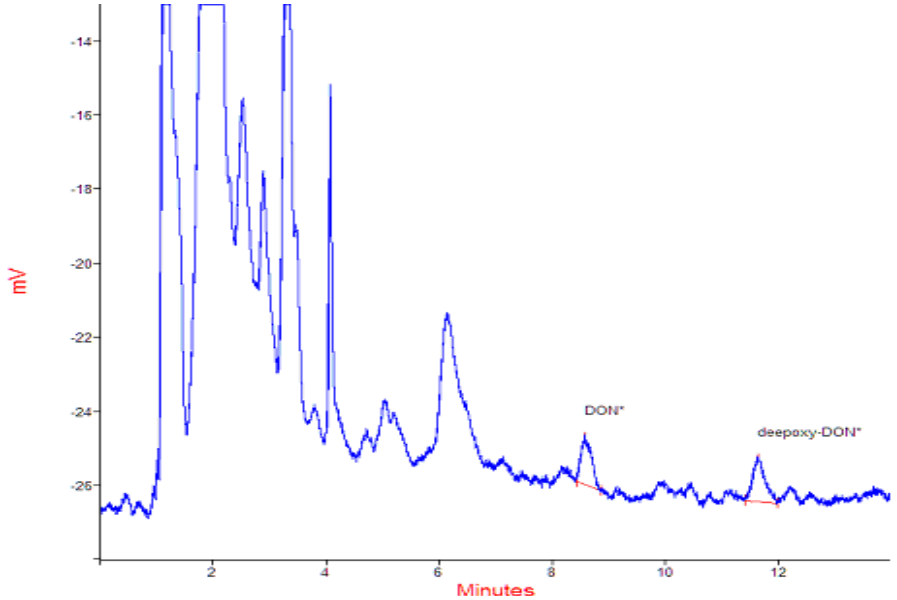

Figure 1 A representative HPLC-UV chromatogram of natural-contaminated plasma containing 4.2 ng DON and 4.1 ng deepoxy-DON/mL after clean-up using immunoaffinity column

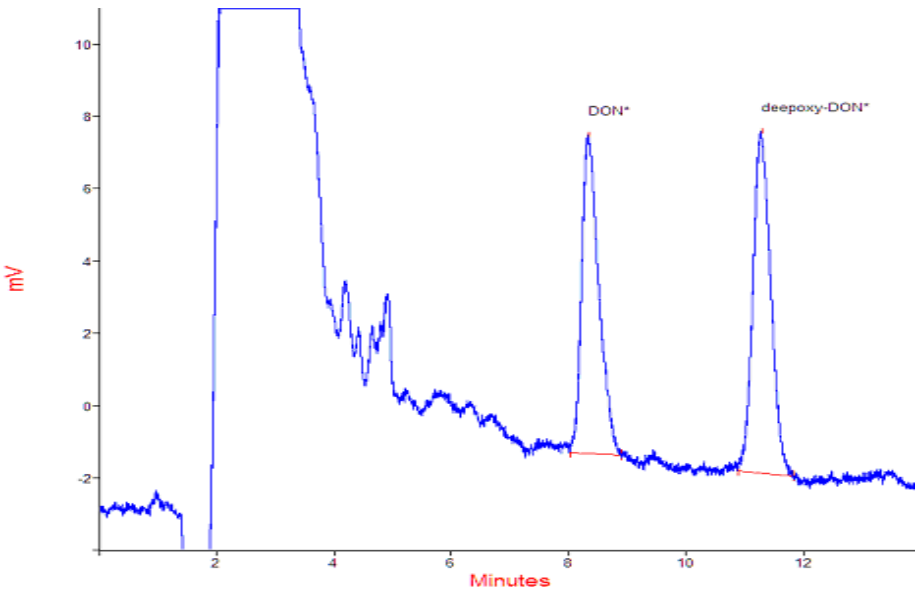

Figure 2 A representative HPLC-UV chromatogram of artificial-contaminated plasma containing 40.7 ng DON/mL and 43.2 ng deepoxy-DON/mL after clean-up using immunoaffinity column

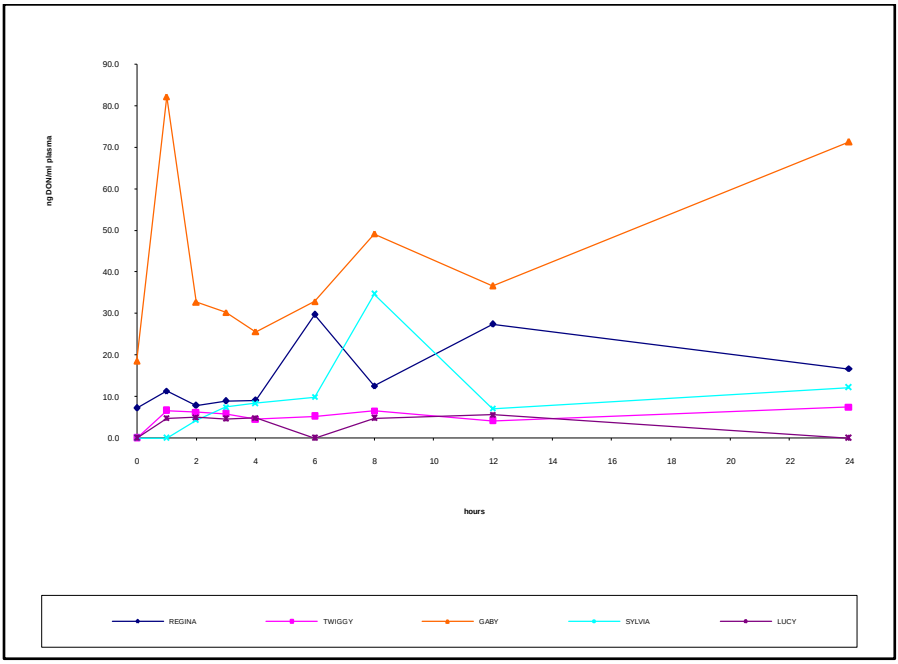

Figure 3 Concentrations of DON in plasma samples at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hours during day one

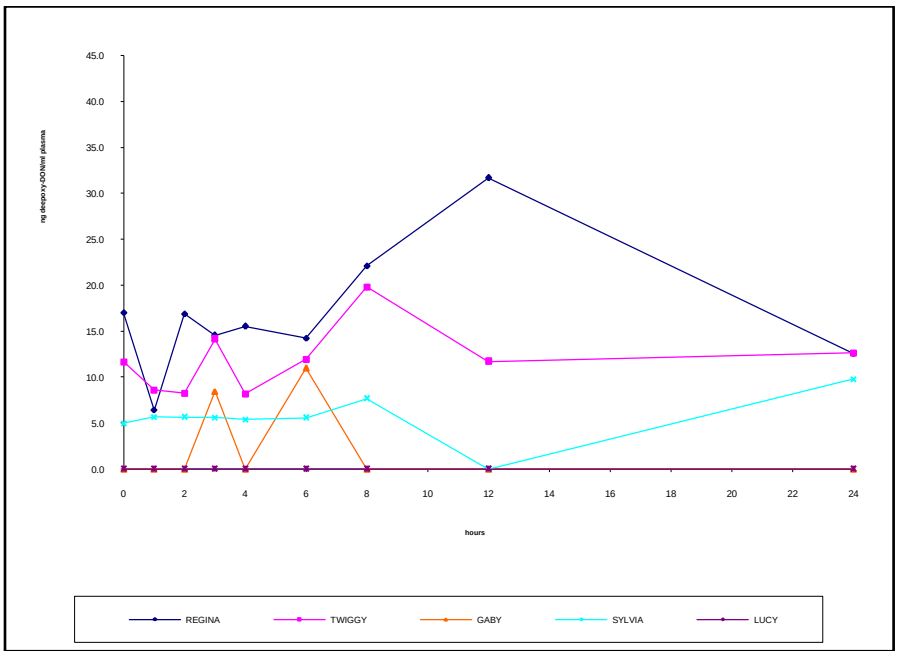

Figure 4 Concentrations of deepoxy-DON in plasma samples at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hours during day one

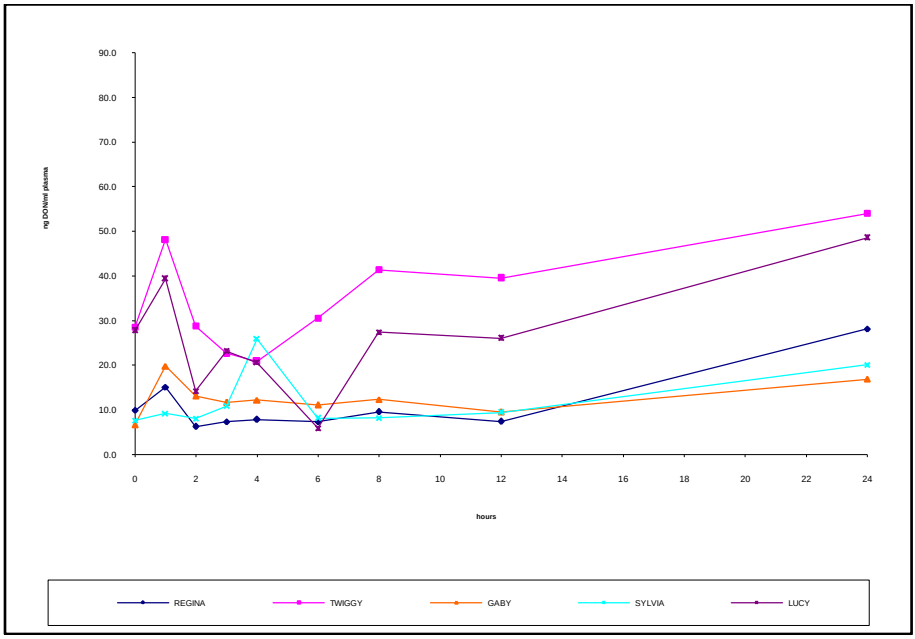

Figure 5 Concentrations of DON in plasma samples at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hours on day 10

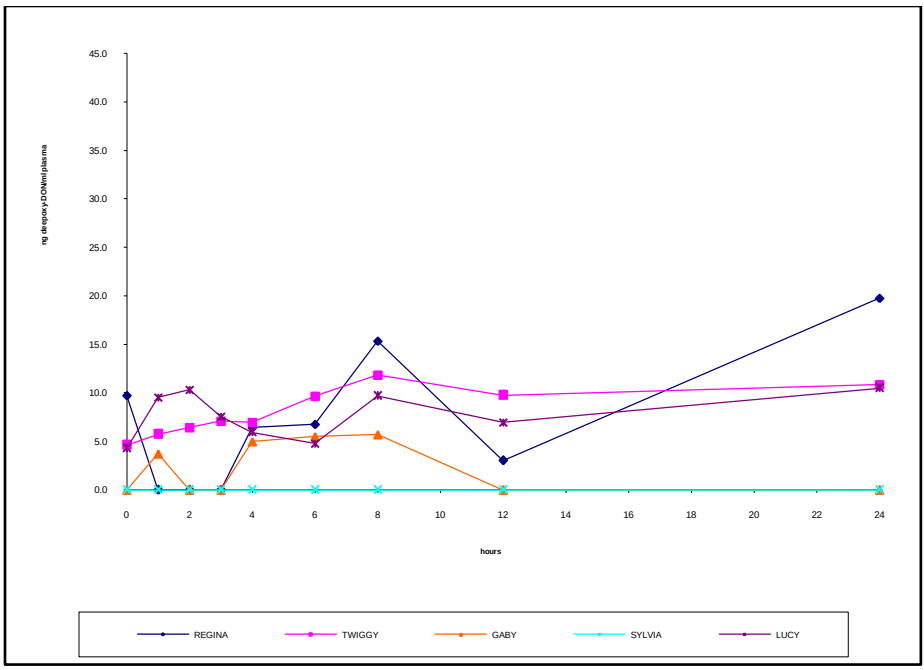

Figure 6 Concentrations of deepoxy-DON in plasma samples at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hours on day 10.

Table 1 Repeatability within-day and between-day of DON analysis in various level of spiked horse's plasma (n=3)

Parameter	10 ng/mL		25 ng/mL		50 ng/mL		100 ng/mL	
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
X_1 (ng/mL)	7.72	7.92	21.27	20.89	42.70	44.89	89.78	85.41
X_2 (ng/mL)	7.47	7.42	20.14	19.30	40.23	40.77	84.67	91.62
X_3 (ng/mL)	7.69	7.58	21.27	19.38	41.13	39.62	91.62	77.53
Mean values	7.63	7.64	20.89	19.86	41.35	41.76	88.69	84.85
SD	0.13	0.26	0.65	0.90	1.25	2.77	3.60	7.06
RSD within-day (%)	1.76	3.35	3.13	4.51	3.03	6.64	4.06	8.32
RSD between-day (%)	2.40		4.43		4.66		6.27	

Table 2 Repeatability within-day and between-day of deepoxy-DON analysis in various level of spiked horse's plasma (n=3)

Parameter	10 ng/mL		25 ng/mL		50 ng/mL		100 ng/mL	
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
X_1 (ng/mL)	8.07	7.67	20.89	20.56	40.82	42.54	86.41	87.95
X_2 (ng/mL)	6.56	6.76	18.55	19.95	41.87	43.21	88.32	88.44
X_3 (ng/mL)	7.84	6.98	19.70	19.18	40.77	43.98	80.69	79.82
Mean	7.49	7.14	19.71	19.90	41.15	43.24	85.14	85.40
SD	0.81	0.47	1.17	0.69	0.62	0.72	3.97	4.85
RSD within-day (%)	10.84	6.63	5.93	3.49	1.51	1.66	4.66	5.67
RSD between-day (%)	8.55		4.37		3.06		4.65	

Table 3 Summary of DON and deepoxy-DON recoveries in various level of spiked horse's plasma (n=6)

Concentration of analyte (ng/mL plasma)	Recoveries of analyte in spiked plasma samples	
	DON (%)	Deepoxy-DON (%)
10	76.3	73.2
25	81.5	79.2
50	83.1	84.4
100	86.8	85.3
Mean ± SD	81.9 ± 5.3	80.5 ± 6.4
RSD of recoveries (%)	6.4	7.9

Evidences showed that a high variation was found in the glucuronidation degree in blood samples from different animals. Prelusky et al. (1988) demonstrated that only small amounts of DON was present in glucuronide-conjugated form in pig's serum. According to Goyarts and Dänicke (2006), 9.1–60 % of total DON appeared to be conjugated in pig's serum, while Eriksen et al. (2003) observed that 42 % of total DON was in glucuronide-conjugated form. In another study, Dänicke et al. (2005a) found that degree of conjugation of DON in the serum was approximately 33 % ranging from

19 to 45 %. Prelusky et al. (1985) reported a difference in the glucuronidation of DON due to routes of administration, because plasma proportion of conjugated-DON was 73 % after oral gavage compared to 20 % after intravenous application to sheep reflecting the role of extra-hepatic tissues in the conjugation for DON. It has been found that concentration of deepoxy-DON was under the limit of detection in the serum samples of pigs experimentally fed a diet contaminated with DON (Dänicke et al., 2005b) which is in accordance to present study.

In conclusion, the problem of the less selectivity of HPLC-UV detector could be overcome by employing an immunoaffinity column clean-up. The immunoaffinity clean-up coupled with HPLC-UV seems to be an applicable method and successful for the quantification of DON and deepoxy-DON in blood.

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