Eur Food Res Technol (2008) 226:1107–1112
DOI 10.1007/s00217-007-0638-4

ORIGINAL PAPER

Analysis and occurrence of deoxynivalenol (DON) in cocoa

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Received: 20 December 2006 / Revised: 20 March 2007 / Accepted: 26 March 2007 / Published online: 26 April 2007

Abstract The primary objective of this study was to establish a current situation assessment of the possible occurrence of deoxynivalenol in cocoa and cocoa products. Since there was no analytic method for determining DON in cocoa and cocoa products, a special method was developed. The applicability and consistency of the method was confirmed by performing recovery assays on various cocoa products. A special post-column derivatisation procedure was used to increase selectivity and raise sensitivity by a factor of 80. The method's limit of detection (LOD) was thereby reduced to 7 µg/kg; the limit of quantification (LOQ) was 14 µg/kg. The method was used to test 230 samples for possible DON content, ranging from cocoa beans to cocoa bean shells, nibs, cocoa liquor and cocoa powders through to finished cocoa-based products. In the case of cocoa beans and cocoa bean shells, DON content close to the detection limit was only determined in isolated cases. No DON content was detected in nibs, cocoa liquor, cocoa powders and finished cocoa-based products. Analogous to ochratoxin A and aflatoxins, the results show DON is more likely to be found in cocoa bean shells. Separation of shells during cocoa processing can reduce potential DON contents. Since no DON was determined in the fractions relevant for chocolate production, these assays show it does not represent a considerable issue for the cocoa and chocolate industry.

Keywords Deoxynivalenol · Cocoa · Cocoa products · Post-column derivatisation · Method validation

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Introduction

Deoxynivalenol (DON) is one of a group of mycotoxins known as type B trichothecenes. These form a very extensive collection of over 170 mycotoxins whose key structural feature is a cyclic sesquiterpene framework with an epoxy ring (Fig. 1). In general most mycotoxins, as per DON is regarded as being extremely stable with respect to storage, technological processing, and the impact of higher temperatures. DON is particularly formed by the mould species *Fusarium* spp., particularly *Fusarium graminarium* and *F. culmorum*. DON was first isolated from mouldy barley in Japan in 1972 [1–4].

Fusarium toxins have a particular toxicological significance since they can harm the health of both animals and humans. Type B-trichothecenes are seen as the strongest currently known inhibitory substances affecting protein biosynthesis [5, 6]. Due to the nausea it creates, it is also known as vomitoxin (lat. vomito: to vomit) and consequently leads to a lack of growth in affected animals. The chronic intake of small amounts of trichothecenes leads to an increased susceptibility to infectious diseases as a result of the suppression of the immune system. However, based on the results of various animal experiments, a carcinogenic and teratogenic effect of DON can be ruled out. Various studies have shown that DON is to be classified as acutely toxic [1, 3, 6].

The frequency with which DON occurs in certain raw materials and the concentrations found make it one of the world's most significant mycotoxin contaminants [1, 2, 5]. Mycotoxin formation is particularly favoured by moist and warm weather during grain cultivation and the storage of grain possessing high moisture content levels. In addition, positive findings of the toxin have been established in cereal-based foods such as bread, noodles, and beer, as well as in oilseeds such as sunflower seeds, cashews, almonds, etc.

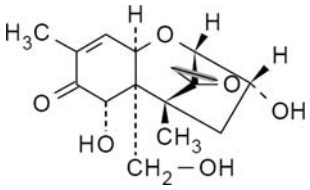

Fig. 1 DON

[7–12]. A higher DON content is to be expected in whole grain products since DON chiefly accumulates in the outer layers of the grain seed husks [13]. To date there are no findings on the occurrence of DON in cocoa and cocoa-containing products.

The objective of our work was to develop a suitable and sensitive method for determining DON in cocoa and cocoa-containing products and to examine a representative number of samples. A great number of HPLC methods have been described for quantitatively determining DON in grain and grain products [14–18]. Experience shows that a simple transfer of described standard testing rules to cocoa and products made thereof is not possible due to many analytical interfering substances. Within the scope of our work, various types of extraction (solid phase extraction and immunoaffinity extraction) and detection methods were tested and a very selective and sensitive method for determining DON in cocoa and cocoa-containing products was developed and validated.

Materials and methods

Samples

The around 230 examined samples comprised a total of 150 cocoa beans and semi-finished cocoa products (75 cocoa beans, 23 cocoa bean shells, 16 cocoa nibs, 20 cocoa powders [partially alkalised], 16 cocoa liquors) from various origins (Africa 98, Asian-Pacific Region 20, Central and South America 32) and 79 cocoa-containing finished products (chocolates, chocolate products, cocoa drink powders, chocolate coatings). Depending on the respective matrix, the sample material weighed between 500 and 1,000 g per sample. Larger sample quantities (<500 g) were divided into subsamples using a Retsch sample divider PT 100 or a Retsch sample splitter RT (Haan, Germany).

Definition of cocoa products (*Theobroma cacao* L.)

Cocoa beans (fermented and dried seeds) are bean-shaped and comparable in size to an almond. They possess a thin shell (cocoa bean shell, seed coat e.g. testa and the remaining pulpe); cocoa nibs (cotyledones without seed coats) are the roasted, shell-free, mostly already broken up cocoa bean kernels; cocoa liquor is made up of finely ground cocoa nibs; cocoa powder is the finely ground cocoa press cake which remains after pressing out the cocoa liquor.

Solvents and reagents

DON standard solution (c = 100 µg/mL) was obtained from Coring System Diagnostix GmbH (Gernsheim, Germany), all other chemicals and solvents were analysis-grade or HPLC-grade and were obtained from Merck (Darmstadt, Germany). Sample clean up was carried out using MycoSep® DON solid phase extraction columns from Coring System Diagnostix GmbH (Gernsheim, Germany) and DONPREP® immunoaffinity columns from R-Biopharm (Darmstadt, Germany).

Standard preparation

The standard solution of DON was diluted to a concentration of 10 µg/mL, this solution was further diluted to 1,000, 500, 100, 50 ng/mL using acetonitrile/water (10/90, v/v).

Sample preparation

Finely ground cocoa and cocoa products (5 g) were weighed and extracted in a mixture of 20 mL of acetonitrile/water (84/16, v/v). The suspension was blended for 1 min at high speed using an Ultra-Turax T25 homogenizer (IKA-Werke, Staufen, Germany). This homogenized solution was centrifuged after cooling (T = 4 °C, 4,000 rpm).

Solid phase extraction (SPE)

An aliquot of the supernatant extract was pipetted into a culture tube of the SPE column (MycoSep® DON column) and the extract was purified by pushing the solid phase through the unpurified sample. The purified extract was evaporated and the residue dissolved in mobile phase acetonitrile/water (8/92, v/v) and injected into the HPLC.

Immunoaffinity clean up (IAC)

For immunoaffinity clean up, 2 mL of the supernatant extract was diluted with 40 mL PBS puffer and the solution was applied to a DONPREP® immunoaffinity column at a flow rate of 2–3 mL/min. After the column had been washed with 10 mL of distilled water, DON was eluted using 3 mL acetonitrile. The extract was evaporated to dryness at 40 °C and the residue was redissolved in 1 mL mobile phase.

HPLC/ultraviolet Detection (UVD)

The HPLC system consisted of a P580 A LPG-pump with a UVD 170 S UV-Detektor (Dionex, Germering, Germany).

Chromatographic separations were performed on an Inertsil ODS-2, 5 µm, 3 × 250 mm i.d. at 35 °C, with a mobile phase of acetonitrile/methanol/water (5/5/90, v/v/v) at a flow rate of 500 µL/min. The injection volume was 100 µL for UV detection at 218 nm.

Post-column derivatisation/fluorescence detection (FD)

Because DON has no fluorescence of its own, it can be derivatised post-column into a fluorescent derivate by means of the so called Hantzsch-synthesis [15]. The HPLC system consisted of a P580 A LPG-pump with a PCX 5200 post-column derivatisation instrument (Pickering, Mountain View, CA, USA), and an RF 2000 fluorescence detector (Dionex, Germering, Germany). Separations were performed on an Inertsil ODS-2, 5 µm, 3 × 250 mm i.d. at 35 °C, with a mobile phase of acetonitrile/water (8/92, v/v) at a flow rate of 400 µL/min. The derivatisation reagents were sodium hydroxide (c_{NaOH} = 0.15 mol/L) and 2 M ammonium acetate (c = 2 mol/L) with methyl acetoacetate (c = 0.03 mol/L). The injection volume was 100 µL. Excitation wavelength was 360 nm and emission wavelength was 470 nm.

Method validation

The post-column-derivatisation/fluorescence-detection method was validated for the following parameters: linearity, recovery, and detection and quantification limits. Recovery and linearity was obtained using the addition method. Cocoa samples (cocoa beans, shells, mass and powder) were spiked with 0 µg/kg–500 µg/kg DON standard. Quantification and detection limits were determined by duplicate injections of spiked cocoa samples based on single-to-noise (S/N) ratios of 10:1 for the quantification limit and 5:1 for the detection limit.

Results and discussion

Method optimisation and method performance

Tests were carried out to see whether sample clean up could be carried out using solid phase extraction columns (MycoSep® DON) and immunoaffinity columns (DON-PREP®) for quantitatively determining DON in cocoa and cocoa products. Figure 2 shows a typical fluorescence chromatogram of a sample extraction using SPE and IACs. Solid phase extraction proved to be insufficiently selective for a matrix containing such a high level of interfering substances as that of cocoa; by contrast, the immunoaffinity column clean up showed positive, reproducible results.

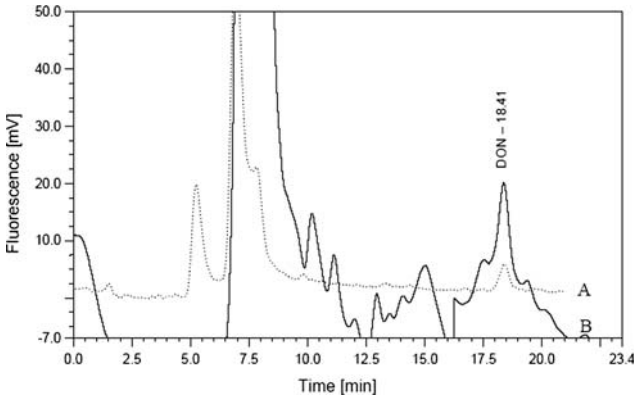

Fig. 2 HPLC chromatogram comparison: sample preparation of a spiked cocoa powder sample (50 ng/g) using IACs [A] and using MycoSep® [B]

Figure 3 shows, by comparison, the detection methods we tested, namely UV detection and fluorescence detection with preceding post-column derivatisation. In comparison to UV detection, fluorescence detection after derivatisation is significantly more selective in testing cocoa powder. Matrix-related interfering substances are hardly detected any longer. The retention time shift, which occurs here is due to the additional path the sample takes through the derivatisation instrument.

In addition to boosting selectivity, the use of post-column derivatisation technology also led to a significant increase in the sensitivity of the method. Derivatisation, in comparison to UV detection, increases the sensitivity of determining the mycotoxin DON by a factor of 80 (cf. Fig. 4). The limit of quantification (LOQ) of the method for sample matrices such as cocoa powder was set at 14 µg/kg, based on an S/N ratio of 10:1, and the limit of detection (LOD) was set at 7 µg/kg, based on an S/N ratio of 5:1.

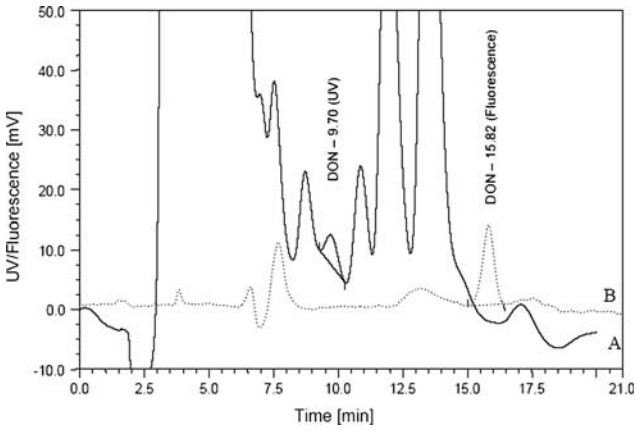

Fig. 3 HPLC chromatogram comparison: spiked cocoa powder sample (150 ng DON/g) without derivatisation and UV detection [A]; spiked cocoa powder sample (150 ng DON/g) with post-column derivatisation and fluorescence detection [B]

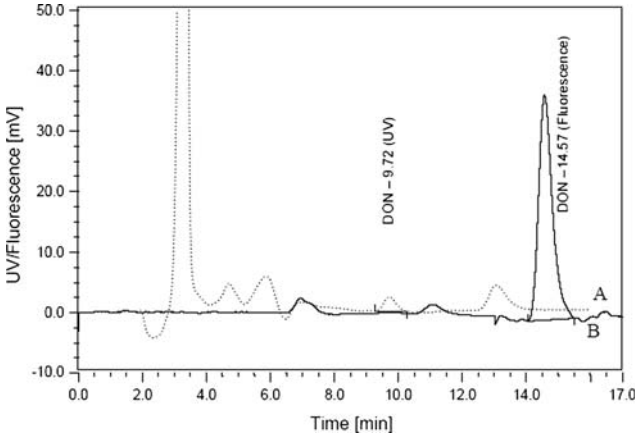

Fig. 4 HPLC chromatogram comparison: 1 µg/mL DON standard solution without derivatisation and UV detection [A]; 1 µg/mL DON standard solution with derivatisation and fluorescence detection [B]

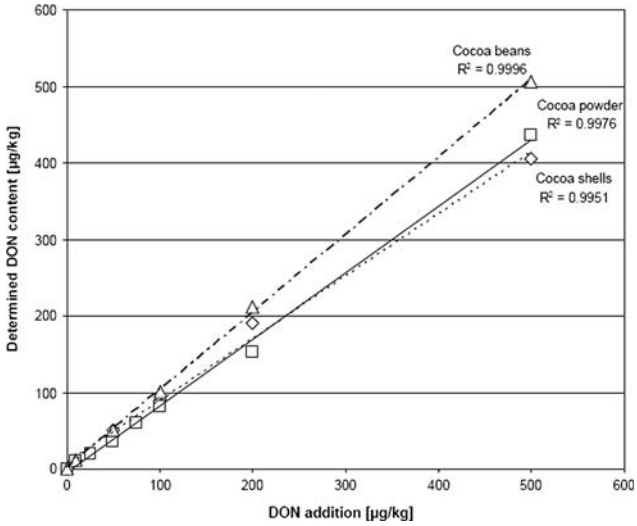

Fig. 5 Linearity range of determining DON in cocoa products (R^2: coefficient of determination)

Method validation

The optimised method for determining DON using IACs and fluorescence detection after derivatisation was validated using four different matrices, namely cocoa bean shells, cocoa powder, cocoa beans, and cocoa liquor. The samples were spiked with defined content levels of DON and prepared and analysed using the optimised extraction method. The results are shown in Table 1. The determined average recovery rates fluctuated between 81% (cocoa powder) and 103% (cocoa beans) with relative standard deviations of between 4 and 13%. The linearity of the method was validated for a concentration range of between 0 and 500 µg of DON per kg of cocoa product (cf. Fig. 5).

Analysis of cocoa product samples

The primary objective of this study was to establish a current situation assessment of the possible occurrence of DON in cocoa and cocoa products. The cocoa bean samples used for testing purposes were selected according to the respective German import shares of their places of origin [19]. A total of 230 samples was tested for possible DON content using the newly developed and optimised method for determining DON in cocoa and cocoa-containing products. The main focus of testing was on cocoa beans (mouldy and unmouldy), cocoa bean fractions, cocoa powders, cocoa liquors and finished cocoa-based products (chocolate, chocolate products, cocoa drink powder, etc.).

Table 2, Figs. 6 and 7 shows the distribution of DON content in the tested cocoa samples, separately listed by product group. DON content above the LOD was determined in 11 of the 75 examined cocoa bean batches. Only one sample had a content level above LOQ, showing a level of 18 µg/kg. Astonishingly the tested, visibly mouldy cocoa beans contained no DON.

Table 3 indicates a comparison of DON contents in the tested cocoa bean batches, shown by continent. This depiction creates the impression that cocoa beans from America are more likely to be contaminated with DON than cocoa beans from Africa and Asia. Given the rather low number of analysed samples used in testing for mycotoxins and going by our experience, this is merely a preliminary but not validated result and should be further examined by taking a larger total number of samples.

Of the tested cocoa bean shell samples, two batches were found to have DON content levels above the LOD. Only one batch was found to have a DON content level distinctly above the LOQ, showing a level of 110 µg/kg. No DON content was detected in cocoa nibs, cocoa liquors cocoa powders and finished cocoa-based products.

Table 1 Results of recovery assays in determining DON in cocoa products

	Spiking range (ng/g)[a]	Average recovery (%)	RSD (%)
Cocoa shells	10–500	96	13
Cocoa beans	10–500	103	4
Cocoa liquor	10–50	97	11
Cocoa powder	10–500	81	9

RSD relative standard deviation

[a] Double determination of four different standard additions

Conclusions

The results suggest that DON may occasionally occur in cocoa beans in very low concentrations. Since no DON

Table 2 Distribution of DON content in the cocoa product samples

Product	*N*	DON (µg/kg)				
		N > LOD (%)	Max	Mean	Median	90th percentile
Cocoa beans	75	11 (15)	18	10	8	12
thereof mouldy	12	0	–	–	–	–
Cocoa bean shells	23	2 (9)	110	60	60	100
Cocoa nibs	16	0	–	–	–	–
Cocoa liquor	16	0	–	–	–	–
Cocoa powder	20	0	–	–	–	–
Finished cocoa-based products	79	0	–	–	–	–

LOD: 7 µg/kg, LOQ: 14 µg/kg, In brackets(): distribution percentage

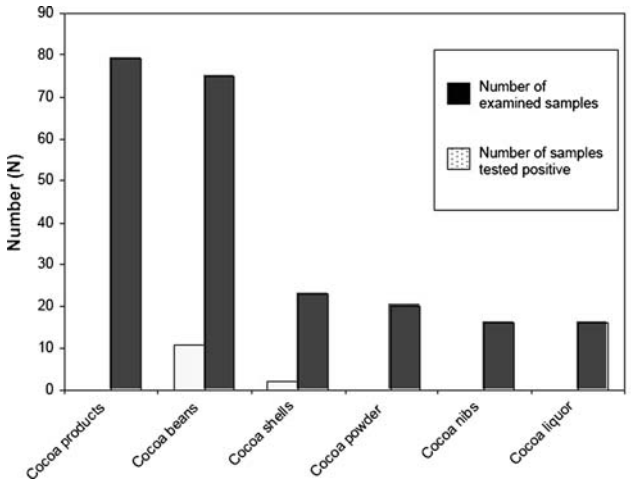

Fig. 6 Proportion of samples that tested positive for DON from the total number of examined samples

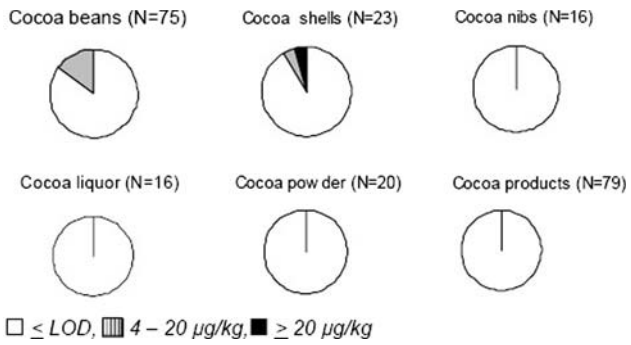

Fig. 7 Distribution of DON content in the cocoa samples and cocoa products; DON values ≤LOD are included in the valuation at half the LOD (4 µg/kg), values between LOD and LOQ are included at half the LOQ (7 µg/kg)

content was found in the tested nibs, DON seems to be largely localised in the outer layers (shell) of the cocoa bean, as are the aflatoxins and OTA [3, 20]. Compared to other foodstuffs (e.g. [21]), DON only occurs very rarely in cocoa, and if so, only in very low concentrations. Our test results allow the conclusion that DON poses no problems for the cocoa and chocolate industry.

Table 3 Comparison of DON content distribution in the tested cocoa bean batches, shown by place of origin

Origin	*N*	DON (µg/kg)				
		N > LOD (%)	Max	Mean	Median	90th percentile
Africa	41	3 (7)	7	–	–	–
America	19	7 (37)	18	11	9	14
Asia	15	1 (7)	7	–	–	–

LOD: 7 µg/kg, LOQ: 14 µg/kg

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