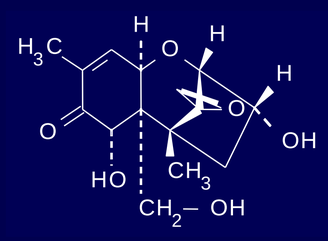
Sensible Method for Determination of DON in Cocoa by means of HPLC-Techniques



LCI Köln

Marion Raters and Reinhard Matissek

LCI
Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie (BDSI) e. V.,
Adamsstr. 52-54, 51063 Köln, www.lci-koeln.de

Introduction

Deoxynivalenol (DON) is one of a group of mycotoxins known as type B trichothecenes and is particularly formed by the mould species *Fusarium* spp., particularly *Fusarium graminarium* and *Fusarium culmorum.* 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]. Positive findings of the toxin especially 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. [2-4].

The primary objective of this study was to establish a current situation assessment of the possible occurrence of DON 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 developed to increase selectivity and raise sensitivity by a factor of 80 (see figure 1-4).

Methods

Sample extraction

10 g sampling material

+ 40 ml acetonitrile/H2O (84/16, v/v)

mix for 1 min. using an Ultraturrax

Clean up / Isolation of DON

centrifuge (4 °C, 4,000 rpm, 10 mins.), bring to room temperature

+ 2 ml filtrate + 38 ml PBS buffer (pH 7.4)

deposit on the immunoaffinity columns and rinse with 10 ml H_2O

detach columns + vacuum dry to the greatest extent practicable

desorption of DON using 3 ml acetonitrile

evaporation of solvent and dissolve in mobile phase

HPLC

UV-Detection

column:	Inertsil ODS 2; 5 µm; 250 x 3 mm
mobile phase:	acetontrile/methanol/water 5/5/90 (v/v/v)
flow:	0.5 ml/min isocratic
injection vol:	100 µl
Detection:	UV-ViS, 218 nm

Fluorescence-Detection
(after post-column derivatisation)

column:	Inertsil ODS 2; 5 µm; 250 x 3 mm
mobile phase:	acetontrilel/water 8/92 (v/v)
flow:	0.4 ml/min isocratic
injection vol:	100 µl
derivatisation	0,15 M NaOH and 2 M NH_4AC with 0,03 M
reagent:	Methylacetoacetat
detection:	λ_{EX} = 360 nm and λ_{EM} = 470 nm

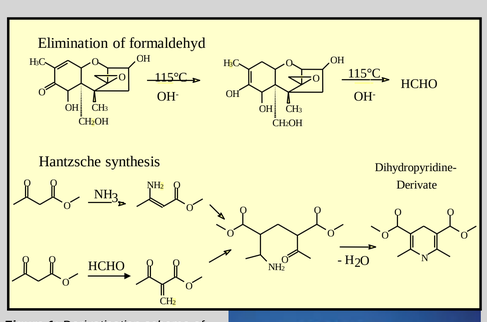


Figure 1: *Derivatisation scheme of DON [5]*

Chromatograms

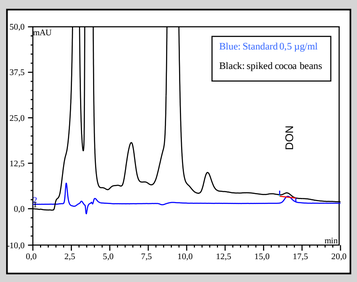


Figure 2: HPLC chromatogram comparison: Spiked cocoa bean sample (500 ng DON/g) [black] and standard solution (0,5 µg/ml) without derivatisation and UV detection [blue]

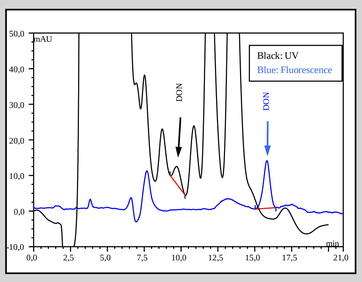


Figure 3: HPLC chromatogram comparison: Spiked cocoa powder sample (150 ng DON/g) without derivatisation and UV detection [black], and with post-column derivatisation and fluorescence detection [blue]

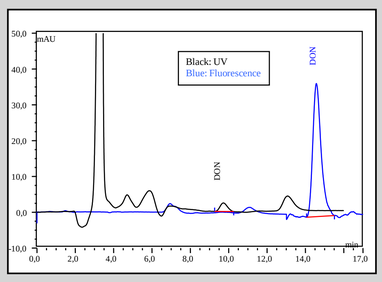


Figure 4: HPLC chromatogram comparison: 1 µg/mL DON standard solution without derivatisation and UV detection [black]; and with derivatisation and fluorescence detection [blue]

Method Performance/Validation

The optimised method for determining DON using IACs and fluorescence detection after derivatisation was validated using three different matrices, namely cocoa bean shells, cocoa powder, and cocoa beans. The samples were spiked with defined content levels of DON, 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 µg and 500 µg of DON per kg of cocoa product (cf. Figure 5).

Table 1: *Performance comparison of DON analytics*

	UV-VIS	Fluorescence (after derivatisation)
Linearity Range	-	14-500 µg/kg
Recovery	81-103 %	81-103 %
RSD	4-13 %	4-13 %
LOD	500 µg/kg	7 µg/kg
LOQ	1,000 µg/kg	14 µg/kg

- Not calculated
* double determination of four different standard additions
RSD: relative standard deviation

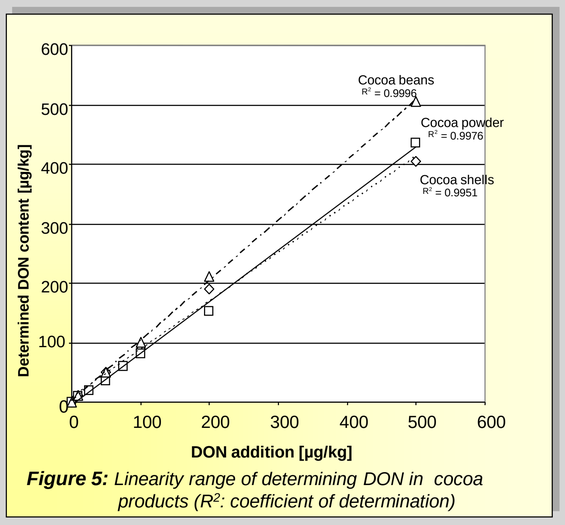


Figure 5: *Linearity range of determining DON in cocoa products (R^2: coefficient of determination)*

DON in Cocoa?

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 and figure 6 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. Of the tested cocoa bean shell samples, 2 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 2: *Distribution of DON content [µg/kg] n the cocoa product samples*

Product	N	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

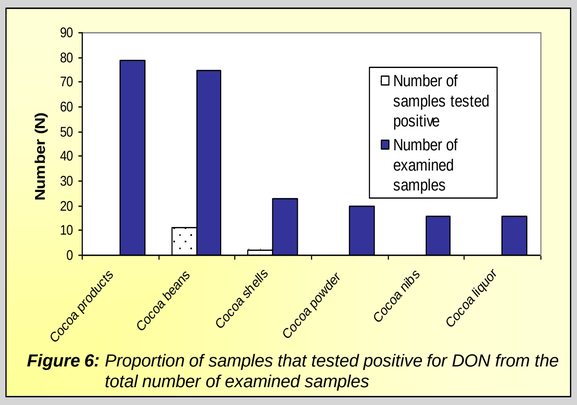


Figure 6: *Proportion of samples that tested positive for DON from the total number of examined samples*

References

[1] Trucksess M (1995) General referee reports-committee on natural toxins, mycotoxins, J AOAC Int 78: 135-141
[2] Müller HM, Reimann J, Schumacher U, Schwadorf K (1997) Fusarium toxins in wheat harvested during six years in an area of southwest Germany, Natural Toxins 5: 24-30
[3] Krska R (1999) Analytik von Fusarium-Mykotoxinen in Europa. Nachr Chem Tech Lab 47: 553-556
[4] Tanaka T, Hasegawa A, Yamamoto S, Lee U-S, Sugiura Y, Ueno Y (1988) Worldwide Contamination of cereals by the Fusarium Mycotoxins Nivalenol, Deoxynivalenol and Zearalenol – 1. Survey of 19 Countries. J Agric Food Chem 36: 979-983
[5] Bossert F, Meyer H, Wehinger E (1981) 4-Arylhydropyridines, a new class of highly active calcium antagonists, Angew Chem 93: 762-770

Conclusion

Since there was no analytic method for determining DON in cocoa and cocoa products, a special method by means of post-column derivatisation and fluorescence detection was developed. The applicability and consistency of the method was confirmed by performing recovery assays on various cocoa products. The method's limit of detection (LOD) was thereby reduced by a factor of 80 to 7 µg/kg; the limit of quantification (LOQ) was 14 µg/kg.

The results suggest that DON may occasionally occur in cocoa beans in very low concentrations. Since no DON 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. Compared to other foodstuffs, 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.

This project was backed by the Foundation of the German Cocoa and Chocolate Industry, Hamburg