LCTech

DONeX™

Deoxynivalenol Clean-Up Columns
3 mL Format

User Manual

Content

1. Introduction

Deoxynivalenol (DON; Figure 1) is a naturally occurring mycotoxin, which is toxic for humans and animals. The Fusarium fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia (European Commission HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL, 1999).

Deoxynivalenol (Vomitoxin) is a mycotoxin produced by fungi of the Fusarium genus, e. g. Fusarium culmorum and Fusarium graminearum, which are abundant in various cereal crops (wheat, maize, barley, oats and rye) and processed grains (malt, beer and bread). Chemically it belongs to the trichothecene type toxins. In contaminated cereals 3- and 15-acetyl DON may occur in significant amounts (10 – 20 %). The fungi producing trichothecenes are soil fungi and are important plant pathogens which grow on the crop in the field (cited in Eriksen and Alexander, 1998). The uptake of DON leads to reduced growth and is associated with higher risk against infection. Biological data refers to various effects of DON on the cellular and subcellular level.

DON inhibits the synthesis of DNA and RNA and protein synthesis at the ribosomal level. The toxin has a haemolytic effect on erythrocytes. An acute dose of DON can induce vomiting (emesis) in pigs, whereas at lower concentrations in the diet it reduces growth and feed consumption (anorexia). Both effects, which are also seen with other trichothecene toxins, are thought to be mediated by affecting the serotonergic activity in the CNS or via peripheral actions on serotonin receptors (Rotter et al., 1996; Eriksen and Alexander, 1998).

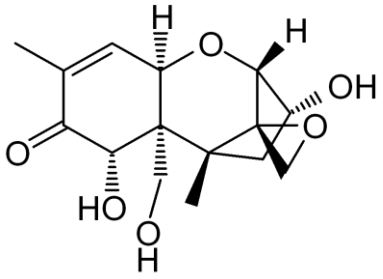


Figure 1: Deoxynivalenol

Due to the toxic effects, the European Community (EC) has set maximum contaminant levels (MCL) in several matrices (Table 1) in the Commission Regulation (EC) No 1126/2007.

Those limits for Deoxnivalenol (DON) are as follows:

Table 1: Maximum contaminant levels (MCL) for Deoxynivalenol in corresponding matrices as regulated by the European Community

** The exemption applies only for maize for which it is evident e.g. through labelling, destination, that it is intended for use in a wet milling process only (starch production)*

Matrix	MCL DON [µg/kg]
Unprocessed cereals other than durum wheat, oats and maize*	1250
Unprocessed durum wheat and oats	1750
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	1750
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed below (**)	750
Pasta (dry)	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based foods and baby foods for infants and young children	200
**Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	750
**Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	1250

2. Information about DONeX

Figure 2: DONeX

The clean-up column DONeX (Figure 2) has been developed for sample clean-up within the food analysis via HPLC with fluorescence detection in combination with highly sensitive post-column derivatization or detection by LC-MS. Alternatively the samples could be measured by UV detection, respectively. The column was developed to reduce matrix interferences to overcome long chromatography and disturbing matrix peaks.

The material within the columns is retaining matrix components and interfering substances, whereas the toxin flows through.

Deoxynivalenol and Nivalenol are passing the column with the same efficiency. The column bed consists of a dry material. The following recoveries of a standard will be obtained when the provided LCTech protocols are applied.

The maximum loading capacity is the equivalent of 4 g matrix. Store the columns dry and at room temperature.

Since the bed material and columns are produced by LCTech, numerous in-house quality tests during the whole production process guarantee a persistent quality. The final release of the columns for sale is done with the quality control test described here:

An equivalent of 4 g matrix is extracted with the extraction solvent and spiked with 1 ppm. Then the extract is loaded onto the column and afterwards the column and sample reservoir are washed as described. The flow-through and wash fraction are collected and pooled. An aliquot of the sample is evaporated to dryness and dissolved in HPLC solvent. The sample is compared to the standard to determine the recovery rate and chromatographic results.

Exemplary recovery rates of various matrices are shown in the following:

Table 2: Various matrices with recoveries (Rec.)

** All matrices were spiked prior to the extraction. Spiking solution was incubated for at least 1 h with matrix before extraction was started. Calculation of recovery is based on subtraction of values obtained without spiking.*

Matrix	Rec [%] DON
Bread	108
Corn	90
Chicken feed	101
Distillers grain	91
Oat flakes	100
Pasta (dried)	105
Poultry feed (cereal based)	100
Rice	104
Rye	91
Wheat	100
Wheat bran	99

Recoveries for Nivalenol are similar to those observed for Deoxynivalenol.

3. Reagents

In the following the chemicals needed are listed and explicitly described. All chemicals used must be of analytical grade; in particular the suitability for fluorescence measurements must be assured!

- Acetonitrile p. a.
- Water HPLC grade.
- Acetic acid (96%) p. a.
- Extraction solution; acetonitrile/water (84/16, v/v)
- HPLC eluent: acetic acid (0,01 M)/acetonitrile (90/10, v/v)
  (using post-column derivatization)
- Ammonium acetate (≥ 98 %)
- Methyl acetoacetate (≥ 99 %)
- Nitrogen for evaporation

4. Apparatus

- DONeX columns (P/N 12792)
- According to the amount and texture of the material to be homogenized, appropriate tools should be used, such as kitchen mixers, lab mills
- Ultraturrax or stirrer for extraction (depending on the extraction tools the extraction time has to be evaluated)
- Beakers 25 mL, 100 mL
- Plaited filters with funnel
- Vacuum manifold (P/N 11048) or peristaltic pump
- 30 mL sample vials (P/N V0030)
- Sample preparation vials with screw cap and sealing ring for DONeX (P/N 10896) compatible with vacuum manifold (P/N 11048) (Figure 3)
- Pipettes 10 mL
- Pipettes 1.0 mL
- Measuring cylinder (100 mL)
- HPLC system consisting of autosampler with injector, pump, column oven,
- and fluorescence detector; PC with chromatography software
- Pickering Laboratories post-column derivatization system PINNACLE PCX (P/N 1153-1072)

Figure 3: LCTech offers the vacuum manifold EluVac (P/N 11048) for sample preparation with various clean-up columns.

Also shown here: the practical sample reservoirs

5. Extraction and Clean-up with DONeX Clean-up Columns

ATTENTION! In order to handle the procedure successfully, the following instructions must be kept!

Matrix load:	**In general 4 g matrix/column should not be exceeded**
Matrix load/sample volume:	**max. 4 g matrix/20 mL crude extract**
Sample volume:	**20 mL/column (lower sample volume increases the risk of concentration changes due to adsorption of solvent by the column bed material, in those cases an prior equilibration of the column with 84/16 acetonitrile/water might be helpful)**

Check that the column did not adsorb humidity due to wrong storage conditions. The column performance could be reduced.

Finally, the necessary matrix amount is determined by the sensitivity of the detection (preferably with fluorescence and post-column derivatization or LC-MS or UV) and the further processing after the elution (concentration or dilution, injection volume).

To ensure a successful processing of your samples, please refer to the chapter “Important Parameters and Troubleshooting”!

Table 3 shows an overview about the matrix loads of various matrices:

Table 3: Various matrices, matrix load/column

** All matrices were spiked prior to the extraction. Spiking solution was incubated for at least 1 h with matrix before extraction was started. Calculation of recovery is based on substraction of values obtained without spiking.*

Matrix	Ladung [g]
Barley	4
Corn	4
Durum wheat	4
Wheat	4
Wheat bran	4
Rye	4
Breakfast cereals (containing nuts)	4/2
Bread	4/2
Rice	4
Oat flakes	4/2
Pasta (dried)	4
Poultry feed (cereal based)	4
Distiller’s grain	4
Chicken feed	4

This procedure is recommended, if no interferences from matrix compounds are expected, as this might be the case for most cereals (e.g. wheat, corn, barley, durum, rice, oat).

For matrices containing high sugar concentration a reduced matrix amount is recommended.

10 g of a thoroughly homogenized sample is extracted with 50 mL of the extraction solution (acetonitrile/water, 84/16, v/v) in a blender jar at high speed, e.g. with an Ultraturrax. For using a magnetic stirrer a 30 min extraction was used with excellent recoveries.

Pass the extract through a plaited filter.

20 mL of the filtered extract is applied on the DONeX clean-up column by a maximum flow rate of 10 mL/min. Attention: High flow rates provoke back pressure.

The flow-through must be kept for further analysis, because it contains the toxin.

The sample flow through the column could be achieved by light overpressure or using a vacuum manifold.

The sample reservoir is washed with 10 mL acetonitrile/water (84/16, v/v) and the washing solution is applied on the column the flow-through is pooled with the first sample and mixed homogeneously.

Evaporate an aliquot (e.g. 7.5 mL representing 1 g of matrix) of the flow-through to dryness and dissolved in an appropriate amount of the HPLC solvent.
Dilute the final sample to your requirements and measure directly by HPLC

6. Post-Column Derivatization

After separation on an HPLC column (see next chapter) deoxynivalenol can be measured by sensitive and specific techniques, either fluorescence or LC-MS or by UV detection, the latter one being less sensitive.

Due to the partially low values for tolerable limits of deoxynivalenol in food and the low intrinsic fluorescence of the toxin, it is recommended to use post-column derivatization for the detection. Thus the sensitivity of the measurement is significantly enhanced and in combination with state-of-the-art HPLC systems and fluorescence detectors, respectively the specificity of the analysis could be significantly increased. Furthermore lower matrix loads can be applied in general, yielding in an optimized and faster overall process.

In order to be able to measure DON at low ppb levels, it is recommended to use post-column derivatization, which in this case has a double specificity.

Figure 4: PINNACLE PCX, system for post-column derivatization

6.1. Method Description

Deoxynivalenol and Nivalenol are subsequently separated on a reversed phase column and derivatized via the PINNACLE PCX in a two-step reaction (see reaction scheme below, Figure 5). Technically the trichothecene is cleaved to formaldehyde in the first step. In the second step formaldehyde reacts with ammonium acetate and methyl acetoacetate in a Hantzsch reaction to a fluorescing lutidine derivative. Afterwards the measurement is performed with fluorescence spectrometry.

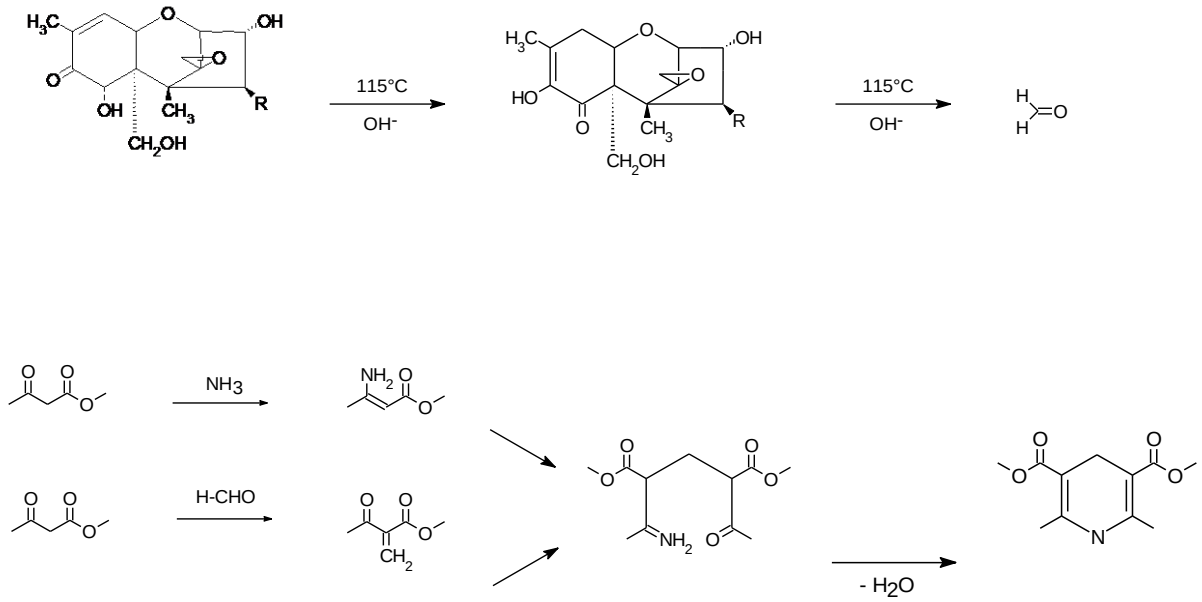


Figure 5: Reaction scheme of the derivatization of Deoxynivalenol to a fluorescent compound

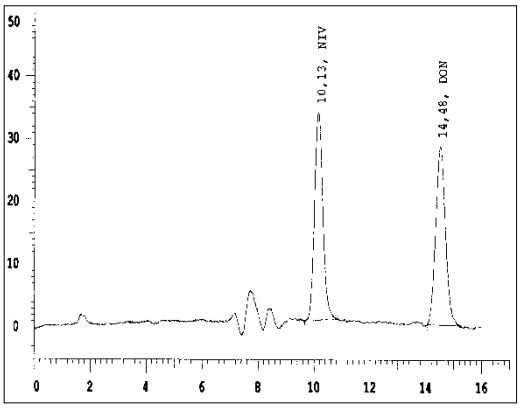


Figure 6: Chromatogram of a DON/NIV standard (50 ng each)

6.2. Parameters for Post-Column Derivatization

Post-Column Derivatization	
Pinnacle PCX	Dual pump
Column oven	35 °C
Reactor volume	Special reactor
Reactor temperature	115 °C
Reagent 1	0.15 N NaOH
Reagent 2	2 M ammonium acetate, 0.03 M methyl acetoacetate
Reagent flow	0.3 mL/min

The PINNACLE PCX needed for the derivatization of the deoxynivalenol is offered by LCTech (P/N 1153-1072)

7. HPLC Analysis

For the Deoxynivalenol analysis an isocratic HPLC can be used. Thus the analysis can be run without expensive eluent and temperature gradients, respectively. Independently thereof it is advantageous to use a fluorescence detector model which is state-of-the-art in combination with post-column derivatization to increase sensitivity and specificity.

7.1. HPLC Conditions and Parameters for Fluorescence Detection

The following conditions and settings, respectively, can be used as a first indication:

Eluent:	Acetic acid (0.01 M) / acetonitrile 90/10 (v/v)
Flow rate	1.0 mL/min
Injection volume	10 – 100 µL
HPLC Column	150 x 4.6 mm; RP C18 (P/N 10522) with guard column 8 x 4 mm (P/N 10523; holder P/N 10750)
Column temperature	33 °C
Excitation wavelength	360 nm
Emission wavelength	470 nm

7.2. HPLC Conditions for UV Detection

The Deoxynivalenol could be detected using HPLC and UV. Clean-up of the toxin using the clean-up column DONeX enables detection of Deoxynivalenol in various matrices in the range from approx. 0.5 ppm to above 10ppm. For analysis of low amounts of toxin at least 3-4 gram matrix equivalents should be prepared by evaporation.

The following conditions and settings, respectively, can be used as a first indication:

Eluent:	HPLC water / acetonitrile 95/5 (v/v)
Flow rate	1.0 mL/min
Injection volume	100 µL
HPLC Column	150 x 4.6 mm; RP C18 (LCTech P/N 10522) with guard column 8 x 4 mm (P/N 10523; holder P/N 10750)
Column temperature	33 °C
UV detector	218 nm

8. Important Parameters and Troubleshooting

As with every procedure, sources of errors or possibilities of errors may occur during the mycotoxin analysis. These are starting with the sampling and end with the evaluation of the obtained chromatographic data.

For the sampling please refer to the corresponding regulations.

In case of a malfunction of the HPLC or post-column derivatization system contact the service of the respective supplier.

In the following some possible reasons or parameters are discussed, which may lead to suboptimal results for the DONeX column.

- **Weighing, Concentration, Calculation Errors and Mistake**
  First of all, the materials used have to be checked. Were new solutions or chemicals used? Are the used chemicals indeed the correct ones?
  Are the used measuring devices correct? Was a new protocol applied or were new calculations conducted? Did a new coworker do the job?

- **DONeX - Shelf Life and Storage**
  Were the DONeX columns stored at room temperature and low humidity conditions and has the shelf life not expired?

- **Homogenization and Extraction**
  Was the spiked sample sufficiently homogenized and extracted?

- **Clogging, Matrix Load/Initial Weight, Filtration and Pressure**
  A plugging of the column may have various reasons. The most common error is loading the column with too high a matrix load to improve the sensitivity or using too low volumes of extracting solution per amount of matrix. If the sample is properly filtered, a clogging of the column can be avoided. For matrices containing high sugar contents a reduction of the matrix amount is strongly recommended, because clean-up effect could be reduced by sugars which interfere with the chromatographic detection.

  As a rule the columns should not be loaded with more than 4 g matrix per column, whereas the matrix load itself should not exceed 2 g per 10 mL of extraction solution.

  If higher initial weights are required, this has to be checked individually.

  In all cases, independently of the application of a gentle vacuum or positive pressure, a flow rate of 10 mL/min must not be exceeded. Otherwise the purification effect of the column is diminished.

In any case it is advisable to filter the extract prior to loading it onto the column.

For all samples that still lead to a clogging, despite obeying all recommendations mentioned above, the sample weight must be reduced or the amount of extraction solution per sample must be increased. In general resulting losses of sensitivity can be compensated by increasing the injection volume or a previous concentration.

If the columns are stored at low temperature, the column must be sealed in the original bag to avoid the adsorption of water which may lead to clogging of the column and reduces performances.

- **Flow Rate and Running to Dryness**
  The maximum flow rate of the columns is 10 mL/min. Higher rates lead to lowered purification effects and renders the quality of the chromatography.
  Running to dryness of the column has to be avoided until the washing solution has passed through the column and the flow-through fraction is taken.

- **Analyte Concentration and Sample Volume**
  The DONeX column can be loaded with a minimum of 50 ng per gram matrix deoxynivalenol and the linearity of recovery is given in the range from 50 ppb to 10 ppm.

  In order to get an optimal result, it is advisable to dilute the crude extract in case of high results (above 10 ppm).

- **Concentration and Injection Volume**
  In most of the cases recent HPLC systems with the corresponding fluorescence or mass detectors will have no sensitivity problems in the DON analysis.

  Should a higher sensitivity be requested anyway and the detector is already set to its limit, the matrix amount should not be increased initially. First dissolve the column eluate in the mobile phase with a volume as low as possible in order to still get a proper injection.

  If low injection volumes are applied, higher volumes will increase the sensitivity. Basically it is an advantage, when the injected sample and the mobile phase of the eluent have the same composition! This is especially important when the injection volume is increased.
  Using the LCTech HPLC analytical column an injection volume of 100 µL should not be exceeded!

  Only if all previously mentioned measures were not successful, an increase of the matrix amount should be taken into consideration!

9. Literature

1) COMMISSION REGULATION (EC) No 1881/2006 of 19 December **2006** setting maximum levels for certain contaminants in foodstuffs

2) COMMISSION REGULATION (EC) No 1126/2007 of 28 September **2007** setting maximum levels for certain contaminants in foodstuff

3) European Commission, Regulation 856/2005, European Union, Brussels, Off. J. Eur. Union L143/3 (**2005**) 143/8

4) Sano, S. Matsutani, M. Suzuki, S. Takitani, J. Chromatogr., **1987,** 410, 427 – 436

5) R. Schuhmacher, R. Krska, J. Weingaertner, M. Grasserbauer, Fresenius J. Anal. Chem. **1997**, 395, 510 – 515.

6) R. Krska, J. Chromatogr. A **1998**, 815, 49 – 57

7) Eriksen GS, Alexander J (eds.), **1998**. Fusarium toxins in cereals – a risk assessment. Nordic Council of Ministers; TemaNord 1998: 502, pp. 7-27 and 45-58; Copenhagen

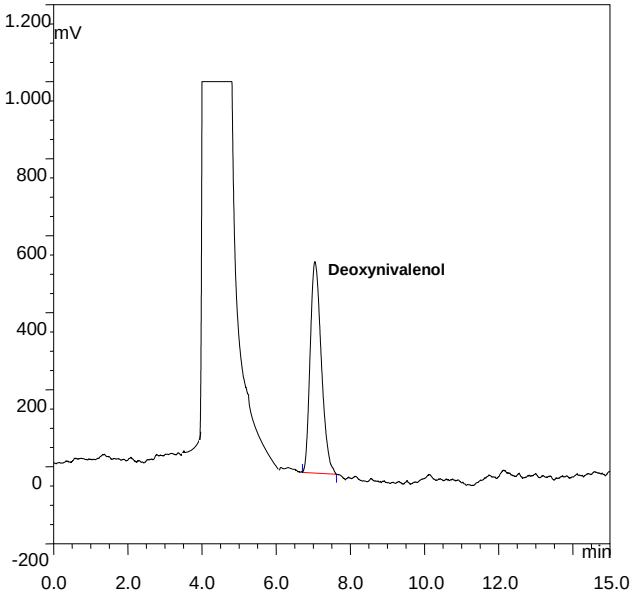
8) Rotter BA, Prelusky DB, Pestka JJ, **1996**. Toxicology of Deoxynivalenol (Vomitoxin).

9) J Toxicol Environ Health 48: 1-34. **1996**

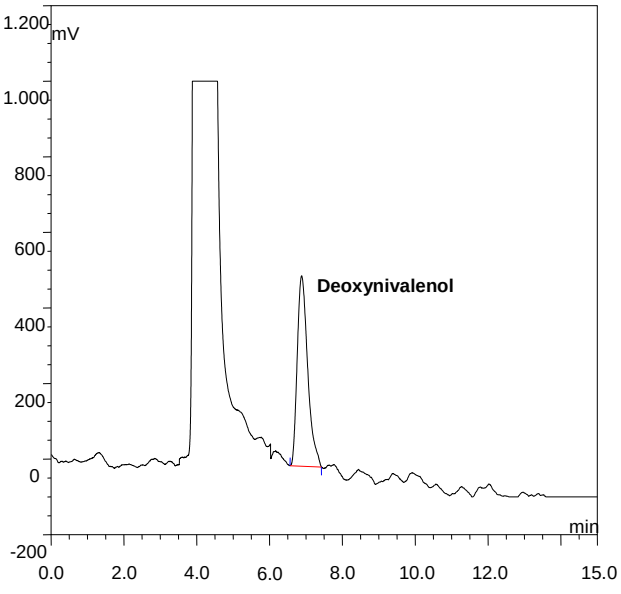
10. Annex

10.1. Chromatograms Showing Various Matrices Analyzed by Post-Column Derivatization and Fluorescence Detection

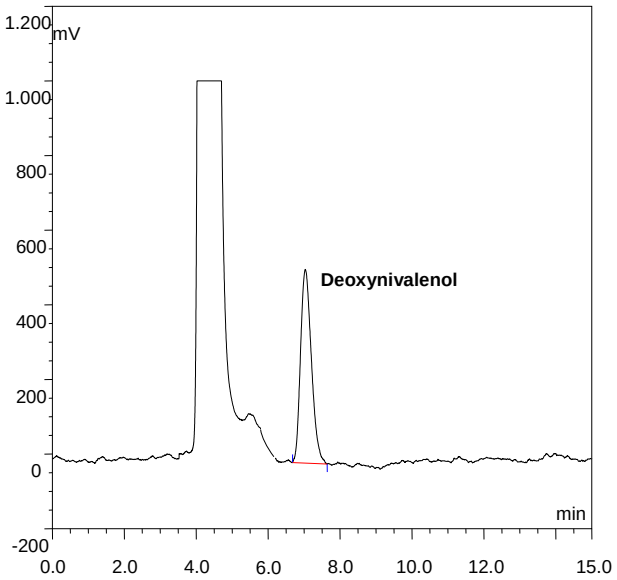
In the following some exemplary chromatograms are shown, which were obtained using DONeX columns and post-column derivatization and detection by fluorescence.



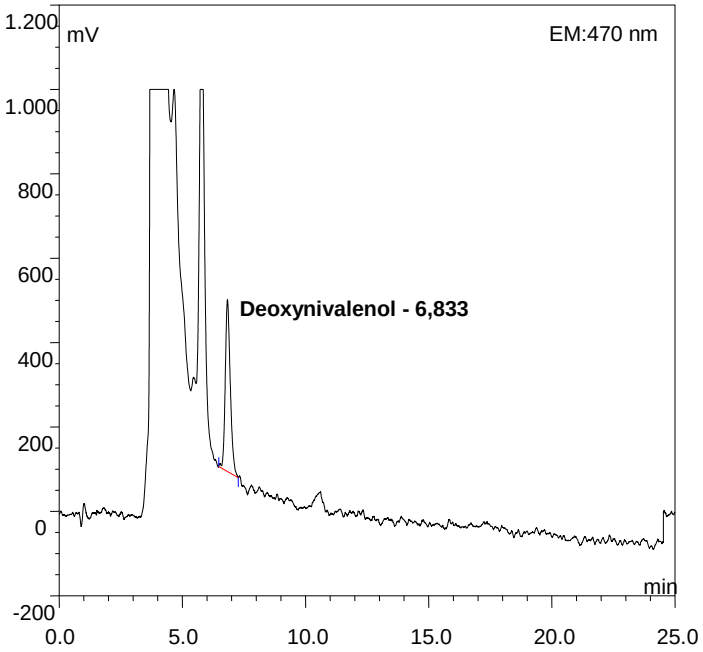
Corn spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



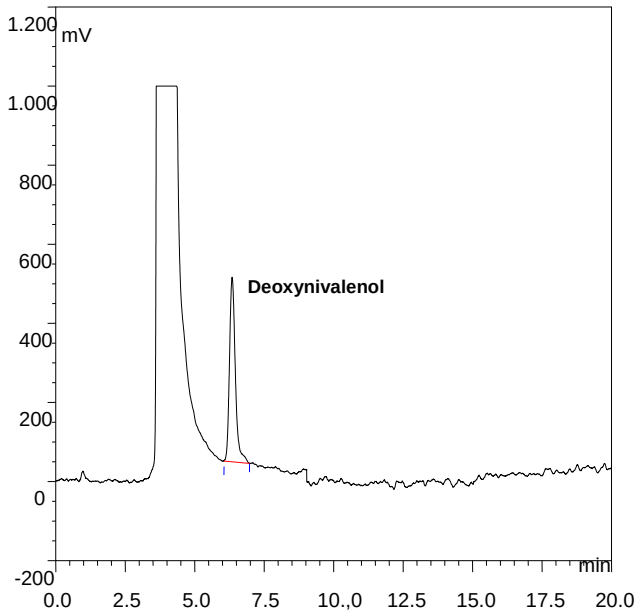
Rye spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



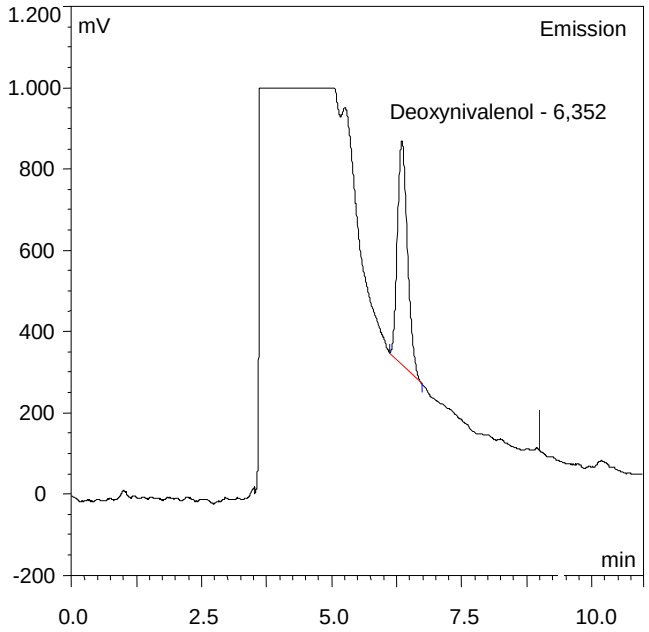
Wheat spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



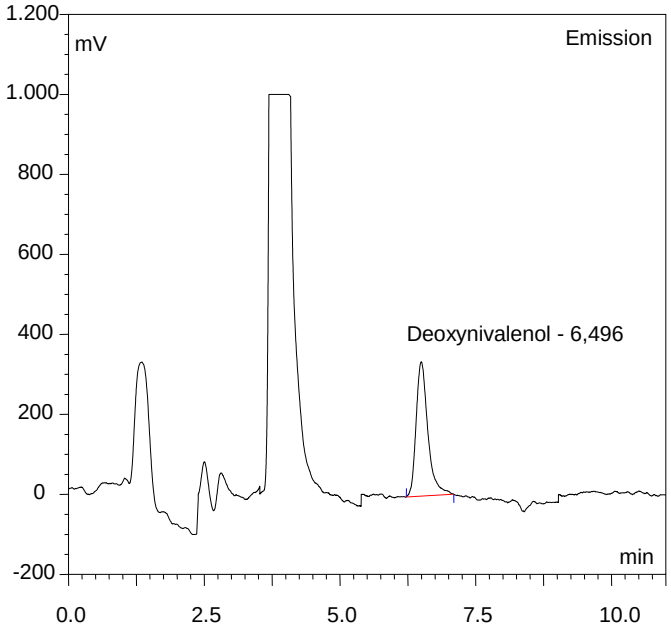
Distiller's grain spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 40 µL injected (0.020 g matrix equivalents represents 20 ng DON injected).



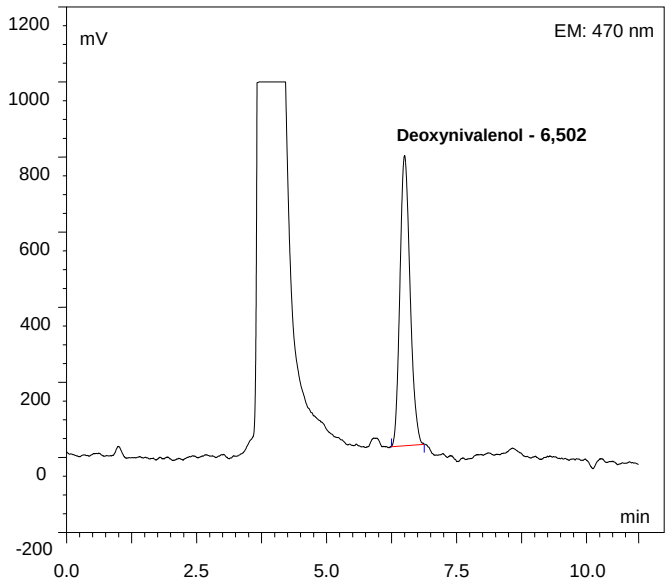
Pasta spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



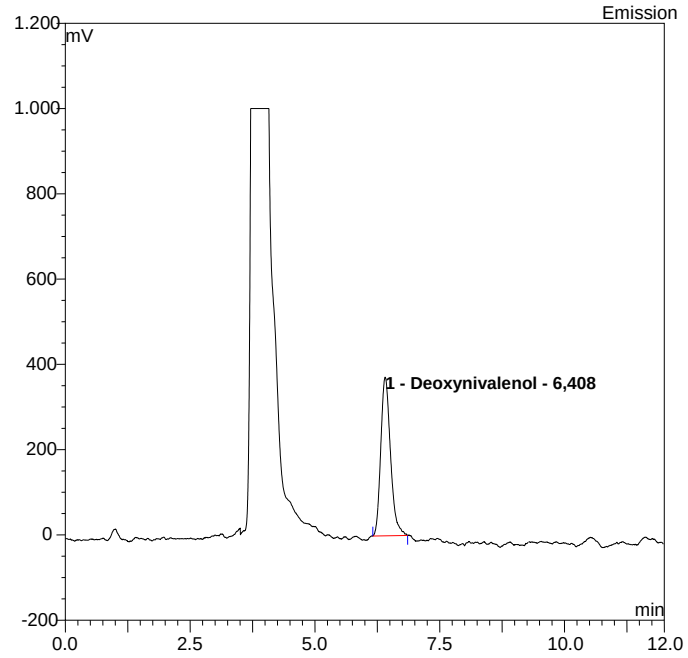
Breakfast cereals (containing nuts and raisins) spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



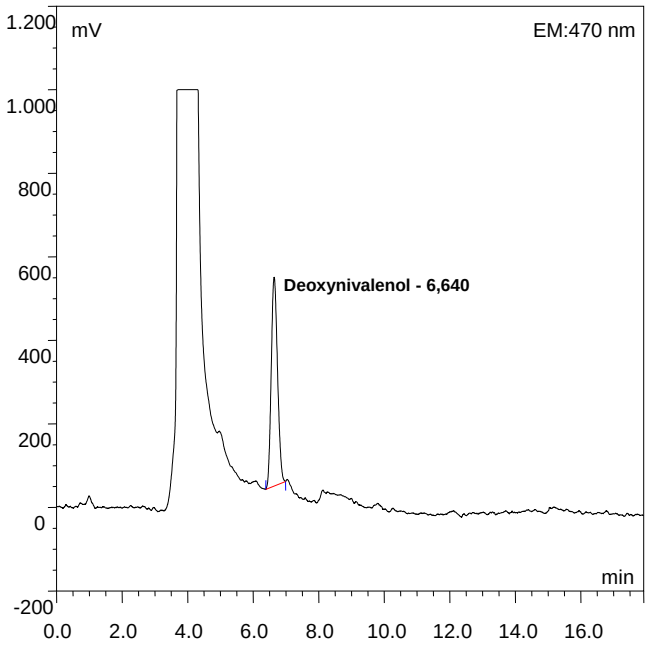
Barley spiked with 0.1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 0.75 mL HPLC solvent, 100 µL injected (0.133 g matrix equivalents represents 13.3 ng DON injected).



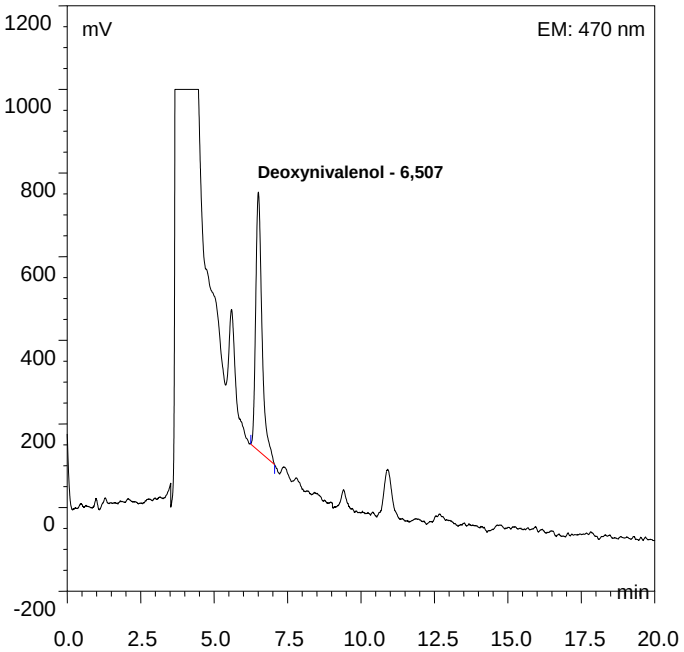
Poultry feed (cereal based) spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 40 µL injected (0.02 g matrix equivalents represent 20 ng DON injected).



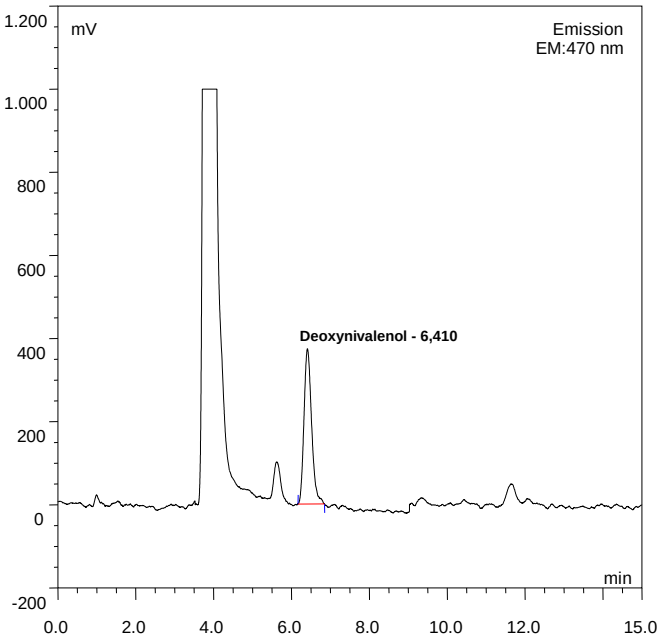
Oat flakes spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



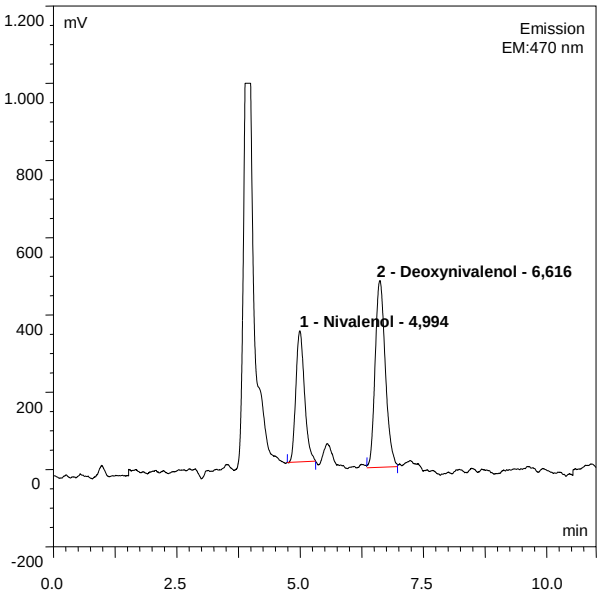
Wheat bran spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 40 µL injected (0.020 g matrix equivalents represents 20 ng DON injected).



Poultry feed spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 40 µL injected (0.02 g matrix equivalents represents 20 ng DON injected).



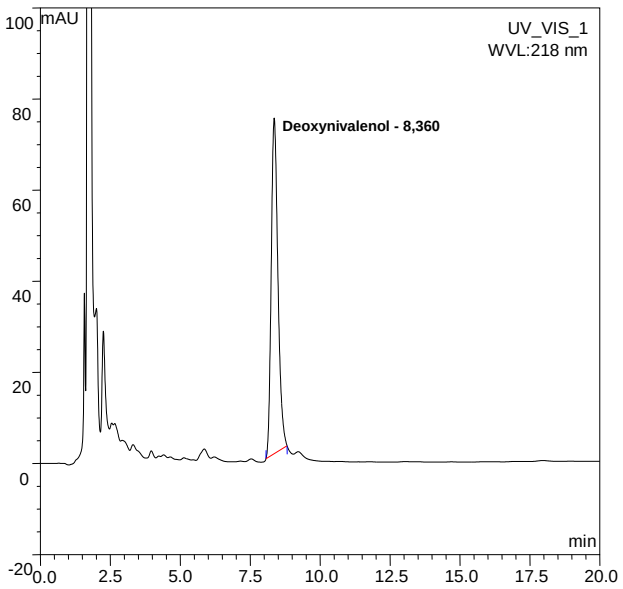
Rice, spiked with 1 ppm, extracted and purified, evaporated sample (1 g) is resolved in 2 mL HPLC solvent, 50 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).



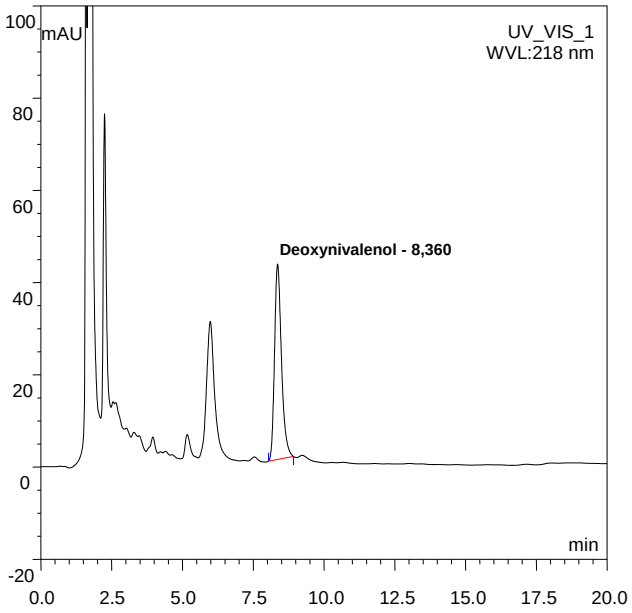
Simultaneous purification of Deoxynivalenol and Nivalenol using DONeX column, toxin amount per g sample 1 ppm, sample spiked with 1 ppm Nivalenol and Deoxynivalenol.

Chromatogram represents 0.02 g matrix equivalents.

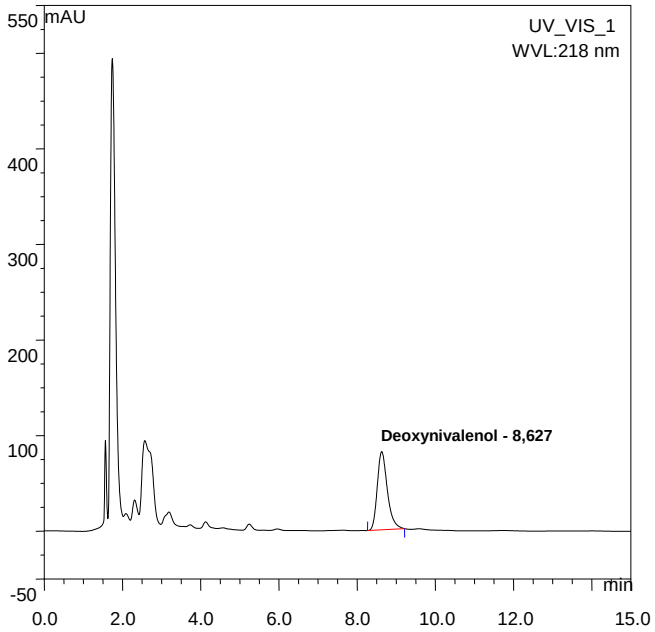
10.2. Chromatograms Showing Various Matrices Analysed by UV Detection



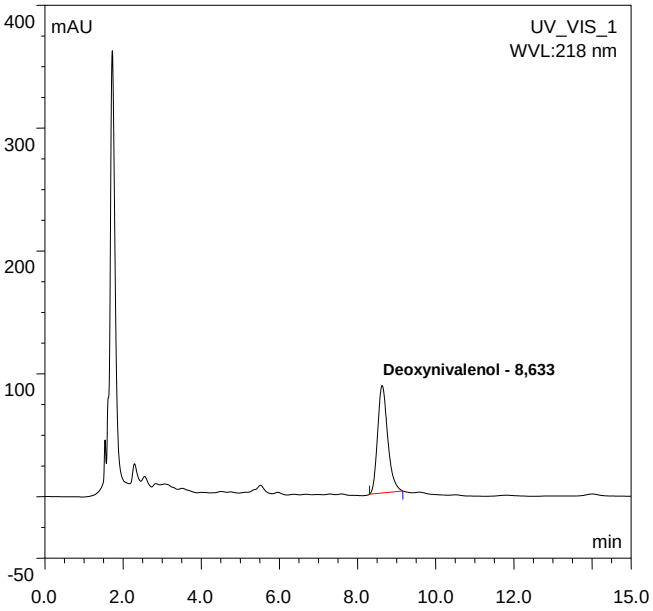
Barley, spiked with 10 ppm, extracted, evaporated sample (1 g) is resolved in 1 mL HPLC solvent, 100 µL injected (0.1 g matrix equivalents represents 100 ng DON injected).



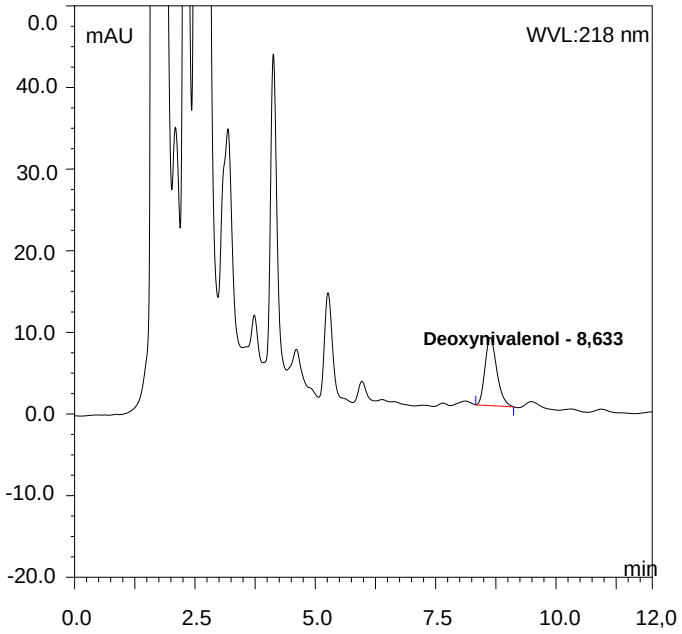
Barley spiked with 5 ppm, extracted, evaporated sample (1 g) is resolved in 1 mL HPLC solvent, 100 µL injected (0.1 g matrix equivalents represents 50 ng DON injected).



Pasta, spiked with 10 ppm, extracted, evaporated sample (1 g) is resolved in 1 mL HPLC solvent, 100 µL injected (0.1 g matrix equivalents represents 100 ng DON injected).



Rice spiked with 10 ppm, extracted, evaporated sample (1 g) is resolved in 1 mL HPLC solvent, 100 µL injected (0.1 g matrix equivalents represents 100 ng DON injected).



Pasta spiked with 1ppm, extracted, evaporated sample (1 g) is resolved in 4 mL HPLC solvent, 100 µL injected (0.025 g matrix equivalents represents 25 ng DON injected).

Any Questions?
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Version History

Versions-No.	Page	Description	Name	Date
1.6	alle	Handbuch neu formatiert	JU	10.01.2019
1.7	Alle	Neues CD angewandt	AM	18.04.2023