Determination of deoxynivalenol in maize and wheat by high performance liquid chromatography and immunoaffinity column clean-up

WARNING: *Deoxynivalenol should be handled as a very toxic substance. Gloves and safety glasses should be worn at all times and all standard and sample preparation stages should be carried out in a fume cupboard. Acetonitrile and methanol are flammable and hazardous solvents. Observe appropriate safety precautions for handling such solvents.*

SUMMARY

Deoxynivalenol (DON) is extracted from wheat sample by blending with water. The aqueous extract is filtered and cleaned up by immunoaffinity column. DON is eluted with methanol and quantified by reversed-phase HPLC with UV detection.

REAGENTS

- **Methanol**, HPLC grade.
- **Acetonitrile**, HPLC grade.
- **Purified water**, Milli-Q purification system.
- **Water**, HPLC grade.
- **HPLC mobile phase** (methanol:water, 15:85, v/v). Mix 15 parts per volume of methanol with 15 parts per volume of HPLC water. The exact amount of methanol used will depend on the HPLC column chosen for analysis and shall be adjusted if necessary. Filter through 0.45 µm filter and degas before use (e.g. with helium).
- **HPLC wash solvent** (methanol:water, 50:50, v/v). Mix 50 parts per volume of methanol with 50 parts per volume of HPLC water.
- **Immunoaffinity columns.** The immunoaffinity column shall contain antibodies raised against DON. The column shall have a total binding capacity of not less than 1000 ng of DON and shall give a recovery of not less than 80% when 500 ng of DON are applied in 1 mL to 2 mL of water.
- **Deoxynivalenol**, purity not less than 97% mass fraction.
- **Deoxynivalenol stock solution** (ca 250 µg/mL). Add 4.0 mL acetonitrile to approximately 5 mg of DON (in crystal form) to obtain a solution with a concentration of ca 1.25 mg/mL. Alternatively, available commercial DON solutions can be used. Store this solution in a freezer at ca -18°C. A solution stored in this way is stable for 12 months.
- Dilute 800 µL of of the 1.25 mg/mL solution to 4.0 mL acetonitrile to obtain a solution containing ca 250 µg/mL of DON (stock solution). Dilute 200 µL of the ca 250 µg/mL solution to 2.0 mL with acetonitrile to obtain a DON solution with a concentration of ca 25 µg/mL. To determine the exact concentration (c_{DON}), record the absorption curve between a wavelength of 200 nm and 270 nm, e.g. in 5 nm steps, of the ca 25 µg/mL solution in a 1 cm quartz cell with acetonitrile as reference. Identify the maximum absorption (i.e. 220 nm) and calculate the mass concentration of solution (c_{DON}), in micrograms per milliliter, µg/mL, using the following equation:

$$c_{DON} = 1000 \times A_{max} \times M / \varepsilon \times \delta$$

where:

A_{max} is the absorption determined at the maximum of the absorption curve;

M is the relative molecular mass of DON (M = 296.3 g mol^{-1});

ε is the relative molar absorption coefficient of DON in acetonitrile (ε = 6,805 L mol^{-1} cm^{-1});

δ is the path length of the quartz cell in centimetres.

- Calculate the mass concentration of the stock solution ($c_{stock\ sol.}$), in micrograms per milliliter, µg/mL, using the following equation:

$$c_{stock\ sol.} = c_{DON} \times 10$$

Store the stock solution in a freezer at ca -18°C. A solution stored in this way is stable for 12 months. Confirm the concentration of the solution if it is older than six months.

- **Deoxynivalenol spiking solution** (100 µg/mL). Pipette an aliquot (equivalent to 500 µg of DON) of the DON stock solution in a 5 mL volumetric flask. Dilute to the mark with acetonitrile. Store this solution in a freezer at ca -18°C. A solution stored in this way is stable for 12 months.
- **Deoxynivalenol standard solution** (10 µg/mL). Pipette 500 µL of the spiking solution (100 µg/mL) in a 5 mL volumetric flask. Dilute to the mark with acetonitrile. Store this solution in a freezer at ca -18°C. A solution stored in this way is stable for 12 months.

APPARATUS

Usual laboratory equipments and, in particular, the following:

- **Microbalance**, capable to measure 0.0001 g.
- **Laboratory balance**, capable to measure 0.01 g
- **High speed blender** or homogenizer.
- **Vortex mixer** or equivalent.
- **Glass vials**, approximately 4 mL, with polytetrafluoroethylene (PTFE)-lined screw cap, or appropriate sealable screw cap.
- **Volumetric flasks**, 5 mL and 10 mL volume, with at least 0.5% accuracy.
- **Vacuum manifold** to accommodate immunoaffinity columns.
- **Filter paper**, fast flow, pore size 20-25 µm (e.g. Whatman no. 4 or equivalent).
- **Glass microfibre filters,** fast flow, pore size 1.6 µm (e.g. Whatman GF/A or equivalent).
- **Solvent evaporator** (heating block).
- **Calibrated microliter syringe(s) or microliter pipette(s)**.
- **HPLC apparatus**, consisting of:
  - **injection system**, a syringe-loading injection valve with 100 µL injection loop or equivalent;

- **HPLC pump**, capable of maintaining a volume flow rate of 1.0 mL/min;
- **analytical reverse phase separating column**, for example stainless steel (150 mm length, 4.6 mm inner diameter) packed with 5 µm C_{18} reverse-phase material preceded by a suitable corresponding reverse-phase guard column or guard filter (0.5 µm). Columns of different dimensions or stationary phases can be used provided that they ensures a baseline resolved resolution of the DON peak from all other peaks;
- **UV (or DAD) detector,** set at 220 nm;
- **data system**.

- **UV spectrometer**, with suitable quartz cells.

PROCEDURES

- **Extraction.** Weight, to the nearest 0.1 g, 25 g of ground sample into a 500 mL blender jar. Add 100 mL of water and homogenize at high speed for 3 min. Filter the extract through filter paper and glass microfibre filter.
- **Immunoaffinity colunm clean-up.** Prepare the immunoaffinity column according to the suppliers instructions. Connect the immunoaffinity column to the vacuum manifold and attach the reservoir to the immunoaffinity column. Add 2.0 mL (equivalent to 0.5 g wheat) of the filtered extract and pass through the immunoaffinity column at a flow rate of about 1 drop per second. The immunoaffinity column shall not be allowed to run dry. Wash the immunoaffinity column with 5 mL water at 1-2 drops per second flow rate. Dry the column passing air through it. Place a vial under the immunoaffinity column and elute DON into the vial with 2 mL methanol. After loading methanol allow elution solvent to pass slowly into the column. Stop the flow and wait 1 min before eluting DON from the column at a flow rate of 1 drop per second.
- **Preparation of the sample test solution**. Place the vial in a heating block and evaporate the eluate to dryness under a gentle stream of nitrogen, e.g. at approximately 50°C. Re-dissolve the dried residue in 500 µL HPLC mobile phase, mix well for example with a vortex mixer and store at 4°C until HPLC analysis.
- **HPLC analysis**. Inject 50 µL of reconstituted extract (equivalent to 0.05 g wheat) into the chromatographic apparatus using the mobile phase at a flow rate of 0.5-1.0 mL/min. The exact amount of methanol used in the mobile phase and the flow rate will depend on the HPLC column chosen for analysis and shall be adjusted if necessary to achieve good separation of DON. The following chromatographic conditions have proved to produce adequate separation (see Figure 1).

  Chromatographic conditions:
  - Flow rate: 0.5 mL/min
  - Injected amount: 50 µL (equivalent to 0.05 g of sample)
  - Column: reversed-phase C18 (Phenomenex Synergi™, 150 × 3.0 mm, 4 µm particles)
  - Mobile phase: water:methanol (85:15, v/v)
  - Detector: UV or DAD (λ = 220 nm)

  Using the HPLC conditions shown above, DON usually elutes with a retention time of about 10 min (see Figure 1).

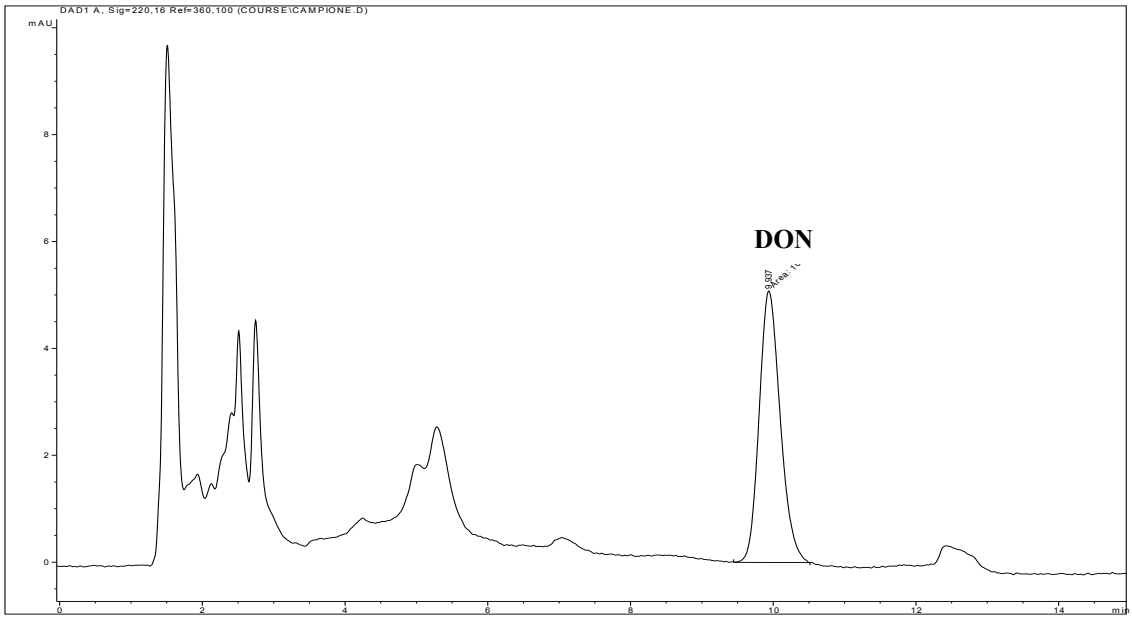

Figure 1. Chromatogram relevant to a naturally contaminated wheat sample containing 1020 µg/kg of DON.

After 15 minutes of run, a washing of the column with HPLC wash solvent (methanol:water, 50:50, v/v) for 10 minutes is recommended. Re-equilibrate the column with methanol:water, 15:85, v/v for 7 min.

Mobile phase prepared with acetonitrile and water have also been shown to be suitable alternatives (generally water:acetonitrile, 90:10, v/v). Such mobile phase can be used if sufficient separation is achieved.

- **Spiking procedure.** To determine the recovery use the DON spiking solution (100 µg/mL). The spiking level should be within the calibration range. Add the spiking solution to a test portion of material previously shown not to contain DON and leave to stand for 30 min before adding the extraction solvent.

CALIBRATION CURVE

- Prepare five HPLC calibration solution from the 10 µg/mL DON working solution. For normal practice evaporate the following amount of working solution in 10 mL volumetric flasks in accordance to Table 1. Fill the flasks up to the mark with HPLC mobile phase.

Table 1 – Preparation of HPLC calibration solutions

	Std 1	**Std 2**	**Std 3**	**Std 4**	**Std 5**
µL of 10 µg/mL DON standard solution	100	500	1000	1500	2000
Final DON concentration in the calibration solution (ng/mL)	100	500	1000	1500	2000
DON mass concentration in the sample (µg/kg)	100	500	1000	1500	2000

Prepare a calibration curve at the beginning of every day of the analysis and whenever chromatographic conditions change. Inject 50 µL of each calibration solution into the HPLC apparatus and plot peak area values of DON calibration solutions against the DON concentrations in the calibration solution.

IDENTIFICATION

Identify DON peak in the test solution by comparing the retention time with the peak of the standard substance in the chromatogram.

DETERMINATION

Obtain the DON concentration in the injected sample from the regression analysis, i.e. integrate the peak area relevant to DON, and compare the results with the corresponding values for the standard substance with the nearest peak area, or use the calibration curve. If the content of DON in the samples falls outside the calibration range, appropriate dilution shall be performed. In this case calculation shall be reconsidered accordingly.

CALCULATION

Read off from the calibration curve, the amount in nanogram per millilitre of DON ($\mathbf{m_{DON}}$) in the aliquot of test solution injected into the HPLC column. Calculate the DON mass concentration, c_{DON}, in micrograms per kilogram, µg/kg, using the equation:

$$\mathbf{c_{DON} = m_{DON} \times V_1 \times V_2 \ / \ V_3 \times m_s}$$

where:

$\mathbf{m_{DON}}$ is the concentration of DON, in nanograms per millilitre, in the aliquot of test solution injected on column determined from the calibration curve;

$\mathbf{V_1}$ is the volume, in millilitre, of the solvent used for extraction (here: 100 mL);

$\mathbf{V_2}$ is the final volume, in millilitre, of test solution (here: 0.5 mL);

$\mathbf{V_3}$ is the volume, in millilitre, of the extract loaded onto the immunoaffinity column (here: 2.0 mL);

$\mathbf{m_s}$ is the mass, in grams, of the ground sample to be analyzed (here: 25 g).

PRECISION

Details of inter-laboratories studies on the precision of the method are given in:

- CEN EN 15891 (2010). Foodstuffs – Determination of deoxynivalenol in cereals, cereal products and cereal based foods for infants and young children – HPLC method with immunoaffinity column cleanup and UV detection.
- MacDonald S.J., Chan D., Brereton P., Damant A. and Wood R. (2005). Determination of deoxynivalenol in cereals and cereal products by immunoaffinity column cleanup with liquid chromatography: interlaboratory study. *Journal of AOAC International*, 88, 1197-1204.
- Neumann G., Lombaert G.A., Kotello S. and N. Fedorowich N. (2009). Determination of deoxynivalenol in soft wheat by immunoaffinity column cleanup and LC-UV detection: interlaboratory study. *Journal of AOAC International*, 92, 181-189.