




DONtest HPLC & DONtest WB

Instruction Manual (for HPLC use)

VICAM®

A Waters Business

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1.1 INTENDED USER

DONtest HPLC™ and DONtest WB™ are quantitative methods intended for use by trained customers in the food processing industry who need to test samples for the presence of DON (also known as deoxynivalenol or vomitoxin) in parts per million (ppm), with a method that is safe, simple, fast and which works reproducibly and accurately.

1.2 PRINCIPLE

Deoxynivalenol (DON) is also known as vomitoxin because of its impact on livestock through interference with animal growth and acceptance of feed. DON has been implicated in moldy corn toxicosis of swine. DON is often present with other mycotoxins and has been isolated from grains and feeds throughout the world at levels as high as 92 ppm. Because of concerns about DON, the United States FDA has instituted advisory levels of 1 ppm for wheat products for human consumption, 5 ppm for grain products for most animal feeds and 10 ppm for grain products for cattle feed. The European Community has regulations for DON levels that range from less than 0.2 ppm in processed cereal-based foods for infants and young children to less than 1.75 ppm for unprocessed wheat.

To measure DON levels, samples are prepared by mixing with an extraction solution, followed by blending and filtering. The extract is then applied to the DONtest HPLC or DONtest WB column, which contains antibodies specific to DON. At this stage, the DON binds to the antibody on the column. The column is then washed to rid the immunoaffinity column of impurities from the extract. By passing an eluting solution through the column, the DON is removed from the antibody. This eluting solution can then be injected into an HPLC system for precise measurement. These steps are outlined in section 1.7 (DONtest HPLC Overview) and section 1.8 (DONtest WB Overview).

1.3 APPLICABILITY

DONtest HPLC and DONtest WB have been optimized for quantitative measurement of DON in wheat. Contact VICAM Technical Services Department for information on new methods.

1.4 LIMITATIONS

This test has been designed for use with the procedure and reagents described on the following pages. Do not use materials beyond the expiration date. Deviation from these instructions may not yield optimum results.

1.5 SAMPLING

Mycotoxins do not occur in every kernel in a lot and may only occur in a small percentage of the kernels in a lot. Because of the wide range in mycotoxin concentrations among individual kernels in a contaminated lot, variation from sample to sample can be large. It is important to obtain a representative sample from a lot. Product should be collected from different locations in a static lot based on a probing pattern. The probe should draw from the top to the bottom of the lot. The samples obtained from the probes should be ground and mixed well and a subsample taken for testing. For further information on grain sampling, refer to the following FGIS publications:

FGIS Aflatoxin Handbook
FGIS Grain Inspection Handbook, Book 1, Grain Sampling
FGIS Mechanical Sampling Systems Handbook

These can be viewed online at:

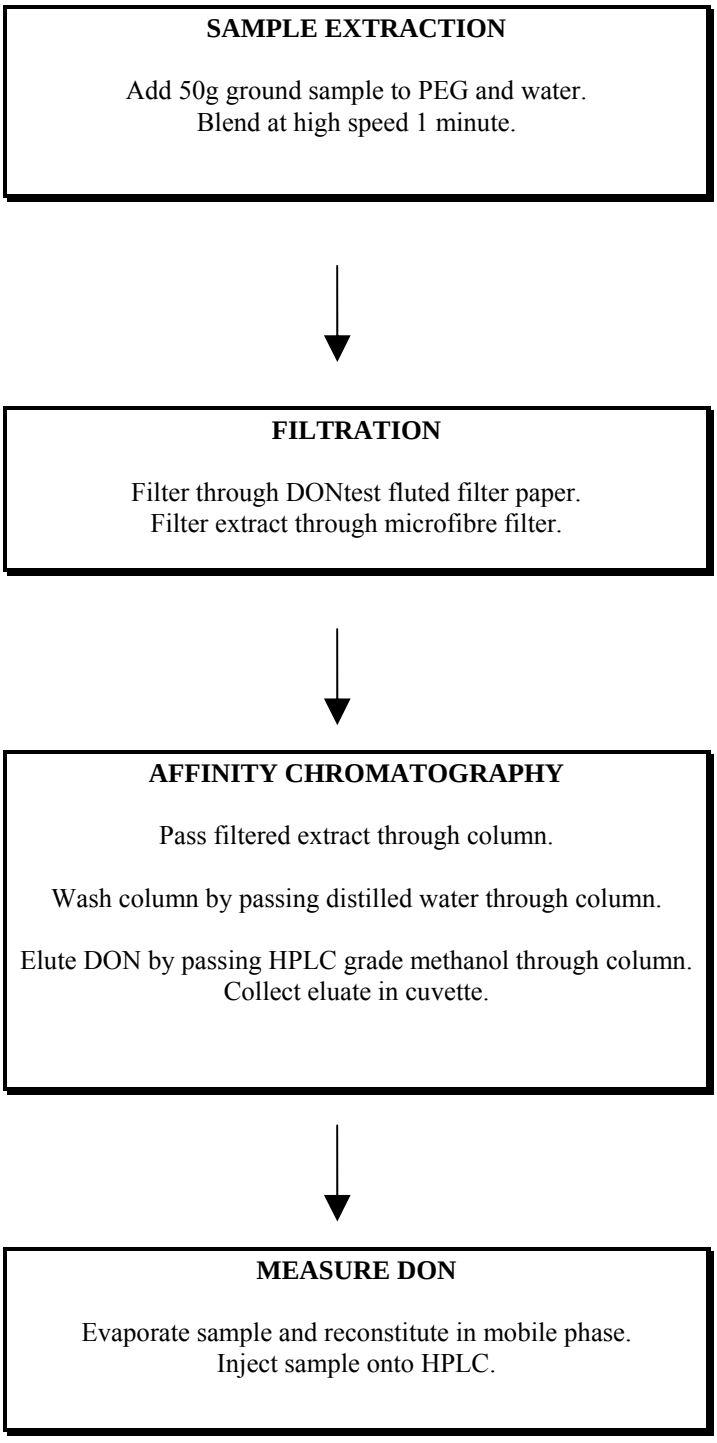
http://www.usda.gov/gipsa/reference-library/handbooks/handbooks.htm
http://www.usda.gov/gipsa/reference-library/brochures/sampling.pdf

Information on sampling can also be found in European Commission Directive 2005/38/EC dated June 6, 2005.

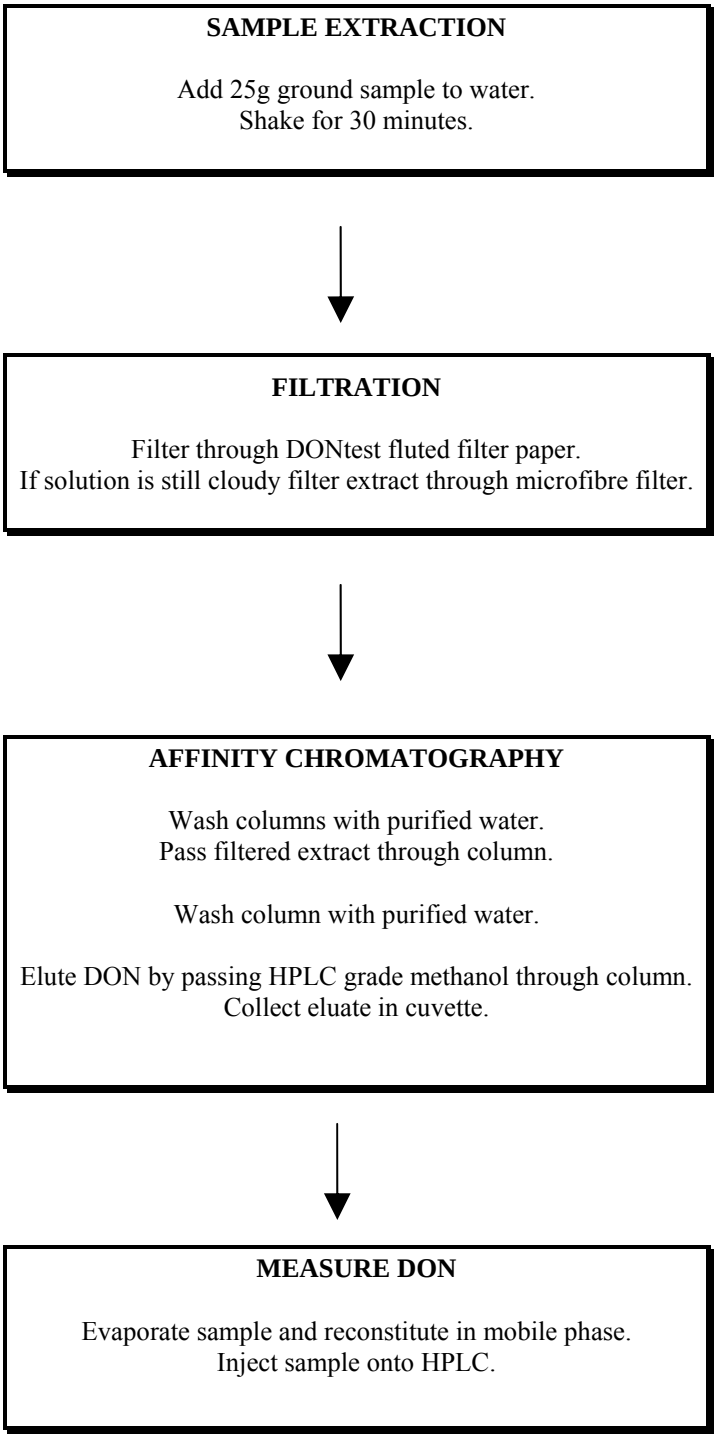
1.6 SHELF LIFE AND STORAGE CONDITIONS

Store at refrigerated temperature (2-8°C) until the expiration date printed on the label. Freezing may damage the affinity columns. It is recommended that columns be at room temperature (18-25°C) for usage.

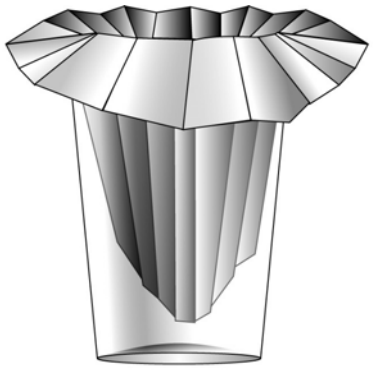
1.7 DONTEST HPLC™ OVERVIEW



1.8 DONTEST WB™ OVERVIEW



2.1 PREPARATION OF FILTRATION STEPS



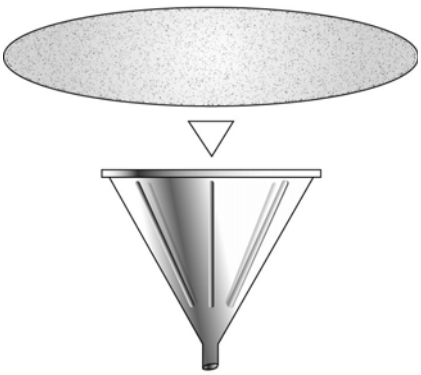
DONtest™ Fluted Filter

The first filtration step is a simple gravity filtration through DONtest fluted filter paper to separate the sample extract solution from the coarse particulate sample solids. The filtrate is collected in a clean container or graduated cylinder.

1. Open one DONtest fluted filter carefully and insert into clean container. (Optional: a large funnel may be used to hold the filter).

2. Fold edges of filter over rim of cup to hold in place. Maintain the fluted folds of the filter paper to maximize surface area. This will increase speed of filtration.

3. It is not necessary to wait for all the extract to pass through the filter before continuing.



Microfibre Filter

The second filtration step is the gravity filtration of the extract through a microfibre filter. This removes any precipitates in the extract and assures that the extract will easily pass through the affinity column. Microfibre filtration is performed just prior to affinity chromatography.

1. Place a small funnel in top outlet of syringe barrel or clean collecting cup.

2. Place one microfibre filter gently into small funnel by pressing filter into funnel with index finger. Be careful not to rip or puncture the filter.

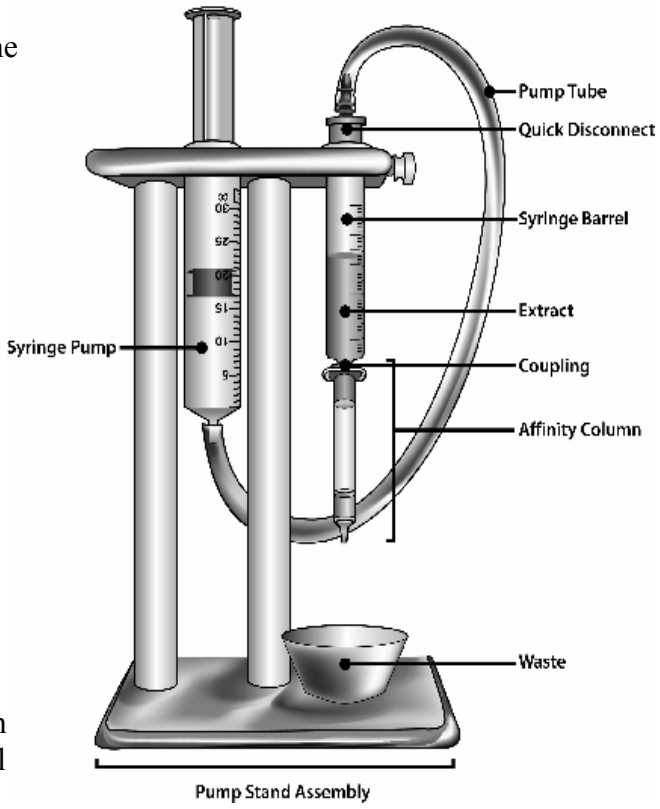
2.2 PUMP STAND SETUP

DONtest HPLC and DONtest WB affinity chromatography is easily performed with the affinity column attached to a pump stand. The stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. A large plastic syringe with tubing and coupling provides air pressure to manually push liquids through the column. An adjustable air pump (VICAM part #20650) can be attached to the pump tube instead of the large pump syringe barrel to operate without using hand pressure. Double (part # 21040), four-position (part #21045) and 12 position (part # G1104) pump stands are available for running multiple samples at one time. Alternatively, a vacuum manifold can be used to draw the extract through the column.

1. Remove large top cap from column.
2. Cut bottom 1/8 inch off the end of the top cap with scissors or sharp blade. This provides a reusable coupling for attaching the DONtest HPLC column. When using DONtest WB columns order part G1118 (WB Column Coupling).
3. Attach column to coupling and place waste collection cup under column outlet. Keep bottom cap on column.
4. Measure desired amount of diluted filtered extract into glass syringe barrel.
5. Pull up on the plastic syringe piston.
6. Inset coupling on end of tube into syringe barrel. Remove column bottom cap.
7. Apply pressure to piston of plastic syringe to push liquid through the column. Maintain a flow rate of 1-2 drops per second. Push all liquid through the column. Repeat for wash and elution steps (see procedures).

Note: Avoid pulling up on plastic syringe piston while coupling is attached to glass syringe barrel. This may displace the antibody coated support beads and affect test results.

Affinity Column Syringe Barrel Connection



2.3 CLEANING EQUIPMENT

Before Starting Testing

To eliminate background fluorescence make sure the equipment is clean and not contaminated with materials that might cause background fluorescence. This is particularly important when using brand new equipment or equipment that has not been used for a long period of time.

Before using the equipment, it should be washed with a mild detergent solution and then rinsed thoroughly with purified water. This includes the glass syringe barrels used for sample reservoirs. Other pieces of equipment that need to be cleaned with detergent before using are graduated cylinders, funnels and blender jars. Repipettors need only to be rinsed before use with methanol.

Between Assays:

After each assay, the blender jar assembly needs to be washed with a mild detergent solution and rinsed thoroughly with purified water. This cleaning procedure must be performed for any equipment that will be reused to hold, collect or transfer sample extracts.

Do not wash methanol repipettor with soap. Methanol repipettor needs only to be refilled with methanol.

In between each assay, the syringe barrel reservoir can be rinsed with methanol followed by a rinse with purified water. This will be sufficient to prevent cross-contamination of samples.

Other Important Precautions

Use only equipment specified by VICAM. Avoid contact of any test reagents or solutions (such as methanol, water, extract, column eluate) with rubber or soft flexible plastic. These materials may leach contaminating absorbent materials into the sample and thereby affect results.

3.0 MATERIALS AND EQUIPMENT FOR DONTEST HPLC™ PROCEDURE FOR WHEAT

MATERIALS REQUIRED

Description	**Part #**
DONtest HPLC Columns (25/box)	G1005
DONtest HPLC Columns (50/box)	G1006
DONtest Fluted Filter Paper, 24 cm (100)	31242
Microfibre Filters, 1.5µm, 11 cm (100)	31955
Kim Wipes Tissues (1 box)	31967
Disposable Cuvettes (250)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers (25)	36010
Distilled, reverse osmosis or deionized water	
Acetonitrile (4 x 4 L)	G1130
Polyethylene Glycol (MW=8000)	G1015

EQUIPMENT REQUIRED

Description	**Part #**
Graduated cylinder, 250 mL	20250
Wash Bottle, 500 mL	20700
Cuvette Rack	21010
Filter Funnel, 65 mm (10)	36020
Filter Funnel, 105 mm (4)	36022
Digital Scale with AC Adapter	20100
Commercial Blender with Stainless Steel container	20200
2-Position Pump Stand w/ Air Pump (10 mL)	21040
or 4-Position Pump Stand w/2 Air Pumps (10 mL)	21045
or 12-Position Pump Stand w/6 Air Pumps (10 mL)	G1104
Vortex Mixer	23040
Speed vacuum or equivalent evaporator	
1mL and 300 µl Pipettor with pipette tips	

SUGGESTED BUT NOT REQUIRED

Description	**Part #**
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501

4.0 DONTEST HPLC™ PROCEDURE FOR WHEAT

4.1 HPLC Set Up: HPLC Conditions 1 were used in the development of this method. Other HPLC conditions are also suitable and may give better results (i.e. HPLC conditions 2).

4.2 Sample Extraction:

4.2.1 Grind sample so that 95% by weight passes through a 20-mesh sieve. Do not grind sample to a fine powder.
4.2.2 Place 50g ground sample into a blender jar.
4.2.3 Add to jar 10g polyethylene glycol 8000 (PEG) and 200 mL purified water.
4.2.4 Cover blender jar and blend at high speed for 1 minute.
4.2.5 Remove cover from jar and pour extract into DONtest™ fluted filter paper. Collect filtrate in a clean vessel.

4.3 Extract Filtration:

4.3.1 Place a gently folded microfibre filter inside a small funnel and set funnel in clean glass syringe barrel. Use a new microfibre filter for each test.
4.3.2 Filter extract through glass microfibre filter into a clean container.

4.4 Column Chromatography:

4.4.1 Measure 1mL (1 mL = 0.25 grams sample equivalent) filtered extract into the glass syringe barrel on the pump stand and pass the 1 mL extract completely through DONtest HPLC affinity column at a rate of about 1 drop/second until air comes through column.
4.4.2 Pass 5 mL of purified water through the column at a rate of about 1 drop/second until air comes through the column.
4.4.3 Place glass cuvette (VICAM part # 34000) under DONtest HPLC column and dispense 1.0 mL HPLC grade methanol with methanol dispenser into glass syringe barrel.
4.4.4 Elute affinity column by passing 1.0 mL HPLC grade methanol through column at a rate of 1 drop/second and collecting all of the sample eluate (1.0 mL) in a glass cuvette.

4.5 Sample preparation for HPLC injection:

4.5.1 Evaporate standards (see section 7.3) and sample eluate to dryness.
4.5.2 Reconstitute dried sample and standards with 300µl of HPLC mobile phase.
4.5.3 Separately inject 50 µl of each reconstituted sample and standards.

4.6 Assay Range: 0 – 5 ppm

4.7 Limit of detection: < 0.1 ppm

4.8 Recovery: > 84% in spiked wheat extracts

5.0 MATERIALS AND EQUIPMENT REQUIRED FOR DONTEST WB™

CONSUMABLES REQUIRED

Description	Part #
DONtest WB Columns (25/box)	G1065
DONtest WB Columns (50/box)	G1066
VICAM Fluted Filter Paper, 24 cm (100)	31240
Kim Wipes Tissues (1 box)	31967
Disposable Cuvettes (250)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers (25)	36010
Distilled, reverse osmosis or deionized water	
Acetonitrile (4 x 4 L)	G1130

EQUIPMENT REQUIRED

Description	Part #
Graduated cylinder, 250 mL	20250
Wash Bottle, 500 mL	20700
Cuvette Rack	21010
Filter Funnel, 105 mm (4)	36022
Digital Scale with AC Adapter	20100
Mechanical Shaker	
2-Position Pump Stand w/ Air Pump (10 mL)	21040
or 4-Position Pump Stand w/2 Air Pumps (10 mL)	21045
or 12-Position Pump Stand w/6 Air Pumps (10 mL)	G1104
Vortex Mixer	23040
Speedvac or equivalent evaporator	
1mL Pipettor with pipette tips	

SUGGESTED BUT NOT REQUIRED

Description	Part #
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501
Microfibre Filters, 1.5µm, 11 cm (100)	31955
Filter Funnel, 65 mm (10)	36020

6.0 DONTEST WB™ PROCEDURE FOR WHEAT

6.1. HPLC Set Up: HPLC Conditions 3 were used in the development of this method. Other HPLC conditions may also be suitable.

6.2. Sample Extraction and Filtration:

6.2.1 Place 25g of coarsely ground sample and 100 mL of purified water into a sealed container and shake for 30 minutes on a mechanical shaker.

6.2.2 Pour extract into VICAM fluted filter paper. Collect filtrate in a clean vessel.

6.2.3 If extract is still cloudy filter extract through a microfibre filter. Place a gently folded microfibre filter inside a small funnel and set funnel in clean glass container. Filter extract through glass microfibre filter into the glass container.

6.3 Column Chromatography:

6.3.1 Pass column buffer through the immunoaffinity column until air comes through column. Pass 1 mL of purified water through the column until air comes through column.

6.3.2 Measure 1mL filtered extract (1 mL = 0.25 grams sample equivalent) into the glass syringe barrel on the pump stand and pass the 1 mL extract completely through DONtest WB affinity column at a rate of about 1 drop/second until air comes through column.

6.3.3 Pass 5 mL of purified water through the column at a rate of about 1 drop/second until air comes through the column.

6.3.4 Place glass cuvette (VICAM part # 34000) under DONtest WB column and dispense 2.0 mL HPLC grade methanol into glass syringe barrel.

6.3.5 Allow methanol to flow into and infiltrate column bed and then remove any air pressure from column. Allow column to sit for approximately 5 minutes. Elute remaining methanol at a rate of 1 drop/second and collecting all of the sample eluate in a glass cuvette.

6.4 Sample preparation for HPLC injection:

6.4.1 Evaporate standards (see section 7.3) and sample eluate to dryness.

6.4.2 Reconstitute dried sample and standards with 500µl or 1.0 mL of HPLC mobile phase.

6.4.3 Separately inject 70 µl of each reconstituted sample and standards.

6.5 Assay Range: 0 – 5 ppm

6.6 Limit of detection: < 0.04 ppb

6.7 Recovery: > 77% in both spiked wheat extracts and naturally contaminated wheat

7.0 HPLC INFORMATION

7.1 HPLC CONDITIONS

HPLC Conditions 1:
- Column: reverse phase C18, 4.6 x 75 mm (3 μm)
- Mobile phase: acetonitrile:water (10:90 by volume) degassed, isocratic
- Flow rate: 0.6mL/min
- Injection volume: 50 μL
- Lamp: deuterium or mercury lamp
- Detection: 218 nm
- Sample loop: 200 μL
- Retention time: 5-6 minutes

HPLC Conditions 2:
- Column: reverse phase C18, 3.9 x 300 mm (4 μm) (Waters part # WAT011695)
- Mobile phase: acetonitrile:water (10:90 by volume) degassed, isocratic
- Flow rate: 0.6 mL/min.
- Injection volume: 50 μL
- Lamp: deuterium or mercury lamp
- Detection: 218 nm
- Sample loop: 200 μL
- Retention time: 10-11 minutes

HPLC Conditions 3:
- Column: reverse phase Synergi 4 μm, Hydro-RP, 250 x 4.6 mm, with precolumn, Phenomenex Co.
- Column temperature: 30°C
- Mobile phase: acetonitrile:water (10:90 by volume) degassed, isocratic
- Flow rate: 1.2 mL/min.
- Injection volume: 70 μL
- Detection: 220 nm, Diode array detector
- Retention time: 5.6 – 5.8 minutes

*****DISCLAIMER
Although specific equipment and HPLC columns are listed in this document, there are a number of equally suitable components that can also be used.

7.2 HPLC STANDARD PREPARATION (FOR 0.25 GRAM EQUIVALENT PROCEDURE)

We use the Supelco deoxynivalenol standard product # 46911 which comes in sealed ampules at an deoxynivalenol concentration of approximately 200µg/mL in ethyl acetate:methanol (95:5). This standard is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of deoxynivalenol.

7.2.1 Prepare Working Solutions of DON

DON-working solution 1 (10µg/mL):
50µl of DON Standard (200µg/mL) + 950 µl Milli-Q water

DON-working solution 2: (1µg/mL):
100µl of DON-working solution 1 (10µg/mL) + 900 µl Milli-Q water

7.2.2 Prepare DON Standards

0.1 ppm (µg/g) X 0.25 g sample equivalent = 0.025 µg
0.025 µg ÷ 1µg/mL (DON-working solution 2) = 0.025mL = 25µl
Add 25 µl DON-working solution **2** to 975µl methanol

0.5 ppm (µg/g) X 0.25 g sample equivalent = 0.125 µg
0.125 µg ÷ 1µg/mL (DON-working solution 2) = 0.125mL = 125µl
Add 125 µl DON-working solution **2** to 875µl methanol

5 ppm (µg/g) X 0.25 g sample equivalent = 1.25 µg
1.25 µg ÷ 10µg/mL (DON-working solution 1) = 0.125mL = 125µl
Add 125 µl DON-working solution **1** to 875µl methanol

7.2.3 As described in the procedure, samples and standards should be evaporated, reconstituted and injected into the HPLC.

7.2.4 Graph the ppm value of the standards vs. HPLC peak area. Calculate the equation of the resulting line. The HPLC peak area of the unknown sample is then plugged into the equation of this line to calculate the ppm value of the sample. This calculation can be done with the software provided by an HPLC manufacturer. In addition, this calculation can be done using Microsoft EXCEL software.

7.3 SPIKING WHEAT WITH DON

We use the Supelco deoxynivalenol standard product # 46911 which comes in sealed ampules at an deoxynivalenol concentration of approximately 200µg/mL in ethyl acetate:methanol (95:5). This standard is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of deoxynivalenol.

0.5 ppm spike

0.5 ppm (µg/g) X 50g wheat = 25 µg
25 µg ÷ 200µg/mL DON standard = 0.125mL = 125 µl
Add 125µl DON standard to 50g wheat

2.0 ppm spike

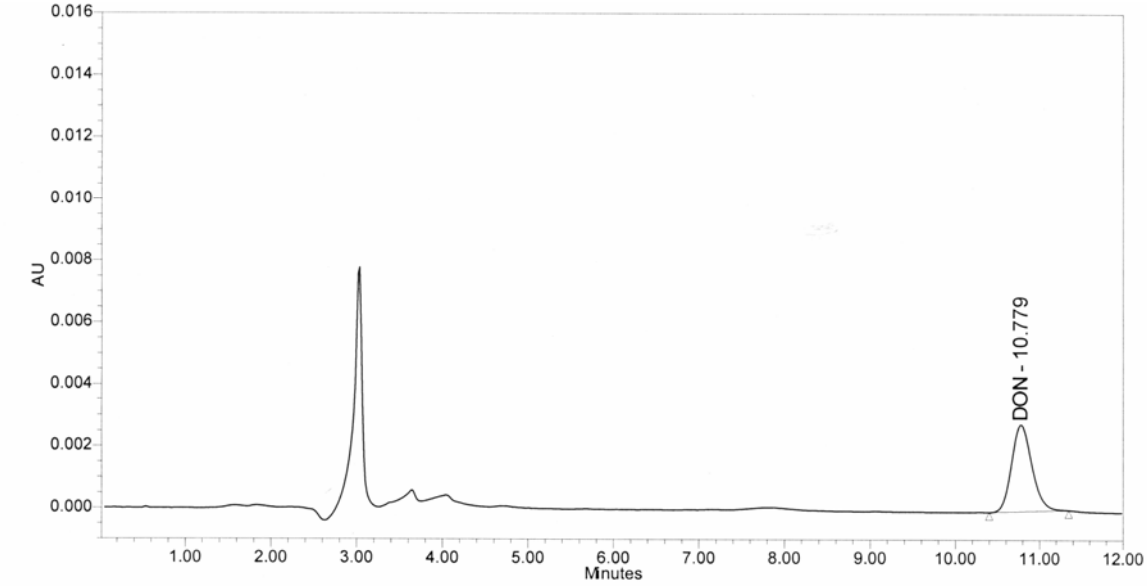
2.0 ppm (µg /g) X 50g wheat = 100 µg
100 µg ÷ 200µg/mL DON standard = 0.5mL = 500 µl
Add 500µl DON standard to 50g wheat

NOTE: Divide volume of DON added by 2 if spiking 25 g of sample.

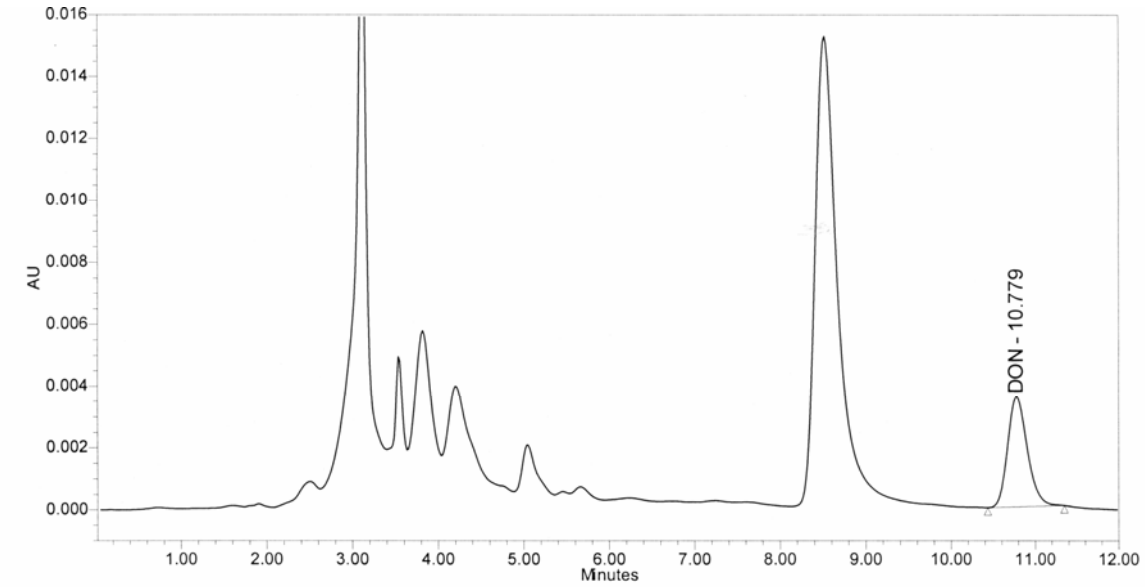
The best accuracy is obtained when spiking is done with a Hamilton Syringe, but an adjustable Pipetman with replaceable plastic tips can also be used. Spike the samples in a fume hood and let them dry for at least 30 minutes before testing.

7.4 REPRESENTATIVE HPLC CHROMATOGRAMS

1 ppm DON standard



1.11ppm Naturally Contaminated Wheat



Chromatograms were generated using HPLC Conditions 2 and DONtest HPLC columns.

8.0 GENERAL PRECAUTIONS, TROUBLESHOOTING AND LIMITATIONS

8.1 General Precautions:

Always use good, clean equipment and reagents (HPLC grade methanol for sample elution and purified, reverse osmosis or deionized water).

Make sure methanol dispensing tube is primed and free of air bubbles before dispensing.

Perform test from beginning to end without interruptions.

Load sample on column immediately after microfibre filtration.

Use only equipment specified by VICAM. Avoid contact of any test reagents or solutions (such as methanol, extract or column eluate) with rubber or soft flexible plastic. These materials may leach absorbent materials into the sample.

Be careful to load 1 mL sample extract over the immunoaffinity column and to wash with 5mL water only. Loading larger volumes and washing the column with larger volumes can lead to a lower percent recovery.

Maintain a slow and steady flow rate through the immunoaffinity column (1 drop/second) during sample loading.

8.2 Troubleshooting:

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low recovery	Low recoveries may be caused by deviation from recommended procedure	1. Be sure to follow VICAM's protocol carefully 2. Do not use more than 1mL sample load or more than 5mL water column wash
	Wrong extraction solution	1. Water must be used to extract DON to achieve optimum recoveries 2. Do not pass sample over the column with any solvent (i.e. No methanol or acetonitrile)
Variable results	Variable results may be caused by non-uniform sampling	Select a representative portion of sample for testing
Slow filtration	Wrong filter	Be sure to use to use the correct filter paper. Use VICAM part # 31242 for DONtest HPLC column procedure and part # 31240 for DONtest WB column procedure

	Did not pour the entire extract onto filter	Pour entire blender contents into fluted filter. This will speed filtration.
	Not adding polyethylene glycol 8000 (PEG) during extraction step if using DONtest HPLC column procedure.	Make sure to add polyethylene glycol 8000 (PEG) to the sample when blending if performing DONtest HPLC column procedure.

8.3 References and Other Published Procedures

Cahill, L.M., Kruger, S.C., McAlice, B.T., Ramsey, C.S., Prioli, R, Kohn, B., *Journal of Chromatography A.*, Quantification of deoxynivalenol in wheat using an immunoaffinity column and liquid chromatography. 1999 Oct 22; 859(1): 23-8.

Janes, W., Schuster, M., *Mycotoxin Research*., Determination of Deoxynivalenol(DON) in Blood, Bile, Urine and Excrement Samples from Swine Using Immunoaffinity Chromatography and LC-UV-Detection. 2001 Vol. 17: 88-95

MacDonald, S., D. Chan, et al., *AOAC international*., Determination of Deoxynivalenol in Cereals and Cereal Products by Immunoaffinity Column Cleanup with Liquid Chromatograph: Interlaboratory Study. 2005 88(4): 1197-1204.

8.4 Disposal of Materials Containing DON:

1. Collect all suspected DON containing material in a waste bucket. This includes extract, waste collected from column loading and washing steps, and eluate.

2. Add approximately 5% bleach (commercially available in drugs stores and supermarkets) by volume. For example, if waste volume is 100 mL, add approximately 5 mL of bleach.

3. Wait 30 minutes for bleach to react with DON.

4. Make the neutralization permanent by adding approximately 5% acetone by volume. For example, if waste volume is now 105 mL, add approximately 5 mL of acetone.

5. Wait 30 minutes for neutralization to become irreversible.

6. Discard material according to local, state and federal regulations.

9.0 TECHNICAL ASSISTANCE

If the desired results are not obtained, verify that the methods adhere to the procedure presented in this manual.

For assistance please contact your local distributor or VICAM Technical Services:

Phone: 800-338-4381 Canada, Mexico and the United States

508-482-4935 All International and United States customers

Fax: 508-482-4972

E-mail: techservice@vicam.com

10.0 LIABILITY

Only individuals with appropriate training should perform the test described in this instruction manual. Materials should be handled and disposed of properly.

The analytical methods described above have been developed by VICAM to be used exclusively with the reagents in this test. The user assumes all risk in using DONtest HPLC and DONtest WB analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that DONtest HPLC and DONtest WB products conform to VICAM's printed specifications and quality control standards. VICAM will at its option repair or replace any product or part thereof which proves to be defective in workmanship or material. VICAM's undertaking to repair or replace such products is exclusive and is in lieu of all warranties whether written, oral expressed, or implied, including any implied warranty of merchantability or fitness for a particular purpose. VICAM shall have no liability for anticipated or lost profits or any loss, inconvenience or damage whether direct, incidental, consequential or otherwise, to person or property, or for strict liability or negligence arising from or in connection with the use of these assay procedures or DONtest HPLC and DONtest WB product.

The foregoing notwithstanding, protocols and other products developed by VICAM are periodically improved and revised in order to maximize reliability and optimize customer use and satisfaction. When an improved, new or substitute version of a protocol and product is available, VICAM shall not be held liable or responsible for any earlier protocol or product, even if use of earlier product or protocol be within the expiration date. Please inform yourself about any new protocols by either e-mailing, faxing or phoning VICAM or your local VICAM distributor.

VICAM shall not be liable or responsible for any unsatisfactory or faulty results or performance involving the use of VICAM protocols or products if the testing or sampling in question is not conducted properly. The customer is solely and fully responsible for educating oneself about the proper testing and sampling procedures using VICAM protocols and products.

11.0 ORDERING INFORMATION

To place an order contact your local VICAM distributor or VICAM at:

In the United States:

Phone:	877-228-4244	Canada and the United States
	800-338-4381	Mexico
	417-725-6588	International and United States customers
Fax:	417-725-6102	
e-mail:	vicam@vicam.com	