




DON-NIV WB

Instruction Manual (for HPLC use)

VICAM®

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1.0 INTRODUCTION

1.1 INTENDED USER

DON-NIV™ WB and its testing procedure 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) and Nivalenol (NIV). The method described in the manual is safe, simple, and works reproducibly and accurately.

1.2 PRINCIPLE

Deoxynivalenol (DON) and nivalenol (NIV) are type B trichothecenes. DON is one of the most common mycotoxins that are found in grains such as wheat, barley, oats, rye, and maize. Although no indication of carcinogenic, mutagenic or teratogenic effects is reported, a study showed that humans could suffer a range of symptoms including abdominal pains, dizziness, headache, vomiting, diarrhea and blood in the stool when consuming food with high concentrations of DON. Studies in animals indicate that chronic consumption of DON could result in reduced feed intake, slowed weight gain, increased susceptibility to disease and even animal death. Acute affects include intestinal disorders and vomiting. There are also reproductive effects in swine including abortion, still births and weak offspring.

The FDA has established advisory levels for DON in foods and animal feeds. The level on finished wheat products, e.g. flour, bran, and germ that may potentially be consumed by humans is 1 ppm. The level of DON in grains and grain by-products destined for animals is 5 ppm.

It has been reported that NIV is a co-contaminate with DON in wheat and barley. Studies indicate that NIV has similar toxicity to DON and may be linked to lung and brain hemorrhage and bone marrow injury through disruption of DNA and protein synthesis.

To measure DON and NIV levels, samples are prepared by mixing with an extraction solution, followed by blending and filtering. The extract is then applied to the DON-NIV WB column, which contains antibodies specific to DON and NIV. At this stage, the DON and NIV bind 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 and NIV are 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 section (DON-NIV WB Overview).

1.3 APPLICABILITY

DON-NIV WB has been optimized for quantitative measurement of DON and NIV 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:

USDA GIPSA DON Handbook Chapter 3 (Publication date: 8-30-04)

FGIS Grain Inspection Handbook, Book 1, Grain Sampling

FGIS Mechanical Sampling Systems Handbook

These can be viewed online at:

www.gipsa.usda.gov. Click on “Federal Grain Inspection” then “Publications” on the left.

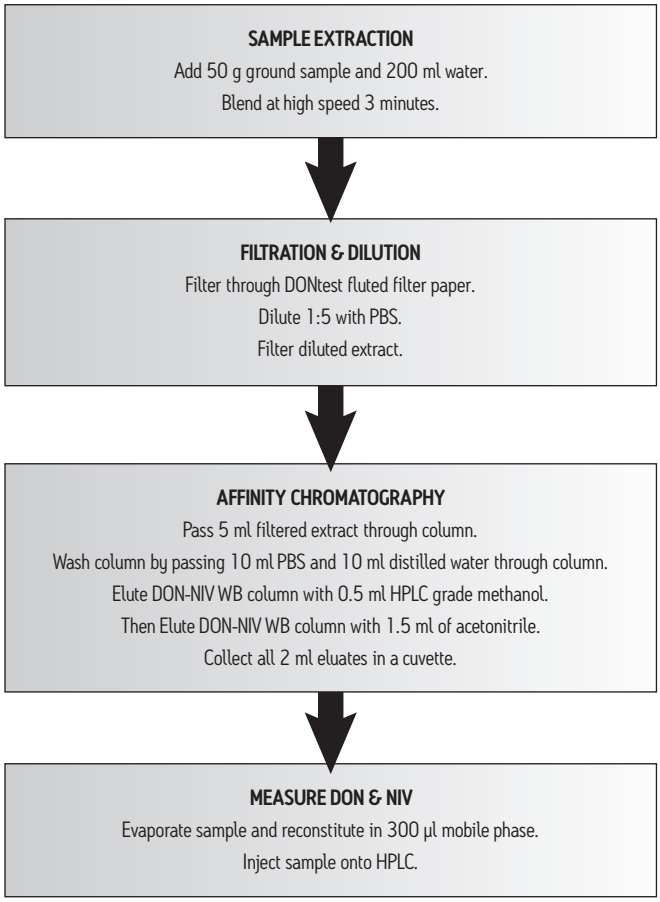
http://www.gipsa.usda.gov/publications/fgis/handbooks/don/chapter_03.pdf

European community sampling procedures can be found in Commission Regulation EC No 401/2006 of 23 February 2006.

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 DON-NIV WB OVERVIEW



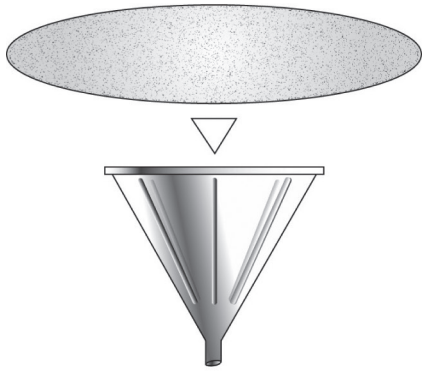
2.0 EQUIPMENT CALIBRATION AND PREPARATION

2.1 PREPARATION OF FILTRATION STEPS

DONtest™ Fluted Filter

The first filtration step is a simple gravity filtration through DON-NIV WB 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 DON-NIV WB fluted filter carefully and insert into clean container. (Optional: a large funnel (part #36022) 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.



Second Filtration

The second filtration step is the gravity filtration of the extract through a VICAM filter #600001106. This removes any precipitates in the extract and assures that the extract will easily pass through the affinity column. The second 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 filter gently into small funnel by pressing filter into funnel with index finger. Be careful not to rip or puncture the filter.

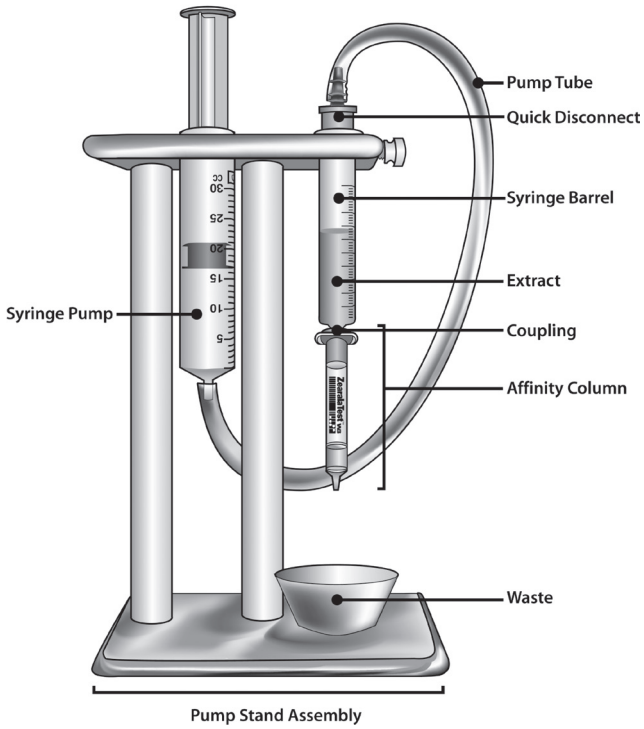
2.2 PUMP STAND SETUP

DON-NIV 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. When using DON-NIV 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.

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.

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 DON-NIV WB PROCEDURE FOR WHEAT

MATERIALS REQUIRED

DESCRIPTION	PART #
DON-NIV WB Columns (25/box)	176002933
DONtest Fluted Filter Paper, 24 cm (100)	31242
Filter (25 pk)	600001106
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, 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	
1 ml and 300 ml Pipettor with pipette tips	

4.0 DON-NIV WB 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 60-70% passes through a 20-mesh sieve. Do not grind sample to a fine powder.

4.2.2 Place 50 g ground sample into a blender jar.

4.2.3 Add 200 ml purified water.

4.2.4 Cover blender jar and blend at high speed for 3 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 Dilution & Filtration:

4.3.1 Take 10 ml above filtrate (step 4.2.5) and mix well with 40 ml PBS.

4.3.2 Place a gently folded filter inside a small funnel and set funnel in clean glass syringe barrel.

4.3.3 Filter the diluted extract through VICAM filter #600001106 into the glass syringe barrel or into a clean container.

4.4 Column Chromatography:

4.4.1 Measure 5 ml (5 ml = 0.25 gram sample equivalent) filtered extract into the glass syringe barrel on the pump stand and pass the 5 ml extract completely through DON-NIV WB affinity column by gravity or at a rate of about 1 drop/2 seconds until air comes through column.

4.4.2 Wash DON-NIV WB affinity column with 10 ml PBS and 10 ml of purified water by gravity or at a rate of about 1 drop/second. Gently blow out water residue at the end of last wash.

4.4.3 Place glass cuvette (VICAM part #34000) under DON-NIV WB column and add 0.5 ml HPLC grade methanol directly into column headspace.

4.4.4 Elute affinity column by passing 0.5 ml HPLC grade methanol through column by gravity.

4.4.5 Elute affinity column by passing 1.5 ml HPLC grade acetonitrile through column by gravity.

4.4.6 Blow out remaining liquid, collecting all the sample eluate (2.0 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 300 µl of HPLC mobile phase.

4.5.3 Separately inject 50 µl of each reconstituted sample and standards.

4.6 Assay Range: 0 – 8 ppm (2000 ng)

4.7 Limit of Detection: DON: 0.03 ppm or lower and NIV: 0.04 ppm or lower

4.8 Recovery: DON > 90% (0 – 8 ppm) and NIV > 90% (0 – 5 ppm) in spiked wheat

5.1 HPLC CONDITIONS

HPLC Conditions 1:

- Column: reverse phase C_{18}, 3.9 x 300 mm (4 mm) (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: NIV 6-7 min, DON 10-11 minutes

HPLC Conditions 2:

- Column: reverse phase C_{18}, 3.9 x 300 mm (4 mm) (Waters part #WAT011695)
- Mobile phase: acetonitrile: water (10:90 by volume) degassed, gradient
- Flow rate: 0.6 ml/min.
- Injection volume: 50 µl
- Lamp: deuterium or mercury lamp
- Detection: 218 nm
- Sample loop: 200 µl
- Run time: 19-20 minutes
- Retention time: NIV 6-7 min; DON 10-11 min; acetyl-DONs 17-18 min

Programmed Flow

Accelerate to 10.0 mL/min in:

Pump Mode Gradient 2.00 min (5.00 mL/min/min)

	Time	Flow	%A	%B	%C	%D	Curve
1		0.60	90.0	0.0	10.0	0.0	
2	1.00	0.60	90.0	0.0	10.0	0.0	11
3	11.00	0.60	90.0	0.0	10.0	0.0	11
4	12.00	0.70	70.0	0.0	30.0	0.0	1
5	18.00	0.60	90.0	0.0	10.0	0.0	11

Solvent A = Water

Solvent C = ACN

5.2 HPLC STANDARD PREPARATION FOR DEOXYNIVALENOL (FOR 0.25 GRAM EQUIVALENT PROCEDURE)

We use the Supelco deoxynivalenol standard product #46911 which comes in sealed ampules at a deoxynivalenol concentration of approximately 200 µg/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.

5.2.1 Prepare Working Solutions of DON

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

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

5.2.2 Prepare DON Standards

0.1 ppm (µg/g) X 0.25 g sample equivalent = 0.025 µg
0.025 µg ÷ 1 µg/ml **(DON-working solution 2)** = 0.025 ml = 25 µl
Add 25 µl **DON-working solution 2** to 1975 µl methanol

0.5 ppm (µg/g) X 0.25 g sample equivalent = 0.125 µg
0.125 µg ÷ 1 µg/ml **(DON-working solution 2)** = 0.125 ml = 125 µl
Add 125 µl **DON-working solution 2** to 1875 µl methanol

5 ppm (µg/g) X 0.25 g sample equivalent = 1.25 µg
1.25 µg ÷ 10 µg/ml **(DON-working solution 1)** = 0.125 ml = 125 µl
Add 125 µl **DON-working solution 1** to 1875 µl methanol

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

5.2.4 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.

$$\textbf{Concentration of Sample (ppm)} = \frac{\textbf{Peak Area of Sample}}{\textbf{Peak Area of Standard}} \times \textbf{Concentration of Standard (ppm)}$$

5.3 SPIKING WHEAT WITH DEOXYNIVALENOL

We use the Supelco deoxynivalenol standard product #46911 which comes in sealed ampules at a deoxynivalenol concentration of approximately 200 µg/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 50 g wheat = 25 µg

25 µg ÷ 200 µg/ml DON standard = 0.125 ml = 125 µl

Add 125 µl DON standard to 50 g wheat

2.0 ppm spike

2.0 ppm (µg/g) X 50g wheat = 100 µg

100 µg ÷ 200 µg/ml DON standard = 0.5 ml = 500 µl

Add 500 µl DON standard to 50 g 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.

5.4 HPLC STANDARD PREPARATION FOR NIVALENOL (FOR 0.25 GRAM EQUIVALENT PROCEDURE)

We use the Sigma nivalenol standard product #34131 which comes in two 1 ml-sealed ampules at nivalenol concentration of approximately 100 µg/ml in acetonitrile. This standard is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of nivalenol.

5.4.1 Prepare Working Solutions of NIV

NIV-working solution 1 (5 µg/ml):

50 µl of NIV Standard (100 µg/ml) + 950 µl Milli-Q water

NIV -working solution 2: (0.5 µg/ml):

100 µl of **NIV -working solution 1** (5 µg/ml) + 900 µl Milli-Q water

5.4.2 Prepare NIV Standards

0.1 ppm (µg/g) X 0.25 g sample equivalent = 0.025 µg

0.025 µg ÷ 0.5 µg/ml (**NIV-working solution 2**) = 0.05 ml = 50 µl

Add 50 µl **NIV-working solution 2** to 1950 µl methanol

0.5 ppm (µg/g) X 0.25 g sample equivalent = 0.125 µg

0.125 µg ÷ 0.5 µg/ml (**NIV-working solution 2**) = 0.25 ml = 250 µl

Add 250 µl **NIV-working solution 2** to 1750 µl methanol

5 ppm (µg/g) X 0.25 g sample equivalent = 1.25 µg

1.25 µg ÷ 5 µg/ml (**NIV-working solution 1**) = 0.25 ml = 250 µl

Add 250 µl **NIV-working solution 1** to 1750 µl methanol

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

5.4.4 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.

5.5 SPIKING WHEAT WITH NIVALENOL

We use the Sigma nivalenol standard product #34131 which comes in two 1 ml-sealed ampules at nivalenol concentration of approximately 100 µg/ml in acetonitrile. This standard is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of nivalenol.

0.5 ppm spike

0.5 ppm (µg/g) X 50 g wheat = 25 µg

25 µg ÷ 100 µg/ml NIV original standard = 0.25 ml = 250 µl

Add 250 µl NIV standard to 50 g wheat

1.0 ppm spike

1.0 ppm (µg/g) X 50 g wheat = 50 µg

50 µg ÷ 100 µg/ml NIV original standard = 0.5 ml = 500 µl

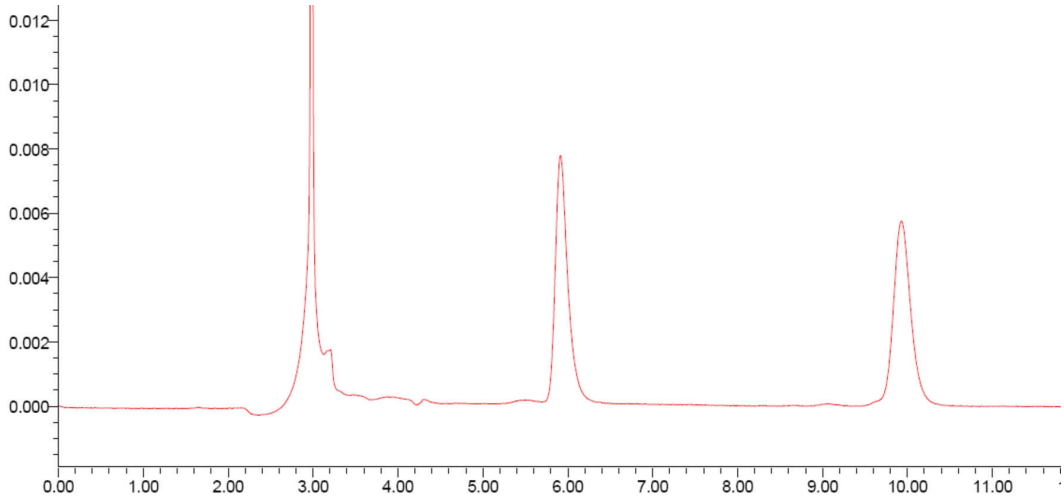
Add 500 µl NIV standard to 50 g wheat

NOTE: Divide volume of NIV 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.

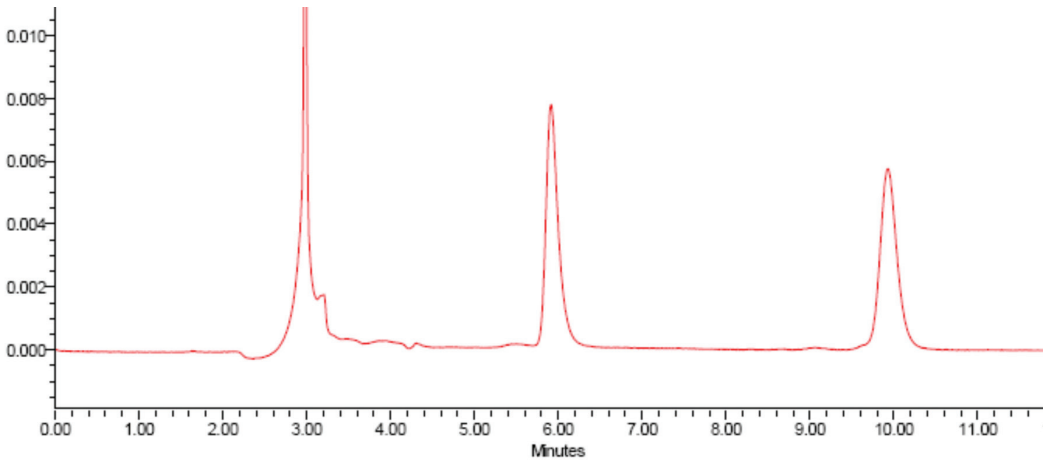
5.6 REPRESENTATIVE HPLC CHROMATOGRAMS

1.0 ppm DON and NIV standard (Retention time: NIV ~ 6min, DON ~10 min.)



Chromatograms were generated using HPLC Conditions 2 and DON-NIV WB columns.

1.0 ppm DON and NIV spiked Wheat



6.0 GENERAL PRECAUTIONS, TROUBLESHOOTING AND LIMITATIONS

6.1 General Precautions:

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

Perform test from beginning to end without interruptions.

Load sample on column immediately after second 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.

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

6.2 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. If organic solvent is used during extraction, dilute organic solvent before passing extract through DON-NIV column.
	Wrong extraction solution	1. Water is needed for extract DON from sample. 2. Do not pass sample over column with more than 15% organic solvent.
	Flow rate is too fast	1. One drop per second when passing diluted extract through DON-NIV column. 2. Elute DON-NIV column by gravity.
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.
	Did not pour the entire extract onto filter	Pour entire blender contents into fluted filter. This will speed filtration.

6.3 Disposal of Materials Containing DON:

1. Collect all suspected DON and NIV 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 and NIV
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.

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 International and United States customers

Fax: 508-482-4972

E-mail: techservice@vicam.com

8.0 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 DON-NIV WB and DON-NIV WB analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that DON-NIV WB and DON-NIV 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 DON-NIV WB and DON-NIV WB product.

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