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INTRODUCTIONS

1.1 INTENDED USER

Myco6in1™ LC/MS/MS is a quantitative method for the simultaneous determination of aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, T-2 and HT-2 toxins. Samples are purified by the VICAM Myco6in1™ LC/MS/MS immunoaffinity column before being quantitated by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

While LC/ESI-MS/MS was used to generate validation data in corn, other methods of quantitation may be used. The column is designed to capture and allow quantitation of the mycotoxins listed. Any measurement method that is adequate to quantitate these mycotoxins could, in principle, be applied to the immunoaffinity column methanol eluate.

1.2 PRINCIPLE

Samples are prepared by extracting sequentially with PBS and then methanol:water solutions. This double extraction results in improved recoveries of fumonisin B1, fumonisin B2 and DON as compared to a single methanol:water extraction. Extracts are diluted and filtered.

The methanol:water extract is passed through the Myco6in1™ LC/MS/MS immunoaffinity column. The column is washed with PBS to remove any traces of methanol present and then the PBS extract is passed over the same Myco6in1™ LC/MS/MS column. Removing the methanol present in the Myco6in1™ LC/MS/MS column before loading the PBS extract improves the recovery of deoxynivalenol.

The column is washed with distilled water to remove the PBS and any matrix interfering compounds. Toxins are eluted from the column with two applications of methanol or other eluting solution. The column eluate is dried down, reconstituted in an appropriate buffer and quantitated by LC/ESI-MS/MS.

These steps are outlined in section 1.7, Myco6in1™ LC/MS/MS Procedure Overview.

1.3 APPLICABILITY AND APPROVALS

Myco6in1™ LC/MS/MS has been optimized for quantitative measurement of aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in corn. The procedure discussed in the Lattanzio paper (see References) meets European Committee for Standardization (CEN) criteria for mycotoxin analysis methods. Assistance in analyzing commodities not listed in this manual can be obtained by contacting our Technical Services Department.

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 United States Federal Grain Inspection Service (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

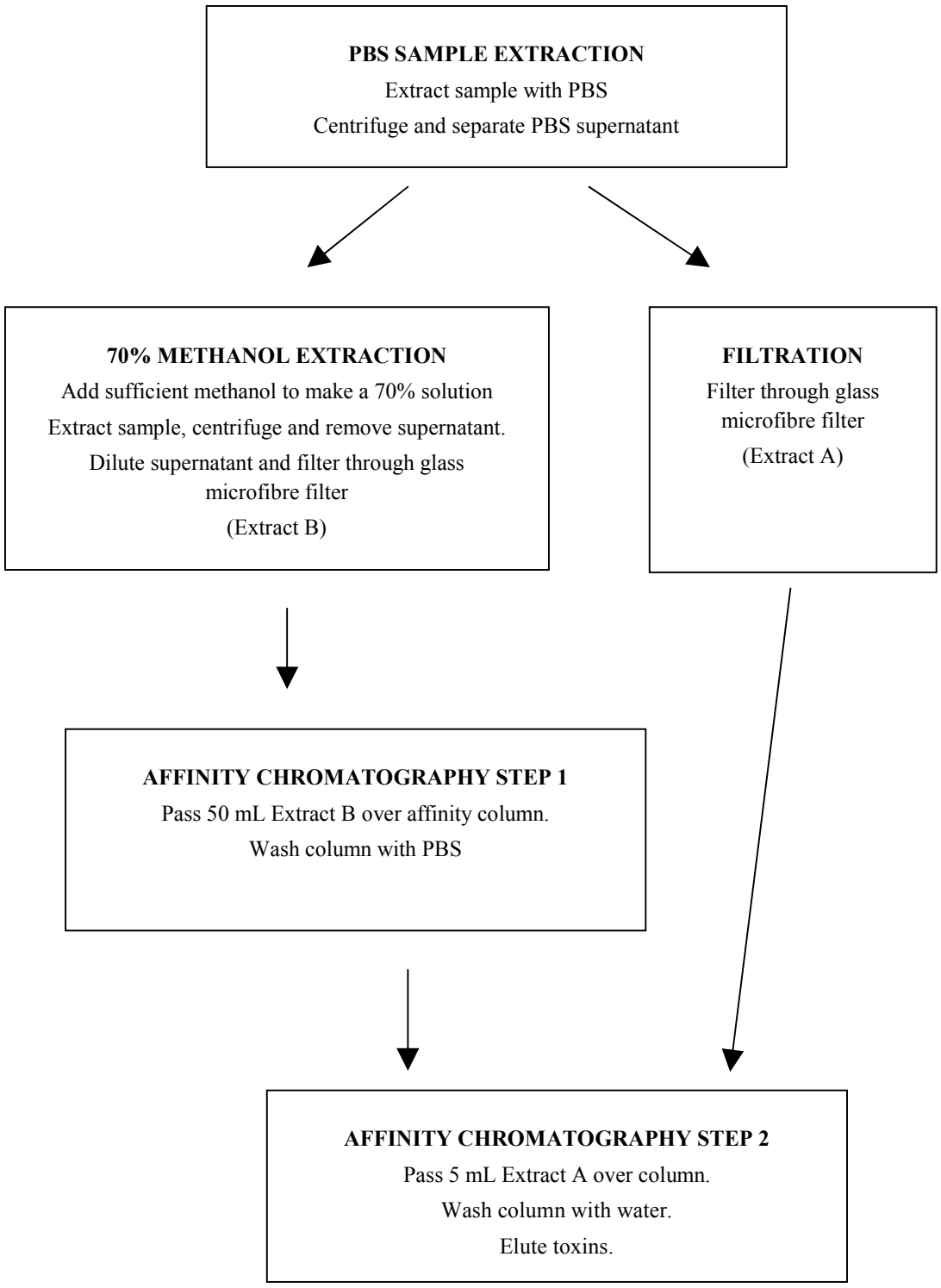
http://www.usda.gov/gipsa/reference-library/brochures/sampling.pdf

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 Myco6in1™ LC/MS/MS columns at refrigerated temperature (2 - 8°C) up until the expiration date on the box of columns. Columns are good for one year from production date. It is recommended that all reagents and columns be at room temperature (18 – 22°C) for usage.

1.7 MYCO6IN1™ LC/MS/MS PROCEDURE OVERVIEW



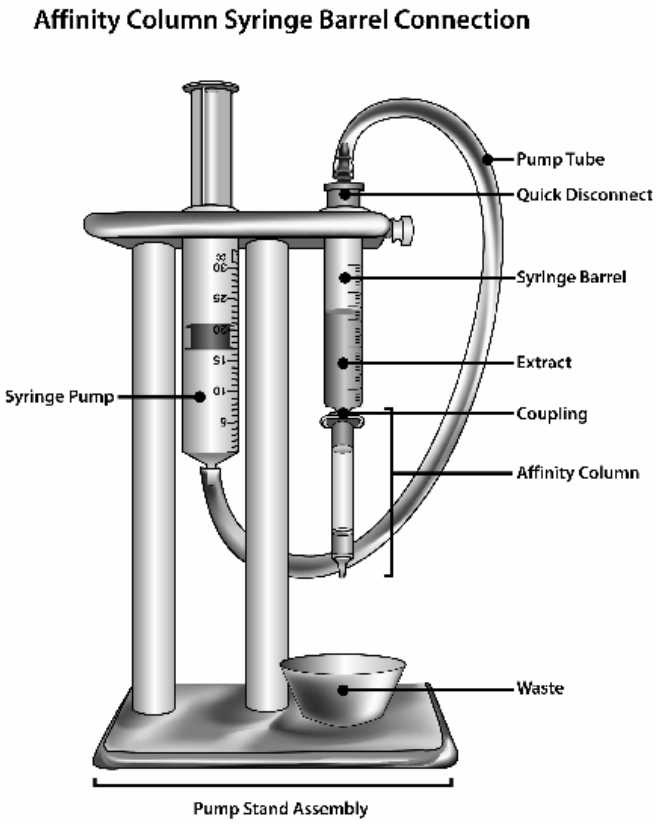
2.1 PUMP STAND SETUP

Myco6in1™ LC/MS/MS affinity chromatography is easily performed with the Myco6in1™ LC/MS/MS 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 #20650) can be attached to the pump tube instead of the large pump syringe barrel to operate without using hand pressure. Double position pump stands (VICAM # 21030), four-position pump stands with aquarium pumps (VICAM #21045), and twelve-position pump stands with aquarium pumps (VICAM # G1104) are available for running multiple samples at one time.

When using a pump stand:

1. Remove large top cap from the column.
2. VICAM WB Column Coupling (part # G1118) provides a reusable coupling for attaching the column to the syringe barrel reservoir.
3. Place waste collection cup under column outlet. Keep bottom cap on column.
4. Place desired amount of extract in 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 the flow rate specified in the procedure. 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.



2.2 CLEANING EQUIPMENT

Before Starting *Myco6in1™ LC/MS/MS* Testing

Before using the equipment, it should be washed with a mild detergent solution and then rinsed thoroughly with purified water. Wash new syringe barrel for pump stands using a brush with soap and water. Then rinse with purified water and methanol before using . Other pieces of equipment that need to be cleaned with detergent before using are graduated cylinders, funnels and extraction containers. Rinse all equipment carefully to remove detergent residue from glassware since mass spectrometry can be adversely affected by the presence of trace amounts of detergent.

Between Assays:

After each assay, any equipment that will be reused to hold, collect or transfer samples or sample extracts needs to be washed with a mild detergent solution and rinsed thoroughly with purified water.*

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. After a number of samples have been tested, the glass syringe barrel should be washed with a brush and detergent and rinsed well with water.

Other Important Precautions

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

3.0 REAGENT PREPARATION

Phosphate Buffered Saline (pH 7.4)

0.20 g KCl

0.20 g KH_2PO_4

2.92 g $Na_2HPO_4 \cdot 12H_2O$

8.00 g NaCl

Dissolve in 900 mL purified water. Adjust to pH 7.4 with 0.1M HCl or 0.1M NaOH and dilute to 1000 mL.

Commercial buffered saline tablets may also be used. A 10X concentrate of PBS may also be purchased from VICAM (# G1113). 10X PBS Concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X concentrate with 900 mL purified water.

* More details on decontamination can be found in JAOAC **48**, 681 (1965); Am. Hyg. Assoc. J. **42**, 398 (1981); and IARC Sci. Publ. No. 37, IARC, Lyon, France, 1980.

4.1 MATERIALS AND EQUIPMENT REQUIRED

Materials Required

Description	Part #
Myco6in1™ LC/MS/MS Columns (25/box)	100000176
Phosphate Buffered Saline, 10X concentrate (150 mL)	G1113
Disposable Cuvettes (250)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers (25)	36010
Whatman GF/A microfibre filters	
Acetic acid, glacial (HPLC grade)	
Ammonium acetate (mass spectrometry grade)	
Distilled, reverse osmosis or deionized water	

Equipment Required

Description	Part #
Graduated Cylinder, 50 mL	20050
Digital Scale with AC Adapter	20100
Micro-pipettor, 1.0 mL	G4033
Micro-pipette Tips for 1 mL Micro-pipettor (100)	20656
Graduated Cylinder, 250 mL	20250
Wash Bottle, 500 mL	20700
Cuvette Rack	21010
Single Position Pump Stand	21020
or 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
VICAM WB Column Coupling (6 per pack)	G1118
Filter Funnel, 65 mm (10 per pack)	36020
Orbital shaker	
Centrifuge capable of obtaining 3000 x g Relative Centrifugal Force	
LC/MS/MS System	

Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, deoxynivalenol, zearalenone, T-2 and HT-2 toxins are available for sale from Sigma-Aldrich (www.sigmaaldrich.com). VICAM has also obtained T-2 and HT-2 from Trilogy Analytical Laboratories (www.trilogylab.com). The sale of some of these mycotoxins may be restricted in certain countries.

4.2 MYCO6IN1™ LC/MS/MS PROCEDURE FOR CORN

1.0 LC/MS/MS Conditions:

Equipment: Validation of this procedure was performed using the equipment listed in the Lattanzio reference in section 5. Although specific equipment, columns and conditions are listed in that paper, any measurement method that is adequate to quantitate these mycotoxins could, in principle, be applied to the immunoaffinity column methanol eluate.

2.0 Sample Extraction, Dilution and Filtration:

2.1 Add 50 mL PBS to 10 g ground corn and shake for 60 minutes on an orbital shaker.

2.2 Centrifuge sample at 3000*g* for 10 minutes. Remove the PBS extract and reserve the solid material for further extraction.

2.3 Filter the PBS extract through a Whatman GF/A glass microfibre filter (**extract A**).

2.4 Add 35 mL of HPLC grade methanol to the remaining solid material and shake again for 60 minutes on an orbital shaker.

2.5 Centrifuge sample at 3000*g* for 10 minutes and remove the methanol:PBS extract.

2.6 Dilute 10 mL of the methanol:PBS extract with 90 mL of PBS. Mix and then filter this through a Whatman GF/A glass microfibre filter (**extract B**).

3.0 Column Chromatography

3.1 Remove large top cap from column. VICAM WB Column Coupling (part # G1118) provides a reusable coupling for attaching the column to the syringe barrel reservoir on the pumpstand.

3.2 Pass 50 mL of **extract B** completely through Myco6in1™ LC/MS/MS affinity column at a rate of about 1-2 drops/second until air comes through column.

3.3 Pass 20 mL of PBS through the column at a rate of about 2 drops/second to wash any remaining methanol from the column.

3.4 Pass 5 mL of **extract A** completely through Myco6in1™ LC/MS/MS affinity column at a rate of about 1-2 drops/second until air comes through column.

3.5 Pass 20 mL of purified water through the column at a rate of about 2 drops/second to remove any PBS residue and matrix interfering compounds.

3.6 Place glass cuvette (VICAM # 34000) under Myco6in1™ LC/MS/MS column. Add 1.5 mL HPLC grade methanol into the column headspace and reattached column to the glass syringe barrel.

3.7 Elute column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate in the glass cuvette. When most of the methanol has passed through the column but the top of the resin bed is not yet dry, stop applying air pressure to the column and allow it to sit undisturbed for 5 minutes.

3.8 Add an additional 1.5 mL HPLC grade methanol into the column headspace and reattached column to the glass syringe barrel. Continue to elute column at a rate of 1 drop/second and collect all of the sample eluate in the same glass cuvette.

3.9 Dry down eluate under an air stream at 50°C. Reconstitute with 200 µL of methanol:water (40:60) containing 1 mM ammonium acetate and 0.1% acetic acid. Analyze 20 µL (equivalent to 100 mg corn sample) by LC/MS/MS.

4.0 Limit of Detection: (values are dependent upon the detection system used)

Toxin	Limit of Detection (ppb)
Aflatoxin B1	0.6
Aflatoxin B2	0.3
Aflatoxin G1	0.4
Aflatoxin G2	0.8
Deoxynivalenol	4.2
Fumonisin B1	1.1
Fumonisin B2	0.4
Ochratoxin-A	0.6
T-2*	1.5
HT-2*	1.9
Zearalenone	0.7

5.0 Recovery:

Toxin	Spiking level (ppb)	% Recovery	% RSD
Aflatoxin B1	10	104	10
Aflatoxin B2	2	98	6
Aflatoxin G1	6	102	6
Aflatoxin G2	2	95	7
Deoxynivalenol	500	79	0.2
Fumonisin B1	500	101	13
Fumonisin B2	250	96	8
Ochratoxin-A	20	82	11
T-2*	100	nd	nd
HT-2*	100	180	1
Zearalenone	100	81	9

* Corresponding to the sum of HT-2 + hydrolyzed T-2. See Lattanzio reference for details.

5.0 REFERENCES AND ALTERNATE PROCEDURES

Lattanzio, V., Solfrizzo, M., Powers, S., Visconti, A., Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup, *Rapid Commun. Mass Spectrometry* 2007; **21**: 3253-3261.

An alternative procedure for elution of the bound toxins from the column has been found to improve recovery. The modification to the procedure given in section 3.0 above is:

3.6 An eluting solution of methanol:water (80:20) containing 0.5% acetic acid should be freshly prepared every day before use. Place a silanized glass cuvette under Myco6in1™ LC/MS/MS column. Add 1.5 mL of eluting solution directly into column headspace and allow this to elute into the silanized glass cuvette by gravity. Once the eluting solution runs through resin bed do not apply any air pressure, and allow the column to sit undisturbed for 5 minutes.

3.7 After 5 minutes add another 1.5 mL of eluting solution directly into column headspace. Allow the column to flow by gravity and collect the eluate into the same cuvette (~3 mL eluate at this step in total).

3.8 Once the second 1.5 mL of eluting solution completely runs through the resin bed, place a new silanized glass cuvette under the column and add **two separate** 1.5 mL portions of eluting solution directly into column headspace. Allow this to elute into the second cuvette by gravity. Apply strong air pressure once elution is finished to collect all the eluate. Elution volume in this cuvette is ~3 mL, but the total volume for each sample/spike is ~ 6 mL).

3.9 Dry all eluates under a vacuum with medium heat (40 °C) for approximately 3.5 hours. **Adjust volume between sample cuvettes during drying process because elute in one cuvette might dry faster than the other.**

3.10 Add 100 µL of methanol:water (40:60) containing 1 mM ammonium acetate and 0.1% acetic acid to each cuvette (each sample has two cuvettes) and vortex well. Allow the cuvettes to sit for 5 minutes, vortexing occasionally during this time. Mix the two eluate cuvettes for each sample (total 200 µL for each sample). Analyze 20 µL (equivalent to 100 mg corn sample) by LC/MS/MS.

6.0 TECHNICAL ASSISTANCE

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

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

International and United States customers
Telephone: 617-926-7045

Fax: 617-923-8055

e-mail: techservice@VICAM.com

7.0 LIABILITY

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 Myco6in1™ LC/MS/MS analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that Myco6in1™ LC/MS/MS 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 Myco6in1™ LC/MS/MS 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.

All VICAM products are protected by worldwide patents and trademarks.

8.0 ORDERING INFORMATION

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

In the United States:
Telephone: 877-288-4244

Canada and the United States
Telephone: 800-338-4381

Mexico
Telephone: 617-926-7045

International and United States customers
Fax: 617-923-8055

E-mail: vicam@vicam.com

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