

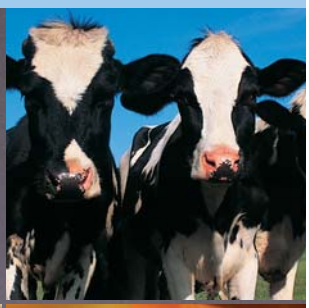

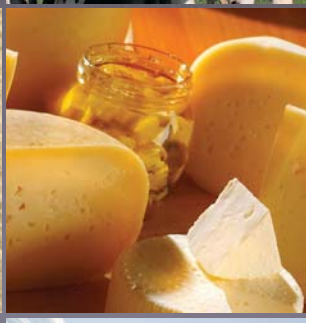


Afla M1

Instruction Manual

VICAM®

A Waters Business

34 MAPLE STREET, MILFORD MA 01757 USA
TEL: 800.338.4381, 508.482.4935
FAX: 508.482.4972 EMAIL: VICAM@VICAM.COM

Afla M_1 Instruction Manual (HPLC, UPLC, and LC/MS)

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1.1 INTENDED USER

Afla M_1™ is a quantitative method for the detection of aflatoxin M_1 in milk. VICAM's advanced technology permits the measurement of aflatoxin M_1 without the use of toxic solvents like chloroform or methylene chloride. Afla M_1™ aflatoxin testing is used in a wide variety of locations from milk processing quality-control laboratories to government testing laboratories—anyplace where quick, easy-to-perform, and highly accurate aflatoxin analysis can prevent contamination and improve the quality of the milk supply.

1.2 PRINCIPLE

Mycotoxins are toxic fungal metabolites that are hazardous to human health and cause economic losses due to disease or reduced production efficiency in livestock. Aflatoxin M_1 is a metabolite of aflatoxin B_1, which is present in the milk of animals that ingest feed contaminated with aflatoxin B_1. Afla M_1™ is a fast, simple, safe, and highly accurate method of quantitatively measuring aflatoxin M_1 in powdered and liquid milk.

Samples are prepared by centrifuging the milk and separating out the fat layer. The skim portion is then applied to the Afla M_1™ column, which contains specific antibodies that selectively bind to aflatoxin M_1. Once the the aflatoxin is bound to the antibody on the column, the column is washed with water to flush out matrix impurities. To elute the aflatoxin from the antibody, an acetonitrile/methanol solution is passed through the column. The aflatoxin M_1 concentration of the sample can then be measured by analyzing a portion of the eluate with high performance liquid chromatography (HPLC), UPLC, or UPLC/MS/MS. These steps are outlined in section 1.5, Afla M_1™ Procedure Overview. The antibodies on the VICAM Afla M_1 column will also bind aflatoxin M_2.

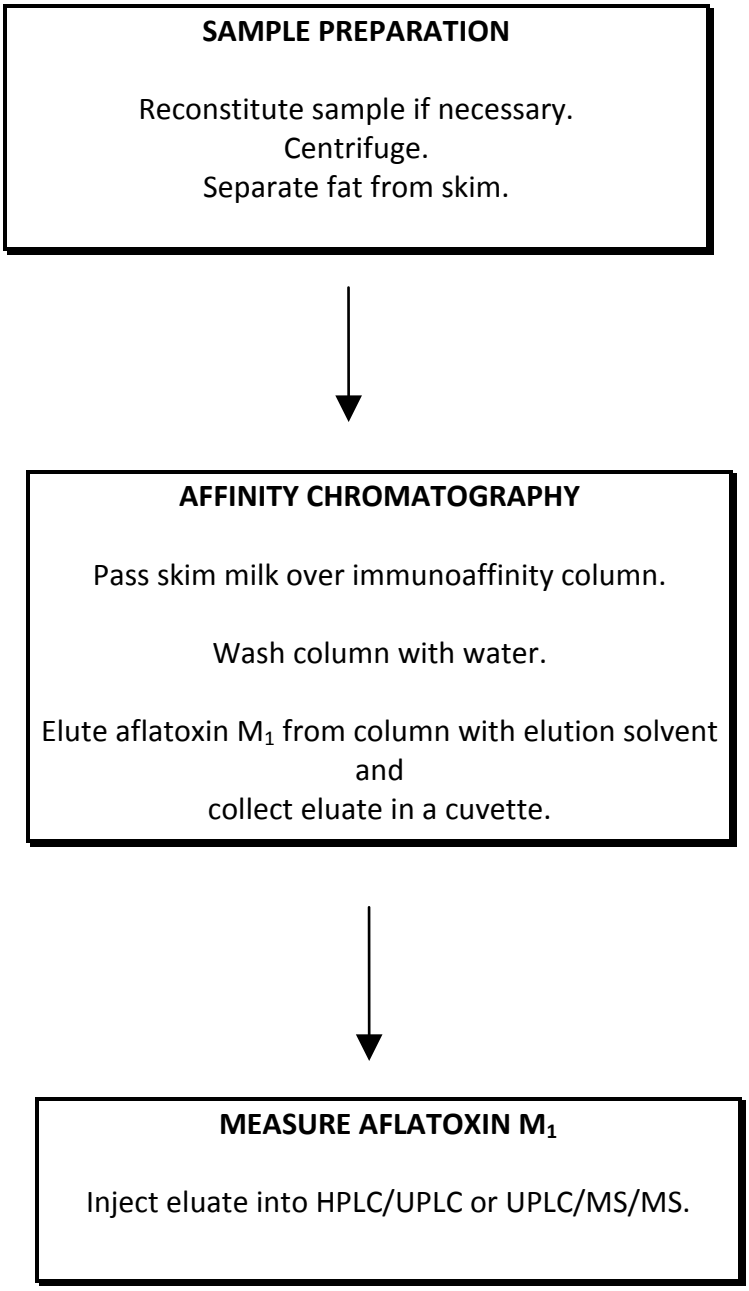
1.3 LIMITATIONS

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. Deviations from these instructions may reduce the test's capacity to yield optimal results.

1.4 SHELF LIFE AND STORAGE CONDITIONS

Store at room temperature. Storage at temperatures above 30°C for prolonged periods of time may reduce shelf life. If storage temperatures above 30°C are anticipated, store columns and reagents in the refrigerator (2–8°C). Do not freeze them. When ready for use, reagents should be at room temperature (20–25°C).

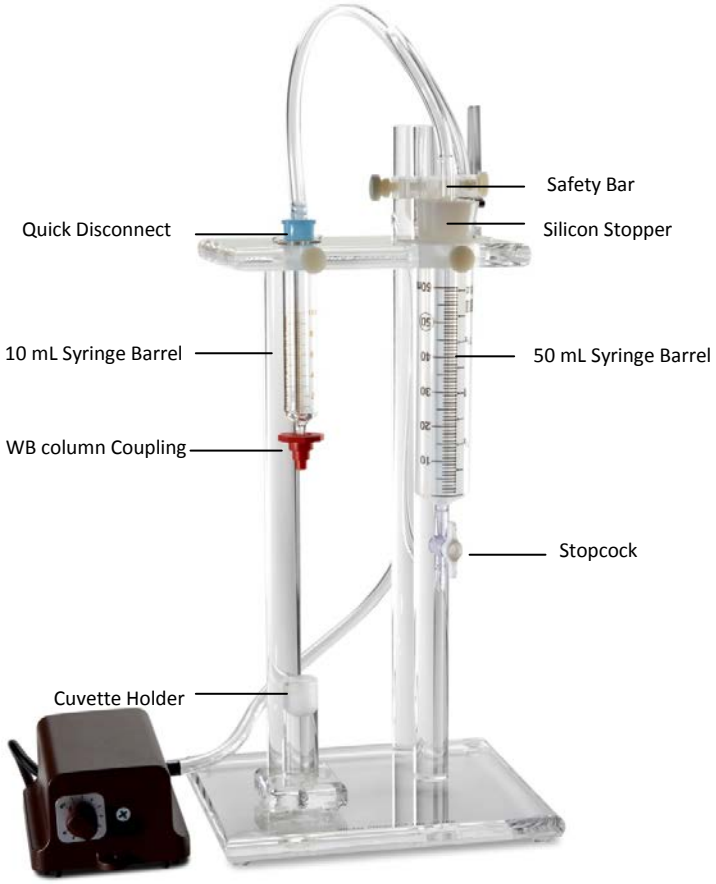
1.5 AFLA M_1™ PROCEDURE OVERVIEW



2.1 PUMP STAND SETUP

Afla M_1 immunoaffinity chromatography procedure is easily performed with the Afla M_1™ affinity column attached to a specially designed Afla M_1 pump stand with a positive pressure pump (VICAM # G1106). The stand has a 50 mL syringe barrel that functions as the sample reservoir and a 10 mL glass syringe barrel that holds the reagents used for the wash and elution steps. An aquarium pump with an attached tube and coupling provides the air pressure required to push liquids through the affinity column. Alternatively, a vacuum manifold can be used to pull liquid through the Afla M_1 column if needed. Frequently, liquids will flow through the Afla M_1 column by means of gravity alone.

1. Place a 50 mL syringe barrel in the large hole in the syringe barrel holder at the top of the stand. Secure by tightening the adjacent thumb screw. Attach WB column coupling (VICAM part # G1118) to outlet at the bottom of syringe barrel.
2. Place a 10 mL glass syringe barrel in small hole in the syringe barrel holder. Secure by tightening the adjacent thumb screw. Attach WB column coupling (VICAM part # G1118) to outlet at the bottom of the syringe barrels.
3. Remove large top cap from affinity column.
4. For chromatography, attach column to column coupling.
5. Place waste collection cup under column.
6. The sample will be poured into the 50 mL syringe barrel for chromatography. Sample may flow onto the column at 1 drop/second by gravity. If it does not, milk can be passed through the column by application of positive pressure from the aquarium pump. DO NOT EXCEED RECOMMENDED FLOW RATES. This can result in decreased recovery. If the sample flows too fast, use a stopcock (VICAM part # G1117) to reduce the flow rate to 1 drop/second.
7. If positive pressure is desired, insert stopper attached to tubing into top of the 50 mL syringe barrel. Lower safety bar on top of the syringe barrel stopper and tighten thumb screw to secure it. Pump pressure is controlled by turning the adjusting knob on the pump.
8. Transfer column and coupling to 10 mL syringe barrel for washing and elution, so that milk residue will not carry over from the 50 mL syringe barrel into wash and elution fluids.
9. Wash as described in procedure.
10. Elute as described in procedure.

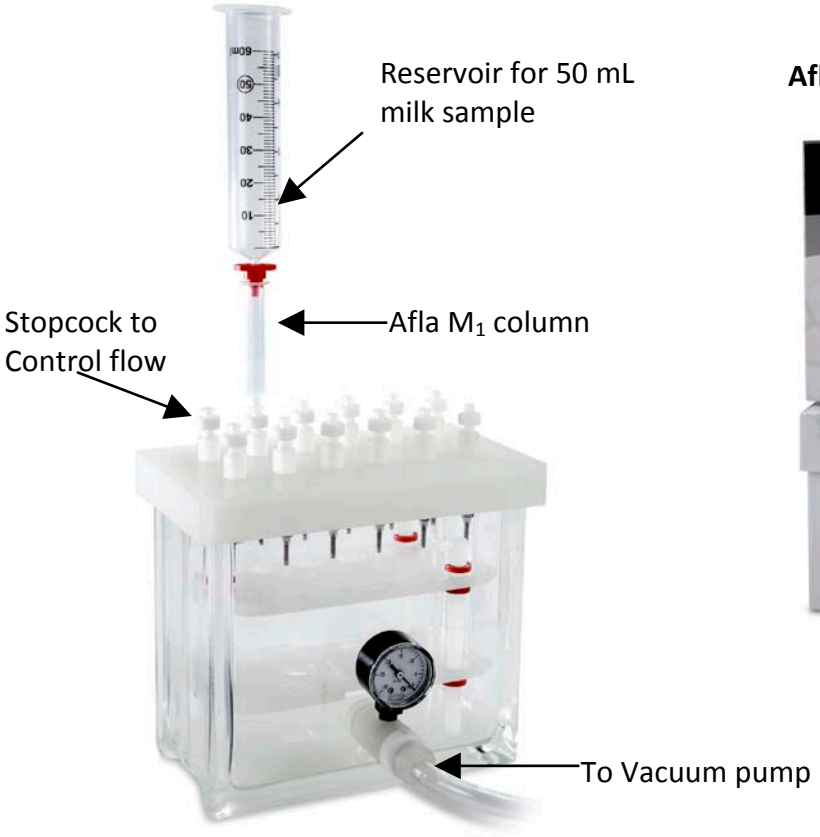


VICAM Part # G1106

Afla M_1 Pump Stand with Positive Pressure Pump

VICAM Part # G1107

12-Position Vacuum Manifold Setup for Afla M_1



Afla M_1 HPLC kits (VICAM Part # G1043)

2.2 CLEANING EQUIPMENT

Before Starting Afla M_1™ Testing

To eliminate background fluorescence, make sure the equipment is clean and not contaminated with fluorescent materials such as dust, fingerprints, lubricant, and fibers. This is particularly important when using brand-new equipment or equipment that has not been used for a long time.

Before using equipment, wash it with a mild detergent solution, and then rinse thoroughly with purified water. Syringe barrels designed for use with a piston plunger may be treated with a lubricant. To remove it, wash new syringe barrels before using with a brush and soap and water, and then rinse with purified water and methanol. Graduated cylinders and beakers also need to be cleaned with detergent before use.

Between Assays:

After each assay, the beakers and graduated cylinders need to be washed with a mild detergent solution and rinsed thoroughly with purified water. The same 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. After a number of samples have been tested, the syringe barrel should be washed with a brush and detergent and rinsed well with water.

It is not recommended to wash and reuse the cuvettes. These cuvettes are designed for one-time use and should be discarded.

Other Important Precautions

Use only equipment specified by VICAM. 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 fluorescent materials into the sample and thereby affect results. When running mass spectrometry tests, avoid use of detergents.

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

3.1 PREPARATION OF ELUTION SOLUTION

The Afla M_1™ procedure uses an acetonitrile: methanol solution to elute aflatoxin M_1 off the column.

To prepare elution solution:

Use HPLC-grade acetonitrile and methanol when preparing elution solutions.

Solution desired (acetonitrile:methanol)	Acetonitrile (mL)	Methanol (mL)	Total Volume (mL)
3:2	30	20	50

CAUTION: This solution is flammable. Keep container tightly capped when not in use.
Prepare every week or as needed. The formula above will prepare approximately 50 mL of solution. Solution volume may be increased or decreased as needed provided the proportions of reagents is kept consistent.

3.2 PREPARATION OF HPLC MOBILE PHASE

The Afla M_1™ procedure uses a water: acetonitrile: methanol solution as the mobile phase for the HPLC .

To prepare mobile phase :

Use HPLC-grade acetonitrile, methanol, and water when preparing solutions.

Solution desired (water:acetonitrile:methanol)	Water (mL)	Acetonitrile (mL)	Methanol (mL)	Total Volume (mL)
68:24:8	680	240	80	1000 (1 liter)

CAUTION: This solution is volatile. Keep container covered.
Prepare every week or as needed. The formula above will prepare approximately 1 liter of solution. Solution volume may be increased or decreased as needed provided the proportions of reagents are kept consistent.

3.3 BACKFLUSH

Backflushing will increase the time the elution solvent is in contact with the antibodies in the Afla M_1 column, ensuring that all toxins are eluted. By gently pushing and pulling a syringe with plunger (VICAM part # 600001145) with an attached coupling (VICAM part # G1118) placed on top of the Afla M_1 immunoaffinty column during elution, the elution solvent will move back and forth through the column to fully wet the resin. Repeat this process at least three times.

4.0 MATERIALS AND EQUIPMENT

MATERIALS REQUIRED

Description	Part #
Afla M_1™ columns (25 per box)	G1007
Afla M_1 HPLC Kit (100 columns and 4 sets stds)	G1043
Afla M_1 HPLC Kit (250 columns and 10 sets stds)	G1039
Fluted filter paper	31240
Disposable cuvettes (250)	34000
Methanol, HPLC grade (4 x 4 L)	35016
Disposable plastic beakers (25)	36010
Purified water (distilled, reverse osmosis or deionized water)	
Acetonitrile (4 x 4 L)	G1130

EQUIPMENT REQUIRED

Description	Part #
Graduated cylinder, 50 mL	20050
Wash bottle, 500 mL	20700
Digital scale with AC adapter	20100
Afla M_1 single-position pump stand with pump (for single sample)	G1106
Afla M_1 4-position pump stand with 2 pumps (for multiple samples)	G1107
4-position pump stand w/2 air pumps (10 mL)	21045
12-position pump stand w/6 air pumps (10 mL)	G1104
Micropipettor, 1 mL	G4033
Micropipette tips for 1 mL micropipettors (100)	20656
50 mL centrifuge tubes	
Centrifuge capable of obtaining 2000 x g relative centrifugal force*	
Nitrogen evaporator	
HPLC system as specified in procedure	

SUGGESTED BUT NOT REQUIRED

Description	Part #
Vortex mixer	23040

* NOTE: The rpm value that corresponds to the specified g force will vary depending on the centrifuge rotor. Use a nomogram to identify the rpm corresponding to the specified g force for your centrifuge rotor. Rotor purchases typically include nomograms developed by the manufacturer.

5.1 CHOOSING A METHOD:

Four methods of determining aflatoxin M_1 in milk are listed below. All of these methods are accurate and will give excellent results. The original method and AOAC official method use a 50 mL milk sample with HPLC. Newer methods using more sensitive detectors and instrumentation can be run by passing a smaller amount of milk sample over the Afla M_1 immunaffinity column. This approach delivers faster results, enabling an operator to test more samples in a shorter period of time. In addition, immunoaffinity column cleanup can be used with LC/MS/MS as listed in section 6.0 and referenced in section 5.7. Published methods are available for cheese, curd, and yogurt as well as milk.

5.2 PREPARATION OF POWDERED MILK SAMPLES

1.0 Milk Powder – Prepare as instructed by the manufacturer. If no instructions given, follow the steps listed below:

1.1 Add 10 g milk powder to a 250 mL beaker.
1.2 Heat 100 mL purified water to 30–40°C.
1.3 Add 80 mL preheated water in small amounts to the milk powder.
1.4 Mix continually until a homogeneous mixture is obtained.
1.5 Transfer milk mixture to a 250 mL measuring cylinder, and bring the volume to 100 mL with the remaining preheated water.
1.6 Centrifuge two 50 mL samples at 2,000 x g for 15 minutes.
1.7 Separate fat (top) layer from defatted (bottom) layer. Use defatted (skim) milk for further analysis. If fat layer is not clearly separated or milk is passing slowly through the column, centrifuge at 10,000 x g for 10 minutes or at 5,000 x g up to an 1 hour.

2.0 Certified Reference Milk
Institute for Reference Materials and Measurements (IRMM) samples currently available through Sigma-Aldrich

2.1 Add 10 g milk powder to a beaker.
2.2 Heat 100 mL purified water to 50–60°C.
2.3 Add 60 mL preheated water to the milk powder. Stir 10 minutes on a stir plate.
2.4 Transfer to a measuring cylinder, and bring the volume to 100 mL with the remaining preheated water.
2.5 Centrifuge two 50 mL samples at 5,000 x g for 15 minutes.
2.6 Freeze centrifuged sample for 15 minutes.
2.7 Separate fat (top) layer from defatted (bottom) layer. Use defatted (skim) milk for further analysis.

Follow column chromatography methods for liquid milk outlined in sections 5.3–5.6.

5.3 AFLA M_1™ HPLC PROCEDURE (50 ML SAMPLE EQUIVALENT)

1.0 HPLC Setup:

1.1 Column: reverse phase Spherisorb ODS-2, 4.6mm x 250 mm, 5 µm (Waters part # PSS831915). A shorter 150mm HPLC column can also be used giving shorter retention times.
1.2 Mobile phase: water:acetonitrile:methanol (68:24:8)
1.3 Flow rate: 1.0 mL/min.
1.4 Fluorescence detector: Waters 474 scanning fluorescence detector, excitation 360 nm, emission 440 nm
1.5 Retention time: about 11 minutes (about 6 minutes for shorter column)

2.0 Sample Preparation:

2.1 Measure two 50 mL of fluid milk into a 50 mL cylinder.
2.2 Centrifuge two 50 mL samples at 2000 x g for 15 minutes.
2.3 Separate fat (top) layer from defatted (bottom) layer. Use defatted (skim) milk for further analysis. Filter if necessary to remove fat particles. If fat layer is not clearly separated or milk is passing slowly through the column, centrifuge at 10,000 x g for 10 minutes or 5,000 x g for up to 1 hour.

3.0 Column Chromatography

3.1 Pass 50 mL of defatted (skim) milk completely through Afla M_1™ affinity column at a rate of about 1 drop/second until air comes through column. It should take 20 minutes for milk to flow through the column. The flow may need to be slowed down using the stopcock.
3.2 Remove column from syringe barrel, and fill the column head space with water. Transfer column to a clean glass syringe barrel, and pass 10 mL of purified water through the column at a rate of 1–2 drops/second.
3.3 Repeat step 3.2 once more until air comes through column.
3.4 Elute affinity column by passing 1.25 mL acetonitrile:methanol (3:2) elution solution through column very slowly at a rate of 1 drop for every 2–3 seconds. Make sure elution solution fills any air pockets in the resin. Collect all of the sample eluate (1.25 mL) in a glass cuvette. Backflush technique can be used to enhance elution. (Please see Backflush section 3.3 for more information.)
3.5 Elute affinity column again by passing 1.25 mL purified water through column at a rate of 1 drop for every 2–3 seconds. Collect all of the sample eluate (1.25 mL) in the same glass cuvette (2.5 mL total volume).
3.6 Vortex cuvette and inject 100 µL eluate into HPLC.

4.0 Recovery: Average recovery of 80% total aflatoxin M_1 over 0.010–3 ppb range.

5.4 AFLA M_1™ HPLC AOAC Method 2000.08 (50 ML SAMPLE EQUIVALENT)

1.0 HPLC Setup:

Multiple HPLC conditions are possible. See AOAC official method 2008.08 or

Sylviane Dragacci, Frederic Grosso, Jöel Gilbert, "Immunoaffinity Column Cleanup with Liquid Chromatography for Determination of Aflatoxin M_1 in Liquid Milk: Collaborative Study," *Journal of AOAC International* 84, no. 2 (2001): 437–443.

VICAM's Afla M_1column has a capacity of at least 100 ng aflatoxin M_1 with a recovery of at least 80% when 50 mL of a 80 ng/L aflatoxin M_1 solution is applied to the column (4 ng).

2.0 Warm milk to 37°C in a water bath and stir gently to disperse fat. Centrifuge liquid milk at 2000 x g to separate fat and discard the upper fat layer. Filter skim (lower layer) through one or more filter papers Whatman 4 or equivalent, collecting 50 mL.

3.0 Column Chromatography

3.1 Pass 50 mL of defatted (skim) milk completely through Afla M_1™ affinity column at a rate of about 2–3mL/min until air comes through column. Column may be able to flow by gravity. It should take 20 minutes for milk to flow through the column. The flow may need to be slowed down using the stopcock.

3.2 Remove column from syringe barrel, and fill the column head space with water. Transfer column to a clean glass syringe barrel, and pass 10 mL of purified water through the column at a rate of 1–2 drops/second.

3.3 Repeat step 3.2 once more (20 mL water total) until air comes through column.

3.4 Transfer column to dry clean syringe barrel. Elute affinity column by passing 4 mL pure acetonitrile through column very slowly at a rate of 1 drop for every 2–3 seconds, collecting in a silanized glass tube. Make sure acetonitrile fills any air pockets in the resin. Allow acetonitrile to be in contact with column at least 60 seconds. Backflush technique can be used to enhance elution. (Please see Backflush section 3.3 for more information.)

3.5 Evaporate to dryness under nitrogen. Silanized tubes can be used to prevent toxin from drying permenantly to tubes.

3.6 Reconstitute in 200 µl mobile phase.

3.7 Inject 50 µL reconstituted sample into HPLC.

4.0 Recovery: Greater than 80%

5.5 AFLA M_1™ HPLC PROCEDURE (10 ML SAMPLE EQUIVALENT)

1.0 HPLC Setup:

1.1 Instrument: Waters Alliance e2695
1.2 Column: Waters Spherisorb 5 µm, reverse phase, 4.6 x 250 mm (part # PSS831915). A shorter 150mm HPLC column can also be used giving shorter retention times.
1.3 Detection: Waters Multi Fluorescence Detector 2475
1.4 Detection wavelength: 360 nm excitation and 440 nm emission
1.5 Mobile phase: water:acetonitrile:methanol (68:24:8) isocratic
1.6 Flow rate: 1 mL/min.
1.7 Retention time: about 11 minutes (about 6 minutes with shorter column)

2.0 Sample Preparation:

2.1 Measure 50 mL of milk into conical vial.
2.2 Centrifuge milk for 10 minutes at 10,000 x g or up to 1 hour at 5,000 x g.
2.3 Remove upper fat layer (top), and collect defatted milk sample (bottom layer). Filter if necessary to remove fat particles.

3.0 Afla M_1 Affinity Chromatography

3.1 Set up syringe column assembly.
3.2 Attach stopcock to bottom of Afla M_1 column making sure stopcock is on the off (no flow) position.
3.3 Pipette 10 mL defatted milk into syringe barrel.
3.4 Slowly release stopcock, and allow milk to flow through at 1 drop per second.
3.5 Wash column by filling column headspace (aprox. 2 mL) and transferring column to a clean 10 mL glass syringe barrel. Fill syringe barrel with purified water, 12 mL total water. Let pass through by gravity.
3.6 Add 10 mL of purified water to syringe barrel when there are approximately 2 mL water left in column headspace from step 3.5. Let pass through by gravity. Blow out excess water with air.
3.7 Elute column with 1.25 mL acetonitrile: methanol (3:2 v/v) by gravity (about 1 drop for every 2–3 seconds or slower) and collect in cuvette. Make sure elution solution fills any air pockets in the resin. Collect all of the sample eluate (1.25 mL) in a glass cuvette. Backflush technique can be used to enhance elution. (Please see Backflush section 3.3 for more information.)
3.8 Elute column again by passing 1.25 mL Milli-Q water by gravity, and collect in the same cuvette (2.5 mL total volume).
3.9 Vortex well and inject 100 uL of eluate into HPLC.

4.0 Limit of Detection: 0.00625 ppb (6.25 ppt)

5.0 Recovery: Average of 93% over assay range of 0.025–3ppb.

5.6 AFLA M_1™ RAPID UPLC PROCEDURE (2 ML SAMPLE EQUIVALENT)

1.0 UPLC Setup:

- **1.1** Instrument: Waters ACQUITY UPLC H-Class System
- **1.2** Column: ACQUITY UPLC HSS T3 1.8 µm (2.1 x 100 mm) (Waters part # 18600359)
- **1.3** Detection: AQUITY FLR Detector with **large volume flow cell** (Waters part # 205000609)
- **1.4** Detection wavelength: 365 nm excitation and 435 nm emission
- **1.5** Mobile phase: water:acetonitrile:methanol (68:24:8) isocratic
- **1.6** Flow rate: 0.4 mL/min.

2.0 Sample Preparation:

- **2.1** Measure 50 mL of milk into conical vial.
- **2.2** Centrifuge milk for 10 minutes at 10,000 x g or up to 1 hour at 5,000 x g.
- **2.3** Remove upper fat layer and collect defatted milk sample (bottom layer). Filter if necessary to remove fat particles.

3.0 Afla M_1 Affinity Chromatography

- **3.1** Pour out PBS from Afla M_1™ column headspace.
- **3.2** Pipette 2 mL defatted milk into column headspace, attach column to syringe barrel outlet, and let milk pass through by gravity.
- **3.3** Wash column by filling column headspace (approx. 2 mL) with methanol:water (15:85 v/v), and let pass through by gravity.
- **3.4** Wash column again by filling column headspace with methanol:water (15:85 v/v), reattach column to syringe barrel outlet, and fill syringe with 10 mL of methanol:water (15:85 v/v). Let pass through by gravity.
- **3.5** Blow out excess water with air.
- **3.6** Elute column with 2 mL methanol (1 drop per 2–3 seconds), and collect into silanized cuvette. Backflush technique can be used to enhance elution. (Please see Backflush section 3.3 for more information.)
- **3.7** Dry elution under steady stream of nitrogen at 40°C.
- **3.8** Reconstitute dried eluate with 400 uL of mobile phase, and inject 10 uL into UPLC.

4.0 Limit of detection: 0.005 ppb

5.0 Recovery: Average 91% over 0.01–2.0 ppb range

5.7 OTHER PUBLISHED PROCEDURES FOR AFLA M_1™ COLUMN

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MINAS CHEESE

Prado, G.; Oliveira, M.; Pereira, M.; Abrantes, F.; Santos, L.; Veloso, T. Aflatoxin M_1 in Samples of “Minas” Cheese Commercialized in the City of Belo Horizonte—Minas Gerais/Brazil. *Ciencia e Tecnologia de Alimentos* 20, no. 3 (2000): 398–400.

SOFT AND PARMESEAN CHEESE

Prado, G.; de Oliviera, M.; de Carvalho, E.; Veloso, T.; de Sousa, L.; Cardoso, A. Aflatoxin M_1 in Soft and Parmesan Cheese by Immunoaffinity Column and Liquid Chromatography. *Revista do Instituto Adolfo Lutz* 60, no. 2 (2001): 147–151.

5.8 SPIKING MILK SAMPLES

A Hamilton syringe is preferred for spiking samples and preparing standards, but an adjustable micropipettor with disposable plastic tips can also be used. The Supelco aflatoxin M_1 standard product # CRM46319 comes in sealed ampoules. The concentration of this aflatoxin standard stock solution is approximately 10 µg/mL (10 ng/µl) in acetonitrile. The certificate of analysis will show the exact concentration. An opened ampoule should be able to be used for up to two weeks when stored at 2–8°C. Use only HPLC-grade solvents when preparing aflatoxin solutions. Aflatoxins are subject to light degradation. Keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil. The use of non-acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Before use, soak the glassware in a dilute acid (e.g., sulphuric acid, 2 mol/L) for several hours, then rinse thoroughly with distilled water to remove all traces of acid (check with pH paper to confirm acid removal).

Prepare Aflatoxin M_1 Spiking Solutions

Prepare a 1.0 ng/µL aflatoxin standard by adding 100 µL of the 10 ng/µL aflatoxin M_1 standard to 900 µL acetonitrile.

Prepare a 0.1 ng/µL aflatoxin standard by adding 100 µL of the 1.0 ng/µL aflatoxin M_1 solution to 900 µL acetonitrile.

Prepare a 0.01 ng/µL aflatoxin standard by adding 100 µL of the 0.1 ng/µL aflatoxin M_1 solution to 900 µL acetonitrile.

Spiking Milk with Aflatoxin M_1 at 0.1 and 0.05 ppb Levels

0.1 ppb (ng/mL) x 50 g (mL) milk = 5 ng
5 ng ÷ 0.1 ng/µL = 50 µL
Add 50 µL of the 0.1 ng/µL aflatoxin M_1 solution to 50 mL defatted milk

0.05 ppb (ng/mL) x 50 g (mL) milk = 2.5 ng
2.5 ng ÷ 0.1 ng/µL = 25 µL
Add 25 µL of the 0.1 ng/µL aflatoxin M_1 solution to 50 mL defatted milk

5.9 PREPARING HPLC STANDARDS

These instructions are specific for preparing the HPLC standards for VICAM's Afla M_1 HPLC procedure in section 5.3 using a 50 mL sample of milk (50 mL sample equivalent).

Prepare **HPLC standard diluent** by mixing equal volumes of eluting solution and HPLC quality water.

1.0 ppb (ng/mL) x 50 mL milk = 50 ng
50 ng ÷ 1.0 ng/µL (aflatoxin M_1 spiking solution) = 50 µL
50 µL 1.0 ng/µL aflatoxin M_1 solution added to 2.45 mL standard diluent

0.1 ppb (ng/mL) x 50 mL milk = 5 ng
5 ng ÷ 0.1 ng/µL (aflatoxin M_1 spiking solution) = 50 µL
50 µL 0.1 ng/µL aflatoxin M_1 solution added to 2.45 mL standard diluent

0.05 ppb (ng/mL) x 50 mL milk = 2.5 ng
2.5 ng ÷ 0.1 ng/µL (aflatoxin M_1 spiking solution) = 25 µL
25 µL 0.1 ng/µL aflatoxin M_1 solution added to 2.475 mL standard diluent

0 ppb (ng/mL) use 2.5 mL HPLC standard diluent.

For AOAC method 2000.08 (section 5.4), dry down standards in silanized vials and reconstitute with the same volume of mobile phase as the samples

5.10 PREPARING UPLC STANDARDS

These instructions are specific for preparing the UPLC standards for VICAM's Afla M_1 UPLC procedure section 5.6 using a 2 mL sample of milk (2-gram sample equivalent).

1.0 ppb (ng/mL) x 2 mL milk = 2 ng
2 ng ÷ 0.1 ng/µL (aflatoxin M_1 spiking solution) = 20 µL
20 µL 0.1 ng/µL aflatoxin M_1 solution added to 380 uL mobile phase

0.1 ppb (ng/mL) x 2 mL milk = 0.2 ng
0.2 ng ÷ 0.01 ng/µL (aflatoxin M_1 spiking solution) = 20 µL
20 µL 0.1 ng/µL aflatoxin M_1 solution added to 380 uL mobile phase

0.05 ppb (ng/mL) x 2 mL milk = 0.1 ng
0.1 ng ÷ 0.01 ng/µL (aflatoxin M_1 spiking solution) = 10 µL
10 µL 0.1 ng/µL aflatoxin M_1 solution added to 390 uL mobile phase

0 ppb (ng/mL) mobile phase (10 uL) was injected into UPLC as a (0 ppm) blank standard.

10 uL injected into UPLC

*Standards were not dried down to avoid inconsistency when reconstituting

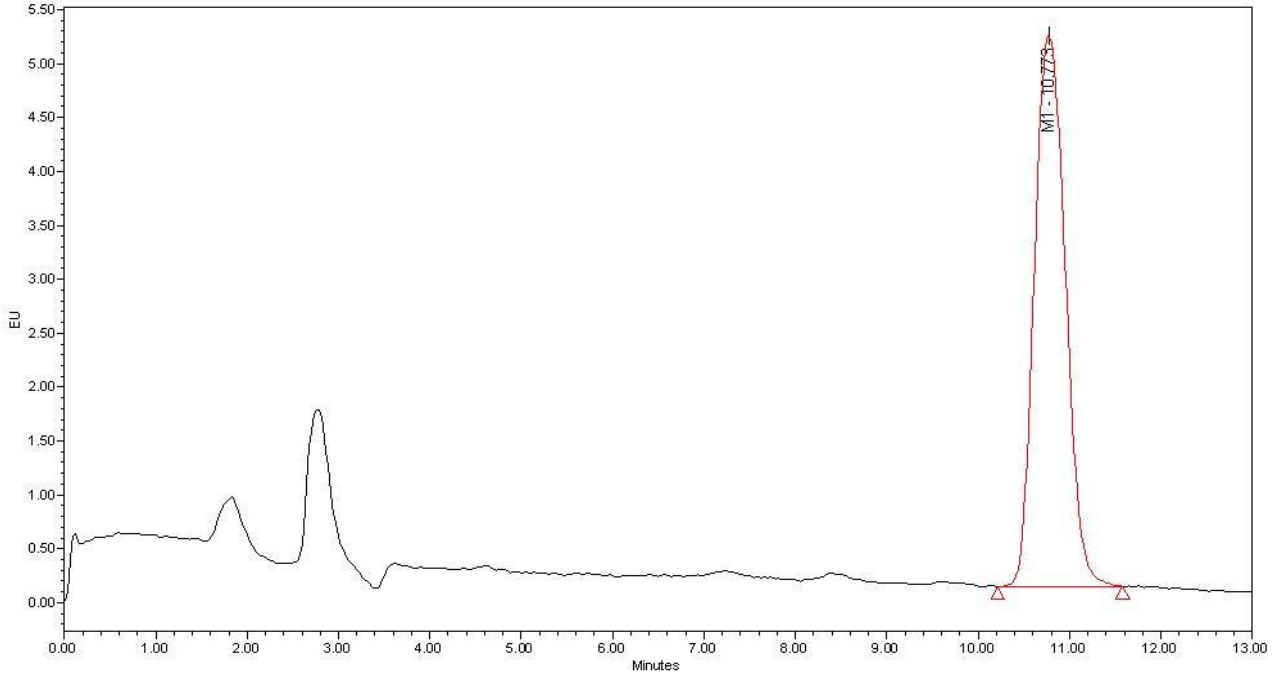
5.11 CALCULATING AFALATOXIN M_1 CONCENTRATION IN POWERED MILK SAMPLES

According to European Commission regulation (EC) No 1881/2006 notes 4 and 10, results in powdered milk are to be reported ready to use **after** reconstitution according to the manufacturer's instructions if marketed as such (liquid milk or liquid baby formula).

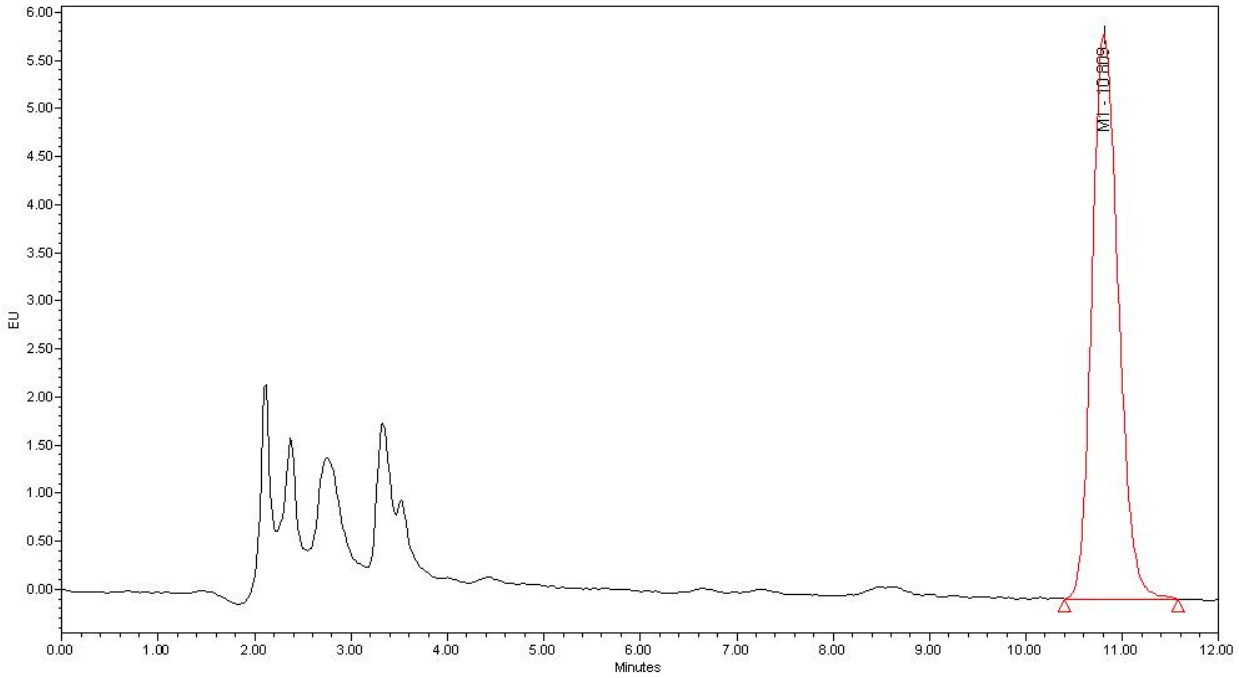
In the case of powdered milk to be used in food products, not as liquid milk, the results are to be reported in the dry matter, so multiplied by 10 from the results in the liquid if the powder was diluted 1:10 in water.

5.12 REPRESENTATIVE HPLC CHROMATOGRAMS

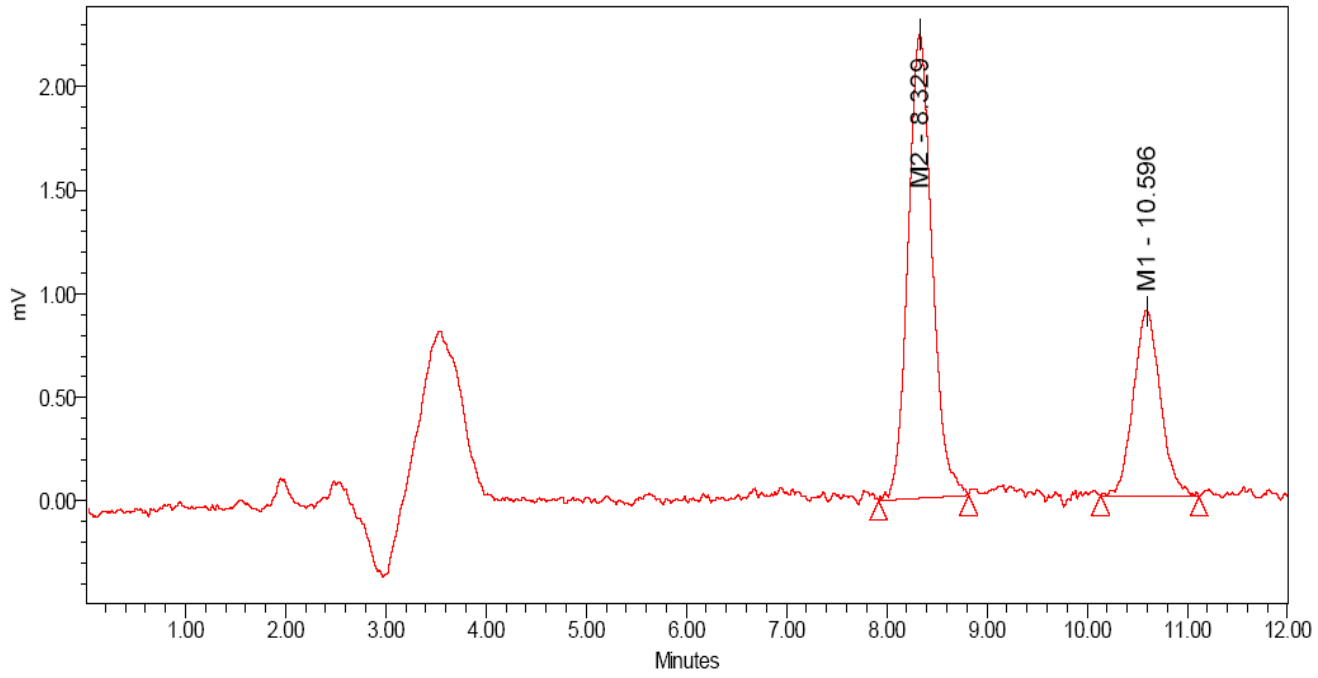
0.05 ppb aflatoxin M_1 standard



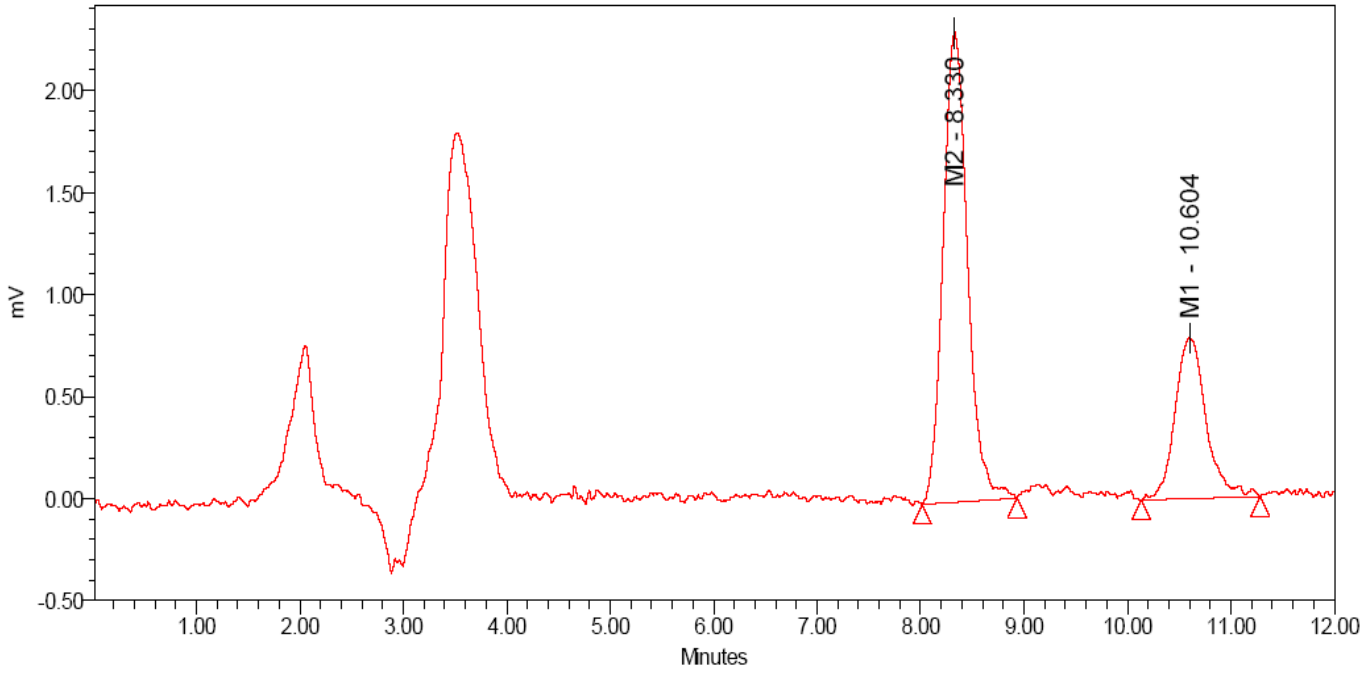
0.05 ppb aflatoxin M_1 contaminated milk



0.05 ppb aflatoxin M_1 and M_2 standard

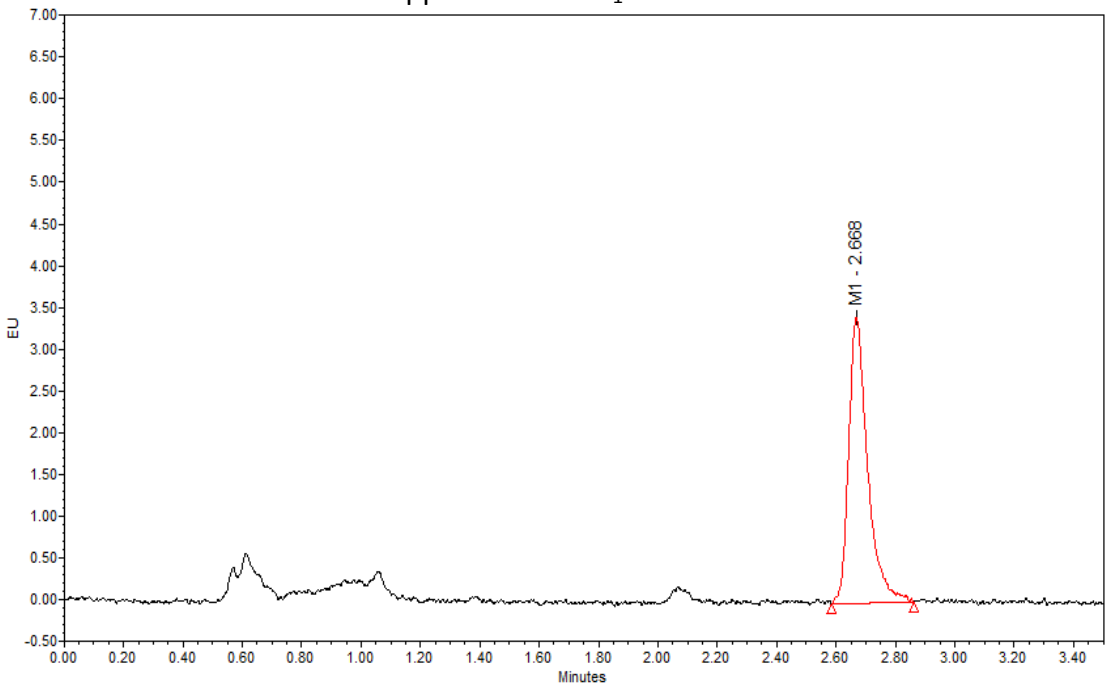


0.05 ppb aflatoxin M_1 and M_2 spiked milk

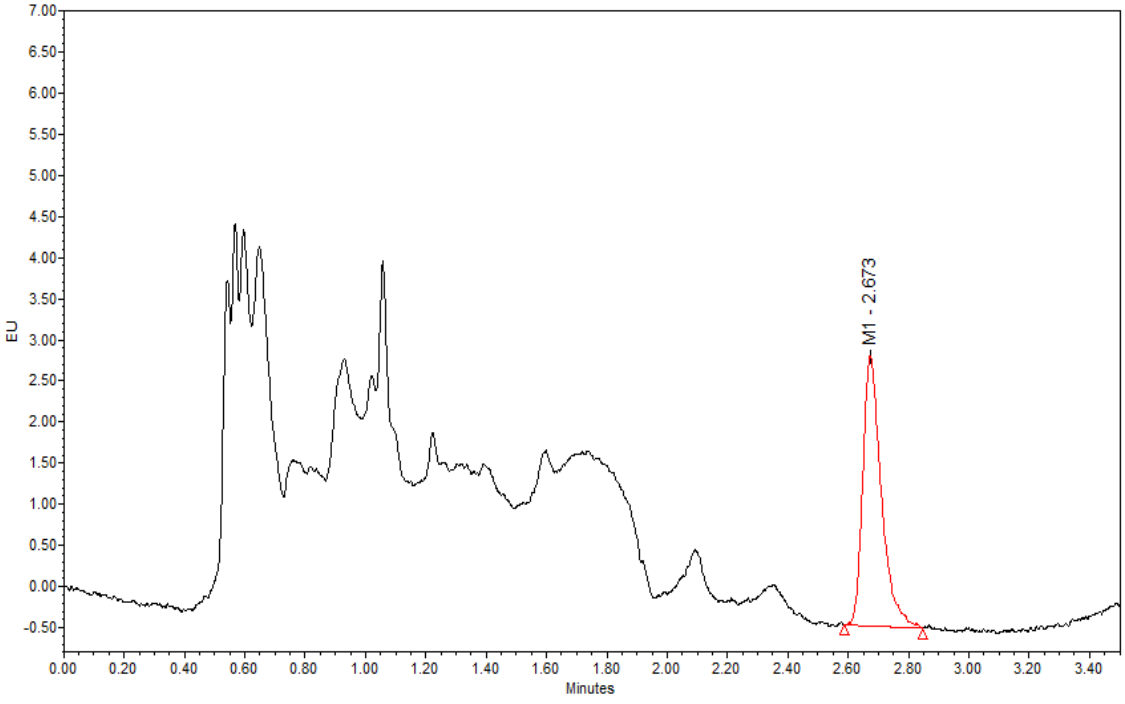


5.13 REPRESENTATIVE UPLC CHROMATOGRAMS

0.025 ppb aflatoxin M_1 standard



0.025 ppb aflatoxin M_1 contaminated milk



6.0 LC MS Conditions:

UPLC/MS Conditions

UPLC condition		**MS condition**	
UPLC system	ACQUITY UPLC	MS system	Quattro Premier XE
Column	ACQUITY UPLC HHS T3, 2.1 x 100 mm, 1.8 µm	Ionization mode	ESI positive
Column temp	40°C	Collision gas (argon)	3.5 x 10^{-3} mbar
Mobile phase A	H_2O water (A)	Capillary voltage	3.2 kV
Mobile phase B	Acetonitrile (B)	Cone voltage	40 V
Elution	1. 0.5 min. linear gradient from 20% to 85% B 2. Hold 85% B for 2.5 min. 3. 0.1 min. from 85% B to 100% B 4. Hold 100% B for 0.9 min.	Extractor voltage	3 V
		Source temp	120°C
Flow rate	0.5 mL/min.	Desolvation temp	390°C
Injection volume	10 µL	Desolvation gas	900 L/hr
Retention time	2.6min	Cone gas	48 L/hr

7.0 GENERAL PRECAUTIONS

Perform test from beginning to end without interruptions.

Load sample on column immediately after centrifugation.

Mix the eluate in the cuvette very well before injecting eluate into LC.

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

Maintain a slow and steady flow rate through the Afla M_1™ column (1 drop/second) during sample loading. Elute the column at a rate of 1 drop for every 2–3 seconds. Flow may need to be slowed down using a stopcock.

When using a UPLC with a fluorescence detector, be sure to use the LARGE VOLUME FLOW CELL (Waters part # 205000609).

8.0 TROUBLESHOOTING

Problem: Cloudy eluate
Solution:

Centrifuge at the specified g force for the length of time indicated in the procedure. The rpm value that corresponds to the specified g force will vary depending on the centrifuge rotor. Use a nomogram to identify the rpm corresponding to the specified g force for your centrifuge rotor. These are usually supplied with the rotor.

Separate defatted portion from fat portion immediately after centrifuge has stopped, to avoid re-mixing. The bottom layer must be taken without disturbing the top layer of fat. To do this, pierce the bottom of a plastic centrifuge tube with an 18-gauge syringe needle and draw the defatted portion into the syringe barrel.

Filter eluate through 0.2 µm, 25 mm nylon membrane syringe filter (VICAM part # G2007) before injecting into HPLC. Test a sample first through the nylon membrane syringe to make sure toxin is not binding to the filter.

Problem: Low recovery
Solution:

Pass milk through the column no faster than 1–2 drops/second. Flow may need to be slowed down using a stopcock.

Elute the column with elution solution at an **even slower** rate by means of gravity or at a flow rate of 1 drop/2–3 seconds. Make sure all resin beads are wet with elution solution and no air is in the resin. Backflushing (see section 3.3) may improve recovery.

Aflatoxin M_1 can bind to glass when drying down. Best results are obtained when eluate is inject directly without drying. If drying down, use silanized cuvettes to prevent toxin from binding to the glassware.

Problem: Slow flow through column
Solution:

Make sure to centrifuge with enough force to clearly separate skim (bottom) from fat (top). Milk may need to be centrifuged longer or at a higher speed: 10,000 x g for 10 minutes or 5,000 x g for up to an hour.

Filter skim milk after centrifugation.

Milk can also be centrifuged warm (37°C) for better separation of skim from fat layer.

9.0 TECHNICAL ASSISTANCE

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

Phone:	+1-800-338-4381	United States
	+1-508-482-4935	International and United States customers
e-mail:	techservice@vicam.com	

10.0 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 Afla M_1™ analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that Afla M_1™ products conform to VICAM's printed specifications and quality control standards. VICAM will at its option repair or replace any product or part thereof that 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, orally 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 Afla M_1™ product.

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11.0 ORDERING INFORMATION

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

In the United States:

Phone:	+1-877-228-4244	Canada and the United States
	+1-417-725-6588	all International and United States customers
Fax:	+1-417-725-6102	
e-mail:	vicam@vicam.com	