CitriTest HPLC

Instruction Manual

VICAM®

A Waters Business

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1.1 INTENDED USER

CitriTest™ HPLC is a quantitative method for the detection of citrinin in Koji Red Rice and corn. VICAM's advanced biotechnology permits detection of citrinin without the use of toxic solvents like chloroform or methylene chloride. CitriTest HPLC is intended for use by trained operators who need to test samples for the presence of citrinin in parts per billion (ppb) with a method that is reliable, rapid and quantitative.

1.2 PRINCIPLE

Citrinin is a naturally occurring fungal metabolite produced by several species of the genera *Penicillium* and *Aspergillus* which causes kidney and liver damage. Citrinin has been found to be mutagenic in hepatocytes and has been implicated as a potential cause of human Endemic Balkan Nephropathy as well as porcine nephropathy.

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

1.3 APPLICABILITY

CitriTest HPLC has been optimized for quantitative measurement of citrinin in Koji Red Rice and corn. The procedures contained in this manual represent all those developed as of the publication date. Assistance in using CitriTest HPLC for other commodities can be obtained by contacting our Technical Assistance 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 grain in a lot and may only occur in a small percentage of the grains in a lot. Because of the wide range in mycotoxin concentrations among individual grains 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

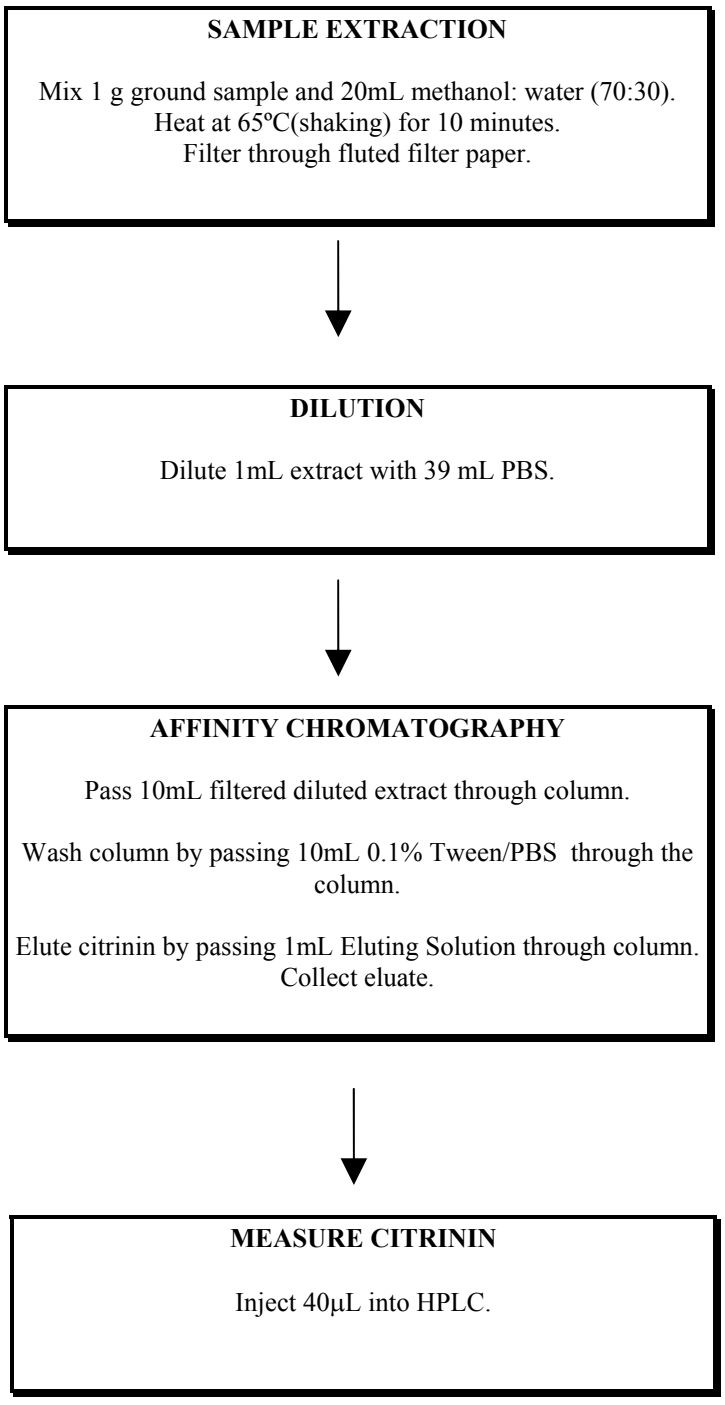
These can be viewed online at:

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

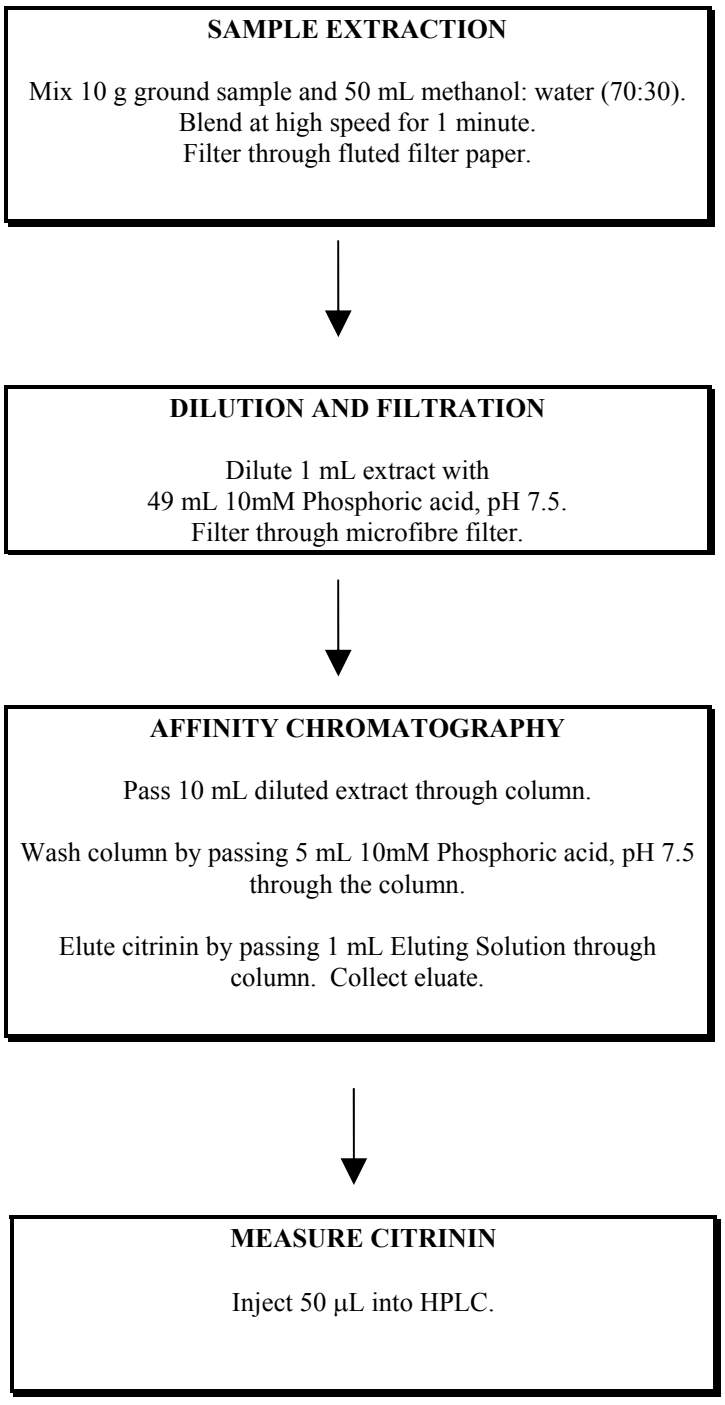
1.6 SHELF LIFE AND STORAGE CONDITIONS

Store 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 the columns be at room temperature (18 – 22°C) for usage.

1.7 CITRITEST ™ HPLC PROCEDURE FOR RED RICE OVERVIEW



1.8 CITRITEST HPLC PROCEDURE FOR CORN OVERVIEW



2.1 PREPARATION OF FILTRATION STEPS

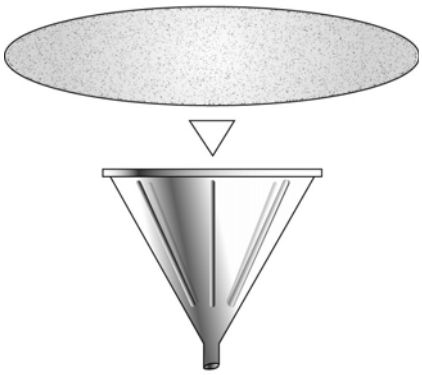
Fluted Filter

The first filtration step is a simple gravity filtration through 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 fluted filter carefully and insert into clean container. (Optional: a 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.



Microfiber 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.

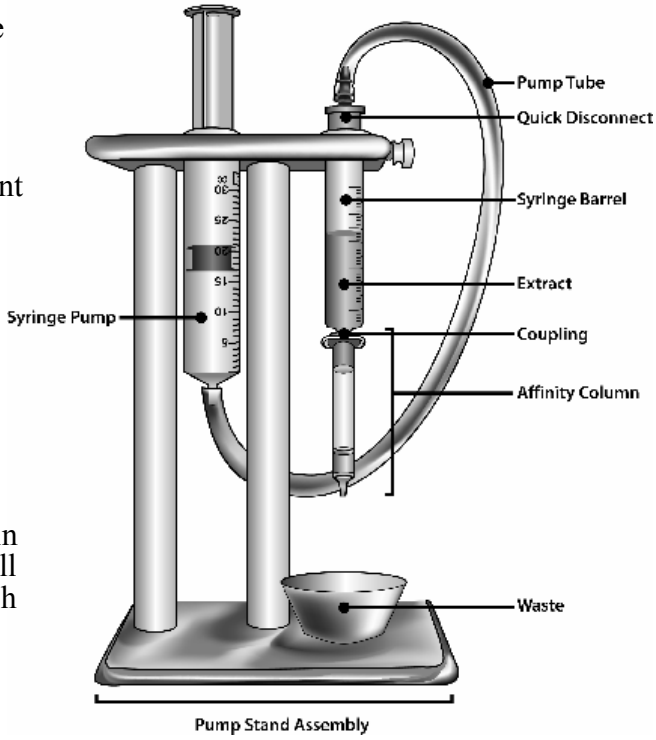
2.2 PUMP STAND SETUP

CitriTest HPLC affinity chromatography is easily performed with the CitriTest HPLC affinity column attached to a pump stand. The stand has a 10mL 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 # 21030), four-position (part #21045) and 12 position (part # G1103) 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. Place WB Column Coupling (G1118) on glass syringe tip. Remove large top cap from column.
2. Attach column to coupling and place waste collection cup under column outlet. Keep bottom cap on column.
3. Pour extract into microfibre filter (see previous section) and collect desired amount of extract in glass syringe barrel using markings on the syringe barrel to measure extract.
4. Pull up on the plastic syringe piston.
5. Inset coupling on end of tube into syringe barrel. Remove column bottom cap.
6. 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 CitriTest™ HPLC 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 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 the same way before using are graduated cylinders, funnels and blender jars. Bottle dispensers need only to be rinsed thoroughly with methanol before use.

Between Assays:

After each assay, the blender jar assembly needs 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

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 large number of samples have been tested, the glass syringe barrel should be washed with a brush and mild detergent then rinsed 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, extract, column eluate) with rubber or soft flexible plastic. These materials may leach contaminating fluorescent materials into the sample and thereby affect results.

3.1 PREPARATION OF EXTRACTION SOLUTION

Use reagent grade or better (i.e. HPLC grade) methanol when preparing extraction solution. Extraction solution can be made at different total volumes than shown below as long as the ratio of methanol and water remains consistent.

Solution desired	Methanol (mL)	Purified Water (mL)	Total Volume (mL)
methanol: water (70:30)	700	300	1000 (1 liter)

Prepare extraction solution every week or as needed.

CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

3.2 PREPARATION OF DILUTION/WASH SOLUTIONS

3.2.1 10mM Phosphoric Acid

1.376mL 85% o-phosphoric acid, HPLC grade (Fisher #A260)
Bring to 2000mL with purified water.

Adjust pH to 2.5 or 7.5 using sodium hydroxide solution.
Filter through sterile bottle top filter (Corning #430015).

3.2.2 1X PBS

A 10X PBS concentrate can be purchased from VICAM (# G1113). The 10X PBS concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X PBS concentrate with 900 mL purified water.

Alternatively, a 1X PBS solution can be prepared as follows:

8.0 g NaCl
1.2 g Na_2HPO_4
0.2 g KH_2PO_4
0.2 g KCl
dissolve in approximately 990 mL purified water
adjust pH to 7.0 with concentrated HCl
bring to 1 liter with purified water

3.2.3 0.1% Tween/PBS

A 10X concentrate of 0.1% Tween-20/PBS may be purchased from VICAM (# G1112). The 10X concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X concentrate with 900 mL purified water.

Alternatively, a 1X 0.1%Tween-20/PBS Wash Buffer solution can be prepared as follows:

Measure 1 ml Tween-20
bring to 1 liter with 1X PBS.

3.3 PREPARATION OF ELUTING SOLUTION

Use HPLC grade methanol when preparing eluting solution. Eluting solution can be made at different total volumes than shown below as long as the ratio remains consistent.

Prepare 0.1% o-Phosphoric Acid by bringing 1.0 mL o-phosphoric acid (HPLC grade, Fisher #A260) to 1 liter with purified water. The pH of the solution does not need to be adjusted.

Solution desired	Methanol (mL)	10mM Phosphoric acid, pH 2.5 (mL)	Total Volume (mL)
Methanol: 10mM Phosphoric acid (70:30)	700	300	1000 (1 liter)

Solution desired	Methanol (mL)	0.1 %Phosphoric acid	Total Volume (mL)
Methanol:0.1% Phosphoric Acid, 70:30	700	300	1000 (1 liter)

Prepare elution solution every week or as needed.

CAUTION: Elution solvent is flammable. Keep container tightly capped when not in use.

4.1 MATERIALS & EQUIPMENT REQUIRED FOR HPLC PROCEDURES

Consumables Required

Description	**Part #**
CitriTest™ HPLC Columns (25 per box)	G1070
VICAM Fluted Filter Paper, 24 cm (100)	31240
Microfibre Filters, 1.5µm, 11cm (100) *for corn procedure*	31955
Disposable Cuvettes (250 per pack)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers	36010
Acetonitrile (4 x 4 L)	G1130
Micro-pipette Tips for 1mL Micro-pipettor (100)	20656
10X concentrate of PBS (150 mL) *for red rice procedure*	G1113
10X concentrate of 0.1% Tween/PBS (150 mL) *for red rice procedure*	G1112
o-phosphoric acid, HPLC grade (Fisher #A260)	
Distilled , reverse osmosis or deionized water	

Equipment Required

Description	**Part #**
Graduated Cylinder, 50ml	20050
Graduated Cylinder, 250ml	20250
Digital Scale with AC Adapter	20100
Commercial Blender with Stainless Steel Container	20200
Cuvette Rack	21010
2-Position Pump Stand w/ Air Pump (10mL)	21040
or 4-Position Pump Stand w/2 Air Pumps (10mL)	21045
or 12-Position Pump Stand w/6 Air Pumps (10mL)	G1104
Vortex Mixer	23040
WB Column Coupling (6)	G1118
Filter Funnel, 65mm (10 per pack) *for corn procedure*	36020
Filter Funnels, 105mm (4 per pack)	36022
Micro-pipettor, 1.0mL	G4033

4.2 CITRITEST ™ HPLC PROCEDURE FOR RED RICE

1.0 General Set Up:

1.1 Prepare mobile phase every week or as needed.
1.2 Prepare methanol:water (70:30 by volume) solution every week or as needed.
1.3 Prepare PBS and 0.1% Tween/PBS every week or as needed.
1.4 Prepare Eluting Solution (Methanol:0.1% Phosphoric Acid, 70:30) every week or as needed.

2.0 HPLC Conditions:

2.1 Column: Waters Sunfire C18, 4.6 X 50mm, 2.5µm (#186003417), preceded by a Sunfire C18, 4.6 X 20mm, 2.5µm guard column (#186003413).
2.2 Mobile Phase: Solution A = 0.1% Phosphoric acid
Solution B = Acetonitrile (HPLC Grade)

Time	Mobile Phase A	Mobile Phase B
0 - 1 min	60%	40%
1 - 7 min linear gradient	10%	90%
7 - 9 min	10%	90%
9 - 10 min linear gradient	60%	40%
10 - 12 min	60%	40%

2.3 Injection volume: 40µL
2.4 Flow rate: 1.0 mL/min
2.5 Fluorescence detector: Waters 2475, excitation = 350nm, emission = 500nm
2.6 Retention time: ~4.3 minutes

3.0 Sample extraction:

3.1 Weigh 1.0 g ground sample and place in a 50 mL polypropylene conical tube.
3.2 Add 20 mL methanol:water (70:30).
3.3 Heat at 65 ºC for 30 minutes shaking vigorously for 30 seconds every 10 minutes.
3.4 Pour extract into fluted filter paper. Collect filtrate in a clean 50 mL polypropylene conical tube.

4.0 Extract Dilution:

4.1 Pipette 1mL filtered extract into a clean vessel.
4.2 Dilute with 39mL of PBS. Mix well.

5.0 Column Chromatography:

5.1 Pass 10mL filtered diluted extract (10mL = 0.0125 g sample equivalent) completely through CitriTest™ column at a rate of about 1-2 drops/second.
5.2 Wash column by passing 10 mL of 0.1% Tween/PBS through the column at a rate of about 1-2 drops/second.
5.3 Elute column with 1mL Eluting Solution (Methanol:0.1% Phosphoric Acid, 70:30) at a rate of about 1 drop/second.
5.4 Vortex cuvette. Inject samples and standards into HPLC.

6.0 Limit of Quantitation: 10ppb

7.0 Assay **Range:** 10 – 500ppb

8.0 Recovery: Mean % recovery values range from 81% to 91%.

4.3 CITRITEST ™ HPLC PROCEDURE FOR CORN (0.04 g SAMPLE EQUIVALENT)

1.0 General Set Up:

1.1 Prepare methanol: water (70:30) extraction solution every week or as needed.
1.2 Prepare 10mM Phosphoric acid, pH 2.5 and pH 7.5 every week or as needed.
1.3 Prepare Eluting Solution (Methanol:10mM Phosphoric acid, 70:30) every week or as needed.

2.0 HPLC Conditions:

2.1 Column: Sunfire C18 2.0µm, 4.6mm x 50 mm stainless steel column (Waters #186003417) fitted with a guard column (Waters #186003413).
2.2 Mobile phases:
Solution A = 10mM Phosphoric acid, pH 2.5
Solution B = acetonitrile, HPLC grade
Solution D = water
2.3 Gradient: 0 - 10 min = 80% A, 20% B
10.01 -14 min gradient = 30% A, 70% B
14.01 - 18 min gradient = 80% A, 20% B
2.4 Injection volume: 50µL
2.5 Flow rate: 0.5ml/min.
2.6 Fluorescence detector: Waters 2475 fluorescence detector, excitation 350 nm, and emission 500 nm.
2.7 Retention time: ~12 minutes

3.0 Sample Extraction:

3.1 Place 10g ground sample into a blender jar.
3.2 Add to jar 50ml methanol: water (70:30).
3.3 Cover blender jar and blend at high speed for 1 minute.
3.4 Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

4.0 Extract Dilution:

4.1 Transfer 1ml filtered extract into another clean vessel.
4.2 Dilute extract with 49mL 10mM Phosphoric acid, pH 7.5. Mix well.
4.3 Filter dilute extract through microfibre filter (Vicam part # 31955). Collect in a clean vessel.

5.0 Column Chromatography:

5.1 Pipet 10ml (10ml = 0.04 g sample equivalent) filtered extract and pass completely through CitriTest™ HPLC affinity column at a rate of about 1-2 drops/second until air comes through column.
5.2 Pass 5ml of 10mM Phosphoric acid, pH 7.5 through the column at a rate of 1 - 2 drops/second until air comes through the column.
5.3 Place glass cuvette (Vicam part # 34000) under CitriTest™ HPLC column and add 1.0ml Eluting Solution (Methanol:10mM Phosphoric acid, 70:30) into glass syringe barrel.
5.4 Elute CitriTest HPLC column at a rate of 1 drop/second or slower and collect all of the sample eluate (1.0ml) in a glass cuvette
5.5 Vortex cuvette. Inject samples and standards into HPLC.

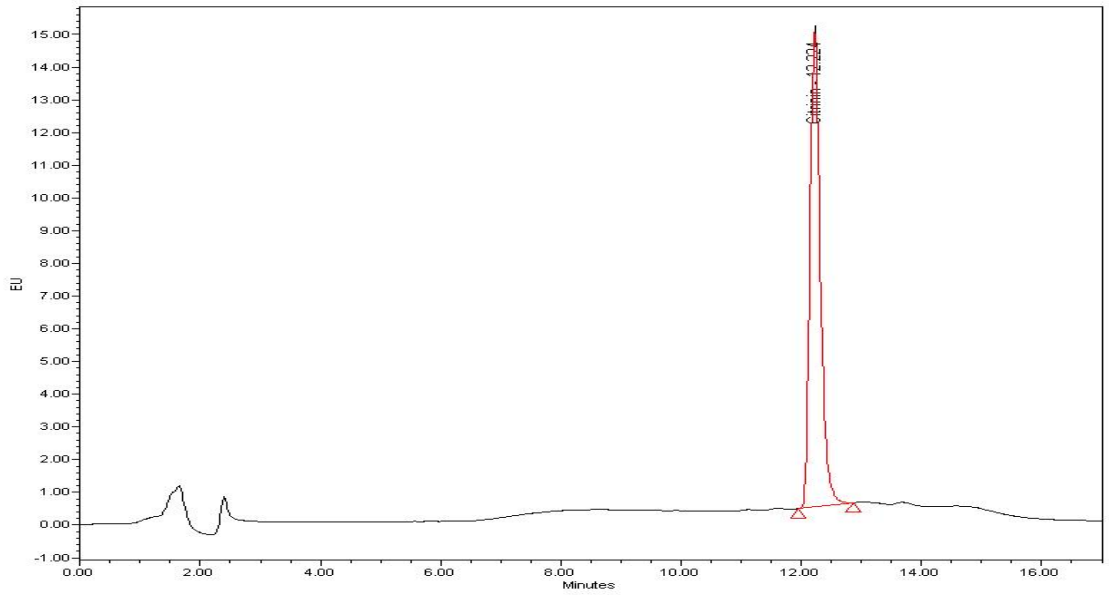
6.0 Limit of Quantitation: 10ppb

7.0 Assay Range: 10 – 500ppb

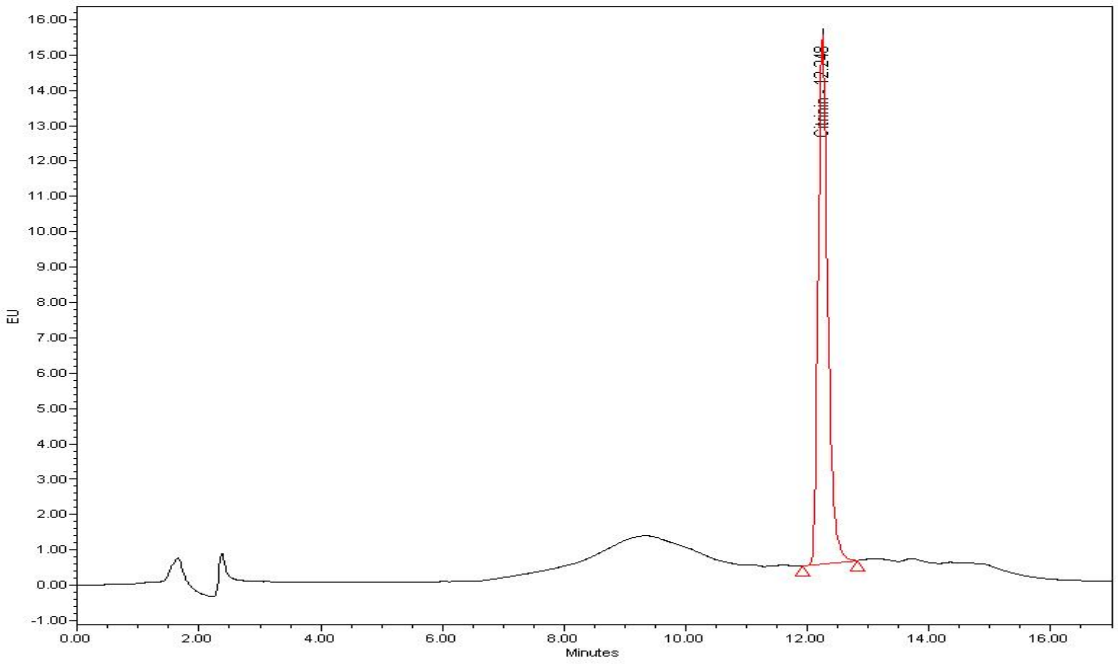
8.0 Recovery: >70%

4.4 REPRESENTATIVE HPLC CHROMATOGRAMS :

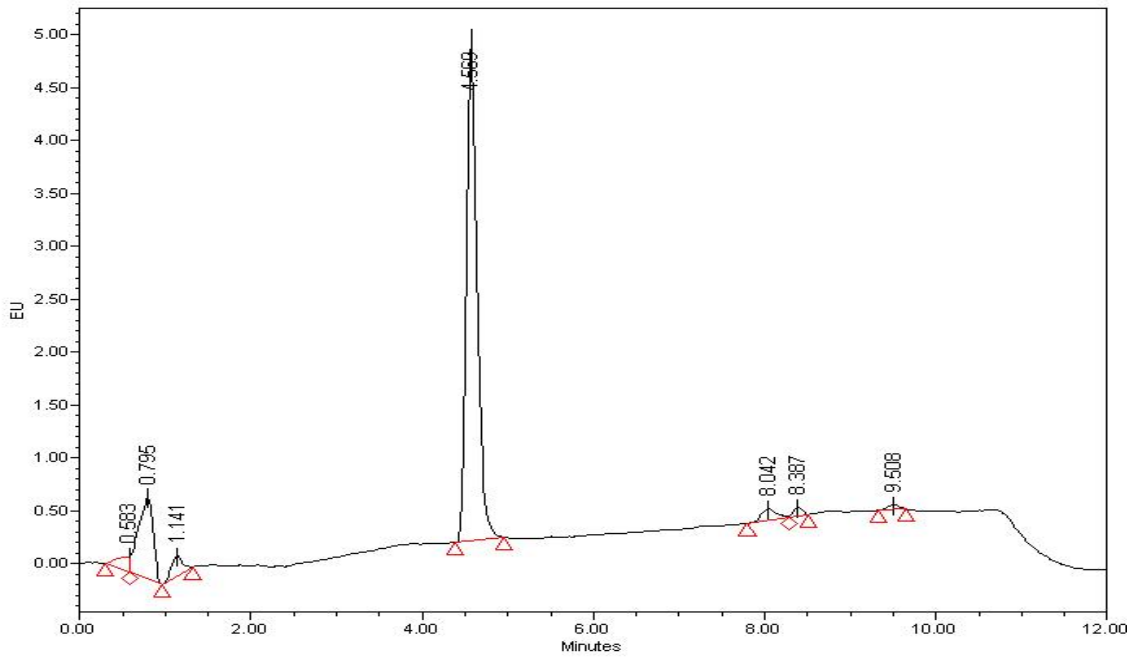
200ppb Citrinin Standard (corn procedure)



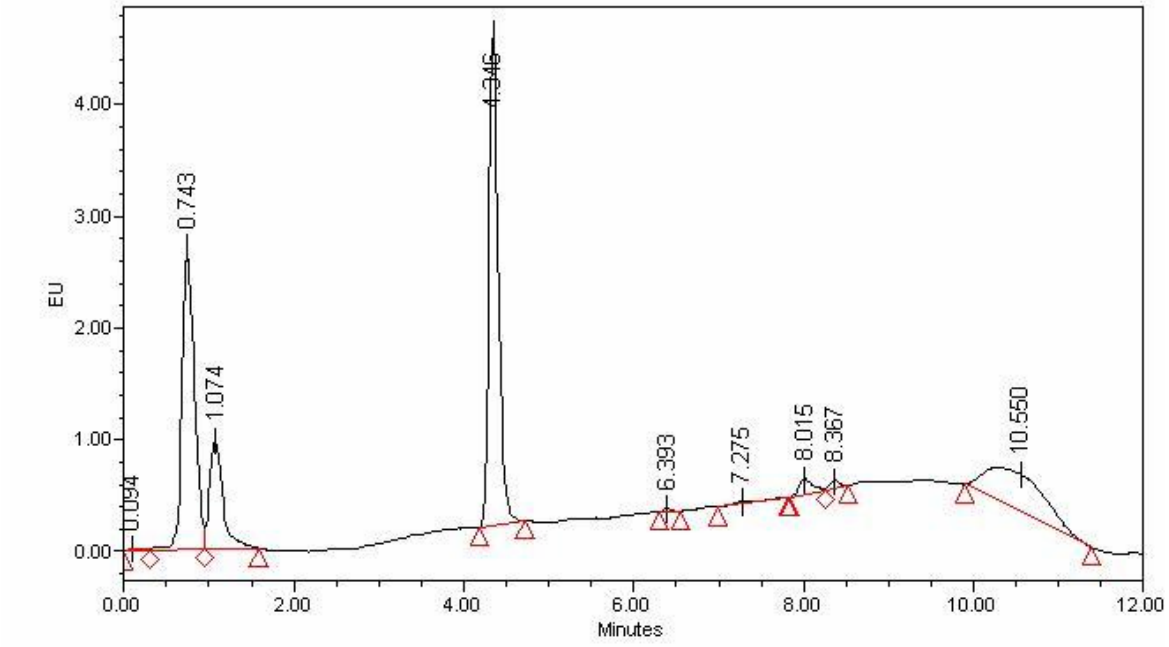
200ppb Spiked Corn Sample:



200ppb Citrinin Standard (red rice procedure)



200ppb Spiked Red Rice Extract:



4.5 HPLC STANDARD PREPARATION AND SAMPLE SPIKING

A Hamilton Syringe is preferred for spiking samples and preparing standards, but an adjustable micropipettor with disposable plastic tips can also be used. Use only HPLC grade methanol when preparing citrinin solutions.

4.5.1. Citrinin solutions

- Prepare a 5mg/mL stock solution by weighing 5mg of citrinin (Sigma C1017) and diluting in 1mL of HPLC grade methanol. Use an amber vial or protect contents from light. This stock solution should be stored at 4°C.
- Prepare a 100ng/µL citrinin solution by adding 20µL of the 5mg/mL stock solution to 980µL methanol.
- Prepare a 10ng/µL citrinin solution by adding 100µL of the 100ng/µL citrinin solution to 900µL methanol.
- Prepare a 1ng/µL citrinin solution by adding 100µL of the 10ng/µL citrinin solution to 900µL methanol.
- Prepare a 0.1ng/µL citrinin solution by adding 100µL of the 10ng/µL citrinin solution to 900µL methanol.
- Prepare a 0.01ng/µL citrinin solution by adding 100µL of the 0.1ng/µL citrinin solution to 900µL methanol.

4.5.2. Spiking corn with citrinin at 100ppb level

100ppb (ng/g) X 10g corn = 1000ng
1000ng ÷ 10ng/µL = 100µL
Add 100µL of the 10ng/µL citrinin solution to 10g corn

Allow the spiked sample to dry in a hood for at least 30 minutes before assaying.

If spiking a red rice sample adjust the volumes needed to accommodate the 1.0 gram of red rice sample used.

4.5.3. Prepare HPLC standards for corn procedure (0.04 gram equivalent)

10ppb (ng/g) X 0.04 g sample equivalent = 0.4ng
0.4ng ÷ 0.01ng/µL citrinin solution = 40µl
Add 40µL of 0.01ng/µL citrinin solution to 960µL Eluting Solution

25ppb (ng/g) X 0.04 g sample equivalent = 1.0ng
1.0ng ÷ 0.01ng/µL citrinin solution = 100µl
Add 100µL of 0.01ng/µL citrinin solution to 900µL Eluting Solution

50ppb (ng/g) X 0.04 g sample equivalent = 2.0ng
2.0ng ÷ 0.01ng/µL citrinin solution = 200µl
Add 200µL of 0.01ng/µL citrinin solution to 800µL Eluting Solution
200ppb (ng/g) X 0.04 g sample equivalent = 8.0ng
8.0ng ÷ 0.1ng/µL citrinin solution = 80µl
Add 80µL of 0.1ng/µL citrinin solution to 920µL Eluting Solution

500ppb (ng/g) X 0.04 g sample equivalent = 20ng
20ng ÷ 0.1ng/µL citrinin solution = 200µl
Add 200µL of 0.1ng/µL citrinin solution to 800µL Eluting Solution

Standards should be injected into the HPLC along with the samples. Make a graph of ppb level of the standards vs. peak area. The peak areas of the unknown samples are then plugged into the equation of this line to calculate the ppb value of the samples. This calculation can be done with the software provided by an HPLC manufacturer. In addition, this calculation can be done using Microsoft Excel software.

If preparing HPLC standards for the red rice procedure adjust the volumes used to reflect a 0.0124 gram sample equivalent procedure.

5.0 TECHNICAL ASSISTANCE

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

6.0 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 CitriTest HPLC analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that CitriTest HPLC 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 CitriTest HPLC 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 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.

7.0 ORDERING INFORMATION

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

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

Instruction Manual # GN-MC9561-1 Rev. B