






OchraTest and OchraTest WB

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

TABLE OF CONTENTS

1.1 INTENDED USER

OchraTest™ and OchraTest WB™ are quantitative methods for the detection of ochratoxin A in a variety of commodities. These products are safe and simple. Sample clean up can be performed in less than 15 minutes. The methods listed in this manual are intended for customers with HPLC systems.

1.2 PRINCIPLE

Ochratoxin is a mycotoxin produced by the fungus *Aspergillus ochraceous* and also by several species of *Penicillium* fungi. Ochratoxin has been known to cause kidney damage and decreased egg production in chickens. It is an immunosuppressant and is considered a potential carcinogen.

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

1.3 APPLICABILITY

OchraTest has been optimized for quantitative measurement of ochratoxin A in many commodities. The Table of Contents lists the testing protocols developed for specific commodities as of the publication date of this manual. Assistance in measuring ochratoxin in commodities not listed in this manual can be obtained by contacting our Technical Assistance Department.

OchraTest™ immunoaffinity columns can be used with AOAC Official Methods for the measurement of ochratoxin in baby food; barley, beer, wine; green coffee and roasted coffee. References for these methods are in section 4.10.

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. Do not freeze columns or reagents.

1.5 SAMPLING

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

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

These can be viewed online at http://www.gipsa.usda.gov. Click on the link for "Handbooks".

1.6 SHELF LIFE AND STORAGE CONDITIONS

Store at room temperature (18 - 22°C). Storage at temperatures above 30°C for prolonged periods of time may reduce shelf life. If storage temperatures above 30°C are anticipated, all components may be stored in the refrigerator (2 - 8°C). Do not freeze columns or reagents. It is recommended that reagents should be at room temperature (18 - 22°C) for usage.

2.1 PUMP STAND SETUP

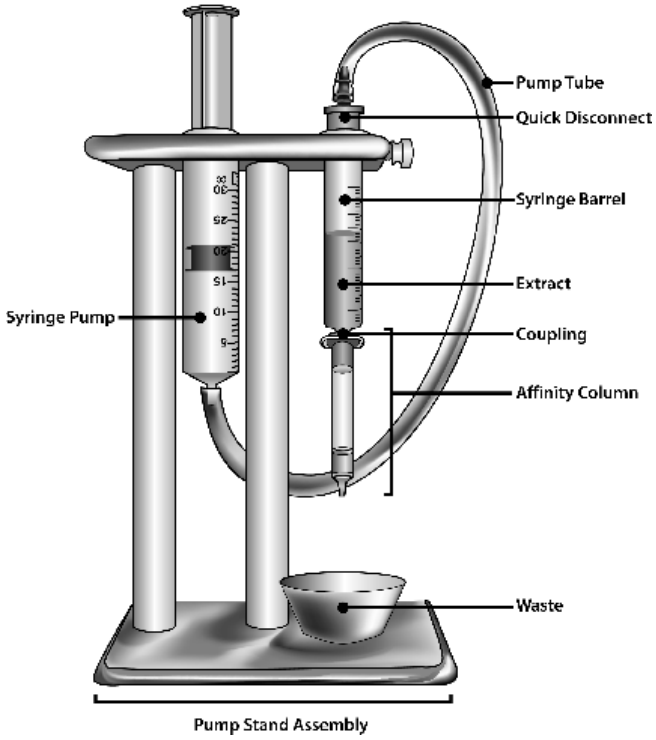
OchraTest™ and OchraTest WB™ affinity chromatography is easily performed with the affinity column attached to a pump stand (part # 21020). The stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. A large plastic syringe with tubing and coupling provides air pressure to manually push liquids through the column. An adjustable air pump (part #20650) can be attached to the pump tube instead of the syringe pump barrel to operate without using hand pressure. Double position pump stand with air pumps (part # 21040), four-position pump stand with air pumps (part #21045) and 12 position pump stand with air pumps (part #G1104) are available for running multiple samples at one time. Alternatively, a vacuum manifold can be used to pull liquid through the column.

When using a pump stand:

1. Remove large top cap from column.
2. Cut bottom 1/8 inch off the end of the top cap with scissors or sharp blade. This provides a reusable coupling for attaching the OchraTest™ column. When using OchraTest WB™ columns order part G1118 (WB Column Coupling).
3. Pour extract after microfiber filtration into glass syringe barrel reservoir.
4. Pull up on the plastic syringe piston.
5. Insert 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



2.2 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. The syringe barrels are treated with a lubricant for use with a piston plunger. Wash new syringe barrel for pump stands using a brush with soap and water. Then rinse with purified water and methanol before using to remove lubricant. Other pieces of equipment that need to be cleaned with detergent before using are graduated cylinders, funnels and blender jars. Repipetters need only to be rinsed 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.

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.

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.

Note: Some blender jar lids are lined with waxed cardboard. These liners are not resistant to methanol and water solutions and will breakdown when used for sample extraction. The extract will then become contaminated with materials, which may cause background fluorescence. Lids with cardboard liner should not be used.

3.0 REAGENT PREPARATION

Prepare solutions every week or as needed. All formulas below will prepare approximately 1 liter of solution. Solution volume may be increased or decreased as needed provided the proportion of reagents is kept consistent.

1. 1% sodium bicarbonate

   10 g $NaHCO_3$
   bring to 1000 mL with purified water

2. 3% sodium bicarbonate

   30 g $NaHCO_3$
   bring to 1000 mL with purified water

3. methanol:1% sodium bicarbonate (70:30)

   700 mL methanol
   300 mL 1% sodium bicarbonate

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

4. methanol:3% sodium bicarbonate (50:50)

   500 mL methanol
   500 mL 3% sodium bicarbonate

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

5. acetonitrile:water (60:40)

   600 mL acetonitrile
   400 mL purified water

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

6. methanol:water (80:20)

   800 mL methanol
   200 mL water

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

7. 2.5% sodium chloride, 0.5% sodium bicarbonate

   25 g NaCl
   5 g $NaHCO_3$
   bring to 1000 mL with purified water

8. PBS

   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

   A 10X concentrate of PBS may also be purchased from VICAM (part # 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.

9. PBS/0.01% Tween-20 Wash Buffer

   0.1 mL Tween 20
   1000 mL PBS

   A 10X concentrate of 0.01% Tween-20/PBS may also be purchased from VICAM (part # G1114). 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.

10. 1% PEG/5% NaHCO3, pH 8.3.

    10 g PEG
    50 g NaHCO3
    dissolve in approximately 950 mL purified water
    adjust pH to 8.3
    bring to 1 liter with purified water

4.1 MATERIALS AND EQUIPMENT FOR HPLC PROCEDURES

Different procedures require different reagents. Please consult the specific procedure to determine which reagents are required.

Materials

Description	Part #
OchraTest™ Columns, Fluorometer & HPLC (25 per box)	13012
Or OchraTest WB™ Columns (25/box)	G1033
Or OchraTest WB™ Columns (50/box)	G1034
VICAM Fluted Filter Paper, 24 cm (100)	31240
Microfibre Filters, 1.5µm, 11 cm (100)	31955
Disposable Cuvettes (250 per pack)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Polyethylene glycol (PEG) —PEG 8000	G1015
10X Concentrate of PBS, 150 mL	G1113
10X Concentrate of 0.01% Tween-20/PBS	G1114
Noniodized sodium chloride (salt, NaCl)	G1124
Acetonitrile, HPLC Grade (4 x 4L)	G1130
Sodium bicarbonate	
Glacial acetic acid —99% purity	
Distilled, reverse osmosis or deionized water	

Equipment

Description	Part #
Graduated Cylinder, 50 mL	20050
Graduated Cylinder, 250 mL	20250
Digital Scale with AC Adapter	20100
Commercial Blender with Stainless Steel Container	20200
Wash Bottle, 500 mL	20700
Cuvette Rack	21010
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
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501
Disposable Plastic Beakers	36010
Filter Funnel, 65 mm (10 per pack)	36020
Filter Funnels, 105 mm (4 per pack)	36022
WB Column Coupling (6)	G1118
Adjustable Micro-pipettor, 1.0 mL	
Micro-pipette Tips for 1 mL Micro-pipettor (100)	20656

4.2 OCHRATEST™ HPLC PROCEDURE FOR GREEN COFFEE (0 - 50 PPB)

1.0 HPLC Set up:
See HPLC condition #1

2.0 Sample Extraction:

2.1 Weigh 25 g ground sample and place in blender jar. (Use 500 mL glass blender jar, VICAM #20300, or smaller jar for best blending).
2.2 Add to jar 50 mL methanol: 1% sodium bicarbonate (70:30).
2.3 Cover blender jar and blend at high speed for 1 minute.
2.4 Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.0 Extract Dilution

3.1 Transfer 10 mL filtered extract into another clean vessel.
3.2 Dilute extract with 40 mL of PBS/0.01% Tween-20 Wash Buffer. Mix well.
3.3 Filter dilute extract through 1.5µm glass microfibre filter (VICAM # 31955) into a clean vessel.

4.0 Column Chromatography

4.1 Pass 10 mL filtered diluted extract (10 mL = 1 g sample equivalent) completely through OchraTest™ affinity column at a rate of about 1 drop/second until air comes through column.
4.2 Pass 10 mL of PBS/0.01% Tween-20 Wash Buffer through the column at a rate of 1-2 drops/second until air comes through the column.
4.3 Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.
4.4 Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL HPLC grade methanol into glass syringe barrel.
4.5 Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate (1.5 mL) in a glass cuvette.
4.6 Add 1.5 mL of purified water to eluate. Vortex. Inject 200 µL onto HPLC.

5.0 Limit of Detection: 0.25 ppb

6.0 Recovery: The percentage recovery using this method is higher at lower levels (mean = 82% for 0.25 - 2.0 ppb range) than at higher levels (mean = 71% for 4.0 - 50 ppb range).

7.0 Note: AOAC method 2004.10 for ochratoxin testing in green coffee can also be used. See Reference section for more details.

4.3 OCHRATEST™ HPLC PROCEDURE FOR CORN, MILO & FEEDS (0 - 100 PPB)

1.0 HPLC Set up:
See HPLC condition #2

2.0 Sample Extraction:
- **2.1** Weigh 50 g ground sample with 5 g salt (NaCl) and place in blender jar.
- **2.2** Add to jar 100 mL methanol:water (80:20)
- **2.3** Cover blender jar and blend at high speed for 1 minute.
- **2.4** Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.0 Extract Dilution
- **3.1** Transfer 10 mL filtered extract into another clean vessel.
- **3.2** Dilute extract with 40 mL of PBS buffer. Mix well.
- **3.3** Filter dilute extract through 1.5µm glass microfibre filter (VICAM # 31955) into a clean vessel.

4.0 Column Chromatography
- **4.1** Pass 10 mL filtered diluted extract (10 mL = 1 g sample equivalent) completely through OchraTest™ affinity column at a rate of about 1 drop/second until air comes through column.
- **4.2** Pass 10 mL of PBS buffer through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.3** Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.4** Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL HPLC grade methanol into glass syringe barrel.
- **4.5** Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate (1.5 mL) in a glass cuvette.
- **4.6** Add 1.5 mL of purified water to eluate. Vortex. Inject 100-200 µL onto HPLC.

5.0 Limit of Detection: 0.25 ppb

6.0 Recovery: Greater than 85% from corn extract spiked at 20 and 100 ppb.

4.4 OCHRATEST™ HPLC PROCEDURE FOR WHEAT (0 - 100 PPB)

1.0 HPLC Set up:
See HPLC condition #3

2.0 Sample Extraction:
- **2.1** Weigh 50 g ground sample and place in blender jar.
- **2.2** Add to jar 100 mL acetonitrile:water (60:40)
- **2.3** Cover blender jar and blend at high speed for 1 minute.
- **2.4** Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.0 Extract Dilution
- **3.1** Transfer 10 mL filtered extract into another clean vessel.
- **3.2** Dilute extract with 40 mL of PBS buffer. Mix well.
- **3.3** Filter dilute extract through 1.5µm glass microfibre filter (VICAM # 31955) into a clean vessel.

4.0 Column Chromatography
- **4.1** Pass 10 mL filtered diluted extract (10 mL = 1 g sample equivalent) completely through OchraTest™ affinity column at a rate of about 1 drop/second until air comes through column.
- **4.2** Pass 10 mL of PBS through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.3** Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.4** Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL HPLC grade methanol into glass syringe barrel.
- **4.5** Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate (1.5 mL) in a glass cuvette.
- **4.6** Add 1.5 mL of purified water to eluate. Vortex. Inject 30 – 200 µL into HPLC

5.0 Limit of Detection: 0.25 ppb

6.0 Recovery: Greater than 80% recovery over the 0.25 - 100 ppb range.

4.5 OCHRATEST™ HPLC PROCEDURE FOR RAISINS (0 - 160 PPB)

1.0 HPLC Set up:
See HPLC condition #4

2.0 Sample Extraction:

2.1 Weigh 80 g homogenized sample (50 g sample + 30 mL water) into a blender jar.

2.2 Add to jar 140 mL methanol and 30 mL water. (This produces 200 mL of 70% methanol when the water used to homogenize the raisin sample is considered).

2.3 Cover blender jar and blend at high speed for 1 minute.

2.4 Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.0 Extract Dilution

3.1 Transfer 10 mL filtered extract into another clean vessel.

3.2 Dilute extract with 40 mL of PBS/0.01% Tween 20. Mix well.

4.0 Column Chromatography

4.1 Pass 20 mL filtered diluted extract (20 mL = 1 g sample equivalent) completely through OchraTest™ affinity column at a rate of about 1 drop/second until air comes through column.

4.2 Pass 10 mL of PBS/0.01% Tween-20 Wash Buffer through the column at a rate of 1-2 drops/second until air comes through the column.

4.3 Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.

4.4 Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL **HPLC grade methanol: acetic acid (98:2, v:v)** into glass syringe barrel.

4.5 Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol:acetic acid through the column and collecting all of the sample eluate (1.5 mL) in a glass cuvette.

4.6 Add 1.5 mL of purified water to eluate. Vortex. Inject 100 µL onto HPLC.

5.0 Limit of Detection: 0.1 ppb

6.0 Recovery: Greater than 72% recovery over the 0.5 - 160 ppb range.

4.6 OCHRATEST™ AOAC HPLC PROCEDURE FOR WINE AND BEER

1.0 HPLC Set up:

See HPLC condition #5

2.0 Sample Preparation:

2.1 Cool beer at $+4^0$C for 30 min to prevent fast foam formation. Degas by sonicating for 1 hour.

2.2 Pour 10 mL wine or beer into a 100 mL conical flask.

2.3 Add 10 mL diluting solution (1% PEG/5% NaHCO3, pH 8.3). Mix vigorously.

2.4 Filter through 1.5µm glass microfibre filter (VICAM cat # 31955) if solution is cloudy or if solid residue is formed after dilution.

3.0 Immunoaffinity Column Cleanup

3.1 Pass 10 mL diluted solution (10 mL = 5 mL sample equivalent) completely through OchraTest™ affinity column at a rate of about 1 drop/second. Do not permit the immunoaffinity column to run dry.

3.2 Wash the immunoaffinity column with 5 mL washing solution (2.5% NaCl/0.5% $NaHCO_3$) at a flow rate of 1-2 drops/second.

3.3 Wash the immunoaffinity columns with 5 mL purified water at a flow rate of 1–2 drops/second until air comes through the column.

3.4 Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add **2.0 mL HPLC grade methanol** into glass syringe barrel.

3.5 Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate (2.0 mL) in a glass cuvette.

3.6 Evaporate the eluate to dryness at 50°C under Nitrogen.

3.7 Redissolve eluate immediately in 250 µL HPLC mobile phase (water:acetonitrile:glacial acetic acid, 99:99:2, v:v:v, pH 3.2) and store at +4°C in dark until HPLC analysis. Inject 100 µL into HPLC.

4.0 Assay range:

Commodity	Ochratoxin A
White wine	0.1 – 2.0 ng/mL
Red wine	0.2 – 3.0 ng/mL
Beer	0.2 –2.0 ng/mL

5.0 Recovery:

Commodity	% Recovery
White wine	88.2 – 105.4%
Red wine	84.3 – 93.1%
Beer	87.2 – 95.0%

4.7 OCHRATEST WB™ HPLC PROCEDURE FOR ROASTED AND SOLUBLE COFFEE

1.0 HPLC Set up:
See HPLC condition #6

2.0 Sample Extraction:
- **2.1** Weigh 12.5 g ground sample and place in blender jar.
- **2.2** Add to jar 250 mL methanol:3% sodium bicarbonate (50:50, v:v).
- **2.3** Cover blender jar and blend at high speed for 5 minutes.
- **2.4** Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.
- **2.5** Filter extract through 1.5µm glass microfibre filter (VICAM # 31955) into a clean vessel.
- **2.6** Dilute 4 mL of filtered extract with 96 mL PBS.

3.0 Column Chromatography
- **3.1** Pass 50 mL filtered diluted extract completely through OchraTest WB™ affinity column at a rate of about 1-2 drops/second until there is about 10mL left in the syringe barrel. Gravity flow rate is acceptable. Add the remaining 50mL and pass through column. Do not let column run completely dry. A total of 100 mL diluted extract (100 mL = 0.2 gram sample equivalent) will be passed through the column. Gently blow air through the column to remove any remaining liquid.
- **3.2** Take column off the syringe barrel and put 3 mL of purified water directly into the column headspace. Reattach the column to the glass syringe barrel and fill the syringe barrel with 7 mL of purified water. Pass this through the column at a rate of 1-2 drops/second. Gravity flow rate is acceptable. Gently blow air through the column to remove any remaining liquid.
- **3.3** Take column off the syringe barrel and add 3.0 mL HPLC grade methanol into the column headspace. Place a glass cuvette under the column and allow the column to elute by gravity pressure. Once 1 - 2 mL of methanol has passed through the column take the column off the syringe barrel again and add 1.0 mL more HPLC grade methanol into the column headspace. Allow the column to elute by gravity pressure and collect all the sample eluate in the glass cuvette. A total of 4 mL of methanol is used to elute the column. Gently blow air through the column to remove any remaining methanol.
- **3.4** Dry down the eluate in a rotary evaporator at 45ºC.
- **3.5** Reconstitute in 200 µL HPLC mobile phase and inject 30 µL onto HPLC.

4.0 Limit of Detection: 0.2 µg/kg

5.0 Recovery: Average of 87.7% from a spiked extract over the range of 0-10 ppb.

4.8 OCHRATEST WB™ HPLC PROCEDURE FOR CORN (0 – 300 PPB)

1.0 HPLC Set up:
See HPLC condition #1

2.0 Sample Extraction:
- **2.1** Weigh 50 g ground sample with 5g salt (NaCl) and place in blender jar.
- **2.2** Add to jar 100 mL methanol:water (80:20).).
- **2.3** Cover blender jar and blend at high speed for 1 minute.
- **2.4** Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.0 Extract Dilution
- **3.1** Transfer 10 mL filtered extract into another clean vessel.
- **3.2** Dilute extract with 40 mL of PBS Buffer. Mix well.
- **3.3** Filter dilute extract through 1.5µm glass microfibre filter (VICAM # 31955) into a clean vessel.

4.0 Column Chromatography
- **4.1** Pass 10 mL filtered diluted extract (10 mL = 1 g sample equivalent) completely through OchraTest WB™ affinity column at a rate of about 1 drop/second until air comes through column.
- **4.2** Pass 10 mL of PBS Buffer through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.3** Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.4** Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL HPLC grade methanol into glass syringe barrel.
- **4.5** Elute OchraTest™ column at a rate of 1 drop/second by passing the methanol through the column and collecting all of the sample eluate (1.5 mL) in a glass cuvette.
- **4.6** Add 1.5 mL of purified water to eluate. Vortex. Inject 100 µL onto HPLC.

5.0 Limit of Detection: 0.25 ppb

6.0 Recovery: Average of 102% from a spiked extract over the range of 0-300 ppb.

4.9 OCHRATEST™ WB HPLC PROCEDURE FOR LICORICE EXTRACT OR POWDER (0 – 500 PPB)

1.0 HPLC Set up:
See HPLC condition #7

2.0 Sample Dilution:
- **2.1** Weigh 2 g sample into a clean vessel. Add 100 mL 3% sodium bicarbonate. Stir until the entire sample is dissolved.
- **2.2** Pipet 10.0 mL of dissolved sample into another clean vessel. Dilute with 30 mL of 0.1% Tween/PBS. Mix well.

4.0 Column Chromatography
- **4.1** Pass the entire 40 mL diluted extract (40 mL = 0.2 g sample equivalent) completely through OchraTest WB™ affinity column at a rate of about 1-2 drops/second until air comes through column.
- **4.2** Pass 10 mL of PBS Buffer through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.3** Pass 10 mL purified water through the column at a rate of 1-2 drops/second until air comes through the column.
- **4.4** Place glass cuvette (VICAM part # 34000) under OchraTest™ column and add 1.5 mL HPLC grade methanol into the OchraTest™ column headspace.
- **4.5** Elute OchraTest™ WB column at a rate of 1 drop/second and collect all of the sample eluate in the glass cuvette. Leave the glass cuvette under the column.
- **4.6** Add 1.5 mL of water to the OchraTest WB column headspace and elute the column at a rate of 1 drop/second and collect all of the sample eluate in the same glass cuvette.
- **4.7** Vortex sample eluate. Inject 100 µL into HPLC.

5.0 Limit of Detection: 0.1 ppb

6.0 Recovery: Average of 79% from a spiked extract over the range of 0-300 ppb.

4.10 OTHER PUBLISHED HPLC PROCEDURES FOR OCHRATEST™

BABY FOOD (AOAC Method 2000.16)

Burdaspal, P., Legarda, T.M. and Gilbert, J*., Journal of AOAC International*, Determination of ochratoxin A in baby food by immunoaffinity column cleanup with liquid chromatography: interlaboratory study, **84** (5) 1445-1452.

BARLEY (AOAC Method 2000.03)

Entwisle, A.C., Williams, A.C., Mann, P.J. and Slack, P.T*., Journal of AOAC International*, Liquid Chromatographic Method with Immunoaffinity Column Cleanup for Determination of Ochratoxin A in Barley: Collaborative Study, **83** (6) 1377-1383.

BEER

Scott, P.M. and Kanhere, S.R., *Food Additives and Contaminants*, Determination of Ochratoxin A in beer, **12** (4) 591-598.

Visconti, A., Pascale, M. and Centonze, G., *Journal of Chromatography A*, Determination of ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography, **888** (2000) 321-326.

BEER AND WINE (AOAC Method 2001.01)

Visconti, A., Pascale, M. and Centonze, G., *Journal of AOAC International,* Determination of Ochratoxin A in Wine and Beer by Immunoaffinity Column Cleanup and Liquid Chromatographic Analysis with Fluorometric Detection: Collaborative Study, **84** (6) 1818-1827*.*

Visconti, A., Pascale, M. and Centonze, G., *Journal of Chromatography A*, Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography, **864** (1999) 89-101.

CURRANTS, RAISINS, SULTANAS, DRIED FRUIT AND FIGS

MacDonald SJ, Anderson S, Brereton P, Wood R., *Journal of AOAC International,* Determination of ochratoxin A in currants, raisins, sultanas, mixed dried fruit, and dried figs by immunoaffinity column cleanup with liquid chromatography: interlaboratory study, **86** (6) (2003 Nov-Dec) 1164-71.

Lombaert, G. A., Pellaers, P., Neumann, G., Kitchen, D., Huzel, V., Trelka, R., Kotello, S. and Scott, P. M., *Food Additives and Contaminants*, Ochratoxin A in dried vine fruits on the Canadian retail market, **21** (6) (June 2004) 578-585.

COFFEE, ROASTED

Sibanda, L., De Saeger, S. and Van Peteghem, C., *Journal of Chromatography A*, Optimization of solid-phase clean-up prior to liquid chromatographic analysis of ochratoxin A in roasted coffee, **959** (2002) 327-330.

COFFEE, ROASTED (AOAC Method 2000.09)

Entwisle, A.C., Williams, A.C., Mann, P.J., Russell, J. and Slack, P.T*., Journal of AOAC International*, Combined Phenyl Silane and Immunoaffinity Column Cleanup with Liquid Chromatography for Determination of Ochratoxin A in Roasted Coffee: Collaborative Study, **84** (2) 444-450.

COFFEE, ROASTED AND SOLUBLE

Lombaert, G. A., Pellaers, P., Chettiar, M., Lavalee, Scott, P. M. and Lau, B. P.-Y., *Food Additives and Contaminants*, Survey of Canadian retail coffees for ochratoxin A, **19** (9) (2002) 869-877.

COFFEE, SOLUBLE (INSTANT)

Pittet, A., Tornare, D., Huggett, A., and Viani, R*., Journal of Agricultural and Food Chemistry*, Liquid Chromatographic Determination of Ochratoxin A in Pure and Adulterated Soluble Coffee Using an Immunoaffinity Column Cleanup Procedure, **44** (11) 3564-3569.

GREEN COFFEE (AOAC Method 2004.10)

Vargas EA, dos Santos EA, Pittet A, Correa TB, da Rocha AP, Diaz GJ, Gorni R, Koch P, Lombaert GA, MacDonald S, Mallmann CA, Meier P, Nakajima M, Neil RJ, Patel S, Petracco M, Prado G, Sabino M, Steiner W, Stroka J, Taniwaki MH, Wee SM*., Journal of AOAC International*, Determination of ochratoxin A in green coffee by immunoaffinity column cleanup and liquid chomatography: collaborative study. 2005 May-Jun; **88** (3): 773-9.

HAM

Chiavaro, E., Lepiani, A., Colla, F., Bettoni, P., Pari, E., and Spotti, E*., Food Additives and Contaminants*, Ochratoxin A determination in ham by immunoaffinity clean-up and a quick fluorometric method, **19** (6) 575-581.

RICE AND DRIED FRUITS

Zinedine, A., Soriano, J, M. , Juan, C., Mojemmi, B., Moltó, J. C., Bouklouze, A., Cherrah, Y., Idrissi, L., El Aouad, R. and Mañes, J., *Food Additives and Contaminants*, Incidence of ochratoxin A in rice and dried fruits from Rabat and Salé area, Morocco, **24**:3, 285-291.

WINE AND GRAPE JUICE

Rosa, C. A. R., Magnoli, C. E., Fraga, M. E., Dalcero, A. M., and Santana, D. M. N, *Food Additives and Contaminants*, Occurrence of ochratoxin A in wine and grape juice marketed in Rio de Janeiro, Brazil, 21 (4) 358-364.

URINE

Pascale, M. and Visconti, A., *Mycopathologia,* Rapid method for the determination of ochratoxin A in urine by immunoaffinity column clean-up and high-performance liquid chromatography, **152,** 91-95.

5.1 HPLC CONDITIONS

HPLC Condition 1

1.1 Column: 4 µm, 3.9 x 150 mm C18 column Nova-Pak® (Waters WAT086344).
1.2 Mobile phase: acetonitrile:water:acetic acid (49.5 : 49.5 : 1 by volume)
1.3 Flow rate: 0.8 mL/min.
1.4 Injection volume: 30 - 200 µL
1.5 Fluorescence detector: Waters 474 Scanning Fluorescence Detector
1.6 Detection wavelength: 333 nm excitation and 477 nm emission

HPLC Condition 2

2.1 Column: 5µm, 5 mm x 125 mm C18 column (Merck 50943, LichroCart 125-4, Lichrosphere 100 RP-18).
2.2 Mobile phase: acetonitrile:0.012M sodium phosphate solution pH 7.5 (60:40), 1 g/L hexadecyltrimethyl ammonium bromide (C-tab)
2.3 Flow rate: 0.8 mL/min.
2.4 Fluorescence detector: Kratos FS950 Fluoromat
2.5 Detection wavelength: 360 nm excitation and 440 nm emission

HPLC Condition 3

3.1 Column: 5µm, 5 mm x 125 mm C18 column (Merck 50943, LichroCart 125-4, Lichrosphere 100 RP-18).
3.2 Mobile phase: water: acetonitrile:acetic acid (49.5:49.5:1, v:v:v) degassed
3.3 Flow rate: 0.9 mL/min.
3.4 Injection volume: 30 - 200 µL
3.5 Fluorescence detector: Waters 470 Scanning Fluorescence detector
3.6 Detection wavelength: 333 nm excitation and 477 nm emission

HPLC Condition 4

4.1 Column: Agilent Zorbax; 3.5 µm, Eclipse XDB-C18, 4.6x75mm
4.2 Mobile phase: Water: Acetonitrile: Acetic Acid (99: 99: 2)
4.3 Flow rate: 0.8 mL/min
4.4 Fluorescence detector: excitation 333 nm and emission 460 nm
4.5 Column temperature: room temperature
4.6 Injection volume: 100 µL

HPLC Condition 5

- **5.1** Column: Stainless steel (150 X 4.6 mm id) packed with 5 mm C18 reversed-phase material preceded by a reversed-phase guard column (i.e., 20 x 4.6 mm id, 5 µm particle size) or guard filter (i.e., 0.5 mm, Rheodyne). Columns of different dimensions may be used, if they adequately resolve the Ochratoxin A peak from all other peaks.
- **5.2** Mobile phase: Water:Acetonitrile:glacial acetic acid (99:99:2, v:v:v), pH 3.2.
- **5.3** Flow rate: 1 mL/min.
- **5.4** Fluorescence detector: Fitted with a flow cell and set at 333 nm (excitation) and 460 nm (emission) indicating a peak from $\geq$ 0.02 ng of Ochratoxin.
- **5.5** Injection volume: 100 µL
- **5.6** Retention times: approximately 6 minutes.

HPLC Condition 6

- **6.1** Column: Spherisorb ODS2 5µm, 4.6 x 250mm C18 column (Waters PSS831915) preceded by a Nova Pak C18 4µm 3.9 x 20mm guard column (Waters WAT044380).
  Columns of different dimensions may be used, if they adequately resolve the Ochratoxin A peak from all other peaks.
- **6.2** Mobile Phase: Acetonitrile:Methanol:Water:Acetic acid (35:35:29:1, v:v:v:v).
- **6.3** Flow rate: 0.8 mL/min.
- **6.4** Fluorescence detector: 332nm (excitation) and 476nm (emission)
- **6.5** Injection volume: 30µL
- **6.6** Retention time: approximately 8.5 minutes

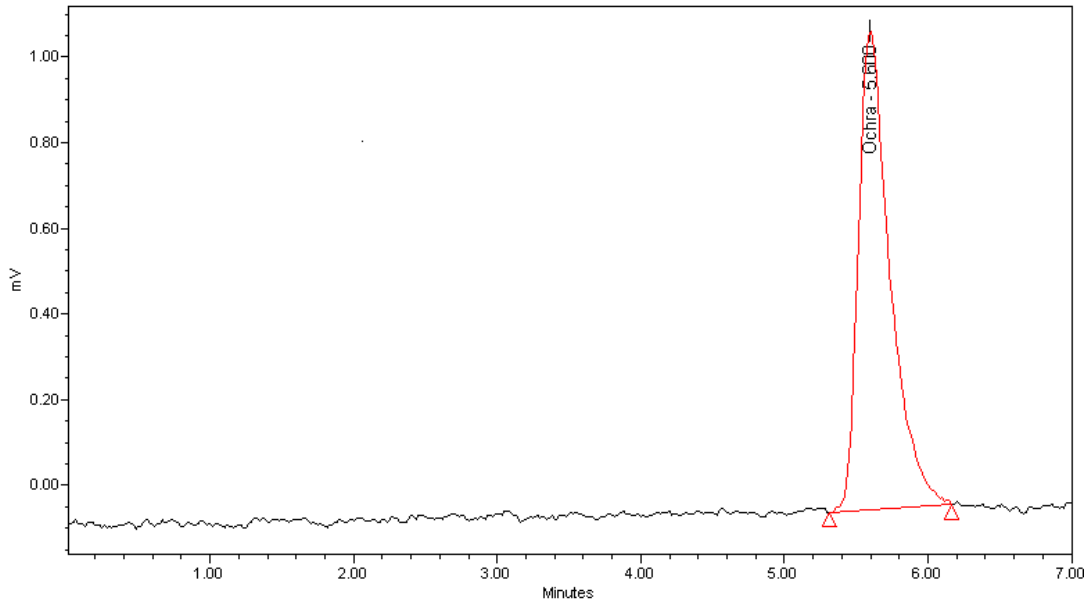
HPLC Condition 7

- **7.1** Column: Spherisorb ODS 2, 4.6 X 250mm, 5µm (Waters PSS831915), preceded by a C18, 3.9 X 20 mm guard column.
- **7.2** Mobile Phase: Water:Acetonitrile:Acetic Acid (49.5:49.5:1 by volume)
- **7.3** Injection volume: 100 µL
- **7.4** Flow rate: 1.0 mL/min
- **7.5** Fluorescence detector: Waters 2475, excitation = 333nm, emission = 477nm
- **7.6** Column temperature: 25ºC
- **7.7** Retention time: approximately 11 minutes

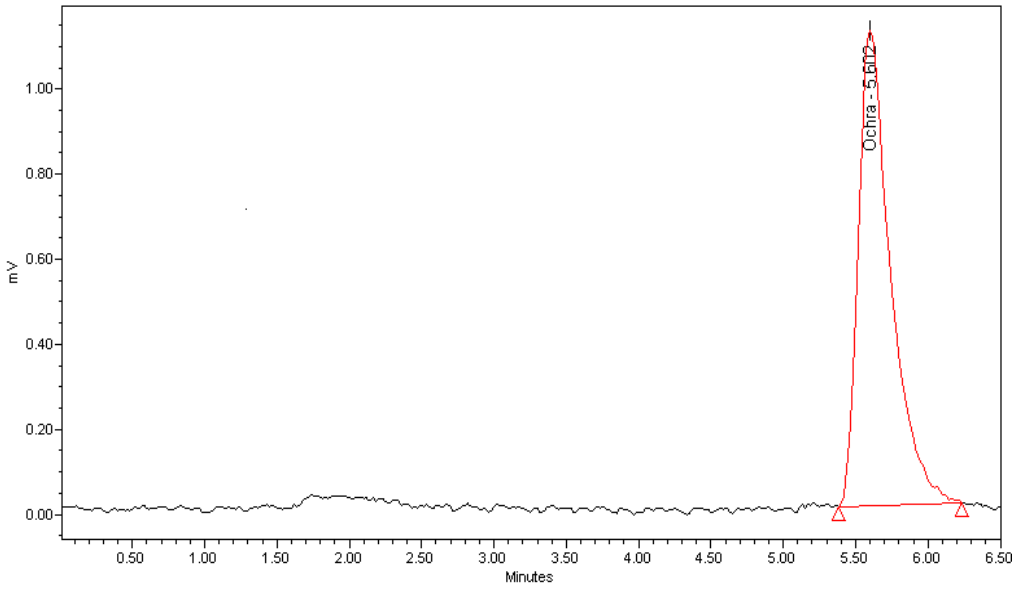
*****DISCLAIMER
Although specific equipment and HPLC columns are listed in this document, there are a number of equally suitable components that can also be used. Mention of trade names or commercial products does not constitute endorsement or recommendation by VICAM

5.2 OCHRATEST™ REPRESENTATIVE CHROMATOGRAMS FOR CORN (Using HPLC Condition 1)

20ppb Ochratoxin A standard



20ppb Ochratoxin A spiked corn sample



5.3 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. The Supelco Ochratoxin A standard product # 46912 comes in sealed ampules. The concentration of this ochratoxin standard stock solution is about 50 ng/µL in benzene:acetic acid (99:1) and is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of ochratoxin. Adjust the calculations below for the exact concentration of the standard that you are using. An opened ampule can be used for up to two months when stored at 2 – 8 °C.

5.3.1. Ochratoxin working solutions

Working Solution A
Prepare a 5.0 ng/µL ochratoxin solution by adding 100 µL of the 50 ng/µL ochratoxin standard stock solution to 900 µL ethanol.

Working Solution B
Prepare a 0.1 ng/µL ochratoxin solution by adding 50 µL of **working solution A** to 2450 µL ethanol.

Working Solution C
Prepare a 0.01 ng/µL ochratoxin solution by adding 100 µL of **working solution B** to 900 µL ethanol.

5.3.2. Preparing HPLC standards for 1 gram sample equivalent procedures (for example green coffee and wheat)

20 ppb (ng/g) X 1.0 g sample equivalent = 20 ng
20 ng ÷ 0.1 ng/µL ochratoxin solution = 200 µL
Add 200 µL of **working solution B** to 1300 µL methanol

10 ppb (ng/g) X 1.0 g sample equivalent = 10 ng
10 ng ÷ 0.1 ng/µL ochratoxin solution = 100 µL
Add 100 µL of **working solution B** to 1400 µL methanol

5.0 ppb (ng/g) X 1.0 g sample equivalent = 5 ng
5 ng ÷ 0.1 ng/µL ochratoxin solution = 50 µL
Add 50 µL of **working solution B** to 1450 µL methanol

1.0 ppb (ng/g) X 1.0 g sample equivalent = 1 ng
1 ng ÷ 0.01 ng/µL ochratoxin solution = 100 µL
Add 100 µL of **working solution C** to 1400 µL methanol

All standards should be treated exactly the same as the sample eluates. Refer to the specific procedure for instructions as to whether to dry down and reconstitute or dilute samples and standards before injection onto the HPLC.

5.3.3. Spiking 25 gram sample (for example green coffee) with ochratoxin at 20 and 50 ppb levels

20 ppb (ng/g) X 25 g sample = 500 ng
500 ng ÷ 5.0 ng/µL ochratoxin standard = 100 µL
Add 100 µL of **working solution A** to 25 g of sample

50 ppb (ng/g) X 25 g sample = 1250 ng
1250 ng ÷ 5.0 ng/µL ochratoxin standard = 250 µL
Add 250 µL of **working solution A** to 25 g of sample

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

5.3.4. Spiking 50 gram sample (for example wheat) with ochratoxin at 5 and 20 ppb levels

5 ppb (ng/g) X 50 g sample = 250 ng
250 ng ÷ 5.0 ng/µL ochratoxin standard = 50 µL
Add 50 µL of **working solution A** to 50 g of sample

20 ppb (ng/g) X 50 g sample = 1000 ng
1000 ng ÷ 5.0 ng/µL ochratoxin standard = 200 µL
Add 200 µL of **working solution A** to 50 g of sample

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

6.1 GENERAL PRECAUTIONS

1. Ochratoxin may be lost if eluate is passed through nylon disc filter.
2. If wheat does not blend properly in 100 mL acetonitrile: water (60:40) use 200 mL acetonitrile:water. Double the extract volume passed through the column to 20 mL to keep the same gram equivalency.

6.2 TROUBLESHOOTING

1. **Problem:** False high readings

   Solution:

   Make sure to use the correct HPLC procedure.

   Check calculation for spiked sample and standard curve.

2. **Problem:** False low readings

   Solution:

   Make sure extraction solution is made correctly and is less than one week old.

   Make sure to use correct pH buffer for dilution and first wash step.

   Maintain the recommended flow rates through the affinity column during sample passing, washing and elution.

   Make sure to use the correct HPLC procedure.

   Check calculation for spiked sample and standard curve.

7.0 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	International and United States customers
Fax:	508-482-4972	
E-mail:	techservice@vicam.com	

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

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

9.0 ORDERING INFORMATION

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

In the United States:

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

Manual Number: G9551 REV B

Effective Date: December 8, 2008