HBI Ion Labs	Standard Operating Procedure **Limit of Citrinin by LC-MS**	**SOP Number** **D-778**	**Revision** **3**
		Effective Date	**Page** **Page 1 of 9**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
MMA 10/20/25	AJS 10/20/25	21-Oct-2025
Title: QC Microbiologist II	**Title: QC Lab Manager**	**Title: QA/QC Director** ①

① Approved by Frank Rolvag, QC Manager HBI Americas in absence of Nicole Matley. 21-Oct-2025

1.0 Purpose

The purpose of this procedure is to define the method for evaluating the limit of citrinin in raw materials and finished products by LC-MS.

2.0 Scope

The method is applicable to all raw materials and finished products with limits in the range of 2 ppb – 40 ppb being tested in the QC laboratory at Ion Labs.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep SOP aligned with current practices.

4.0 Definitions

4.1 **LC-MS** – Liquid Chromatography with detection by Mass Spectrometry

4.2 **HPLC** – High Performance Liquid Chromatography

4.3 **QC** – Quality control

4.4 **CofA** – Certificate of analysis

4.5 **ACN** – Acetonitrile

4.6 **H_2O** – Deionized water

4.7 **PVDF** – Polyvinylidene fluoride

5.0 References

5.1 PRTCL-20-0109, Protocol, Validation of an Analytical Method for the Limit of Citrinin by LC-MS

5.2 RPT-21-0022, Report, Estimation of Uncertainty

6.0 Supplies

6.1 Chemicals

6.1.1 Citrinin reference standard, 100 µg/mL solution with expanded uncertainty of no more than 0.6%, CAS #518-75-2 (or equivalent)

6.1.2 ACN (**LC-MS grade**), CAS #75-05-8 (or equivalent)

6.1.3 ACN (**HPLC grade**), CAS #75-05-8 (or equivalent)

6.1.4 Formic acid (**LC-MS grade**), CAS #64-18-6 (or equivalent)

6.1.5 Ethanol (**ACS/HPLC grade**), Catalog #241HPLC200 (or equivalent)

6.2 Glassware / Disposables

6.2.1 Volumetric glassware as required for standard and sample preparations

6.2.2 Tips for adjustable pipets

6.2.3 250 µL gas-tight syringe

6.2.4 10-mL plastic syringe and 0.45 µm PVDF syringe filter

6.2.5 HPLC vials, 2mL with screw-cap enclosures and septa

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, and column oven coupled with Agilent Ultivo mass spectrometer using MassHunter software for instrument control and data processing.

6.3.2 Analytical balance

6.3.3 Wrist action shaker

6.3.4 Adjustable pipets

7.0 Procedure

7.1 Mobile Phase and Diluent Preparation

7.1.1 Mobile Phase A (0.1% formic acid in H_2O)

7.1.1.1 Transfer 1000 mL of H_2O to a suitable container.

7.1.1.2 Add 1.0 mL of LC-MS grade formic acid.

7.1.1.3 Mix well.

7.1.2 Mobile Phase B (0.1% formic acid in ACN)

7.1.2.1 Transfer 1000 mL of LC-MS grade ACN to a suitable container.

7.1.2.2 Add 1.0 mL of LC-MS grade formic acid.

7.1.2.3 Mix well

7.1.3 Diluent

7.1.3.1 Use HPLC grade ACN.

7.2 Stock Standard Preparation (200 ng/mL)

7.2.1 **Citrinin is light sensitive. Prepare all standards and samples in low actinic glassware**.

7.2.2 Transfer 200 µL of the reference standard solution to a 100-mL volumetric flask by gas-tight syringe.

7.2.3 Dilute to volume with HPLC grade ACN.

7.3 Spiking Solution Preparation (5 ng/mL)

7.3.1 Transfer 2.5 mL of the *Stock Standard* to a 100-mL volumetric flask.

7.3.2 Dilute to volume with HPLC grade ACN

7.4 Sample Preparation

7.4.1 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, then follow preparation procedure as described below, maintaining concentration within the linear range listed above

7.4.2 Sample Weight

7.4.2.1 Based on the limit level, calculate the required sample weight.

7.4.2.2 Example for a sample with 30 ppb limit:

Sample Wt (g) = 50 ÷ Limit (ppb)

Sample Wt (g) = 50 ÷ 30 ppb

Sample Wt (g) = 1.667 g

7.4.3 Unspiked Sample

7.4.3.1 Weigh the required amount of sample (within 0.01g) into a 100-mL low actinic volumetric flask.

7.4.3.2 Add 65 mL of *Diluent*.

7.4.3.3 Shake on a wrist action shaker for 30 minutes.

7.4.3.4 Dilute to volume with *Diluent*.

7.4.3.5 Sonicate for 5 minutes.

7.4.3.6 Filter an aliquot using a 0.45 µm PVDF membrane discarding the first 2-3 mL before collecting a portion in a HPLC vial for analysis.

7.4.4 Spiked Sample #1 (Spike Concentration = 250 pg/mL)

7.4.4.1 Weigh the required amount of sample (within 0.01g) into a 100-mL low actinic volumetric flask.

7.4.4.2 Add 5.0 mL of *Spiking Solution*.

7.4.4.3 Add 60 mL of *Diluent*.

7.4.4.4 Shake on a wrist action shaker for 30 minutes.

7.4.4.5 Dilute to volume with *Diluent*.

7.4.4.6 Sonicate for 5 minutes.

7.4.4.7 Filter an aliquot using a 0.45 µm PVDF membrane discarding the first 2-3 mL before collecting a portion in a HPLC vial for analysis.

7.4.5 Spiked Sample #2 (Spike Concentration = 500 pg/mL)

7.4.5.1 Weigh the required amount of sample (within 0.01 g) into a 100-mL low actinic volumetric flask.

7.4.5.2 Add 10.0 mL of *Spiking Solution.*

7.4.5.3 Add 55 mL of *Diluent*.

7.4.5.4 Shake on a wrist action shaker for 30 minutes.

7.4.5.5 Dilute to volume with *Diluent*.

7.4.5.6 Sonicate for 5 minutes.

7.4.5.7 Filter an aliquot using a 0.45 µm PVDF membrane discarding the first 2-3 mL before collecting a portion in a HPLC vial for analysis.

7.5 HPLC Parameters

7.5.1 Column: Agilent AdvanceBio EC-C18, 2.7 µm x 2.1 mm x 100 mm

7.5.2 Column Temperature: 35 °C

7.5.3 Flow rate: 0.25 mL/min

7.5.4 Injection Volume: 5 µL

7.5.5 Run Time: 6.5 minutes

7.5.6 Gradient:

Time (min)	%A	%B
0.0	55	45
3.5	10	90
3.6	55	45
6.5	55	45

7.6 MS Parameters

7.6.1 Ion Source: AJS ESI

7.6.2 Gas Temperature: 300 °C

7.6.3 Gas Flow: 12.0 L/min

7.6.4 Nebulizer Pressure: 35 psi

7.6.5 Sheath Gas Temperature: 350 °C

7.6.6 Sheath Gas Flow: 12.0 L/min

7.6.7 Capillary Voltage (Positive Setpoint): 4000 V

7.6.8 Nozzle Voltage (Positive Setpoint): 400 V

7.6.9 Time Segments:

Start Time (min)	Scan Type
0	MRM

7.6.10 Acquisition Parameters

Compound Name	ISTD	Precursor (m/z)	MS1 res	Product (m/z)	MS2 res	Dwell (ms)	Fragmentor (V)	CE (V)	Polarity
Citrinin	no	251.2	unit	205.2	unit	400	70	29	+
Citrinin	no	251.2	unit	233.2	unit	200	70	17	+

7.7 Recommended Sequence

7.7.1 Make at least 2 injections of ACN.

7.7.2 Make a single injection of the *Unspiked Sample.*

7.7.3 Make a single injection of *Spiked Sample #1.*

7.7.4 Make a single injection of *Spiked Sample #2.*

7.7.5 Repeat steps 7.7.2 – 7.7.4, and calculate the average measured concentration from the two replicate measurements.

7.8 System Suitability Requirements

7.8.1 No significant (>5%) interference are present in the diluent injection.

7.8.2 The coefficient of determination (R^2) for a least squares linear regression of the calibration points is no less than 0.99.

7.8.3 The difference in measured concentration between the two replicates is no more than 10% of the limit level.

7.8.4 If results are below LOD, then the system suitability requirements do not apply to the injection that is below LOD.

7.9 Column Wash and Storage

7.9.1 Wash and store the column in Acetonitrile

8.0 Example Calculation

8.1 The calculation may be performed automatically by chromatographic software.

8.2 Plot the data obtained from the unspiked and spiked samples with spike concentration on the x-axis and peak area on the y-axis.

8.3 Perform linear regression of the data to obtain the equation $y = mx + b$.

8.4 Calculate the concentration of citrinin in the sample:

$$\text{Citrinin (ppb)} = \frac{10^{-6}\,\frac{\mu g}{pg} \times 100\ \text{mL} \times \text{b}}{\text{SW} \times \text{m}}$$

b y-intercept of the linear regression

SW Sample weight of the unspiked sample in g

m slope of the linear regression (mL/pg)

8.5 For results below the detection limit of 2 ppb, the mean of the *Unspiked Samples* will be reported as "< 2 ppb".

8.6 For results below the quantitation limit of 6 ppb, the mean of the *Unspiked Samples* will be reported as "< 6 ppb".

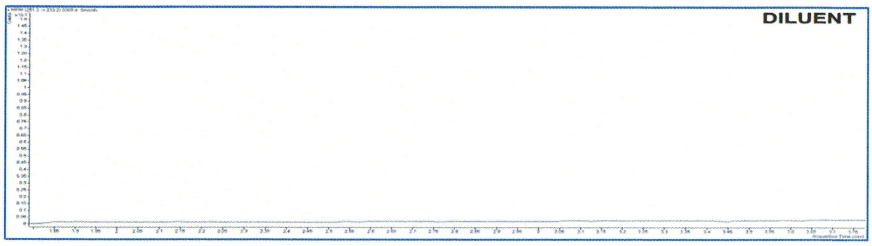
9.0 Reporting Results

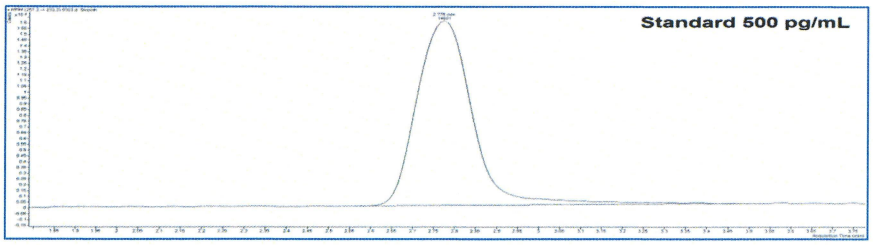
9.1 The expanded uncertainty of the method is 7.1% with a coverage factor of 2.

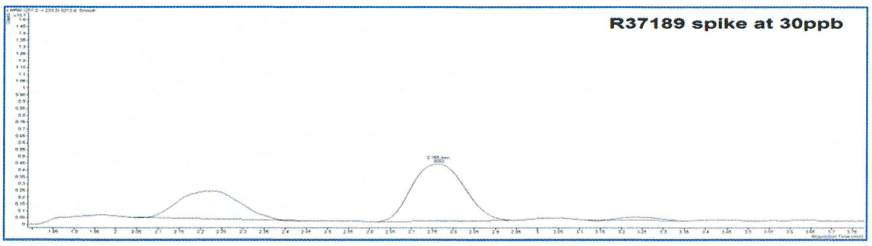
9.1.1 Results should include the expanded uncertainty of the method along with the coverage factor in the following format:

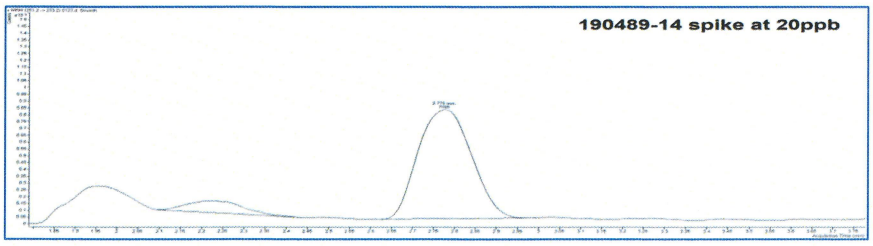
9.1.1.1 10 ppb citrinin, U = ± 1 ppb k = 2

10.0 Representative Chromatograms









11.0 Troubleshooting

11.1 Citrinin can be difficult to wash from the injection system and/or column resulting in a persistent carry-over peak. The carry-over peak can be greatly reduced or eliminated by performing one or more injections of 100 µL of ethanol.

12.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/08/21	New	N/A	S. Sassman
1	11/09/21	Added 17025:2017 requirements	CC-21-0427	J. Sassman
2	06/06/23	Add instruction to follow test details for product specific sample prep, change scope to include anything with a limit in the range 2 – 40 ppb, add option to use alternate reference standard, require filtration of samples instead of settling by gravity, fix calculation so that units are clear.	CC-23-0274	S. Sassman
3	10/16/25	Added statement to say if results are below LOD, then system suitability requirements do not apply. Updated reporting criteria. Added CAS and Catalog numbers to the supplies list.	CC-25-0265	M. Autrey