STUDY TITLE

Validation of Method GPL-MTH-099 for the Determination of NF-180, QP-1-1, and QP-1-7 in Soils and Spray Application Targets by LC-MS/MS

GUIDELINE REQUIREMENTS

OPPTS 860.1340 Residue Analytical Method
OECD ENV/JM/MONO(2007)17
SANTE/11945/2015
SANCO/3029/99 rev.4

II.   MATERIALS

A.   Equipment

The equipment that was used is listed below:

- Autosampler vials, clear with Teflon lined caps: 2-mL
- Balance, Analytical, Mettler Toledo XS204
- Balance, Top Loading, Mettler Toledo MS3002S/03
- Bottles, HDPE: Nalgene, 250-mL
- Centrifuge, Eppendorf 5810
- Centrifuge Tubes, graduated, Corning Falcon® plastic, 15 mL
- Filter paper: Advantec 5A, 185 mm
- Funnels, Pyrex®, 60° Powder, 100 mm
- Graduated Mixing Cylinders: 250-mL
- Mechanical shakers, Eberbach platform
- Micropipette, Drummond Wiretrol® disposable micropipettes: 200 μL
- Pipettes, VWR glass serological, 5 mL
- Syringes, HSW plastic, 5 mL
- Syringe filter, 25 mm, 0.45 μm PTFE

- 1-L glass jars with Teflon-lined lids (used for extraction of spray pads)
- Whatman #3 filter paper, 9 or 15-cm (used for preparation of spray pad laboratory QC samples)

- Instrumentation: Sciex Triple Quad 6500+ LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SIL-20AC HT Autosamplers, Rheodyne switching valves, Analyst Data System Ver. 1.6, and MPX™ Driver Ver. 1.2.

B.   **Reagents and Standards**

The following chemicals were used:

Chemical	Grade	Manufacturer	Supplier	Part Number
Acetonitrile	Optima	Fisher	Fisher	A996-4
Acetic Acid	ACS	Fisher	Fisher	A38S-500
Water	HPLC	Fisher	Fisher	W5-4

Preparation of Reagent Solutions:

Reagents and solutions were prepared according to the procedures described in the analytical method in Appendix B.

Test/Reference Substances

The test/reference substances were received in good condition on June 14, 2016. Test/reference substances were received from Lehigh Agricultural & Biological Services, Inc., Hamburg, PA. Safety Data Sheets and certificates of analysis were received for each compound in the shipment. A copy of the certificates of analysis for each test/reference substance is located in Appendix D of this report. The following table contains detailed information for the analytical reference substances used in this study.

Analytical Standard	CAS #	Lot #	Purity (%)	Expiration Date
NF-180	N/A	11426-T.Kinoshita	98.3	June 15, 2020
QP-1-1	N/A	31-13227-R.WADA	99.8	October 8, 2017
QP-1-7	N/A	31-16149-D.SATO	98.2	May 16, 2018

Upon receipt, the neat standards were stored in a freezer set to maintain $\leq$ -10 °C (frozen), when not in use.

Preparation of Standard Solutions

The reference substances were used in the preparation of the fortification and calibration standard solutions. All standard solutions were prepared according to the procedures described in the analytical method in Appendix B.

III.   **METHODS**

A.   **Analytical Method**

The analytical method was based on conditions supplied by the sponsor. Method flow charts showing an outline of the analysis procedures are contained in Appendix C. Analytes for the method validation were NF-180, QP-1-1, and QP-1-7 in soils, and NF-180 only in spray pads.

The method validation trials, including additional testing for extract stability and matrix effect, were conducted between November 8, 2016, and February 7, 2017. The soil samples were extracted and analyzed in two total analytical sets, each consisting of one soil type, California and Iowa. The spray pad samples were extracted and analyzed in one set. Each validation set consisted of one reagent blank sample, two control samples, five LOQ laboratory fortification samples, and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number; the sample set designation and a sample number (e.g., 694MV01-1).

B. **Analytical Procedure for Soils**

1.   Control Matrixes

Control matrix samples of homogenized soils were used for the validation. Control soil samples CA-0005 and IA-0005 were sourced from a companion terrestrial field dissipation study, AA160718. The matrix control samples were inspected prior to use in this study to confirm the correct matrix type and that they were in suitable condition for this study. There was no response in the soil control matrix samples in the chromatograms corresponding to the retention time of NF-180, QP-1-1 and QP-1-7. A representative subsample of each soil type, CA (California) and IA (Iowa), each 0 – 7.5 cm depth, were analyzed at Agvise Laboratories for soil characterization in compliance with GLPs. Soil characterization results are located in Appendix E.

2.   Preparation of Samples

Sub-samples (20 g) of the control soil matrix were measured into 250-mL HDPE Nalgene bottles.

3.   Fortifications

Method validation samples were fortified as described in the table below. Fortifications were performed as listed in the following table:

Analyte(s)	Sample Amount (g)	Fortification Level (ppm)	Amount and Concentration of Spiking Solution Used
NF-180	20	LOQ (0.002)	0.2 mL of 0.2 µg/mL
	20	10x LOQ (0.02)	0.2 mL of 2 µg/mL
QP-1-1	20	LOQ (0.002)	0.2 mL of 0.2 µg/mL
	20	10x LOQ (0.02)	0.2 mL of 2 µg/mL
QP-1-7	20	LOQ (0.002)	0.2 mL of 0.2 µg/mL
	20	10x LOQ (0.02)	0.2 mL of 2 µg/mL

4.   Extraction

All soil samples were extracted according to the procedures described in the analytical method in Appendix B. Briefly, samples were extracted twice via shaking in a solution of acetonitrile/water/acetic acid. Extracts were gravity filtered, and brought up to a known volume with water. Aliquots of the extracts were diluted with a solution of acetonitrile/water, syringe-filtered and vialed for analysis by LC-MS/MS.

C.   **Analytical Procedure for Spray Pads**

1.   Control Matrixes

Control matrix samples consisting each consisting of one 15-cm Whatman #3 filter paper were used for the validation.

2.   Preparation of Samples

Filter papers were placed into 1-L glass jars.

3.   Fortifications

Method validation samples were fortified as described in the table below. Fortifications were performed as listed in the following table:

Analyte(s)	Sample (each)	Fortification Level (µg/sample)	Amount and Concentration of Spiking Solution Used
NF-180	1	LOQ (40)	2 mL of 20 µg/mL
	1	10x LOQ (400)	2 mL of 200 µg/mL

4.   Extraction

All spray pad samples were extracted according to the procedures described in the analytical method in Appendix B. Briefly, samples were extracted via shaking in a solution of acetonitrile/water/acetic acid. Aliquots of the extract were syringe-filtered, diluted with a solution of acetonitrile/water as needed, and vialed for analysis by LC-MS/MS.

D.   **Instrumentation**

1.   Description

Instrument: Sciex Triple Quad 6500+ LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SIL-20AC HT Autosamplers, Rheodyne Switching Valves

Data System: Analyst Chromatography Data System version 1.6.3, Sciex, MPX™ Driver version 1.2

HPLC Conditions:

HPLC Column: Phenomenex Kinetex 2.6-μm C18 100 Å
                (100 x 3 mm), Part Number: 00D-4462-Y0
Column Temperature: 40 °C
Column Flow: 0.5 mL/minute
Injection Volume: 10 μL
Mobile Phase A: 0.1% Acetic Acid in Acetonitrile
Mobile Phase B: 0.1% Acetic Acid in Water

HPLC Gradient:

Time (min.)	% A	% B
0.00	30	70
0.15	30	70
5.00	95	5
5.90	95	5
6.00	30	70
7.00	30	70

Retention Times:

Analyte	Time (min.)
NF-180	4.6
QP-1-1	5.6
QP-1-7	3.4

MS/MS Conditions:

MS Sample Introduction: Electrospray Ionization
Scan Type: MRM
Polarity: Positive (Unit/Unit Resolution)

IS = 3500, TEM = 500, CUR = 35, GS1 = 40,
GS2 = 40, EP = 10, CAD = 12, CXP = 10

Analyte, Transition Ions	Dwell Time (msec)	DP	CE
NF-180 (348.1/330.1)	200	50	30
NF-180 (348.1/180.1)	100	50	30
QP-1-1 (330.1/180.2)	200	50	35
QP-1-1 (330.1/314.2	100	50	45
QP-1-7 (378/332)	200	85	32
QP-1-7 (378/314.1)	100	85	44

E. **Potential Interferences**

    1.   Matrix Interference

        The detection technique is highly selective for this method. No interferences from the matrix were observed in the unfortified or fortified samples. An assessment of matrix effect was completed as part of the validation study. Aliquots of control soil matrix extracts were fortified and analyzed against calibration standards prepared in solvent. No matrix effects were observed, as shown in the acceptable recovery results of the fortified samples and in the matrix effect testing conducted in this study.

    2.   Reagent and Solvent Interference

        High purity solvents and reagents were used for this assay. There was no response in the reagent blank sample in the chromatograms corresponding to the retention time of the analytes. This indicates there were not any reagent or solvent interferences.

    3.   Labware Interference

        This method uses mostly disposable labware. No interferences from the labware used were observed.

F. **Confirmatory Techniques**

The method validation set was run by LC-MS/MS with monitoring of two ion transition pairs. As this method is highly selective, no additional confirmatory technique was used.

G. **Time Required for Analysis**

Approximately 6 hours were required to prepare a single soil or spray pad analysis set (13 samples) from the time samples were prepared to LC-MS/MS analysis.

Automated LC-MS/MS analysis was performed overnight. Approximately an additional 1-2 hours were spent on data calculation and tabulation the following day.

H. **Modification or Potential Problems**

There were no modifications to the method. No potential problems were encountered.

I.   **Methods of Calculation**

Analyst Chromatography Data System version 1.6.3, a product of AB Sciex, was used to acquire, integrate and calculate the analyte concentrations in ng/mL using the linear regression function with 1/x weighting. The calibration was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient and other parameters of the standard curve were calculated. Calibration standards were injected at a maximum interval of four sample injections, as well as at the beginning and end of the injection sequence. Seven different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of the analytes detected in method validation sample extracts were interpolated from the standard calibration curve.

In soil, the concentration as ppm (µg/g) of residues found in the samples was calculated with Microsoft® Excel using the equations in the examples below.

$$\textit{Sample Conc. (ppm)} = \frac{\textit{Concentration from Curve (ng/mL) x Aliquot Factor x Final Volume (mL) x 1 µg}}{\textit{Sample Amount (g) x 1000 ng}}$$

The aliquot factor was determined as follows:

$$\textit{Aliquot Factor} = \frac{\textit{Extraction Volume (200 mL)}}{\textit{Aliquot Volume (5 mL)}} = 40$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\textit{\% Recovery} = \frac{\textit{(Sample Concentration, ppm) x 100}}{\textit{(Fortification amount, ppm)}}$$

An example calculation in soil for an NF-180 laboratory fortification (primary ion) in set 694MV01, sample 694MV01-4 LOQ sample fortified at 0.00202 ppm, is as follows:

$$\textit{standard curve equation: } y = 3.28 \times 10^{6}(x) + (643)$$

$$\textit{where } x = \textit{NF-180 concentration in ng/mL and}$$

$$y = \textit{peak response} = 290987.0$$

$$\textit{NF-180 concentration from the curve} = 0.0884 \textit{ ng/mL}$$

$$\textit{Sample Conc. (ppm)} = \frac{\textit{0.0884 ng/mL x 40 x 10 mL x 1 µg}}{\textit{20.01 g x 1000 ng}} = 0.00177 \textit{ ppm}$$

$$\textit{\% Recovery} = \frac{\textit{(0.00177 ppm) x 100}}{\textit{(0.00202 ppm)}} = 87.6\%$$

In spray pads, the concentration as µg/sample of residues found in the samples was calculated with Microsoft® Excel using the equations in the examples below.

$$Sample\ Conc.\ (\mu g/sample) = \frac{Concentration\ from\ Curve\ (ng/mL)\ x\ Final\ Volume\ (mL)\ x\ 1\ \mu g}{1\ Sample\ x\ 1000\ ng}$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{(Sample\ Concentration,\ \mu g/sample)\ x\ 100}{(Fortification\ amount,\ \mu g/sample)}$$

An example calculation in spray pads for an NF-180 laboratory fortification (primary ion) in set 694MV04, sample 694MV04-4 LOQ sample fortified at 39.9 µg/sample, is as follows:

standard curve equation: $y = 1.57\ x\ 10^6\ (x) + (5.85\ x\ 10^3)$

where x = NF-180 concentration in ng/mL and

y = peak response = 1336259.0

NF-180 concentration from the curve = 0.846 ng/mL

$$Sample\ Conc.\ (ppm) = \frac{0.846\ ng/mL\ x\ 50500\ mL\ x\ 1\ \mu g}{1\ sample\ x\ 1000\ ng} = 42.7\ \mu g/sample$$

$$\%\ Recovery = \frac{(42.7\ \mu g/sample)\ x\ 100}{(39.9\ \mu g/sample)} = 107\%$$

Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

J.   Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), percent relative standard deviation and 95% confidence interval for analyte recovery data. Linear regression analysis (with 1/x weighting) was applied to LC-MS/MS calibration curves for the determination of slope, y-intercept and correlation coefficient values.

D. **Limits of Quantitation and Detection**

The LOQ was determined as the lowest fortification level at which acceptable recovery data was obtained. In soil the LOQ was equal to the lowest validated concentration, i.e. 0.002 ppm. In spray pads the LOQ was equal to the lowest validated concentration, i.e. 40 µg/sample. The LOQs for each analyte in matrix are listed below.

The limit of detection (LOD) was calculated for all analytes in soil, and were all estimated to be $\leq$ 0.0003 ppm. The LOD was calculated by using the measured residue values generated for each analyte at the 0.002 ppm level (the established LOQ), which represents a set of 5 replicates. The calculations were performed using the statistical procedure described in the Handbook of Environmental Analysis, fourth edition, by Roy-Keith Smith, Genium Publishing, 1999. The $t_{0.99}$ value for a set of 5 replicates is 3.747.

$$LOD = t_{0.99} \times S$$

$t_{0.99}$ = the one-tailed statistic at the 99% confidence level for n replicates
S = the standard deviation of recovery results from n samples fortified at the established LOQ (0.002 ppm).

The statistically calculated LOD value for QP-1-7 was estimated to be 0.0001 ppm. However, the LOD for this analyte was also listed as 0.0003 ppm for method consistency. The LOD for NF-180 was not determined for spray pads since the procedure was not intended as a residue method.

The LOQs and LODs for each analyte are listed below.

Analyte	Matrix	LOQ (ppm)	LOD (ppm)
NF-180	Soil	0.002	0.0003
QP-1-1	Soil	0.002	0.0003
QP-1-7	Soil	0.002	0.0003
Analyte	**Matrix**	**LOQ (µg/sample)**	**LOD (µg/sample)**
NF-180	Spray Pad	40	N/A

E.   Selectivity and Specificity

There was no observed response in the unfortified samples in the region of the chromatograms at the retention time of the analytes. These results indicate that the method is selective for both the analytes in almond hulls and almond nutmeat matrixes. The method is specific for each analyte due to the use of two different MS/MS transition ion pairs.

F.   Extract Stability

Sample extracts from the method validation trials were stored frozen. Selected soil procedural recovery extracts were removed from storage and reanalyzed at 28 days following extraction to determine extract stability.

G.   Evaluation of Matrix Effect

An assessment was conducted on the possible effects on the quantitation of the residues by the matrix components present in the final extracts. Matrix-matched standards were prepared in final extracts of untreated control samples. Untreated control sample extracts were selected from analytical sets 694MV01 and 694MV02 for California and Iowa soils, respectively. These samples were previously analyzed in method validation sets and found to have no detectable residues. The concentration of the matrix-matched standards was run at one level, within the range of the solvent calibration standards. Matrix matched standards were prepared and analyzed in duplicate. Matrix effects were considered to be insignificant if there is <20% difference in response compared to the calibration standards prepared in solvent.

H.   <u>**Limitations**</u>

The method has been tested in two sources of soil and a single type of spray pad. It can be assumed that the method may be applicable to other matrix types not tested in these validations provided successful recovery tests are conducted at relevant fortification levels.

V.   CONCLUSION

The reference method was successfully validated using LC-MS/MS. GPL-MTH-099 was successfully validated at for the determination of NF-180, QP-1-1 and QP-1-7 in soil and application verification spray pads.

VI.   CHANGES TO THE PROTOCOL

Two amendments to the study protocol was issued by the Study Director and approved by a representative of the Sponsor. Protocol Amendment 1 documented a change in Study Monitor. Protocol Amendment 2 removed the validation for tank mix samples due to a change in Sponsor requirements. This amendment also clarified that matrix effect testing was not necessary for spray pad samples. These amendments did not have a negative effect on the results or the integrity of the study.