

VALENT U.S.A. CORPORATION
VALENT TECHNICAL CENTER
Dublin, California

Determination of Residues of Clothianidin in Crops

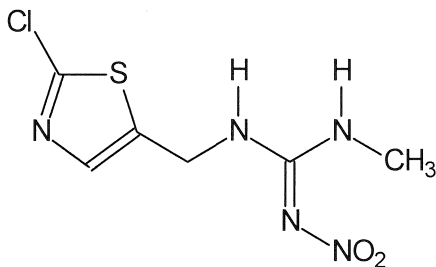
Method: **RM-39C-1**

Date: December 8, 2008

This method describes the determination of clothianidin [(*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine, CAS Number 210880-92-5] in crops. Briefly, the method involves an extraction with acidified water (0.1% formic acid), and centrifugation to remove the solids. An aliquot of the supernatant is adsorbed on Celite® and then extracted with hexane/ethyl acetate. The sample is evaporated to dryness, redissolved in 0.1% formic acid in water and analyzed by high performance liquid chromatography/mass spectrometry (LC/MS-MS).

This method is based on the analytical method used for a variety of crops, originally developed by Morse Laboratories (Morse Laboratories Method 164), and modified by Golden Pacific Laboratories (References 1 and 2). This method includes a modification to the cleanup step, and it has been validated for use on figs and pomegranates.

I. ANALYTICAL STANDARDS



Clothianidin

Standard Solutions

Stock Solution, 1 mg/mL:

Accurately weigh 0.050 g (correct the amount for chemical purity to ensure a 1.0 mg/mL solution), and transfer to a 50 mL volumetric flask. Dilute to volume with acetonitrile. Store the Stock Solution in a refrigerator when not in use.

Intermediate Solution, 10 µg/mL:

Transfer a 1.0 mL aliquot of the 1 mg/mL stock solution to a 100 mL volumetric flask, and dilute to volume with acetonitrile. Store this solution in a refrigerator when not in use.

Fortification Solution, 0.2 µg/mL:

Transfer a 2.0 mL aliquot of the 10 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water. Store this solution in a refrigerator when not in use.

Analytical Standard Solutions:

0.05 µg/mL: Transfer a 25 mL aliquot of the 0.2 µg/mL Fortification solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.02 µg/mL: Transfer a 40 mL aliquot of the 0.05 µg/mL Fortification solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.01 µg/mL: Transfer a 50 mL aliquot of the 0.02 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.005 µg/mL: Transfer a 50 mL aliquot of the 0.01 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.002 µg/mL: Transfer a 40 mL aliquot of the 0.005 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.001 µg/mL: Transfer a 50 mL aliquot of the 0.002 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

0.0005 µg/mL: Transfer a 50 mL aliquot of the 0.001 µg/mL solution to a 100 mL volumetric flask, and dilute to volume with 0.1% formic acid in water.

Store these Analytical Solutions refrigerated when not in use. Additional dilutions and/or alternate concentrations may be used to generate appropriate standards.

III. REAGENTS and MATERIALS

Acetonitrile – pesticide quality
Celite ® 545 – Baker Analyzed Reagent, J.T. Baker
Hexane – pesticide quality
Ethyl Acetate – pesticide quality
Formic Acid, ASC, 96%+
Water, HPLC grade

IV. EQUIPMENT

Balances, Analytical and Top Loading
Centrifuge tubes, 50 mL polypropylene
Centrifuge, Beckman GPKR Centrifuge
Robot Coupe® Food Chopper R25T

Pipettor, Automatic - capable of accurately dispensing 0.5 to 2.5 mL volumes, Rainin
 Pipettor, Automatic - capable of accurately dispensing 20 to 250 μ L volumes, Rainin
 Polytron[®] homogenizer, model PCU11
 Round bottom boiling flasks, 100 mL
 Volumetric Flasks - 50 and 100 mL
 Note: Equivalent equipment may be also used.

V. SOLUTIONS

0.1% Formic Acid in Water – dilute 1 mL of formic acid in 1 L HPLC grade water.
 0.05% Formic Acid in Acetonitrile – dilute 0.5 mL of formic acid in 1 L acetonitrile.
 0.05% Formic Acid in Water – dilute 0.5 mL of formic acid in 1 L HPLC grade water..
 Hexane/Ethyl Acetate 1/1, v/v – mix 500 mL of cyclohexane with 500 mL of ethyl acetate

V. INSTRUMENTATION

High Performance Liquid Chromatograph/Mass Spectrometer (LC/MS-MS)

Applied Biosystems API 4000 mass spectrometer with a Turbo Spray interface and an Agilent 1200 HPLC binary pump system (or equivalent). Conditions shown below are suggested for these analyses, however other conditions may be used as appropriate.

HPLC Conditions:

Column: Synergi 2.5 μ Fusion-RP, 50 mm x 2.0 mm
 (Phenomenex part # 00B-4423-B0)
 Mobile Phases: Water, 0.05% formic acid
 Acetonitrile, 0.05% formic acid
 Flow Rate: 500 μ L/minute
 Injection Volume: 15 μ L

Gradient Program:

<u>Time (mins.)</u>	<u>% Water, 0.05% FA</u>	<u>% ACH, 0.05% FA</u>
0	90	10
4.0	90	10
6.0	10	90
7.5	10	90
8.0	90	10
12.0	100	0

MS-MS Conditions:

Interface: TurboSpray Ionization (ESI)

Period 1, Experiment 1

Relative Start Time: 0 minutes

Scan Type: MRM

Polarity:	Negative
Q1 Mass:	247.9 m/z
Q3 Mass:	164.8 m/z
Dwell (msec):	300.0
Collision Gas (CAD):	8.00
Curtain Gas (CUR):	20.0
Ion Source Gas 1 (GS1):	30.0
Ion Source Gas 2 (GS2):	30.0
Ion Spray Voltage (IS):	-4000.0
Temperature (TEM):	450.0
Interface Heater (ihe):	on
Declustering Potential (DP):	-45.0
Entrance Potential (EP):	-5.0
Collision Energy (CE):	-24.0
Collision Cell Exit Potential (CXP):	-10.0

Retention Times: Clothianidin: 5.0 minutes

VI. ANALYTICAL PROCEDURES

1. Sample Setup

Macerate or grind the sample using the Robot Coupe® food chopper. Weigh 2 g (± 0.1 g) of the sample into a 50 mL centrifuge tube. At this point, if required by the testing facility, a control sample to be used for method recoveries may be fortified with clothianidin (*see Note 1*).

2. Extraction

Add 20 mL of 0.1% formic acid in water and homogenize the sample for one minute using a Polytron® sample homogenizer, then centrifuge the sample for five minutes at approximately 2500 rpm.

3. Sample Cleanup

Transfer a 2.0 mL aliquot of the sample extract to a clean 50 mL centrifuge tube containing approximately 1.5 g (5 mL) of Celite, and mix well by tapping and/or shaking to break up any clumps formed. Add 15 mL of hexane/ethyl acetate and shake the sample for one minute. Centrifuge the sample for 5 minutes at approximately 2500 RPM. Decant the supernatant into a 100 mL round-bottom flask, and repeat the extraction/centrifugation once more with an additional 15 mL of hexane/ethyl acetate. Combine the extracts, and evaporate the sample to dryness using a rotary evaporator with a water bath at $<40^{\circ}\text{C}$. Redissolve the sample in 2.0 mL of 0.1% formic acid in water.

4. Sample Analyses

Transfer a portion of the sample to an autosampler vial for analysis. Condition the LC/MS-MS instrument with at least three injections of a sample extract before starting the analyses. Include within the analytical sequence five to seven standard concentrations to establish the response of the instrument. [A typical set of linearity standards would include a 0.0005, 0.001, 0.002, 0.005, and 0.010 $\mu\text{g/mL}$, covering an expected residue range of 0.005 to 0.10 ppm]. A sample sequence is typically constructed with the following order: conditioning sample extract injections, a reference standard (i. e., the 0.002 $\mu\text{g/mL}$ standard), a set of 1 to 3 sample extracts, a linearity standard, another set of 1 to 3 sample extracts, ..., and the reference standard. The sequence must begin and end with the reference standard, and have at least one additional reference standard analyzed within the sequence. If the peak response for an analyte in a sample extract is greater than the peak response of the highest linearity standard, the sample extract must be diluted with 0.1% formic acid and the diluted sample analyzed.

Residue data is calculated using a weighted ($1/x$) linear fit equation. In cases where the LC/MS-MS instrument is not linear, a polynomial curve generated from the standards injected may be used (See Section 5 for standard curve calculations). To check the validity of the standard injections and the response curve, the responses for each standard are fit into the curve equations, and the back-calculated amounts detected for each standard must be within $\pm 10\%$ of the labeled concentration ($\pm 15\%$ for the smallest standard concentration). In addition, the coefficient of variation of the reference standard responses must be 10% or less for the set to be acceptable.

5. Residue Calculations

The amount of clothianidin in the sample is calculated using a response curve drawn from the linearity standard analyzed with each set of samples. The standard data is entered into a Microsoft Excel[®] spreadsheet as concentration versus response to provide a weighted linear response curve or a second order polynomial curve.

Weighted Linear Response Calculations:

The weighted linear response curve is determined by replicating the standard entries in the data set prior to performing a linear regression in Excel. An example of a typical standard set with the number of entries (to provide weighting relative to the highest standard concentration) is shown below:

Standard Concentration	Number of Entries in Data Set (highest standard conc. \div standard conc.)
0.010 $\mu\text{g/mL}$	1
0.005 $\mu\text{g/mL}$	2
0.002 $\mu\text{g/mL}$	5
0.001 $\mu\text{g/mL}$	10
0.0005 $\mu\text{g/mL}$	20

Determine the slope and y-intercept of the linear regression curve using Excel's functions =SLOPE(*data range*) and =INTERCEPT(*data range*).

The analyte concentration in the standards and the final sample extract is calculated from the weighted linear fit using the equation:

$$\text{Concentration, } \mu\text{g/mL} = [(\text{Analyte Response} - y\text{-intercept}) \div \text{slope}]$$

Second Order Polynomial Curve Calculations:

A second order polynomial curve can be determined using Excel by entering the standard concentration ($\mu\text{g/mL}$) and response (area) for each linearity standard injection and one reference standard injection into a spreadsheet. Using Excel's graphing function, draw a curve plotting the response on the 'x-axis' and the standard concentration on the 'y-axis'. Add a polynomial, second order trendline, and display the curve equation on the graph.

Calculate the $\mu\text{g/mL}$ concentration for each injection by using the curve constants a, b and c from the equation, and the area response from each injection using the equation:

$$\mu\text{g/mL} = (a * \text{area}^2) + (b * \text{area}) + c$$

Residue Calculations (ppm)

The ppm residue in the sample is calculated from the $\mu\text{g/mL}$ concentration found in the extract, using the initial sample weight and the final volume, taking into account any aliquots taken during sample workup. If the sample is further diluted prior to analysis, a Dilution Factor is used:

$$\text{Residue, ppm} = \frac{\text{Concentration } (\mu\text{g/mL})}{[\text{Initial Sample Wt (g)} / \text{Extraction Vol (mL)}] * [\text{Aliquot Vol (mL)} / \text{Final Vol (mL)}]} * DF$$

Percent recoveries for the fortified samples are calculated by first subtracting the ppm residue found in the untreated sample (if any) from the ppm residue found in the fortified sample, and dividing by the fortification level. Alternatively, the peak area of the untreated sample may be subtracted from the fortified sample.

$$\text{Percent Recovery} = (\text{fort sample ppm} - \text{utc ppm}) \div \text{fortification level}$$

VIII. ANALYTICAL LIMITS

The Limit of Detection (LOD) is calculated from the smallest linearity standard injected divided by the final sample concentration. For this method, the LOD is 0.005 ppm, based on an initial 2 g sample, an extraction volume of 20 mL, a 2.0 mL aliquot, a final sample volume of 2.0 mL and using a 0.0005 $\mu\text{g/mL}$ linearity standard as the lowest linearity standard injected.

$$\text{LOD} = \frac{0.0005 \mu\text{g/mL}}{(2 \text{ g} / 20 \text{ mL}) * (2 \text{ mL} / 2 \text{ mL})} = 0.005 \mu\text{g/g (ppm)}$$

The Limit of Quantitation (LOQ) is defined as the lowest fortification level tested resulting in acceptable recoveries. Untreated fig and pomegranate samples were fortified at 0.01 ppm and provided acceptable recoveries, validating the LOQ at 0.01 ppm.

IX. DISCUSSION

The method was validated for use on figs and pomegranates. Untreated samples were fortified with clothianidin at 0.01 ppm (Limit of Quantitation, 2x Limit of Detection) and at 0.05 ppm (10x LOD), then analyzing the sample extracts by LC/MS-MS. The mean recovery for clothianidin in figs was 87.2 ± 4.9 %, and in pomegranates the mean recovery was 80.1 ± 2.8 %. Example chromatograms of the analyses are shown in Figures 1 through 9. The recovery data are presented in Attachments 1 and 2.

X. RESIDUE CONFIRMATION

Clothianidin residues may be confirmed by analyzing samples and monitoring for additional mass spectral fragmentation ions. Additional fragmentation ions for negative mode ionization are 110.9 and 149.9 m/z, and these can be included in the analyses to confirm clothianidin residues. Alternate HPLC columns have been used in other methods, and may also be used to confirm residues. These columns include a Phenomenex Luna C18(2), 5 μ m x 15 cm x 0.46 cm and a Phenomenex Synergi Polar-RP, 4 μ m. 50 mm x 2 mm.

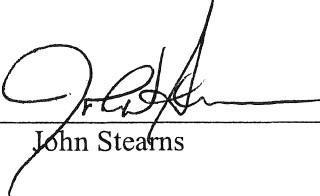
XI. NOTES


1. Valent Standard Operating Procedure VR-002 specifies that fortified control samples are to be analyzed with each set of samples. If the testing facility does not require concurrent analysis of fortified control samples, or if an untreated control (UTC) sample is not available, this method requirement may be waived. Typically, samples are fortified at 0.01 ppm and 0.05 ppm. For example, add 0.10 mL of the 0.2 μ g/mL Fortification Standard solution to the 2.0 g sample for a 0.01 ppm fortification. Additional fortification levels may be desired, based on the residues detected in treated samples, and should include a level greater than the largest residue detected. Method recoveries must be 70 to 120% to be acceptable, unless approved by the supervising chemist responsible for the analysis.

XII. REFERENCES

1. "Clothianidin Residues on Cantaloupe from Trials Conducted in the United States in 2006 and 2007", Stewart Agricultural Research Services Inc. Study No. SARS-06-78, E.R. Stewart, 2007.
2. "Clothianidin Residues on Cotton and Cotton Processed Commodities from Trials Conducted in the United States in 2006 and 2007", Stewart Agricultural Research Services Inc. Study No. SARS-06-85, E.R. Stewart, 2008.

XII. SIGNATURES

Written by:  Date 18-DEC-08
John Stearns

Approved by:  Date 12/18/2008
Allan Rose, Laboratory Manager


Reviewed by:  Date Dec. 18, 2008
Michelle Chen, QAU

Figure 1 – 0.0005 µg/mL Clothianidin standard

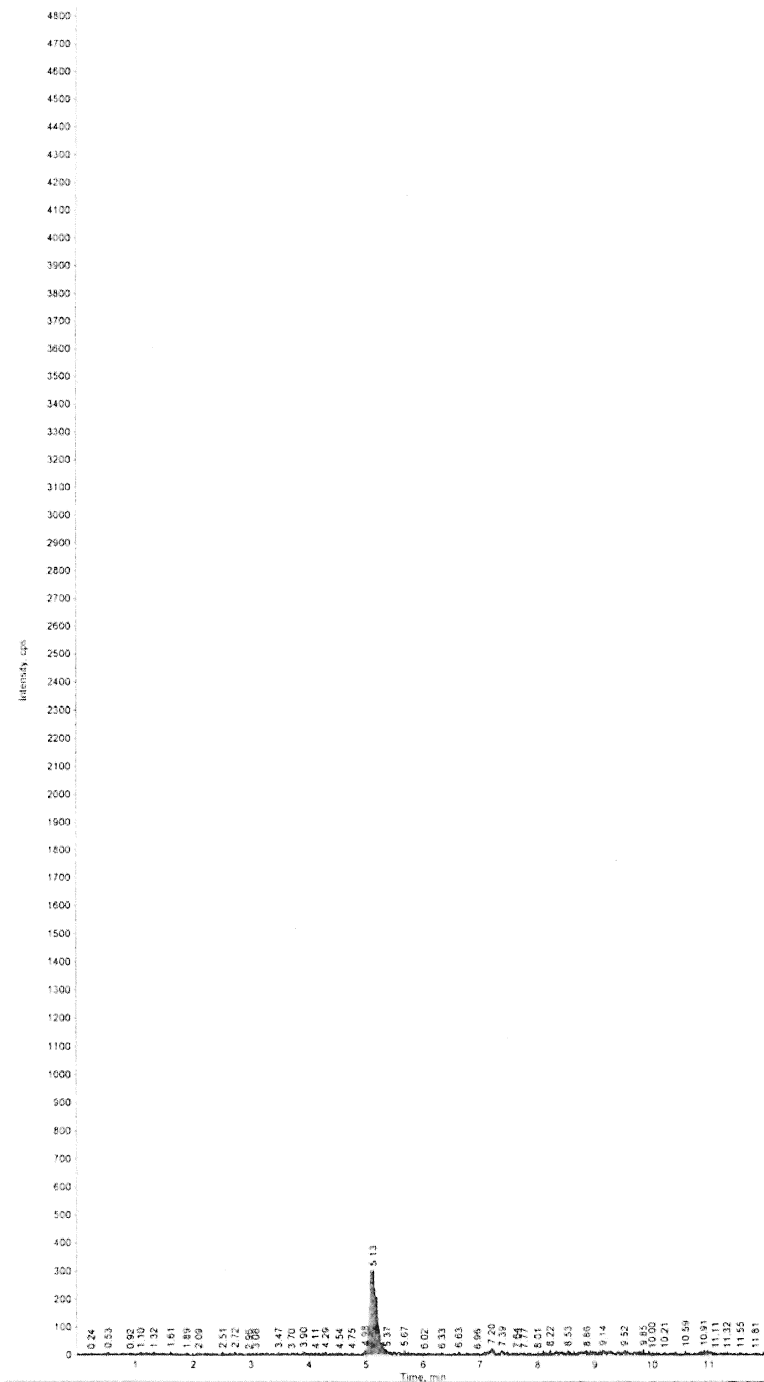


Figure 2 – 0.002 µg/mL Clothianidin standard

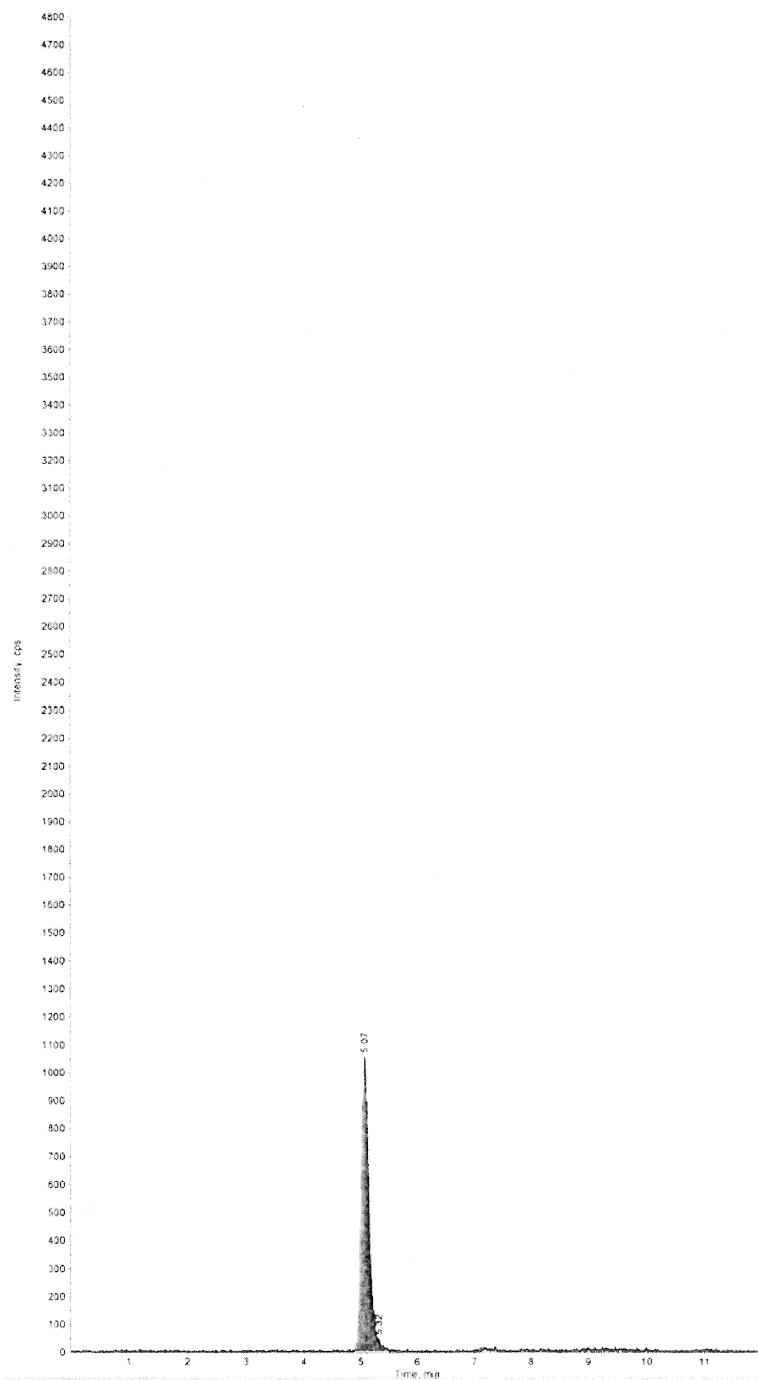


Figure 3 – 0.010 µg/mL Clothianidin standard

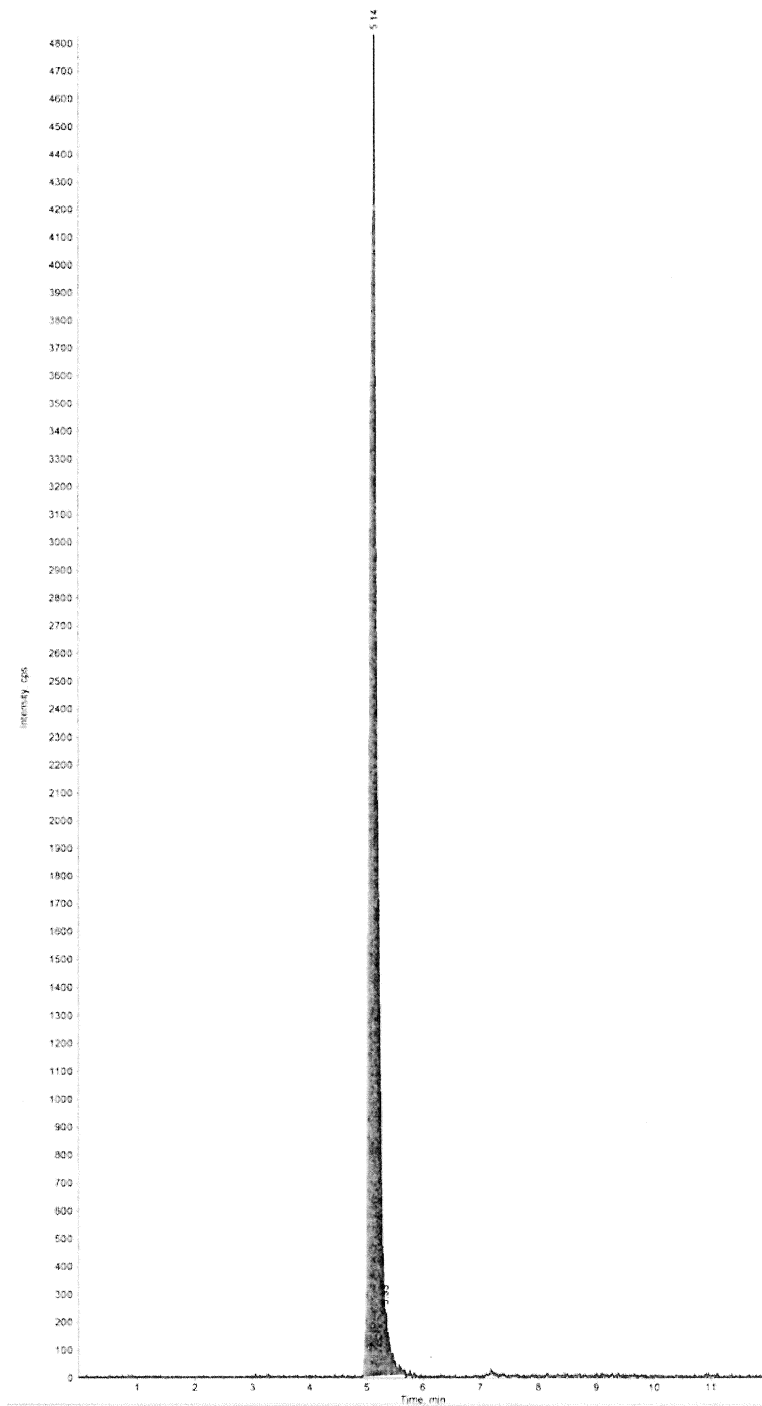


Figure 4 – Untreated Figs, 0.10 g / 1.0 mL final concentration

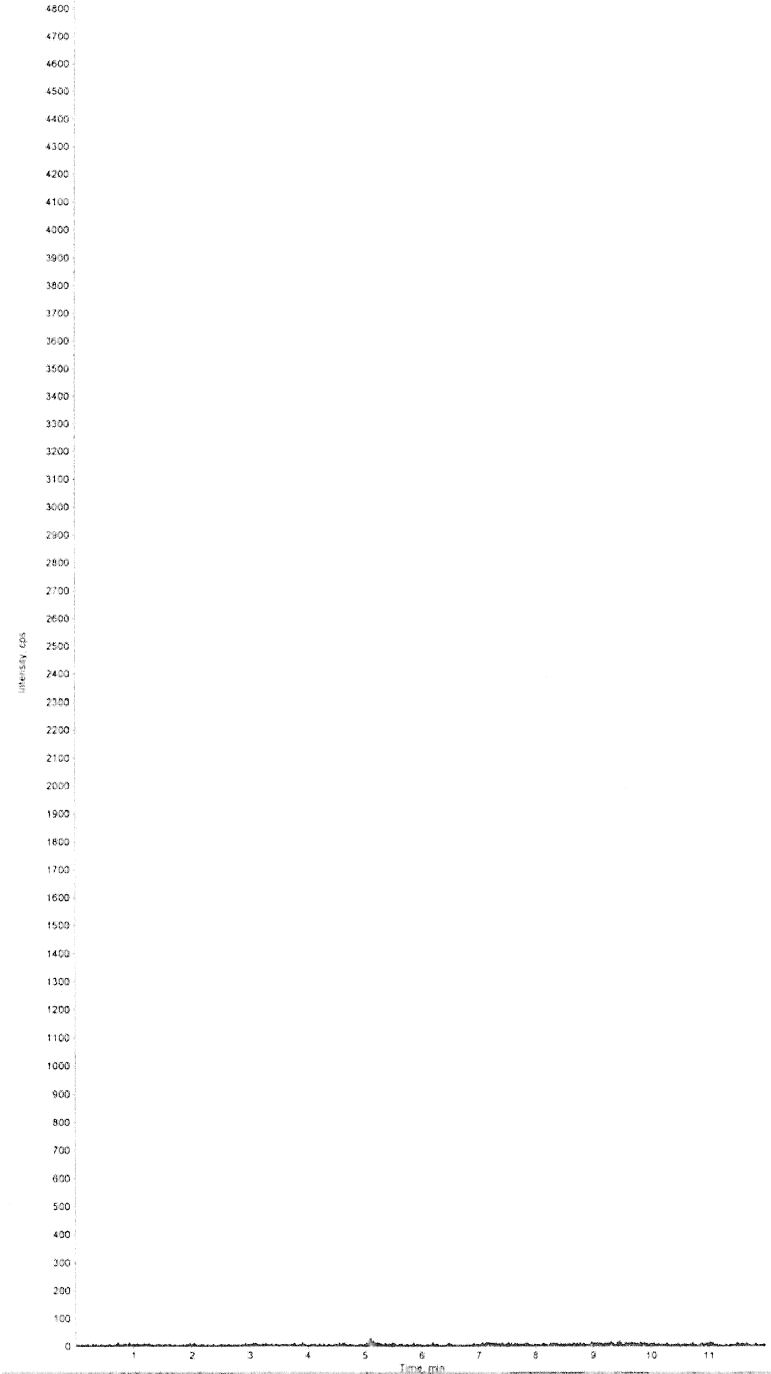


Figure 5 – Figs, Fortified with 0.01 ppm Clothianidin, 0.10 g / 1.0 mL final concentration

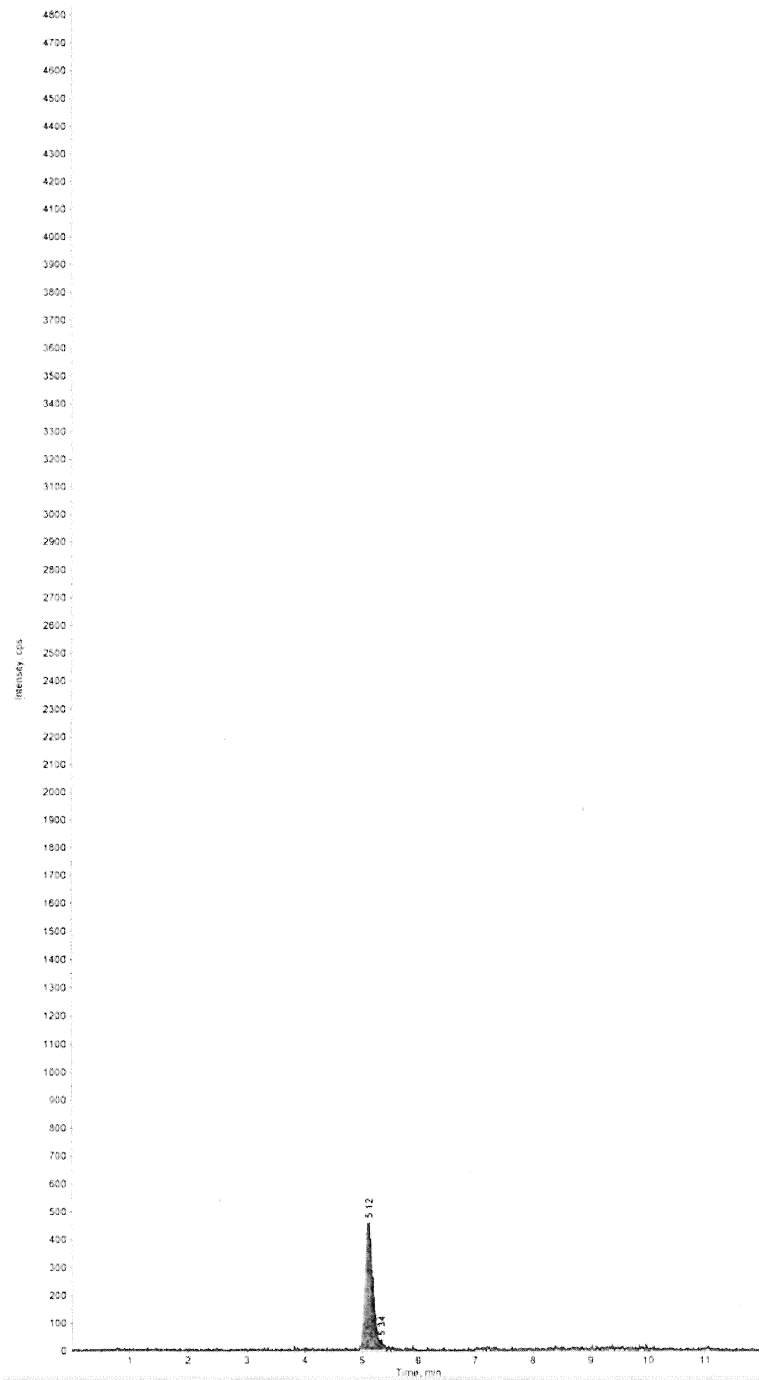


Figure 6 – Figs, Fortified with 0.05 ppm Clothianidin, 0.10 g / 1.0 mL final concentration

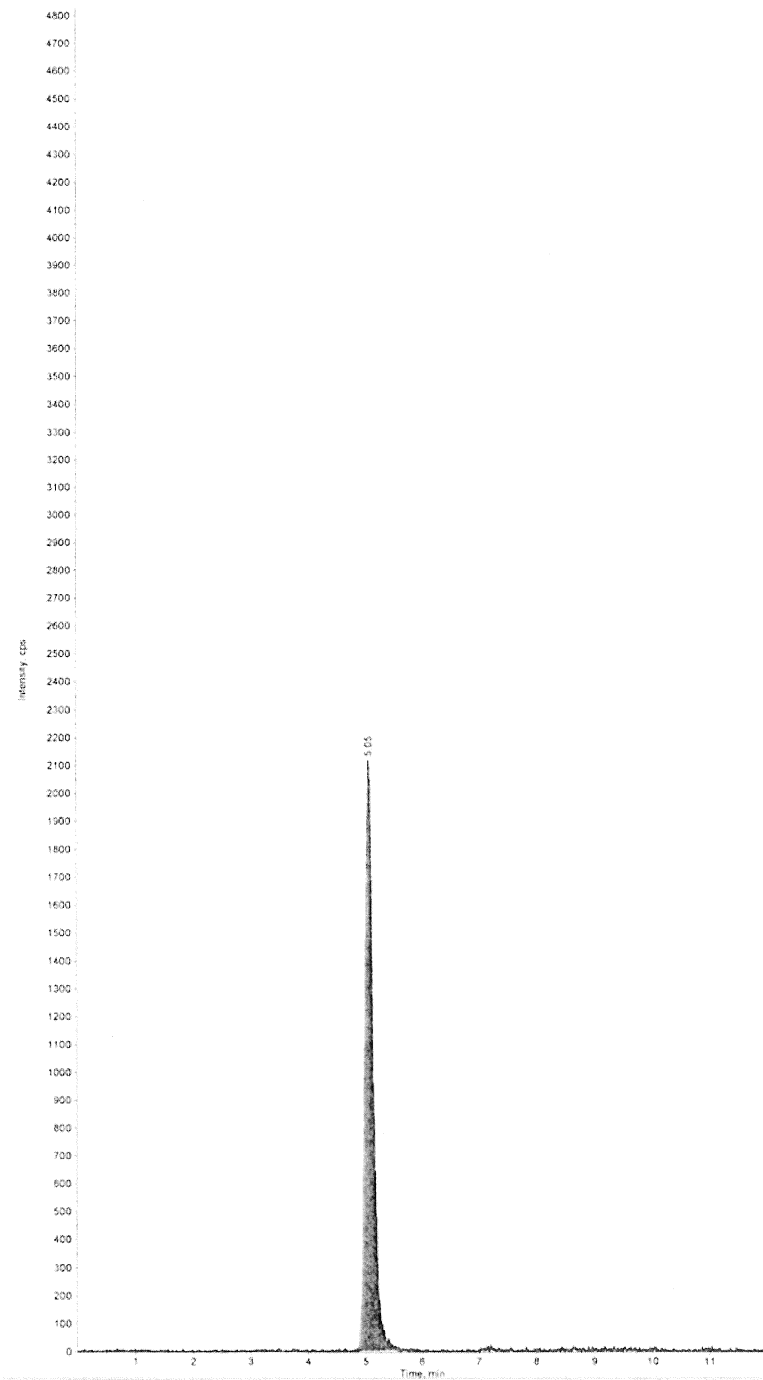


Figure 7 – Pomegranates, untreated, 0.10 g / 1.0 mL final concentration

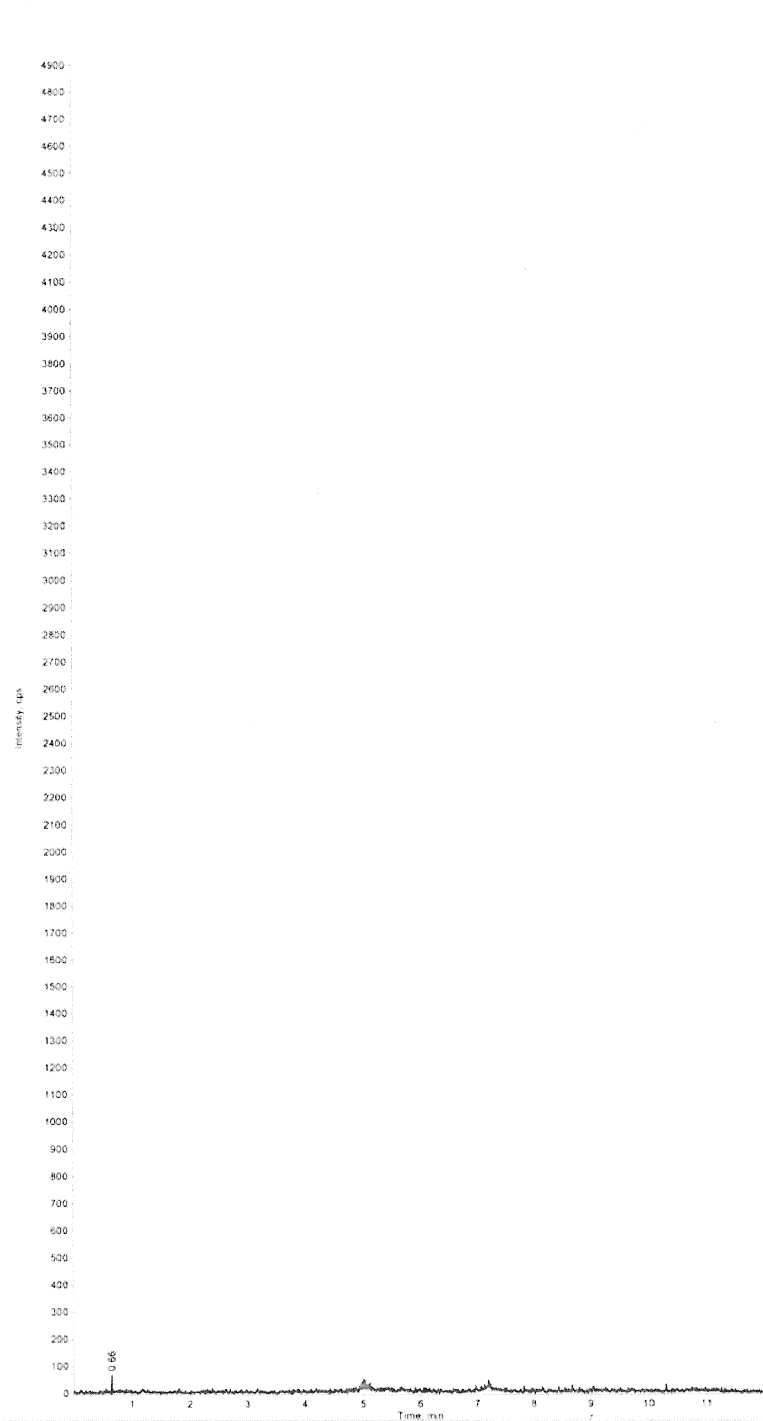


Figure 8 – Pomegranates, Fortified at 0.01 ppm Clothianidin, 0.10 g / 1.0 mL final concentration

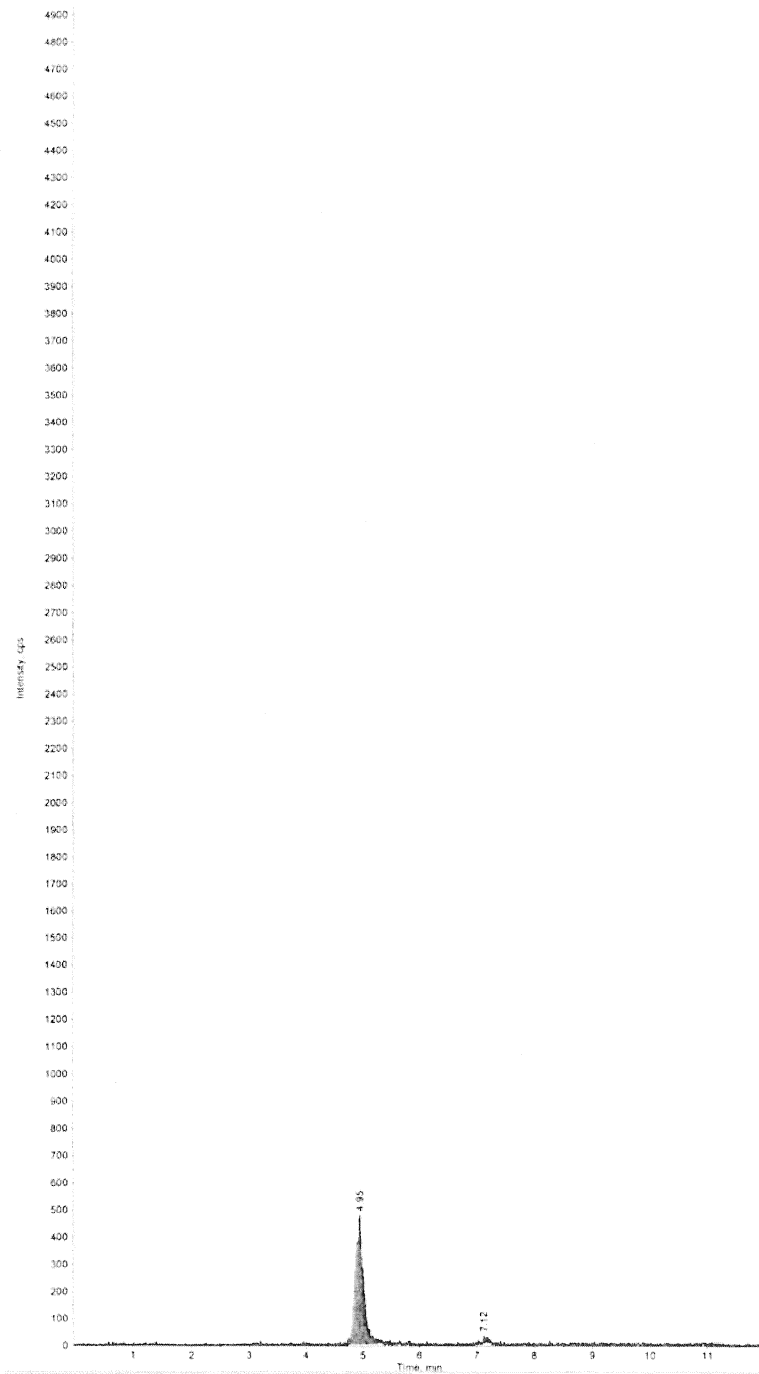
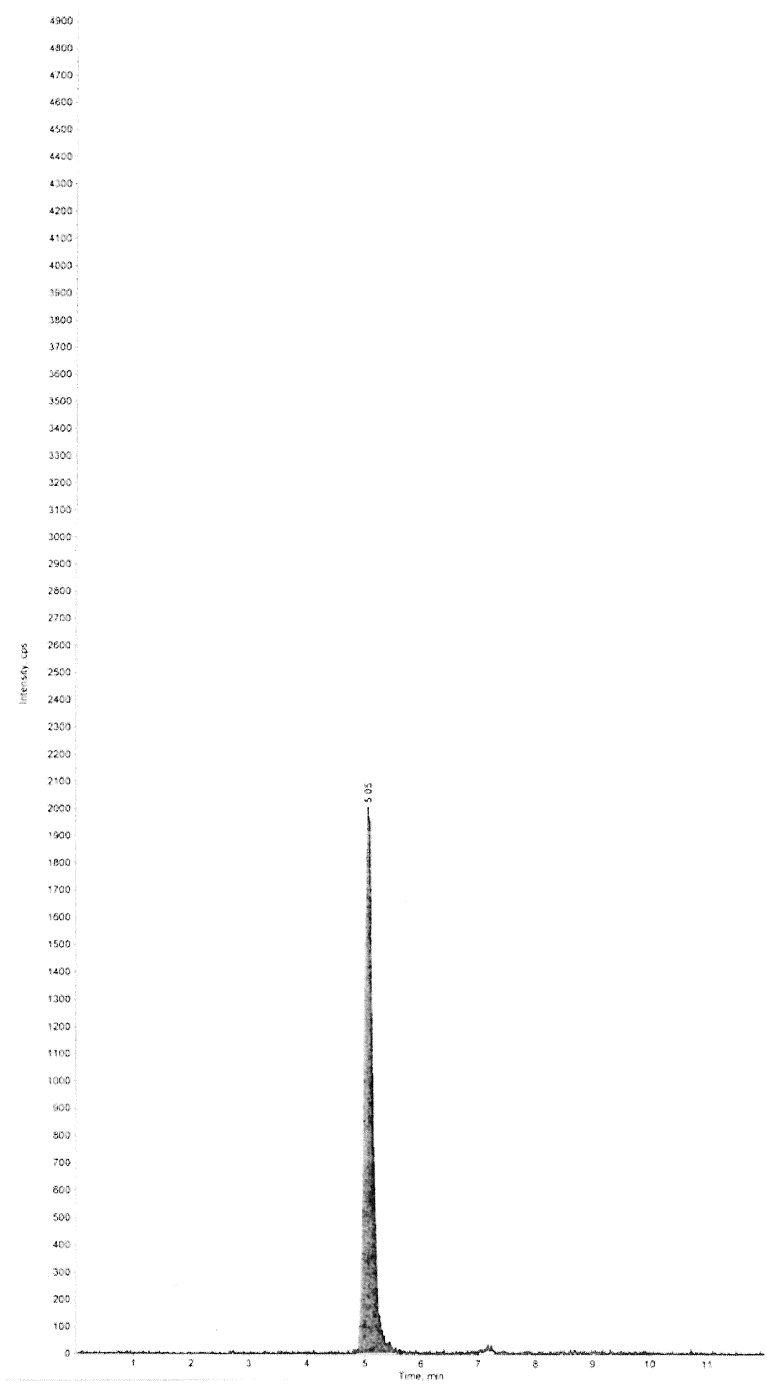


Figure 9 – Pomegranates, Fortified at 0.05 ppm Clothianidin, 0.10 g / 1.0 mL final concentration



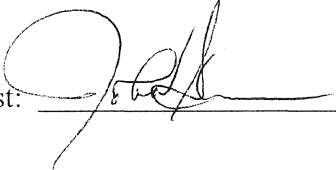
**ATTACHMENT 1
VALENT USA CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CA**

VALIDATION REPORT FOR RESIDUE METHOD **RM-39C-1**

Figs

Sample	Date Extracted	Date Analyzed	Residue (ppm)	% Recovery
Figs, utc	4-Dec-08	4-Dec-08	< LOD ¹	--
Figs, Ft 1 @ 0.01 ppm	4-Dec-08	4-Dec-08	0.0088	87.6
Figs, Ft 2 @ 0.01 ppm	4-Dec-08	4-Dec-08	0.0083	82.7
Figs, Ft 3 @ 0.01 ppm	4-Dec-08	4-Dec-08	0.0095	94.7
Average (n = 3):				88.3%
%CV				6.8%
Figs, Ft 4 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0404	80.8
Figs, Ft 5 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0416	83.2
Figs, Ft 6 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0447	89.4
Figs, Ft 7 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0444	88.7
Figs, Ft 8 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0448	89.5
Figs, Ft 9 @ 0.05 ppm	4-Dec-08	4-Dec-08	0.0442	88.4
Average Grape Recovery (n = 6):				86.7%
%CV:				4.3%
Overall Average (n = 9):				87.2%
Overall % CV:				4.9%

¹ The Limit of Detection (LOD) is 0.005 ppm.

Analyst:  Date 9-DEC-08

Reviewed By:  Date Dec. 17, 2008


ATTACHMENT 2
VALENT USA CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CA


VALIDATION REPORT FOR RESIDUE METHOD RM-39C-1

Pomegranates

Sample	Date Extracted	Date Analyzed	Residue (ppm)	% Recovery
Pomegranates, utc	5-Dec-08	8-Dec-08	< LOD ¹	--
Pomegranates, Ft 1 @ 0.01 ppm	5-Dec-08	8-Dec-08	0.0082	81.6
Pomegranates, Ft 2 @ 0.01 ppm	5-Dec-08	8-Dec-08	0.0077	77.3
Pomegranates, Ft 3 @ 0.01 ppm	5-Dec-08	8-Dec-08	0.0083	83.0
Average (n = 3):				80.6%
%CV				3.7%
Pomegranates, Ft 4 @ 0.05 ppm	5-Dec-08	8-Dec-08	0.0391	78.3
Pomegranates, Ft 5 @ 0.05 ppm	5-Dec-08	8-Dec-08	0.0406	81.2
Pomegranates, Ft 6 @ 0.05 ppm	5-Dec-08	8-Dec-08	0.0394	78.9
Average Grape Recovery (n = 6):				79.5%
%CV:				1.9%
Overall Average (n = 9):				80.1%
Overall % CV:				2.8%

¹ The Limit of Detection (LOD) is 0.005 ppm.

Analyst:  Date 9-12-08

Reviewed By:  Date Dec. 17, 2008

RM-39C-1 Method Modification

An alternate cleanup is being used for sample cleanup due to lack of performance of the Celite cleanup described in the method RM-39C-1.

OASIS HLB Cartridge:

HLB cartridges elute by gravity, and do not need vacuum, and may be left without solvent on top.

- 1) Condition the cartridge with 5 mL of methanol, let drain to top of packing.
- 2) Wash cartridge with 5 mL of deionized or HPLC water, and drain to top of packing.
- 3) Wash cartridge with 5 mL of deionized or HPLC water, and drain to top of packing.

- 4) Add 2.0 mL of sample to cartridge and drain to top of packing.
- 5) Wash sample with 5 mL of Methanol / HPLC water (1/1, v/v) and drain to top of packing.
- 6) Wash sample with 5 mL of Methanol / HPLC water (1/1, v/v) and drain to top of packing.

Discard all of above.

- 7) Elute clothianidin from cartridge with 2 x 5 mL Methanol into a 50 mL round-bottom flask.

Evaporate sample (rotary evaporation with a water bath at <math><40^{\circ}\text{C}</math>) to dryness. Ethyl acetate (10 mL additions) may be added to the sample to assist in removal of any residual water.

Redissolve the sample in 2.0 mL of water/acetonitrile (both with 0.05% formic acid added), 9/1, v/v.



John W. Stearns

18-APR-11

Date