Bayer Method RV-002-S10-02

An Analytical Method for the Determination of Residues of BYI 02960, 6-Chloronicotinic Acid (6-CNA) and Difluoroacetic Acid (DFA) in Soil and Sediment Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of BYI 02960, and its metabolites 6-chloronicotinic acid (6-CNA) and difluoroacetic acid (DFA) in soil and sediment.

Residues of BYI 02960 and its metabolites are extracted from soil and sediment using a mixture of acetonitrile/water (70/30 v/v) with microwave extraction. An isotopic internal standard is added to the sample and an aliquot diluted in 240mM ammonium formate solution. The samples were analyzed for BYI 02960, 6-CNA and DFA by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

An optional clean up step using a Varian Bond Elute C18 solid phase extraction In case of ion suppression or interference peak is included.

This method includes two sets of LC/MS/MS conditions for each analyte, one for quantitation purposes and the second for confirmatory purposes.

The method limit of quantitation (LOQ) in all sample matrices for BYI 02960, and its metabolites 6-CNA and DFA is 5ng/g.

2.0 BACKGROUND

BYI 02960 is an insecticide, currently under development by Bayer CropScience. The analytical method presented in this report is designed to measure residues of BYI 02960 and its metabolites 6-chloronicotinic acid (6-CNA) and difluoroacetic acid (DFA) using isotopically labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

(Functional equivalents may be substituted)

- Various general laboratory glassware and utensils.
- MicroMan pipettors and tips (M50, M250, and M1000).
- Eppendof pipettors and tips (1 mL and 5 mL).
- Eppendof repeater pipette and tips.
- Glass funnels
- IKA Werke HS501 Shaker
- Beckman Allegra 6 Centrifuge
- Milestone Ethos E Microwave Labstation, equipped with a Model 320 Touch Screen Controller and automatic temperature control with fiber optic sensor.

- SeQuant Zic-Hilic 150 mm X 4.6 mm 5 µm particle size (Part No: 150455.0001).
- Phenomenex Gemini C18 150 mm X 2.0 mm 5 µm particle size (Part No: 00F-4435-B0).
- RESTEK Allure Organic Acids 150x4.6mm 5 µm particle size (Part No: 9165565).
- API 4000 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and a CTC PAL autosampler, and Analyst 1.4.1 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

(Functional equivalents may be substituted)

- Acetonitrile (HPLC Grade)
- Acetic Acid, 93% (Reagent Grade)
- Formic Acid 88% (Reagent Grade)
- Ammonium Formate (HPLC Grade)
- Water (HPLC Grade; Fisher No. W7-4)
- 125 mM ammonium formate, pH 3.2; Dissolve 7.9 g of ammonium formate in 1000 mL water. Adjust pH by adding formic acid dropwise while stirring until a pH of 3.2 is reached.
- 200 mM Ammonium Formate, pH 3.2; Dissolve 12.6 g of ammonium formate in 1000 mL water. Adjust pH by adding formic acid dropwise while stirring until a pH of 3.2 is reached.
- 60:40 acetonitrile/125mM ammonium formate, pH 3.2. Combine 400mL of 125 mM ammonium formate, pH 3.2 and 600mL acetonitrile. Mix well.
- 95/5 v/v acetonitrile/water. Combine 950mL water and 50mL acetonitrile. Mix well.
- 0.1% acetic acid in water. Add 1 mL acetic acid to 1000 mL water. Mix well.
- 50/50 acetonitrile/water Combine 500mL water and 500mL acetonitrile. Mix well.
- Fisherbrand 125mL 4oz glass jars (Cat. No. 02-911-455)
- 60mL I-chem vials
- Varian Bond Elute C18, 6 mL, 500 mg (Varian Part No. 12162028).
- Guide needles (Grace Davison Discovery Science, Cat. No. 412450 or Supelco, Cat. No. 57059)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Reference Filter : GF/A Circles 125 mm Cat N° 1820 125 Whatman
- Cationic resin AG-50W-X8 20-50 Mesh-Hydrogen foam (Bio-Rad Part No**.** 142-1421)

<u>Preparation of the washed cationic resin AG-50W-X8 (20-50 Mesh-Hydrogen foam)</u>

1. Weigh about 100 g of resin into a 1 L polypropylene bottle.
2. Add 800 mL of deionized water.
3. Shake/mix for 15 min. Sit the bottle for 5 to 10 min until the solid settles to the bottom of the bottle.
4. Remove the supernatant (water)
5. Repeat step 2-4 a further 2 times.
6. Filter the resin on a GFA filter set into a glass funnel, the washed resin is ready for use.

NOTE: the washed resin can be kept for several days at room temperature in a sealed disposable plastic bottle.

5.0 PREPARATION OF STANDARD SOLUTIONS

BYI 02960, 6-CNA and DFA analytical standards and the isotopic internal standards BYI 02960-$^{13}C_3$, ^{15}N, 6-Chloronicotinic acid-2,4,5-d_3-carboxyl-^{13}C, and difluoroacetic acid-$^{13}C_2$ are needed. These standards may be obtained from Bayer CropScience, 17745 South Metcalf, Stilwell, KS 66085. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Class “A” volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in a refrigerator in amber glass bottles when not in use. Solutions should be allowed to warm to room temperature prior to use. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Stock Standard Solution

Prepare individual ~100µg/mL stock solutions of BYI 02960 and its metabolites 6-CNA and DFA. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01mg. Standards are typically provided in 9.0 to 12.0mg aliquots. The standards are quantitatively transferred to a 100mL volumetric flask using acetonitrile, and diluted to volume with acetonitrile.

Prepare a mixed stock 2.0µg/mL solution containing a mixture of BYI 02960 and its metabolites by taking an appropriate volume (~1.0mL) of each of the primary stock solutions and diluting to 50mL with 50/50 acetonitrile/water.

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

5.2 Fortification Standard Solutions

100ng/mL mixed solution of BYI 02960, 6-CNA and DFA

Transfer 5.0mL of the 2.0µg/mL BYI 2960, 6-CNA and DFA mixed stock standard solution into a 100mL volumetric flask. Dilute to volume with 50/50 acetonitrile/water. Mix well.

5.3 Isotopic Internal Standard Solutions

Prepare individual ~50µg/mL stock solutions of BYI 02960-$^{13}C_3$, ^{15}N, 6-Chloronicotinic acid-2,4,5-d_3-carboxyl-^{13}C, and difluoroacetic acid-$^{13}C_2$. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing

samples to ± 0.01mg. Standards are typically provided in 2.0 to 4.0mg aliquots. The standards are quantitatively transferred to a 50mL volumetric flask using acetonitrile, and diluted to volume with acetonitrile.

Prepare a mixed 500ng/mL internal standard solution containing a mixture of BYI 02960-$^{13}C_3$, ^{15}N, 6-Chloronicotinic acid-2,4,5-d_3-carboxyl-^{13}C, and difluoroacetic acid-$^{13}C_2$ by taking an appropriate volume (~1.0mL) of each of the stock internal standard solutions and diluting to 100mL with 50/50 acetonitrile/water.

5.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.0, 0.3, 0.5, 1.0, 2.0, 4.0, 10.0, 20.0 and 40.0ng/mL of BYI 02960 and its metabolites diluted to 100mL with 60:40 acetonitrile/125mM ammonium formate adjusted to pH 3.2. Before bringing the calibration solutions to volume, add by pipet 0.5mL of the 500ng/mL internal standard solution prepared in acetonitrile/water to each of the calibration solutions. (see Section 5.3 Isotopic Internal Standard Solutions)

Concentration of Standard Solution used for dilution (ng/mL)	Concentration of Internal Standard Solution used for dilution (ng/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Calibration Solution (ng/mL)	Concentration of Internal Standard (ng/mL)
2000	500	2.0	0.5	100	40	2.5
2000	500	1.0	0.5	100	20	2.5
2000	500	0.5	0.5	100	10	2.5
2000	500	0.2	0.5	100	4	2.5
2000	500	0.1	0.5	100	2	2.5
2000	500	0.05	0.5	100	1	2.5
2000	500	0.025	0.5	100	0.5	2.5
2000	500	0.015	0.5	100	0.3	2.5
---	500	---	0.5	100	0	2.5

Further calibration solutions may be prepared as needed. Depending on the analytical range for the plant samples, at least six calibration standards are needed.

6.0 PROCEDURE

6.1 Sample extraction

Appendix 2 shows the analytical scheme for the extraction of BYI 02960, 6-CNA and DFA in soil. The detailed stepwise procedure is as follows:

1. Weigh 20 ± 0.05 grams of soil/sediment into a 125mL glass jar.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile/water (see Section 5.2 Fortification Stock Solutions). Let the fortified samples sit for a minimum of 5 minutes.

3. Add ~50mL of acetonitrile:water(70:30 %v/v) to each sample.

4. Add a magnetic stirrer to each sample and loosely attach the lid in order to minimize the possibility of a pressure build up inside the vessel during the microwave extraction.

   Note: The microwave extraction system monitors the reaction temperature using an automatic fiber optic temperature control system. The temperature sensor and Teflon sleeve are directly inserted into one of samples by piercing a hole in one of the lids. It is recommended that the reaction temperature is monitored using the UTC sample.

5. Load between nine to ten samples onto the microwave carousel. Insert the fiber optic temperature control probe and Teflon sleeve into the UTC sample, and manually rotate the carousel to check that the temperature control probe cable does not catch on any of the samples.

6. Close the microwave door, and program the microwave with the following method:

Step Number	Time Duration	T1 Temperature set point at end of step	E Power Limit (to maintain/ control temperature)	Comments
1	10 min.	70 °C	≤350 W	Ramp from ambient to 70°C
2	5 min.	70 °C	≤350 W	Maintain 70 °C

Ventilation time:	1 minute
QP limit:	60-80% (Shut off limit in case of vapors in oven becoming too concentrated)
Stirrer (speed setting):	Value not used. Manual control overrides program setting.
Rotor control:	On (Rotor rotation is on)
Twist control:	On (Rotor rotates clockwise and then counterclockwise to keep probe cable from twisting)

7. Once the samples have cooled, remove them from the microwave.

8. Add 0.5mL of the 500ng/mL internal standard solution to each sample. Reattach the lid and mix well.

9. Transfer ~0.75mL of the supernatant into a LC vial, dilute with ~0.75mL acetonitrile: 200mM ammonium formate in water adjusted to pH3.2 (50:50 v/v) and analyze by LC/MS/MS.

The following optional sample clean up may be included after step 8 if required.

1. Prepare a 6mL 500 mg Varian Bond Elute C18 cartridge by activating with ~3mL acetonitrile and equilibrating with ~3mL water.
2. Set up a clean 15mL tube to collect the sample extract on the manifold. Transfer ~3mL of the sample from step 8 onto the cartridge. Allow the sample extract to percolate through the cartridge to the clean tube by applying a slight vacuum.
3. Transfer ~0.75mL of the sample from the 15mL tube into a LC vial and dilute with ~0.75mL acetonitrile: 200mM ammonium formate in water adjusted to pH3.2 (50:50 v/v).

6.2 Additional clean-up for DFA confirmatory method

1. Into a 15 mL plastic centrifuge tube, introduce: 1 g of cationic resin AG-50W-X8 washed resin (prepared in Section 4.0), 2.5 mL of acetonitrile and 2.5 mL of the extract solution from Section 6.1, Step 8.

2. Cap the centrifuge tube, and shake for ~15 min on a mechanical shaker.

3. Transfer the tube to a centrifuge and centrifuge at ~2500 rpm for ~5 min.

4. Transfer an aliquot of the supernatant to an LC vial for LC/MS/MS analysis.

7.0 ANALYSIS BY LC-MS/MS

7.1 Analytical Procedure

Step 1. Using the recommended procedures listed below, analyze an aliquot of each of the calibration standard solutions (if necessary, additional standard solutions may be added).

Step 2. Analyze an aliquot of each of the analytical samples.

Note: Up to 20 sample analyses can be made after the analysis of the standard solutions. In the case of over 20 samples, extra standard solutions could be added between sample analyses.

Step 3. Again, analyze an aliquot of each of the calibration standard solutions (and, if necessary, additional standard solutions).

7.2 HPLC Conditions

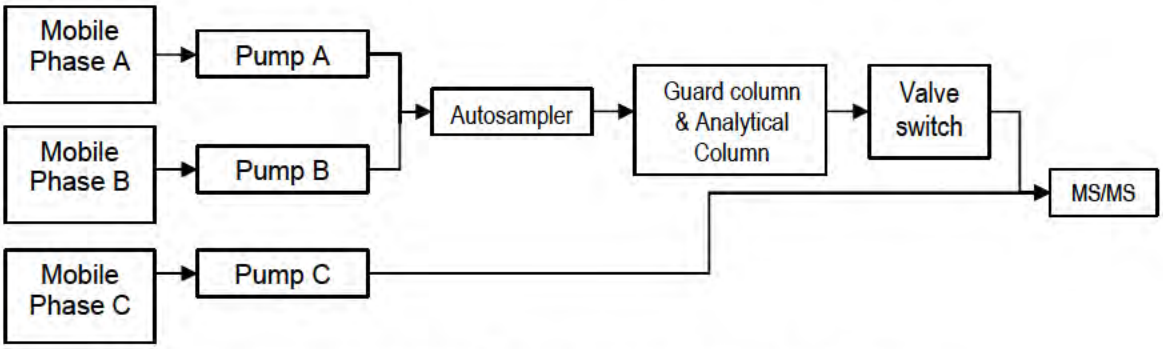
Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. The following recommended conditions were used on an ABSciex API 4000 instrument.

7.2.1 HPLC Conditions for BYI 02960, 6-CNA and DFA analysis

Inject a sample aliquot from Section 6.1, Step 9.

Mobile Phase A: Aqueous 0.1% acetic acid in water
Mobile Phase B: Acetonitrile
Mobile Phase C: Acetonitrile/Water (95/5 v/v)

HPLC column: SeQuant Zic-Hilic 150 mm X 4.6 mm 5 µm particle size
Injection volume: 3-10 µL (Adjust for LC/MS/MS system being used)



Time (min)	Mobile Phase B %	Flow rate (A &B) uL/min
0.0	95	400
0.01	95	400
4.0	75	400
4.1	75	700
4.2	10	700
6.5	10	400
8.6	10	400
9.0	95	400
13	Controller	Stop

Analyte	Approx Retention Time (min)
BYI 02960	3.6
6-CNA	3.7
DFA	5.1

7.2.2 HPLC Conditions for BYI 02960 and 6-CNA - Confirmatory Analysis

Inject a sample aliquot from Section 6.1, Step 9.

Mobile Phase A: 0.5% formic acid in water
Mobile Phase B: 0.1% formic acid in acetonitrile.
HPLC column: Phenomenex Gemini C18 150 mm X 2.0 mm 5 µm particle size.
Injection volume: 5 - 40 µL (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase B %	Flow rate (A &B) uL/min
0.0	10	750
0.5	10	750
3.0	60	750
6.5	95	750
7.0	10	750
8.0	Controller	Stop

Analyte	Approx Retention Time (min)
BYI 02960	3.7
6-CNA	3.9

7.2.3 HPLC Conditions for DFA - Confirmatory Analysis

Inject a sample aliquot from Section 6.2, Step 4.

Mobile Phase A: 0.5% formic acid in water
Mobile Phase B: 0.5% formic acid in acetonitrile.
HPLC column: RESTEK Allure Organic Acids 150x4.6mm 5 µm particle size.
Injection volume: 5 - 20 µL (Adjust for LC/MS/MS system being used)

Start condition: Binary Gradient Total Flow: 0.5 mL/min.

Time (min)	Module	Events	Flow rate (A &B) uL/min
0.0	Pumps	% B	99%
1.0	Pumps	% B	99%
2.2	Pumps	Total Flow	1mL/min
3.0	Pumps	% B	20%
3.01	Pumps	Total Flow	0.5mL/min
5.0	Pumps	% B	20%
5.2	Pumps	% B	99%
6.5	Controller	Stop	

Analyte	Approx Retention Time (min)
DFA	2.1

7.3 Mass Spectrometer Conditions

Note: The analyst should optimize the mass spectrometer conditions to obtain satisfactory system response. The following conditions were used on an API 4000 instrument.

Positive ion mode for BYI 02960

CUR: Curtain Gas	15
CAD: Collision Gas	10
GS1: Ion Source Gas 1	45
GS2: Ion Source Gas 2	45
TEM: Source Temp.	550ºC
IHE: Interface Heater	ON
IS: Ion Transfer Voltage	5500

Negative ion mode for 6-CNA and DFA

CUR: Curtain Gas	50
CAD: Collision Gas	10
GS1: Ion Source Gas 1	45
GS2: Ion Source Gas 2	45
TEM: Source Temp.	550ºC
IHE: Interface Heater	ON
IS: Ion Transfer Voltage	-4500

7.4 Mass Spectrometer Data Collection

Note: The analyst should optimize the mass spectrometer data collection to obtain satisfactory system response. As the HPLC column ages, the retention times of the analytes will change. The time between the ion mode transition from negative mode to positive mode may change with the retention time change. A standard solution should be analyzed before each set of samples to confirm the data collection parameters.

The daughter ions used in this method were chosen due to their optimum sensitivity on the ABSciex API 4000 instrument used for this study. The following recommended ion transitions and conditions were example conditions used on an ABSciex API 4000 instrument:

When using the LC conditions for the analysis of BYI 02960, 6-CNA and DFA described in Section 7.2.1 it is recommended that two separate periods are used in the LC/MS/MS methods. The first period is used for analysis of BYI 02960 and 6-CNA with the second period for the analysis of DFA.

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CE	CXP
BYI02960	+	289	126	50	45	11	40	25
BYI02960 IS	+	295	130	50	45	11	40	25
6_CNA	+	156	112	50	-40	-4	-16	-5
6_CNA IS	+	159	114	50	-40	-4	-16	-5
DFA	-	95.2	51.1	200	-40	-4	-18	-7
DFA IS	-	97.2	52.1	200	-40	-4	-18	-1

8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using ABSciex quantitation software Analyst (Version 1.4.1) using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

After regression coefficients were calculated, the residue in ng/g was determined using the following equation,

$$\text{Residue found (ng/g)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

Where: W = 20g
V_1 = 50mL
V_2 = 0.75mL
V_3 = 1.5mL

Analyst software was used to calculate the amount of BYI 02960 in ng/g for each sample and the percent recovery for the spiked samples.

8.1 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

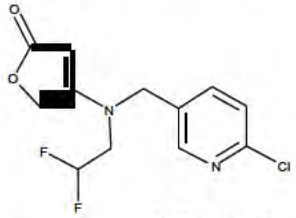
Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 5ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

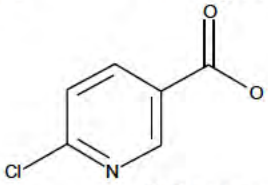
Appendix 1 Test and Reference Substances

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

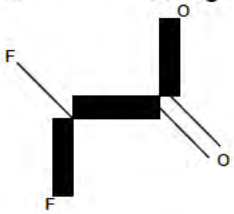
Code Name: BYI 02960
Molecular Formula: $C_{12}H_{11}ClF_2N_2O_2$
Molecular Weight: 288.7 g/mol



Code Name: 6-Chloronicotinic acid (6-CNA)
Molecular Formula: $C_6H_4ClNO_2$
Molecular Weight: 157.6 g/mol

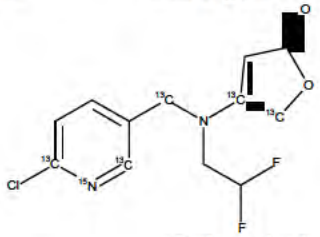


Code Name: Difluoroacetic acid (DFA)
Molecular Formula: $C_2H_2F_2O_2$
Molecular Weight: 96.0 g/mol

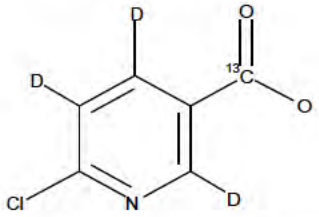


Appendix 1 Test and Reference Substances (Cont'd)

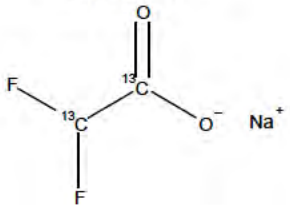
Code Name: BYI 02960 $^{13}C_5{}^{15}N$
Molecular Formula: $C_{12}H_{11}ClF_2N_2O_2$
Molecular Weight: 294.6 g/mol



Code Name: 6-Chloronicotinic acid-2,4,5-d_3-carboxyl-^{13}C
Molecular Formula: $C_2H_2F_2O_2$
Molecular Weight: 161.6 g/mol



Code Name: Difluoroacetate-$^{13}C_2$ (BCS-AB60481-$^{13}C_2$)
Molecular Formula: $C_2HF_2O_2 . Na$
Molecular Weight: 120.0 g/mol



Appendix 2 Extraction Scheme for Soil/Sediment Samples

Note that the steps in italics in the extraction scheme are optional

Weigh an aliquot of soil/sediment into a 125mL glass jar

↓

Add ~50mL of acetonitrile:water(70:30 %v/v)

↓

Microwave extraction. Ramp temperature to 70°C over 10 minutes then hold at 70°C for 5 minutes

↓

Add 0.5mL of the 500ng/mL internal standard solution

↓

Prepare a 6-mL 500 mg Varian Bond Elute C18 cartridges by activating with 3 mL ACN and equilibrating with 3 mL water

↓

Set up clean tube to collect the sample extract on the manifold. Transfer ~3 mL of sample extract to the cartridge. Allow the sample extract to percolate through the cartridge to the clean tube by applying vacuum

Transfer ~0.75mL of the sample to an LC vial

↓

Add ~0.75mL 200mM ammonium formate, pH 3.2 to the LC vial

↓

Mix well and analyze by LC/MS/MS

Additional clean-up for DFA confirmatory method

Mix 1 g of cationic resin AG-50W-X8 washed resin 2.5 mL of acetonitrile and 2.5 mL of the extract solution, shake for ~15 minutes

↓

Centrifuge and transfer an aliquot of the supernatant to an LC vial for LC/MS/MS analysis.