APPENDIX C: ANALYTICAL METHOD STM1906B.00

Determination of Residues of XDE-208 and its Major Metabolites in Water using HPLC/MS/MS

Robert Seymour, Ph.D.

Pyxant Labs Inc.
4720 Forge Road, Suite 108
Colorado Springs, CO 80907
Phone: 719.593.1165
Fax: 719.593.1625
www.pyxant.com

Method: STM1906B.00

Effective: 24 November 2008

Signing Author:

R J Seymour 24 Nov 08

Robert Seymour, Ph.D. Date
Group Leader
Pyxant Labs Inc.

Approved By:

David Robaugh 11/24/08

David Robaugh, Ph.D. Date
Vice President
Pyxant Labs Inc.

TABLE OF CONTENTS

1.0 SCOPE

This method is applicable for the quantitative determination of residues of XDE-208 (Parent) and its major metabolites in water. The validated limits of quantitation (LOQ) are 0.05 ng/mL (ppb) and the limits of detection are 0.015ng/mL for all four analytes.

2.0 PRINCIPLE

Residues of XDE-208 and its metabolites are determined in water after addition of an internal standard mixture and analysis by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

3.0 SAFETY

The analysts must also be acquainted with the potential hazards of the reagents and solvents employed in the laboratory. Such information may be obtained from the MSDS, literature, and other documents such as the laboratory chemical hygiene plan. Disposal of chemicals and reagents must comply with all federal, state, and local regulations.

Formic acid and acetic acid may cause burns to exposed skin and can be harmful if inhaled or ingested. Proper ventilation, eye protection, and personal protective equipment should be worn when handling this reagent.

4.0 GLASSWARE, HARDWARE, AND EQUIPMENT

4.1 Glassware and Hardware

4.1.1 Adjustable Pipettes, variable sizes

4.1.2 96 Deep Well Plate or 2mL autosampler vials

4.1.3 10 mL Volumetric Flask

4.1.4 50 mL Volumetric Flask

4.1.5 100 mL Volumetric Flask

4.1.6 HPLC Column: Phenomenex, Synergi hydro (75x4.6 mm id)

4.2 Equipment

4.2.1 Analytical balance, to ± 0.1 µg, Model No. C35, Cahn Microbalance

4.2.2 Ultra High Purity Water System, Model No. PL5232, ELGA

5.0 ANALYTICAL INSTRUMENTATION

Instrumentation:	Spark Holland Symbiosis Pharma Online SPE LC System or equivalent HPLC system Applied Bio / SCIEX API 4000 LC/MS/MS System Applied Bio / SCIEX Analyst v. 1.4.1 Software

5.1 Typical LC/MS Conditions

Column:	Phenomenex Synergi hydro (75x4.6 mm id)
Column Temperature:	Ambient
Injection Volume:	100 µL , depending on sensitivity and linearity of instrument
Injection Wash Program	1) 700 µL 80:20:0.1, v/v/v, acetonitrile:water:formic acid 2) 700 µL methanol 3) 700 µL water
Run Time:	14.0 minutes
Mobile Phase:	A –Acetonitrile with 0.01% formic acid B –Water with 0.01% formic acid
Flow Rate:	1.0 mL/min. The eluent may be split before entering the source. Whether to split and amount of the split is to be determined depending on sensitivity and linearity of instrument
Gradient for LC only:	Note: Gradient may be modified to obtain better separation or faster equilibration so long as separation is not compromised.

Time, min	A, %	B, %
0:00-0:01	0	100
0:30	0	100
1:00	10	90
6:00	64	36
6:01	100	0
8:00	100	0
8:01	0	100
10:00	0	100

Flow Diverter Program: 1) 0.0→3.0 min: flow to waste
2) 3.0→8.0 min: flow to source
3) 8.0→end of run: flow to waste

5.2 Typical Mass Spectrometry Conditions

Interface:	ESI
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Collision Gas (CAD):	6.0
Curtain Gas (CUR):	20 psi
Ion Source Gas 1 (GS1):	Between 40 and 80 psi depending on the split ratio and instrumentation used.
Ion Source Gas 2 (GS2):	Between 60 and 80 psi depending on the split ratio and instrumentation used.
IS	5500
Temperature (TEM):	Between 500°C and 700°C depending on the split ratio and instrumentation used.

Compound:	Ion, *m/z*		Time, ms	DP/CE/CXP
	Q1	Q3		
XDE-208 quant	278.0	174.0	150	50/11/16
XDE-208 conf	278.0	154.0	150	50/37/16
XDE-208-urea quant	296.0	174.0	150	35/15/16
XDE-208-urea conf	296.0	105.0	150	35/23/16
X11519540 sulfone quant	254.1	175.1	150	60/26/16
X11519540 sulfone conf	254.0	51.1	150	60/123/14
X11579457 sulfoximine quant	253.1	174.1	150	41/13/16
X11579457 sulfoximine conf	253.1	80.0	150	41/17/16
XDE-208-M+3 stable isotope (ISTD)	281.1	177.0	150	26/23/16
XDE-208-M+3 stable isotope (ISTD) Alternative Ions	281.1	106.0	150	26/15/16
XDE-208-urea-M+3 stable isotope (ISTD)	299.0	177.2	150	36/17/16

Note: The XDE-208-M+3 ISTD is used as IS for the XDE-208 quantitation. The Urea M+3 ISTD is used as an IS for the urea, sulfone and sulfoximine metabolites.

Note: The 281.1/106 XDE-208-M+3 stable isotope transition produces slightly less intensity than the 281.1/177 ions, but may also have less background offset on some instruments and may be used instead of the 281.1/177 ions.

6.0 REAGENTS, STANDARDS, AND PREPARED SOLUTIONS

6.1 Materials and Reagents

6.1.1 Acetonitrile, HPLC grade, Burdick & Jackson or equivalent grade

6.1.2 Water, Ultra High Purity (UHP), 18.2 MΩcm or equivalent grade

6.1.3 Formic Acid, HPLC grade, J.T. Baker or equivalent grade

6.2 Standards

Common and code names: XDE-208 (X11422208), 208-Urea (X11719474), 208-Sulfone (X11519540), 208-Sulfoximine (X11579457)

6.2.1 X11422208, DowAgro Sciences, Indianapolis, IN

6.2.2 X11719474, DowAgro Sciences, Indianapolis, IN

6.2.3 X11579457, DowAgro Sciences, Indianapolis, IN

6.2.4 X11519540, DowAgro Sciences, Indianapolis, IN

6.2.5 X11843864 (XDE-208-d_3-ISTD), DowAgro Sciences, Indianapolis, IN

6.2.6 X11817334 (XDE-208 Urea-d_3-ISTD), DowAgro Sciences, Indianapolis, IN

6.3 Prepared Solutions

Prepared solutions may be scaled appropriately.

6.3.2 80:20 acetonitrile/water +0.1 % formic acid, injection wash solvent

6.3.3 Mobile Phase A: Acetonitrile with 0.01% formic acid Add 100 µL of formic acid to 1000 mL of acetonitrile.

6.3.4 Mobile Phase B: Water with 0.01% formic acid. Add 100 µL of formic acid to 1000 mL of ultra high purity water.

7.0 PREPARATION OF STANDARD SOLUTIONS

The following are recommended standard concentrations which may be adapted as appropriate; volumes of solutions may also be changed.

Stock solutions should be prepared in duplicate with two separate weighings of reference standards. One is to be utilized for the preparation of fortification standards and the other for

calibration standards. Prior to any dilutions, an analytical comparison of the stocks should be made to assess their precision and presumed accuracy.

7.1 Preparation of Stock, Intermediate and Spiking (Fortification) Solutions

7.1.1. Weigh 0.0100 g of the XDE-208, the urea metabolite, the sulfone and sulfoximine standards and transfer to separate 10 ml volumetric flasks using acetonitrile. Dilute to volume to produce a 1000 µg/mL solution of each analyte.

7.1.2. Pipet 1.0 mL of each of the 1000-µg/mL solutions into a 10 mL volumetric flask and dilute to volume with acetonitrile to produce a 100 µg/mL mixed standard solution.

7.1.3. Pipet 5.0 mL of the 100-µg/mL solution into a 50 mL volumetric flask and dilute to volume with acetonitrile to produce a 10 µg/mL mixed standard solution.

7.1.4. Pipet 5.0 mL of the 10-µg/mL solution into a 50 mL volumetric flask and dilute to volume with acetonitrile to produce a 1.0 µg/mL mixed standard solution.

7.1.5. Pipet 5.0 mL of the 1.0-µg/mL solution into a 50 mL volumetric flask and dilute to volume with acetonitrile to produce a 0.1 µg/mL mixed standard solution. (10-mL water sample aliquots may be fortified at 400 x LOQ with 200µL of this solution.)

7.1.6. Pipet 5.0 mL of the 1.0-µg/mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 50ng/mL mixed standard solution. (10-mL water sample aliquots may be fortified at at the LOQ with 10µL of this solution and at 10 x LOQ with 100µL of this solution.)

7.1.7. Pipet 1 mL of the 50-ng/mL solution into a 10 mL volumetric flask and dilute to volume with acetonitrile to produce a 5ng/mL mixed standard solution. (10-mL water sample aliquots may be fortified at the LOQ with 100µL of this solution.)

7.2 Preparation of Internal Standard Stocks and IS Fortification Solution

7.2.1. Weigh 0.005-g of XDE-208-M+3 stable isotope standard and XDE-208-urea-M+3 stable isotope standard in separate 50-ml volumetric flasks and dilute to volume with acetonitrile to produce a 100-µg/mL solution of each analyte.

7.2.2. Transfer 1.0-mL of each 100-µg/mL internal standard solution into a 100-mL volumetric flask and dilute to volume with water/acetonitrile/acetic acid (95:5:0.1) to produce a 1-µg/mL mixed internal standard solution.

7.2.2. Transfer 10-mL of the 1-µg/mL mixed internal standard solution into a 100-mL volumetric flask and dilute to volume with water/acetonitrile/acetic acid (95:5:0.1) to produce a 0.1-µg/mL mixed internal standard solution.

7.3 Preparation of Calibration Standards

Dilute the 10, 1 and 0.1-µg/mL standard solutions with a water/acetonitrile/acetic acid (95:5:0.1) solution according to the following table. Add 1-mL of the 1-µg/mL mixed internal standard solution to each flask prior to volume adjustment in order to get solutions with 10-ng/mL of ISTD.

Original Standard Concentration	Aliquot of Spiking Standard	Final Standard Volume	Calibration Solution Final Conc.	Equivalent Sample Concentration in Water
µg/mL	µL	mL	ng/mL	ng/mL
0.01	150	100	0.015	0.015
0.01	500	100	0.05	0.05
0.1	75	100	0.075	0.075
0.1	150	100	0.15	0.15
0.1	250	100	0.25	0.25
0.1	500	100	0.5	0.5
0.1	1000	100	1	1
1	500	100	5	5
1	1000	100	10	10
10	200	100	20	20
10	250	100	25	25
10	350	100	35	35

8.0 SAMPLE PREPARATION

8.1. Sample Preparation

8.1.1 For spikes, take 10-mL of water into polyethylene or glass vials.
8.1.2. Add appropriate aliquots of the spiking solutions to obtain concentrations

ranging from 0.05 to 20 ng/mL (µg/L, ppb)

8.1.3. Mix and transfer a 1-mL aliquot to a 96 deep well plate or autosampler vial.

8.1.4. If a sample needs to be diluted, use HPLC grade water.

8.1.5. Transfer a 1-mL aliquot of each water sample to a 96 deep well plate or autosampler vial.

8.1.6. Add 100-µL of internal standard mix (0.1-µg/mL) into each sample. Mix thoroughly.

8.1.7. Analyze directly by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

9.0 CALCULATIONS

9.1 Analyte Concentration

Generate a best fit regression calibration curve for instrument response ratio versus analyte concentration ratio in the calibration standards. The response may be linear or quadratic with or without weighting depending on the instrumentation and conditions used. Determine the analyte concentration in the final extracts of samples using the regression equation. These calculations should be performed using the Analyst software installed on the LC/MS/MS data system.

The following calculations may be used to determine the analyte concentration in the original commodity.

$$\text{Gross Analyte}\,(\mu g/g) = CF \times \frac{V_f}{V_a} \times \frac{V_i}{W} \times \frac{1\,\mu g}{1000\,ng} \times DF$$

Where:

CF = Analyte concentration in ng/mL
V_f = Volume of final extract (1 mL)
V_i = Volume of initial extract (40 mL)
V_a = Volume of aliquot (2 mL)
W = Weight (g)

Recovery of fortified controls is calculated by the following equation:

$$\%\,\text{Recovery} = \frac{AC}{FC} \times 100$$

Where:

AC = Analyte concentration (µg/g) - concentration found in UTC (µg/g)
FC = Concentration fortified (ppm)

9.2 Percent Recovery

The percent recovery of the QC samples, shown in Equation 5, is determined by dividing the net concentration of analyte found by the fortified concentration.

$$\%\,\text{Recovery} = \frac{AF_{net}}{FC} \times 100 \quad (5)$$

Where:

AF_{net} = net concentration of analyte found (ppm)

FC = fortified concentration (ppm)

9.3 Statistical Treatment of Data

The Analyst® software program will be used to construct calibration curves and determine the best regression fit of the data. The program will also be used to calculate the concentration of the analyte based upon these curves and when applicable, convert these concentrations into the appropriate units. Mean recoveries will be calculated using the "AVERAGE" function of the Microsoft Excel® spreadsheet computer program which divides the sum of the selected cells by the number of determinations. The standard deviations were calculated using the "STDEV" function of the same spreadsheet program which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, is calculated by dividing the standard deviation by the mean, then multiplying by 100.

10.0 NOTES

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and therefore, are not listed.

Operating conditions may be modified to obtain optimal chromatographic separation and performance, if necessary.

11.0 REFERENCES

Residue Chemistry Test Guidelines, OPPTS 860.1340, Residue Analytical Method, U.S. Environmental Protection Agency. U.S. Government Printing Office: Washington, DC, 1996; EPA-712-C-95-174.

Analysis of 208 in Water by LC/MS/MS, (HCK, DAS Brazil, Apr 16th, 2008), Dow AgroSciences, Brazil

APPENDIX D: ANALYTICAL METHOD STM1906B.05

Determination of Residues of XDE-208 and its Major Metabolites in Water using HPLC/MS/MS

Pyxant Labs Inc.
4720 Forge Road, Suite 108
Colorado Springs, CO 80907
Phone: 719.593.1165
Fax: 719.593.1625
www.pyxant.com

ee DES 6JAN10

Method: ~~STM1906B.04~~
STM1906B.05

Effective: May 15, 2009

Signing Author:

15MAY09

Dustin Barr Date
Chemist
Pyxant Labs Inc.

Approved By:

David Robaugh 5/15/09

David Robaugh, Ph.D. Date
Vice President
Pyxant Labs Inc.

TABLE OF CONTENTS

1.0 SCOPE

This method is applicable for the quantitative determination of residues of XDE-208 (Parent) and its major metabolites in water. The validated limits of quantitation (LOQ) are 0.05 ng/mL (ppb) and the limits of detection are 0.015 ng/mL for all four analytes.

2.0 PRINCIPLE

Residues of XDE-208 and its metabolites are determined in water after addition of an internal standard mixture and analysis by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

3.0 SAFETY

The analysts must be acquainted with the potential hazards of the reagents and solvents employed in the laboratory. Such information may be obtained from the MSDS, literature, and other documents such as the laboratory chemical hygiene plan. Disposal of chemicals and reagents must comply with all federal, state, and local regulations.

Formic acid and acetic acid may cause burns to exposed skin and can be harmful if inhaled or ingested. Proper ventilation should be maintained. Eye protection and personal protective equipment should be worn when handling these reagents.

4.0 GLASSWARE, HARDWARE, AND EQUIPMENT

4.1 Glassware and Hardware

4.1.1 Adjustable Pipettes, variable sizes

4.1.2 96 Deep Well Plate or 2mL autosampler vials

4.1.3 HPLC Column: Phenomenex Synergi hydro (4 µm, 75x4.6 mm)

4.1.4 Scirocco Plates: Part Number 186002448

Equipment

4.2.1 Analytical balance, to ± 0.1 µg, Model No. C35, Cahn Microbalance

4.2.2 Ultra High Purity Water System, Model No. PL5232, ELGA

5.0 ANALYTICAL INSTRUMENTATION

Instrumentation:	Spark Holland Symbiosis Pharma Online SPE LC System MDS SCIEX API 4000 LC/MS/MS System MDS SCIEX Analyst 1.4.2 data system

5.1 Typical Online SPE Conditions

SPE Cartridge Tray:	HySphere C18HD 7µm 10x2mm, Part Number 0822.609
SPE Solvation:	acetonitrile, 1 mL at 4 mL/min (SSM A)
SPE Equilibration:	water, 1 mL at 3 mL/min (SSM B)
Sample Extraction:	water, 1 mL at 2 mL/min (SSM B)
SPE Wash 1:	water, 1 mL at 2 mL/min (SSM B)
SPE Elution:	Focus mode using 300 µL of 80/20/0.1, v/v/v, acetonitrile/water/formic acid at 100µL/min
Clamp Flush:	water, 2 mL at 5 mL/min (HPD2)

5.2 Typical LC/MS Conditions

Column:	Phenomenex Synergi Hydro (75x4.6 mm id)
Column Temperature:	Ambient
Injection Volume:	5 to 100 µL depending on sensitivity and linearity of instrument
Injection Wash Program	1) 700 µL 80/20/0.1, v/v/v, acetonitrile/water/formic acid 2) 700 µL methanol 3) 700 µL water
Run Time:	14.0 minutes
Mobile Phase:	A – Water with 0.01% formic acid B – Acetonitrile with 0.01% formic acid
Flow Rate:	1.0 mL/min. The eluent may be split before entering the source. Whether to split and amount of the split is to be determined depending on sensitivity and linearity of instrument
Gradient:	Note: Gradient may be modified to obtain better separation or faster equilibration so long as separation is not compromised.

Time, min	A, %	B, %
0:00	0	100
3:01	0	100
3:05	10	90

8:15	100	0
10:00	100	0
10:15	0	100
14:00	0	100

Flow Diverter Program (optional):
1) 0.0→3.0 min: flow to waste
2) 3.0→8.0 min: flow to source
3) 8.0→end of run: flow to waste

5.3 Typical Mass Spectrometry Conditions

Interface: ESI
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – unit, Q3 – low
Collision Gas (CAD): 6.0
Curtain Gas (CUR): 20 psi
Ion Source Gas 1 (GS1): 50 or between 40 and 80 psi depending on the split ratio and instrumentation used.
Ion Source Gas 2 (GS2): 65 or between 60 and 80 psi depending on the split ratio and instrumentation used.
IS Temperature (TEM): 5000 600 °C or between 500°C and 700°C depending on the split ratio and instrumentation used.

Compound:	Ion, *m/z*		Time, ms	DP/CE/CXP
	Q1	Q3		
XDE-208 quant	278.0	174.0	75	50/11/16
XDE-208 conf	278.0	154.0	20	50/37/16
XDE-208-urea quant	296.0	174.0	75	35/15/16
XDE-208-urea conf	296.0	104.9	20	35/23/16
X11519540 sulfone quant	254.1	175.1	125	60/26/16
X11519540 sulfone conf	254.1	51.2	20	60/123/14
X11579457 sulfoximine quant	253.1	174.1	125	41/13/16
X11579457 sulfoximine conf	253.1	80.0	20	41/17/16
XDE-208-M+3 stable isotope (ISTD) (optional)	281.1	177.0	75	26/23/16
XDE-208-M+3 stable isotope (ISTD) Alternative Ions	281.1	106.0	75	26/15/16
XDE-208-urea-M+3 stable isotope (ISTD)	299.0	177.2	75	36/17/16

Note: The XDE-208-M+3 ISTD is used as IS for the XDE-208 quantitation. The Urea M+3 ISTD

is used as an IS for the urea, sulfone and sulfoximine metabolites.

Note: The 281.1/106 XDE-208-M+3 stable isotope transition produces slightly less intensity than the 281.1/177 ions, but may also have less background offset on some instruments and may be used instead of the 281.1/177 ions.

6.0 REAGENTS, STANDARDS, AND PREPARED SOLUTIONS

6.1 Materials and Reagents

6.1.1 Acetonitrile, HPLC grade, Burdick & Jackson or equivalent grade

6.1.2 Water, Ultra High Purity (UHP), 18.2 MΩcm or equivalent grade

6.1.3 Formic Acid, HPLC grade, J.T. Baker or equivalent grade

6.1.4 Acetic Acid, Glacial, HPLC Grade

6.2 Standards

Common and code names: XDE-208 (X11422208), 208-Urea (X11719474), 208-Sulfone (X11519540), 208-Sulfoximine (X11579457)

6.2.1 X11422208, DowAgro Sciences, Indianapolis, IN

6.2.2 X11719474, DowAgro Sciences, Indianapolis, IN

6.2.3 X11579457, DowAgro Sciences, Indianapolis, IN

6.2.4 X11519540, DowAgro Sciences, Indianapolis, IN

6.2.5 X11843864 (XDE-208-d_3-ISTD), DowAgro Sciences, Indianapolis, IN

6.2.6 X11817334 (XDE-208 Urea-d_3-ISTD), DowAgro Sciences, Indianapolis, IN

6.3 Prepared Solutions

Prepared solutions may be scaled appropriately.

6.3.1 Mobile Phase A: Water with 0.01% formic acid. Add 100 µL of formic acid to 1000 mL of ultra high purity water.

6.3.2 Mobile Phase B: Acetonitrile with 0.01% formic acid. Add 100 µL of formic acid to 1000 mL of acetonitrile.

7.0 PREPARATION OF STANDARD SOLUTIONS

The following are recommended standard concentrations which may be adapted as appropriate; volumes of solutions may also be changed.

Stock solutions should be prepared in duplicate with two separate weighings of reference standards. One is to be utilized for the preparation of fortification and calibration standards standards and the other is used as a check standard. Prior to any dilutions, an analytical comparison of the stocks should be made to assess their precision and presumed accuracy. The check stock should be diluted to produce a high and low standard to check accuracy.

7.1 Preparation of Stock, Intermediate and Spiking (Fortification) Solutions

7.1.1. Weigh 0.0100 g of the XDE-208, the urea metabolite, the sulfone and sulfoximine standards and transfer to separate 10 ml volumetric flasks using acetonitrile. Dilute to volume to produce a 1000 µg/mL solution of each analyte.

7.1.2. Pipet 1000 µL of each of the 1000-µg/mL solutions into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 10 µg/mL mixed standard solution.

7.1.3. Pipet 50 mL of the 10 µg /mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 5 µg /mL mixed standard solution.

7.1.4 Pipet 20 mL of the 10 µg /mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 2 µg /mL mixed standard solution.

7.1.5 Pipet 10 mL of the 10 µg /mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 1 µg /mL mixed standard solution.

7.1.6 Refer to table below for calibration curve dilution scheme.

7.2 Preparation of Internal Standard Stocks and IS Fortification Solution

7.2.1. Weigh 0.0025-g of XDE-208-M+3 stable isotope standard and XDE-208-urea-M+3 stable isotope standard in separate 25-ml volumetric flasks and dilute to volume with acetonitrile to produce a 100-µg/mL solution of each analyte.

7.2.2. Transfer 250-µL of the 100-µg/mL each of the two internal standard solutions into a 100-mL volumetric flask and dilute to volume with acetonitrile to produce a 0.25-µg/mL mixed internal standard solution.

7.2.3 Transfer 10 mL of 250 ng/mL internal standard solution into 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 25 ng/mL

7.3 Preparation of Calibration Standards

Dilute all calibration standards with 95/5/0.1, v/v/v, H_2O/ACN/Acetic Acid

Original Standard Concentration	Aliquot of Spiking Standard	Final Standard Volume	Calibration Solution Final Conc.
µg/mL	µL	mL	ng/mL
0.1	15	100	0.015[1]
0.5	10	100	0.050[1]
0.1	100	100	0.100[1]
0.2	100	100	0.200[1]
0.5	100	100	0.500[1]
0.1	1000	100	1.00[1]
0.2	1000	100	2.00[1]
0.5	1000	100	5.00[1]
0.5	1000	100	5.00*
1	10000	100	100*
2	10000	100	200
5	10000	100	500

* These solutions are also used for fortifications of the QC samples
[1]Add 0.5-mL of the 0.250-µg/mL mixed internal standard solution to each flask prior to volume adjustment in order to get solutions with 1.25-ng/mL of ISTD. .

8.0 SAMPLE PREPARATION

8.1. Sample Preparation

8.1.1 For spiked QC samples, aliquot 10 mL of UTC water, provided by the sponsor from the same site as the samples to be analyzed, into polyethylene tubes. (See notes below for suggested fortification levels.) Add appropriate aliquots of the spiking solutions to obtain concentrations ranging from 0.015, 0.05, and to 4 ng/mL. Vortex for 5 seconds.

8.1.2. If a sample needs to be diluted, use HPLC grade water and dilute the sample prior to addition of the IS.

8.1.3. Transfer a 1-mL aliquot of each QC and water sample to a 96 Deep Well Plate or 2 mL autosampler vials.

8.1.4. Add 50 µL of internal standard mix (25-ng/mL) into each sample or QC aliquot.

8.1.5. Cover and Vortex for 30 seconds.

8.1.6. Analyze directly by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

9.0 CALCULATIONS

9.1 Analyte Concentration

Generate a best fit regression calibration curve for instrument response ratio versus analyte concentration ratio in the calibration standards. The response may be linear or quadratic with or without weighting depending on the instrumentation and conditions used. Determine the analyte concentration in the final extracts of samples using the regression equation. These calculations should be performed using the Analyst software installed on the LC/MS/MS data system.

The following calculations may be used to determine the analyte concentration in the original commodity.

$$\text{Gross Analyte Concentration (ppb)} = CF \times \frac{1}{\rho H_2O} \times DF$$

Where:
CF = Concentration Found (ng/mL)
ρH_2O = 1 g/mL
DF = Dilution Factor

Recovery of fortified controls is calculated by the following equation:

$$\%\,\text{Recovery} = \frac{AC}{FC} \times 100$$

Where:
AC = Gross Analyte Conc. (ppb) – Gross Analyte Conc. UTC (ppb)
FC = Concentration fortified (ppb)

9.2 Statistical Treatment of Data

The Analyst® software program will be used to construct calibration curves and determine the best regression fit of the data. The program will also be used to calculate the concentration of the analyte based upon these curves and when applicable, convert these concentrations into the appropriate units. Mean recoveries will be calculated using the "AVERAGE" function of the Microsoft Excel® spreadsheet computer program which divides the sum of the selected cells by the number of determinations. The standard deviations were calculated using the "STDEV" function of the same spreadsheet program which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, is calculated by dividing the standard deviation by the mean, then multiplying by 100.

10.0 NOTES

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and therefore, are not listed.

Operating conditions may be modified to obtain optimal chromatographic separation and performance, if necessary.

11.0 REFERENCES

Residue Chemistry Test Guidelines, OPPTS 860.1340, Residue Analytical Method, U.S. Environmental Protection Agency. U.S. Government Printing Office: Washington, DC, 1996; EPA-712-C-95-174.

Analysis of 208 in Water by LC/MS/MS, (HCK, DAS Brazil, Apr 16th, 2008), Dow AgroSciences, Brazil