2. INTRODUCTION

The objective of this study was to independently validate the method, ET-002-W13-01 (Appendix A), for the determination of ethephon in water. The method was found to be suitable for the determination of ethephon residues in water based on recovery and precision criteria being met at the LOQ of 0.5 ng/mL and the 10×LOQ level of 5.0 ng/mL in water. This independent laboratory validation (ILV) was conducted to satisfy the requirements of the U.S. EPA Guideline OPPTS 860.1340 (1) and OCSPP 850.6100 (2) as well as OCSPP 850.7100 (3) and Pesticide Regulation Notices 96-1 (4) and 2011-3 (5). This validation report presents the results of the independent laboratory validation for ethephon in water.

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing field samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Bayer CropScience and the Study Director or analyst. Throughout the conduct of the study, any communications between Bayer CropScience and the Study Director and/or the analyst were logged for inclusion in the report. No one from Bayer CropScience was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

3. ANALYTICAL

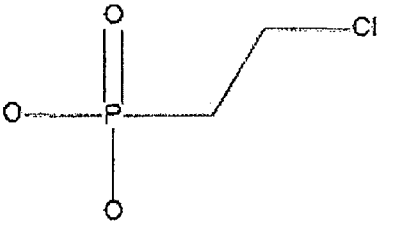
3.1 Sample Receipt, Labeling and Storage

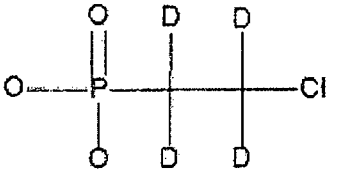
The control water was acquired from a local Colorado pond. The control sample was assigned a unique master logbook (MLB) number 99996057 and stored frozen (-20°C). Characterization data are included in Appendix B.

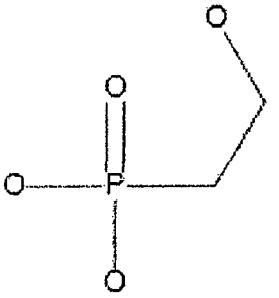
3.2 Preparation of Solutions and Standards

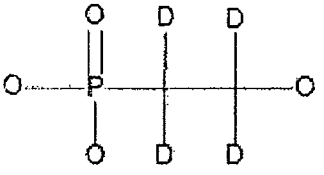
The analysis of ethephon metabolite 2-hydroxyethylphosphonic acid (2-HEPA) was cancelled following preparation of mixed fortification solutions and calibration standards. Reference standard information is therefore included in this report.

The test substances used in this study are described below. Ethephon, 2-HEPA, and the internal standards d_4-ethephon and d_4-HEPA, were shipped from Bayer CropScience and received by Pyxant Labs Inc. on November 20, 2013. The pre-weighed test substances were received in good condition, and stored frozen when not in use. All test substance stock solutions prepared were corrected for purity in the concentration calculations. Certificates of Analysis are included in Appendix C.

Test Substance:	Ethephon
CAS Name:	(2-Chloroethyl)phosphonic acid
CAS No.:	16672-87-0
Lot No.:	0627200304
Purity:	95.9%
Expiration Date:	02Mar2014
Molecular Weight:	144.5 g/mol
Recommended Storage Conditions:	Frozen
Structure:	

Test Substance:	d_4-Ethephon
CAS Name:	(2-Chloroethyl-*1,1,2,2-d*$_4$)phosphonic acid
CAS No.:	Unavailable
Lot No.:	0827200401
Purity:	0.46%
Expiration Date:	27Aug2014
Molecular Weight:	148.5 g/mol
Recommended Storage Conditions:	Frozen
Structure:	

Test Substance:	2-Hydroxyethylphosphonic acid (2-HEPA)
CAS Name:	(2-Hydroxyethyl)phosphonic acid
CAS No.:	22987-21-9
Lot No.:	0714200415
Purity:	95.3%
Expiration Date:	26Jul2016
Molecular Weight:	126.0 g/mol
Recommended Storage Conditions:	Frozen
Structure:	

Test Substance:	d_4-HEPA
CAS Name:	(2- Hydroxyethyl-*1,1,2,2-d*$_4$)phosphonic acid
CAS No.:	Unavailable
Lot No.:	K-2080
Purity:	97.0%
Expiration Date:	12Mar2016
Molecular Weight:	130.1 g/mol
Recommended Storage Conditions:	Frozen
Structure:	

Standard solutions and calibration standard solutions were prepared as described below.

Stock solutions were prepared in 0.1% formic acid in water at a nominal concentration of approximately 100 µg/mL:

Substance Name	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [µg/mL]*
Ethephon	Stock	N839P01-1	10.17	100.00	97.53
2-HEPA	Stock	N839P01-2	14.06	100.00	134.0
d_4-HEPA	Stock	N839P04-1	3.28	50.00	63.63

Substance Name	Solution Type	Solution Lot Number	Pipette [µL]	Dilute In [mL]	Obtain [µg/mL]*
d_4-Ethephon	Stock	N839P04-2	692.5	25.00	100.0

*Resulting concentrations after correcting for purity

Mixed intermediate and fortification solutions were prepared in 0.1% formic acid in water:

From Solution Lot Number	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N839P01-1	97.53	10,253	100.00	10.00	N839P02-1
N839P01-2	134.0	7463			
N839P02-1	10.00	10,000	100.00	1.000	N839P02-2
N839P02-2	1.000	10,000	100.00	0.1000	N839P03-1

The mixed internal standard solutions were prepared in 0.1% formic acid in water:

From Solution Lot Number	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N839P04-2	100.0	10,000	100.00	10.00	N839P05-1
N839P04-1	63.63	15,720			
N839P05-1	10.00	10,000	100.00	1.00	N839P05-2

Mixed calibration standards were prepared in 0.1% formic acid in water. Before bringing to volume, 0.5 mL of the 1 µg/mL mixed internal standard solution was added to each calibration standard:

From Solution Lot Number	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Calibration Solution Final Concentration [ng/mL]	Final Solution Lot Number
N839P02-1	10.0	250	50	50.0	N839P06-6
N839P02-2	1.00	500	50	10.0	N839P06-5
N839P02-2	1.00	250	50	5.00	N839P06-4
N839P02-2	1.00	100	50	2.00	N839P06-3
N839P03-1	0.100	400	50	0.800	N839P06-2
N839P03-1	0.100	100	50	0.200	N839P06-1

3.3 Fortification of Recovery Samples

One ILV trial each of the method was performed for ethephon in water according to the protocol (Appendix D). The trial was comprised of one sample batch, consisting of the following:

1 (one) reagent blank (containing no matrix or analyte)

2 (two) unfortified control samples

5 (five) control samples fortified at LOQ of the method (0.5 ng/mL)

5 (five) control samples fortified at 10×LOQ (5.0 ng/mL)

For preparation of recovery control specimens, appropriate volumes of the fortification standards were added as exemplified below:

Sample Volume	Nominal Target Fortification Level	Fortification Standard Added
10 mL	0.5 ng/mL	0.050 mL of fortification solution: 0.100 µg/mL of ethephon/2-HEPA
	5.0 ng/mL	0.050 mL of fortification solution: 1.00 µg/mL of ethephon/2-HEPA

3.4 Sample Extraction and Analysis

The ILV trials were conducted as described in the analytical method entitled: "An Analytical Method for the Determination of Residues of Ethephon and its Metabolite 2-HEPA in Water Using LC/MS/MS" (Appendix A). Residues of ethephon were extracted from 10-mL water samples by adding approximately 0.0500 mL of formic acid to each sample. Approximately 0.100 mL of the 1.00 µg/mL d_4-ethephon/d_4-HEPA mixed internal standard solution was added to each extract. After shaking to mix, the samples were vialed for analysis by LC/MS/MS.

3.5 Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the chromatographic instrumentation and the cleanup columns to be used. These preliminary studies established that adequate retention time of ethephon and detector sensitivity could be achieved. The confirmatory ion transition was monitored.

The instruments and equipment described below were utilized in the conduct of the independent laboratory validation of the residue analytical method.

3.5.1 Instrumentation

Typical HPLC Conditions

Instrument:	API 5000 UPLC
Column:	Phenomenex Aqua C18 column, 4.6 mm ×150 mm, 3 µm
Injection Volume:	40 µL
Run Time:	4 minutes
Mobile Phase:	A: 0.1% formic acid in water
	B: 0.1% formic acid in acetonitrile
Needle Wash:	A: Acetonitrile
	B: Water
Flow Rate:	1.00 mL/min
Polarity:	Negative

Isocratic:

Time, min	Solvent A, %	Solvent B, %
0.00	95	5
4.00	95	5

Compound		Q1 → Q3 *m/z*	Dwell Time (ms)	DP	EP	CE	CXP	Approximate Retention Time
Ethephon	Quan	143 → 107	100	-51	-10	-13	-10	2.6 minutes
	Qual	107 → 79	100	-80	-10	-15	-7	
d_4- Ethephon	IS	147 → 111	100	-51	-10	-13	-7	2.6 minutes

3.5.2 Materials

Volumetric flasks, various sizes
Graduated cylinders, various sizes
Graduated mixing cylinders, various sizes
Volumetric pipettes, various sizes
Mechanical pipettes, various sizes
20-mL glass vials
2-mL HPLC vials and caps
Phenomenex Aqua C18 column, 4.6 mm ×150 mm, 3 µm, serial number 666355-2

3.5.3 Chemicals

Acetonitrile, HPLC grade, lot numbers SHBD5036V, 53220, 83835, EMD
Water, HPLC grade, lot numbers 53246, 53270, EMD
Formic Acid, lot number K43371964, EMD

3.6 Calculations

Calculations were not modified from the original analytical method.

Using the calibration curve calculated by linear regression, the analyte concentration in the sample extracts in ng/mL was calculated using Equation 1:

$$\text{Concentration found (ng/mL)} = \frac{(y - b)}{m} \quad (1)$$

Where:

y = Ratio of native peak area/internal standard peak area
b = Intercept of the linear regression curve (area ratio)
m = Slope of the linear regression curve (response per ng/mL)

The percent recovery of the fortified samples was calculated using Equation 2:

$$\%\,\text{Recovery} = \frac{R}{T} \times 100 \quad (2)$$

Where:

R = ng/mL of target analyte found in fortified sample
T = theoretical ppb in fortified sample

As an example, the 10×LOQ quality control sample, Pyxant ID P2687B01-010 (Figure 9) resulted in an ethephon recovery of 100%. The calculations for this sample are demonstrated below as a representative example of how all the sample results were calculated for this study. Slight differences between the results obtained here from values displayed in the tables and those produced by the calculation software are due to rounding in the displayed table results versus use of the non-rounded values being carried through by the calculation software.

The linear regression analysis of the calibration curve used in the analysis of ethephon residues in samples from Trial 1 was determined to have the following regression coefficients: m = 0.19921 and b = -2.56E-04 (Figure 1). The analyte peak area ratio was 9.98E-001; therefore the concentration of ethephon in the final extract of this sample was calculated using Equation 1:

$$\text{Concentration found (ng/mL)} = \frac{(0.998 + 2.56E-04)}{0.19921} = 5.01\text{ng/mL} \quad (1)$$

The percent recovery of the sample was calculated using Equation 2:

$$\%\,\text{Recovery} = \frac{5.01\text{ng/mL}}{5.00\text{ng/mL}} \times 100 = 100\% \quad (2)$$

Bayer Method ET-001-W13-01

An Analytical Method for the Determination of Residues of Ethephon and its metabolite 2-HEPA in Water Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of Ethephon and its *metabolite 2-HEPA in water.*

Residues of ethephon and 2-HEPA are amended with an isotopic internal standard and analyzed by direct injection. The samples were analyzed for ethephon and 2-HEPA by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

The method limit of quantitation (LOQ) for ethephon and 2-HEPA is 0.5 ng/mL.

2.0 BACKGROUND

The analytical method presented in this report is designed to measure residues of ethephon and its metabolite 2-HEPA in water 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
- Phenomenex Aqua C18, 150 mm X 4.6 mm, 3 µm particle size, (Part No: 00F-4311-E0)
- ABSciex API 5500 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and a CTC PAL autosampler, and Analyst 1.6.1 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

(Functional equivalents may be substituted)

- Acetonitrile (ACN, Optima Grade, Fisher Part No. A996-4)
- Formic Acid 99% (Acros, Part no. 14793-0010)
- Water (Optima Grade; Fisher Part No. W7-4)
- 0.1% formic acid in water. Add 1 mL formic acid to 1000 mL water. Mix well.
- 0.1% formic acid in ACN. Add 1 mL formic acid to 1000 mL ACN. Mix well.
- 20 mL glass vial (Fisher Part No. 50-949-406)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)

5.0 PREPARATION OF STANDARD SOLUTIONS

Ethephon and 2-HEPA analytical standards and the isotopic internal standards ethephon-d_4 and 2-HEPA-d_4 are needed. These standards may be obtained from Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, North Carolina, 27709. 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 stock solutions should be stored in a freezer when not in use and fortification and calibration standard solutions should be stored in a refrigerator 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 ~100 µg/mL stock solutions of ethephon and 2-HEPA. Standards are typically provided in 9.0 to 12.0 mg aliquots. The standards are quantitatively transferred to a 100 mL volumetric flask using 0.1% formic acid in water and diluted to volume.

5.2 Fortification Standard Solutions

10 µg/mL mixed solution

Prepare a 10 µg/mL mixed solution by taking an appropriate volume (~10.0 mL) each of the ethephon primary stock solution and the 2-HEPA primary stock solution and diluting to 100 mL with 0.1% formic acid in water.

1 µg/mL mixed solution

Transfer 10 mL of the 10 µg/mL mixed standard solution into a 100 mL volumetric flask. Dilute to volume with 0.1% formic acid in water. Mix well.

0.1 µg/mL mixed solution

Transfer 10 mL of the 1 µg/mL mixed standard solution into a 100 mL volumetric flask. Dilute to volume with 0.1% formic acid in water. Mix well.

5.3 Isotopic Internal Standard Solutions

Prepare individual ~100 µg/mL stock solutions of ethephon-d_4 and 2-HEPA-d_4. Standards are typically provided in 2.0 to 5.0 mg aliquots. The standards are quantitatively transferred to a 50 mL volumetric flask using 0.1% formic acid in water and diluted to volume.

10 µg/mL mixed internal standard solution

Prepare a 10 µg/mL mixed internal standard solution by taking an appropriate volume (~10.0 mL) of the ethephon-d_4 and 2-HEPA-d_4 primary stock internal standard solutions and diluting to 100 mL with 0.1% formic acid in water.

1 µg/mL mixed internal standard solution

Transfer 10 mL of the 10 µg/mL mixed internal standard stock standard solution into a 100 mL volumetric flask. Dilute to volume with 0.1% formic acid in water. Mix well.

5.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.2, 0.8, 2, 5, 10, and 50 ng/mL diluted to 50 mL with 0.1% formic acid in water. Before bringing the calibration solutions to volume, add by pipet 0.5 mL of the 1 µg/mL mixed internal standard solution to each of the calibration solutions.

Concentration of Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg /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)
10	1	0.25	0.5	50	50	10
1	1	0.50	0.5	50	10	10
1	1	0.25	0.5	50	5	10
1	1	0.10	0.5	50	2	10
0.1	1	0.40	0.5	50	0.8	10
0.1	1	0.10	0.5	50	0.2	10

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

6.0 PROCEDURE

6.1 Sample extraction

Appendix 2 shows the analytical scheme for the analysis of ethephon and 2-HEPA in water. The detailed stepwise procedure is as follows:

1. Transfer 10 mL of water into a suitable stoppered container.
2. Add 0.050 mL of formic acid to each sample. (Samples must be acidified prior to fortification to ensure no degradation of compounds.)
3. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution. For fortifications at the LOQ add, by pipet, 0.050 mL of the 0.1 µg/mL mixed fortification solution.
4. Add, by pipet, 0.1 mL of the 1.0 µg/mL mixed internal standard solution. Stopper the container and shake well.
5. Transfer an aliquot to a 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 5500 instrument.

Isocratic
95% Mobile Phase A: 0.1% formic acid in water
5% Mobile Phase B: 0.1% formic acid in acetonitrile

HPLC column: Phenomenex Aqua C18, 150 mm X 4.6 mm, 3 µm particle size
Flow rate: 1.0 mL/min
Injection volume: 20 µL (Adjust for LC/MS/MS system being used)
Runtime: 4 minutes

Analyte	Approx Retention Time (min)
2-HEPA	1.8
Ethephon	2.7

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 a ABSciex API 5500 instrument.

Negative ion mode

CUR: Curtain Gas	30
CAD: Collision Gas	12
GS1: Ion Source Gas 1	60
GS2: Ion Source Gas 2	70
TEM: Source Temp.	600°C
IS: Ion Transfer Voltage	-4500

Positive ion mode

CUR: Curtain Gas	30
CAD: Collision Gas	12
GS1: Ion Source Gas 1	60
GS2: Ion Source Gas 2	70
TEM: Source Temp.	600°C
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. 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 5500 instrument used for this study. The following recommended ion transitions and conditions were example conditions used on an ABSciex API 5500 instrument:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CE	CXP
Ethephon	-	142.9	107.0	100	-51	-10	-12	-7
Ethephon IS	-	146.9	111.0	100	-51	-10	-12	-7
Ethephon Confirmatory	-	106.8	78.8	100	-51	-10	-12	-7
2-HEPA	-	124.9	94.9	100	-35	-10	-18	-7
2-HEPA IS	-	128.9	96.9	100	-35	-10	-18	-7
2-HEPA confirmatory	+	127.1	99.0	100	56	10	27	7
2-HEPA IS Confirmatory	+	131.1	99.0	100	56	10	27	7

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 Analyst software (version 1.6) 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/mL was determined using the following equation.

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

Analyst software was used to calculate the amount of ethephon and 2-HEPA in ng/mL 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:

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

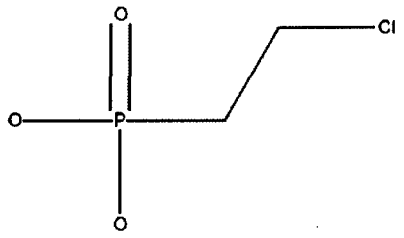
Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

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 0.5 ng/mL or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

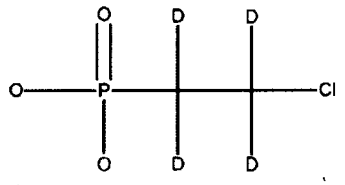
Appendix 1 Test and Reference Substances

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: Ethephon
Molecular Formula: $C_2 H_6 Cl O_3 P$
Molecular Weight: 144.5 g/mol

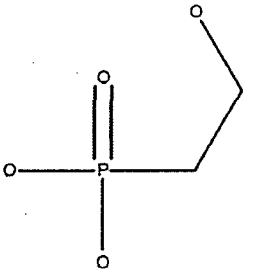


Code Name: Ethephon-d_4
Molecular Formula: $C_2 H_2 Cl D_4 O_3 P$
Molecular Weight: 148.5 g/mol

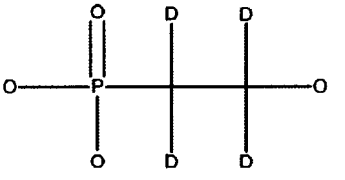


Appendix 1 · Test and Reference Substances (Cont'd)

Code Name: 2-HEPA
Molecular Formula: $C_2 H_7 O_4 P$
Molecular Weight: 126.0 g/mol



Code Name: 2-HEPA-d_4
Molecular Formula: $C_2 H_3 D_4 O_4 P$
Molecular Weight: 130.1 g/mol



Appendix 2 Extraction Scheme for Water Samples

Transfer 10 mL of water into a vial

↓

Add 0.050 mL of formic acid

↓

Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution

↓

Add 0.1 mL of the 1 µg/mL mixed internal standard solution

↓

Mix well

↓

Analyze by LC/MS/MS