II. OBJECTIVE

The purpose of this study was to perform an independent laboratory validation (ILV) of Bayers's Method RV-002-S10-02, entitled "An Analytical Method for the Determination of Residues of BYI 02960, 6-Chloronicotinic Acid (6-CNA) and Difluoroacetic Acid (DFA) in Soil and Sediment" (Appendix I), to satisfy guideline requirements described in the United States Environmental Protection Agency (US EPA) Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods.

III. INTRODUCTION

The EPA Guideline, OPPTS 850.7100, includes a requirement for registrants to validate analytical methods for the determination of residues in soil and sediment at an independent laboratory prior to submission to the EPA. This report details the results of the confirmatory trial of Bayers's Method RV-002-S10-02, (Appendix I), authored by D. Netzband, Bayer CropScience, Stilwell, Kansas. The study was conducted according to EN-CAS Protocol No. 11-0027 entitled "Independent Laboratory Validation (ILV) of Bayer Method RV-002-S10-02 for the Determination of BYI 02960 and its Two Acid Metabolites in Soil and Sediment", included as Appendix II to this report.

The independent validation trial was successful. As described in the protocol, the validation trial consisted of one analysis set for sandy loam soil. The set consisted of one reagent blank, two control samples not fortified with the analytes, and fortifications at both the limit of quantitation (LOQ, 5.0 ppb), and at 10X the LOQ (50 ppb). Each LOQ fortification sample was fortified with 1000 µL of the 100 ng/mL BYI 02960 combined fortification solution. Each 10X fortification sample was fortified with 500 µL of the 2000 ng/mL BYI 02960 combined fortification solution.

The study was initiated on January 17, 2012 when the Study Director signed EN-CAS Protocol # 11-0027. Analytical Standards were initially prepared per GLP guidelines on January 10, 2012, as allowed in the protocol. The experimental start date was January 23, 2012, and the experimental termination date was April 4, 2012.

IV. TEST SYSTEM

Control soil used in the validation study was received (ambient) on June 24, 2011 from AGVISE Laboratories, Inc., Northwood, North Dakota. The sample was assigned an EN-CAS ID number of ET5947. The sample was stored at room temperature. Sample log-in information can be found in the raw data packages associated with this study. Sample storage records are on file at EN-CAS Analytical Laboratories.

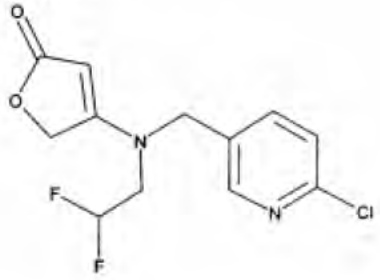
V. TEST AND REFERENCE MATERIALS

Analytical grade BYI 02960, 6-CNA, DFA, BYI 02960-[$^{13}C_5{}^{15}N$], 6-Chloronicotinic acid-2,4,5-d3-carboxyl-^{13}C and BYI 02960-difluoroacetate-$^{13}C_2$ (BCS-AB60481-$^{13}C_2$) standards were received at EN-CAS on December 21, 2011 from Bayer CropScience, Kansas City, MO and were used for preparation of stock, fortification, and calibration standards. The standards were received frozen and were stored under freezer conditions (approximately -10 °C). Characterization of the test/reference materials was performed by Bayer CropScience, Kansas City, MO, which retains the characterization data on file. The Certificates of Analysis of the test/reference materials can be found in Appendix IV.

The following information accompanied the test/reference materials upon receipt at EN-CAS.

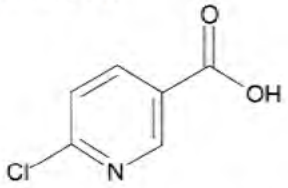
Standard Reference	EN-CAS Number	% Purity	Lot/Batch Number	Expiration Date	Physical Appearance
BYI 02960	ET7562	99.4	NLL 7780-47-4	10/26/13	White powder
6-CNA	ET7560	99.3	K1938	10/14/18	Light y powder
DFA	ET7564	95.8	K1913	9/20/12	Clear l
BYI 02960-[$^{13}C_5{}^{15}N$]	ET7561	99.7	K-1907	3/1/20	White powder
6-Chloronicotinic acid-2,4,5-d3-carboxyl-^{13}C	ET7565	99.5	K1227	8/12/13	Beige Powder
BYI 02960-difluoroacetate-$^{13}C_2$ (BCS-AB60481-$^{13}C_2$)	ET7563	95.0	KATH 15199-1-4	9/20/15	White powder

Report Name: BYI 02960
CAS Nomenclature: 2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]-
CAS Number: 951659-40-8
Molecular Formula: $C_{12}H_{11}ClF_2N_2O_2$
Molecular Weight: 288.6777
Chemical Structure:



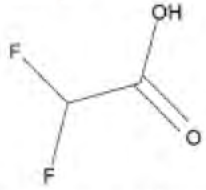
Report Name: 6-Chloronicotinic Acid (6-CNA)
CAS Nomenclature: 6-Chloro-3-pyridinecarboxylic acid
CAS Number: 5326-23-8
Molecular Formula: $C_6H_4ClNO_2$
Molecular Weight: 157.55446

Chemical Structure:

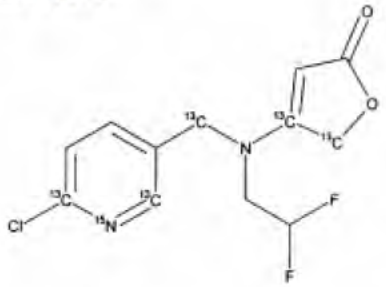


Report Name: Difluoroacetic acid (DFA)
CAS Nomenclature: Acetic acid, 2,2-difluoro-
CAS Number: 381-73-7
Molecular Formula: $C_2H_2F_2O_2$
Molecular Weight: 96.0329

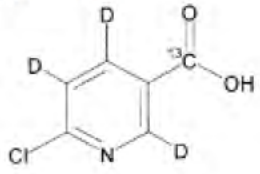
Chemical Structure:



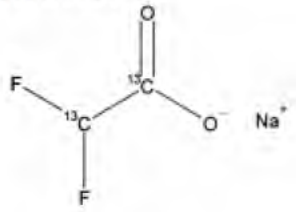
Report Name: BYI 02960-[$^{13}C_5{}^{15}N$]
CAS Nomenclature: NA
CAS Number: NA
Molecular Formula: $C_{12}H_{11}ClF_2N_2O_2$
Molecular Weight: 294.6175
Chemical Structure:



Report Name: 6-Chloronicotinic acid-2,4,5-d3-carboxyl-^{13}C
CAS Nomenclature: 6-Chloro-3-pyridine-2,4,5-d_3-carboxylic-^{13}C acid
CAS Number: NA
Molecular Formula: $C_6HClD_3NO_2$
Molecular Weight: 161.56224
Chemical Structure:



Report Name: BYI 02960-difluoroacetate-$^{13}C_2$ (BCS-AB60481-$^{13}C_2$)
CAS Nomenclature: NA
CAS Number: NA
Molecular Formula: $C_2HF_2O_2.Na$
Molecular Weight: 119.99331
Chemical Structure:



VI. DESCRIPTION OF ANALYTICAL METHOD

Bayers's Method RV-002-S10-02, entitled "An Analytical Method for the Determination of Residues of BYI 02960, 6-Chloronicotinic Acid (6-CNA) and Difluoroacetic Acid (DFA) in Soil and Sediment" (attached as Appendix I) was used for this study. The method is designed to determine individual residues of BYI 02960 and its metabolites, 6-CNA and DFA, in soil and sediment. Procedural recoveries are separately determined for BYI 02960, 6-CNA or DFA by fortifying control samples with a combined standard containing all three analytes.

BYI 02960, 6-CNA or DFA was extracted from a 20-g soil sample by adding approximately 50 mL of 70:30 (v/v) ACN: H_2O and a magnetic stirrer to the 125-mL French square bottle. The lid was loosely attached and the sample loaded onto the microwave carousel. The fiber optic temperature control probe and Teflon® sleeve were inserted into one of the control samples. The microwave was programmed as listed in the method (Appendix I). The sample was allowed to cool before removing from the microwave and 0.5 mL of the 500 ng/mL internal standard solution was added. The lid was reattached and the sample was shaken, by hand, to mix well. Approximtely 0.75 mL of the supernatant was transferred into a LC vial and diluted with approximately 0.75 mL of 50:50 (v/v) ACN:200mM ammonium formate in H_2O (adjusted to pH 3.2). The sample was then analyzed by LC/MS/MS.

Optional Sample Clean-up

NOTE: The clean up is included after the sample has been removed from the microwave and mixed well.

A Varian Bond-Elut 500-mg/6-mL C18 cartridge was conditioned with approximately 3 mL of ACN followed by approximately 3 mL of H_2O. Approximately 3 mL of the sample extract was loaded onto the cartridge, passed

through the cartridge using slight vacuum, and the eluate was collected in a 15- mL graduated tube. The sample tube was rinsed with 5 mL of H_2O, applied to the cartridge, and the eluate collected into the same 15-mL graduated tube. The volume was recorded and the sample was mixed by vortexing. Approximately 0.75 mL of the supernatant was transferred into a LC vial and diluted with approximately 0.75 mL of 50:50 (v/v) ACN:200mM ammonium formate in H_2O (adjusted to pH 3.2). The sample was then analyzed by LC/MS/MS.

DFA Confirmatory Method Clean-up

NOTE: Be sure that the conditioning of the cationic resin has been completed before continuing with the method (see following section).

One gram of cationic resin AG-50W-X8, 2.5 mL of ACN and 2.5 mL of the extract sample was added to a 15-mL plastic centrifuge tube. The tube was capped and the sample was placed on a mechanical shaker for approximately 15 minutes and then centrifuged for approximately 5 minutes at 2500 rpm. An aliquot of the supernatant was transferred to an LC vial for LC/MS/MS analysis.

Conditioning of Cationic Resin

The cationic resin is conditioned by adding 25 g of AG-50W-X8 resin into a 250-mL polypropylene bottle, adding 200 mL of D.I. H_2O and shaking, on a mechanic shaker, for 15 minutes. The resin is allowed to settle (approximately 5 to 10 minutes) and the supernatant is decanted and discarded. Washing the resin with 200 mL of H_2O, mixing, allowing to settle and decanting and discarding the supernatant is repeated two more times. The conditioned resin is vacuum filtered through a GFA filter inside a Buchner funnel, attached to a 500-mL side-arm flask, capped and stored in a 250-mL polypropylene bottle.

The following minor adjustments were made to the method.

1. The amounts used in the preparation of the cationic resin were scaled down, but the proportions were maintained (25 grams of resin instead of 100 grams, 200 mL of D.I. H_2O instead of 800 mL).

2. The cationic resin was stored at room temperature, instead of refrigerated. However, it was used on the same day that it was prepared, and not used thereafter.

3. LC/MS/MS conditions were adapted to EN-CAS equipment. On the Zic-Hilic column, separate injections were performed for the BYI 02960 analyte, instead of switching from positive to negative mode as described in the method.

VII. EXPERIMENTAL DESIGN

A. Establish Method Chromatography and Performance Criteria

1. Preliminary Method Setup

Prior to performing the ILV, EN-CAS determined approximate analyte retention times and instrument detection limits for BYI 02960 and its metabolites. Linear calibration curves were established by injecting standards at five levels ranging from 0.30 µg/mL to 40 µg/mL.

2. Preparation of Stock Standard Solutions

Individual stock standard solutions of BYI 02960 (99.994 µg/mL), 6-CNA (100.04 µg/mL) and DFA (144.179 µg/mL), were prepared in ACN on 1/10/12 (notebook reference PWB # 641/8).

Individual stock standard solutions of BYI 02960-[$^{13}C_5{}^{15}N$] (50 µg/mL), 6-Chloronicotinic acid-2,4,5-d3-carboxyl-^{13}C (49.85 µg/mL) and BCS-AB60481-$^{13}C_2$ (49.21 µg/mL) were prepared in ACN on 1/10/12 (notebook reference PWB # 641/8) and were used for the isotopic internal standard.

3. Preparation of Fortification Standard Solutions

Aliquots of the stocks prepared on 1/10/12 and diluted with 50:50 ACN:H_2O to prepare a 2000 ng/mL fortification solution. The 2000 ng/mL fortification solution was used to prepare a 100 ng/mL fortification solution.

4. Preparation of Stock and Calibration Standard Solutions

Aliquots of the combined 2000ng/mL fortification standard prepared on 1/10/12 were diluted with 60:40 ACN:125nM ammonium formate to generate a series of calibration standards containing 0.0, 0.30, 0.50, 1.0, 2.0, 4.0, 10, 20 and 40 ng/mL, respectively.

Aliquots of the stocks prepared on 1/10/12 for the isotopic internal standard were diluted with 50:50 ACN:H_2O to prepare a 5.0 ng/mL isotopic internal standard solution. The 500 ng/mL isotopic internal standard solution was diluted with 60:40 ACN:125mM ammonium formate to prepare a 2.5 ng/mL isotopic internal standard solution.

All solutions were stored in a refrigerator at 1 °C to 3 °C. All standard solutions were stored in amber bottles. Further information regarding the

preparation of fortification standards and LC calibration standards is located in the file for EN-CAS Study No. 11-0027.

5. Calibration Curve

Standards were injected at the beginning, at the end, and interspersed throughout each run at the following levels: 0.0, 0.30, 0.50, 1.0, 2.0, 4.0, 10, 20 and 40 ng/mL. The calibration curve used was a linear regression curve, $y = mx + b$, where m is the slope and b is the y-intercept. 1/x weighting was used to produce the calibration curve. Example calibration curves are shown in Figures 9, 22, 35, 60, 73 and 86.

6. Chromatography

Example chromatograms of standards, controls, and fortified samples are shown in Figures 1 – 90 for soil.

7 Description of Instrument and Operating Conditions

For all sample analyses, a PE Sciex API 4000 Tandem Mass Spectrometer with a MS detector tandem mode and an Agilent 1100 WPALS Autosampler was used. Detailed operating conditions are listed below:

HPLC Conditions (For Samples With and Without C18 Cleanup)

Column:	Zic-Hilic 4.6 x 150 mm, 5 µm particle size.; ID 265; S/N 146170
Injector:	Agilent: Autosampler 1100 WPALS Pump 1100 QuatPump
Mobile Phase:	Sol 1: ACN Sol 2: 0.1% Acetic Acid
Oven:	FIAtron CH50/CH30 @ 30°C*
Flow Rate:	200 µL/min
Injection Volume:	40 µL (BYI 02960) 1 µL (6-CNA and DFA)

* For the 6-CNA and DFA, ambient temperature was used; no column oven.

Retention Time: BYI 02960 = 4.2 min
6-CNA = 4.5 min
DFA = 5.2 min

Run Time: BYI 02960 = 13 min
6-CNA = 17 min
DFA = 17 min

Standard/Sample Solvent: 60:40 ACN:125mM Ammonium formate (pH 3.2)

Mass Spectrometer Conditions for BYI 02960

LC/MS Instrument: AB-Sciex API4000 Tandem Mass Spectrometer

API Source: Turbo Ion Spray V/L 11.0/4.55 550°C

MS Mode: Tandem (MS/MS) Positive

MS Parameters: CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
12/15/45/45/5500/80/10/28/24

Mass Calibration: Based on PPG masses; 59, 175.133, 616.464, 906.673, 1254.925, 1545.134, 2010.469, 2242.637

Masses Monitored: BYI 02960 = 289 → 126 BYI 02960 IS = 295 → 130

Dwell Time: 50 ms

Gradient Table:

Step	Time	Flow	Sol. 1	Sol. 2
0	0.00	400	5	95
1	0.01	400	5	95
2	4.00	400	25	75
3	4.10	700	25	75
4	4.20	700	90	10
5	6.50	400	90	10
6	8.60	400	90	10
7	9.00	400	5	95
8	13.00	400	5	95

Mass Spectrometer Conditions for 6-CNA and DFA

LC/MS Instrument: AB-Sciex API4000 Tandem Mass Spectrometer

API Source: Turbo Ion Spray V/L 11.0/4.55 550°C

MS Mode: Tandem (MS/MS) Negative

MS Parameters:
6-CNA = CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
10/32/45/45/-4500/-32/-5/-16/-5.5
DFA = CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
10/32/45/45/-4500/-32/-5/-16/-7.5

Mass Calibration: Based on PPG masses; 59, 175.133, 616.464, 906.673, 1254.925, 1545.134, 2010.469, 2242.637

Masses Monitored:
6-CNA = 156 → 112 6-CNA IS = 159 → 114
DFA = 95 → 51 DFA IS = 97 → 52

Dwell Time:
6-CNA = 100 ms
DFA = 300 ms

Gradient Table:

Step	Time	Flow	Sol. 1	Sol. 2
0	0.00	400	5	95
1	0.01	400	5	95
2	4.00	400	25	75
3	4.10	700	25	75
4	4.20	700	90	10
5	6.50	400	90	10
6	8.60	400	90	10
7	9.00	400	5	95
8	17.00	400	5	95

HPLC Conditions (BYI 02960 and 6-CNA Confirmatory)

Column: Gemini 2 x 150 mm,
5 µm particle size.; ID 263; S/N 603547-8

Injector: Agilent: Autosampler 1100 WPALS
Pump 1100 QuatPump

Mobile Phase: Sol 1: 0.1% Formic Acid in ACN
Sol 2: 0.1% Formic Acid in H_2O

Oven: FIAtron CH50/CH30 @ 30°C

Flow Rate: 200 µL/min

Injection Volume: 10 µL

Retention Time: BYI 02960 = 3.5 min
6-CNA = 3.2 min

Run Time: BYI 02960 = 11 min
6-CNA = 11 min

Standard/Sample Solvent: 60:40 ACN:125mM Ammonium formate (pH 3.2)

Mass Spectrometer Conditions (BYI 02960 Confirmatory)

LC/MS Instrument: AB-Sciex API4000 Tandem Mass Spectrometer

API Source: Turbo Ion Spray V/L 11.0/4.55 550°C

MS Mode: Tandem (MS/MS) Positive

MS Parameters: CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
12/15/45/45/5500/80/10/28/24

Mass Calibration: Based on PPG masses; 59, 175.133, 616.464, 906.673, 1254.925, 1545.134, 2010.469, 2242.637

Masses Monitored: BYI 02960 = 289 → 126 BYI 02960 IS = 295 → 130

Dwell Time: 50 ms

Gradient Table:

Step	Time	Flow	Sol. 1	Sol. 2
0	0.00	750	90	10
1	0.50	750	90	10
2	3.00	750	40	60
3	6.50	750	5	95
4	7.00	750	90	10
5	11.00	750	90	10

HPLC Conditions (DFA Confirmatory)

Column: Restek Allure 4.6 x 150 mm,
5 µm particle size.; ID 264; S/N 12010639M

Injector: Agilent: Autosampler 1100 WPALS
Pump 1100 QuatPump

Mobile Phase: Sol 1: 0.5% Formic Acid in ACN
Sol 2: 0.5% Formic Acid in H_2O

Oven: FIAtron CH50/CH30 @ 30°C

Flow Rate: 200 µL/min

Injection Volume: 10 µL

Retention Time: 2.3 min

Run Time: 12 min

Standard/Sample Solvent: 60:40 ACN:125mM Ammonium formate (pH 3.2)

Mass Spectrometer Conditions (6-CNA and DFA Confirmatory)

LC/MS Instrument: AB-Sciex API4000 Tandem Mass Spectrometer

API Source: Turbo Ion Spray V/L 11.0/4.55 550°C

MS Mode: Tandem (MS/MS) Negative

MS Parameters: 6-CNA = CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
10/32/45/45/-4500/-32/-5/-16/-5.5
DFA = CAD/CUR/GS1/GS2/IS/DP/EP/CE/CXP
10/32/45/45/-4500/-32/-5/-19/-7.5

Mass Calibration: Based on PPG masses; 59, 175.133, 616.464, 906.673, 1254.925, 1545.134, 2010.469, 2242.637

Masses Monitored: 6-CNA = 156 → 112 6-CNA IS = 159 → 114
DFA = 95 → 51 DFA IS = 97 → 52

Dwell Time: 6-CNA = 250 ms
DFA = 100 ms

Gradient Table:

6-CNA

Step	Time	Flow	Sol. 1	Sol. 2
0	0.00	750	90	10
1	0.50	750	90	10
2	3.00	750	40	60
3	6.50	750	5	95
4	7.00	750	90	10
5	11.00	750	90	10

DFA

Step	Time	Flow	Sol. 1	Sol. 2
0	0.00	500	1	99
1	1.00	500	1	99
2	2.20	1000	1	99
3	3.00	1000	80	20
4	3.01	500	80	20
5	5.00	500	80	20
6	5.20	500	1	99
7	12.00	500	1	99

B. Quantitation and Example Calculation

Standards were injected at the beginning and end of the run to generate linear regression calibration curves for BYI 02960, 6-CNA and DFA. The calibration curves were produced using 1/x weighting. Quantitation of the amount of BYI 02960, 6-CNA or DFA found in an unknown sample was accomplished by inserting the analyte peak area into the appropriate linear regression equation. From the injected concentration, the residue concentration and percent recovery were calculated for each fortified control sample. Average percent recovery, standard deviation, 95% confidence interval and coefficients of variation (CV) were calculated for the analyte at each fortification level. The residue concentration was determined from the following equations:

1. Calculation of Linear Equation

$$Y = \frac{\text{Sample peak area}}{\text{Internal standard peak area}}$$

Y = Native peak area: isotopic peak area ratio

2. Calculation of Dilution Factor

$$\text{Dilution factor (DF)} = \frac{EV}{G} \times \frac{FV}{AV}$$

G = grams of sample initially extracted (20 g)
EV = Extraction volume (50 mL)
DF = Dilution factor
FV = Final volume (1.5 mL)
AV= Aliquot volume (0.75 mL)

3. Calculation of ppb Found

$$\text{ppb Found} = \frac{(Y-B) \times DF}{M}$$

M = Slope
B = y intercept

NOTE: The curve is generated using 1/x weighting.

4. Calculation of Percent Recovery in Fortification Samples

Where:

$$\%\ \text{Recovery} = \frac{R-S}{FL} \times 100$$

R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
FL = Fortification Level (5.0 ppb or 50 ppb)

5. Example Calculation for a Procedural Recovery Sample

For ET5947-S2 (Low-level BYI 02960 procedural recovery from Set 1-01-AN, fortified at 5.0 ppb) (see Figure 12)

Where:

$$\text{Dilution factor (DF)} = \frac{50\ \text{mL}}{20\ \text{g}} \times \frac{1.5\ \text{mL}}{0.75\ \text{mL}} = 5\ \text{mL/g}$$

$$Y = \frac{2766}{9754} = 0.2836$$

$$\text{ppb Found} = \frac{0.9993 - (-0.00208) \times 5\ \text{mL/g}}{0.281} = 5.08\ \text{ppb}$$

$$\%\ \text{Recovery} = \frac{5.08 - 0.09615}{5.0\ \text{ppb}} \times 100 = 99.7\%$$

VIII. METHOD OBSERVATIONS

A. Problems Encountered

The trial initally showed considerable peak splitting for DFA on the Zic-Hilic column. Therefore, the HPLC injection volume was reduced to minimize peak splitting, also a fresh Zic-Hilic column was used.

B. Critical Steps

There are no steps that must be followed exactly as detailed in the method in order to obtain adequate recoveries.

C. Matrix or Solvent Effects

No problems were detected with matrix or solvent effects.

D. Signal Enhancement or Suppression

No problems were detected.

E. Stability of Solutions

Sample solutions were injected as much as two months after sample preparation for 6-CNA and DFA. Acceptable recoveries seem to indicate good stability of sample solutions for at least that amount of time.

IX. RECOMMENDED CHANGES TO METHOD

Listing the LC/MS column temperatures, even if ambient, would be useful.

X. CONCLUSIONS

The Bayer Method RV-002-S10-02, entitled "An Analytical Method for the Determination of Residues of BYI 02960, 6-Chloronicotinic Acid (6-CNA) and Difluoroacetic Acid (DFA) in Soil and Sediment" was successfully validated in sandy loam soil.

XI. TIME REQUIREMENTS

A set of 13 samples can be prepared in approximately 16 hours and analyzed overnight by LC/MS/MS. Additional time is needed for calculation of results.

XII. CONTACTS WITH SPONSOR

There were several e-mail contacts between the Sponsor and EN-CAS, mainly concerning the test substances and protocol comments. The e-mails are available in the project file.

XIII. CIRCUMSTANCES THAT MAY HAVE AFFECTED THE DATA

No circumstances occurred that might have affected the integrity of raw data for the study.