1. BACKGROUND

The fungicide BYF 14182 is currently being developed by Bayer CropScience.

An analytical method was developed for the analysis of BYF 14182 and its associated metabolites in water and the method was validated in Bayer CropScience Study Number RAELP013[1].

The structures for these compounds are presented in Section 3. This analytical method was prepared based on the results obtained in the validation study.

Typical recovery results are presented in Appendix 3, and the data shown was obtained from the method validation study.

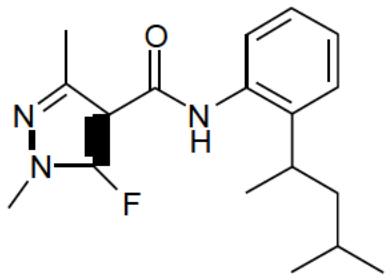
2. PRINCIPLE

The residues of BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP in water are determined by direct injection onto the LC/MS/MS system. Quantification is based on a comparison of peak areas with those of known standards.

The LOQ of the method is 0.1ng/mL (ppb) for BYF 14182 and its metabolites.

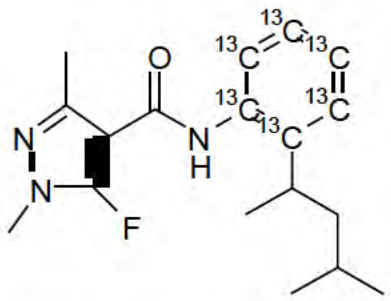
3. COMPOUNDS

Code Name: **BYF 14182**
(Parent Molecule)



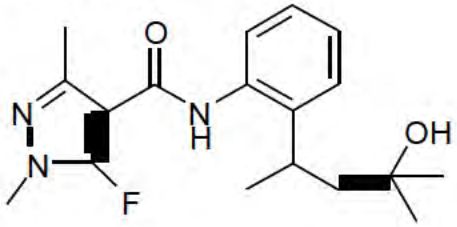
CAS Name: N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula: $C_{18}H_{24}FN_3O$
Molecular Weight: 317 g/mol

Code Name: **BYF 14182 [phenyl-$^{13}C_6$]**
(Parent Molecule, Isotopic Internal Standard)



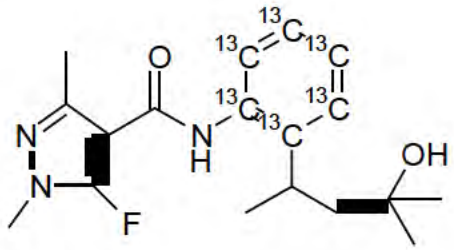
CAS Name: N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula: $^{13}C_6C_{12}H_{24}FN_3O$
Molecular Weight: 323 g/mol

Code Name: **BYF14182-3-hydroxy-butyl**
(Metabolite)



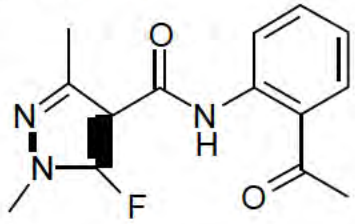
CAS Name: 5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula: $C_{18}H_{24}FN_3O_2$
Molecular Weight: 333 g/mol

Code Name: **BYF14182-3-hydroxy-butyl-$^{13}C_6$**
BCS-AA10006 [phenyl-$^{13}C_6$] BCS-AA10006
(Metabolite, Isotopic Internal Standard)



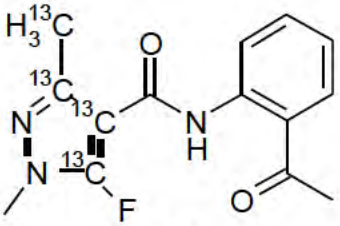
CAS Name: 5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula: $^{13}C_6C_{12}H_{24}FN_3O_2$
Molecular Weight: 339 g/mol

Code Name: **BYF14182-pyrazolyl-AAP**
(Metabolite)



CAS Name: N- (2-acetylphenyl)-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula: $C_{14}H_{14}FN_3O_2$
Molecular Weight: 275 g/mol

Code Name: **BYF14182-pyrazolyl-AAP-$^{13}C_4$**
[3-methyl-^{13}C,pyrazolyl-$^{13}C_3$] BCS-AF73126
(Metabolite, Isotopic Internal Standard)



Molecular Formula: $^{13}C_4C_{10}H_{14}FN_3O_2$
Molecular Weight: 279 g/mol

4. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Eppendorf Reference Series 2000 pipettes (Cat. No.: 05-402-48 and 05-402-50)
- VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex® Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
- National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
- National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
- Acrodisc 0.45µm 13mm syringe filter, Pall Life Sciences, Part No. 4426T
- YMC: Pro C18, 120A, 3µm, 33 x 4.0mm i.d.
- Applied Biosystems PE Sciex 4000 LC/MS/MS System with Analyst Software Version 1.4.1 or higher installed
- Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10A VP Controller
- CTC PAL autosampler
- Perkin Elmer Series 200 column oven
- Fisherbrand 30mL glass jars (Cat. No. 02-911-465)

5. REAGENTS

Use as a guide; equivalents or different manufactures (brands) may be substituted.

- Acetonitrile, Fisher Scientifc Optima, , (Cat. No. A996-4)
- Deionized Water filtered through a Milli-Q water system or Water, Fisher Scientific Optima, (Cat. No.: W7-4)
- Acetic Acid, Guaranteed Reagent, (VRW Cat. No.: EM-AX0073-14)
- Sodium Thiosulfate, Mallinckrodt, (Cat. No. 8100)
- Sodium hypochlorite 13%, Acros, (Cat. No. 219250025)
- Certified analytical reference standards of BYF14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP
- Certified internal standards of BYF 14182 [phenyl-$^{13}C_6$], BYF 14182-3-hydroxybutyl-$^{13}C_6$ (BCS-AA10006-[phenyl$^{13}C_6$], and BYF 14182-pyrazolyl-AAP-$^{13}C_4$ ([3-methyl-^{13}C,pyrazole-$^{13}C_3$] BCS-AF73126)
- Solution of 10ppm sodium thiosulfate: Weigh approximately 100mg of sodium thiosulfate into a 100mL volumetric flask. Dissolve the amount in approximately 50mL of HPLC grade water and make up the volume to the 100mL mark. Mix thoroughly by inverting the flask several times. This solution is 1mg/mL or 1000ppm. Transferring 1 mL of this solution to a 100mL water sample will produce 10ppm concentration of sodium thiosulfate in that sample. Transfer the sodium thiosulfate solution to 100 mL amber bottle and store refrigerated at $\leq$10ºC.
- Solution of HPLC grade water chlorinated with sodium hypochlorite (NaOCl):
  Pipet 128µL of NaOCl (13% chlorine, density 1.209g/mL) into a 100mL volumetric flask. Fill to volume with deionized or HPLC grade water. The resulting free chlorine concentration is 200µg/mL. To simulate a chlorinated finished drinking water add an appropriate amount of this solution to a water sample. For example, add 100 µL of the 200µg/mL free chlorine solution to a 10mL deionized or HPLC grade water sample. The

resulting level of free chlorine is 2µg/mL(ppm). Chlorine is volatile, so this solution should be stored tightly sealed, in the dark under refrigeration at ≤10ºC and should be remade if more than three weeks old.

6. PREPARATION OF ANALYTICAL STANDARDS

NOTE: The following procedure is an example description of how standard solutions may be prepared. Standards may be prepared as mixed solutions by dilution from individual stock solutions or prepared individually. 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.

6.1 Primary Stock Standard Solutions

Prepare individual stock solutions of approximately 100µg/mL BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in 9.0 to 12.0 mg aliquots. The standards are quantitatively transferred to a volumetric flask using acetonitrile. After correction for purity, an appropriate volume of the initial stock solution is used to prepare the primary standard solutions.

Prepare a mixed stock 1.0µg/mL solution containing a mixture of BYF 14182 and its metabolites by taking an appropriate volume of the initial stock solution and diluting to 100mL with acetonitrile.

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

6.2 Fortification Standard Solutions

Prepare a 0.025µg/mL fortification solution containing a mixture of BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP by taking an 2.5mL aliquot of the 1µg/mL standard solution and diluting to 100mL with acetonitrile.

Further dilutions of this mixed fortification solution may be made as needed.

6.3 Isotopic Internal Standard Solutions

Prepare individual stock solutions of approximately 50µg/mL of BYF 14182 [phenyl-$^{13}C_6$], BYF 14182-3-hydroxybutyl-$^{13}C_6$, and BYF 14182-pyrazolyl-AAP-$^{13}C_4$. A similar procedure is followed for preparing internal standards (IS) as preparing primary stock standards. However, these standards are available in limited quantities, so the amount weighed is typically 1.5 to 3.0 mg. The standards are quantitatively transferred to a volumetric flask using acetonitrile.

Prepare a working internal standard solution of 500ng/mL of BYF 14182 [phenyl-$^{13}C_6$], BYF 14182-3-hydroxybutyl-$^{13}C_6$, and BYF 14182-pyrazolyl-AAP-$^{13}C_4$. by taking 1.0 mL of the 50µg/mL stock solution and diluting to 100mL in acetonitrile.

Further dilutions of this mixed fortification solution may be made as needed.

6.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.0, 0.05, 0.1, 0.25, 0.5, 1 and 2.0ng/mL of BYF 14182 and its metabolites diluted to 100mL with 10:90 v/v acetonitrile:deionized water and 1% acetic acid. Before bringing the calibration solutions to volume, add by pipet 0.2mL of the 0.5µg/mL internal standard solution prepared in acetonitrile to each of the calibration solutions. (see Section 6.3 Isotopic Internal Standard Solutions)

Further calibration solutions may be prepared as needed.

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)
100	500	2.0	0.2	100	2.0
100	500	1.0	0.2	100	1.0
100	500	0.5	0.2	100	0.5
100	500	0.25	0.2	100	0.25
10	500	1	0.2	100	0.1
10	500	0.5	0.2	100	0.05
10	500	0.0	0.2	100	0

7. ANALYSIS OF FINISHED DRINKING WATERS (TAP WATERS) CONTAINING FREE CHLORINE

BYF 14182-pyrazolyl-AAP degrades in water containing free chlorine. In order to accurately detect these residues when present in chlorine treated water, these residues would have to be stabilized at the time of sampling the water. Stabilization of residues for BYF 14182-pyrazolyl-AAP can be achieved by adding sodium thiosulfate to the finished water sample at the time of collection. Sodium thiosulfate added to the water sample at 10ppm concentration is sufficient to remove 2ppm of chlorine and stabilize residues of BYF 14182-pyrazolyl-AAP. For example, sample bottles that are used for collecting 100mL samples of treated water should contain 1mL of a 1000ppm solution of sodium thiosulfate. Addition of the sodium thiosulfate to the sample bottles may be performed in the lab prior to transport to the water collection sites to prevent any potential contamination of the bottles in the field. The samples so treated are then analyzed as per the method for non-free chlorine containing waters as described above.

Tap water or HPLC water chlorinated in the lab may be used for a finished drinking water method recovery sample. Appropriate amounts, for example, 100µL of the 1000ppm solution of sodium thiosulfate and 10mL of water, should be used, with the thiosulfate being added *before* the water is spiked with a known amount of a fortification solution to give the desired level of fortification. See Section 5 above for preparing free chlorine and thiosulfate solutions.

8. EXTRACTION

NOTE: This method uses internal standards to determine the concentrations of BYF 14182 and its metabolites present in water. If the concentrations of these components are outside the range of the appropriate calibration curve the analyses will have to be repeated using a reduced sample volume. If a further dilution is made to the final extract, adjust the concentration of internal standard added in step 8.3 so that the final concentration of internal standard present in the final sample is 1ng/mL.

1 Transfer a 25 ± 1mL water sample by graduated cylinder into a 30mL glass jar.

2 Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile (see Section 6.2 Fortification Stock Solutions).

3 Add by pipet 0.05mL of the 0.50ppm internal standard solution prepared in acetonitrile. (see Section 6.3 Internal Standard Solutions).

4 Add by pipet, 250µL of acetic acid. Cap and shake the glass jar.

5 Transfer an aliquot from the jar into an LC vial and cap to await analysis by LC/MS/MS. If necessary, filter the sample using an Acrodisc® 0.45µm syringe filter.

9. ANALYSIS

9.1 Sample Analysis

BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP are analyzed by LC/MS/MS using isotopic internal standards.

Inject an 80 µL aliquot of each test sample (or fortified sample matrix) from step 5 in Section 8 onto the LC/MS/MS under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

9.2 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix I by injecting an aliquot of each LC/MS/MS calibration solution both before and after the sample solutions.

BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP residues are quantified using internal standard linear regression analysis. A separate calibration curve is produced for each set of samples analyzed on the LC/MS/MS. A calibration curve is generated by 1/x weighted linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL using Applied Biosystems Analyst Software (Version 1.4.1), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set.

The standards were fit to the linear equation: $Y = MX + B$

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

The equation shown below is for the calculation of BYF 14182 residues.

After regression coefficients were calculated, the residue in parts per billion was determined. The ng/mL (or ppb) of BYF 14182 in the water was calculated using the following equation,

$$\text{BYF 14182 (ng/mL)} = \frac{(Y - B)}{M}$$

Analyst software was used to calculate the amount of BYF 14182 in ng/mL (or ppb) for each sample and the percent recovery for the fortified samples.

9.3 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 & 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 0.1ng/mL in water or other appropriate level with fortification solutions. Calculate the final residue R for the control (S) and fortified control (R) samples.

10. DISCUSSION

10.1 Method Validation

The method validation has been performed and reported in Bayer CropScience Study RAELP013[1]. The results for both the primary and confirmatory ions are summarized in Appendix 3.

10.2 Independent Laboratory Validation (ILV)

An ILV has been successfully performed on this method. The validation results are summarized in Table 2 of this report

10.3 Time Considerations

A set of fourteen samples can be prepared for analysis in 2-3 hours, analyzed overnight and the data processed the following working day.

11. REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1	RAELP013		**Wade, J.M.**, In House Validation of the Analytical Method for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP In Water Using LC/MS/MS, 2008
2	EL-001-W08-01		**Wade, J.M.,** An Analytical Method for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP In Water Using LC/MS/MS
3	P 614 087006	MR-09/119	**Krebber, R, Marcel, H.,** Independent laboratory validation of method EL-001-W08-01 for the determination of residues of BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP In water Using LC/MS/MS

Table 1 Analytical Method Summary Parameters (DER Table B.1.1)

Summary Parameters for the Analytical Method Used for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP In Water	
Method ID	EL-001-W08-02
Analyte(s)	BYF 14182, BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP
Extraction solvent / Technique	Direct inject
Cleanup Strategies	none
Instrument Detector Column	- Two Shimadzu LC-10AD VP HPLC pumps with a Shimadzu SCL-10 controller and CTC PAL autosampler - Perkin Elmer Series 200 column oven - Applied Biosystems API 4000 MS/MS - YMC: Pro C18, 120A, 3µm, 33 x 4.0mm i.d
Standardization Method	Multi point calibration curve (Internal standard)
Stability of Standard Solutions	Stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤-18°C Fortification and calibration standard solutions are stable for a minimum of 1 month when stored in the dark at ≤4°C
Retention times	BYF 14182 (~2.5 minutes) BYF 14182-3-hydroxy-butyl (~1.3 minutes) BYF 14182-pyrazolyl-AAP (~1.5 minutes)

Table 2 Characteristics for the Analytical Method (DER Table C.1.2)

Characteristics for the the Analytical Method Used for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP In Water	
Analyte(s)	BYF 14182, BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP
Equipment ID	Two Shimadzu LC-10AD VP HPLC pumps with a Shimadzu SCL-10 controller and CTC PAL autosampler oven and ms/ms
Limit of quantitation (LOQ)	0.1ng/mL
MDL - Method Detection Limit (ng/mL)[a]	0.02ng/mL for all analytes
Reliability of the method (ILV) [b]	BYF 14182 Quantitation MRM: 103 ± 4.1 BYF 14182 Confirmatory MRM: 103 ± 4.3 BYF 14182-3-hydroxy-butyl Quantitation MRM: 109 ± 4.5 BYF 14182-3-hydroxy-butyl Confirmatory MRM: 106 ± 5.5 BYF 14182-pyrazolyl-AAP Quantitation MRM: 105 ± 1.8 BYF 14182-pyrazolyl-AAP Confirmatory MRM: 102 ± 3.4
	Detector response was linear within the range of 0 – 2.0ng/mL for all analytes.
Specificity	The analytical method employs a highly specific and selective detector (LC/MS/MS). The control chromatograms generally have no peaks above the chromatographic background. Peaks were well defined and symmetrical.

[a] Data obtained from Study RAELP013[1]
[b] Data obtained from Study MR-09/119[3]

Appendix 1 Instrument Conditions For BYF 14182 and its metabolites

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

NOTE: As the LC/MS/MS system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually affected over time. Therefore, the given LC/MS/MS parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.

Acquisition Parameters

Instrument Used:	Perkin Elmer Sciex API 4000 LC/MS/MS System
Interface:	PE Sciex Turbo Ion Spray Electrospray
Synchronization Mode:	LC Sync
AutoEquilibration:	Off
Acquisition Duration:	4 min. 02 sec.
Periods in File:	2
Acquisition Module:	Acquisition Method
Software Version:	Analyst 1.4.1

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Low
Intensity Thres.:	0.00 cps
Settling Time:	000.0000 msec
MR Pause:	5.0070 msec
MCA:	No

Appendix I (continued)

Analyte (~1.3 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182-3-hydroxy-butyl	334.2	141	75	DP	50	50
				EP	6	6
				CE	30	30
				CXP	10	10

Analyte (~1.3 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF14182-3-hydroxy-butyl (Confirmatory)	334.2	316	75	DP	21	21
				EP	6	6
				CE	13	13
				CXP	8	8

Analyte (~1.3 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182-3-hydroxy-butyl Internal Standard	340.2	152	75	DP	50	50
				EP	6	6
				CE	30	30
				CXP	10	10

Analyte (~1.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182-pyrazolyl-AAP	276.2	140.9	75	DP	46	46
				EP	10	10
				CE	23	23
				CXP	8	8

Analyte (~1.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182-pyrazolyl-AAP (Confirmatory)	276.2	84.0	200	DP	46	46
				EP	10	10
				CE	55	55
				CXP	6	6

Analyte (~1.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182-pyrazolyl-AAP Internal Standard	280.2	144.9	75	DP	46	46
				EP	10	10
				CE	23	23
				CXP	8	8

Appendix I (continued)

Parameter Table	CUR:	35
	GS1:	40
	GS2:	15
	IS:	5500
	TEM:	750
	CAD:	12

Period 2 Experiment 1:	Scan Type:	MRM (MRM)
	Polarity:	Positive
	Scan Mode:	N/A
	Ion Source:	Turbo Spray
	Resolution Q1:	Unit
	Resolution Q3:	High
	Intensity Thres.:	0.00 cps
	Settling Time:	000.0000 msec
	MR Pause:	5.0070 msec
	MCA:	No
	Step Size:	0.00 amu
	Scan Type:	MRM (MRM)

Analyte (~2.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Parameter	Start	Stop
BYF 14182	318.2	141	75	DP	50	50
				EP	14	14
				CE	43	43
				CXP	45	45

Analyte (~2.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Parameter	Start	Stop
BYF 14182 (Confirmatory)	318.2	234	75	DP	75	75
				EP	14	14
				CE	25	25
				CXP	45	45

Analyte (~2.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYF 14182 Internal Standard	324.2	240	75	DP	50	50
				EP	14	14
				CE	43	43
				CXP	45	45

Appendix I (continued)

Parameter Table	CUR:	35
	GS1:	40
	GS2:	15
	IS:	5500
	TEM:	750
	CAD:	12

CTC PAL Autosampler Properties

Inject Details	
Syringe Size (µl):	100
Injection Volume (µl):	80
Flush Details	
VSWII-wash	
Solvent Chase 1_Yes 0_No ()	0
Needle Dip 1_Yes 0_No ()	0
Nr Washes to Waste ()	0
Loop Washes 1_Yes 0_No ()	0
Loop Wash Delay after Inject (s)	20
Nr Loop Wash Vlv Toggles ()	0
Delay Between Vlv Toggles (s)	1
Solvent Chase Vial	Wash1
Solvent Chase Volume (µl)	10
Solvent Chase Airgap (µl)	0
Dip Delay (s)	3
Filling Speed (µl/s)	10
Filling Strokes ()	1
Inject to	LC Vlv1
Injection Speed (µl/s)	5
Pre Inject Delay (ms)	500
Post Inject Delay (ms)	500
Post Clean with Solvent 1 ()	6
Post Clean with Solvent 2 ()	6
Wash Solvent Fill Speed (µl/s)	10

Appendix I (continued)

HPLC Parameters

Pumps Used:	Two Shimadzu LC-10ADVP (High Pressure Mixer) pumps with a Shimadzu SCL-10 controller	
Minimum Pressure:	0.0 psi	
Maximum Pressure:	4000 psi	
Column oven	Perkin Elmer Series 200 column oven	
Column Temperature:	60ºC	
Column:	Manufacturer:	YMC
	Type:	Pro C18
	Particle Size:	3 μm
	Diameter:	4.0 mm
	Length:	33 mm
Note:	This method requires the column to be heated to 60ºC	
	Mobile Phase A:	Water/ Acetonitrile (9/1 v/v) with 0.01% Acetic Acid
	Mobile Phase B:	0.01% Acetic Acid in Acetonitrile

Gradient Program:

Step	Time (min.)	Module	Flow Rate (mL/min)	A(%)	B(%)
0	0.01	Pumps	1.0	80.0	20.0
1	0.10	Pumps	1.0	60.0	40.0
2	1.00	Pumps	1.0	60.0	40.0
3	2.20	Pumps	1.0	25.0	75.0
4	2.30	Pumps	1.0	10.0	90.0
5	3.00	Pumps	1.0	10.0	90.0
6	3.01	Pumps	1.0	80.0	20.0
7	4.00	System Controller	Stop		

Appendix 2 Example Calculation

An example calculation for BYF 14182 from sample RAELP013-LOQ6-raw, which was analyzed during the method validation study is presented below. This sample was fortified with 0.1ng/mL BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP. The chromatogram used in this example is presented in Appendix 4 (Chromatogram 5) and the calibration curve for this analysis is presented in Appendix 5.

The standards were fit to the linear equation: $Y = MX + B$

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

The example shown below is for the calculation of BYF 14182 residues. BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The ng/mL or parts per billion (ppb) of BYF 14182 in water was calculated using the following equation,

$$\text{BYF 14182 found (ng/mL)} = \frac{(Y-B)}{M}$$

V_1	Native Peak Area	IS Peak Area	Y	M	B
25mL	32140.4	44546.7	0.7215	7.09	0.0109

From the above equations:

$$\text{BYF 14182 found} = \frac{(0.7215-0.0109)}{7.09} = 0.1002 \text{ ng/mL}$$

Therefore sample RAELP013-LOQ6-raw contains 0.1002 ng/mL BYF 14182.

The % recovery was calculated 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 samples, real or apparent
T = theoretical ppb in fortified sample

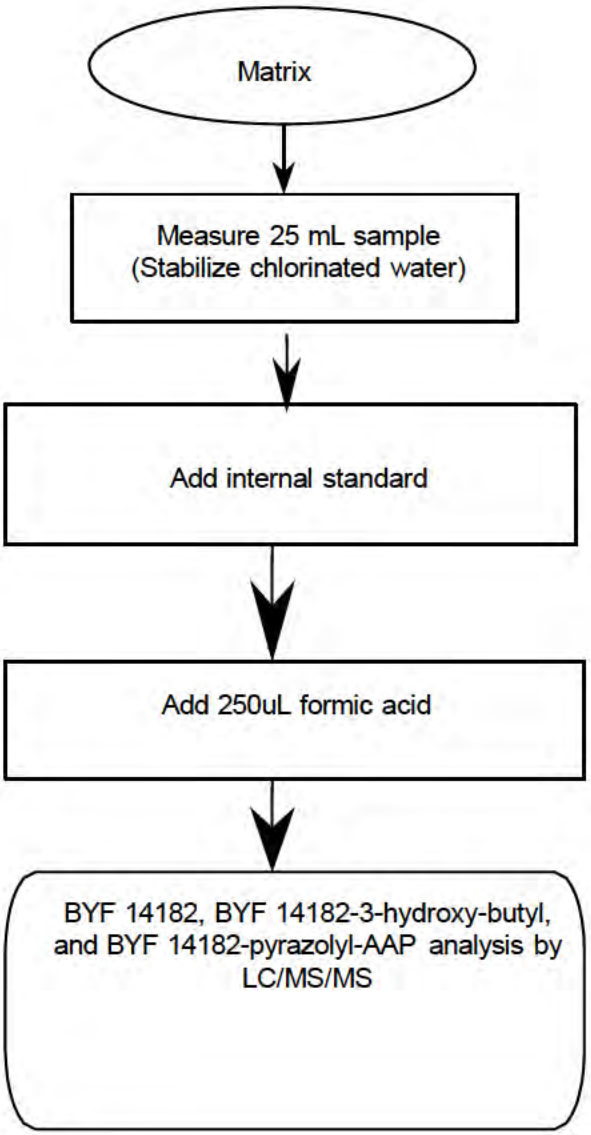
Therefore, for sample RAELP013-LOQ6-raw, this was fortified with 0.1ppb BYF 14182:

R = 0.1002 ng/mL
S = 0.00033ng/mL
T = 0.1 ng/mL

$$\% \text{ BYF 14182 Recovery} = \frac{(0.1002 - 0.00033)}{0.1} \times 100 = 100\%$$

Note: The above calculations were performed using rounded numbers and may vary slightly from the results presented in the raw data.

Appendix 6 Method Flow Chart



Appendix 7 Revision History

Method #	Revision	Description
EL-001-W08-01	01	Method prepared on completion of validation study[1]
EL-001-W08-02	01	Method prepared on completion of ILV study[3]