1 Summary

The method 01073 describes the determination of the active ingredient bixafen in drinking and surface water by HPLC-MS/MS using two MRM transitions.

A validation for drinking water was not necessary because the limit of quantitation for surface water is below the drinking water limit of 0.1 µg/L.

Acetified water samples are diluted with acetonitrile and determined by direct injection into the HPLC-MS/MS instrument using positive ionisation mode without further clean-up. Concentrations were quantified using external matrix-matched standard solutions.

Specificity: Apparent concentrations in control samples were below 0.3 × LOQ. Two MRM transitions were monitored for bixafen (m/z 414 → m/z 394 for quantitation and m/z 414 → m/z 266 for confirmation). Therefore, the HPLC-MS/MS method is highly specific and an additional confirmatory method is not necessary.

Linearity: The correlation between the injected amount of substance and the detector response was linear (1/x weighted) for aqueous standard solutions ranging from 0.04 µg/L to 10 µg/L. The correlation coefficient was ≥ 0.9997 for both MRM transitions.

LOQ and LOD: The limit of quantitation (LOQ) for bixafen is 0.05 µg/L in surface water. The limit of detection (LOD) is 0.02 µg/L.

Recovery Rates (Accuracy): Because of the direct measurement of fortified samples without separate extraction and clean-up steps it is not possible to determine recovery rates in a classical way and therefore, an estimate of the accuracy of the analytical technique was made by an assessment of the linearity of matrix calibration and by determination of the reproducibility of sample analysis.

However, for additional demonstration of the reliability of the method, the validation samples were evaluated like recovery rates. Mean recoveries for each fortification level and the overall mean recoveries were within the 70 % - 110 % range for both MRM transitions (see Table 1).

Repeatability (Precision): Relative standard deviations were below 20 % (see Table 1) for both MRM transitions.

Storage Stability of Analyte: The analyte was stable in aqueous solution when stored in a freezer at ≤ -18 °C for a period of at least 7 days.

2 Introduction and Objective

The objective of the study was to validate the method 01073 for the determination of concentrations bixafen in drinking and surface water by HPLC-MS/MS.

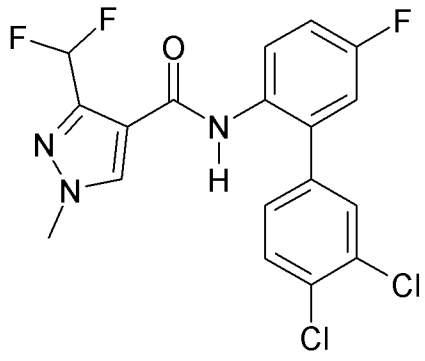
3 Compound

3.1 Reference Item

Generally, only sufficiently characterised and certified substances were used a reference items.

Common name:	**Bixafen**
Chemical name:	N-(3´,4´-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide
Chemical code:	BYF 00587
CAS. No.:	581809-46-3
Mol ID:	5965
Certificate No.:	AZ 14311
Expiry date:	August 2010
Purity:	98.9 %

Structural formula:



Empirical formula: $C_{18}H_{12}Cl_2F_3N_3O$

Molecular weight: 414.21 g/mol

Appearance: white powder

Solubility (20°C):

Water	0.49 mg/L
Acetone	> 250 g/L
Methanol	32 g/L
Ethyl acetate	> 82 g/L
Dichloromethane	102 g/L

4 Experimental Section

4.1 Test System

The analytical method was validated for surface water. A validation for drinking water was not necessary because the limit of quantitation for surface water is below the drinking water limit of 0.1 µg/L.

For method validation surface water from the river Rhine sampled in Leverkusen-Hitdorf was used. Characteristics of the test system are listed in Table 2.

Table 2: Characteristics of the Surface Water from River Rhine, Sampled on 2007-09-12 in Leverkusen-Hitdorf (Germany)

Parameter	Value
Total organic carbon (TOC)	3 mg/L
Dissolved organic carbon (DOC)	3 mg/L
Conductivity	481 µS/cm
pH	7.8
Water hardness	10.7 °dH
Dry residue after filtration	280 mg/L

4.2 Safety

The German guidelines for laboratories of the Employees' Liability Insurance Association, e.g. Bulletin M006 [4] or comparable guidelines in other countries should be observed.

The following chemicals were used, which are classified by the hazardous material regulations. The classification is based on the German guidelines [5] and has to be adapted to the respective national guidelines in case the method is used outside Germany.

Bixafen:	Dangerous to the environment N
Methanol:	Toxic T and easily flammable F
Ammonium formate:	Irritant Xi
Formic Acid 98-100%:	Corrosive C

The pertinent safety instructions must be observed when working with all compounds mentioned in this method (e.g. R- and S-phrases).

4.3 Materials

4.3.1 Apparatus and Reagents

For apparatus and reagents please see Appendix 1.

4.3.2 Stock Solution

The stock solution was prepared by weighing a defined amount of the reference item into a volumetric flask and making up to volume with acetonitrile.

Table 3: Preparation Scheme of Reference Item Stock Solution

Reference Item No.		Mass [mg]	Volume [mL]	Solvent	Final Concentration Required [mg/L]	Final Concentration Actual* [mg/L]
B1	Bixafen	12.25	100	Acetonitrile	100	121.2

*: Concentration is corrected for purity

4.3.3 Standard Solutions/Calibration Standards

Standard solutions (secondary standards) were prepared from the stock solution by dilution with a mixture of river Rhine water/acetonitrile/formic acid or deionizied water/acetonitrile/formic acid (800/200/0.1, v/v/v).

Table 4: Preparation Scheme for Standard Solutions (Secondary Standards)

No.	Target Concentration (µg/L)	Prepared by Removal of [mL]	No. of Solution	Dilution to [mL]	Solvent
B2	1000	0.825	B1	100	acetonitrile
B3	10.0	1.0	B2	100	acetonitrile
B4	10.0	0.10	B2	10	*1
B5	5.0	0.10	B2	20	*1
B6	1.0	0.10	B2	100	*1
B7	0.50	0.050	B2	100	*1
B8	0.10	0.10	B3	10	*1
B9	0.050	0.10	B3	20	*1
B10	0.040	0.10	B3	25	*1
B11	0.020	0.10	B3	50	*1
B12	0.50	0.050	B2	100	*2

*1: River Rhine water/acetonitrile/formic acid 800/200/0.1 (v/v/v)

*2: deionizied water/acetonitrile/formic acid 800/200/0.1 (v/v/v)

4.4 Sample Preparation

Acetified water samples are diluted with acetonitrile and analysed by direct injection into the HPLC-MS/MS instrument or after appropriate dilution with surface water/acetonitrile/formic acid (800/200/0.1, v/v/v).

4.5 Instrumental Analysis

4.5.1 Principle of Measurement

An aliquot of the sample solution was injected into the high performance liquid chromatograph and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electrospray ionisation. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM). The pseudomolecular ions of the analytes ($[M+H]^+$, $[M-H]^-$ or any adducts) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were selected: one product ion (MRM-transition) serving for quantitation and the second for confirmation.

4.5.2 Variations in Instrument Conditions

Variations in equipment or sample characteristics and/or deterioration of system performance may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Instrument parameters and mobile phase may be adjusted to improve separation from unexpected interfering peaks.

Therefore, the given HPLC-MS/MS parameters listed (cf. appendix 2) may require adaptation.

4.5.3 Chromatography

Instrument: Agilent 1100, Agilent Technologies or equivalent
Injector: HTC PAL, CTC Analytics or equivalent
Column: Luna 2.5µ C18(2)-HST, length 50 x 2 mm (or equivalent) with pre-column; Phenomenex or equivalent
Injection Volume: e.g. 100 µL or as needed for the sensitivity
Oven temperature: e.g. 60 °C
Mobile Phase: Bin Pump A: Deionized water / methanol, 900/100, v/v
+ 10 mM Ammonium formate + 120 µL/L formic acid
Bin Pump B: Deionized water / methanol, 100/900, v/v
+ 10 mM Ammonium formate + 120 µL/L formic acid
Iso Pump C: Deionized water / methanol, 500/500, v/v

Time Table:

Time [min]	A [%, v/v]	B [%, v/v]	Into MS	Into Waste
0.0	70	30		0 min – 4.8 min
0.1	70	30		
5.0	5	95	4.8 min – 7.0 min	
8.0	5	95		7.0 min – 12.0 min
8.1	70	30		
12.0	Stop time			

Flow (Column): 0.30 mL/min
Flow (into MS): 0.30 mL/min
Retention times: Bixafen approx. 5.5 min

4.5.4 Detection

The detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in positive ion mode and multiple reaction monitoring (MRM). Unit mass resolution was established and maintained in the mass resolving quadrupoles by maintaining a full width at half-maximum (FWHM) of about 0.7 amu. Optimal collisionally-activated dissociation (CAD) conditions for fragmentation of the pseudomolecular ions of the analytes were applied with nitrogen as the collision gas.

Detector: Ionics EP10+ with turbo-ionspray interface mass selective detector (MS/MS) upgrade of API 365 from Applied Biosystems, Concord, Ontario Canada, Analyst 1.4.1 software versions or any equivalent HPLC-MS/MS System

Interface: Turbo IonSpray (ESI)
Gas Temperature: 380 °C or as needed for the sensitivity

Scan Type: MRM (Multiple Reaction Monitoring)

Table 5: MS/MS Parameters for the Determination of Bixafen

	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)	Polarity
Bixafen Quantitation	414	394	250	21	pos
Bixafen Confirmatory	414	266	250	33	pos

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

4.6 Calculation

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

4.6.1 Calculation of Concentrations

The measured concentration is calculated by comparison of the analyte response to a standard calibration curve (1/x weighted).

The following equation is used for calculation in case of a linear calibration curve of type y = ax + b:

$$C_S = \frac{A_{(S)}\text{Sample} - \text{Intercept (b)}}{\text{Slope (a)}} \cdot \text{Dilution factor}$$

C_S = concentration of the analyte in the sample
$A_{(S)}$Sample = peak area of the analyte in the sample solution
Intercept = point where the calibration curve crosses the y-axis
Slope = slope of the calibration curve

The calculation of concentrations is also possible by comparison of the peak areas of the samples with the peak areas of the external standard solutions.

The concentration in water samples can be calculated according to the following equation:

$$\text{Conc}_{\text{Sample}}\,[\mu g/L] = \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc}_{\text{Standard}}[\mu g/L] \times \text{Dilution Factor}}{\text{Peak Area}_{\text{Standard}}}$$

$\text{Conc}_{\text{Standard}}$ = concentration of the analyte in the standard solution
$\text{Conc}_{\text{Sample}}$ = peak area of the analyte in the sample solution

4.6.2 Calculation of the Repeatability (Precision)

The repeatability or precision of the method is defined as the dispersion of the validation results and is expressed as the relative standard deviation (RSD).

At each fortification level, the relative standard deviation was calculated as follows:

$$\text{Relative Standard Deviation [\%]} = \frac{\text{Standard Deviation}}{\text{Mean of Recoveries}} \times 100$$

Table 13: Summary Parameters for the Analytical Method Used for the Quantitation of Bixafen Residues in Surface Water (DER TABLE B.1.1)

Method ID	01073
Analyte	Bixafen
Extraction solvent/technique	None – direct sample injection
Cleanup strategies	None – direct sample injection
Instrument/Detector/Column	Agilent 1100 LC Ionics EP10+ with turbo-ionspray interface mass selective detector (MS/MS), upgrade from Applied Biosystems API 365, IONICS, Concord, Ontario Canada Luna 2.5µ C18(2)-HST 50 x 2 mm (or equivalent) with pre-column; Phenomenex
Standardization method	Linear regression with 1/x weighting
Stability of std solutions	At least 10 months in refrigerator
Retention times	Bixafen approx. 5.5 min

Table 14: Characteristics for the Analytical Method Used for the Quantitation of Bixafen Residues in Surface Water (DER TABLE C.1.2)

Analytes	Bixafen
Equipment ID	Agilent 1100 LC Ionics EP10+ with turbo-ionspray interface mass selective detector (MS/MS), upgrade from Applied Biosystems API 365, IONICS, Concord, Ontario Canada
Limit of detection (LOD)	Surface water: 0.02 µg/L
Limit of quantitation (LOQ)	Surface water: 0.05 µg/L
Accuracy/Precision	Bixafen: Surface Water 101 ± 3.0 %
Reliability of the Method/[ILV]	An ILV has not been performed on this method
Linearity	The method/detector response was linear (correlation coefficient $r \geq 0.9997$ for all MRM transitions) within the range of 0.04 µg/L – 10 µg/L
Specificity	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well defined and symmetrical.

Appendix 1: Apparatus and Reagents

Apparatus

- Liquid chromatograph, Agilent 1100 column compartment , Agilent 1100 binary pump , Agilent 1100 iso pump , Agilent 1100 degasser , Agilent Technologies, Böblingen, Germany or equivalent
- Autosampler, HTC PAL, CTC Analytics, Switzerland or equivalent
- Mass selective detector (MS/MS), Ionics EP10+ with turbo-ionspray interface upgrade from Applied Biosystems API 365, IONICS, Concord, Ontario Canada
- Chromatography column, Luna 2.5µ C18(2)-HST, length 50 x 2 mm (or equivalent) with pre-column
- Volumetric flasks, 10-mL, 20-mL, 25-mL, 50-mL, 100-mL, 200-mL
- Variable dispenser, 10-mL, 50-mL
- Calibrated variable pipettes, 0.01-mL - 0.1-mL, 0.1-mL - 1-mL, Eppendorf AG, Hamburg, Germany or equivalent
- Brown glass bottles 50-mL, 100-mL
- Small instruments, e.g. Pasteur pipettes, autosampler vials, filter frits for reservoir

Reagents

- Methanol for chromatography, Optigrade, LGC Promochem, D-46485 Wesel, Germany or equivalent
- Water, HPLC grade, purified with a Milli-Q-water system, Millipore Co., Eschborn, Germany or equivalent
- Ammonium formate, Ultra: >99.0%, Fluka, D-89555 Steinheim, Germany or equivalent
- Formic acid, Riedel de Haën, Sigma Aldrich GmbH, D-30926 Seelze or equivalent
- Nitrogen 5.0, 99.9990% purity, as bath, nebulizer, collision, curtain, and turbo gas, Linde AG, Höllriegelskreuth, Germany or equivalent

Appendix 2: Detailed Summary of Chromatographic and Mass Spectrometric Conditions

```
Comment:    Luna 2.5 µm C18(2)-HST   Serial-No. 408443-2 with pre-column
Synchronization Mode:   LC Sync
Auto-Equilibration:     Off
Acquisition Duration:   12min0sec
Number Of Scans:  1176
Periods In File:  1
Acquisition Module:     Acquisition Method
Software version  Analyst 1.4.1

MS Method Properties:
Period 1:
--------------
Scans in Period:  1176
Relative Start Time:    0.00 msec
Experiments in Period:  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
Smart Settling:   Off
Settling Time:    0.0000 msec
MR Pause:   5.0070 msec
MCA:  No
Step Size:  0.00 amu

@Q1 Mass (amu)    Q3 Mass (amu)     Dwell(msec) Param Start Stop
414.06      393.90      250.00      CE    21.00 21.00
                                    CXP   20.00 20.00

@Q1 Mass (amu)    Q3 Mass (amu)     Dwell(msec) Param Start Stop
414.06      265.80      250.00      CE    33.00 33.00
                                    CXP   16.00 16.00

Parameter Table(Period 1  Experiment   1):
NEB:  12.00
CUR:  12.00
CAD:  2.00
IS:   5500.00
TEM:  380.00
DP    86.00
FP    120.00
EP    12.00
CEP   0.00

Agilent 1100 Column Oven Properties
Left Temperature (°C):        60.00
Right Temperature (°C): 60.00
Temperature Tolerance +/- (°C):     1.00
Start Acquisition Tolerance +/- (°C):     0.50
Time Table  (Not Used)
Column Switching Valve  Installed
```

Appendix 2: Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

```
Position for first sample in the batch:  Right (1->2)
Use same position for all samples in the batch

Agilent 1100 LC Pump Method Properties
Pump Model: Agilent 1100 LC Binary Pump
Minimum Pressure (psi): 0.0
Maximum Pressure (psi): 5801.0
Dead Volume (µl): 40.0
Maximum Flow Ramp (ml/min²):  100.0
Maximum Pressure Ramp (psi/sec):    290.0

Step Table:

Step  Total Time(min)   Flow Rate(µl/min) A (%) B (%) TE#1  TE#2  TE#3  TE#4
0     0.00              300               70.0  30.0  open  open  open  open
1     0.10              300               70.0  30.0  open  open  open  open
2     5.00              300               5.0   95.0  open  open  open  open
3     8.00              300               5.0   95.0  open  open  open  open
4     8.10              300               70.0  30.0  open  open  open  open
5     12.00             300               70.0  30.0  open  open  open  open

Left Compressibility:   50.0
Right Compressibility:  115.0
Left Dead Volume (µl):  40.0
Right Dead Volume (µl): 40.0
Left Stroke Volume (µl):      -1.0
Right Stroke Volume (µl):     -1.0
Left Solvent:     A1
Right Solvent:    B1


      Agilent 1100 LC Pump Method Properties
Pump Model: Agilent 1100 LC Isocratic Pump
Minimum Pressure (psi): 0.0
Maximum Pressure (psi): 5801.0
Compressibility:  100.0
Dead Volume (µl): 40.0
Stroke Volume (µl):     -1.0
Maximum Flow Ramp (ml/min²):  100.0
Maximum Pressure Ramp (psi/sec):    290.0

Step Table:
@Step Total Time(min)   Flow Rate(µl/min)
0     0.00              300
1     12.00             300


Primary Flow Rate (µl/min):   200.0
Flow Sensor Calibration Table Index:      0

      Valco Valve Method Properties
Valco Valve Diverter

      Total Time (min)  Position
1     0.1   Waste
2     4.8   MS
3     7.0   Waste
```

Appendix 2: Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

```
CTC PAL Autosampler Method Properties
Loop Volume1 (µl):      100
Loop Volume2 (µl):      100
Injection Volume (µl):  100.000

Method Description:

Syringe:  100ul

01    Analyst LC-Inj
      Air Volume (µl)                                   0
      Pre Clean with Solvent 1 ()                       1
      Pre Clean with Solvent 2 ()                       1
      Pre Clean with Sample ()                          0
      Filling Speed (µl/s)                              10
      Filling Strokes ()                                2
      Inject to                                         LC Vlv1
      Injection Speed (µl/s)                            100
      Pre Inject Delay (ms)                             500
      Post Inject Delay (ms)                            500
      Post Clean with Solvent 1 ()                      2
      Post Clean with Solvent 2 ()                      2
      Valve Clean with Solvent 1 ()                     2
      Valve Clean with Solvent 2 ()                     2
      Replicate Count ()                                1
      Analysis Time (s) ()                              12
```