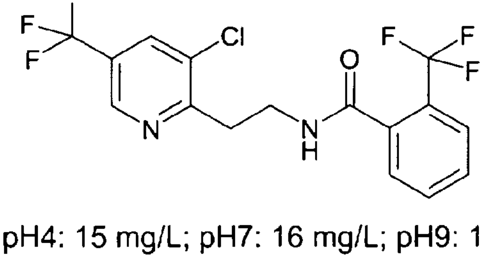
2 Introduction and Objective

The objective of the study was to validate the analytical method 01051 for the determination of concentrations of fluopyram 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:	Fluopyram
Chemical code:	AE C656948
Chemical name:	N-{2-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide
CAS No.:	658066-35-4
Mol ID:	6207
Empirical formula:	$C_{16}H_{11}ClF_6N_2O$
Molecular weight:	396.72 g/mole
Analysis Certificate:	AZ 13703
Expiry Date:	2008-10-24
Purity:	99.4%
Structural formula:	
Water solubility (20 °C):	pH4: 15 mg/L; pH7: 16 mg/L; pH9: 15 mg/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 2004-09-10 in Leverkusen-Hitdorf (Germany)

Parameter	Value
Total organic carbon (TOC)	4 mg/L
Dissolved organic carbon (DOC)	<2 mg/L
Conductivity	507 µS/cm
pH	7.6
Water hardness	10.1 °dH
Dry residue after filtration	54 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.

Acetic acid	Corrosive C
Acetonitrile	Harmful Xn and highly flammable F
Fluopyram	Dangerous for the environment

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 Solutions

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]
A1	Fluopyram	10.49	100	Acetonitrile	100	104.3

*: 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 river Rhine water or deionizied water.

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
A2	Fluopyram, (1000)	0.959	A1	100	*1
A3	Fluopyram, (10)	0.1	A2	10	*1
A4	Fluopyram, (5)	0.1	A2	20	*1
A5	Fluopyram, (1)	1	A3	10	*1
A6	Fluopyram, (0.5)	2	A4	20	*1
A7	Fluopyram, (0.1)	1	A5	10	*1
A8	Fluopyram, (0.05)	2	A6	20	*1
A9	Fluopyram, (0.04)	0.8	A6	10	*1
A10	Fluopyram, (0.02)	1	A6	25	*1
A11	Fluopyram, (0.5)	1.0	A4	10	*2

*1: River Rhine water
*2: deionizied water

4.4 Sample Preparation

The water samples are directly injected into the HPLC or after appropriate dilution with surface water.

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: Aqua 5μ C18; Length 150 mm, i. d. 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. 40 °C

Mobile Phase:
Bin Pump A: Deionized water/acetonitrile/acetic acid 900/100/0.1, v/v/v (Add 0.1 mL acetic acid to a 1 L volumetric flask and make up to volume with water/acetonitrile, 900/100, v/v)
Bin Pump B: Acetonitrile/acetic acid 1000/0.1, v/v (Add 0.1 mL acetic acid to a 1 L volumetric flask and make up to volume with acetonitrile)
Iso Pump C: Deionized water/acetonitrile/acetic acid 500/500/0.1, v/v/v (Add 0.1 mL acetic acid to a 1 L volumetric flask and make up to volume with water/acetonitrile, 500/500, v/v)

Time Table:

Time [min]	A [%, v/v]	B [%, v/v]	Into MS	Into Waste
0.0	55	45	Iso pump	Bin pump
4.5	55	45		
5.0	10	90	Bin pump	Iso pump
7.5			Iso pump	Bin pump
9.0	10	90		
9.1	55	45		
12.5	55	45		
12.5	Stop time			

Flow (Column):	0.5 mL/min	
Flow (into MS):	0.1 mL/min	
Retention time:	Fluopyram	approx. 6.1 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:	Triple Quadrupole Tandem Mass Spectrometer, Ionics EP10+ or any equivalent HPLC-MS/MS System
Interface:	Turbo IonSpray (ESI) Gas Temperature: 300 °C or as needed for the sensitivity
Scan Type:	MRM (Multiple Reaction Monitoring)

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

	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)	Polarity
Fluopyram Quantitation	397	173	250	41	pos
Fluopyram Confirmatory	397	145	250	73	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

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:

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

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 spectrometer, Ionics EP 10+ or equivalent
- Chromatography column, Phenomenex Aqua 5µ C18, 150 x 2 mm, or equivalent with pre-column
- Volumetric flasks, 2-mL, 10-mL, 20-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
- Glass bottles 50-mL, 100-mL
- Small instruments, e.g. Pasteur pipettes, autosampler vials, filter frits for reservoir

Reagents

- Acetonitrile for chromatography, LiChrosolv Merck KGaA, Darmstadt, Germany or equivalent
- Water, HPLC grade, purified with a Milli-Q-water system, Millipore Co., Eschborn, Germany or equivalent
- Acetic acid, Suprapur, Merck KGaA, Darmstadt, Germany or equivalent
- Nitrogen 5.0, 99.9990% purity, as bath, nebulizer, collision, curtain, and turbo gas, Linde AG, Höllriegelskreuth, Germany or equivalent