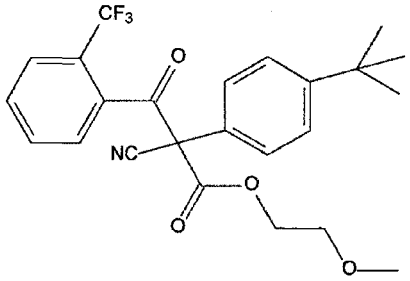
6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Identification	OK-5101
Structure	
Molecular formula	$C_{24}H_{24}F_3NO_4$
Molecular weight	447.5
CAS Number	400882-07-7
Description	Pale yellow powder (determined at NOTOX)
Batch	01H1
Purity	98.4%
Test substance storage	In refrigerator (2-8°C) in the dark
Stability under storage conditions	Stable
Expiry date	16 May 2008

The sponsor is responsible for all test substance data unless determined by NOTOX.

6.1.2. Study specific test substance information

There is no study specific test substance information necessary for this study.

6.2. Test system

Drinking water
Notox tap water was used as test system for drinking water.

Ground water
Water from a groundwater well (Westbroek, The Netherlands) was used as test system for ground water.

Surface water
Water from the river Maas (Ravenstein, The Netherlands) was used as test system for surface water. The parameters pH, hardness, DOC and suspended solids were determined as follows:

pH	6.93
Total hardness	14 German degrees, medium-hard
DOC	4.4 mg/l
Suspended solids	43.6 mg/l

6.3. Test concentrations

Test concentrations Surface water

LOQ: 0.1 µg/L	Conc. Level 1.
10x LOQ: 1.0 µg/L	Conc. Level 2.
see text below: 0.63 µg/L	Conc. Level 3.
10x Conc. level 3: 6.3 µg/L	Conc. Level 4.

For surface water the target limit of quantification (concentration level 1) will be 0.1 µg/L. Concentration level 2 will be based on 10x LOQ. If it's not possible to reach the target concentration level 1 concentration level 3 will be the next target level. This concentration level is based on the toxicity of OK-5101 for aquatic organisms. Because the LC_{50} for Daphnia is >0.063 mg/l which is the lowest concentration in table 1, concentration level 3 will be set to 0.63 µg/l based on the toxicity exposure ratio (SANCO 8075/VI/97 rev.7 08-07-2000).

Table 1 Toxicity data for aquatic organisms

Species	Test / duration	Results	Reference
Fish			
Rainbow trout	acute toxicity 96 hours / flow through	LC_{50} = >0.63 mg/l	[1]
Carp	acute toxicity 96 hours / flow through	LC_{50} = >0.54 mg/l	[2]
Invertebrates			
Daphnia magna	acute toxicity 48 hours / flow through	EC_{50} = >0.063 mg/l	[3]
Alga			
Alga (Selenastrum capricornutum)	growth inhibition test 72 hours / static	E_bC_{50} = >0.3 mg/l E_rC_{50} = >0.3 mg/l	[4]

Test conditions Tap water and ground water

If the established LOQ of the method for surface water is 0.1 µg/L, or less, then the use of this method for tap water and ground water will be demonstrated by analyzing 2 blanks of tap water and ground water. The method can be accepted for both types of matrices if the interference in the blank in each matrix is ≤ 30% of the LOQ in surface water.

If the LOQ of the method for surface water is ≥ 0.1 µg/l then a full validation for specificity, recovery and precision for tap water and ground water will also be performed.

6.4. Reagents

Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore Corp., Bedford, MA, USA
Acetonitrile	HPLC-grade, VWR International, Leuven, Belgium
Methanol	HPLC-grade, VWR International, Leuven, Belgium
Formic acid	p.a. 98-100%, Merck, Darmstadt, Germany

6.5. Analytical method

6.5.1. Chromatography

A high performance liquid chromatographic method with tandem mass spectrometric detection (HPLC-MS/MS) for quantitative analysis of OK-5101 in water was developed. The conditions are described below:

Column	
Stationary phase	Symmetry C18
Dimensions	50 x 2.1 mm; dp = 3.5 µm
Brand	Waters, Milford, MA, USA
Guard column	
Stationary phase	Symmetry C18
Dimensions	10 x 2.1 mm; dp = 3.5 µm
Brand	Waters
Mobile phase	Gradient system A: Milli-Q water with 0.1% Formic Acid B: Methanol with 0.1% Formic Acid

Time (minutes)	% A	% B
0.00	80	20
2.00	80	20
5.00	0	100
9.00	0	100
9.01	80	20
12.0	80	20

Flow	400 µl/min
Injection volume	full loop (1000 µl) overfill with 1500 µl sample
Autosampler temperature	4°C
Detection	Mass Spectrometric detection using an API 3000 mass spectrometer (Applied Biosystems, Sciex, Toronto, ON, Canada)
Ionisation source	Turbo ion spray, positive mode (No split)
Temperature	500°C
Nitrogen flow	7000 ml/min
Acquisition	MS/MS monitoring the reaction: 448.3 → 173.1 amu

In order to prevent the ion source from excessive contamination, the mobile phase eluting during the first 3 minutes of each run was discarded to waste.

6.5.2. Preparation of Solutions

Stock solutions
Standard solutions were prepared in acetonitrile at exactly known concentrations between 877.6 mg/l and 714.0 mg/l. Calibration solutions for the validation tests were obtained by dilution of these standard solutions with 80/20/0.1 (v/v/v) Milli-Q water/Methanol/formic acid (end solution).

Calibration solutions
Six calibration solutions in the concentration range 0.0500 – 2.00 µg/l were prepared from two stock solutions. The end solution of the calibration solutions was 80/20/0.1 (v/v/v) Milli-Q water/Methanol/formic acid .

Accuracy samples
8 ml surface water was spiked with the test substance at nominal concentrations of 0.1 and 1.0 µg/l. thereafter 2 ml of 0.5% Formic acid in Methanol was added and the samples were analysed.

The blank accuracy samples (specificity samples) were prepared and treated similar to the accuracy samples.

Note: the spiking volume was < 5% (v/v) of the sample volume. Nominal concentrations were not corrected for the spiking volume.

6.6. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- Analyst version 1.4.1 (Applied Biosystems, Sciex, Toronto, ON, Canada).

Temperature and/or relative humidity during sample storage and/or performance of the studies were monitored continuously using the following programme:
- REES monitoring system version 1.5 (REES Scientific, Trenton, NJ, USA).

6.7. Validation

The method was validated for specificity, linearity, precision, recovery, limit of quantification (LOQ), matrix effect, stability of the chromatographic system and end solutions and stability of standard solutions.

6.8. Interpretation

Response: Peak area test substance [units]

Response factor:

$$Rf = \frac{response}{c}$$

where:
Rf = response factor [units × l/µg]
c = concentration of test substance [µg/l]

Calibration curve: The response was correlated with the test substance concentration, using linear regression analysis (least squares method; weighting factor ($1/concentration^2$)).

$$R = a\,C + b$$

where:
R = response calibration solution [units]
C = concentration of test substance in calibration solution [µg/l]
a = slope [units × l/µg]
b = intercept [units]

Concentration analysed

$$C = \frac{(R - b)}{a} \times d$$

where:
C = concentration of test substance in sample [µg/l]
R = response sample [units]
a = slope [units × l/µg]
b = intercept [units]
d = dilution factor

Recovery

$$\frac{\text{concentration analysed}}{\text{concentration prepared}} * 100\%$$

Limit of detection (LOD)

The limit of detection is defined as the lowest concentration of test substance that can be distinguished from instrumental noise using the analytical method described.

$$LOD = \frac{3N}{S} \times C$$

where:
C = concentration of test substance in solution [µg/l]
N = noise height [cps]
S = test substance peak height [cps]

6.9. List of deviations

6.9.1. List of protocol deviations

1. Matrix effect was performed in duplicate at two concentration levels instead of in triplicate at one concentration level.
   Evaluation: In total 4 determinations were performed which clearly demonstrate the absence of matrix effect.

2. Stability of the chromatographic system and end solutions was determined using fresh dilutions of the standards as well. Therefore these data can only be used for the determination of the stability of the chromatographic system. Stability of standard solutions was determined from the change in response of a single standard solution over time.
   Evaluation: this approach was chosen due to the relatively long analysis time.

The study integrity was not adversely affected by the deviations.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures (SOPs) were evaluated and filed in the study file. There were no deviations from SOPs that affected the integrity of the study.