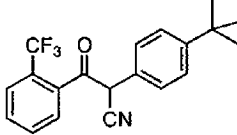


6. MATERIALS

6.1. Test substance

6.1.1. Test substance information

Identification	AB-1
Structure	
Molecular formula	C ₂₀ H ₁₈ F ₃ NO
Molecular weight	345.4
Description	White powder (determined at NOTOX)
Batch	0863NT2
Purity	99.8%
Test substance storage	In freezer (≤ -15°C) in the dark
Stability under storage conditions	Stable
Expiry date	16 June 2009 (allocated by NOTOX, 1 year after receipt of the test substance)

6.1.2. Study specific test substance information

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

6.2. Sediment

Sediment was prepared and treated similarly as during Sediment-Water Chironomid Toxicity test (see NOTOX project 489663).

6.3. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:

- Analyst version 1.4.2 (Sciex, Toronto, Canada)

6.4. List of deviations

6.4.1. List of protocol deviations

1. The back calculated accuracies of the lowest calibration solution was in the range of 85-115% (criterion range is 90-110%) (see paragraph 7.6.2).

Evaluation: The calibration curve obtained was considered to be accurate for the determination of the test substance in the test samples. The responses of the lowest calibration solution were most likely affected by the spread of the analytical method.

The study integrity was not adversely affected by the deviations.

6.4.2. 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.

7. VALIDATION OF AN ANALYTICAL METHOD

7.1. Guideline

The study was based on the following guideline:

European Commission: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, section 4) and Annex III (Part A, section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

7.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).
Acetonitrile	Biosolve, Valkenswaard, The Netherlands.
Acetone	VWR International, Leuven, Belgium
Ammonium formate	Fluka, Buchs, Switzerland.
Formic acid	Merck, Darmstadt, Germany.
Ammonia solution, 25%	Merck.

All reagents were of analytical grade, unless specified otherwise.

7.3. Performance of the study

A high performance liquid chromatographic method with mass spectrometric detection (HPLC-MS/MS) for the quantitative analysis of the test substance in sediment was developed. Validation of the analytical method was performed for the following parameters:

Specificity

A test substance solution and blank accuracy sample were analysed by single injection. The analytical method was found to be specific if the blank chromatogram showed no response for the test substance or a response of < 30% of the limit of quantification.

Linearity

Calibration solutions were analysed in duplicate. The response of the calibration solutions was correlated with concentration using regression analysis with a $1/\text{concentration}^2$ weighting factor. A calibration curve with a coefficient of correlation (r) of > 0.99 and back calculated accuracies of the calibration solutions in the range 90-110% with accuracies of 85-115% for the lowest concentration level was accepted.

Accuracy and repeatability

Accuracy samples were analysed by single injection into the analytical system. The analytical method was considered applicable for the determination of the test substance if the mean accuracy was in the range 70-110% and the coefficient of variation was $\leq 20\%$.

Limit of quantification

The limit of quantification (LOQ) is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of $\leq 20\%$ is demonstrated. The LOQ was obtained from the data of the accuracy- and repeatability test.

Stability of the analytical system and end solutions

Calibration solutions were injected throughout the validation sequence including the beginning and end. The analytical system and/or end solutions were found to be stable if the coefficient of variation on the responses of the solutions was $\leq 20\%$.

Stability of stock solutions

Stock solutions of the test substance were stored at room temperature for at least 12 hours. Additional calibration solutions were prepared and analysed by single injection. The stock solutions were found to be stable if the coefficient of variation on the response factors of the calibration solutions prepared with fresh- and stored stock solutions was $\leq 10\%$.

7.4. Analytical method**7.4.1. Analytical conditions**

Instrument	Acquity UPLC binary solvent manager and Acquity UPLC sample manager (Waters, Milford, MA, USA)
Detector	API 5000 LC/MS/MS system (Applied Biosystems/MDS Sciex, Toronto, Canada)
Column	Acquity UPLC BEH Shield RP-18, 50 mm \times 2.1 mm i.d., dp = 1.7 μ m (Waters)
Column temperature	40°C
Injection volume	10 μ l
Mobile phase	A - 5/95 (v/v) acetonitrile/water containing 2.5 mM ammonium formate and 0.1% (v/v) formic acid B - 95/5 (v/v) acetonitrile/water containing 2.5 mM ammonium formate and 0.1% (v/v) formic acid
Isocratic conditions	40/60 (v/v) mobile phase A / mobile phase B
Flow	0.800 ml/min
MS/MS detection	
Ionisation source	Turbo Ion Spray; negative ion mode
Ion spray voltage	-3500 V
Temperature	450°C
Collision gas	8
Curtain gas	25
Ion source gas 1	55
Ion source gas 2	45
Declustering potential	-145
Entrance potential	-10
Collision energy	-34
Collision cell exit potential	-7
Dwell time	200 msec
Quantitation	<i>m/z</i> 344.2 \rightarrow <i>m/z</i> 304.0

7.4.2. Preparation of solutionsStock- and spiking solutions

Stock solutions of the test substance were prepared in acetonitrile at concentrations of 870 - 1105 mg/l.

Spiking solutions of the test substance were prepared in acetone at a concentration of 5.43 or 54.3 g/l.

Calibration solutions

Five calibration solutions in the concentration range of 0.1 – 10 μ g/l were prepared from two stock solutions. The end solution of the calibration solutions was 50/50 (v/v) acetonitrile/water.

Accuracy samples

60 gram sediment based on dry weight¹ was spiked with the test substance at a target concentration of 100 or 1000 mg/kg. The accuracy samples were extracted at 200 rpm with 100 ml of 50/50 (v/v) acetonitrile/water containing 1% (v/v) ammonium solution. The shaking time was 30 minutes. The samples were centrifuged at 16421 g and 20°C for 5 minutes. Thereafter the samples were filtered through a 0.2 µm Spartan FP 30/0.2 CA-S filter (Whatman, Dassel, Germany) and further diluted with 50/50 acetonitrile/water to obtain concentrations within the calibration range.

The blank accuracy sample was prepared and treated similar to the accuracy samples.

7.5. Formulas

Response (R) Peak area of the test substance [units]

Response factor (R_f)
$$R_f = \frac{R}{C_N}$$

where:

C_N = nominal concentration [$\mu\text{g/l}$]

Calibration curve
$$R = a C_N + b$$

where:

a = slope [units \times $l/\mu\text{g}$]

b = intercept [units]

Analysed concentration (C_A)
$$C_A = \frac{(R - b)}{a} \times \frac{V \times d}{w} \times \frac{1}{1000} \text{ [mg/kg]}$$

where:

w = weight sample [g]

V = volume of extraction solvent [ml]

d = dilution factor

Accuracy
$$\frac{C_A}{C_N} \times 100\%$$

¹ The water percentage of the sediment was 40%. According to this aliquots of 85 gram of the sediment were applied in the study. The volume of the extraction solvent was adjusted for the water content of the samples.