2 TEST AND REFERENCE ITEM

2.1 Test Items

The test items were supplied by Huntingdon Life Sciences and were also used as reference item (analytical standard). All information about the test items was provided by the Supplier.

2.1.1 SL-573

Identification:	SL-573 PAI
Chemical Name:	1-[[1-ethyl-4-[3-(2-methoxyethoxy)-2-methyl-4-(methylsulfonyl)benzoyl]-1*H*-pyrazol-5-yl]oxy]ethyl methyl carbonate
Batch:	20120131
Purity:	99.9%
Physical State/Appearance:	Light yellow powder
Expiry Date:	31 May 2015
Storage Conditions (as provided by the Supplier):	Frozen (-20 °C)
Storage Conditions (as handled by Harlan Laboratories Ltd.):	-20 ± 5°C

2.1.2 MT-2153

Identification:	MT-2153
Chemical Name:	5-Hydroxy-1-ethylpyrazol-4-yl(3-(2- methoxyethoxy)-2-methyl-4-(methylsulfonyl)phenyl]methanone
Batch:	20120615
Purity:	99.5%
Physical State/Appearance:	White powder
Expiry Date:	06 November 2015
Storage Conditions (as provided by the Supplier):	Frozen (-20 °C)
Storage Conditions (as handled by Harlan Laboratories Ltd.):	-20 ± 5°C

3 MATERIALS AND METHODS

Details of the materials and methods that are not specified in the subsequent sections of the present report are described in the appropriate standard operating procedures.

3.1 Definitions and Abbreviations

LC	Liquid Chromatography
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification

3.2 Test System

The test systems were selected by the Sponsor in accordance with the quoted guideline.

3.2.1 Drinking Water

Source:	Harlan Laboratories canteen, 4452 Itingen / Switzerland
Blank Matrix:	Drinking water (freshly sampled tap water)
pH-Value:	6.87
Dissolved organic carbon (DOC):	0.86 mg C/L
Total hardness:	38.4 °fr.H

3.2.2 Ground Water

Source:	4460 Gelterkinden / Switzerland
Blank Matrix:	Ground water (fountain)
Storage Location:	Harlan Laboratories Ltd., 4452 Itingen / Switzerland
Storage temperature:	5 ± 3 °C
pH-Value:	8.15
Total organic carbon (TOC):	0.0 g/L
Total hardness:	18.0 °fr.H
Evaporation residue:	0.21 g/L
Filtration residue:	1.0 mg

3.2.3 Surface Water

Source:	Ergolz, 4415 Lausen / Switzerland
Blank Matrix:	Surface water (river)
Storage Location:	Harlan Laboratories Ltd., 4452 Itingen / Switzerland
Storage temperature:	5 ± 3 °C
pH-Value:	8.23

Total organic carbon (TOC):	0.0 g/L
Total hardness:	39.6 °fr.H
Evaporation residue:	0.56 g/L
Filtration residue:	0.6 mg

3.3 Reagents

ELGA water:	Harlan Laboratories Ltd.
Acetonitrile:	J.T. Baker no. 9017
Ammonium acetate:	Sigma A1542
Formic acid:	Merck, 1-00264
Methanol:	J.T. Baker no. 8402

3.4 Equipment and Materials

Balance:	UMT-2	Mettler Toledo
Laboratory Material:	HPLC vials (2 mL, amber)	BGB
Pipettes:	Piston stroke pipette	Gilson
Evaporator:	-	Barkey
Freezer:	-20 ± 5 °C	Liebherr
Vortex:	Genie 2	Bender & Hobein AG
Refrigerator:	5 ± 3 °C	Liebherr
Ultrasonic Bath:	220	Bandelin Sonorex
SPE cartridges:	Strata X, 6cc, 100 mg	Phenomenex

4 ANALYTICAL METHOD

Concentrations of SL-573 and MT-2153 were determined by liquid chromatography (LC) coupled with tandem mass spectrometric detection (MS/MS). The method was developed at Harlan Laboratories Ltd. under non-GLP conditions.

4.1 Solutions for Fortification and Calibration

4.1.1 Preparation of Stock Solutions

Stock solutions of SL-573 and MT-2153 were prepared separately.

Stock Solution A of SL-573 and MT-2153 (1000 µg/mL):

For example an amount of 7.9553 mg of SL-573 (see Section 2.1) was dissolved in acetonitrile (7.947 mL) using an ultrasonic bath for about 5 minutes.

For example an amount of 7.7978 mg of MT-2153 (see Section 2.1) was dissolved in acetonitrile (7.759 mL) using an ultrasonic bath for about 5 minutes.

Stock Solution B of SL-573 and MT-2153 (1000 µg/mL):

For example an amount of 6.1922 mg of SL-573 (see Section 2.1) was dissolved in acetonitrile (6.186 mL) using an ultrasonic bath for about 5 minutes.

For example an amount of 6.6300 mg of MT-2153 (see Section 2.1) was dissolved in acetonitrile (6.597 mL) using an ultrasonic bath for about 5 minutes.

The stock solutions were stored frozen (-20 ± 5 °C) until completion of the analyses.

4.1.2 Preparation of Fortification Solutions

A defined volume of the stock solution A of SL-573 and MT-2153 was diluted with acetonitrile to obtain fortification solutions with a concentration of 10 µg/mL, 0.18 µg/mL and 0.018 µg/mL as described in the table below.

Fortification solution	Aliquot [µL]	Aliquot taken from	Final volume [mL]	Solvent	Final concentration [µg/mL]
1F	100	stock solution A of SL-573 and MT-2153	10	acetonitrile	10
2F	180	1F	10		0.18
3F	1000	2F	10		0.018

These solutions were stored frozen (-20 ± 5 °C) until completion of the analyses.

4.1.3 Preparation of Intermediate Solutions

A defined volume of the stock solution B of SL-573 and MT-2153 was successively diluted with methanol/water; 5 mM ammonium acetate (20/80, v/v) to obtain three intermediate solutions with concentrations of 10, 1 and 0.1 µg/mL.

Intermediate solution	Aliquot [µL]	Aliquot taken from	Final volume [mL]	Solvent	Final concentration [µg/mL]
1I	100	stock solution B of SL-573 and MT-2153	10	methanol/water; 5 mM NH_4Ac (20/80, v/v)	10
2I	1000	1I	10		1
3I	1000	2I	10		0.1

These solutions were kept in the refrigerator (5 ± 3 °C) until completion of the analyses.

4.1.4 Preparation of Calibration Solutions

Defined volumes of the intermediate solutions were diluted with methanol/water; 5 mM ammonium acetate (20/80, v/v) to obtain additional intermediate calibration solutions in the range of 2 ng/mL to 75 ng/mL. These solutions were kept in the refrigerator (5 ± 3 °C) until completion of the analyses.

The intermediate calibration solutions were further diluted using control extract of the matrix to obtain matrix matched standard solutions in the range of 0.2 ng/mL to 7.5 ng/mL. These solutions were freshly prepared before use.

4.2 Fortification

In order to demonstrate the validity of the analytical method, untreated drinking, ground and surface water samples were fortified with fortification solutions of SL-573 and MT-2153 prior to the extraction and extracted as described in Section 4.3. Five separate samples of each fortification level were prepared:

0.01 µg/L: 100 µL of the fortification solution 3F (0.018 µg/mL) were added to 180 mL of an untreated drinking, ground or surface water sample

0.10 µg/L: 100 µL of the fortification solution 2F (0.18 µg/mL) were added to 180 mL of an untreated drinking, ground or surface water sample

4.3 Sample Work up

1. 180 mL untreated drinking, ground or surface water were transferred into an appropriate vessel
2. the fortification samples were spiked accordingly to the spike system (see Section 4.2)
3. the samples were vortexed

4. the SPE cartridge was conditioned using 5 mL of methanol and 5 mL of water
5. the sample was loaded onto the SPE cartridge (flow rate approximately 2 mL/min)
6. the sample was eluted using 2 x 3 mL of acetonitrile and 2 x 2 mL of methanol
7. the eluate was evaporated to dryness under N_2
8. the residue was reconstituted in 3.0 mL of methanol/water; 5 mM ammonium acetate (20/80, v/v)
9. an aliquot was transferred into an amber HPLC-vial and final solutions were measured by LC/MS/MS.

4.4 LC/MS/MS Conditions

Instrumentation

MS Detector: API 5000, MDS Sciex, Toronto/Canada
Software: *ANALYST*

LC Pumps: High pressure gradient system consisting of two Shimadzu LC-10AD pumps and a Shimadzu SCL System Controller
LC Injector: CTC PAL

Sample Injection

Wash Solvent: 1: water / methanol / formic acid (80+20+0.5, v/v/v)
2: water / acetonitrile / methanol (10+45+45, v/v/v)

Washing Procedure: 2 x syringe and 2 x injection port with each solvent

Injection Volume: 50 µL

Chromatographic Separation

Analytical Column: Xbridge C8 (Waters) [2.1 mm x 50 mm; 3.5 µm]

Mobile Phases A: water / methanol; 5 mM ammonium acetate (95+5, v/v)
B: water / methanol; 5 mM ammonium acetate (5+95, v/v)

Gradient Program:

Time [min]	**0**	**2.5**	**4.5**	**4.6**	**5.4**	**5.5**	**5.9**	**6.0**	**7.0**
A [%]	80	40	25	0	0	100	100	80	80
B [%]	20	60	75	100	100	0	0	20	20
Flow [µL/min]	300	300	300	300	300	300	300	300	300

Detection

Ionization: Pneumatically and thermally assisted electro spray ionization (ESI)
Source: Sciex Turbo-V-Source

Spray Voltage: 4500 V
Heater Gas Temperature: 300 °C
Gases: Nebulizer (air), heater (air), curtain (N_2), collision (N_2)

Scan Mode: Multiple reaction monitoring (MRM)

Analyte	Ion Polarity	*m/z* → *m/z*	Dwell time [ms]	CE [eV]
SL-573	$[M+H]^+$	485.3 → 383.3	100	17
SL-573	$[M+H]^+$	485.3 → 409.3	900	13.5
MT-2153	$[M+H]^+$	383.3 → 111.2	300	59
MT-2153	$[M+H]^+$	383.3 → 325.2	700	25

Resolution Q1: Unit resolution
Resolution Q3: Unit resolution

4.5 Data Acquisition, Calculation and Quantification

Acquisition and peak calculations were performed with the software ANALYST. Quantification of the analytes was performed using the regression model:

y = b * x + a, weighting 1/y (1)

y = Area [counts]
x = Final concentration of analyte in extract [ng/mL]
a = Intercept
b = Slope

4.5.1 Calculation

The results are calculated by external calibration using peak areas.

Individual residue levels in the specimen are calculated as shown in the following equation 2:

$$R = \frac{x \cdot V_F}{V_{sample}} \quad (2)$$

R = Recovered residue of analyte [µg/L]
x = Final concentration of analyte in extract [ng/mL] (calculated from equation 1)
V_F = Final sample volume [mL]
V_{sample} = Volume of sample [mL]

Recoveries are calculated as shown in the following equation 3:

$$Rec = \frac{R}{F} \cdot 100\% \qquad (3)$$

Rec = Recovery [%]
R = Residue of analyte [µg/L]
F = Fortification level [µg/L]

Note: The tabulated values represented rounded-off results obtained by calculations based on the exact data.

4.5.2 Example of Calculation

The calculation is exemplified with internal sample ID 111 (SL-573 in drinking water, primary mass transition, see Table 1). Numerical data in the tables represent rounded-off results obtained by calculations based on the exact data. Therefore, manual recalculation may slightly differ in values.

For example, the correlation of the calibration row (SL-573) of the recovery analysis of 20 November 2014 is calculated to be:

$$y = 4805 + 334836 \cdot x \quad \text{or} \quad x = \frac{y - 4805}{334836} \qquad (4)$$

For a peak area of y = 180295 (counts) for the internal sample ID 111 the concentration of SL-573 in sample solution is calculated to be 0.5241 ng/mL.

The residue of SL-573 in the sample is calculated according to equation 2 using:

R = Recovered residue of analyte [µg/L]
x = Final concentration of analyte in extract (here: 0.5241 ng/mL)
V_F = Final sample volume (here: 3.0 mL)
V_{sample} = Volume of sample (here: 180 mL)

$$R = \frac{0.5241 \cdot 3}{180} = 0.008735 \text{ µg/L}$$

The residue of SL-573 in the sample is calculated to be 0.008735 µg/L.

The recovery of SL-573 in the sample is calculated according to equation 3 using:

Rec = Recovery [%]
R = Calculated residue of SL-573 in the sample of 0.008735 µg/L
F = Fortification level (here: 0.01 µg/L)

$$Rec = \frac{0.008735}{0.01} \text{ x } 100\% = 87\%$$

The recovery of SL-573 in this drinking water sample is calculated to be 87%.

5.8 Matrix Effects

The final sample solution of an untreated control surface, ground or surface sample was fortified with SL-573 and MT-2153 and analyzed by LC/MS/MS. The area counts were compared to an equivalent solution prepared without matrix.

Significant matrix influences for the determination of SL-573 in drinking, ground and surface water using this LC/MS/MS method were observed. For MT-2153 no significant matrix influences in drinking, ground and surface were observed. However, matrix matched standard solutions were used for analysis of SL-573 and MT-2153.