4 Introduction

The aim of the study was to demonstrate that the proposed method [1] for water analysis can be reproduced, and that method performance characteristics meet the requirements as laid down in SANCO/825/00 [2].

The following parameters were determined during validation: recovery and repeatability at 0.10 µg/L (LOQ) and 1.0 µg/L level, and specificity. This was done for three types of water: surface water, drinking water and groundwater. The surface water sample was characterised by determination of total hardness, TOC, pH and silt content.

Aliquots of water samples were fortified with the test items. For the determination of all test items, two extraction methods were required. DPX-E2Y45 and degradation products IN-F9NO4, IN-EQW78, IN-GAZ70 and IN-ECD73 were extracted using liquid-liquid extraction. IN-F6L99 was extracted using solid phase extraction (SPE). The extracts were analysed, separately, using LC/MS/MS analysis with atmospheric pressure chemical ionisation (APCI). From the data obtained, the validation parameters were determined and tested against the criteria. Two validation runs were performed of which the first validation run (liquid-liquid extraction) was not successful due to low recoveries.

The study was conducted in compliance with study plan P6449 [5] and study plan amendment P6449/A01 [6] and in compliance with the OECD principles of Good Laboratory Practice (GLP) [3].

5 Test system

5.1 Sample

The validation was carried out using three types of water: drinking water (tap water, Zeist), groundwater (Netherlands), surface water (local river/channel, Netherlands). Groundwater en surface water samples were stored at 2-10 °C from April 2005 until use. Groundwater and surface water samples were received from Water Boards. However, no GLP compliance is claimed for the sampling of these water samples. In the event water samples contained sediment, the samples were centrifuged and the water was decanted into a clean container prior to analysis. No filtration or purification was performed before analysis.

5.2 Test items

DPX-E2Y45

Lot	: 100
Purity	: 99.2%
Storage	: Desiccator (room temperature)
Expiration date	: 27 February 2006

IN-ECD73

Lot	: 003
Purity	: 99.8 %
Storage	: Desiccator (room temperature)
Expiration date	: 11 February 2006

IN-EQW78

Lot	: 004
Purity	: 99.8%
Storage	: Desiccator (room temperature)
Expiration date	: 14 January 2007

IN-F9NO4

Lot	: 001
Purity	: 95.6%
Storage	: Desiccator (room temperature)
Expiration date	: 9 July 2006

IN-GAZ70

Lot	: 001
Purity	: 96.0%
Storage	: Desiccator (room temperature)
Expiration date	: 7 July 2006

IN-F6L99

Lot	: 003
Purity	: 97.7%
Storage	: room temperature

Expiration date : 17 March 2006

Any analysis for the identity, quality and purity of each batch of the test substance, with supporting documentation, is the responsibility of the Sponsor.

5.3 Chemicals

Formic acid, purity 98-100 % (Batch No. UN1779, Merck)
Acetonitrile (Batch No. 38172 (run 1), Batch No. 39678 (run 2), Biosolve)
DMSO (Batch No. 32K0063 (run 1), Batch No. 32K6003 (run 2), Sigma)
Ethyl acetate (Batch No. 39562, Biosolve)
n-Hexane, picograde (Batch No. 501403, Promochem)
Methanol (Batch No. 39425, Biosolve)

5.4 Materials

SPE cartridges (OASIS HLB, 6 ml, 0.5 g, Waters).

5.5 Validation sample set

For each type of water the validation set consisted of:

- 1 Reagent Blank (LLE or SPE without water sample)
- 2 control samples (water without analytes)
- 5 samples fortified at LOQ (0.10 µg/L)
- 5 samples fortified at 10 x LOQ (1.0 µg/L)
- 1 control sample fortified post sample purification to assess matrix effects

6 Procedures and methods

6.1 Preparation of individual solutions

6.1.1 Stock solutions DPX-E2Y45, IN-GAZ70, IN-EQW78 and IN-ECD73

Standard stock solutions of each compound were prepared by accurately weighing ca. 10 mg into 10 ml volumetric flasks. The compounds were dissolved in DMSO (concentration approx. 1000 µg/mL).

6.1.2 Diluted stock solutions (100 µg/mL)

100 µg/mL solutions were prepared by accurately pipetting 1.0 mL of the 1000 µg/mL stock standard into a 10 mL volumetric flask. The solutions were diluted to 10.0 ml with acetonitrile. The solutions were stored in the refrigerator (2-10 °C) for a maximum period of 6 months.

6.1.3 Stock solutions IN-F9N04 and IN-F6L99

Standard stock solutions of each compound were prepared by accurately weighing ca. 10 mg into 100 ml volumetric flasks. The compounds were dissolved in acetonitrile (concentration approx. 100 µg/mL). The solutions were stored in the refrigerator (2-10 °C) for a maximum period of 6 months.

6.2 Preparation of multi-analyte standard solutions

6.2.1 1.0 µg/mL multi-analyte intermediate standard

A 1.0 µg/mL multi-analyte intermediate standard was prepared by pipetting 1.0 mL of each 100 µg/mL standard solution into a 100 mL volumetric flask. The solution was filled up to 100 with acetonitrile and mixed. The solution was stored in the refrigerator (2-10 °C) for a maximum period of 6 months.

6.2.2 0.10 µg/mL multi-analyte intermediate standard

A 0.10 µg/mL multi-analyte intermediate standard was prepared by pipetting 1.0 mL of each 1.0 µg/mL intermediate standard solution into a 10 mL volumetric flask. The solution was filled up to 10 mL with acetonitrile and mixed. The solution was stored in the refrigerator (2-10 °C) for a maximum period of 6 months.

6.3 Preparation and stability of calibration solutions

Two sets of calibration solutions were prepared, both containing all 6 test items. For LC/MS/MS analysis of DPX-E2Y45, IN-F9NO4, IN-EQW78, IN-GAZ70, and IN-ECD73 solutions in acetonitrile/aqueous formic acid were used. For LC/MS/MS analysis of IN-F6L99 solutions in aqueous formic acid were used.

The calibration standards were prepared by pipetting the volumes of the 1.0 µg/mL and 0.10 µg/mL DPX-E2Y45, IN-F9NO4, IN-EQW78, IN-GAZ70, IN-ECD73, and IN-F6L99 fortification standard solutions, shown in Table 1, into separate volumetric flasks of 10.0 mL.

Table 1: Preparation calibration standards for LC/MS/MS analysis.

Desired standard concentration (ng/mL)	Volume of 0.10 µg/mL multi-analyte standard (µL)	Volume of 1.0 µg/mL multi-analyte standard (µL)
20	-	200
10	-	100
5	-	50
1	100	-
0.5	50	-

Calibration set 1 (for DPX-E2Y45, IN-F9NO4, IN-EQW78, IN-GAZ70, and IN-ECD73 analysis)
The appropriate amount of 50:50 acetonitrile:0.01 M aqueous formic acid solution was added to dilute to 10.0 mL. The calibration standards were stored in the refrigerator (2-10 °C) for a maximum period of one week.

Calibration set 2 (for IN-F6L99 analysis)
The appropriate amount of 0.01 M aqueous formic acid solution was added to dilute to 10.0 mL. The calibration standards were stored in the refrigerator (2-10 °C) for a maximum period of one week.

The solutions were vortex mixed before transferring into autosampler vials for injection.

6.4 Sample fortification

6.4.1 *Sample fortification for LLE*

Glass containers (bottles or tubes) were filled with aliquots of 50.0 mL of water. Fortification was done to each bottle individually using multi-analyte standards in acetonitrile. To obtain a concentration in water of 0.10 µg/L, 50 µL of 0.10 µg/mL solution was added to the bottles. To obtain a concentration of 1.0 µg/L, 50 µL of 1.0 µg/mL solution was added to the bottles.

6.4.2 *Sample fortification for SPE*

Glass containers (bottles or tubes) were filled with aliquots of 25.0 mL of water. Fortification was done to each bottle individually using multi-analyte standards in acetonitrile. To obtain a concentration in water of 0.10 µg/L, 25 µL of 0.10 µg/mL solution was added to the bottles. To obtain a concentration of 1.0 µg/L, 25 µL of 1.0 µg/mL solution was added to the bottles.

6.5 Control samples

For each type of water two control samples were pre-treated using LLE and SPE to investigate the specificity of the methods. The control samples consisted of blank tap water, blank surface water and blank groundwater (without any analytes).

6.6 Control samples fortified after sample pre-treatment

6.6.1 *Sample fortification after LLE (validation run 1)*
Blank tap water, surface water and groundwater were pre-treated using LLE after which the extract was evaporated to dryness. The extract was reconstituted in 1.0 ml of acetonitrile spiked with ca.50 ng/mL DPX-E2Y45 and degradation products using a vortex and sonication for 5 min. Add 1.0 mL of 0.01 M aqueous formic acid solution. Vortex for 30 seconds.

6.6.2 *Sample fortification after LLE (validation run 2)*
Blank tap water, surface water and groundwater were pre-treated using LLE after which the extract was evaporated to dryness. The extract was reconstituted in 1.0 ml of acetonitrile spiked with ca.20 ng/mL DPX-E2Y45 and degradation products except IN-F6L99 using a vortex and sonication for 5 min. Add 1.0 mL of 0.01 M aqueous formic acid solution. Vortex for 30 seconds.

6.6.3 *Sample fortification after SPE*
Blank tap water, surface water and groundwater were pre-treated using SPE after which the extract was evaporated to dryness. The extract was reconstituted in 0.5 ml of methanol spiked with ca.50 ng/mL DPX-E2Y45 and degradation products using a vortex and sonication for 5 min. Add 1.5 mL of 0.01 M aqueous formic acid solution. Vortex for 30 seconds.

6.7 Extraction and purification procedures

Glassware was scrubbed with a brush using a laboratory soap solution, rinsed two to five times with tap water, rinsed with distilled or deionized water, and finally rinsed with acetone or another suitable solvent and allowed to air dry prior to each use.

Because of the tendency of the analytes to adhere to surfaces when in water, analyte-contaminated glassware, such as stock standard volumetric flasks were not washed in common wash areas. Contaminated glass ware was thoroughly rinsed with acetonitrile prior to following normal glassware cleaning procedures.

6.7.1 *DPX-E2Y45, IN-F9NO4, IN-EQW78, IN-GAZ70 and IN-ECD73*

1. Accurately measure 50.0 mL of water into a glass container. Fortify if necessary. Mix the sample. Transfer into a 250 mL separation funnel.
2. Measure 50 mL of ethyl acetate using the same glass container. Mix the ethyl acetate and transfer it to the same 250 mL separation funnel
3. Measure 50 mL of hexane using the same glass container. Mix the hexane and transfer it to the same 250 mL separation funnel.
   Set aside the glass container to collect the water after the partition step.
4. Mix the separation funnel for approximately 30 seconds by hand and allow the phases to separate. Transfer the water (lower layer) back into the original glass. Transfer the upper layer (hexane/ethyl acetate) into a 250 ml graduated mixing cylinder.
   Care should be taken to avoid any water transferring into the mixing cylinder.

5. Transfer the water layer back into the 250 mL separation funnel. Using the glass container, measure 25 mL of ethyl acetate and mix. Add 25 mL of hexane and transfer the ethyl acetate/hexane into the 250 mL separation funnel.
6. Mix the separation funnel for approximately 30 seconds by hand and allow the phases to separate. Discard the water (lower layer). Transfer the ethyl acetate/hexane phase (upper layer) into the same 250 mL graduated mixing cylinder as used following the first partition.
   Care should be taken to avoid any water transferring into the mixing cylinder.
   Dilute the extract to 150 mL using ethyl acetate and mix the extract by hand.
7. Transfer a 50 mL aliquot of the extract into a glass tube and reduce to dryness using nitrogen gas at 50-55°C. Reconstitute the extract in 1.0 mL of acetonitrile by mixing using a vortex and sonication for 5 min. Add 1.0 mL of 0.01 M aqueous formic acid solution. Vortex for 30 seconds.
8. Using a disposable syringe, filter the resulting solution through a 0.2 µm Acrodisc filter into an autosampler vial and analyze by APCI-LC/MS/MS.

Extracts were stored at 2-10 °C until LC/MS/MS analysis.

6.7.2 IN-F6L99

1. Accurately measure 25 mL of water into a 50 mL glass container. Fortify sample if necessary. Mix the sample. Add 250 µl of glacial acetic acid and mix.
2. Attach a 6 mL, 0.5-g Oasis HLB cartridge [Waters] to an SPE manifold. Precondition the cartridge with 5 mL of methanol followed by 10 mL of purified water.
   Do not let the cartridge go to dryness.
3. Pass the 25 mL sample through the SPE cartridge, discarding the eluate. After the extract has passed through, rinse the glass container with 2x5 mL of purified water which is passed through the cartridges.
   Vacuum may be used to keep a steady slow flow of approximately 2-5 mL/min. Do not allow steady streams.
4. After the sample and the rinses have passed through the cartridges, dry the cartridge using vacuum for 5 minutes.
5. Elute IN-F6L99 by the addition of 30 mL of acetone. Measure acetone using the original glass container. Load the solution onto the cartridge and collect the eluate in a glass tube (approximately 2-3 mL/min).
   Vacuum may be used to maintain the flow rate, no steady streams.
6. Evaporate the extract to dryness using a flow of nitrogen at approximately 50-55°C.
7. Add 0.5 ml of methanol into the tube. Vortex and sonicate for 5 minutes. Pipette 1.5 mL of 0.01 M aqueous formic acid solution into the centrifuge tube and vortex.
8. Using a disposable syringe, filter the resulting solution through a 0.2 µm Acrodisc filter into an autosampler vial and analyze by APCI-LC/MS/MS.

Extracts were stored at 2-10 °C until LC/MS/MS analysis.

6.8 Analysis

6.8.1 LC/MS/MS analysis for DPX-E2Y45,IN-F9NO4, IN-EQW78, IN-GAZ70 and IN-ECD73

The LC/MS/MS equipment and conditions are given below.

HPLC pump:	Surveyor (ThermoElectron)
Autosampler:	Surveyor (ThermoElectron)
Guard column:	RP-C18, 4 mm x 3 mm i.d., 5 µm (Phenomenex)
LC column:	C18, 150 mm x 4.6 mm i.d., 3 µm (Phenomenex)
Column temp.:	40 °C
Autosampler temp.:	ca. 10 °C
Flow rate:	1.0 mL/min
Mobile phase A:	0.01 M aqueous formic acid
Mobile phase B:	Methanol
Injection volume:	100 µl (first validation run) and 40 µl (second validation run)

Gradient:

Time (min)	Mobile phase A	Mobile phase B
0	40	60
0.5	40	60
2.0	20	80
5.0	2	98
8.0	2	98
8.1	40	60
12.0	40	60

MS/MS:	TSQ Quantum (ThermoElectron)	
Interface :	APCI	
Ion mode :	positive	
Ions monitored:	DPX-E2Y45	484.000 → 285.827
		484.000 → 452.878
	IN-F9N04	470.020 → 452.808
		470.020 → 285.804 (not used for quantification)
	IN-GAZ70	450.100 → 306.974
		450.100 → 413.921
	IN-EQW78	466.100 → 75.800
		466.100 → 187.944
		466.100 → 207.014
	IN-ECD73	244.250 → 209.046 (not used for quantification)
		279.085 → 209.037 (not used for quantification)
		279.085 → 243.986

6.8.2 LC/MS/MS analysis for IN-F6L99

The LC/MS/MS equipment and conditions are given below.

HPLC pump:	Surveyor (ThermoElectron)
Autosampler:	Surveyor (ThermoElectron)
Guard column:	RP-C18, 4 mm x 3 mm i.d., 5 µm (Phenomenex)
LC column:	C18, 150 mm x 4.6 mm i.d., 3 µm (Phenomenex)

Column temp.: 40 °C
Autosampler temp.: ca. 10 °C
Flow rate: 1.0 mL/min
Mobile phase A: 0.01 M aqueous formic acid
Mobile phase B: Methanol
Injection volume: 75 µl

Gradient:

Time (min)	Mobile phase A	Mobile phase B
0	90	10
0.5	90	10
5.5	20	80
5.8	10	90
8.8	10	90
9.0	90	10
12.0	90	10

MS/MS: TSQ Quantum (ThermoElectron)
Interface: APCI
Ion mode: positive
Ions monitored: IN-F6L99 204.013 → 66.123 (not used for quantification)
204.013 → 172.891

6.9 Quantification

Quantification was performed using the average response factor of the standards analyzed concurrently with the samples.

The Average Response Factor (RF_{Ave}) was calculated as follows:

$$RF_{Ave} = \frac{(\text{Conc. A} \div \text{Area A}) + (\text{Conc. B} \div \text{Area B}) + (\text{Conc. X} \div \text{Area X})}{\text{total number of standards injected}}$$

µg/L found was calculated as follows:

$$\mu\text{g/L Found} = \frac{(\text{Peak Area}) \times (RF_{Ave}) \times (\text{Aliquot Factor}) \times (\text{Final Volume})}{(\text{Sample Volume})}$$

The percent recovery found was calculated as follows:

$$\% \text{ Recovery} = \frac{(\text{ppb Found})}{(\text{Fortification level})} \text{ x } 100$$

6.10 Acceptance criteria

The acceptation criteria were based on the EU guidance documents on residue analytical methods [2, 4].

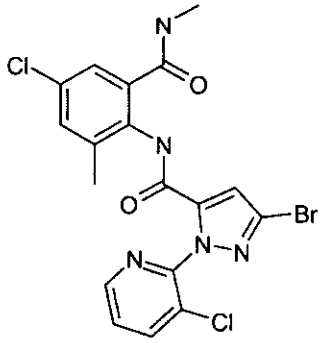
6.11 Deviations from the study plan

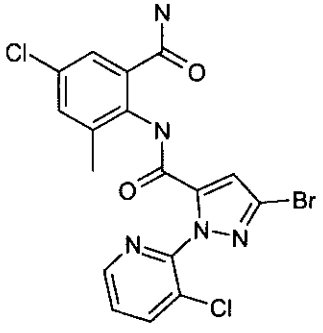
- Study plan amendment P6449/A01 [6] did not clearly state whether Frank van Schaik or Brigitte Buscher would be Study Director after 19 September 2005. Frank van Schaik has been Study Director from 29 August 2005 until 19 September 2005.

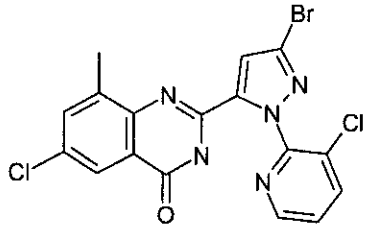
- On the request of the sponsor, in the second validation run 40 µl of the extracts was injected into the LC-MS system (instead of 100 µl).

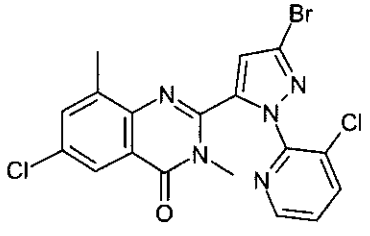
Appendix A

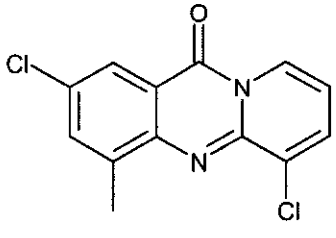
Structure and properties of DPX-E2Y45 and metabolites

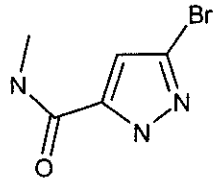
Structure	
DPX Number	DPX-E2Y45
CAS Chemical Name	3-Bromo-*N*-[4-chloro-2-methyl-6-[methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide
CAS Number	500008-45-7
Formula	$C_{18}H_{14}BrCl_2N_5O_2$
Molecular Weight	483.1511
Monoisotopic Weight	480.9708

Common Name	None
Structure	
DPX Number	IN-F9NO4
Formula	C17H12BrCl2N5O2
Molecular Weight	469.1242
Monoisotopic Weight	466.9581

Common Name	None
Structure	
DPX Number	IN-GAZ70
Formula	$C_{17}H_{10}BrCl_2N_5O$
Molecular Weight	451.1089
Monoisotopic Weight	448.9446

Common Name	None
Structure	
DPX Number	IN-EQW78
Formula	$C_{18}H_{12}BrCl_2N_5O$
Molecular Weight	465.1358
Monoisotopic Weight	462.9602

Common Name	None
Structure	
DPX Number	IN-ECD73
Formula	$C_{13}H_8Cl_2N_2O$
Molecular Weight	279.1248
Monoisotopic Weight	278.0014

Common Name	None
Structure	
DPX Number	IN-F6L99
Formula	$C_5 H_6 Br N_3 O$
Molecular Weight	204.0263
Monoisotopic Weight	202.9694