2 Introduction

The purpose of this study was to validate an analytical method supplied by the Sponsor for the determination of residues of DPX-E2Y45 and its metabolites in soil. The method (DuPont-10814) includes two extraction procedures. The first procedure involves extraction of readily extractable residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in soil. The second procedure involves extraction of more difficult to extract residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in soil.

All work was conducted in accordance with EU Guidelines 91/414/EEC and SANCO/825.00 revision 7, "Guidance Document on Residue Analytical Methods".

The analytical work described in this report was conducted in the Department of Bioanalytical Chemistry, Inveresk, Tranent, EH33 2NE, Scotland, according to the following schedule:

Study Initiation Date:	14 May 2003
Experimental Start Date:	14 May 2003
Experimental Completion Date:	12 August 2004
Study Completion Date:	See Authentication page for date of Study Director's signature

Experiments were conducted in accordance with Inveresk Protocol No. 303358 and Amendments 1-9.

All data generated and recorded during this study, including a copy of the final report, will be shipped to E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, DE 19714-0030, USA for archiving under GLP conditions*. Certified copies of all data generated and recorded during this study will be stored in the Scientific Archives of Inveresk for 5 years after issue of the final report. At the end of the 5 year period the Sponsor will be consulted regarding the disposal, transfer or continued storage of copied data.

*This excludes site-specific data, which will be maintained at the site where the work was performed.

3 Experimental Procedure

3.1 Materials

3.1.1 Analytical Standards

The analytical standard, DPX-E2Y45 (Batch No's. DPX-E2Y45-25 and 100) was received from the Sponsor on 07 April 2003 and 24 June 2003 respectively. The DPX-E2Y45 standard was a white neutral powder with purity values of 99.0% and 99.2% and expiry dates of 04 February 2003 and 27 February 2005 respectively. DPX-E2Y45-25 was used for analysis until 01 August 2003.

The analytical standard, IN-EQW78 (Batch No. 002) was received from the Sponsor on 07 April 2003 and 30 June 2003. The IN-EQW78 standard was an off white neutral powder with a purity of 97.2% and an expiry date of 10 February 2005.

The analytical standard, IN-ECD73 (Batch No. 003) was received from the Sponsor on 07 April 2003 and 30 June 2003. The IN-ECD73 standard was a bright yellow powder with a purity of 99.8% and an expiry date of 11 February 2005.

The analytical standard, IN-F6L99 (Batch No. 003) was received from the Sponsor on 18 April 2003. The IN-F6L99 standard was a white neutral powder with a purity of 97.7% and an expiry date of 17 March 2005.

The analytical standard, IN-GAZ70 (Batch No. 001) was received from the Sponsor on 27 February 2004 and 29 March 2004. The IN-GAZ70 standard was tan neutral granules with a purity of 96.0% and an expiry date of 07 July 2004. The expiry date was updated to 07 July 2006 after recertification.

The analytical standards were stored in the dark at ambient room temperature when not in use.

Copies of each of the Certificates of Analysis for all analytical standards are presented in Appendix 1.

3.1.2 Control Samples

3.1.2.1 Control Soil Used in Phase 1

Control soil was obtained by Inveresk from the following sources:

Inveresk Project No.	DuPont Reference No.	Location of Trial
682634	12787	Seville, Spain
683287	12792	Alsace, France
683271	12791	Burgundy, France
683292	12793	Bologna, Italy

The soil was stored at *ca* -20°C or ambient temperature.

3.1.2.2 Control Soil Used in Phase 2

Control soil was obtained by Inveresk from the following sources:

Inveresk Project No.	DuPont Reference No.	Location of Trial
682634	12787	Seville, Spain
683271	12791	Burgundy, France
684694	14441	Lleida, Spain

The soil was stored at *ca* -20°C or ambient temperature.

3.2 Analytical Methodology

The method of analysis was supplied by the Sponsor (DuPont-10814).

The Inveresk validated versions are presented in Appendices 2 and 3 in IS0 78/2 format. The analysis of unknown soil samples should be completed using the procedure described in Appendix 3.

The methods include a conventional extraction procedure for the determination of readily extractable residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 and exhaustive extraction procedures for the determination of more difficult to extract residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70.

The conventional extraction procedure involves extraction using an acetonitrile:aqueous formic acid solution (90:10, v/v). An aliquot from the extract is removed and cleaned up using Waters Oasis HLB SPE cartridges (analysis of IN-F6L99). The remaining extract is cleaned up using SAX SPE cartridges and Waters Oasis HLB SPE cartridges (analysis of DPX-E2Y45, IN-EQW78 and IN-ECD73). The final extracts are quantified by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

The exhaustive extraction procedure involves extraction using acetonitrile:aqueous formic acid solution (90:10, v/v) and acetonitrile:hydrochloric acid solution (99:1, v/v and 95:5, v/v). An aliquot is removed and cleaned up using SAX SPE cartridges and Waters Oasis HLB SPE cartridges. The final extracts are quantified for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

The conventional extraction procedure and exhaustive extraction procedure were validated with respect to system suitability and linearity, assay accuracy and precision, matrix effects and assay specificity.

3.3 Method Validation

During Phases1 and 2 of this study, the experimental procedure followed for the exhaustive extraction method was identical. The only experimental difference was that an additional compound (IN-GAZ70) was added to the procedure. Analytical standards were therefore prepared for IN-GAZ70 during Phase 2 and the appropriate ions were

monitored for IN-GAZ70 during the LC-MS/MS analysis for Phase 2. The exhaustive procedure, complete with analysis for IN-GAZ70, is the proposed method for monitoring DPX-E2Y45 and metabolites in soil.

3.3.1 System Suitability and Linearity

During Phase 1 (validation of the conventional and exhaustive extraction procedure) mixed standard solutions of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 were prepared as detailed in Appendix 2 (Sections 5.4.4 and 5.4.5). Standards were prepared over the range of *ca* 0.1 to 25 ng/mL for DPX-E2Y45, IN-EQW78 and IN-ECD73 and *ca* 0.05 to 10 ng/mL for IN-F6L99.

During Phase 2 (validation of the exhaustive extraction procedure to include IN-GAZ70) mixed standard solutions of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 were prepared as detailed in Appendix 3 (Section 5.4.4). Standards were prepared over the range of *ca* 0.1 to 25 ng/mL for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70.

The standard solutions were injected (25 µL) onto the LC-MS/MS system. The detector response for DPX-E2Y45, IN-EQW78, IN-ECD73, IN-F6L99 and IN-GAZ70 was plotted against the nominal concentration to generate a calibration curve. Calculated concentrations of the injected standards were determined using least squares linear regression with a weighting factor (1/x), the origin being excluded from the regression analysis. Individual calibration standards should be within ±15% of nominal values across the calibration range and within ±20% at the LLOQ. Calibration standards outside this range should be excluded.

3.3.2 System Precision

The system precision was determined by 10 replicate injections (25 µL) onto the LC-MS/MS system of a *ca* 5 ng/mL mixed standard containing DPX-E2Y45, IN-EQW78 and IN-ECD73, and 10 replicate injections (25 µL) onto the LC-MS/MS system of a *ca* 2 ng/mL IN-F6L99 standard. The system precision for IN-GAZ70 was determined by 10 replicate injections (25 µl) on to the LC-MS/MS system of a ca 5.0 ng/mL mixed standard. The coefficient of variation (precision) was determined by the following equation:

$$\text{CV (precision)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

CV = Coefficient of variation (%)
SD = Standard deviation

3.3.3 Assay Accuracy and Precision

3.3.3.1 Phase 1 - Conventional Extraction Procedure

The accuracy and precision of the conventional method was determined in 4 different soils. These soils are detailed in Section 3.1.2.1. Control samples of each of the 4 soil types were fortified with DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 at the following fortification levels.

Fortification Level (µg/kg)	Replicates
Control	2
0.5 (LOQ)	5
5.0	5
70	3

3.3.3.2 Phase 1 - Exhaustive Extraction Procedure

The accuracy and precision of the exhaustive method was determined in 4 different soils. These soils are detailed in Section 3.1.2.1. For each type of soil the accuracy and precision was determined using soil pellets (obtained from soil extracted using the conventional extraction procedure) and soil which had not been previously extracted. Soil pellets and fresh soil for each of the 4 soil types were fortified with DPX-E2Y45, IN-EQW78 and IN-ECD73 at the following fortification levels.

Fortification Level (µg/kg)	Replicates
Control	2
0.5 (LOQ)	5
5.0	5
70	3

3.3.3.3 Phase 2 - Exhaustive Extraction Procedure

The accuracy and precision of the exhaustive method was determined in 3 different soils. These soils are detailed in Section 3.1.2.2. For each type of soil the accuracy and precision was determined using soil which had not been previously extracted. Fresh soil from Seville and Burgundy were fortified with IN-GAZ70 at the fortification levels shown below. Fresh soil from Lleida was fortified with DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 at the fortification levels shown below.

Fortification Level (µg/kg)	Replicates
Control	2
0.5 (LOQ)	5
5.0	5
70	3

3.3.3.4 Acceptable Method Performance Criteria

Each sample was analysed following Inveresk Method Nos. 0335 A or B (Appendices 2 and 3).

The recovery rate was defined as the ratio of the determined concentration over the *actual concentration (expressed as a percentage). The criteria for acceptance was that* for each fortification level the mean recovery was between 70-110%, with 80 % or greater of the individual recoveries between 70-110%. The acceptance criteria for the coefficient of variation (relative standard deviation) of the mean determined concentration was ≤ 20%.

3.3.4 Matrix Effects

3.3.4.1 Phase 1 - Conventional Extraction Procedure

In order to determine matrix effects, control extracts from each of the 4 soil types detailed in Section 3.1.2.1 were fortified at *ca* 5 µg/kg (equivalent to 10 x LOQ concentration) and analysed on the LC-MS/MS system as per the method in Appendix 2.

3.3.4.2 Phase 1 - Exhaustive Extraction Procedure

In order to determine matrix effects, control extracts from soil pellets and fresh soil for each of the 4 soil types detailed in Section 3.1.2.1 were fortified at *ca* 5 µg/kg (equivalent to 10 x LOQ concentration) and analysed on the LC-MS/MS system as per the method in Appendix 2.

3.3.4.3 Phase 2 - Exhaustive Extraction Procedure

In order to determine matrix effects, control extracts from fresh soil for each of the 3 soil types detailed in Section 3.1.2.2, were fortified at *ca* 5 µg/kg (equivalent to 10 x LOQ concentration) and analysed on the LC-MS/MS system as per the method in Appendix 3.

The criteria for acceptance for all matrix effect samples was that the response for the samples would be within the range of 85 -115%.

3.3.5 Assay Specificity

3.3.5.1 Phase 1 - Conventional Extraction Procedure

Control samples from each of the 4 soil types, detailed in Section 3.1.2.1, were analysed following Analytical Method No. 0335A described in Appendix 2. A minimum of 2 control chromatograms were examined for possible interferences at the retention times of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99. The method was considered to be specific if residues detected did not exceed one third of the LOQ.

3.3.5.2 Phase 1 - Exhaustive Extraction Procedure

Control soil pellets and control fresh soil for each of the 4 soil types detailed in Section 3.1.2.1, were analysed following Analytical Method No. 0335A described in Appendix 2. A minimum of 2 control chromatograms were examined for possible interferences at the retention times of DPX-E2Y45, IN-EQW78 and IN-ECD73. The method was considered to be specific if residues detected did not exceed one third of the LOQ.

3.3.5.3 Phase 2 - Exhaustive Extraction Procedure

Control fresh soil from each of the 3 soil types detailed in Section 3.1.2.2 were analysed following Analytical Method No. 0335B described in Appendix 3. A minimum of 2 control chromatograms were examined for possible interferences at the retention times of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70. The method was considered to be specific if residues detected did not exceed one third of the LOQ.

3.3.6 Validation of Extraction Procedure for Analysis of Petri Dishes Without Soil

A method for the extraction of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 from petri dishes containing no soil was validated.

The analytical method for the analysis of petri dish samples without soil involves extraction with acetonitrile:aqueous formic acid (90:10,v/v). The petri dishes were placed in plastic pots (complete with sealing lids), submerged in extraction solvent and sonicated. The samples were shaken by hand and diluted within the calibration range (using theoretical recovery of 100%). The final extracts were then quantified for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 by liquid chromatography with

tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

The accuracy and precision of the analytical method was determined by the extraction of four control glass petri dishes fortified with DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 at 50 µg/dish and four control glass petri dishes fortified with DPX-E2Y45 only, at 600 µg/dish.

The specificity of the method was established by extraction of 2 unfortified control glass petri dishes.

3.3.7 Acceptable Method Performance Criteria

The samples were analysed following Section 8.3 of Inveresk Method No. 0335 B (Appendix 3).

The recovery rate was defined as the ratio of the determined concentration over the actual concentration (expressed as a percentage). The criteria for acceptance was that for each fortification level the mean recovery was between 70-110%, with 80 % or greater of the individual recoveries between 70-110%. The acceptance criteria for the coefficient of variation (relative standard deviation) of the mean determined concentration was ≤ 20%.

The method was considered to be specific if there were no interferences at the *retention times of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 which exceeded* one third of the LOQ.

3.3.8 Homogenisation Procedure

The homogenisation procedure was validated for each of the soils detailed in Section 3.1.2.1 and the Lleida soil (Inveresk Project No. 684694) detailed in Section 3.1.2.2. The soil from each location was assessed to determine the most appropriate homogenisation procedure. Soil from depths 0-5, 5-15, 15-30, 30-50, 50-70 and 70-90 cm were assessed (with the exception of Lleida soil since only soil from the 15-30 cm depth was supplied by the Sponsor for the homogenisation test). Depending upon the characteristics of the soil (clay content, moisture content *etc*) different techniques were applicable. Dry samples could be mixed by hand whilst wet samples needed to be air-dried and then sieved to attain a homogenous sample.

Once the most appropriate method had been established, 5 cores (or 6 cores, if appropriate) from the selected horizon were weighed and a known amount of Evans Blue dye was mixed through one of the cores using a Hobart Bowl Cutter to produce a test core containing blue dye. The sample was homogenised as appropriate. Six sub-samples (*ca* 10 g) were taken from the composite sample and the Evans Blue dye was extracted with distilled water and quantified using a spectrophotometer.

The precision of the homogenisation procedure was determined by calculating the percentage coefficient of variation (CV,%) for the absorbances obtained. The

homogenisation procedure used was considered acceptable if the percentage coefficient of variation was ≤20%.

3.3.9 Homogenisation Procedure for Large Soil Samples

A homogenisation procedure was also validated for large soil samples (samples exceeding 1 kg). A composite control soil sample from DuPont 14330 (Inveresk Project No. 206928, site located in Lleida) consisting of replicate cores from a depth of 0-20 cm was used to validate the procedure. Since the soil from Inveresk Project No. 684694 (site located in Lleida) had already been assessed it was recognised that soil should be air dried and then sieved. Due to the weight of the sample, (approximately 5 kg) procedures which involved sieving only a portion of the sample were tested.

Approximately 1 kg of the composite sample was removed and a known amount of Evans Blue dye was added to this aliquot and mixed well using a Hobart Bowl Cutter to produce a sub-sample containing blue dye. The sub-sample containing blue dye was then mixed well by hand and *ca* 0.5 kg and 1.0 kg aliquots were removed and sieved using a sieve with a mesh size of 4 mm. Further aliquots of *ca* 0.5 kg and 1.0 kg were taken from the composite sample and sieved using a sieve with a mesh size of 2 mm. Six sub-samples (*ca* 10 g) were taken from each sieved sample and the Evans Blue dye was extracted with distilled water and quantified using a spectrophotometer.

The precision of the homogenisation procedure was determined by calculating the percentage coefficient of variation (CV,%) for the absorbances obtained. The homogenisation procedure used was considered acceptable if the percentage coefficient of variation was ≤20%.

3.3.10 Data Calculations

Data manipulations carried out during this study were performed using Analyst Versions 1.1 and 1.3.

Appendix 2 Analytical Method No. 0335A Determination of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in Soil

ASSAY:	DPX-E2Y45, IN-EQW78, IN-ECD73, IN-F6L99	ANALYTICAL METHOD No.: 0335A Version 1
MATRIX:	Soil	ISSUED: 29 January 2004 APPROVAL: Gwen C Syme

1 Scope

Analytical method no. 0335A is suitable for the determinatior of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in soil.

2 Field Application

The method described is suitable for the determination of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in soil over the range of 0.5 to 70 µg/kg.

3 Definitions

Reference Material: DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 analytical standards.

Control Sample: Soil known to be free from DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 residues above one third of the limit of quantification (LOQ).

Fortified Sample: Control soil to which a known amount of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 has been added.

4 Principle

The determination of DPX-E2Y45 and its metabolites is carried out using two extraction procedures (conventional and exhaustive). The conventional extraction procedure is used for the determination of readily extractable residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 and the exhaustive extraction procedure is used for the determination of more difficult to extract residues of DPX-E2Y45, IN-EQW78 and IN-ECD73.

The conventional extraction procedure involves extraction using an acetonitrile:aqueous formic acid 90:10 v/v solution. An aliquot from the extract is removed and cleaned up using Waters Oasis HLB SPE cartridges (analysis of IN-F6L99). The remaining extract is cleaned up using SAX SPE cartridges and Waters Oasis HLB SPE cartridges (analysis of DPX-E2Y45, IN-EQW78 and IN-ECD73). The final extracts are quantified by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

Appendix 2
(continued)

The exhaustive extraction procedure involves extraction using acetonitrile:aqueous formic acid (90:10, v/v) and acetonitrile:hydrochloric acid (99:1, v/v and 95:5, v/v) solutions. An aliquot is removed and cleaned up using SAX SPE cartridges and Waters Oasis HLB SPE cartridges. The final extracts are quantified for DPX-E2Y45, IN-EQW78 and IN-ECD73 by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

5 Reagents and Materials

5.1 General

The following reagents were used for the validation of the analytical method. Alternative suppliers and grades of these reagents may be employed, provided adequate analytical sensitivity and resolution are achieved.

Water	HPLC	BDH
Formic Acid (98/100%)	Analytical	Fisher
Hydrochloric Acid (32%)	Analytical	Fisher
Methanol	HPLC	Rathburn
Ethyl Acetate	HPLC	Rathburn
Acetonitrile	HPLC	Rathburn
Dimethylsulfoxide	Analytical	Fisher

Standard laboratory safety precautions should be adhered to when using reagents and materials described in this method. Laboratory coats and safety glasses must be worn at all times.

5.2 Materials

The DPX-E2Y45 analytical standard, Batch Nos. DPX-E2Y45-25 and 100, were received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had expiry dates of 04 February 2003 and 27 February 2005, respectively.

The IN-EQW78 analytical standard, Batch No. 002, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 10 February 2005.

The IN-ECD73 analytical standard, Batch No. 003, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 11 February 2005.

The IN-F6L99 analytical standard, Batch No. 003, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 17 March 2005.

Appendix 2
(continued)

Control soil was obtained by Inveresk from the following sources:

Inveresk Project No.	DuPont Reference No.	Location of Trial
682634	12787	Seville, Spain
683287	12792	Alsace, France
683271	12791	Burgundy, France
683292	12793	Bologna, Italy

5.3 Supplier Details

The following provides further details of our materials and equipment suppliers. All are located in the UK unless stated otherwise.

1. BDH Chemicals Limited, Poole, Dorset, BH15 1TD.
2. Applied Biosystems, Warrington, Cheshire, WA3 7QH.
3. Fisher Chemicals, Loughborough, Leicestershire, LE11 0RG.
4. Jouan Limited, Ilkeston, Derbyshire, DE7 4RA.
5. Mettler Toledo, Beaumont Leys, Leicestershire, LE4 1AW.
6. Perkin Elmer, Beaconsfield, Buckinghamshire, HP9 1QA.
7. Rathburn Chemicals Limited, Walkerburn, Scotland, EH43 6AU.
8. Techne Cambridge Limited, Duxford, Cambridge, CB2 4PZ.
9. IKA, supplied by Fisher Scientific, Loughborough, Leicestershire, LE11 0RG.
10. Buchi, supplied by Fisher Scientific, Loughborough, Leicestershire, LE11 0RG.
11. Zymark, Runcorn, Cheshire, WA7 3AZ.
12. Decon Laboratories, Hove, East Sussex, B3 3LY.
13. Phenomenex UK Limited, Macclesfield, Cheshire, SK10 2BN.
14. Waters, Watford, Hertfordshire, WD1 8YW.
15. Varian, Southgate, London, N14 6JS.

Appendix 2
(continued)

5.4 Preparation of Reagents

5.4.1 Solutions

5.4.1.1 0.01 M Aqueous Formic Acid

Dilute 460 mg of concentrated formic acid (98/100%) to 1 L in a volumetric flask using HPLC grade water. Store solution at room temperature and use within one week of preparation.

5.4.1.2 0.2% Aqueous Formic Acid Solution

Add 2 mL of concentrated formic acid (98/100%) to a 1L volumetric flask and make to volume with HPLC grade water. Store solution at room temperature and use within one week of preparation.

5.4.1.3 0.2% Formic Acid Extraction Solvent

Combine 100 mL of 0.2% aqueous formic acid solution with 900 mL of acetonitrile in a 1L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.1.4 1% Hydrochloric Acid Extraction Solution

Dilute 5 mL of concentrated hydrochloric acid (32%) to 500 mL in a volumetric flask using acetonitrile. Store solution at room temperature and use within one week of preparation.

5.4.1.5 5% Hydrochloric Acid Extraction Solution

Dilute 25 mL of concentrated hydrochloric acid (32%) to 500 mL in a volumetric flask using acetonitrile. Store solution at room temperature and use within one week of preparation.

5.4.1.6 SAX Conditioning Solution

Combine 300 mL of acetonitrile, 700 mL of HPLC grade water and 5.0 mL of concentrated formic acid in a 1L Duran bottle. Store solution at room temperature and use within one week of preparation.

Appendix 2
(continued)

5.4.1.7 Injection Solvent A

Combine 500 mL of 0.01M aqueous formic acid with 500 mL of acetonitrile in a 1 L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.1.8 Injection Solvent B

Combine 900 mL of 0.01M aqueous formic acid with 100 mL of acetonitrile in a 1 L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.2 Calibration Standard Solution

Accurately weigh approximately 10 mg of DPX-E2Y45 and dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-EQW78, dissolve in 5 mL of dimethylsulfoxide and then dilute to 10 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 1000 µg/mL. Further dilute IN-EQW78 *ca* 1000 µg/mL to give a stock calibration standard solution of *ca* 100 µg/mL by accurately pipetting 1 mL of *ca* 1000 µg/mL solution into a 10 mL volumetric flask. Dilute to 10 mL with acetonitrile and mix well.

Accurately weigh approximately 10 mg of IN-ECD73, dissolve in 8 mL of dimethylsulfoxide and then dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

Accurately weight approximately 10 mg of IN-F6L99 and dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

5.4.3 Preparation of Mixed Calibration Standard Solutions

Accurately pipette 1.0 mL of each of the *ca* 100 µg/mL standards from Section 5.4.2 into a 100 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed stock calibration standard solution of *ca* 1.0 µg/mL.

Accurately pipette 1.0 mL of the *ca* 1.0 µg/mL mixed stock calibration standard solution into a 10 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed stock calibration standard solution of *ca* 0.1 µg/mL. This dilution scheme and subsequent dilutions are presented in Sections 5.4.4 and 5.4.5.

Appendix 2
(continued)

5.4.4 Dilution of Calibration Standards for DPX-E2Y45, IN-EQW78 and IN-ECD73

Dilutions are prepared from the mixed stock calibration standard solutions as detailed in Section 5.4.3 using injection solvent A to give mixed calibration standards ranging from 0.1 to 25 ng/mL for DPX-E2Y45, IN-EQW78 and IN-ECD73, as detailed below:

Standard Reference	Volume Taken (mL)	Solution Used	Final Volume (mL)	Solution Concentration (ng/mL)
A	-	-	-	100000 (1000000*)
B**	1.0	A	10	100000
C	1.0	A	100	1000
D	1.0	C	10	100
E	0.250	C	10	25
F	0.100	C	10	10
G	0.050	C	10	5
H	0.200	D	10	2
I	0.100	D	10	1
J	0.050	D	10	0.5
K	0.025	D	10	0.25
L	0.100	F	10	0.1

* Concentration of calibration standard stock IN-EQW78 only.
**This dilution step refers to IN-EQW78 only.

Solvent for Std A:	Acetonitrile (DPX-E2Y45) and dimethylsulphoxide and acetonitrile (IN-EQW78 and IN-ECD73)
Solvent for Std B-D:	Acetonitrile
Solvent for Std E-L:	Injection Solvent A
Expiration for Std A:	3 months after preparation (IN-EQW78 and IN-ECD73) and 6 months after preparation (DPX-E2Y45)***
Expiration for Std B-D:	6 months after preparation***
Expiration for Std E-L:	One week after preparation
Storage Conditions:	*ca* +4°C for all solutions

*** Note: the expiry date has since been revised to 3 months from preparation. The standards used throughout the validation were assigned with expiry dates of 6 months and were considered to be suitable for use for this study.

Appendix 2
(continued)

5.4.5 Dilution of Calibration Standards for IN-F6L99

Dilutions are prepared from the mixed stock calibration standard solution as detailed in Section 5.4.3 using injection solvent B to give mixed calibration standards ranging from 0.05 to 10 ng/mL for IN-F6L99 as detailed below:

Standard Reference	Volume Taken (mL)	Solution Used	Final Volume (mL)	Solution Concentration (ng/mL)
A	-	-	-	100000
B	1.0	A	100	1000
C	1.0	B	10	100
D	0.100	B	10	10
E	0.050	B	10	5.0
F	0.020	B	10	2.0
G	0.100	C	10	1.0
H	0.050	C	10	0.5
I	0.025	C	10	0.25
J	0.010	C	10	0.1
K	0.100	E	10	0.05

Solvent for Std A-C: Acetonitrile
Solvent for Std D-K: Injection Solvent B
Expiration for Std A: 6 months after preparation***
Expiration for Std B-C: 6 months after preparation***
Expiration for Std D-K: One week after preparation
Storage Conditions: *ca* +4°C for all solutions

*** Note: the expiry date has since been revised to 3 months from preparation. The standards used throughout the validation were assigned with expiry dates of 6 months and were considered to be suitable for use for this study.

5.4.6 Quality Control Standard Solution

Accurately weigh approximately 10 mg of DPX-E2Y45 and dilute to 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-EQW78, dissolve in 5 mL of dimethylsulfoxide and then dilute to 10 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 1000 µg/mL. Further dilute IN-EQW78, *ca* 1000 µg/mL to give a stock quality control standard solution of *ca* 100 µg/mL. Accurately pipette 1 mL of *ca* 1000 µg/mL solution into a 10 mL volumetric flask. Dilute to 10 mL with acetonitrile and mix well.

Appendix 2
(continued)

Accurately weigh approximately 10 mg of IN-ECD73 and dissolve in *ca* 8 mL of dimethylsulfoxide then dilute to a final volume of 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-F6L99 and dilute to 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

5.4.7 Preparation of Mixed Quality Control Standard Solutions

Accurately pipette 1.0 mL of each of the *ca* 100 µg/mL quality control standards from Section 5.4.6 into a 100 mL volumetric flask and dilute to volume using acetonitrile.

Mix well to give a mixed stock quality control standard solution of *ca* 1.0 µg/mL. The resulting solution has an expiration date of 6 months if stored at *ca* +4°C.

Accurately pipette 1.0 mL of the mixed stock quality control standard solution of *ca* 1.0 µg/mL into a 10 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed quality control standard solution of *ca* 0.1 µg/mL. The resulting solution has an expiration date of 6 months if stored at *ca* +4°C.

6 Apparatus

The following items may be substituted with components of equivalent specification and performance:-

Laboratory Glassware:	Standard Analytical Glassware, Fisher
Centrifuge:	Jouan GR422
TurboVap:	Zymark TurboVap 11
Driblock:	Techne DB3A
SPE Tank:	Isolute VacMaster
Vacuum Pump :	*Vacuubrand ME4C*
Vortex Mixer:	Fisons
Ultrasonic Bath:	Decon FS4006
Flat Bed Shaker:	IKA Labortecnik HS 250
Bowl Cutter:	Hobart FP61

Appendix 2
(continued)

Balances:	Mettler PM300, PE3000, AG245
Water Bath:	Buchi B-490
Solid Phase Cartridges:	Oasis HLB 1 g/20 cc (Waters, PN 186000117) SAX Mega Bond Elut 1 g/6 mL (Variar, PN 1225-6013)

LC-MS/MS System:

HPLC Pump:	Perkin Elmer (Series 200)
*Autosampler:	Perkin Elmer (Series 200)
Cooling Unit:	Perkin Elmer (Peltier)
*Autosampler:	CTC Analytics (HTS PAL)
Column Oven:	Perkin Elmer (Series 200)
Degasser:	Perkin Elmer (Series 200)
Mass Spectrometer:	Applied Biosystems API 4000 with APCI Source
Data System:	Analyst Versions 1.1 and 1.3.

* Note: 2 different autosamplers were used throughout this study

7 *Sampling and Samples*

7.1 Test Sample

Soil sample free from DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99.

7.2 Test Portion

For the conventional extraction procedure and the exhaustive extraction procedure (fresh samples), accurately weigh 10 g of soil into a 250 mL plastic bottle.

For the exhaustive extraction procedure (pellet), use fresh soil samples (10 g) which have previously been extracted using the conventional extraction procedure (pellet) generated from Sections 8.1.1 to 8.1.5.

7.3 Blank Test

In each batch of samples analysed at least 1 test sample should be a control sample.

Appendix 2
(continued)

7.4 Fortified Samples

Conventional Extraction Procedure

To verify the accuracy and precision of the method on each analytical occasion, a minimum of one fortified recovery sample for every 10 samples in the batch (at least one of these should be at the LOQ) should be analysed.

To 10 g test portions of soil, QC standard solutions should be added as follows.

Recovery Level (µg/kg)	Fortification
0.5 (LOQ)	50 µL of *ca* 0.1 µg/mL mixed QC Standard
5.0	50 µL of *ca* 1.0 µg/mL mixed QC Standard
70	700 µL of *ca* 1.0 µg/mL mixed QC Standard

After fortification, samples should be allowed to air dry in a fumehood for 10 min to allow fortification solvent to evaporate before being analysed as detailed in Section 8.

Exhaustive Extraction Procedure

To verify the accuracy and precision of the method on each analytical occasion, a minimum of one fortified recovery sample for every 10 samples in the batch (at least one of these should be at the LOQ) should be analysed.

To 10 g test portions of soil, QC standard solutions should be added as shown below:

Recovery Level (µg/kg)	Fortification
0.5 (LOQ)	50 µL of *ca* 0.1 µg/mL mixed QC Standard
5.0	50 µL of *ca* 1.0 µg/mL mixed QC Standard
70	700 µL of *ca* 1.0 µg/mL mixed QC Standard

The fortified samples should then be analysed as detailed Section 8.

8 Procedure

8.1 Conventional Extraction Procedure

8.1.1 Add 10 mL of HPLC grade water to the sample and shake vigorously by hand for *ca* 30 s. Then place in the refrigerator for *ca* 18 h at ca +4°C.

8.1.2 Remove the sample from the refrigerator and add 50 mL of 0.2% formic acid extraction solvent. Cap and shake at *ca* 300 motions/min cn a flat bed shaker for *ca* 60 min.

Appendix 2
(continued)

8.1.3 Centrifuge sample at *ca* 3000 r.p.m. for 10 min.

8.1.4 Decant the supernatant into a 250 mL graduated cylinder with stopper. If preparing extracted control samples for use in the exhaustive extraction procedure, the supernatant can be discarded.

8.1.5 Repeat steps 8.1.2 and 8.1.3, combining extracts in the same 250 mL graduated cylinder. Save the remaining pellet for optional use in the exhaustive extraction procedure.

8.1.6 Adjust the volume of each extract to 120 mL with the 0.2% formic acid extraction solvent and mix the extract thoroughly.

8.1.7 Pipette 40 mL of the extract into a 50 mL centrifuge tube. This aliquot will be used for the analysis of IN-F6L99 (Aliquot B).

8.1.8 Dilute the remaining 80 mL to 220 mL using HPLC grade water. This aliquot will be used for the analysis of DPX-E2Y45, IN-EQW78 and IN-ECD73 (Aliquot A).

8.2 Exhaustive Extraction Procedure

8.2.1 Add 25 mL of 0.2% formic acid extraction solvent to the sample and shake vigorously by hand. Place sample on flat bed shaker for 15 min at ca 300 motions/min.

8.2.2 Centrifuge the sample at *ca* 3000 r.p.m. for 10 min and decant the supernatant into a 250 mL graduated mixing cylinder with stopper.

8.2.3 Add 25 mL of 1% hydrochloric acid extraction solution to the sample and shake vigorously by hand. Place sample on flat bed shaker for 15 min at *ca* 300 motions/min.

8.2.4 Place sample in a water bath set to *ca* 60°C for 30 min.

8.2.5 Remove sample from water bath and slowly open cap to release any pressure. Centrifuge sample at *ca* 3000 r.p.m. for 10 min. Decant the supernatant into the same 250 mL graduated mixing cylinder with stopper.

8.2.6 Add 50 mL of 5% hydrochloric acid extraction solution to the sample and shake vigorously by hand. Place sample on flat bed shaker for 5 min at *ca* 300 motions/min.

8.2.7 Place sample in a water bath set at *ca* 60°C for 1 h.

Appendix 2
(continued)

8.2.8 Remove sample from water bath and slowly open cap to release any pressure. Place sample on flat bed shaker for 10 min at *ca* 300 motions/min.

8.2.9 Centrifuge the sample at 3000 r.p.m. for 10 min and decant the supernatant into the same 250 mL graduated mixing cylinder.

8.2.10 Adjust the volume of each extract to 100 mL with 1% hydrochloric acid extraction solution and mix the extract thoroughly.

8.2.11 Pipette 35 mL aliquot into 250 mL graduated mixing cylinder with stopper, make up to 115 mL with HPLC grade water and mix thoroughly.

8.3 Analyte Purification Procedure for Conventional Extraction Procedure (Aliquot A - DPX-E2Y45, IN-EDQW78 and IN-ECD73) and Exhaustive Extraction Procedure (fresh and pellet).

8.3.1 Condition Oasis HLB cartridge (1 g/20 cc) with 5 mL of methanol followed by 20 mL HPLC grade water (do not let cartridges go to dryness).

8.3.2 Condition SAX cartridge (1 g/6 mL) with 15 mL of SAX conditioning solution (do not let cartridges go to dryness).

8.3.3 Using an adaptor, stack the SAX cartridge above the Oasis HLB cartridge. A sample reservoir may be placed above the SAX cartridge to simplify loading the extract.

8.3.4 Load 55 mL of the 220 mL extract (Aliquot A) from the conventional extraction procedure or 40 mL of the 115 mL extract from the Exhaustive Extraction Procedure through the joined cartridges at a flow rate of approximately 4-5 mL/min. Discard the eluate.

8.3.5 Rinse the graduated cylinder with 2 x 10 mL of SAX conditioning solution and pass through the cartridges. Discard the eluate.

8.3.6 Ensure the SAX cartridges are completely dry by applying a vacuum for *ca* 30 s once all extracts and rinses have passed through cartridges. Remove SAX cartridge. Continue the vacuum for a further 5 min to ensure Oasis HLB cartridges are completely dry.

8.3.7 Elute DPX-E2Y45, IN-EQW78 and IN-ECD73 by addition of 20 mL acetonitrile followed by 25 mL ethyl acetate. A vacuum may be required to start the flow but should be turned off once the flow has started. Collect the eluate in a glass 50 or 250 mL TurboVap tube.

Appendix 2
(continued)

8.3.8 Evaporate the extract to dryness using a flow of nitrogen in a TurboVap at approximately 50°C and reconstitute as follows:

Conventional Extraction Procedure

Add 1 mL acetonitrile, ultrasonicate for 5 min and add 1 mL 0.01M aqueous formic acid. Ultrasonicate for a further 5 min and remove an aliquot for LC/MS/MS analysis.

Samples of higher concentrations may be diluted to ensure the sample response is within the calibration range. Dilutions should be made using the 50:50 Acetonitrile:0.01 M aqueous formic acid solution.

Exhaustive Extraction Procedure

Add 0.5 mL acetonitrile, ultrasonicate for 5 min and add 0.5 mL 0.01M aqueous formic acid. Ultrasonicate for a further 5 min, vortex for *ca* 30 seconds and remove an aliquot for LC-MS/MS analysis.

Samples of higher concentrations may be diluted to ensure the sample response is within the calibration range. Dilutions should be made using the 50:50 Acetonitrile:0.01 M aqueous formic acid solution.

8.4 Analyte Purification Procedure for Conventional Extraction Procedure (Aliquot B – IN-F6L99)

8.4.1 Remove a 10 mL aliquot from the 40 mL extract (Section 8.1.7) and evaporate to approximately 0.1 mL under a stream of nitrogen using a driblock at *ca* 35°C.

8.4.2 Dilute the extract to 10 mL using HPLC grade water and vortex for *ca* 30 seconds.

8.4.3 Condition Oasis HLB cartridge (1 g/20 cc) with 5 mL methanol followed by 20 mL HPLC grade water (do not let cartridges go to dryness).

8.4.4 Load the 10 mL extract onto the cartridge. Discard the eluate.

8.4.5 Rinse the tube with 2 x 5 mL HPLC grade water and pass through cartridge.

8.4.6 Dry cartridge under vacuum for 5 min.

8.4.7 Elute IN-F6L99 by addition of 30 mL acetonitrile. A vacuum may be required to start the flow but should be turned off after the flow has started. Collect the eluate in a glass 50 or 250 mL TurboVap tube.

Appendix 2
(continued)

8.4.8 Evaporate the extract to dryness using a flow of nitrogen in a TurboVap at approximately 50°C.

8.4.9 Add 0.2 mL of acetonitrile, ultrasonicate for 5 min and add 1.8 mL 0.01M aqueous formic acid. Ultrasonicate for a further 5 min, vortex mix for *ca* 30 seconds and remove an aliquot for LC/MS/MS analysis. Samples of higher concentrations may be diluted to ensure the sample response is within the calibration range.

8.5 LC-MS/MS Conditions for all Extraction Procedures

HPLC System:	Refer to Section 6
Guard Column:	Phenomenex C18(ODS), 3 µm, 4.6. x 2.0 mm
Analytical Column:	Phenomenex C18(2), 3 µm, 4.6 x 150 mm
Mobile Phase:	A = 0.01M Aqueous Formic Acid B = Methanol
Injection Volume:	25 µL
Flow Rate:	1.0 mL/min
Autosampler Temperature:	*ca* + 4°C
Column Temperature:	*ca* 40°C

Appendix 2
(continued)

LC Gradient for Conventional and Exhaustive Extraction Procedure (Aliquot A – DPX-E2Y45, IN-EQW78 and IN-ECD73)

The following gradient program was used:

Time (min)	%A	%B
0.0	40	60
0.5	40	60
2.0	20	80
5.0	2	98
8.0	2	98
8.5	40	60
11.5	40	60

LC Gradient for Conventional Extraction Procedure (Aliquot B – IN-F6L99)

The following gradient program was used:

Time (min)	%A	%B
0.0	90	10
0.5	90	10
5.5	20	80
5.8	10	90
8.8	10	90
9.0	90	10
11.0	90	10

MS/MS Conditions for Conventional and Exhaustive Extraction Procedure

Ionisation Mode: APCI, Positive

MS/MS Gas Settings:

Gases	DPX-E2Y45 All Transitions	IN-EQW78 All Transitions	IN-ECD73 All Transitions	IN-F6L99 All Transitions
Curtain Gas Setting (CUR)	15	15	15	10
CAD Gas Setting (CAD)	3	3	3	3
Nebuliser Current (NC)	3	3	3	3
Nebuliser Temperature (°C)	600	600	600	600
Ion Source Gas 1 (GS1)	30	30	30	20

Appendix 2
(continued)

MS/MS Voltages for Conventional and Exhaustive Extraction Procedure

Voltages	DPX-E2Y45		IN-EQW78	
	284.0 → 177.0 amu	484.0 → 452.8 amu	466.0 → 188.0 amu	466.0 → 186.0 amu
Declustering Potential (DP)	58	58	58	58
Entrance Potential (EP)	-10.3	-10.3	-10.3	-10.3
Focusing Lens 1 (IQ1)	-10.8	-10.8	-10.8	-10.8
Prefilter (ST)	-17	-17	-17	-17
Focusing 2 (IQ2)	-20	-20	-20	-20
Collision Energy (CE)	34	21	45	45
Collision Cell Exit Potential (CXP)	29	15	4	4

Voltages	IN-ECD73		IN-F6L99	
	279.1 → 244.1 amu	244.0 → 209.0 amu	204.0 → 172.9 amu	204.0 → 66.0 amu
Declustering Potential (DP)	58	58	50	50
Entrance Potential (EP)	-10.3	-10.3	-10.0	-10.0
Focusing Lens 1 (IQ1)	-10.8	-10.8	-11.0	-11.0
Prefilter (ST)	-17	-17	-17	-17
Focusing 2 (IQ2)	-20	-20	-50	-50
Collision Energy (CE)	35	40	35	35
Collision Cell Exit Potential (CXP)	14	18	14	14

Q1 Resolution = Unit
Q3 Resolution = Unit
CAD/Curtain Gas: Nitrogen

Note: the gas settings and voltages specified may change after system re-optimisation which may be required when there is a decrease in system sensitivity.

Masses Monitored:	DPX-E2Y45	284.0 → 177.0 amu	Target Ion
		484.0 → 452.8 amu	Confirmatory Ion
	IN-EQW78	466.0 → 188.0 amu	Target Ion
		466.0 → 186.0 amu	Confirmatory Ion
	IN-ECD73	279.1 → 244.1 amu	Target Ion
		244.0 → 209.0 amu	Confirmatory Ion
	IN-F6L99	204.0 → 172.9 amu	Target Ion
		204.0 → 66.0 amu	Confirmatory Ion

Dwell/Scan Time: *300 ms on each transition*

Pause Time: 10 ms

Smart Setting: Off

Setting Time: 0 ms

Appendix 2
(continued)

9 Method of Calibration

9.1 Interpolation from the calibration line

System linearity and precision are established by injecting mixed calibration standards prepared in Sections 5.4.4 and 5.4.5. Calibration standards are run on each analytical occasion to demonstrate system linearity.

The calibration line should be linear with an intercept approaching zero and a percentage difference from the line not greater than ± 15 % at all levels except for the lowest calibration level, where ± 20 % is acceptable.

10 Expression of Results

The DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 peak areas are calculated for each of the calibration standards, quality control samples, controls and unknown test samples. A calibration curve is then obtained by weighted least squares linear regression analysis (1/x) of the plot of peak area versus the concentration of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in each calibration standard. A smoothing factor of 2 was applied to all chromatographic peaks. The calibration curve should not be forced through zero.

The calculated concentration in each sample is corrected for the proportion of sample taken through the extraction, sample weight and the sample final volume. The calculation is detailed below:

Concentration of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in sample:-

$$\text{Sample A} = \frac{\text{PR A} - c}{m} \times \text{Correction Factor}$$

where PR A	=	Peak response for analyte in Sample A
c	=	Intercept on y-axis from regression analysis
m	=	Slope of the line from regression analysis

Appendix 2
(continued)

Conventional Extraction Procedure

Correction Factor (Aliquot A): $$\frac{\binom{120}{80}^{+} x \binom{220}{55}^{+} x2^{++}}{10^{+++}}$$

\+ Procedural Cut in Method (mL)
++ Final volume (mL)
+++ Sample weight (g)

Correction factor = 1.2 if no further dilutions performed.

Correction Factor (Aliquot B): $$\frac{\binom{120}{40}^{+} x \binom{40}{10}^{+} x2^{++}}{10^{+++}}$$

\+ Procedural Cut in Method (mL)
++ Final volume (mL)
+++ Sample weight (g)

Correction factor = 2.4 if no further dilutions performed.

Exhaustive Extraction Procedure

Correction Factor: $$\frac{\binom{100}{35}^{+} x \binom{115}{40}^{+} x1^{++}}{10^{+++}}$$

\+ Procedural Cut in Method (mL)
++ Final volume (mL)
+++ Sample weight (g)

Correction factor = 0.8214 if no further dilutions performed.

The analytical method is considered to be acceptable if the mean batch recoveries are between 70-110% of the theoretical value and there is no significant interference from the control.

Appendix 2
(continued)

10.1 Quantification of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 using calibration standards interspersed throughout the run

On occasions it may be necessary to quantify residues of DPX-E2Y45, IN-EQW78, IN-ECD73 or IN-F6L99 using calibrations standards interspersed throughout the run due to fluctuation of the detector response.

The extracts are analysed as per the method detailed in Section 8.5. The samples interspersed with appropriate calibration standards. A typical run sequence would involve injection of a calibration line to establish linearity and then injection of a single calibration standard (at a level which is deemed to be appropriate for the expected residue levels in the samples), followed by *ca* 4 samples then another calibration standard at the same concentration as the preceding standard. The residues detected are calculated using the equation detailed below.

Concentration of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-F6L99 in

sample (µg/kg) =

$$\frac{\text{Sample Response}}{\text{Mean Standard Response}} \times \text{Standard Concentration} \times \frac{\text{Sample Final Volume}}{\text{Sample Weight}} \times \text{Correction Factor}$$

Sample Response	=	Detector response (peak area, counts)
Mean Standard Response	=	Mean detector response (peak area, counts) for bracketing standards
Standard Concentration	=	Concentration of bracketing standard solution (ng/mL)
Sample Final Volume	=	Volume of final sample extract (mL)
Sample Weight	=	Weight of the sample (g)
Correction Factor	=	Accounts for any procedural cuts in the method

It should be noted that in this report, the calculations were performed electronically using data which had not been rounded. Consequently attempts to reproduce these calculations with tabulated data, which has been rounded, may produce slight variations in the results.

Appendix 3 Analytical Method No. 0335B Determination of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in Soil

ASSAY:	DPX-E2Y45, IN-EQW78, IN-ECD73, IN-GAZ70	ANALYTICAL METHOD No.: 0335B Version 1
MATRIX:	Soil	ISSUED: 29 June 2004 APPROVAL: *Gwen C Syme*

1 Scope

Analytical Method no. 0335B is suitable for the determinatior of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in soil. The analysis of unknown soil samples should be completed using the procedure described below. This analytical method is also suitable for the determination of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in petri dishes without soil.

2 Field Application

The method described is suitable for the determination of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in soil over the range of 0.5 to 70 µg/kg. The method for petri dishes without soil is suitable for the determination of DPX-E2Y45 over the range of 50 µg/dish to 600 µg/dish and for IN-EQW78, IN-ECD73 and IN-GAZ70 at 50 µg/dish.

3 Definitions

Reference Material:	DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 analytical standards.
Control Sample:	Soil (or petri dish) known to be free from DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 residues above one third of the limit of quantification (LOQ).
Fortified Sample:	Control soil (or petri dish) to which a known amount of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 has been added.

4 Principle

The determination of DPX-E2Y45 and its metabolites is carried out using the exhaustive extraction procedure. The exhaustive extraction procecure is used for the determination of more difficult to extract residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in soil.

The exhaustive extraction procedure involves extraction using acetonitrile:aqueous formic acid (90:10, v/v) and acetonitrile:hydrochloric acid (99:1, v/v and 95:5, v/v) solutions. An aliquot is removed and cleaned up using SAX SPE cartridges and

Appendix 3
(continued)

Waters Oasis HLB SPE cartridges. The final extracts are quantified for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70

by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

Residues of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 are determined in petri dishes (without soil) by extraction with acetonitrile:aqueous formic acid, 90:10, (v/v) solution. The extracts are diluted prior to analysis and quantified for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 by liquid chromatography with tandem mass spectrometry employing atmospheric pressure chemical ionisation in positive mode.

5 Reagents and Materials

5.1 General

The following reagents were used for the validation of the analytical method. Alternative suppliers and grades of these reagents may be employed, provided adequate analytical sensitivity and resolution are achieved.

Water	HPLC	BDH
Formic Acid (98/100%)	Analytical	Fisher
Hydrochloric Acid (32%)	Analytical	Fisher
Methanol	HPLC	Rathburn
Ethyl Acetate	HPLC	Rathburn
Acetonitrile	HPLC	Rathburn
Dimethylsulfoxide	Analytical	Fisher

Standard laboratory safety precautions should be adhered to when using reagents and materials described in this method. Laboratory coats and safety glasses must be worn at all times.

5.2 Materials

The DPX-E2Y45 analytical standard, Batch Nos. DPX-E2Y45-25 and 100, were received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had expiry dates of 04 February 2003 and 27 February 2005, respectively.

The IN-EQW78 analytical standard, Batch No. 002, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 10 February 2005.

Appendix 3
(continued)

The IN-ECD73 analytical standard, Batch No. 003, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 11 February 2005.

The IN-GAZ70 analytical standard, Batch No. 001, was received from E.I. du Pont de Nemours and Company. The standard was stored at ambient temperature in the dark and had an expiry date of 07 July 2004. The expiry date was updated to 07 July 2006 after recertification.

Control soil was obtained by Inveresk from the following sources:

Inveresk Project No.	DuPont Reference No.	Location of Trial
682634	12787	Seville, Spain
683271	12791	Burgundy, France
684694	14441	Lleida, Spain

5.3 Supplier Details

The following provides further details of our materials and equipment suppliers. All are located in the UK unless stated otherwise.

1. BDH Chemicals Limited, Poole, Dorset, BH15 1TD.
2. Applied Biosystems, Warrington, Cheshire, WA3 7QH.
3. Fisher Chemicals, Loughborough, Leicestershire, LE11 0RG.
4. Jouan Limited, Ilkeston, Derbyshire, DE7 4RA.
5. Mettler Toledo, Beaumont Leys, Leicestershire, LE4 1AW.
6. Perkin Elmer, Beaconsfield, Buckinghamshire, HP9 1QA.
7. Rathburn Chemicals Limited, Walkerburn, Scotland, EH43 6AU.
8. Techne Cambridge Limited, Duxford, Cambridge, CB2 4PZ.
9. IKA, supplied by Fisher Scientific, Loughborough, Leicestershire, LE11 0RG.
10. Buchi, supplied by Fisher Scientific, Loughborough, Leicestershire, LE11 0RG.
11. Zymark, Runcorn, Cheshire, WA7 3AZ.
12. Decon Laboratories, Hove, East Sussex, B3 3LY.
13. Phenomenex UK Limited, Macclesfield, Cheshire, SK10 2BN.

Appendix 3
(continued)

14. Waters, Watford, Hertfordshire, WD1 8YW.

15. Varian, Southgate, London, N14 6JS.

5.4 Preparation of Reagents

5.4.1 Solutions

5.4.1.1 0.01 M Aqueous Formic Acid

Dilute 460 mg of concentrated formic acid (98/100%) to 1 L in a volumetric flask using HPLC grade water. Store solution at room temperature and use within one week of preparation.

5.4.1.2 0.2% Aqueous Formic Acid Solution

Add 2 mL of concentrated formic acid (98/100%) to a 1L volumetric flask and make to volume with HPLC grade water. Store solution at room temperature and use within one week of preparation.

5.4.1.3 0.2% Formic Acid Extraction Solvent

Combine 100 mL of 0.2% aqueous formic acid solution with 900 mL of acetonitrile in a 1L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.1.4 1% Hydrochloric Acid Extraction Solution

Dilute 5 mL of concentrated hydrochloric acid (32%) to 500 mL in a volumetric flask using acetonitrile. Store solution at room temperature and use within one week of preparation.

5.4.1.5 5% Hydrochloric Acid Extraction Solution

Dilute 25 mL of concentrated hydrochloric acid (32%) to 500 mL in a volumetric flask using acetonitrile. Store solution at room temperature and use within one week of preparation.

Appendix 3
(continued)

5.4.1.6 SAX Conditioning Solution

Combine 300 mL of acetonitrile, 700 mL of HPLC grade water and 5.0 mL of concentrated formic acid in a 1L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.1.7 Injection Solvent A

Combine 500 mL of 0.01M aqueous formic acid with 500 mL of acetonitrile in a 1 L Duran bottle. Store solution at room temperature and use within one week of preparation.

5.4.2 Calibration Standard Solution Preparation for the Analysis of Soil Samples and Petri Dishes (without soil)

Accurately weigh approximately 10 mg of DPX-E2Y45 and dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-EQW78, dissolve in 5 mL of dimethylsulfoxide and then dilute to 10 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 1000 µg/mL. Further dilute IN-EQW78 *ca* 1000 µg/mL to give a stock calibration standard solution of *ca* 100 µg/mL by accurately pipetting 1 mL of *ca* 1000 µg/mL solution into a 10 mL volumetric flask. Dilute to 10 mL with acetonitrile and mix well.

Accurately weigh approximately 10 mg of IN-ECD73, dissolve in 8 mL of dimethylsulfoxide and then dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-GAZ70, dissolve in 8 mL of dimethylsulfoxide and then dilute to 100 mL with acetonitrile. Mix well to give a stock calibration standard solution of *ca* 100 µg/mL.

5.4.3 Preparation of Mixed Calibration Standard Solutions for the Analysis of Soil Samples and Petri Dishes (without soil)

Accurately pipette 1.0 mL of each of the *ca* 100 µg/mL standards from Section 5.4.2 into a 100 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed stock calibration standard solution of *ca* 1.0 µg/mL.

Accurately pipette 1.0 mL of the *ca* 1.0 µg/mL mixed stock calibration standard solution into a 10 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed stock calibration standard solution of *ca* 0.1 µg/mL. This dilution scheme and subsequent dilutions are presented in Section 5.4.4.

Appendix 3
(continued)

5.4.4 *Dilution of Calibration Standards for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 for the Analysis of Soil Samples and Petri Dishes (without soil)*

Dilutions are prepared from the mixed stock calibration standard solutions as detailed in Section 5.4.3 using injection solvent A to give mixed calibration standards ranging from 0.1 to 25 ng/mL for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70, as detailed below:

Standard Reference	Volume Taken (mL)	Solution Used	Final Volume (mL)	Solution Concentration (ng/mL)
A	-	-	-	*100000 (1000000*)*
B**	1.0	A	10	100000
C	1.0	A	100	1000
D	1.0	C	10	100
E	0.250	C	10	25
F	0.100	C	10	10
G	0.050	C	10	5
H	0.200	D	10	2
I	0.100	D	10	1
J	0.050	D	10	0.5
K	0.025	D	10	0.25
L	0.100	F	10	0.1

* Concentration of calibration standard stock IN-EQW78 only.
**This dilution step refers to IN-EQW78 only.

Solvent for Std A:	Acetonitrile (DPX-E2Y45) and dimethylsulphoxide and acetonitrile (IN-EQW78, IN-ECD73 and IN-GAZ70)
Solvent for Std B-D:	Acetonitrile
Solvent for Std E-L:	Injection Solvent A
Expiration for Std A:	3 months after preparation (IN-EQW78, IN-ECD73 and IN-GAZ70) and 6 months after preparation (DPX-E2Y45)***
Expiration for Std B-D:	6 months after preparation***
Expiration for Std E-L:	One week after preparation
Storage Conditions:	*ca* +4°C for all solutions

*** Note: the expiry date has since been revised to 3 months from preparation. The standards used throughout the validation were assigned with expiry dates of 6 months and were considered to be suitable for use for this study.

5.4.5 *Quality Control Standard Solution Preparation for fortification of Soil Samples*

Accurately weigh approximately 10 mg of DPX-E2Y45 and dilute to 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

Appendix 3
(continued)

Accurately weigh approximately 10 mg of IN-EQW78, dissolve in 5 mL of dimethylsulfoxide and then dilute to 10 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 1000 µg/mL. Further dilute IN-EQW78, *ca* 1000 µg/mL to give a stock quality control standard solution of *ca* 100 µg/mL. Accurately pipette 1 mL of *ca* 1000 µg/mL solution into a 10 mL volumetric flask. Dilute to 10 mL with acetonitrile and mix well.

Accurately weigh approximately 10 mg of IN-ECD73 and dissolve in *ca* 8 mL of dimethylsulfoxide then dilute to a final volume of 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

Accurately weigh approximately 10 mg of IN-GAZ70, dissolve in 8 mL of dimethylsulfoxide and then dilute to 100 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 100 µg/mL.

5.4.6 Preparation of Mixed Quality Control Standard Solutions for fortification of Soil Samples

Accurately pipette 1.0 mL of each of the *ca* 100 µg/mL quality control standards from Section 5.4.5 into a 100 mL volumetric flask and dilute to volume using acetonitrile.

Mix well to give a mixed stock quality control standard solution of *ca* 1.0 µg/mL. The resulting solution has an expiration date of 6 months if stored at *ca* +4°C.

Accurately pipette 1.0 mL of the mixed stock quality control standard solution of *ca* 1.0 µg/mL into a 10 mL volumetric flask and dilute to volume using acetonitrile. Mix well to give a mixed quality control standard solution of *ca* 0.1 µg/mL. The resulting solution has an expiration date of 6 months if stored at *ca* +4°C.

5.4.7 Quality Control Standard Solution Preparation for fortification of Petri Dishes (without soil)

The stock quality control solutions prepared in Section 5.4.5 are used for fortification for the petri dishes (without soil).

In addition, accurately weigh approximately 10 mg of DPX-E2Y45 and dilute to 20 mL with acetonitrile. Mix well to give a stock quality control standard solution of *ca* 500 µg/mL. The resulting solution has an expiration date of 6 months if stored at *ca* +4°C.

Appendix 3
(continued)

6 Apparatus

The following items may be substituted with components of equivalent specification and performance:-

Laboratory Glassware:	Standard Analytical Glassware, Fisher
Centrifuge:	Jouan GR422
TurboVap:	Zymark TurboVap 11
Driblock:	Techne DB3A
SPE Tank:	Isolute VacMaster
Vacuum Pump:	Vacuubrand ME4C
Vortex Mixer:	Fisons
Ultrasonic Bath:	Decon FS4006
Flat Bed Shaker:	IKA Labortecnik HS 250
Bowl Cutter:	Hobart FP61
Balances:	Mettler PM300, PE3000, AG245
Water Bath:	Buchi B-490
Solid Phase Cartridges:	Oasis HLB 1 g/20 cc (Waters, PN 186000117) SAX Mega Bond Elut 1 g/6 mL (Varian, PN 1225-6013)

LC-MS/MS System:

HPLC Pump:	Perkin Elmer (Series 200)
*Autosampler:	Perkin Elmer (Series 200)
Cooling Unit:	Perkin Elmer (Peltier)
*Autosampler:	CTC Analytics (HTS PAL)
Column Oven:	Perkin Elmer (Series 200)
Degasser:	Perkin Elmer (Series 200)
Mass Spectrometer:	Applied Biosystems API 4000 with APCI Source
Data System:	Analyst Versions 1.1 and 1.3.

* Note: 2 different autosamplers were used throughout this study

Appendix 3
(continued)

7 Sampling for Analysis of Soil Samples

7.1 Test Sample

Soil sample free from DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70.

7.2 Test Portion

Accurately weigh 10 g of fresh soil into a 250 mL plastic bottle.

7.3 Blank Test

In each batch of samples analysed at least 1 test sample should be a control sample.

7.4 Fortification of Soil Samples

To verify the accuracy and precision of the method on each analytical occasion, a minimum of one fortified recovery sample for every 10 samples in the batch (at least one of these should be at the LOQ) should be analysed.

To 10 g test portions of soil, QC standard solutions should be added as shown below:

Recovery Level (µg/kg)	Fortification
0.5 (LOQ)	50 µL of *ca* 0.1 µg/mL mixed QC Standard
5.0	50 µL of *ca* 1.0 µg/mL mixed QC Standard
70	700 µL of *ca* 1.0 µg/mL mixed QC Standard

The fortified samples should then be analysed as detailed Section 8.

8 Procedure

8.1 Exhaustive Extraction Procedure for Analysis of Soil Samples

8.1.1 Add 25 mL of 0.2% formic acid extraction solvent to the sample and shake vigorously by hand. Place sample on flat bed shaker for 15 min at ca 300 motions/min.

8.1.2 Centrifuge the sample at *ca* 3000 r.p.m. for 10 min and decant the supernatant into a 250 mL graduated mixing cylinder with stopper.

8.1.3 Add 25 mL of 1% hydrochloric acid extraction solution to the sample and shake vigorously by hand. Place sample on flat bed shaker for 15 min at *ca* 300 motions/min.

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(continued)

8.1.4 Place sample in a water bath set to *ca* 60°C for 30 min.

8.1.5 Remove sample from water bath and slowly open cap to release any pressure. Centrifuge sample at *ca* 3000 r.p.m. for 10 min. Decant the supernatant into the same 250 mL graduated mixing cylinder with stopper.

8.1.6 Add 50 mL of 5% hydrochloric acid extraction solution to the sample and shake vigorously by hand. Place sample on flat bed shaker for 5 min at *ca* 300 motions/min.

8.1.7 Place sample in a water bath set at *ca* 60°C for 1 h.

8.1.8 Remove sample from water bath and slowly open cap to release any pressure. Place sample on flat bed shaker for 10 min at *ca* 300 motions/min.

8.1.9 Centrifuge the sample at 3000 r.p.m. for 10 min and decant the supernatant into the same 250 mL graduated mixing cylinder.

8.1.10 Adjust the volume of each extract to 100 mL with 1% hydrochloric acid extraction solution and mix the extract thoroughly.

8.1.11 Pipette 35 mL aliquot into a 250 mL graduated mixing cylinder with stopper, make up to 115 mL with HPLC grade water and mix thoroughly.

8.2 Analyte Purification Procedure Exhaustive Extraction Procedure

8.2.1 Condition Waters Oasis HLB cartridge (1 g/20 cc) with 5 mL of methanol followed by 20 mL HPLC grade water (do not let cartridges go to dryness).

8.2.2 Condition SAX cartridge (1 g/6 mL) with 15 mL of SAX conditioning solution (do not let cartridges go to dryness).

8.2.3 Using an adapter, stack the SAX cartridge above the Water Oasis HLB cartridge. A sample reservoir may be placed above the SAX cartridge to simplify loading the extract.

8.2.4 Load 40 mL of the 115 mL extract from 8.1.11 through the joined cartridges at a flow rate of approximately 4-5 mL/min. Discard the eluate.

8.2.5 Rinse the graduated cylinder with 2 x 10 mL of SAX conditioning solution and pass through the cartridges. Discard the eluate.

Appendix 3
(continued)

8.2.6 Ensure the SAX cartridges are completely dry by applying a vacuum for *ca* 30 s once all extracts and rinses have passed through cartridges. Remove SAX cartridge. Continue the vacuum for a further 5 min to ensure Water Oasis HLB cartridges are completely dry.

8.2.7 Elute DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 by addition of 20 mL acetonitrile followed by 25 mL ethyl acetate. A vacuum may be required to start the flow but should be turned off once the flow has started. Collect the eluate in a glass 50 or 250 mL TurboVap tube.

8.2.8 Evaporate the extract to dryness using a flow of nitrogen in a TurboVap at approximately 50°C and reconstitute as detailed in 8.2.9.

8.2.9 Add 0.5 mL acetonitrile, ultrasonicate for 5 min and add 0.5 mL 0.01M aqueous formic acid. Ultrasonicate for a further 5 min, vortex for *ca* 30 seconds and remove an aliquot for LC-MS/MS analysis. Samples of higher concentrations may be diluted to ensure the sample response is within the calibration range.

8.3 Analysis of DPX-E2Y45 and its Metabolites in Petri Dishes (without soil)

8.3.1 Place the bottom half of the petri dish (smaller diameter) inside in a 1 litre plastic pot (pot complete with sealing lid). Note: for extraction of petri dishes from the field the upper half of the petri dish (larger diameter) should also be extracted (remove the upper half of the dish, invert it, place the bottom half of the petri dish inside).

8.3.2 Fortify as appropriate. On each analytical occasion, a minimum of one fortified recovery sample for every 10 samples in the batch, should be extracted (at least one of these should be at the LOQ). The fortification levels are detailed below. Only the bottom half should be fortified and then left to dry for *ca* 20 min.

Recovery Level (µg/dish)	Fortification
50	500 µL of *ca* 100 µg/mL DPX-E2Y45 QC standard 50 µL of *ca* 1000 µg/mL IN-EQW78 QC standard 500 µL of *ca* 100 µg/mL IN-ECD73 QC standard 500 µL of *ca* 100 µg/mL IN-GAZ70 QC standard
600	1.2 mL of *ca* 500 µg/mL DPX-E2Y45 QC standard

Appendix 3
(continued)

8.3.3 Add 500 mL of the acetonitrile:aqueous formic acid (90:10, v/v) solution to the pot. Note : if the petri dishes are larger in size, an additional aliquot of extraction solvent may be added until the petri dish is fully submerged. The total volume of extraction solvent added should be recorded

8.3.4 Place the pot in a sonic bath and sonicate for *ca* 20 min.

8.3.5 Remove the pot from the sonic bath. Shake the pot by hand for *ca* 1 min.

8.3.6 Remove an aliquot of the extract and dilute it to a concentration which falls within the calibration range (using a theoretical recovery of 100%). The sample should be initially diluted with water until the composition is 50:50 acetonitrile:water. The extract should be diluted to the final volume with 50:50 acetonitrile:0.01 M aqueous formic acid solution.

8.4 LC-MS/MS Conditions for Analysis of Soil Samples and Petri Dishes Without Soil

HPLC System:	Refer to Section 6
Guard Column:	Phenomenex C18(ODS), 3 µm, 4.6. x 2.0 mm
Analytical Column:	Phenomenex C18(2), 3 µm, 4.6 x 150 mm
Mobile Phase:	A = 0.01M Aqueous Formic Acid B = Methanol
Injection Volume:	25 µL
Flow Rate:	1.0 mL/min
Autosampler Temperature:	*ca* + 4°C
Column Temperature:	*ca* 40°C

Appendix 3
(continued)

LC Gradient for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70

The following gradient program was used:

Time (min)	%A	%B
0.0	40	60
0.5	40	60
2.0	20	80
5.0	2	98
8.0	2	98
8.5	40	60
11.5	40	60

MS/MS Conditions for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70

Ionisation Mode: APCI, *Positive*

MS/MS Gas Settings:

Gases	DPX-E2Y45 All Transitions	IN-EQW78 All Transitions	IN-ECD73 All Transitions	IN-GAZ70 All Transitions
Curtain Gas Setting (CUR)	15	15	15	15
CAD Gas Setting (CAD)	3	3	3	3
Nebuliser Current (NC)	3	3	3	3
Nebuliser Temperature (°C)	600	600	600	600
Ion Source Gas 1 (GS1)	30	30	30	30

MS/MS Voltages for DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70

Voltages	DPX-E2Y45		IN-EQW78	
	284.0 → 177.0 amu	484.0 → 452.8 amu	466.0 → 188.0 amu	466.0 → 186.0 amu
Declustering Potential (DP)	58	58	58	58
Entrance Potential (EP)	-10.3	-10.3	-10.3	-10.3
Focusing Lens 1 (IQ1)	-10.8	-10.8	-10.8	-10.8
Prefilter (ST)	-17	-17	-17	-17
Focusing 2 (IQ2)	-20	-20	-20	-20
Collision Energy (CE)	34	21	45	45
Collision Cell Exit Potential (CXP)	29	15	4	4

Appendix 3
(continued)

Voltages	IN-ECD73		IN-GAZ70	
	279.1 → 244.1 amu	244.0 → 209.0 amu	451.9 → 416.0 amu	449.9 → 414.10 amu
Declustering Potential (DP)	58	58	70	62
Entrance Potential (EP)	-10.3	-10.3	-11	-11
Focusing Lens 1 (IQ1)	-10.8	-10.8	-10.8	-10.8
Prefilter (ST)	-17	-17	-17	-17
Focusing 2 (IQ2)	-20	-20	-20	-20
Collision Energy (CE)	35	40	35	30
Collision Cell Exit Potential (CXP)	14	18	12	35

Q1 Resolution = Unit
Q3 Resolution = Unit
CAD/Curtain Gas: Nitrogen

Note: the gas settings and voltages specified may change after system re-optimisation which may be required when there is a decrease in system sensitivity.

Masses Monitored:	DPX-E2Y45	284.0 → 177.0 amu	Target Ion
		484.0 → 452.8 amu	Confirmatory Ion
	IN-EQW78	466.0 → 188.0 amu	Target Ion
		466.0 → 186.0 amu	Confirmatory Ion
	IN-ECD73	279.1 → 244.1 amu	Target Ion
		244.0 → 209.0 amu	Confirmatory Ion
	IN-GAZ70	451.9 → 416.0 amu	Target Ion
		449.9 → 414.1 amu	Confirmatory Ion

Dwell/Scan Time: 300 ms on each transition

Pause Time: 10 ms

Smart Setting: Off

Setting Time: 0 ms

Appendix 3
(continued)

9 Method of Calibration

9.1 Interpolation from the calibration line

System linearity and precision are established by injecting mixed calibration standards prepared in Section 5.4.4. Calibration standards are run on each analytical occasion to demonstrate system linearity.

The calibration line should be linear with an intercept approaching zero and a percentage difference from the line not greater than ± 15 % at all levels except for the lowest calibration level, where ± 20 % is acceptable.

10 Expression of Results

10.1 Quantification of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 using calibration curve

The DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 peak areas are calculated for each of the calibration standards, quality control samples, controls and unknown test samples. A calibration curve is obtained by weighted least squares linear regression analysis (1/x) of the plot of peak area versus the concentration of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in each calibration standard. A smoothing factor of 2 was applied to all chromatographic peaks. The calibration curve should not be forced through zero.

The calculated concentration in each sample is corrected for the proportion of sample taken through the extraction, sample weight and the sample final volume. The calculation is detailed below:

Concentration of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 in sample:-

$$\text{Sample A} = \frac{\text{PR A} - c}{m} \times \text{Correction Factor}$$

where PR A	=	Peak response for analyte in Sample A
c	=	Intercept on y-axis from regression analysis
m	=	Slope of the line from regression analysis

Appendix 3
(continued)

Exhaustive Extraction Procedure

Correction Factor: $$\frac{\binom{100}{35}^{+} \times \binom{115}{40}^{+} \times 1^{++}}{10^{+++}}$$

$^{+}$ Procedural Cut in Method (mL)
$^{++}$ Final volume (mL)
$^{+++}$ Sample weight (g)

Correction factor = 0.8214 if no further dilutions performed.

The analytical method is considered to be acceptable if the mean batch recoveries are between 70-110% of the theoretical value and there is no significant interference from the control.

10.2 Quantification of DPX-E2Y45, IN-EQW78, IN-ECD73 and IN-GAZ70 using calibration standards interspersed throughout the run

On occasions it may be necessary to quantify residues of DPX-E2Y45, IN-EQW78, IN-ECD73, or IN-GAZ70 using calibrations standards interspersed throughout the run due to fluctuation of the detector response.

The extracts are analysed as per the method detailed in Section 8.4. The samples interspersed with appropriate calibration standards. A typical run sequence would involve injection of a calibration line to establish linearity and then injection of a single calibration standard (at a level which is deemed to be appropriate for the expected residue levels in the samples), followed by *ca* 4 samples then another calibration standard at the same concentration as the preceding standard. The residues detected are calculated using the equation detailed below.

Concentration of DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 in sample µg/kg (or ng/dish for petri dishes without soil) =

$$\frac{\text{Sample Response}}{\text{Mean Standard Response}} \times \text{Standard Concentration} \times \frac{\text{Sample Final Volume}}{\text{Sample Weight}} \times \text{Correction Factor}$$

Sample Response	=	Detector response (peak area, counts)
Mean Standard Response	=	Mean detector response (peak area, counts) for bracketing standards

Appendix 3
(continued)

Standard Concentration	=	Concentration of bracketing standard solution (ng/mL)
Sample Final Volume	=	Volume of final sample extract (mL)
Sample Weight	=	Weight of the sample (g)
Correction Factor	=	Accounts for any procedural cuts in the method

It should be noted that in this report, the calculations were performed electronically using data which had not been rounded. Consequently attempts to reproduce these calculations with tabulated data, which has been rounded, may produce slight variations in the results.