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Watch list method

Analytical method for the determination of compounds selected for the first Surface water watch list

Validation report, according to ISO 17025 requirements

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Abstract

The validation of an analytical method is a necessary step in controlling the quality of quantitative analysis. Method validation is an established process which provides documentary evidence that a system fulfils its pre-defined specification, or shows that an analytical method is acceptable for its intended purpose. The purpose of the present study was to develop and validate analytical procedures for the quantitative determination in surface water of substances selected in the first watch list. Two different methods were developed and validated:

- a multi-residual method based on SPE-LC-MS/MS analysis, using OASIS HLB as sorbent material for the extraction of 1 litre water samples and—quantitative determination of EE2, E2, E1, diclofenac, azithromycin, clarythromycin, methiocarb acetamiprid, clothianidin, imidacloprid, thiacloprid, thiametoxam and oxadiazon;
- a multi-residual method based on LLE-GC-MS, using hexane as an extraction solvent—for the extraction of 0.01 litre water samples and quantitative determination of BHT, EHMC and Triallate.

The calibration curves, working ranges, recoveries, detection and quantification limits, trueness as well as repeatability were determined. The uncertainty budget was estimated based on in-house validation data.

1 Introduction

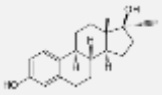
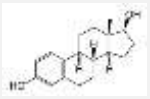
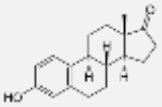
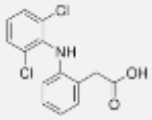
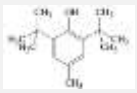
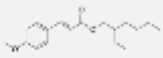
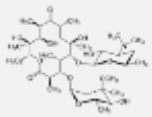
The Commission Implementing Decision (EU) 2015/495 of 20 March 2015 established a first watch list of substances for EU-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council.

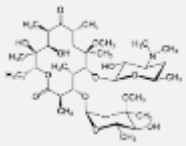
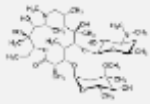
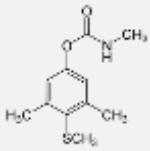
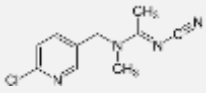
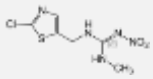
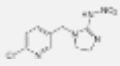
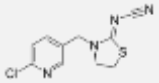
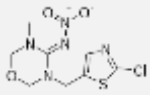
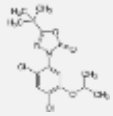
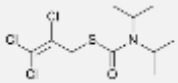
Up to 10 groups of substances have been selected for which EU-wide monitoring data are to be gathered for the purpose of supporting future prioritisation exercises in accordance with Article 16(2) of Directive 2000/60/EC of the European Parliament and of the Council.

Ten substances/groups of substances have been selected for which EU-wide monitoring data are to be gathered for the purpose of supporting future prioritisation exercises in accordance with Article 16(2) of Directive 2000/60/EC of the European Parliament and of the Council.

The substances are listed in Table 1.

Table 1: Substances on the first watch list

Name of substance	CAS number	EU number(*)	Formula	Maximum acceptable method detection limit (ng/l)
17-α-Ethinylestradiol (EE2)	57-63-6	200-342-2		0.035
17-β-Estradiol (E2)	50-82-2,	200-023-8		0.4
Estrone (E1)	53-16-7	200-023-8		0.4
Diclofenac	15307-79-6	239-348-5		10
2.6-Ditert-butyl-4-methylphenol (BHT)	128-37-0	204-881-4		3 160
2-Ethylhexyl 4-methoxycinnamate	5466-77-3	226-775-7		6 000
Erythromycin	114-07-8	204-040-1		90

Name of substance	CAS number	EU number(*)	Formula	Maximum acceptable method detection limit (ng/l)
Clarythromycin	81103-11-9			90
Azythromycin	83905-01-5	617-500-5		90
Methiocarb	2032-65-7	217-991-2		10
Acetamiprid	135410-20-7/ 160430-64-8			9
Clothianidin	210880-92-5	433-460-1		9
Imidacloprid	105827-78-9/ 138261-41-3	428-040-8		9
Thiacloprid	111988-49-9			9
Thiamethoxam	153719-23-4	428-650-4		9
Oxadiazon	19666-30-9	243-215-7		88
Triallate	2303-17-5	218-962-7		670

(*): European Union number not available for all substances.

For each substance a maximum acceptable method detection limit (LOD), expressed as ng/l in whole water, was established which corresponded to the substance-specific predicted no-effect concentration (PNEC) in the relevant matrix.

In accordance with (1) of the Commission Implementing Decision (EU) 2015/495 of 20 March 2015, *'the method detection limit should be at least as low as the substance-specific PNEC for each substance in the relevant matrix. If new information leads to a*

decrease in the PNEC for particular substances, the maximum acceptable method detection limit might have to be lowered while those substances remain on the list. The analytical methods are not considered to entail excessive costs.'

In the methods validation described in this report, calibration ranges have been established which include PNEC values in the higher part of the curves in order to have the possibility to further lower the limit of detections. This could preserve the method validity and the collected datasets in case of future PNEC values decreases.

This approach was not applicable for 17α-ethynyl estradiol, because today's state-of-the-art analytical techniques allow us to reach sensitivity levels just close to its PNEC (i.e. 0.035 ng/l).

Considering the huge difference among PNEC values (and consequently among maximum acceptable method detection limits) and chemical and physical properties of the selected compounds, two different methods have been developed and validated:

- a multi-residual method based on SPE-LC-MS/MS analysis, using OASIS HLB as sorbent material for the extraction of 1 litre water samples and quantitative determination of EE2, E2, E1, diclofenac, azithromycin, clarythromycin, methiocarb acetamiprid, clothianidin, imidacloprid, thiacloprid, thiametoxam and oxadiazon. Neither pH modification nor any other sample pre-treatment was performed in order to allow the extraction of all the selected compounds;
- a multi-residual method based on LLE-GC-MS, using hexane as the extraction solvent—for the extraction of 0.01 litre water samples and quantitative determination of BHT, EHMC and Triallate.

The present document consists of three sections:

- experimental set-up of method validation and results;
- 'Supplementary information' specifying chemicals, laboratory equipment, instrumental parameters and extraction procedures;
- 'Annex 1' statistical evaluation on experimental dataset.

2 Experimental set-up of methods validation

Different experiments were carried out for the characterisation of the developed procedures in terms of linearity and working range, limit of detection and quantitation, recovery, trueness, repeatability, intermediate precision and uncertainty budget.

In our approach, a calibration curve created from freshly prepared standards and quality control samples (QCs) in MilliQ water were run on five different days. Some of the experiments were used in the evaluation of different parameters.

Specifications for all standard and sample solutions prepared and used for the method development are found in the section 'Supplementary Information'.

The analyte/internal standard peak area ratios were used as target parameters for quantitation. A weighted (1/c) least-square regression analysis of data was performed in order to determine the calibration curve parameters and the coefficient of determination (R^2).

The equation obtained with the linear regression method is as follows:

$$X = \frac{Y - B}{A}$$

where:

X = analyte concentration

Y = peak area ratio = $\frac{\text{analyte peak area}}{\text{I.S. peak area}}$

A = slope

B = intercept.

2.1 Selectivity

Selectivity of quantitative determination was accomplished by relative retention times and by operating in multiple reaction monitoring (MRM) mode using LC-MS/MS and in selected ion monitoring (SIM) mode using GC-MS.

At least two MRM transitions or two selected fragment ions were recorded for each compound.

2.2 Limits of detection and quantification

The limits of detection and quantification were estimated both in MilliQ and surface water by analysing blank samples belonging to the respective calibration curves.

The mean value of blank samples (b) and the relative standard deviation (RSD) served for LOD and LOQ estimations, in accordance with the following equations:

LOD = b + 3SD;

LOQ = b + 10SD.

Limits of quantification of the developed procedure should be at least as low as the maximum acceptable method detection limits stated in the Commission Implementing Decision (EU) 2015/495.

2.3 Linearity study

The calibration standards in MillliQ water (six different spiking levels, including a blank sample) were freshly prepared and processed on each day of validation. Table 2 indicates the covered calibration ranges and the level of internal standard used for analytical determination.

Table 2: Studied calibration ranges

Analyte	Calibration standard Concentration (ng/l)					Internal standard Conc (ng/l)
	E	D	C	B	A	
EE2	0.56	0.28	0.14	0.07	0.035	1
E2	3.2	1.6	0.8	0.4	0.2	1
E1	3.2	1.6	0.8	0.4	0.2	1
Diclofenac	80	40	20	10	5	1.1
BHT	6320	3160	1580	790	395	1800
EHMC	12000	6000	3000	1500	750	2000
Clarythromycin	180	90	45	22.5	11.25	1
Azythromycin	180	90	45	22.5	11.25	1
Methiocarb	20	10	5	2.5	1.25	1.1

Analyte	Calibration standard Concentration (ng/l)					Internal standard Conc (ng/l)
	E	D	C	B	A	
Acetamiprid	18.08	9.04	4.52	2.26	1.13	1.1
Clothianidin	18.08	9.04	4.52	2.26	1.13	1.2
Imidacloprid	18.08	9.04	4.52	2.26	1.13	1
Thiacloprid	18.08	9.04	4.52	2.26	1.13	1.1
Thiamethoxam	18.08	9.04	4.52	2.26	1.13	1.3
Oxadiazon	176	88	44	22	11	1.1
Triallate	1339.84	669.92	334.96	167.48	83.74	2000

The relationship (goodness of fit) between peak area ratios of analyte/IS and concentrations in the concentration range investigated was assessed by the coefficient of determination (R^2) and by the shape of the distribution of residuals around the horizontal axis.

The acceptance criteria set for calibration curves were:

- $R^2 \geq 0.9900$ calculated over five calibration curves; and
- random dispersion of residuals around the horizontal axis, proving the pertinence of the linear regression model to interpret the data.

2.4 Matrix comparison

In the determination of the 16 selected compounds in water samples, calibration curves prepared in MilliQ water were compared with those prepared in surface water (i.e. Ispra Bay, Varese, Italy). This comparison study was formulated to identify whether or not a significant matrix effect occurs for all or some of the analytes.

For this purpose, five calibration curves in MilliQ water and three calibration curves in surface water were determined on five different days. Analysis of covariance (ANCOVA) was first used to compare the calibration curve within each water type to check the stability over several days. Calibrations were then compared between water types to assess whether a statistically significant change occurred in terms of slopes and intercepts.

The ANCOVA is a statistical tool that can be used to compare regression curves (slopes and intercepts). The ANCOVA is an extension of the analysis of variance (ANOVA) that provides a means of statistically controlling the (linear) effect of one or more continuous variables that are not part of the main experimental manipulation but have an influence on the dependent variable (Field et al., 2012). These variables are called *covariates* and should be measured on an interval or ratio scale. A one-way ANCOVA evaluates whether population averages of the dependent variable are the same across all levels of a factor (independent variable), adjusting for differences in the covariate. The factor divides individuals into two or more groups or levels, while the covariate and the dependent variable differentiate individuals based on quantitative dimensions. The one-way ANCOVA is used to analyse data from several types of studies, including studies that investigate the differences among calibration curves in order to check their stability (2), evaluate comparison between matrix types (3), and to compare different measurement procedures (4).

ANCOVA makes the same assumptions as ANOVA with two additional considerations (points 1 and 5):

1. independence: the covariate variable is independent of the groups (i.e. the covariant and independent variables are independent);

2. normality: the residuals must be normally distributed around the regression line for each group;
3. homogeneity of variance (homoscedasticity): the variance must be equal for both groups around their respective regression lines;
4. linearity: the relationship between the dependent variable (y) and the covariate (x) is linear for each factor;
5. homogeneity of regression slopes: the regression lines for these individual factors are assumed to be parallel (they have the same slope).

2.5 Repeatability and intermediate precision

Three QCs were freshly prepared in MilliQ water and analysed on three different occasions at two spiking levels for a total of 9 independent sample preparations. Table 3 summarises the spiking levels studied for each analyte.

Table 3: Level of quality control samples

Analyte	QC concentration (ng/l)	
	QC H	QC L
EE2	0.42	0.0525
E2	2.4	0.3
E1	2.4	0.3
Diclofenac	60	7.5
BHT	4500	450
EHMC	9360	936
Clarythromycin	135	16.9
Azythromycin	135	16.9
Methiocarb	15	1.9
Methiocarb	15	1.9
Acetamiprid	13.6	1.7
Clothianidin	13.6	1.7
Imidacloprid	13.6	1.7
Thiacloprid	13.6	1.7
Thiamethoxam	13.6	1.7
Oxadiazon	132	16.5
Triallate	100.8	1005

The acceptance criterion for the RSD of the repeatability and intermediate precision was set to 30% at both spiking levels.

2.6 Extraction variability of trueness

Due to the absence of Certified Reference Material (CRM) in the market, the trueness was evaluated as extraction variability of target analytes in spiked samples. The average concentrations found in spiked samples were compared to the added (theoretical) concentrations in order to estimate the extraction variability as slope of the regression line, expressed as a percentage. Values in the range 80-120 % were considered satisfactory.

2.7 Recovery

Recovery was evaluated by extracting and analysing in triplicate 1-litre MilliQ water samples spiked, before extraction, with native analytes only. The internal standard was then added to the extracts at the end of the sample preparation with the aim of allowing an estimation of analyte loss during processing.

The recovery was evaluated by comparing the ratios analyte/IS in spiked samples to the same ratios obtained by analysing a standard solution containing native compounds and the labelled solution at the same concentration levels.

The spiking levels studied for each analyte are reported in Table 4.

Table 4: Spiking levels for recovery evaluation

Analyte	Spiking level for recovery evaluation (ng/l)
EE2	0.035
	10
E2	10
	0.035
E1	10
	0.035
Diclofenac	11.6
BHT	450
	4500
EHMC	936
	9360
Clarythromycin	10.4
Azythromycin	10.2
Methiocarb	11
Acetamiprid	13.8
Clothianidin	12.8
Imidacloprid	10
Thiacloprid	10.8
Thiamethoxam	9.8
Oxadiazon	12.2
Triallate	100.8
	1005

3 Validation procedure and results

3.1 Selectivity

3.1.1 LC-MS/MS

For the identification of selected analytes, the two most abundant MRM transition ions from the precursor ion were chosen and monitored. The first was used for quantitation purposes, whereas the second ('qualifier') was used to confirm the presence of the target compound in the sample. The quantitated analyte was identified by comparing the retention time of the corresponding standard and the ratio between two ions recorded (± 30 %), in the standard and water samples.

The selected mass transitions used for quantification and confirmation were reported in Table 27 and 28.

3.1.2 GC-MS

For the identification of BHT, EHMC and triallate, SIM was used and two selected ions among the most abundant were recorded, one for quantitation purposes and the other for confirmation.

The quantitated analytes were identified by comparing the retention time of the corresponding standard and the presence of peak on both selected ions.

The selected ions used for quantification and confirmation are reported in Table 30.

3.2 Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection and quantification were estimated by analysing blank samples in the respective matrix.

The mean values of the blank samples (b) and standard deviation (SD) were calculated using the data output from these experiments. LOD and LOQ were estimated according to the formula reported in 2.3.

The results of the LOD and LOQ estimation are shown in Table 5, both for MilliQ and surface water.

Table 5: LOD and LOQ

Matrix	MilliQ water			Surface water		
Analyte	Nr of blanks analysed	LOD (ng/l)	LOQ (ng/l)	Nr of blanks analysed	LOD (ng/l)	LOQ (ng/l)
EE2	4	0.01	0.03	3	0.03	0.07
E2	5	0.05	0.13	3	0.04	0.09
E1	5	0.01	0.02	3	0.09	0.1
Diclofenac	4	0.47	1.09	3	1	2.6
BHT	5	21.53	42.64	3	19.6	39.6
EHMC	5	25.48	60.57	3	30.4	69.1
Clarythromycin	2	0.13	0.33	3	2.1	4.6
Azythromycin	2	0.59	1.34	3	1.3	2.6

Matrix	MilliQ water			Surface water		
Analyte	Nr of blanks analysed	LOD (ng/l)	LOQ (ng/l)	Nr of blanks analysed	LOD (ng/l)	LOQ (ng/l)
Methiocarb	4	0.07	0.17	3	0.01	0.02
Acetamiprid	4	0.04	0.09	3	0.08	0.2
Clothianidin	4	0.41	1.07	3	0.06	0.1
Imidacloprid	5	0.11	0.27	3	0.5	1.0
Thiacloprid	4	0.03	0.05	3	0.04	0.05
Thiamethoxam	4	0.66	1.6	3	0.5	1
Oxadiazon	4	0.2	0.4	3	0.4	1
Triallate	5	15.41	31.60	3	22.9	49.2

LODs and LOQs resulted to be below the established Maximum Detection Limits (MDLs) indicated in the Commission Implementing Decision (EU) 2015/495 both in case of MilliQ and surface water.

However, special care is recommended when evaluating these methodological parameters in the presence of matrix components which could interfere with analytes determination.

The overall sensitivity of developed procedure could be affected by the real matrix, even in cases where the regression analysis did not show any statistical difference.

This contribution becomes even more crucial when the LOD and LOQ are strictly in the range of MDL, as it is clearly shown by EE2 analysis.

LOD and LOQ were estimated to be 0.01 and 0.03ng/l in MilliQ water.

EE2 analysis in surface water showed a baseline noise increase compared to MilliQ water. Consequently LOD and LOQ were estimated to be about 0.03 and 0.07ng/l, respectively, as showed in Figure 33 and 34.

Nevertheless, the recommendations about MDL for this compound were fully met.

As rule of thumb, a proper verification of sensitivity parameters using real matrix samples should always be performed to guarantee the reliability of produced datasets.

Figure 1: Chromatogram of EE2 extracted from 1 litre MilliQ water

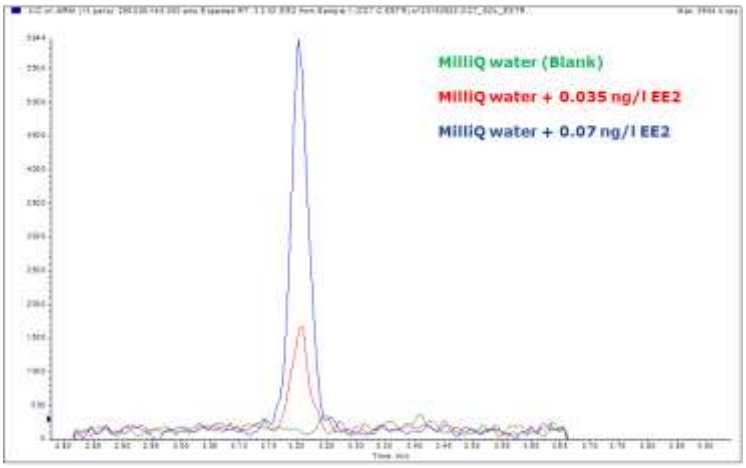
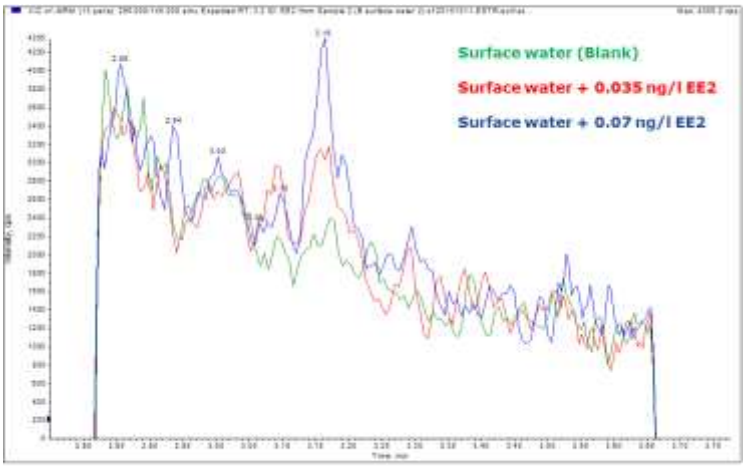


Figure 2: Chromatogram of EE2 extracted from 1 litre surface water



3.3 Linearity study

The linearity of the whole procedures in MilliQ water was studied in calibration ranges reported in Table 6.

Table 6: Calibration ranges and maximum acceptable method detection limit (ng/l)

Analyte	Calibration range (ng/l) in MilliQ water	Maximum acceptable method detection limit (ng/l)
EE2	0.035-0.56	0.035
E2	0.2-3.2	0.4
E1	0.2-3.2	0.4
Diclofenac	5-80	10

Analyte	Calibration range (ng/l) in MilliQ water	Maximum acceptable method detection limit (ng/l)
BHT	375-6000	3160
EHMC	780-12480	6000
Clarythromycin	11.25-180	90
Azythromycin	11.25-180	90
Methiocarb	1.25-20	10
Acetamiprid	1.13-18.08	9
Clothianidin	1.13-18.08	9
Imidacloprid	1.13-18.08	9
Thiacloprid	1.13-18.08	9
Thiamethoxam	1.13-18.08	9
Oxadiazon	11-176	88
Triallate	83.75-1340	670

In order to verify the linearity of the calibration curve, a blank sample spiked only with labelled IS and five spiked MilliQ water samples were extracted and analysed on three different days. The calibration curves are illustrated in Figure 1.

Figure 3: EE2 calibration curve

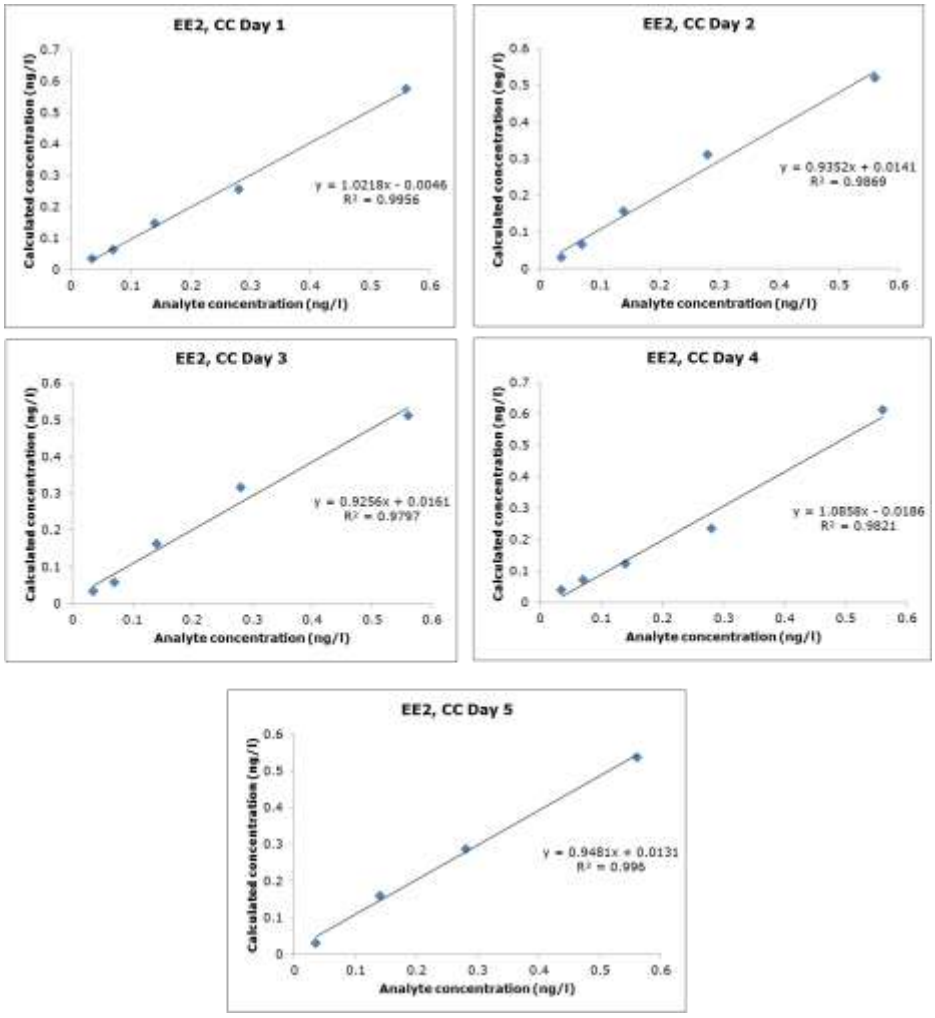


Figure 4: E2 calibration curve

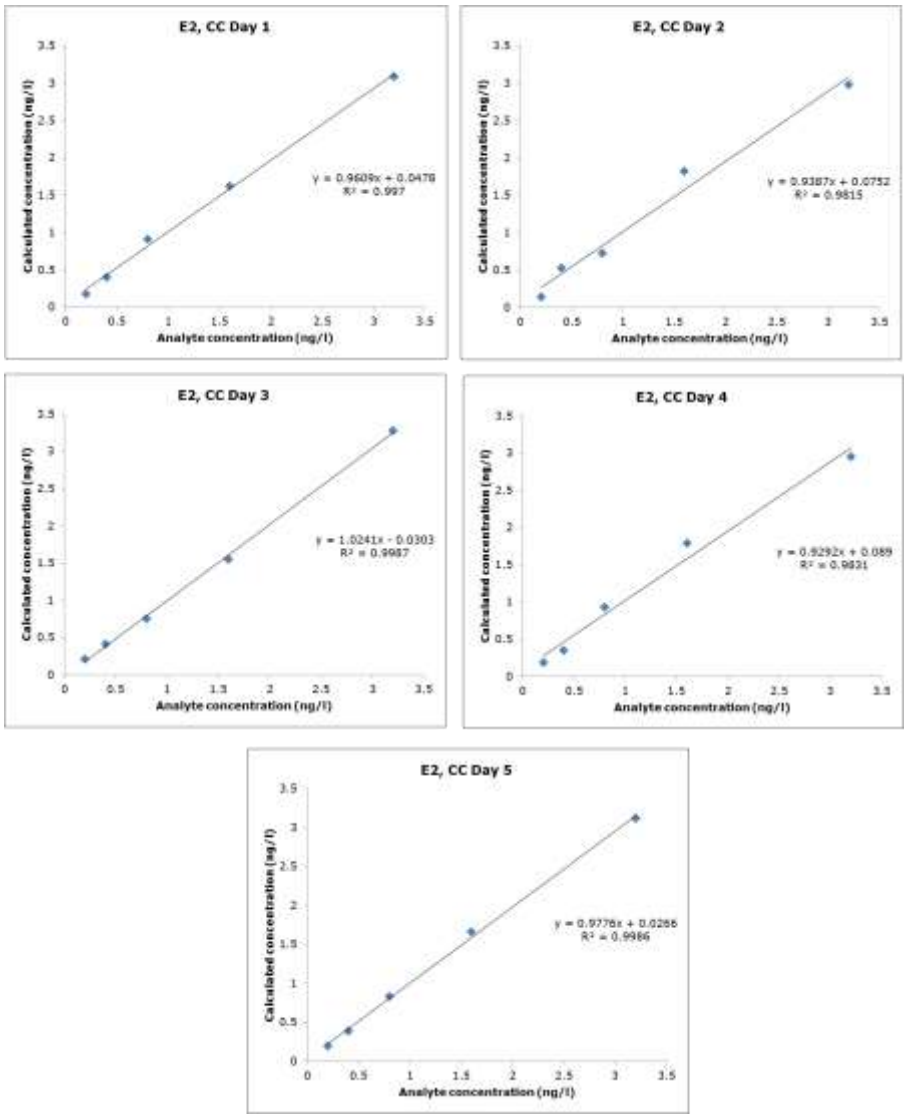


Figure 5: E1 calibration curve

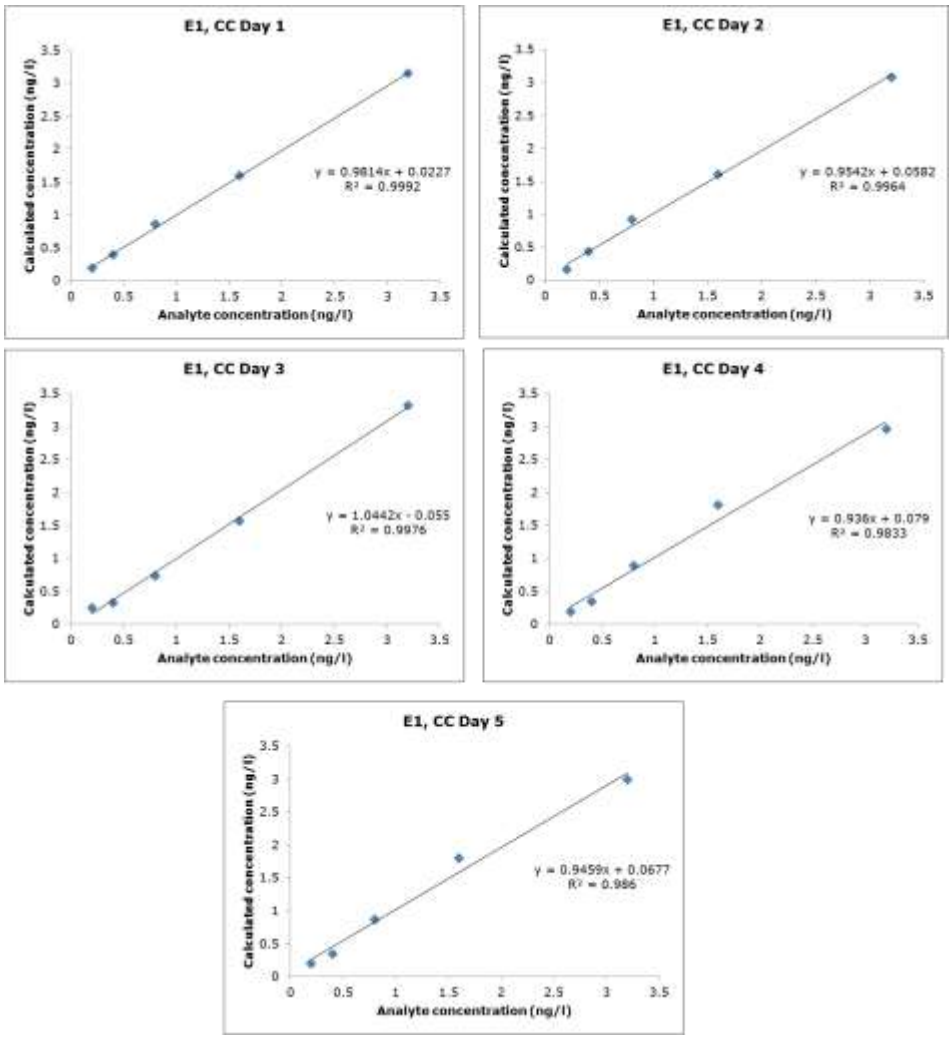


Figure 6: Diclofenac calibration curves

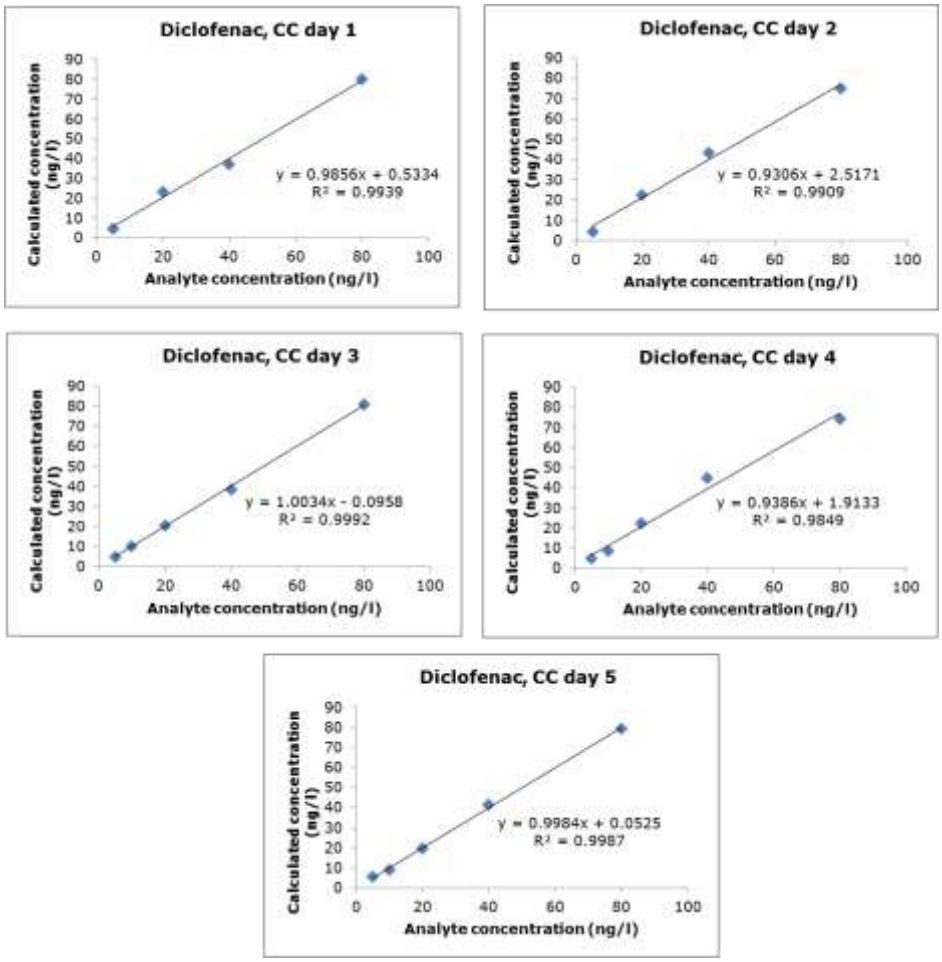


Figure 7: BHT calibration curves

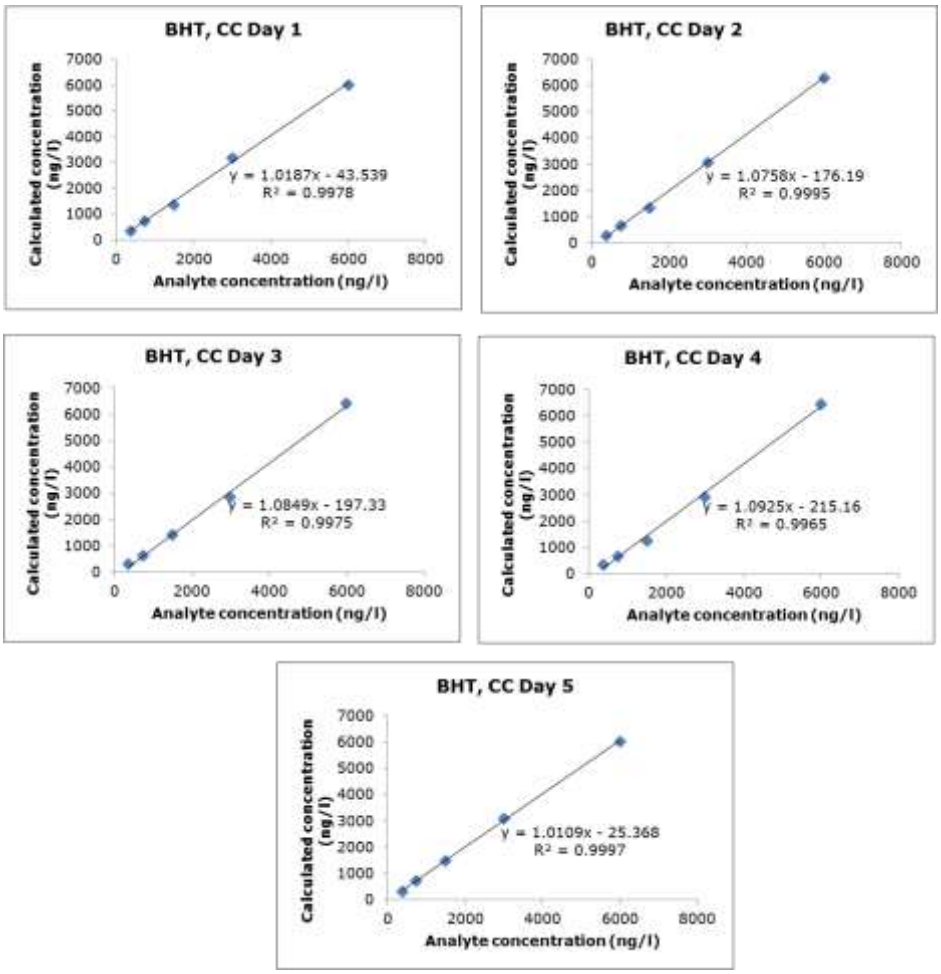


Figure 8: EHMC calibration curves

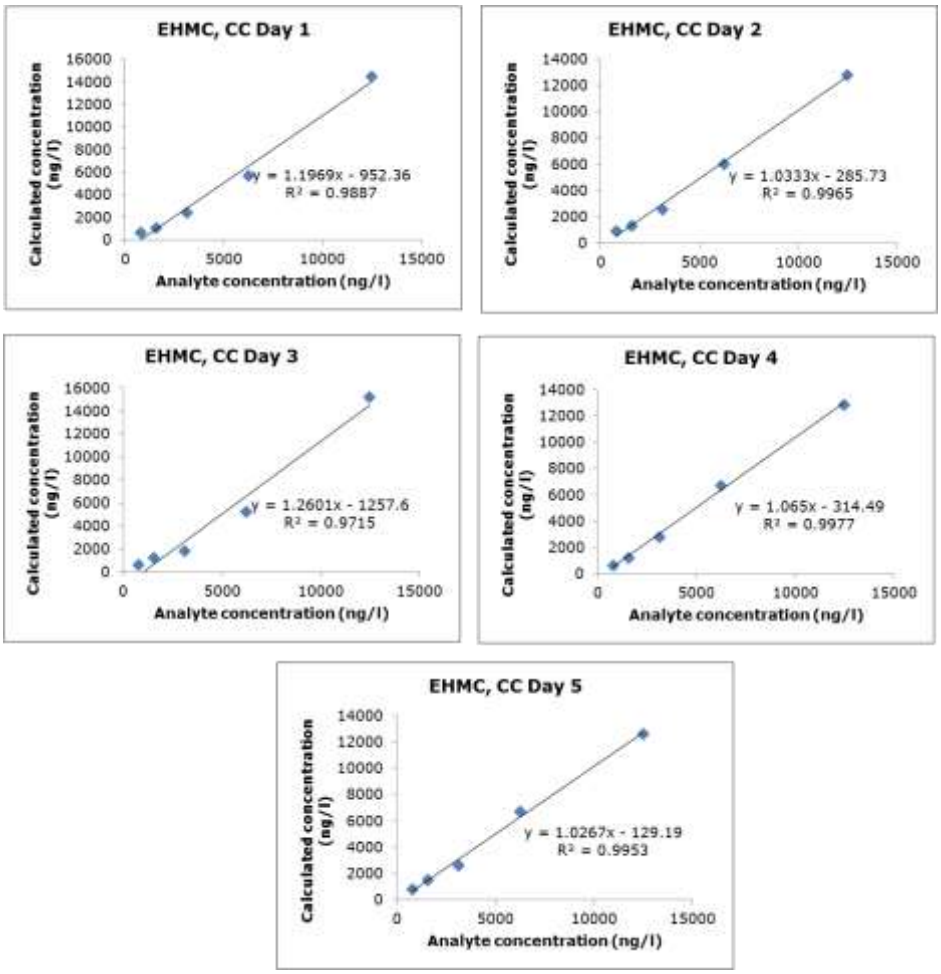
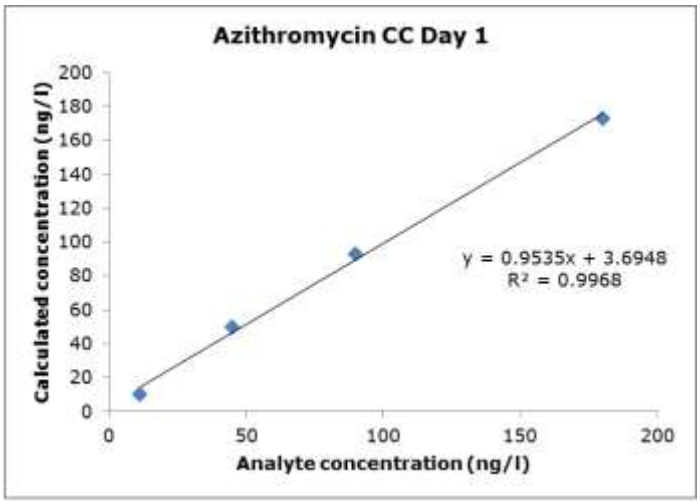


Figure 9: Azithromycin calibration curves



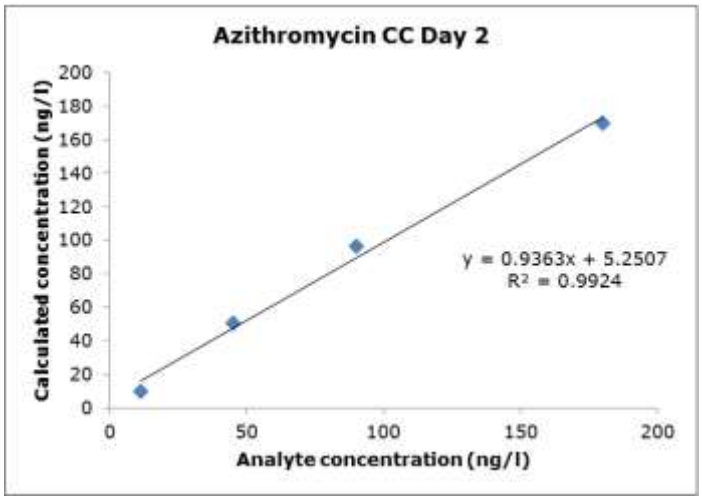
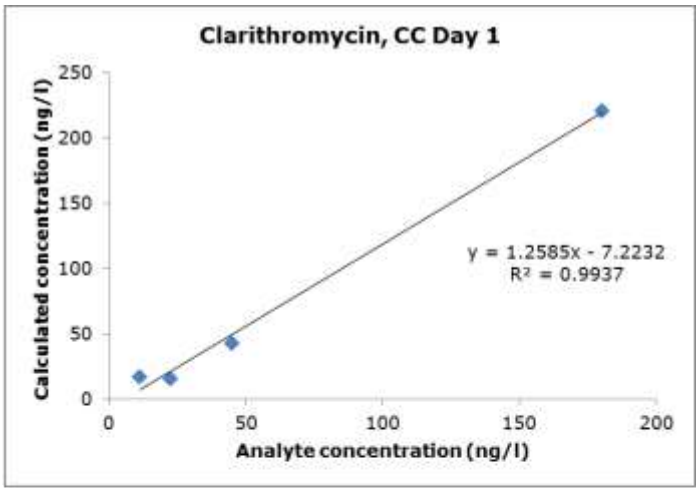


Figure 10: Clarithromycin calibration curves



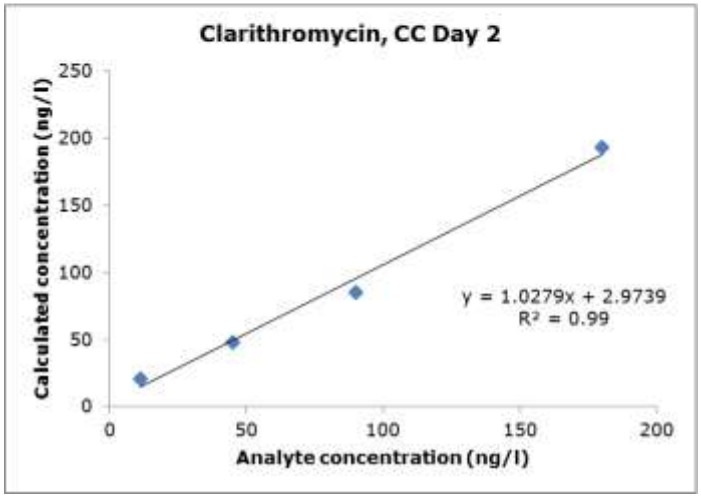


Figure 11: Methiocarb calibration curves

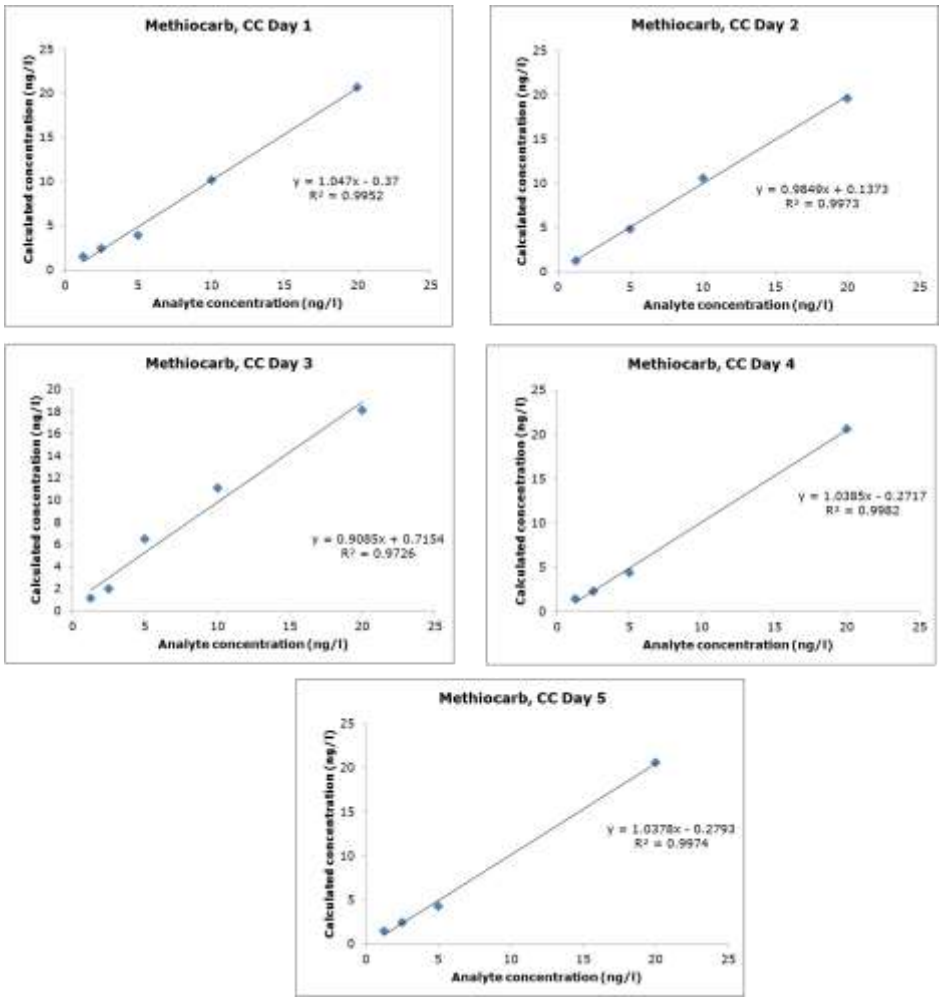


Figure 12: Acetamiprid calibration curves

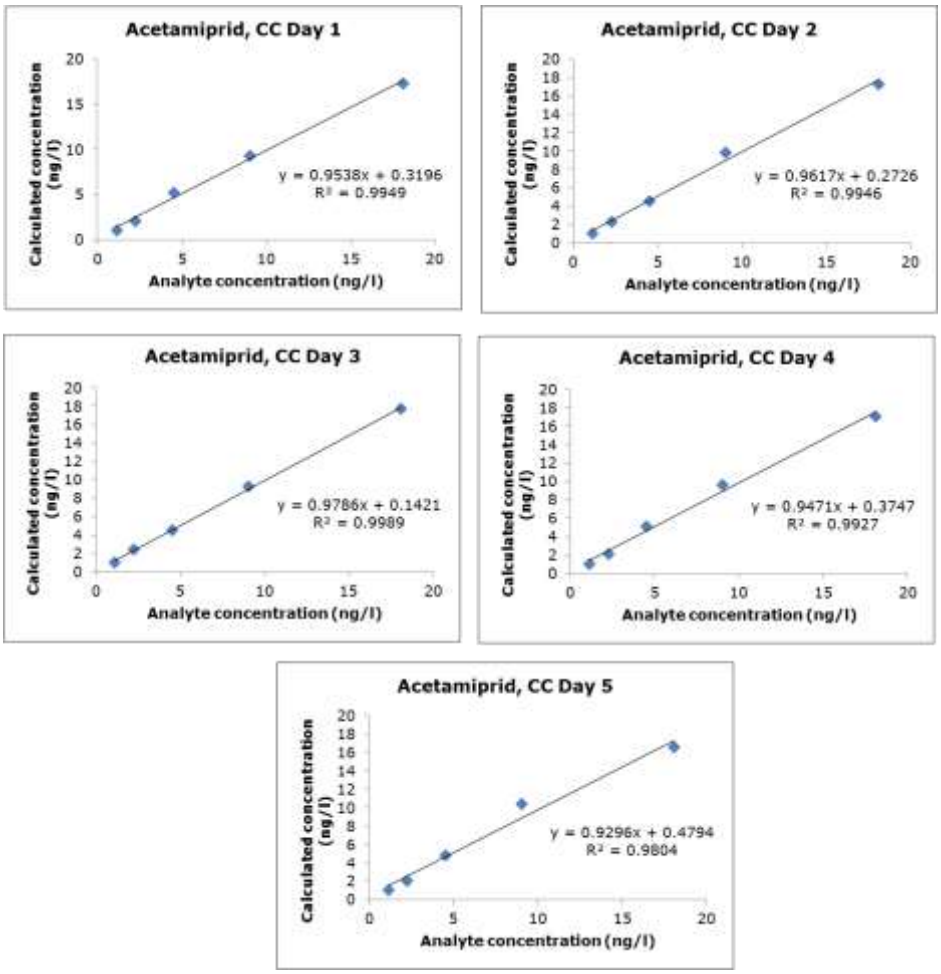


Figure 13: Clothianidin calibration curves

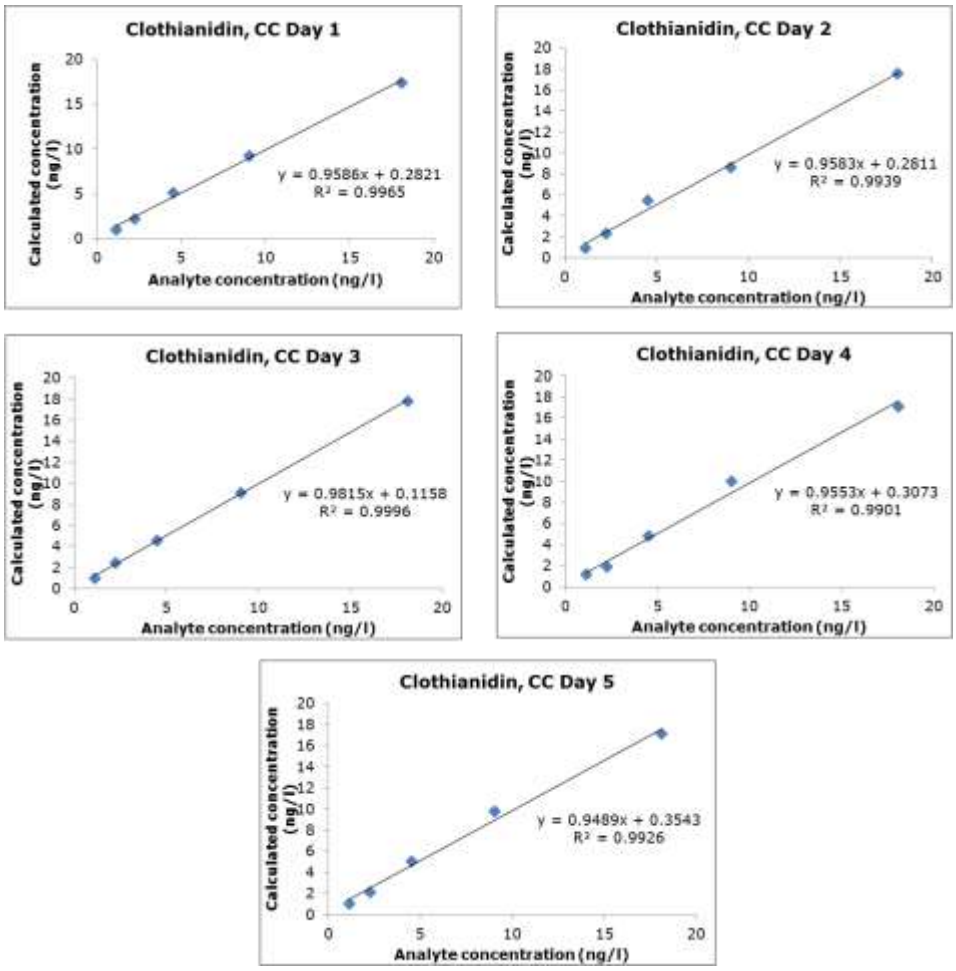


Figure 14: Imidacloprid calibration curves

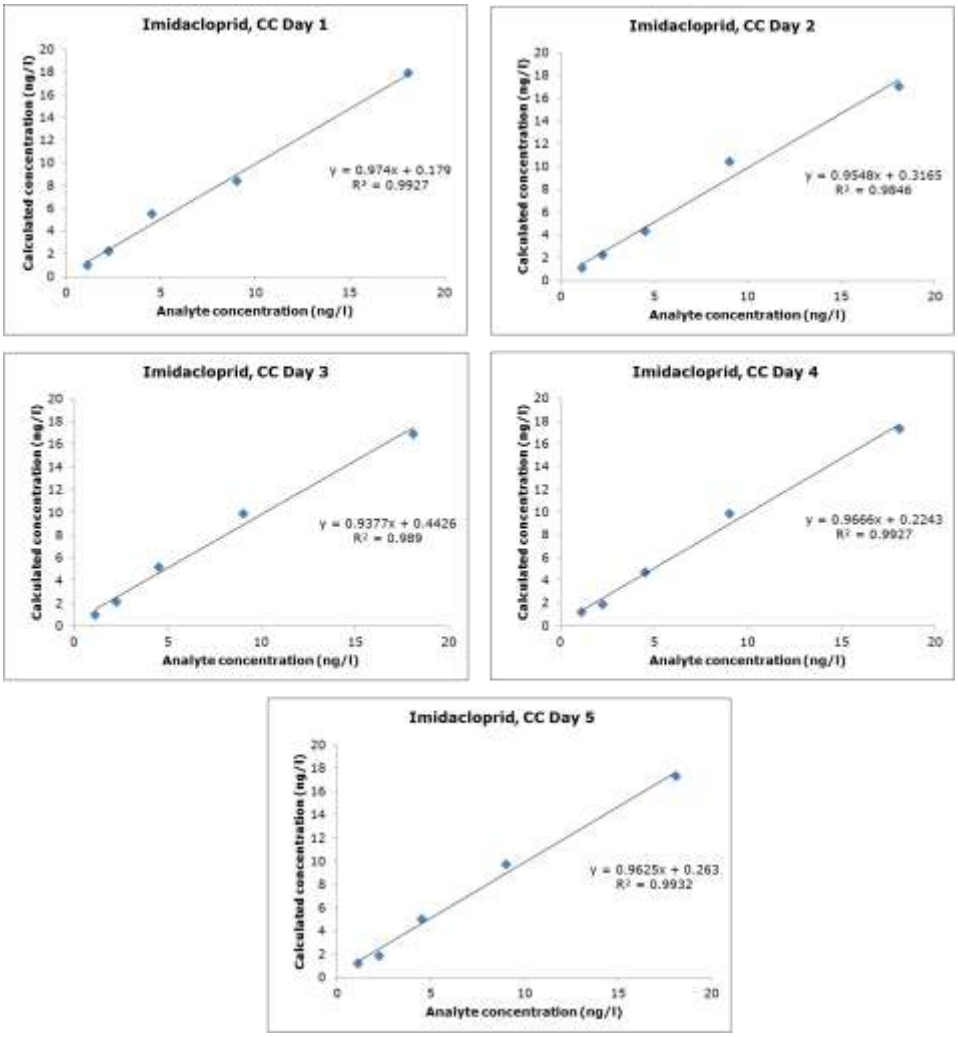


Figure 15: Thiacloprid calibration curves

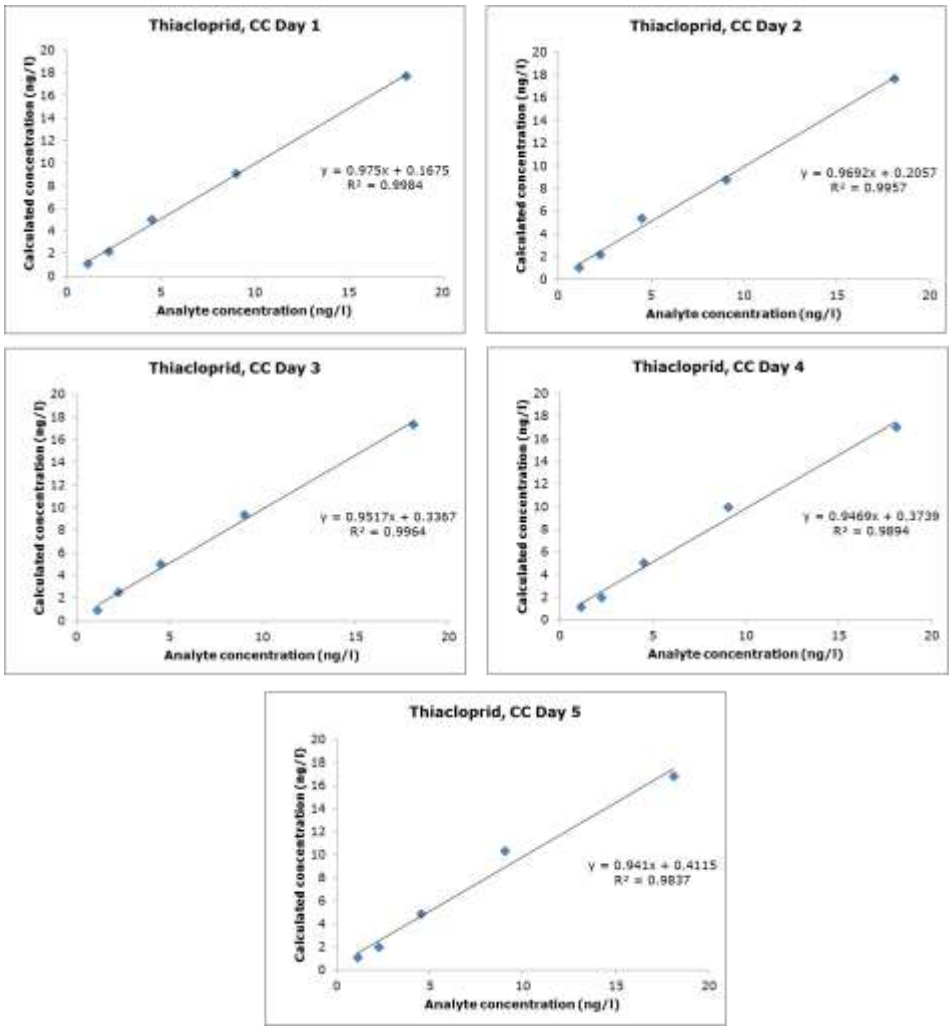


Figure 16: Thiamethoxam calibration curves

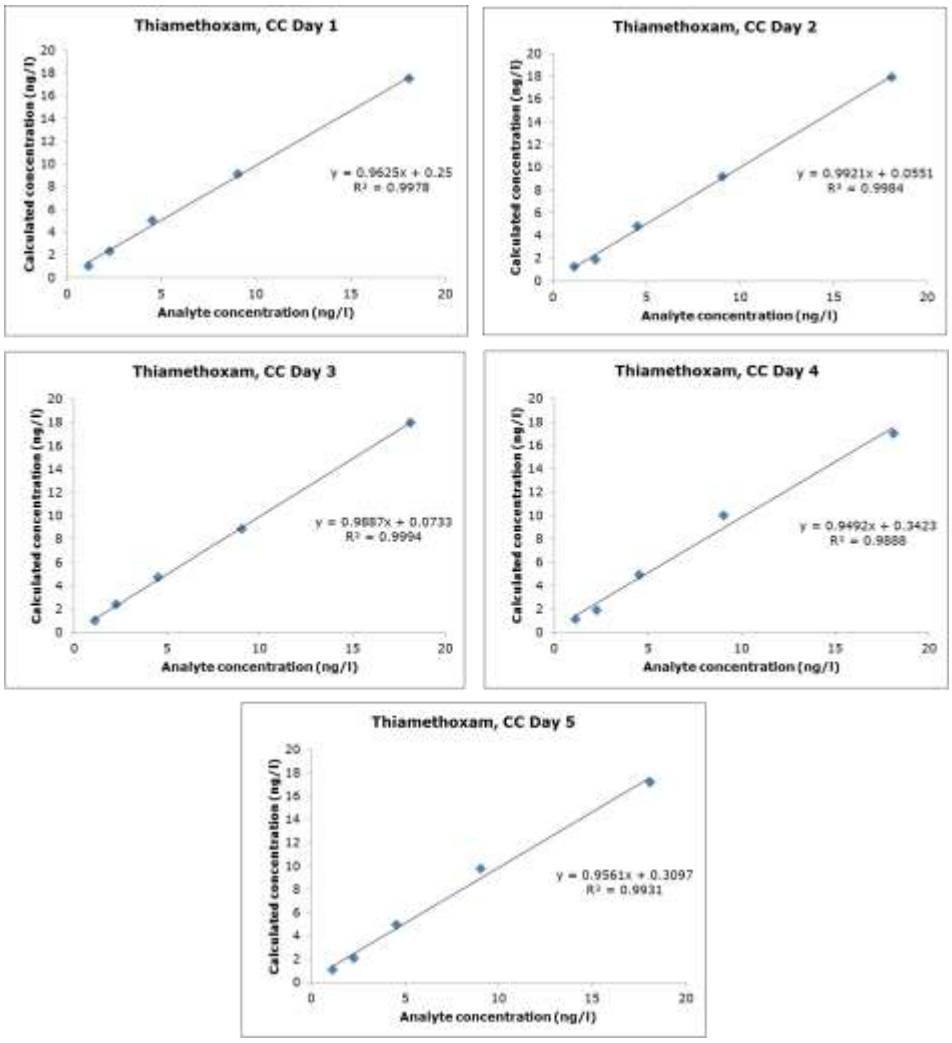


Figure 17: Oxadiazon calibration curves

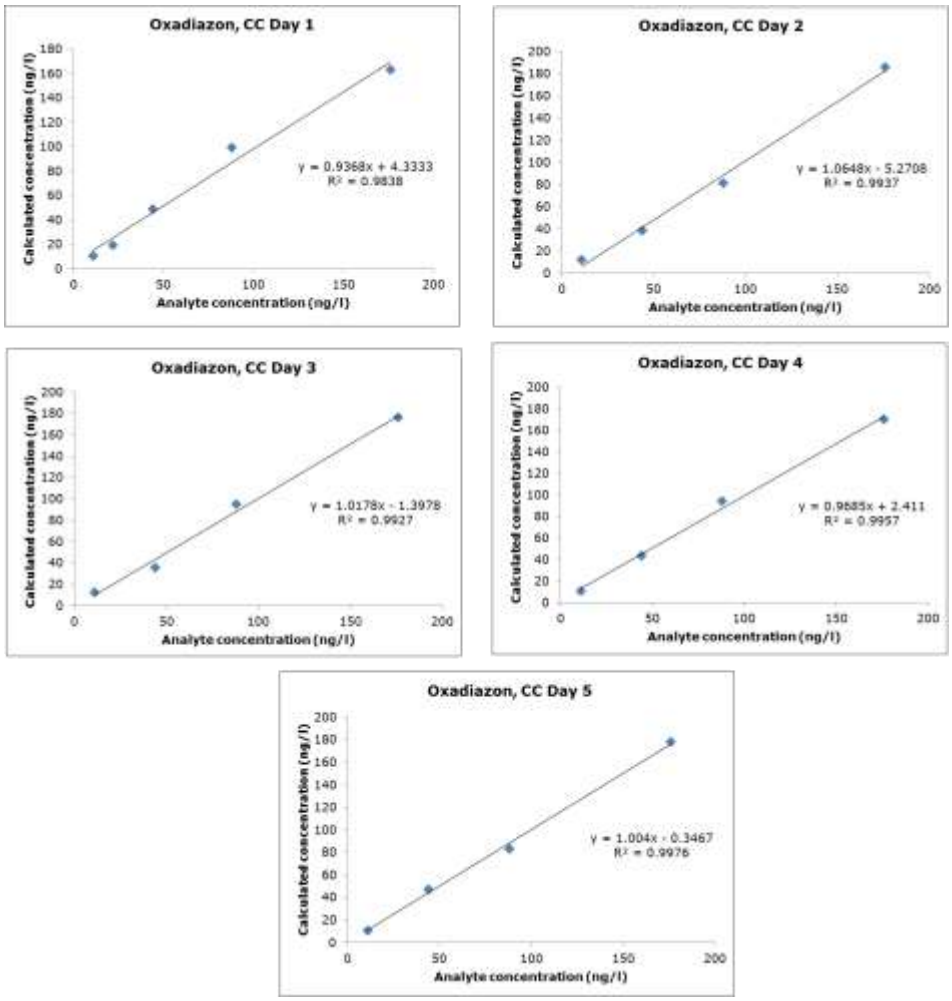


Figure 18: Triallate calibration curves

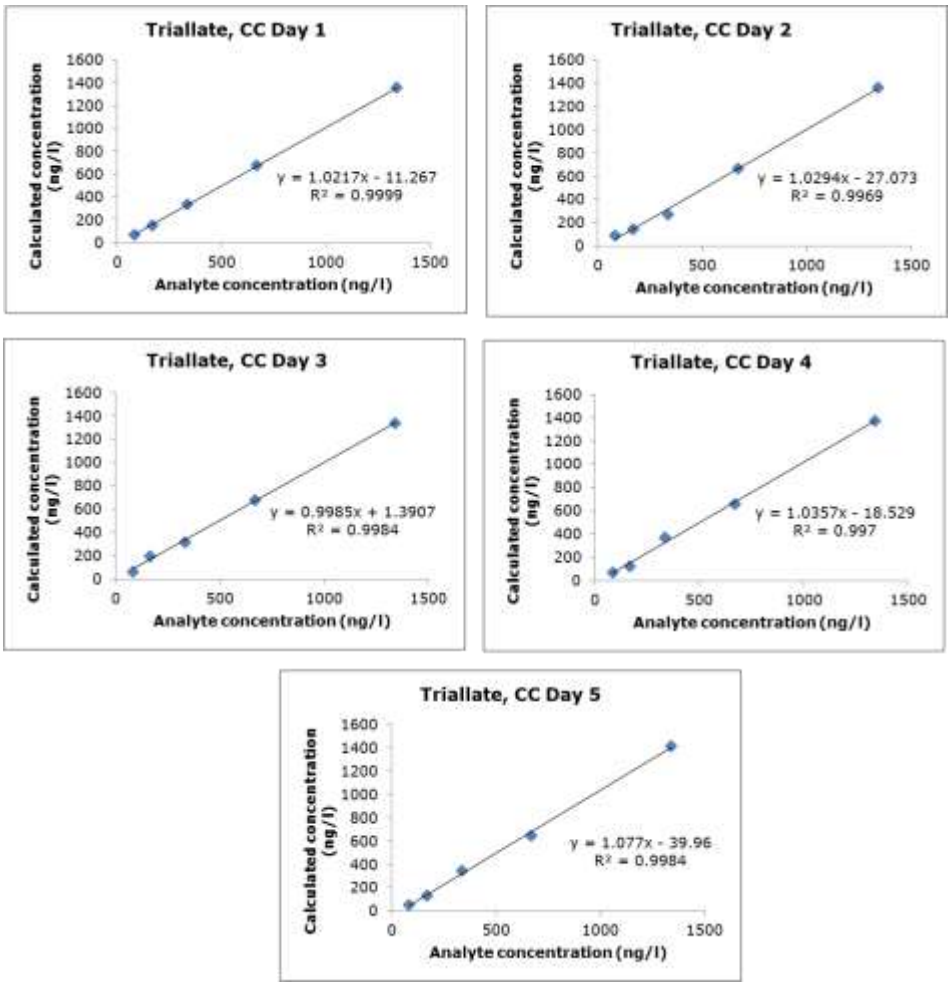


Table 7 summarises the coefficients of determination on five days of validation, together with the mean values and the RSDs for each selected compound.

Table 7: Coefficient of determination (R^2) values for calibration curves on different days

Analyte	R^2 Day 1	R^2 Day 2	R^2 Day 3	R^2 Day 4	R^2 Day 5	Mean R^2	RSD %
EE2	0.9956	0.9820	0.9960	0.9870	0.9960	0.9913	0.6
E2	0.9940	0.9970	0.9815	0.9987	0.9831	0.9921	0.8
E1	0.9933	0.9992	0.9964	0.9976	0.9833	0.9939	0.7
Diclofenac	0.9815	0.9939	0.9909	0.9992	0.9849	0.9987	0.6
BHT	0.9978	0.9995	0.9975	0.9965	0.9997	0.9982	0.1
EHMC	0.9887	0.9965	0.9715	0.9977	0.9953	0.9900	1.1
Clarythromycin	0.9937	0.9900	0.9950	0.9965	na	0.9935	0.3
Azythromycin	0.9968	0.9924	na	na	na	0.9946	0.3
Methiocarb	0.9952	0.9973	0.9726	0.9982	0.9974	0.9921	1.1
Acetamiprid	0.9949	0.9946	0.9989	0.9927	0.9804	0.9932	0.7
Clothianidin	0.9965	0.9939	0.9996	0.9996	0.9901	0.9959	0.4
Imidacloprid	0.9927	0.9846	0.9890	0.9927	0.9932	0.9904	0.4
Thiacloprid	0.9984	0.9957	0.9964	0.9894	0.9894	0.9938	0.4
Thiamethoxam	0.9978	0.9984	0.9994	0.9888	0.9931	0.9955	0.4
Oxadiazon	0.9838	0.9937	0.9927	0.9957	0.9976	0.9927	0.5
Triallate	0.9999	0.9969	0.9984	0.9970	0.9984	0.9981	0.1

For all analytes, the R^2 respect the set performance criteria of > 0.9900.

The study of the distribution of residuals revealed shapes heterogeneously distributed around the horizontal axis, proving the pertinence of the linear regression model for interpreting the data. The residual plots are shown in the following figures.

Figure 19: EE2 residual plot

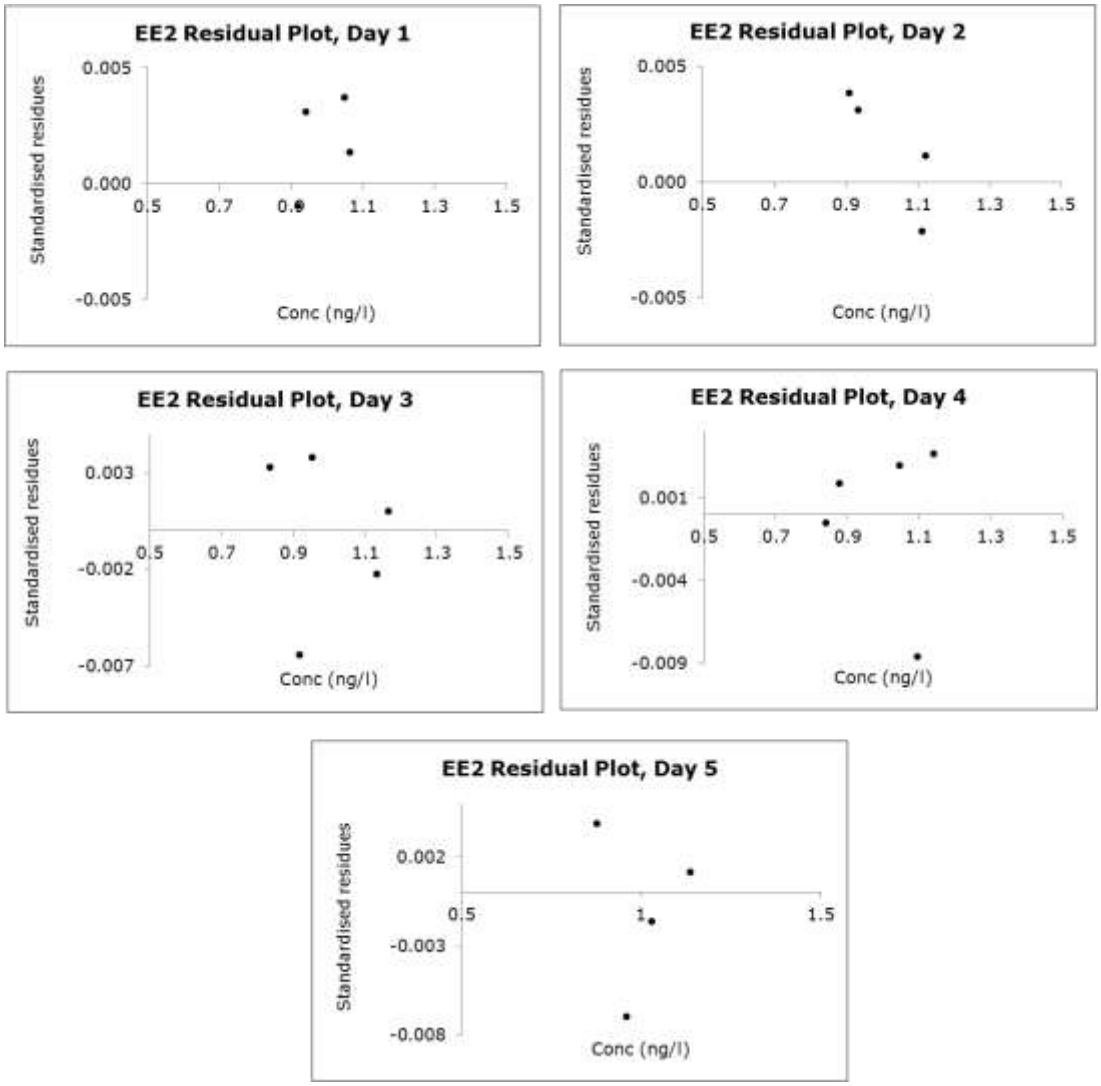


Figure 20: E2 residual plot

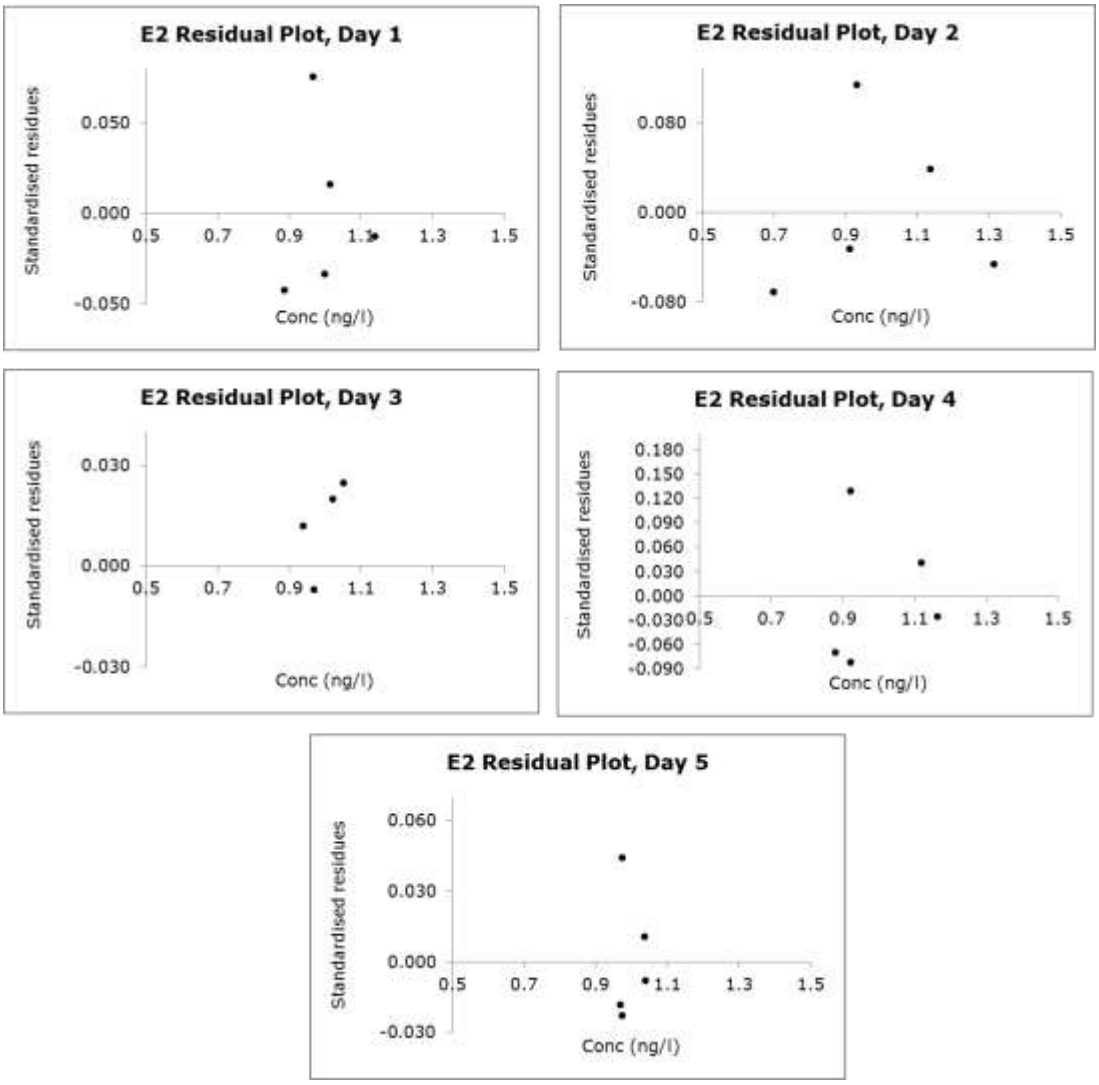


Figure 21: E1 residual plot

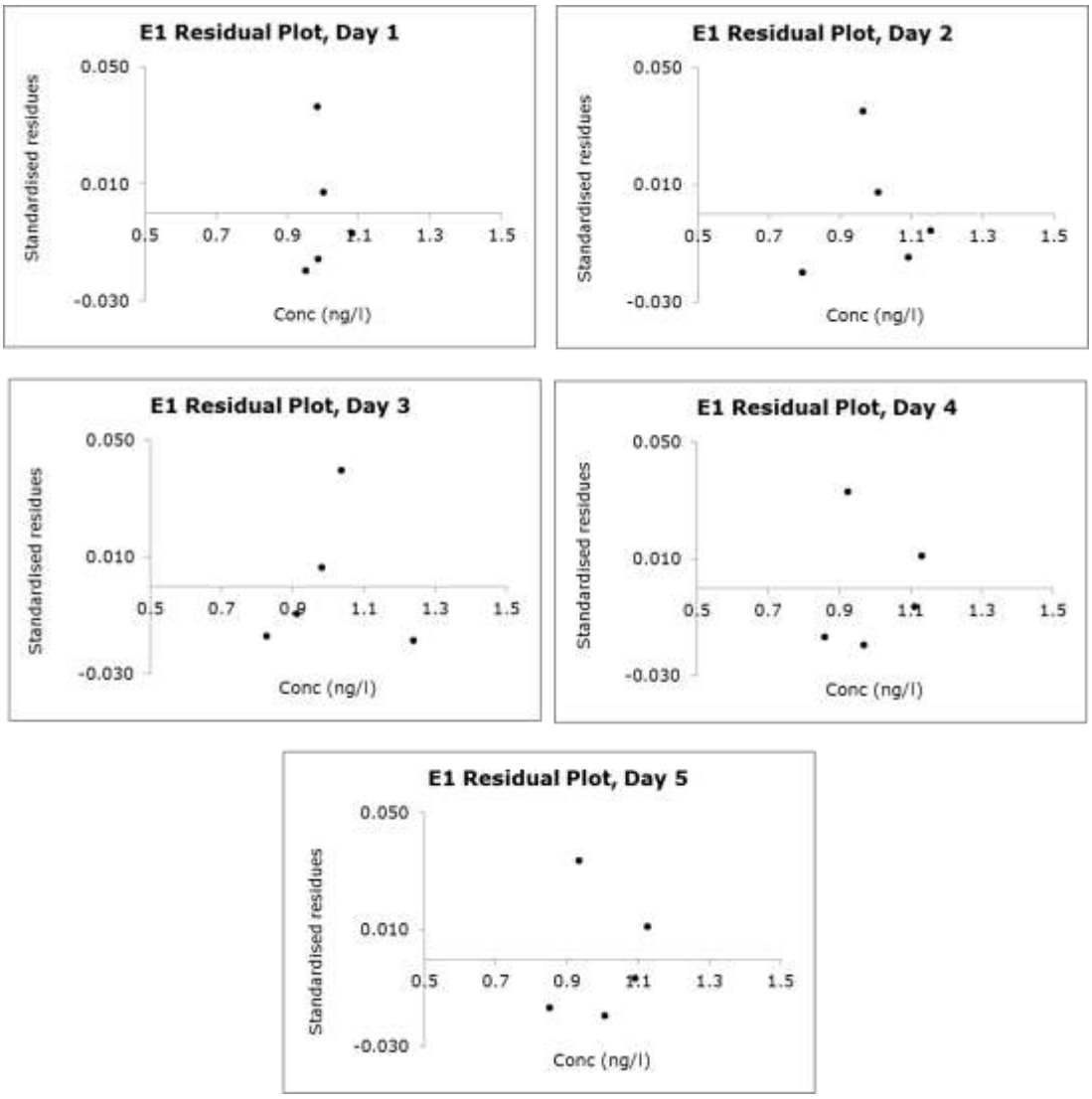


Figure 22: Diclofenac residual plot

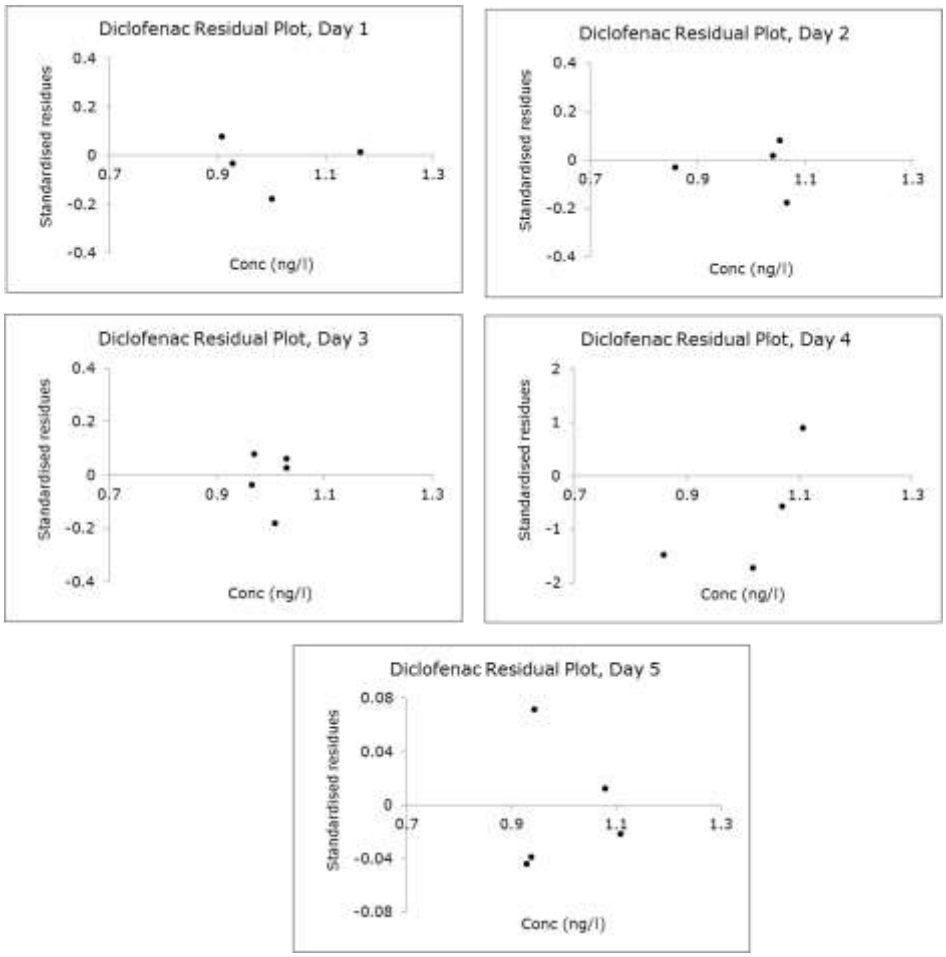
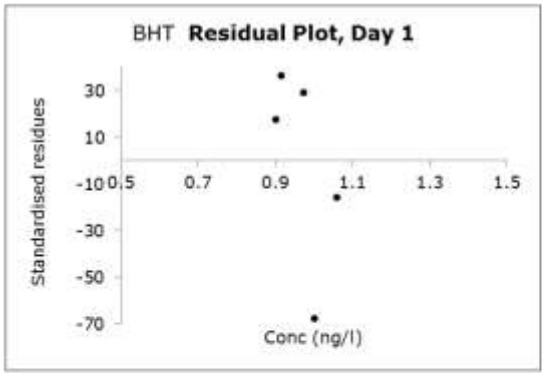
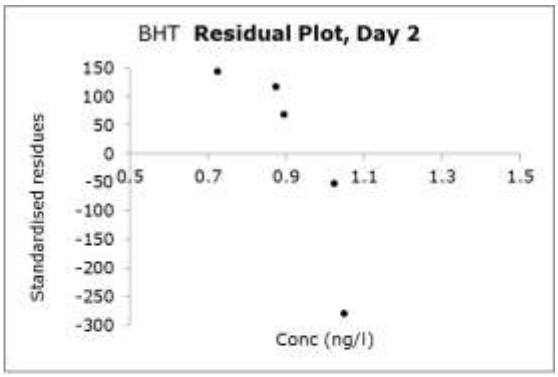
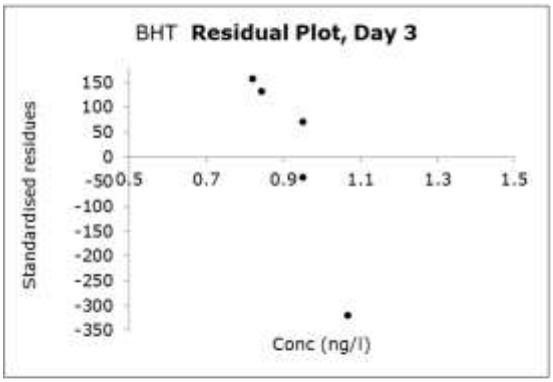
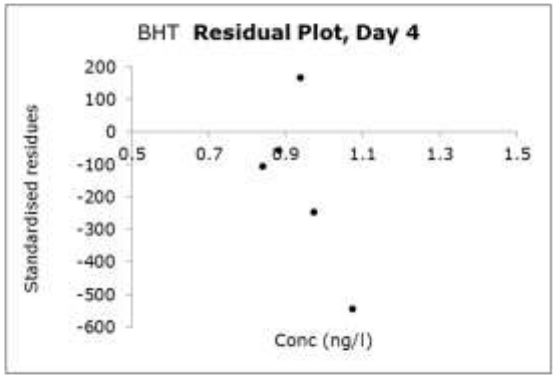


Figure 23: BHT residual plot









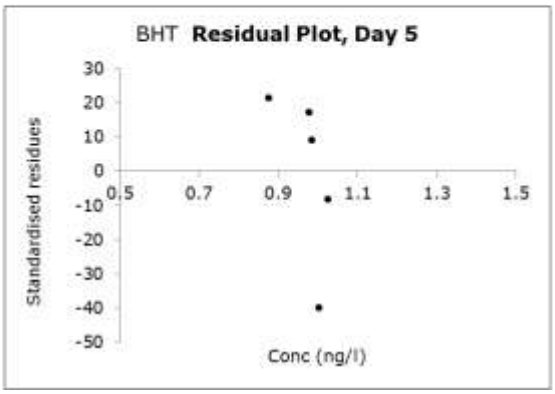
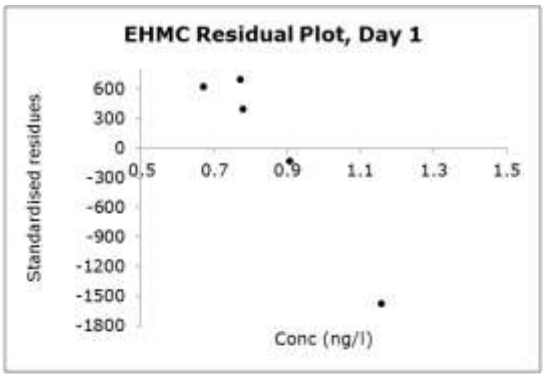
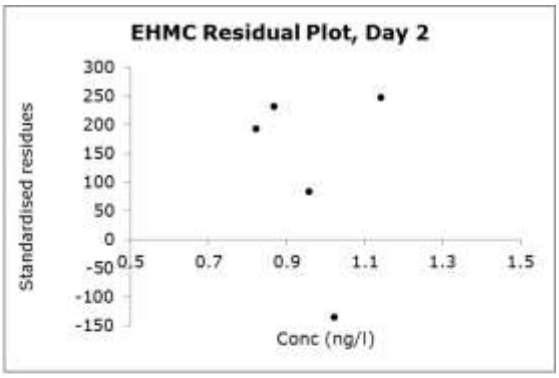
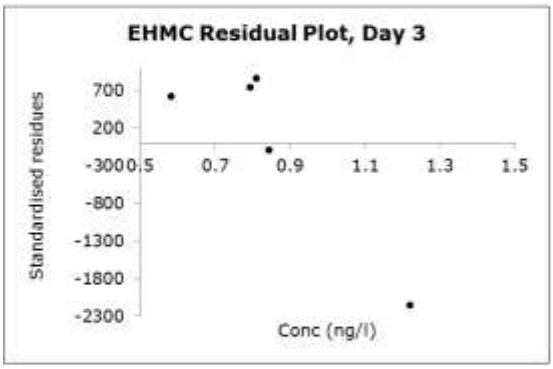
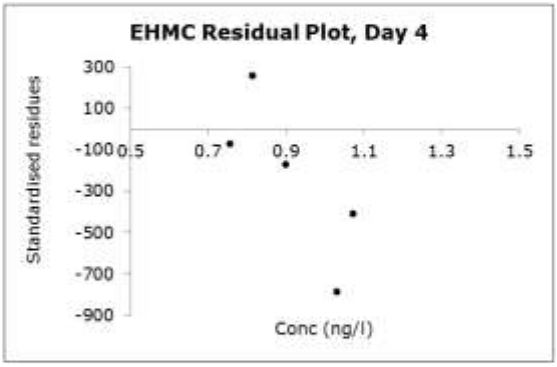


Figure 24: EHMC residual plot









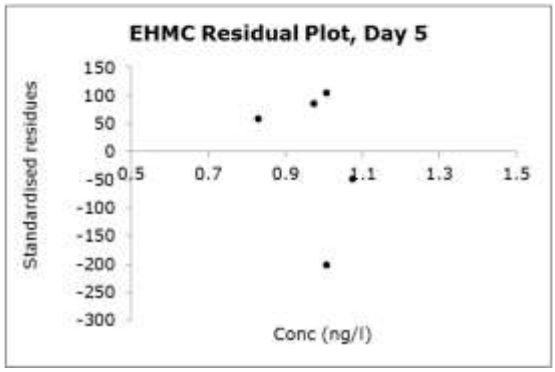


Figure 25: Azithromycin residual plot

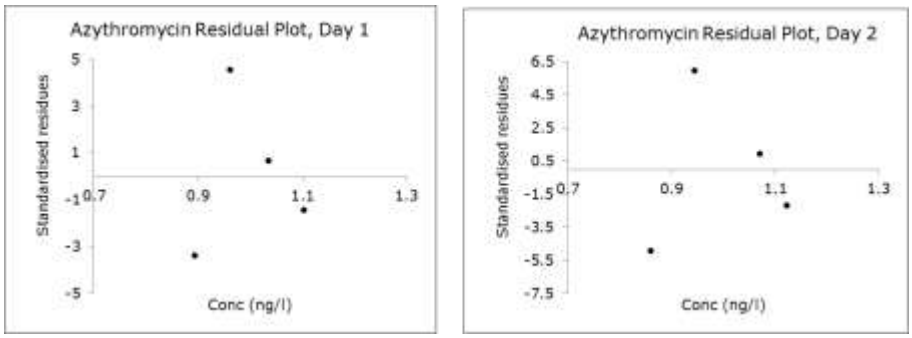


Figure 26: Clarithromycin residual plot

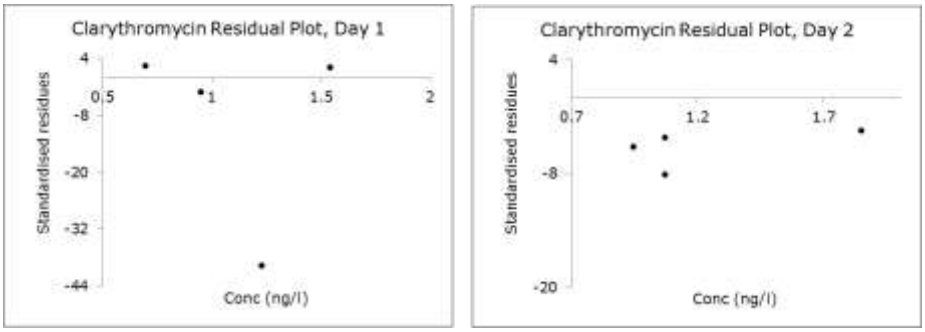


Figure 27: Methiocarb residual plot

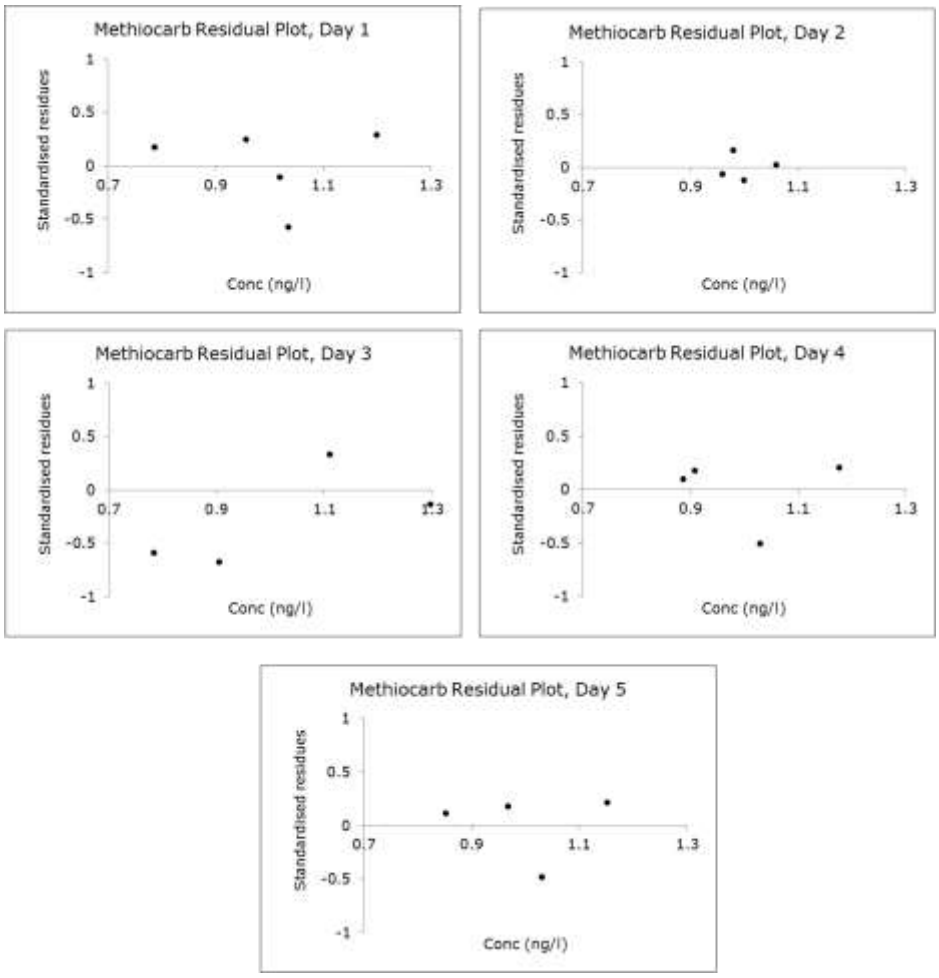


Figure 28: Acetamiprid residual plot

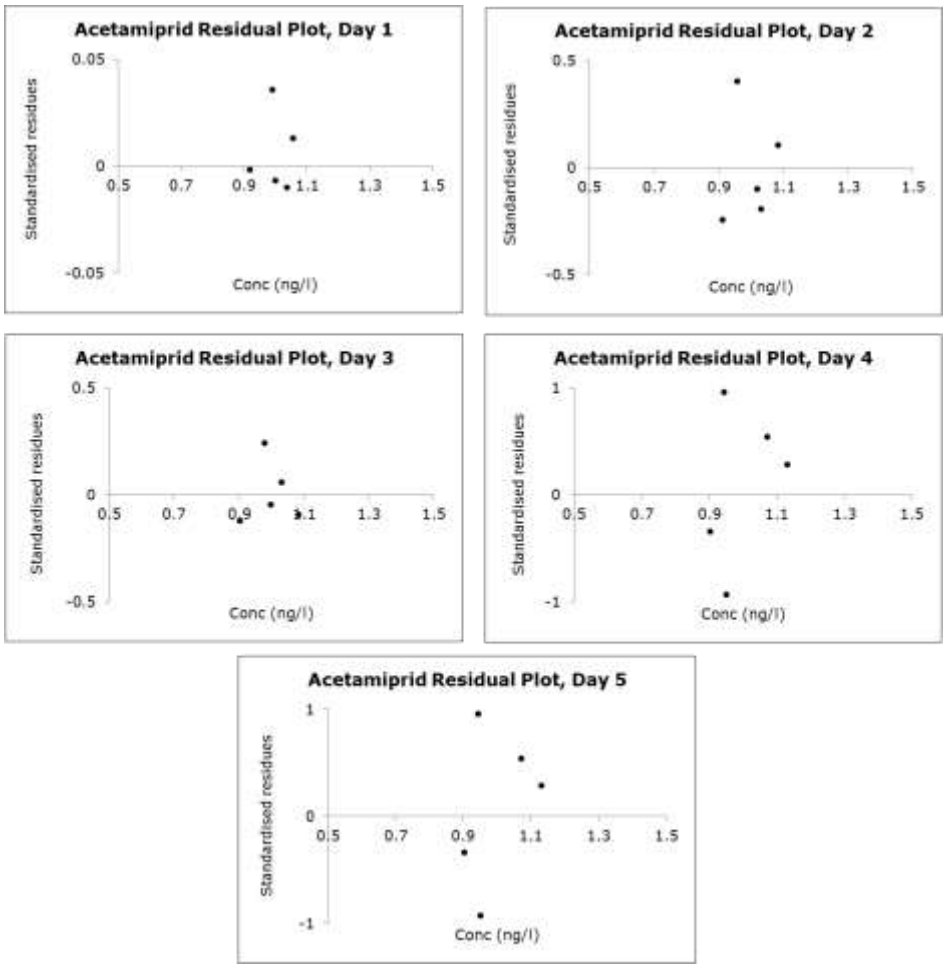


Figure 29: Clothianidin residual plot

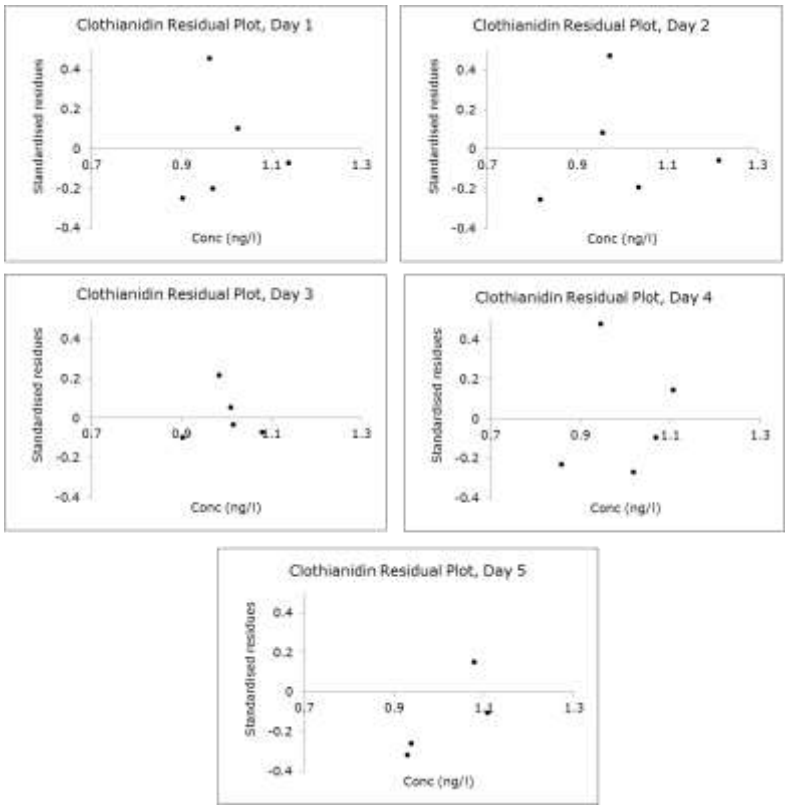


Figure 30: Imidacloprid residual plot

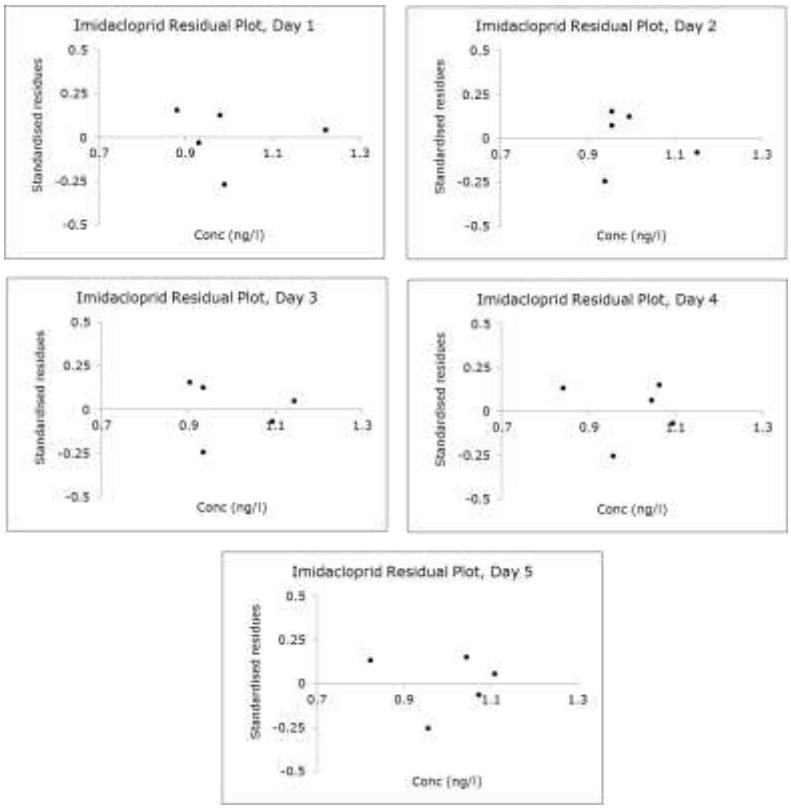


Figure 31: Thiacloprid residual plot

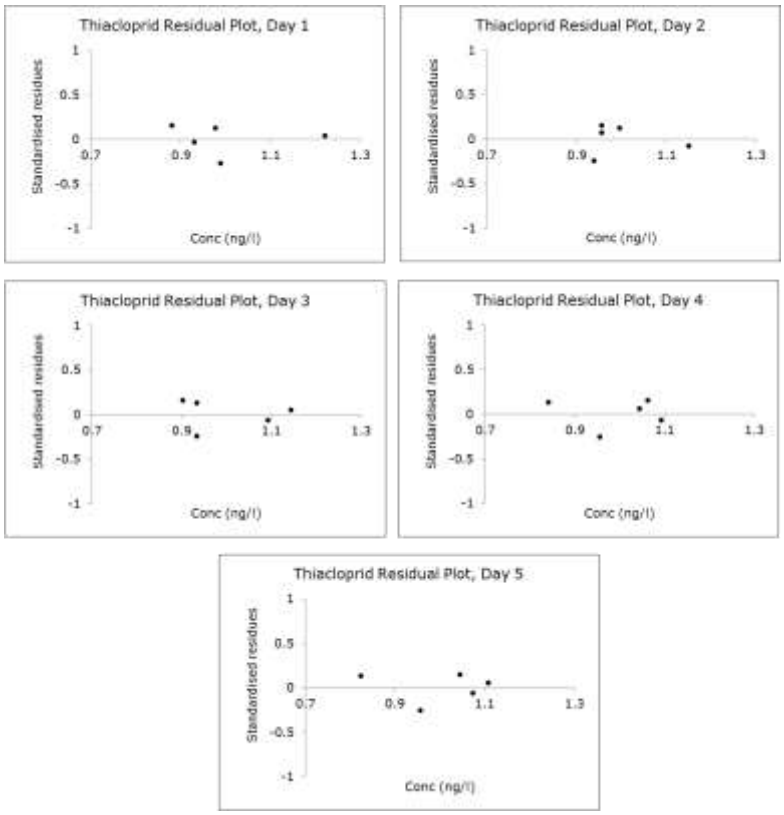


Figure 32: Thiamethoxam residual plot

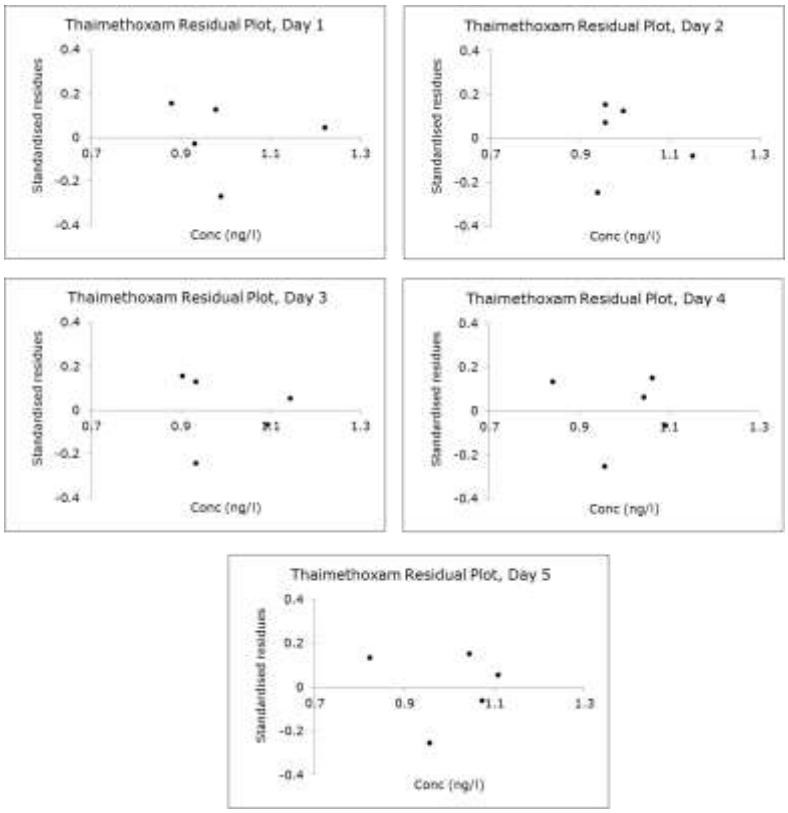


Figure 33: Oxadiazon residual plot

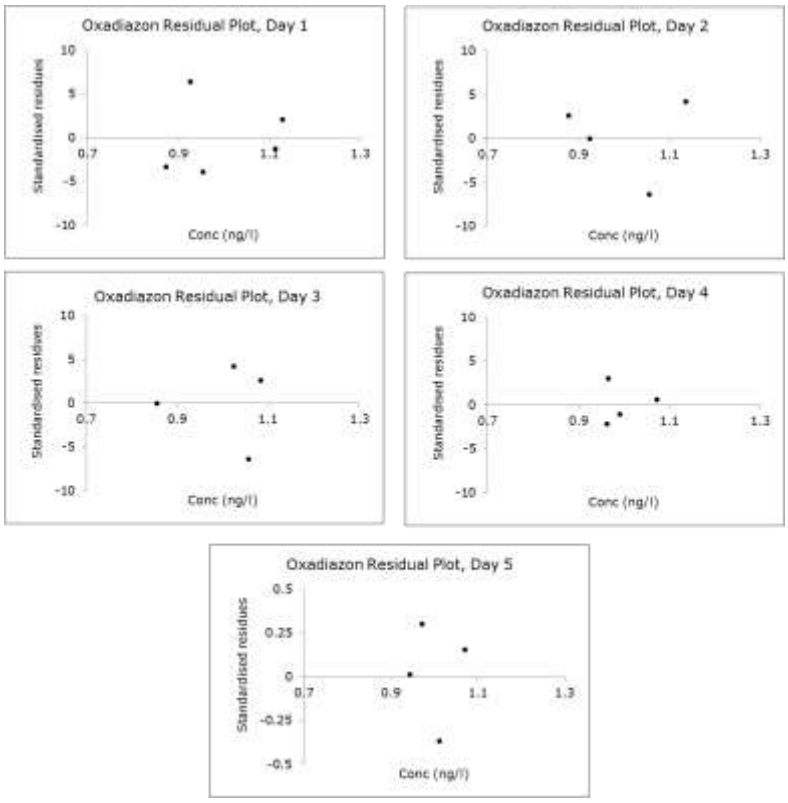
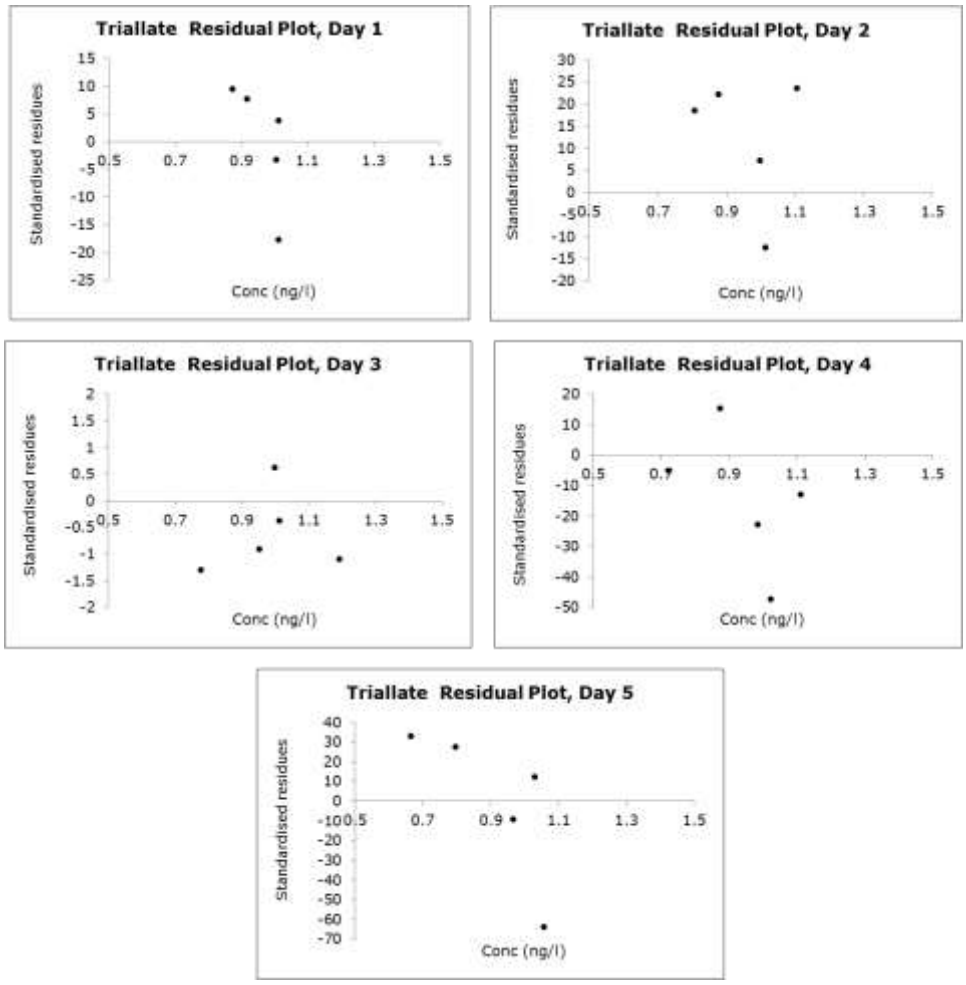


Figure 34: Triallate residual plot



3.3.1 Working range

The working range, defined as the range of concentrations for which the chosen calibration curve is valid, was determined by the lowest and the highest calibration points in the respective calibration curve and matrix. Table 8 summarises the working ranges established in the procedure for the selected analytes both in MilliQ and surface water.

Table 8: Working ranges of the analytical method

Analyte	Working range (ng/l) in MilliQ water	Working range (ng/l) in surface water
EE2	0.035-0.56	0.07-0.56
E2	0.2-3.2	0.2-3.2
Estrone	0.2-3.2	0.2-3.2
Diclofenac	5-80	5-80
BHT	375-6000	375-6000
EHMC	780-12480	780-12480
Clarythromycin	11.25-180	11.25-180

Azythromycin	11.25-180	11.25-180
Methiocarb	1.25-20	1.25-20
Acetamiprid	1.13-18.08	1.13-18.08
Clothianidin	1.13-18.08	1.13-18.08
Imidacloprid	1.13-18.08	1.13-18.08
Thiacloprid	1.13-18.08	1.13-18.08
Thiamethoxam	1.13-18.08	1.13-18.08
Oxadiazon	11-176	11-176
Triallate	83.75-1340	83.75-1340

In case of EE2 determination in surface water, the lowest point of the calibration curve changed to 0.07 ng/l.

In case of analytical determinations of concentration values included between the lowest point of the calibration curve and the estimated LOQ, an accurate verification of the validity of the linear model for data interpolation is recommended.

It can be easily accomplished by analysing samples spiked at the opportune level.

3.4 Matrix comparison

The assumption verification and the ANCOVA analysis were carried out using the R software (5); the R code used for the analysis and the full computations are given in the Annex 1. A summary of the results is reported here.

3.4.1 Verification of ANCOVA assumption

3.4.1.1 Independence

This assumption tests the independence of the covariate variable (concentrations of the standard) among groups (days). The full R outputs are given in Table 31 (MilliQ water), Table 32 (surface water) and Table 33 (matrix comparison) of the Annex 1.

Table 9, provides summary results of the independence test.

Since concentration levels of the covariate are equal for all days the computed p-value, resulting from the independence test, is 1 for all cases. With p-values greater than 0.05 (95% level of confidence), the hypothesis of independence is accepted for all the compounds in the three specified cases.

Table 9: Summary results of the independence test

Compounds	MilliQ water	Lake water	Matrix comparison
17-α-Ethinyl estradiol	True	True	True
17-β-Estradiol	True	True	True
Estrone	True	True	True
Diclofenac	True	True	True

Compounds	MilliQ water	Lake water	Matrix comparison
BHT	True	True	True
EHMC	True	True	True
Clarythromycin	True	True	True
Azythromycin	True	True	True
Methiocarb	True	True	True
Acetamiprid	True	True	True
Clothianidin	True	True	True
Imidacloprid	True	True	True
Thiacloprid	True	True	True
Thiamethoxam	True	True	True
Oxadiazon	True	True	True
Triallate	True	True	True

3.4.1.2 Normality

To inspect if the distribution of residuals is normal, the quantile-quantile (Q-Q) plot is used. This graph plots the cumulative values of the data against the cumulative probability of a normal distribution. Each value is compared to the expected value that the score should have in a normal distribution and they are plotted against one another.

If the residuals follow the normal distribution, then the points on the Q-Q plot will fall approximately on a straight line; deviations from the line show deviations from normality. Only significant departures from the line suggest violations of normality.

When the sample size is small, as in the case under analysis, non-normality can be hard to detect.

QQ-plots are given in Table 34 (MilliQ water), Table 35 (surface water) and Table 36 (matrix comparison) of Annex 1. No significant deviation from normality is verified for all the analysed compounds in all three examined cases.

3.4.1.3 Homogeneity of variance

Levene's test was used to determine if the variance in the outcome variable changes across groups. The full R output is given in Table 37 (MilliQ water), Table 38 (surface water) and Table 39 (matrix comparison) of Annex 1. Table 10 gives summary results of the homogeneity of variance test.

For all the selected compounds in all the examined cases, Levene's test results were non-significant, with p-values always higher than 0.05 (95% confidence level). This means that the variances are very similar and the hypothesis of homogeneity of variances is accepted.

Table 10: Summary results of the homogeneity of variance test

Compounds	MilliQ water	Surface water	Matrix comparison
17-α-Ethinyl estradiol	True	True	True
17-β-Estradiol	True	True	True
Estrone	True	True	True
Diclofenac	True	True	True
BHT	True	True	True
EHMC	True	True	True
Clarythromycin	True	True	True
Azythromycin	True	True	True
Methiocarb	True	True	True
Acetamiprid	True	True	True
Clothianidin	True	True	True
Imidacloprid	True	True	True
Thiacloprid	True	True	True
Thiamethoxam	True	True	True
Oxadiazon	True	True	True
Triallate	True	True	True

3.4.1.4 Linearity

The assumption of linearity is checked by a simple inspection of the calibration scatterplots for each day separately. No outliers should occur.

Calibration graphs reported in 3.2 provide a positive response for the linearity assumption.

3.4.1.5 Homogeneity of regression slopes

This assumption is verified by examining the scatter plot for each experimental condition (factor) with the covariate on one axis and the outcome on the other. The regression line for each of these scatter plots is then calculated, and the homogeneity of regression slopes is accepted if slopes are similar across factors.

Calibration graphs reported in 3.3 show that slopes of the regression lines computed in different days are similar.

3.4.2 Results of the ANCOVA analysis

ANCOVA was applied in order to compare slopes and intercepts of regression curves in the following three cases:

a. five-day calibration curves for compounds analysed in MilliQ water;

b. three-day calibration curves for compounds measured in surface water;

c. two calibration curves, one in MilliQ water and one in surface water, for each compound, taken from the first two cases after accepting the equality of regression curves over days.

All statistical analyses were performed using R software (5). The R code used for the ANCOVA analysis and the full R outputs are given in the Annex 1.

3.4.2.1 Case a: MilliQ water

The ANCOVA model was performed specifying five different slopes and five different intercepts (one a day). For the compounds Azythromycin and Clarythromycin, only two days were inspected.

Based on the output of the ANCOVA computation, the hypothesis of equal slopes and the hypothesis of equal intercepts of regression lines were both accepted with p-values greater than 0.05 (95% confidence level). Full R output is given in Table 40 of Annex 1.

Results confirm that the day on which the calibration curve was computed did not influence the output variable (concentration of the analyte) for all the selected compounds.

3.4.2.2 Surface water

The ANCOVA model was performed with three different slopes and three different intercepts (one a day). Full R output is given in Table 41 of Annex 1.

From the ANCOVA results, choosing a confidence level of 95%, the hypothesis of equal slopes and intercepts between the regression lines was accepted (p-value>>0.05).

Again, this indicates that the day on which the calibration curve was computed did not influence the output variable (concentration of the analyte) for all the selected compounds.

3.4.2.3 MilliQ water v surface water

After having tested the comparability of the calibration curved over days in the MilliQ water and surface water separately, it is possible to compare the calibration curves between the two water types. In this case, the ANCOVA will give us information about the effect of the matrix type.

To compare the curves for the two waters, the first day calibration curve for each matrix type was used for the ANCOVA computation. The model was thus computed with two slopes and two intercepts. Full R output is given in Table 42 of Annex 1.

Results show that the hypothesis of equal slope and equal intercept between the regression lines were both accepted with a 95% confidence level (p-value>>0.05).

The two calibration curves deriving from the analysis in MilliQ water and surface water respectively and for all the selected compounds can, in conclusion, be assumed to be coincident at a level of confidence of 95%. This implies that the matrix type has no significant effect on calibration curves for the considered analyte.

3.4.3 Conclusion of ANCOVA analysis

From the ANCOVA analysis, for all the selected compounds, the calibration curves determined in MilliQ and in surface waters are coincident (same slopes and same intercepts).

For method validation purposes, the equivalence of the calibration curves in the two different matrices means that no new method validation needs to be carried out when the matrix type changes.

Nevertheless, although results show slopes and intercepts of calibration curves to be coincident, LOD and LOQ values can be affected when changing from MilliQ to surface water matrix.

As a consequence, proper checks of sensitivity performance of the entire analytical procedure is always recommended.

3.5 Repeatability and intermediate precision

For repeatability and intermediate precision, three QCs at two concentration levels were tested on three different days. Using one-way ANOVA, the results obtained are shown in Table 11.

Table 11: RSDs of repeatability and intermediate precision

Analyte	Spiking level (ng/l)	RSD of repeatability measurements	RSD of intermediate precision measurements
EE2	0.0525	11.6	4.7
	0.42	4.8	9.7
E2	0.3	6.7	2.8
	2.4	2.9	2.8
E1	0.3	11.1	10.2
	2.4	6.1	5
Diclofenac	7.5	8.6	8.7
	60	9.8	6.2
BHT	450	8.2	11.8
	4500	4.4	5.1
EHMC	936	5.6	16.5
	9360	3.1	13.2
Clarythromycin	16.9	8	1.9
	135	5.5	4
Azythromycin	16.9	22	10
	135	8.3	10.1
Methiocarb	1.88	4.7	6.4
	15	3.2	7.2
Acetamiprid	1.7	6.2	10.3
	13.6	4.6	11
Clothianidin	1.7	10.2	7.4
	13.6	8	7.4
Imidacloprid	1.7	9.3	5.1
	13.6	9.7	4
Thiacloprid	1.7	6.7	9.3
	13.6	3.9	10.8

Thiamethoxam	1.7	8.9	2.2
	13.6	7.4	8.2
Oxadiazon	16.5	2	5.8
	132	5.4	2.7
Triallate	101	9.9	5.8
	1005	12.1	4

3.6 Extraction variability of trueness

The extraction variability of trueness has been evaluated using the data from the standard addition experiments (i.e. three QCs at low and high concentration levels, extracted and analysed on three different days, for a total of nine independent replicates).

Using the LINEST function provided by Excel, regression lines, obtained using the 'least-square method', were calculated, interpolating QCs back-calculated concentrations and the corresponding theoretical values.

The extraction variability was determined as slope % and is listed in Table 12.

Table 12: Results of the extraction variability

Analyte	Slope	Extraction variability
EE2	1.016	101.6
E2	0.9807	98.07
E1	1.059	105.9
Diclofenac	0.9832	98.32
BHT	1.0501	105.01
EHMC	1.0592	105.92
Clarythromycin	1.0352	103.52
Azythromycin	1.0583	105.83
Methiocarb	0.8381	83.81
Acetamiprid	1.1069	110.69
Clothianidin	1.0161	101.61
Imidacloprid	1.0397	103.97

Analyte	Slope	Extraction variability
Thiacloprid	1.088	108.8
Thiamethoxam	1.0381	103.81
Oxadiazon	0.9282	92.82
Triallate	0.8863	88.63

3.7 Recovery

The results of the recovery experiments, carried out using analyte-spiked MilliQ water and according to section 2.7, are listed in Table 13.

Table 13: Recovery

Analyte	Spiking level (ng/l)	Mean recovery (%)	RSD (%)
EE2	0.035	112.4	8.8
	10	112.6	12.5
E2	0.035	100.2	1.6
	10	101.3	5.1
E1	0.035	98.2	4.6
	10	115.7	3.6
Diclofenac	11.6	96.5	25.8
BHT	450	97.4	14.5
	4500	98.4	10.1
EHMC	936	69	6
	9360	101.8	14.1
Azythromycin	10.4	81.4	24
Clarythromycin	10.2	80.9	49
Methiocarb	11	97.4	10.5
Acetamiprid	13.8	101.4	8.0
Clothianidin	12.8	89.3	10
Imidacloprid	10	90.7	8.4
Thiacloprid	10.8	95.1	7.7
Thiamethoxam	9.8	92.0	11.4

Analyte	Spiking level (ng/l)	Mean recovery (%)	RSD (%)
Oxadiazon	12.2	99.2	24.4
Triallate	100.8	73.6	11.4
	1005	76.1	3.8

Recovery of oestrogens has been tested at 0.035 and 10 ng/l levels.

Concerning EE2, the evaluation of recovery at LOD level (i.e. 0.035 ng/l) had the aim of confirming the applicability of the procedure developed, considering the challenging level of sensitivity to be reached.

Considering E2 and E1, spiking level of 0.035ng/l is one order of magnitude below the established maximum acceptable method detection limit (i.e. 0.4ng/l). Even considering the reduced reliability of these results, being below the studied working ranges, they clearly indicate the possibility of further decreasing the limits of detections for the selected oestrogens.

Recovery of azithromycin and clarithromycin was tested at 10ng/l. This value is only slightly below the working range studied (i.e. 11.25-180ng/l), not significantly affecting the reliability of the results obtained.

Recovery of BHT, EHMC and Triallate have been evaluated at low and high levels of QCs.

3.8 Uncertainty estimation

The estimation of measurement uncertainty was carried out following a top-down approach based on in-house validation data. The data derived from the validation of the method includes the sample preparation, standard dilution, and chromatographic and mass spectrometric detection variability. This approach takes into account the RSD of repeatability, the intermediate precision and the trueness measurements. The uncertainty of prepared standard stock solution is also considered.

The expanded uncertainty was calculated using the following formula:

$U = k\sqrt{(u_{Tness})^2 + (u_{Rep})^2 + (u_{ip})^2 + (u_{Std})^2}$ where:

U is the expanded relative uncertainty,

k is the coverage factor (k=2),

u_{Tness} is the relative standard uncertainty of trueness estimation,

u_{Rep} is the relative standard uncertainty of repeatability,

u_{ip} is the relative standard uncertainty of intermediate precision, and

u_{Std} is the relative standard uncertainty related to calibration standards including weighing, purity and dilution contributions.

3.8.1 Uncertainty of trueness

u_{Tness} is the standard relative uncertainty associated with trueness.

It is equal to the uncertainty of the extraction variability and calculated from the ratio between the relative uncertainty of slope and the slope, provided by LINEST function applied to standard addition experiment data.

Uncertainty of Trueness = Uncertainty of the extraction variability (%)

$$u_{Tness} = \frac{u_{slope}}{slope} x100$$

3.8.2 Uncertainty of repeatability and intermediate precision

u_{Rep} and u_{Ip} are the standard relative uncertainties related to repeatability and intermediate precision measurements respectively. Individual contributions are calculated according to the following equations:

$$u_{Rep} = \sqrt{\frac{(RSD_{Rep})^2}{n_{Rep}}} \text{ and}$$

$$u_{ip} = \sqrt{\frac{(RSD_{Ip})^2}{n_{days}}}$$

where:

RSD_{Rep} standard deviation of repeatability measurements,

RSD_{Ip} standard deviation of intermediate precision measurements,

n_{Rep} number of total replicates for repeatability measurements, and

n_{days} number of days for intermediate precision measurements.

3.8.3 Uncertainty of standard

$u_{Std}u_{Std}$ is the standard relative uncertainty associated with analytical standards used, and is calculated as follows:

$$u_{Std} = \sqrt{(u_{analyte})^2 + (u_{flask})^2 + (u_{balance})^2}$$

$$u_{std} = \sqrt{(u_{analyte})^2 + (u_{flask})^2 + (u_{balance})^2}$$

Uncertainty as reported in the certificates of analysis of used analytical standards are summarised in Table 14.

Table 14: Uncertainty of analytical standard

Analyte	Uncertainty as stated in CoA
EE2	99.96±1.02µg/ml(k=2) 1.02/99.96=0.0102 = U u=0.0102/2=0.005
E2	Purity 100% u=0
E1	99.0±1 µg/ml 1/99.0=0.01=U, k=2 u=0.01/2=0.005

Analyte	Uncertainty as stated in CoA
Diclofenac	U=±0.5%=0.005, k=2 u=0.005/2=0.0025
BHT	100±1 µg/ml 1/100=0.01=U, k=2 u=0.01/2=0.005
EHMC	Purity 98.9% u=1.1/100=0.011
Azithromycin	Titration 95.2% u=4.8/100=0.048
Clarithomycin	Purity 99.5% u=0.5/100=0.005
Methiocarb	Purity 99.5% u=0.5/100=0.005
Acetamiprid	Purity 99.9% u=0.1/100=0.001
Clothianidin	Purity 99.9% u=0.1/100=0.001
Imidacloprid	100.1±1.02 µg/kg (k=2) U=1.02/100.1=0.01 u=0.01/2=0.005
Thiacloprid	Purity 99.9% u=0.1/100=0.001
Thiamethoxam	Purity 99.6% u=0.4/100=0.004
Oxadiazon	Purity 99.9% u=0.1/100=0.001
Triallate	Purity 98.8% u=1.2/100=0.012

u_{Flask} is the uncertainty related to the volumetric flask. The tolerance of the class A 10-ml volumetric flask (given by the manufacturer) is set to 0.04ml. As this value is not correlated with confidence level or distribution information, a rectangular distribution is assumed.

For the uncertainty estimation, the relative tolerance value (i.e. 0.4%) must by divided by √3, giving a value of 0.231 for u_{Flask}.

$u_{Syringe}$ is the uncertainty related to the withdrawal of the standard solution using a 1 000-µl Hamilton syringe. As these syringes are manufactured to be accurate within ± 1% of the nominal value and this value is not correlated with confidence level or distribution information, a rectangular distribution is assumed. For the uncertainty estimation the relative uncertainty (i.e. 1ml/1000ml*100=0.1%) must by divided by $\sqrt{3}$, giving a value for $u_{Syringe}$ equal to 0.058.

$u_{Balance}$ is the contribution from the weight of standards, and it is due to the linearity uncertainty of the balance from the calibration certificate. From balance linearity (± 0.03 mg), a rectangular distribution is assumed to obtain a standard uncertainty; this contribution is considered twice, once for the tare and once for the gross weight. According to this approach, the $u_{Balance}$ as RSD % is:

$$u_{Balance} = 2x\sqrt{\left(\frac{0.03}{\sqrt{3}}\right)^2} = 0.035$$

$$u_{Balance} = \frac{0.035mg}{10mg}\% = 0.35\%.$$

As the repeatability and trueness of the measurement were estimated for two different concentration levels, the uncertainty can also be estimated separately for low and high concentration levels.

3.9 Final uncertainty budget

Table 15 reports the detailed uncertainty budgets (contributions from trueness, repeatability, intermediate precision and standard purity) and results of uncertainty estimations at low and high concentration levels for each compound studied.

The data are based on 95% confidence level (k=2), nine replicates (n_1=9) on three different days (n_2=3) for the evaluation of the uncertainty budget of validation and on single replicate (n_1=1) in a single day (n_2=1) for the uncertainty budget of method application.

Table 15: Uncertainty budget and estimated uncertainty of measurements

Analyte	k, n_1, n_2	Conc (ng/l)	U_{Tness} (%)	u_{Rep} (%)	u_{Ip} (%)	u_{Std} (%)	Expanded relative uncertainty (U, %)
EE2	2, 9, 3	0.0525	3.7	3.9	2.7	0.3	12
		0.42	3.7	1.6	5.6	0.3	14
	2, 1, 1	0.0525	3.7	11.6	4.7	0.3	26
		0.42	3.7	4.8	9.7	0.3	23
E2	2, 9, 3	0.3	1.5	2.2	1.6	0.3	6
		2.4	1.5	1	1.6	0.3	5
	2, 1, 1	0.3	1.5	6.7	2.9	0.3	15
		2.4	1.5	2.9	2.8	0.3	9

Analyte	k, n_1, n_2	Conc (ng/l)	U_{Tness} (%)	u_{Rep} (%)	u_{Ip} (%)	u_{Std} (%)	Expanded relative uncertainty (U, %)
E1	2, 9, 3	0.3	3.5	3.7	5.9	0.3	16
		2.4	3.5	2	2.9	0.3	10
	2, 1, 1	0.3	3.5	11.1	10.2	0.3	31
		2.4	3.5	6.1	5	0.3	17
Diclofenac	2, 9, 3	7.5	4.3	2.9	5	0.4	14
		60	4.3	3.3	3.6	0.4	13
	2, 1, 1	7.5	4.3	8.6	8.7	0.4	26
		60	4.3	9.8	6.2	0.4	25
BHT	2, 9, 3	450	2.3	2.7	6.8	0.3	15
		4500	2.3	1.5	2.9	0.3	8
	2, 1, 1	450	2.3	8.2	11.8	0.3	29
		4500	2.3	4.4	5.1	0.3	14
EHMC	2, 9, 3	936	4.4	1.9	9.5	0.3	21
		9360	4.4	1	7.6	0.3	18
	2, 1, 1	936	4.4	5.6	16.5	0.3	36
		9360	4.4	3.1	13.2	0.3	29
Azithromycin	2, 9, 3	16.875	4.5	2.7	1.1	0.3	21
		135	4.5	1.8	2.3	0.3	16
	2, 1, 1	16.875	4.5	8	1.9	0.4	49
		135	4.5	5.5	4	0.4	45
Clarithromycin	2, 9, 3	16.875	2.5	7.4	5.8	0.3	8
		135	2.5	2.8	5.8	0.3	8
	2, 1, 1	16.875	2.5	22.1	10	0.3	17
		135	2.5	19.4	10.1	0.3	15
Methiocarb	2, 9, 3	1.875	2.6	1.6	3.7	0.3	10
		15	2.6	1.1	4.2	0.3	10
	2, 1, 1	1.875	2.6	4.7	3.2	0.3	17
		15	2.6	6.4	7.2	0.3	17
Acetamiprid	2, 9, 3	1.695	4	2.1	5.9	0.3	15
		13.56	4	1.5	6.4	0.3	15
	2, 1, 1	1.695	4	6.2	10	0.3	25
		13.56	4	4.6	11	0.3	25
Clothianidin	2, 9, 3	1.695	3.9	3.4	4.3	0	13
		13.56	3.9	2.7	4.3	0.3	13
	2, 1, 1	1.695	3.9	10.2	7.4	0.3	26
		13.56	3.9	8	7.4	0.3	23
Imidacloprid	2, 9, 3	1.695	4.8	3.1	2.9	0.3	13

Analyte	k, n_1, n_2	Conc (ng/l)	U_{Tness} (%)	u_{Rep} (%)	u_{Ip} (%)	u_{Std} (%)	Expanded relative uncertainty (U, %)
		13.56	4.8	3.2	2.3	0.3	12
	2, 1, 1	1.695	4.8	9.3	5.1	0.3	23
		13.56	4.8	9.7	4	0.3	23
Thiacloprid	2, 9, 3	1.695	3.8	2.2	5.4	0.3	14
		13.56	3.8	1.3	6.2	0.3	15
	2, 1, 1	1.695	3.8	6.7	9.3	0.3	24
		13.56	3.8	3.9	10.8	0.3	24
Thiamethoxam	2, 9, 3	1.695	3.9	3	1.3	0.3	10
		13.56	3.9	2.5	4.7	0.3	13
	2, 1, 1	1.695	3.9	8.9	2.2	0.3	20
		13.56	3.9	7.4	8.2	0.3	23
Oxadiazon	2, 9, 3	16.5	2.3	0.7	3.3	0.3	8
		132	2.3	1.8	1.6	0.3	7
	2, 1, 1	16.5	2.3	2	5.8	0.3	13
		132	2.3	5.4	2.7	0.3	13
Triallate	2, 9, 3	100.8	2.3	3.3	3.3	0.3	11
		1005	2.3	4	2.3	0.3	10
	2, 1, 1	100.8	2.3	9.9	5.8	0.3	23
		1005	2.3	12.1	4	0.3	26

4 Conclusions

SPE-LC-MS/MS and LLE-GC-MS multi-compound methods developed and described in this report are fit for purpose for the quantitative determination of environmental contaminants selected in the first watch list for surface water monitoring.

Appropriately cross-validated and applied, they will enable MS laboratories to collect environmental data in support of future prioritisation exercises in accordance with Article 16(2) of the Directive 2000/60/EC of the European Parliament and of the Council.

Based on EE2 results it is recommended that LOD and LOQ be evaluated individually on each real sample analysed, accounting for various types of matrix interferences.

LOD value in real samples can be obtained from the S/N (usually a S/N of 3:1 is applied).

5 References

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List of abbreviations and definitions

Chemical elements are identified by their respective symbols as defined by the International Union of Pure and Applied Chemistry (IUPAC).

Throughout this report, the following abbreviations and symbols are used:

ANOVA	Analysis of variance
ANCOVA	Analysis of Covariance
BHT	2.6-Ditert-butyl-4-methylphenol
CAD	Collision Gas
CUR	Curtain Gas
CRM	Certified reference material
CXP	Collision Cell Exit Potential
DG	Directorate-General
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinyl estradiol
EC	European Commission
EHMC	2-Ethylhexyl-methoxycinnamate
EI	Electron Impact
EP	Entrance Potential
EU	European Union
GC	Gas chromatography
GS1	Ion Source gas 1
GS2	Ion Source gas 2
HLB	Hydrophilic-lipophilic balance
IES	Institute for Environment and Sustainability
IS	Internal standard/Ion Transfer voltage
ISO	International Organisation for Standardisation
JRC	Joint Research Centre
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
PPG	Polypropylene glycol
PS	Priority substances
QC	Quality control sample
R^2	Coefficient of determination
RSD	Relative standard deviation
RT	Room temperature / retention time
SD	Standard deviation
S/N	Signal to Noise
SPE	Solid-phase extraction
TEM	Temperature
UHPLC	Ultra-high-pressure liquid chromatography
WFD	Water Framework Directive

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SUPPLEMENTARY INFORMATION

General recommendation

It was observed that an operator's use of an ordinary face cream containing EHMC heavily influenced the analysis results of said compound even though gloves were worn during all handling. It is therefore highly recommended that operators verify that any body creams used do not contain any EHMC. This problem has also been reported in earlier studies of the compound (Kameda et al., Environmental Pollution, 159, (2011), 1570-1576).

For estrogen analysis, it is highly recommended that the aqueous sample be stored in the dark at 4 C and to perform extraction and analysis as soon as possible, within 48 hours from collection (Gabet, V. et al., *Trends in Anal. Chem*., 26, 11, 2007, 1113-1131).

1 Chemicals

1.1 Standards

Native and labelled standards were commercially purchased and their technical data are summarised in Table 16 and Table 17.

Table 16: Analytical standards

Analytev (unlabelled)	CAS	Batch	Purity	Expiry date	Supplier
EE2	57-63-6	SDEE-021	≥ 98 %	6/25/2024	CIL
E2	50-28-2	PR-25021	≥ 98 %	11/01/2018	CIL
E1 100 µg/ml in acetonitrile	53-16-7	SDDF-016	≥ 98 %	10/04/2023	CIL
Diclofenac	15307-79-6	30226	99.5 %	11/02/2017	Dr Ehrenstorfer
BHT 100 µg/ml in nonane	128-37-0	SDDDE-023	≥ 98 %	7/19/2023	CIL
EHMC	5466-77-3	BCBK1010V	98.9 %	See Product Dating Information Statement from Sigma	Sigma-Aldrich
Azythromycin	83905-01-5	446421/1 V	95.2 %	See Product Dating Information Statement from Sigma	Sigma-Aldrich
Clarythromycin	81103-11-9	084M4134V	99.5 %	04/30/2016	Sigma-Aldrich
Methiocarb	2032-65-7	SZDB302XV	99.5 %	10/29/2018	Sigma-Aldrich
Acetamiprid	135410-20-7	SZBC110XV	99.9 %	04/19/2017	Sigma-Aldrich
Clothianidin	210880-92-5	SZBD053XV	99.9 %	02/22/2017	Sigma-Aldrich
Imidacloprid 100 µg/ml in methanol	13826-41-3	SCIK-006	≥ 98 %	01/26/2019	CIL
Thiacloprid	111988-49-9	SZDB234XV	99.9 %	08/22/2017	Sigma-Aldrich

Analytev (unlabelled)	CAS	Batch	Purity	Expiry date	Supplier
Thiamethoxam	153719-23-4	SZBC031XV	99.6 %	01/31/2017	Sigma-Aldrich
Oxadiazon	19666-30-9	SZBD324XV	99.9 %	11/20/2018	Sigma-Aldrich
Triallate	2303-17-5	SZBX301XV	98.8 %	10/28/2018	Sigma-Aldrich

Table 17: Labelled analytical standards

Labelled analogues	Batch	Purity	Expiry date	Supplier
EE2 (2,4,16,16-d_4)	PR-24836	97-98 %	Stable if stored at RT away from light and moisture	CIL
E2 (2,4,16,16-d_4)	PR-10457	95-97 %	Two years after receipt if stored at RT away from light and moisture	CIL
E1 (2,3,4-$^{13}C_3$) 50 µg/ml in methanol	I-19311	≥ 99 %	Stable if stored frozen (-20° C) and protected from light	CIL
Diclofenac-(acetophenyl ring $^{13}C_6$)	SZBE136XV	99.6 %	See Product Dating Information Statement from Sigma	Sigma-Aldrich
BHT (d_{21})	I-17754	98 %	Stable if stored at RT away from light and moisture	CIL
Erythromycin (n,N-dimethyl-$^{13}C_2$) 100 µg/ml in MTBE	SDEJ-012	≥ 90 %	12/19/2024	CIL
Acetamiprid-d_3	1438678 V	99.7 %	See Product Dating Information Statement from Sigma	Sigma-Aldrich
Clothianidin-d_3	BCBN8335V	99.1 %	01/31/2018	Sigma-Aldrich
Imidacloprid (4,4,5,5-d_4) 100 µg/ml in methanol	SCIK-005	≥ 98 %	01/26/2019	CIL
Thiacloprid d_4	T242A150303	99.8 %	03/03/2018	Analytical Standard Solutions
Thiamethoxam d_3	1438684 V	99.1 %	05/31/2016	Sigma-Aldrich
p-Terphenyl-d_{14}	PAHSSB1011	> 98 %	03/01/2017	Wellington Lab.

1.2 Materials and reagents

Methanol, code 701091.1612, (LC-MS) PAI, Panreac Química, Barcelona (Spain).

Acetonitrile, code 701881.1612, (LC-MS) PAI, Panreac Química, Barcelona (Spain).

Ammonium acetate 99.99+ %, metal basis, code 431311-50g, Aldrich.

Ammonium hydroxide solution ≥ 25 %, code 44273-100 ml, Fluka.

Hexane, code 34412-2.5L, for analysis of dioxins, furans and PCBs, Fluka.

Acetone, code 1.00012.2500, SupraSolv, Merck.

Toluene, code 1.08389.2500, SupraSolv, Merck.

MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA).

OASIS HLB cartridges 6CC (0.2g), code WAT106202, Waters, Milford, MA, USA.

1.3 Reagent solutions for LC-MS/MS

Mobile phase A1: CH_3COONH_4 10 mM pH 3: 0.077 g of CH_3COONH_4 was dissolved in 1 l MilliQ water and adjusted to pH 3 with CH_3COOH.

Mobile phase B1: Methanol: 1000 ml methanol was degassed using ultrasonic bath for 20 seconds.

Mobile phase A2: 0.1 % NH_4OH: 1.96ml NH_4OH 25 % was dissolved in 0.5 l water and degassed using ultrasonic bath for 20 seconds.

Mobile phase B2: Acetonitrile: 1000 ml acetonitrile was degassed using ultrasonic bath for 20 seconds.

UHPLC Autosampler strong washing solution: 900 ml of water and 100 ml of methanol were mixed and degassed using ultrasonic bath for 20 seconds.

UHPLC Autosampler weak washing solution: 100ml of water and 900 ml of methanol were mixed and degassed using ultrasonic bath for 20 seconds.

UHPLC Seal washing solution: same as UHPLC Autosampler weak washing solution.

UHPLC-MS/MS Reconstituting solution for LC-MS/MS analysis: 900ml water was mixed with 100 ml actonitrile.

2 Standard solutions

2.1 Standard solutions of native compounds

Whenever available, analytical standards in solution were purchased.

For chemical standards purchased as solid, stock standard solutions were prepared in methanol, as described below.

Diclofenac stock standard solution (1160 µg/ml): 11.6 mg of diclofenac was dissolved with 10 ml methanol in a 10-ml volumetric flask.

EHMC stock standard solution (1040 µg/ml): 10.4 mg of EHMC was dissolved with 10 ml methanol in a 10-ml volumetric flask.

EHMC working standard solution (10.4 µg/ml): 0.1 ml of EHMC stock standard solution was diluted with methanol in a 10-ml volumetric flask.

Clarithromycin stock standard solution (1020 µg/ml): 10.2 mg of Clarithromycin was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Azithromycin stock standard solution (1040 µg/ml): 10.4 mg of Azithromycin was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Methiocarb stock standard solution (1100 µg/ml): 11 mg of Methiocarb was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Acetamiprid stock standard solution (1380 µg/ml): 13.8 mg of Acetamiprid was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Clothianidin stock standard solution (1 280 µg/ml): 12.8 mg of Clothianidin was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Thiacloprid stock standard solution (1080 µg/ml): 10.8 mg of Thaicloprid was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Thiametoxam stock standard solution (980 µg/ml): 9.8 mg of Thiamethoxam was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Working standard solution for spiking 1 l MilliQ water samples for linearity study was prepared according to the scheme reported below:

Triallate stock standard solution (1340 µg/ml):13.4 mg of triallate was dissolved with 10 ml methanol in a 10-ml volumetric flask.

Triallate working standard solution (1.34 µg/ml): 0.01 ml of Triallate stock standard solution was diluted with 10 ml methanol in a 10-ml volumetric flask.

Two different sets of working solution were prepared. The first included the analytes monitored by LC-MS/MS and the second included the compounds to be analysed by GC-MS.

Working standard solution for LC-MS/MS were prepared according to the scheme described below:

Standard Solution E for LC-MS/MS

The volumes of stock standard solution indicated in Table 18 were diluted with methanol into a total volume of 10 ml using a volumetric flask.

Table 18: Preparation of working standard solution E for LC-MS

Analyte for LC-MS analysis	Stock sol. Conc (µg/ml)	Withdrawn volume (ml)	Standard solution E Conc. (ng/ml)
EE2	100	0.0056	56
E2	100	0.0320	320
E1	100	0.0320	320
Diclofenac	1160	0.0690	8000
Clarythromycin	1020	0.1765	18000
Azythromycin	1040	0.1731	18000
Methiocarb	1100	0.0182	2000
Acetamiprid	1380	0.0131	1808
Clothianidin	1280	0.0141	1 808
Imidacloprid	100	0.1808	1808
Thiacloprid	1080	0.0167	1808
Thiamethoxam	980	0.0184	1808
Oxadiazon	1220	0.1443	17600

Working standard solution for GC-MS were prepared according to the scheme described below:

Standard Solution E for GC-MS

The volumes of stock standard solution indicated in Table 19 were diluted with methanol into a total volume of 10 ml using a volumetric flask.

Table 19: Preparation of working standard solution E for GC-MS

Analyte for GC-MS analysis	Stock sol. Conc (µg/ml)	Withdrawn volume (ml)	Standard solution E Conc. (ng/ml)
BHT	100	0.06	600
EHMC	10.4	1.2	1248
Triallate	1.34	1	134

Standard Solution D, C, B and A

Consecutive serial dilutions 1:1 from standard solutions E, both for LC-MS/MS and for GC-MS, originated standard solutions D, C, B and A according to Table 20 for LC-MS/MS and Table 21 for GC-MS.

Table 20: Preparation of diluted working standard solutions for LC-MS/MS

Analyte	Standard Sol. D (ng/ml)	Standard Sol. C (ng/ml)	Standard Sol. B (ng/ml)	Standard Sol. A (ng/ml)
EE2	28	14	7	3.5
E2	160	80	40	20
E1	160	80	40	20
Diclofenac	4000	2000	1000	500
BHT	300	150	75	37.5
EHMC	624	312	156	78
Clarythromycin	9000	4500	2250	1125
Azythromycin	9000	4500	2250	1125
Methiocarb	1000	500	250	125
Acetamiprid	904	452	226	113
Clothianidin	904	452	226	113
Imidacloprid	904	452	226	113
Thiacloprid	904	452	226	113
Thiamethoxam	904	452	226	113
Oxadiazon	8800	4400	2200	1 00
Triallate	67	33.5	16.75	8.375

Table 21: Preparation of diluted working standard solutions for GC-MS

Analyte	Standard Sol. D (ng/ml)	Standard Sol. C (ng/ml)	Standard Sol. B (ng/ml)	Standard Sol. A (ng/ml)
BHT	300	150	75	37.5
EHMC	624	312	156	78
Triallate	67	33.5	16.75	8.375

2.2 Standard solutions of labelled analogues

Whenever available, stock standard solutions of labelled analogues were purchased.

For labelled standards purchased as solid, stock standard solutions were prepared in methanol, as described below:

2.2.1 Labelled analogues mixture for LC-MS/MS determination

EE2 d_4 stock standard solution (0.1mg/ml): 1 mg 17α-ethynyl estradiol d_4 was dissolved with methanol in a 10-ml flask.

E2 d_4 stock standard solution 1(5 mg/ml): 5 mg of 17β-estradiol d_4 was dissolved in methanol in a 10-ml volumetric flask.

E2 d_4 stock standard solution 2 (0.1 mg/ml): 0.02 ml of 17β-estradiol d_4 5 mg/ml was diluted with methanol into total volume of 10-ml using a volumetric flask.

Diclofenac $^{13}C_6$ stock standard solution (0.11 mg/ml): 1.1 mg of diclofenac $^{13}C_6$ was dissolved with methanol in a 10-ml volumetric flask.

Acetamiprid d_3 stock standard solution (0.11 mg/ml): 0.1 mg of acetamiprid d_3 was dissolved with methanol in a 10-ml volumetric flask.

Clothianidin d_3 stock standard solution (0.12 mg/ml): 1.2 mg of clothianidin d_3 was dissolved with methanol in a 10-ml volumetric flask.

Thiacloprid d_4 stock standard solution (0.11 mg/ml): 1.1 mg of thiacloprid d_4 was dissolved with methanol in a 10-ml volumetric flask.

Thiamethoxam d_3 stock standard solution (0.13 mg/ml): 1.3 mg of thiamethoxam d_3 was dissolved with methanol in a 10-ml volumetric flask.

Interna Standard Working Solution for LC-MS/MS

The volumes of individual stock standard solution indicated in 7 were diluted with methanol into total volume of 10 ml using a volumetric flask.

Table 22: Preparation of internal standard working solution for LC-MS/MS

Compound	Withdraw (ml)	Final Volume(mL) MeOH	Working Internal Standard Sol. Conc (ng/ml)
EE2-d_4	0.01	10	100
E2-d_4	0.01	10	100
E1 $^{13}C_3$	0.02	10	100
Diclofenac $^{13}C_6$	0.01	10	110
Erythromycin $^{13}C_2$	0.01	10	100
Acetamiprid-d_3	0.01	10	110
Clothianidin-d_3	0.01	10	120
Imidacloprid-d_4	0.01	10	100
Thiacloprid-d_4	0.01	10	110
Thiamethoxam-d_3	0.01	10	130

2.2.2 Labelled analogues solutions for GC-MS determination

Whenever available, stock standard solutions of labelled analogues were purchased.

For BHT d_{21}, stock standard solution was prepared in methanol, as described below:

BHT d_{21} stock standard solution (0.18 mg/ml): 1.8 mg of BHT d_{21} was dissolved with methanol in a 10-ml volumetric flask.

Internal Standard working solutions for GC-MS determination was prepared according to the dilution scheme reported inTable 23.

Table 23: Preparation of internal standard working solution for GC-MS

Compound	Withdraw (ml)	Final volume (mL) acetone	Working Internal Standard Sol. Conc (ng/ml)
BHT-d_{21}	0.02	20	180
p-Terphenyl-d_{14}	0.4	10	200

3 Apparatus

Analytical balance: Model AX204, Mettler-Toledo SpA.

Automatic pipettes: Eppendorf research (Milan, Italy).

Microsyringes: Microliter Syringes, Hamilton (Reno, CA, USA).

Autosampler vials for LC-MS: Micro-V vials target Dp clear, 1.5 ml, 12x22 mm National Scientific (Germany).

Volumetric flasks: Grade A various sizes, Duran®.

Volumetric pipettes: Grade A various sizes, Duran®.

Dionex Autotrace AT280 automated SPE system (Thermo Scientific, Waltham, MA, USA).

TurboVap II (Caliper Life Science, Mountain View, CA, USA).

Vortex Genius, Ika, Staufen, Germany.

Horizontal shaker, GFL 3018.

4 Instrumental equipment and conditions

4.1 LC-MS/MS equipment and conditions

Pumps:	Binary Solvent Manager, Model UPB, Waters (Milford, MA, USA).
Autosampler:	Sample Manager, Model UPA, Waters (Milford, MA, USA).
Detector:	QTRAP 5500, Applied Biosystems MDS SCIEX, (Foster City, CA, USA) equipped with Turbo V™ ion source.
Flow rate:	400 µl/min.
Injection volume:	5 µl in ESI_WL2015_All_SCHED.dam method and 30 µl in ESI_WL2015_EstrogenSCHED.dam method.
Analytical column:	Hypersil GOLD, 1.9 µm, 50 x 2.1 mm, Thermo Scientific (for both methods).

Two different UHPLC-MS/MS methods were developed and optimised for the quantification of selected chemicals. Methods are named as follows:

- ESI_WL2015_EstrogenSCHED.dam, and
- ESI_WL2015_All_SCHED.dam.

The method ESI_WL2015_All_SCHED.dam was used for the quantification of diclofenac, azithromycin, clarithromycin, methiocarb, acetamiprid, clothianidin, imidacloprid, thiacloprid, thiametoxamm, oxadiazon. By polarity switching, using Analyst 1.6 scheduling

algorithm, this accomplished the quantification of the selected compounds in positive polarity with the exception of diclofenac which was quantified in negative polarity.

Chromatography was performed in gradient mode according to the scheme described in Table 24.

Table 24: Gradient scheme for ESI_WL2015_All_SCHED.dam method

Time	A: CH_3COONH_4, 10 mM ph 3	B: MeOH	Flow (ml/min)
0	90	10	0.4
0.1	90	10	0.4
5	10	90	0.4
5.1	90	10	0.4
8	90	10	0.4

Under these conditions, the selected analytes eluted at the retention time are listed in Table 25.

Table 25 Retention time in ESI_WL2015_All_SCHED.dam method

Analyte	RT (minutes)
Diclofenac	5.4
Azythromycin	4.8
Clarythromycin	4.8
Methiocarb	4.9
Acetamiprid	3
Clothianidin	2.7
Imidacloprid	2.7
Thiacloprid	3.3
Thiamethoxam	2.3
Methiocarb	4.9
Oxadiazon	6

The run time was about 8 minutes.

The method ESI_WL2015_EstrogenSCHED.dam was used for the quantification of EE2, E2 and E1 in negative polarity.

Chromatography was performed in gradient mode according to the scheme described in Table 26.

Table 26: Gradient scheme for ESI_WL2015_EstrogenSCHED.dam method

Time (minutes)	A: 0.1% NH_4OH	B: AcN	Flow (ml/min)
0	90	10	0.4

0.5	90	20	0.4
1	60	40	0.4
5	10	90	0.4
6	10	90	0.4
6.5	90	10	0.4
12	90	10	0.4

Under these conditions, monitored analytes eluted at the following retention times: EE2 at 3.2 min, E2 at 3.1 min, E1 at 3.3 min. The run time was 12 minutes.

An AB Sciex QTRAP5500 mass spectrometer equipped with Turbo V™ ion source was used. The instrument was previously tuned and calibrated in electrospray mode using polypropylene glycol (PPG). Prior to analysis, all the specific parameters were optimised infusing a 1 µg/ml standard solution of analyte and IS.

The eluent from the column was introduced directly into the ion source. The rapid desolvation and vaporisation of the droplets minimises thermal decomposition and preserves their molecular identity. The data were collected using the software programme Analyst 1.6.

All calculations were based on chromatographic peak area ratios for the multiple reaction monitoring (MRM) precursor-product ion transitions for analyte to the precursor-product ion transition of the IS. Analyst 1.6 software was used for data acquisition and data processing.

Statistical calculations were performed using Excel software.

The general operating conditions were as follows:

Scan Type: Scheduled MRM

Polarity: Positive/Negative

Ion Source: Turbo Spray

Resolution Q1: Unit

Resolution Q3: Unit

MR Pause: 5.0000 msec

Table 27 summarises MRM selected transitions, retention time, operative instrumental parameters and internal standard of ESI_WL2015_All_SCHED.dam method.

Table 27: LC-MS/MS parameters ESI_WL2015_All_SCHED.dam method

ESI_WL2015_All_SCHED.dam								
Analyte MRM								
Q1	**Q3**	**RT (min)**	**Analyte ID**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**	**Internal Standard**
294	250	5.4	Diclofenac	-42	-10	-16	-11	
294	214	5.4	Diclofenac 1	-42	-10	-29	-11	
300	256	5.4	Diclofenac $^{13}C_6$	-173	-10	-15	-11	

ESI_WL2015_All_SCHED.dam								
Analyte MRM								
Q1	Q3	RT (min)	Analyte ID	DP (V)	EP (V)	CE (V)	CXP (V)	Internal Standard
300	220	5.4	Diclofenac $^{13}C_6$ 1	-173	-10	-29	-11	
749.6	591.4	4.8	Azythromycin	200	10	40	13	Erythromycin $^{13}C_2$
749.6	573.3	4.8	Azythromycin 1	200	10	47	13	
748.5	590.5	4.8	Clarythromycin	100	10	28	13	Erythromycin $^{13}C_2$
748.5	558.5	4.8	Clarythromycin 1	100	10	31	13	
736	578	4.4	Erythromycin $^{13}C_2$	130	10	26	13	
736	560	4.4	Erythromycin $^{13}C_2$ 1	130	10	26	13	
736	130	4.4	Erythromycin $^{13}C_2$ 2	160	10	36	13	
226	169	4.9	Methiocarb	30	10	12	13	Thiacloprid-d_4
226	121	4.9	Methiocarb 1	30	10	25	13	
223	126	3	Acetamiprid	80	10	29	13	Acetamiprid-d_3
223	73	3	Acetamiprid 1	80	10	76	13	
225	128	3	Acetamiprid 2	80	10	29	13	
225	75	3	Acetamiprid 3	80	10	74	13	
226	126	3	Acetamiprid-d_3	80	10	27	13	
226	73	3	Acetamiprid-d_3 1	80	10	80	13	
226	190	3	Acetamiprid-d_3 2	80	10	19	13	
250	132	2.7	Clothianidin	50	10	26	13	Clothianidin-d_3
250	169	2.7	Clothianidin 1	50	10	16	13	
252	134	2.7	Clothianidin 2	50	10	24	13	
253	172	2.7	Clothianidin-d_3 1	50	10	18	13	
253	132	2.7	Clothianidin-d_3 1	50	10	23	13	
256	209	2.7	Imidacloprid	60	10	21	13	Imidacloprid-d_4
256	175	2.7	Imidacloprid 1	60	10	27	13	
260	213	2.7	Imidacloprid-d_4	60	10	26	13	
260	179	2.7	Imidacloprid- d_4 1	60	10	29	13	
253	126	3.3	Thiacloprid	100	10	27	13	Thiacloprid-d_4
253	90	3.3	Thiacloprid 1	100	10	55	13	
255	128	3.3	Thiacloprid 2	77	10	28	13	
255	90	3.3	Thiacloprid 3	77	10	53	13	
257	126	3.3	Thiacloprid-d_4	100	10	28	13	
257	73	3.3	Thiacloprid-d_4 1	100	10	83	13	
257	90	3.3	Thiacloprid-d_4 2	100	10	54	13	
292	132	2.3	Thiamethoxam	60	10	35	13	Thiamethoxam-d_3
292	211	2.3	Thiamethoxam 1	60	10	18	13	

ESI_WL2015_All_SCHED.dam								
Analyte MRM								
Q1	Q3	RT (min)	Analyte ID	DP (V)	EP (V)	CE (V)	CXP (V)	Internal Standard
295	214	2.3	Thiamethoxam-d_3	70	10	19	13	
295	132	2.3	Thiamethoxam-d_3 1	70	10	30	13	
345	220	6	Oxadiazon	90	10	28	13	Thiacloprid-d_4
345	303	6	Oxadiazon 1	90	10	21	13	

Table 28: LC-MS/MS parameters ESI_WL2015_EstrogenSCHED.dam

ESI negative (ESI_WL2015_EstrogenSCHED.dam)								
Analyte MRM								
Q1	Q3	RT (min)	Analyte ID	DP (V)	EP (V)	CE (V)	CXP (V)	Internal standard
295	67	2	EE2	-100	-10	-70	-11	EE2 d_4
295	145	3.2	EE2	-100	-10	-70	-11	
295	143	3.2	EE2 1	-100	-10	-50	-11	
299	145	3.2	EE2 d_4	-100	-10	-60	-11	
299	187	3.2	EE2 d_4 1	-100	-10	-45	-11	
271	145	3.1	E2	-83	-10	-60	-11	E2 d_4
271	143	3.1	E2 1	-83	-10	-78	-11	
275	147	3.1	E2 d_4	-100	-10	-55	-11	
275	187	3.1	E2 d_4 1	-100	-10	-50	-11	
269	145	3.3	E1	-100	-10	-53	-11	E1 $^{13}C_3$
269	143	3.3	E1 1	-100	-10	-74	-11	
272	146	3.3	E1 $^{13}C_3$	-150	-10	-88	-11	
272	148	3.3	E1 $^{13}C_3$ 1	-150	-10	-50	-11	

Further operative instrumental parameters were optimised as follows:

Curtain gas (CUR)	25
Collision gas (CAD)	Medium
Temperature (TEM)	550
Ion Transfer Voltage (IS)	-4500
Entrance Potential (EP)	10.00
Collision cell Exit Potential (CXP)	-11.00
Ion Source gas 1 (GS1)	55
Ion Source gas 2 (GS2)	45

4.2 GC-MS equipment and conditions

Autosampler:	CTC Analytics GC PAL
Gas chromatograph:	Thermofisher Trace 1 310
Analytical column:	Agilent HP-5 MS UI, length 30 m, diameter 0.25 mm, film: 0.25 µm
Mass spectrometer:	Thermofisher Ion Trap ITQ 1 100

Table 29: GC-MS parameters

GC-MS PARAMETERS	
Temperature programme	100°C for 1 min.; 10°C/min to 300°C; 300°C for 5 min.
Column flow (ml/min)	1
Splitless (min)	1
Injection volume (µl)	2
Ionisation	EI at 70 eV
Scan mode	Full Scan 50-500 amu
Max Ion Time (msec)	25
Carrier gas	Helium
Injector PTV	100°C for 0.2 sec.; 14.5°C/sec. to 300°C; 300°C for 5 min.
Split flow (ml/min)	50
GC-MS interface T (°C)	300
Source temperature T (°C)	250
Damping gas flow (ml/min)	1.5
MicroScans nr.	2

Trace Finder 3.0 was used for data acquisition and data processing.

Statistical calculations were performed using Excel software

The selected ions used for quantification and the ISs are reported in Table 15.

Table 30: GC-MS selected ions and retention times

Analyte ID		Selected ions		
	RT (min)	Quan Mass	Conf. Mass	Internal Standard
BHT-d_{21}	8.25	222	240	
BHT	8.4	205	220	BHT-d_{21}
Triallate	11.93	268	270	p-terphenyl-d_{14}
p-terphenyl-d_{14}	15.38	244	243	
EHMC	16.46	178	161	p-terphenyl-d_{14}

5 Preparation of calibration standards and water samples for LC-MS analysis

5.1 Calibration standards and Quality Control samples (QCs)

Corresponding water samples were produced by adding 0.01 ml of standard solutions A-E respectively in 1 l MilliQ water (calibration ranges as indicated in Table 2 *'Studied calibration ranges'* in the report) and then spiked with 10 µl of IS working solution.

5.2 Water sample extraction

SPE OASIS HLB cartridges were conditioned with 10 ml methanol followed by 10 ml water. The water samples, spiked with 10 µl IS working solution, were loaded at 5 ml/min and successively the cartridges were dried under nitrogen for 30 minutes. The sorbent was eluted with 10 ml methanol (3 ml/minute), the eluent evaporated to dryness under a gentle stream of nitrogen and then reconstituted with 0.1 mL water: acetonitrile, 9:1, % v/v.

6 Preparation of calibration standards and water samples for GC-MS analysis

6.1 Calibration standards and Quality Control samples (QCs)

Corresponding water samples were produced by adding 0.1 ml of standard solutions A-E respectively in 10 ml MilliQ water (calibration ranges as indicated in Table 2 *'Studied calibration ranges'* in the report) in a 60 ml glass vial and then spiked with 0.1 ml of IS working solutions.

6.2 Water sample extraction

0.1 ml of BHT d_{21} and p-terphenyl d_{14} working solutions were added to 10 ml water samples which were then extracted twice with 10 ml hexane, using a horizontal shaking table. To the hexane extracts 0.1 ml toluene weas added as keeper and evaporated to approximately 0.1 ml. It is important that samples never reach complete dryness, as this will result in a complete loss of the BHT and Triallate.

ANNEX 1

ANCOVA TEST FOR THE EVALUATION OF THE MATRIX COMPARISON IN FIRST WATCH LIST SELECTED COMPOUNDS IN SURFACE WATERS

1 Introduction

The R code used for the ANCOVA analysis and the full R outputs are described below.

ANCOVA was performed using the R software (R Core Team, 2014) with the following variables specifications:

- *Std*, the covariate variable = the concentration of the standard solution used to compute the calibration curve. Five concentration levels were used;
- *Computed*, the dependent variable = the computed concentration of the compound obtained from the peak area;
- *Day*, the factor = the fixed factor which corresponds to the calibration day in cases a and b, and to the matrix type for case c.

ANCOVA was performed to establish whether, for each level of the factor, all calibration curves have equal slopes and intercepts. This means verifying whether or not the factor has a significant effect on the dependent variable, 'cleaned' by the effect of the covariate variable.

Depending on the case, the factor can have five, three or two levels. In case a, the five levels are given by the five different days on which the calibration curves are determined in MilliQ water. In case b, the three levels are the two calibration curves determined in surface water. In case c, the two levels correspond to the calibration curves determined in both MilliQ and surface water, after having verified the day-to-day stability of calibration curves in each water type separately.

Null hypotheses

The first null hypothesis of ANCOVA is that the slopes of the regression lines are all equal; in other words, the regression lines are parallel to each other. Once the null hypothesis of parallel regression lines is accepted, it is possible to test the second null hypothesis: the intercepts of the regression lines are all the same.

2. Verification of the ANCOVA assumptions

2.1. Independence

The R code applied to each compound separately is the following:

```
> independence<-aov(Std~Day, Data),
> summary(independence).
```

Full R outputs for this command are given in Table 31, 32 and 33 for MilliQ water, surface water and matrix comparison, respectively.

Table 31: R output of the independence test for the MilliQ water

Chemical	R output – Case a: Milli-Q water					
17-α-Ethinyl estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	36.17	1.447		
17-β-Estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	36.17	1.447		
Estrone		Df	Sum Sq	Mean Sq	F value	Pr(>F)

Chemical	R output – Case a: Milli-Q water					
	Day	4	0	0	0	1
	Residuals	25	36.17	1.447		
Diclofenac		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	22604	904.2		
BHT		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1.3e+07	5085938		
EHMC		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	55e+07	2.2e+07		
Clarythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	45764	4576		
Azythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	45764	4576		
Methiocarb		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1413	56.5		
Acetamiprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1157	46.28		
Clothianidin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1157	46.28		
Imidacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1157	46.28		
Thiacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1157	46.28		
Thiamethoxam		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	1157	46.28		
Oxadiazon		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1

Chemical	R output – Case a: Milli-Q water					
	Residuals	25	109404	4376		
Triallate		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	4	0	0	0	1
	Residuals	25	6341882	253675		

Table 32: R output of the independence test for the surface water

Chemical	R output – Case b: lake water					
17-α-Ethinyl estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	0.6646	0.0443		
17-β-Estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	21.7	1.447		
Estrone		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	21.7	1.447		
Diclofenac		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	13563	904.2		
BHT		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	7.6e+07	5085938		
EHMC		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	3.3e+08	2.2e+07		
Clarythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	68646	4576		
Azythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	68646	4576		
Methiocarb		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	847.7	56.51		
Acetamiprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	694.2	46.28		

Chemical	R output – Case b: lake water					
Clothianidin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	694.2	46.28		
Imidacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	694.2	46.28		
Thiacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	694.2	46.28		
Thiamethoxam		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	694.2	46.28		
Oxadiazon		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	65642	4376		
Triallate		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	2	0	0	0	1
	Residuals	15	3805129	253675		

Table 33: R output of the independence test for the matrix comparison

Chemical	R output – Case c: Matric effect					
17-α-Ethinyl estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	0.443	0.0443		
17-β-Estradiol		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	14.47	1.447		
Estrone		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	14.47	1.447		
Diclofenac		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	9042	904.2		
BHT		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	5.1e+07	5.1e+06		
EHMC		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	2.2e+08	2.2e+07		
Clarythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	45764	4576		
Azythromycin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	45764	4576		
Methiocarb		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	565.1	56.51		
Acetamiprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	462.8	46.28		
Clothianidin		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	462.8	46.28		
Imidacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1

Chemical	R output – Case c: Matric effect					
	Residuals	10	462.8	46.28		
Thiacloprid		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	462.8	46.28		
Thiamethoxam		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	449	44.9		
Oxadiazon		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	43762	4376		
Triallate		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	Day	1	0	0	0	1
	Residuals	10	2536753	253675		

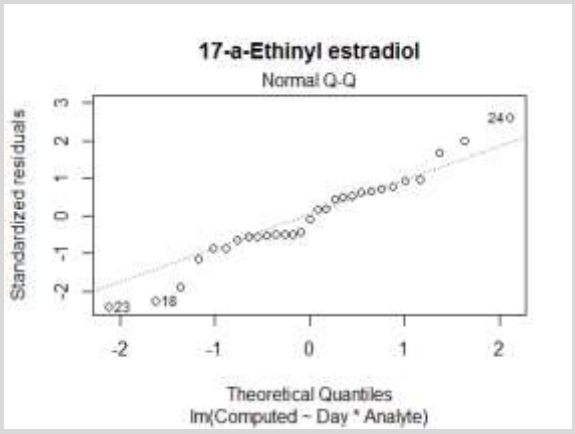
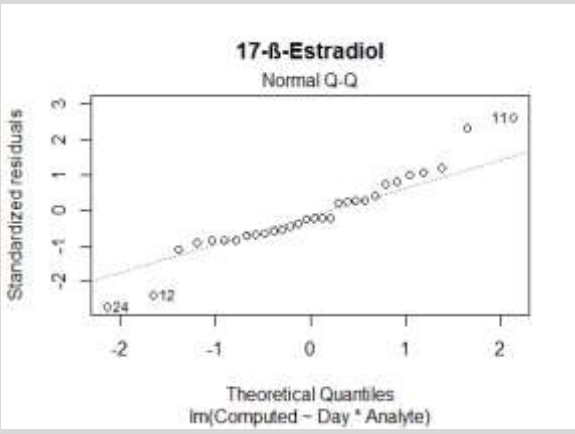
2.2. Normality

QQ-plots are plotted by typing the following R code:

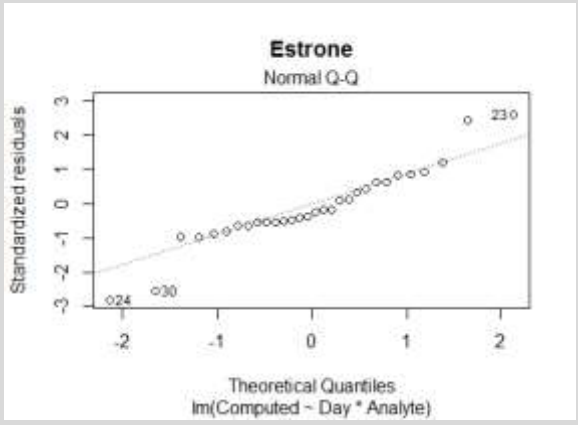
```
> Res.lm = lm(Computed~Day*Std, Data)
> plot(Res.lm, main='Thiamethoxam')
```

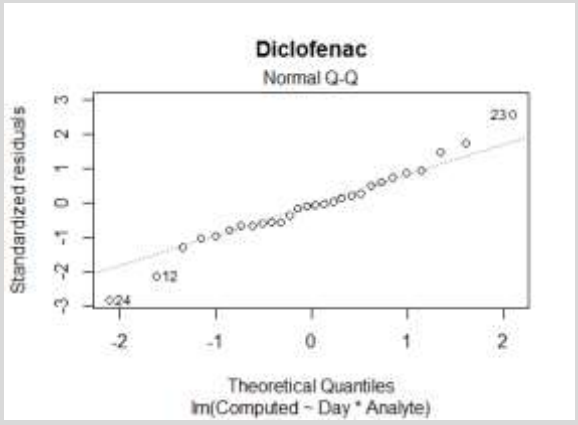
QQ-plots are given in Table 34, Table 35 and Table 36 for MilliQ water, surface water and matrix comparison respectively.

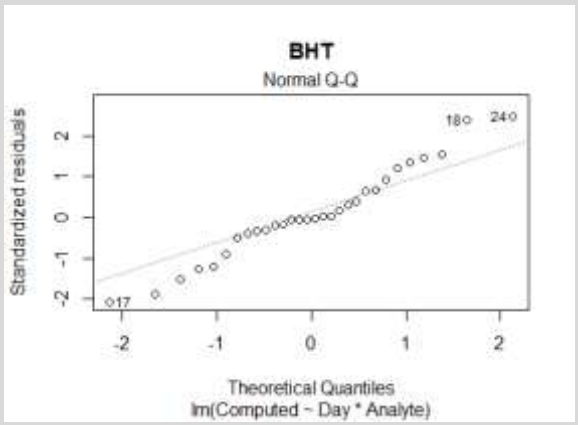
Table 34: R output of the normality test for the MilliQ water

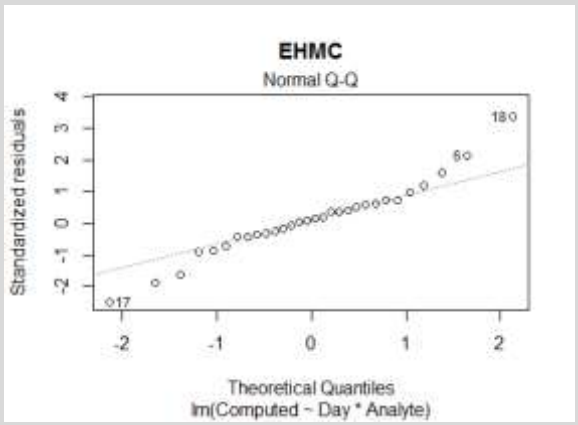
QQ-plots — Case a: MilliQ water	
	

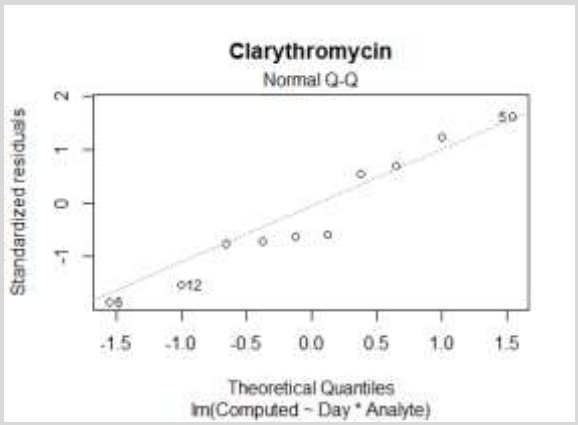
QQ-plots — Case a: MilliQ water

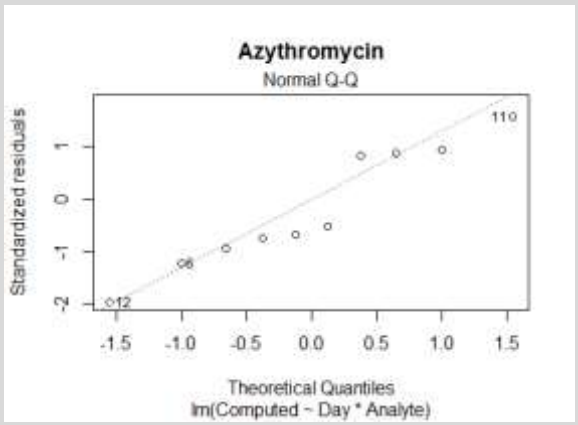




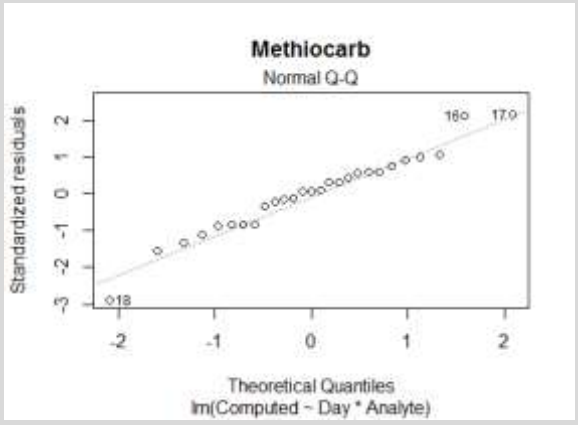


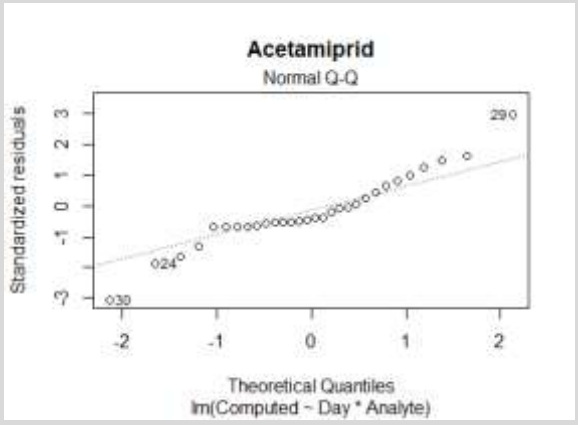


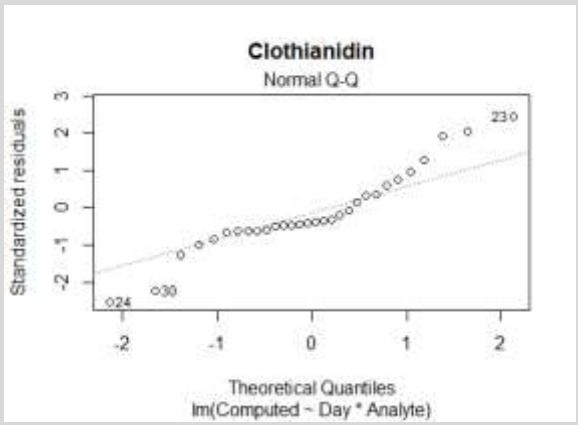


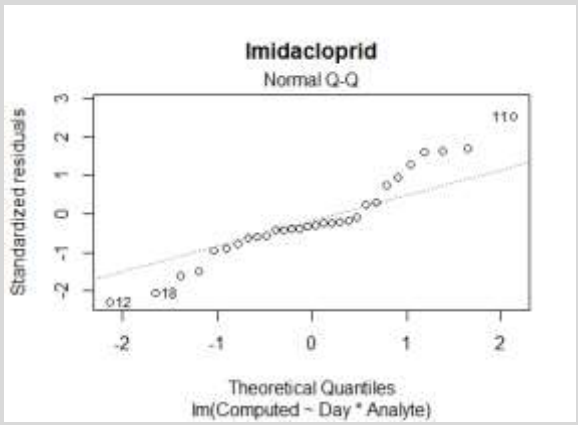


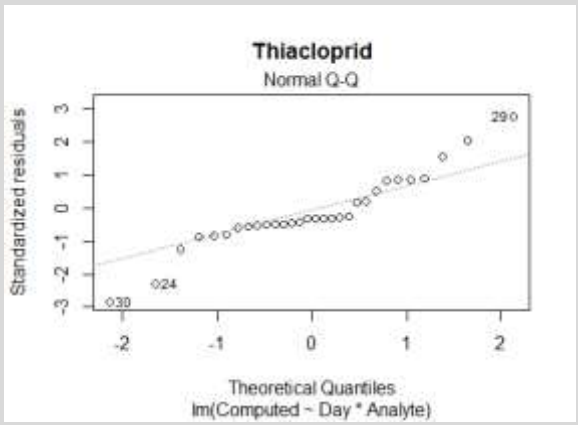
QQ-plots — Case a: MilliQ water

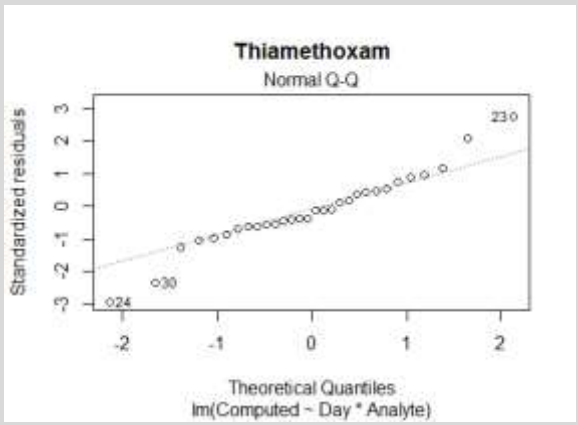




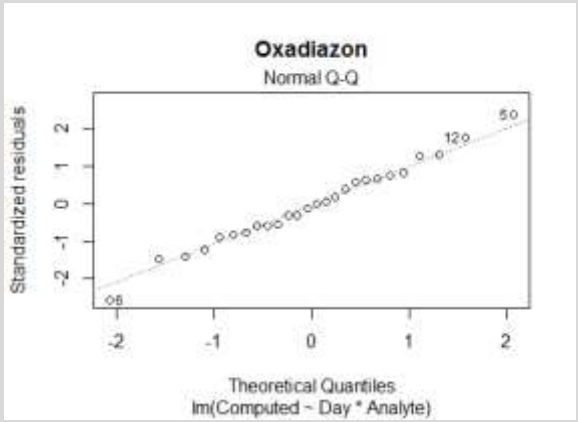








QQ-plots — Case a: MilliQ water



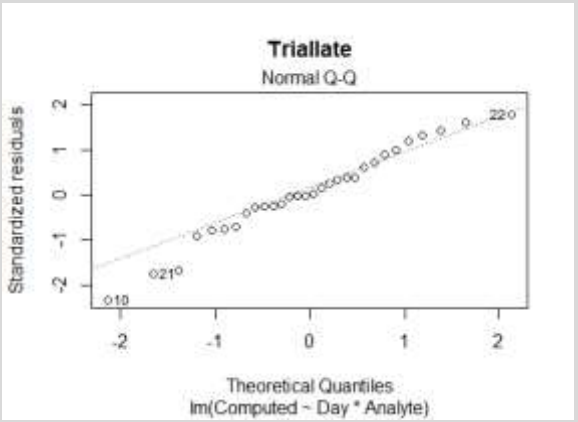
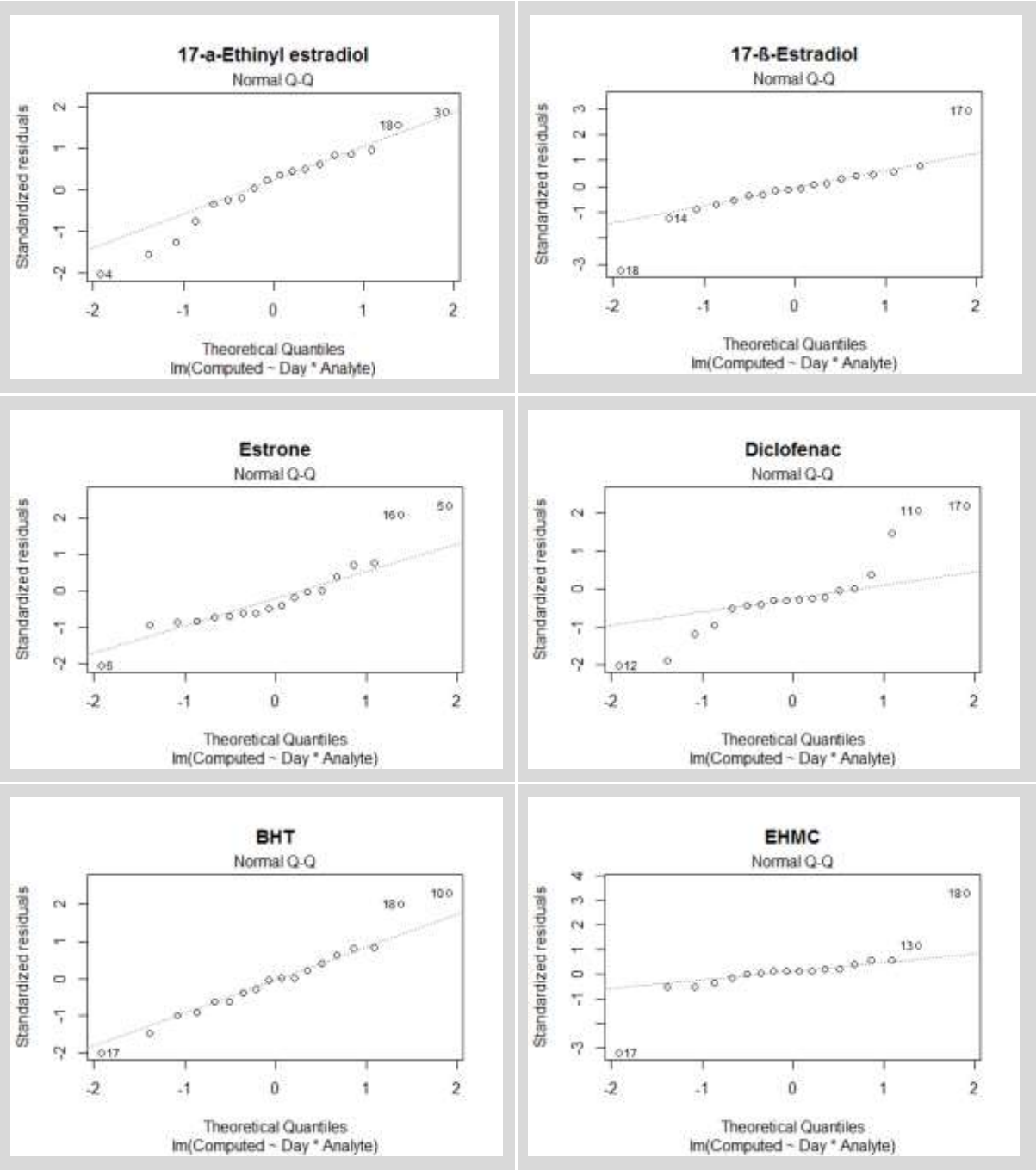
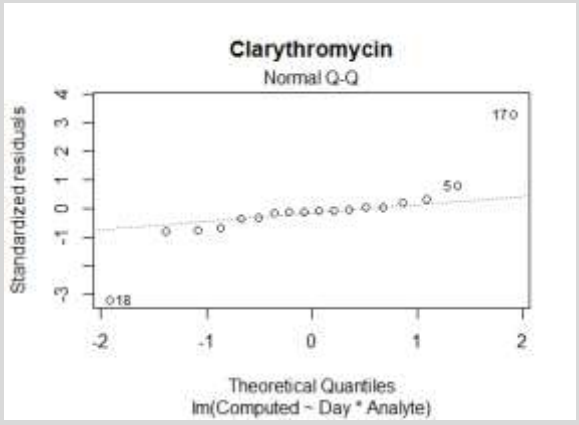


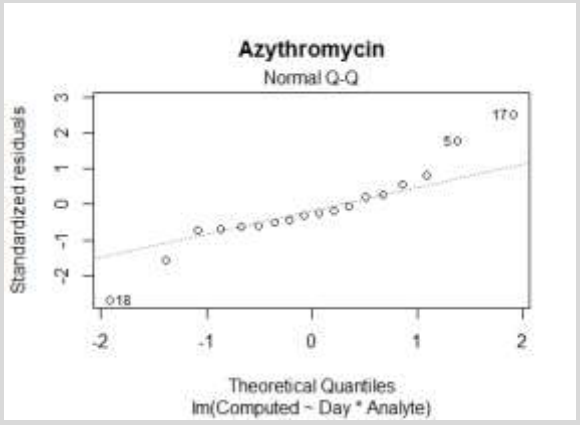
Table 35: R output of the normality test for the surface water

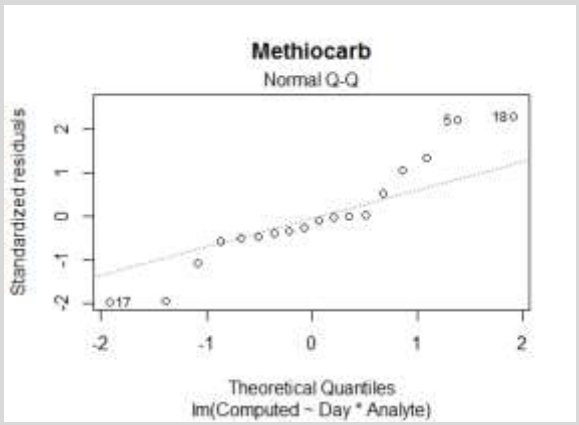
QQ-plots — Case b: surface water

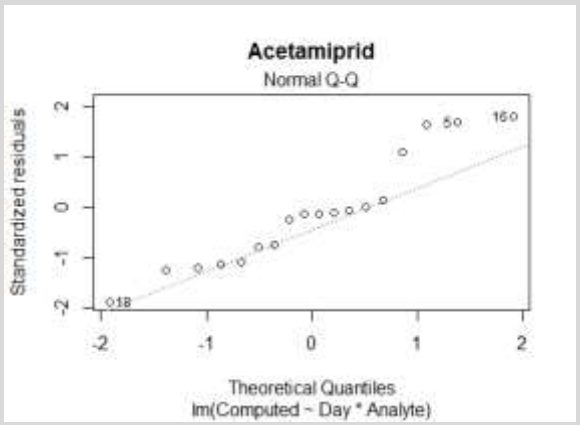


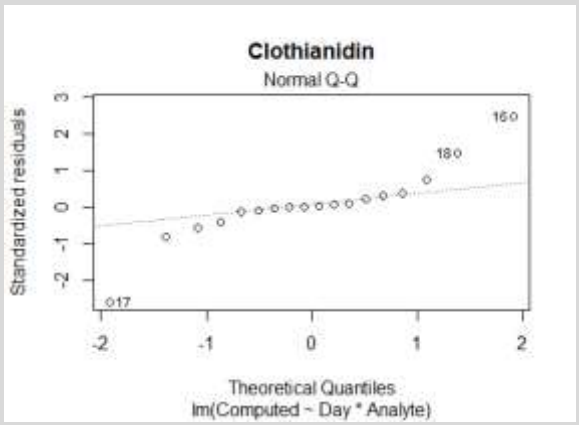
QQ-plots — Case b: surface water

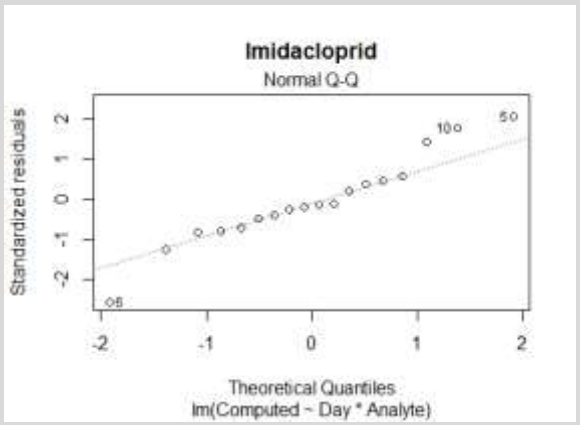




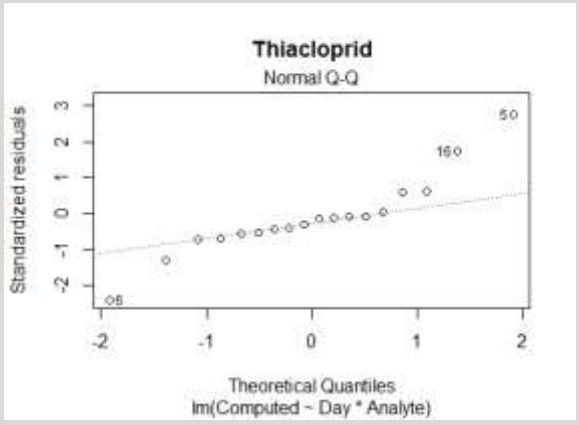


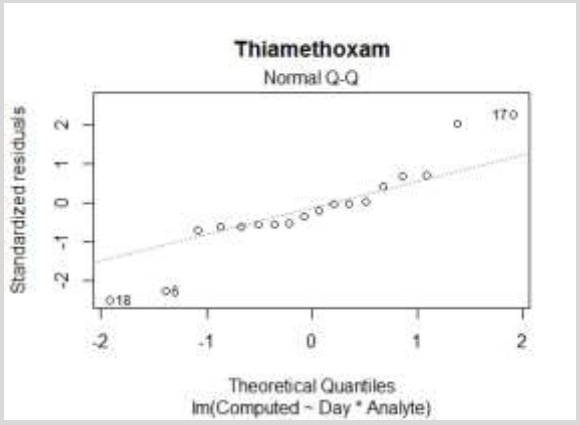


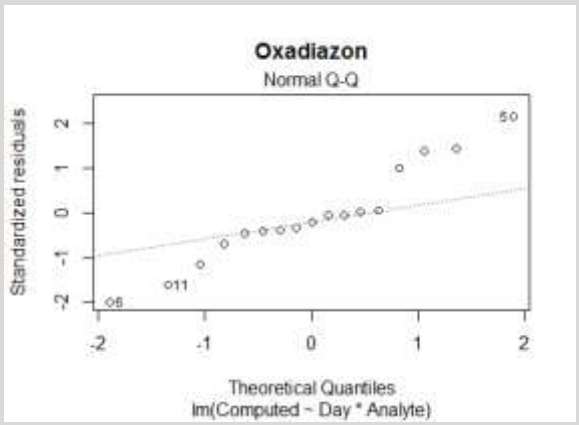




QQ-plots — Case b: surface water







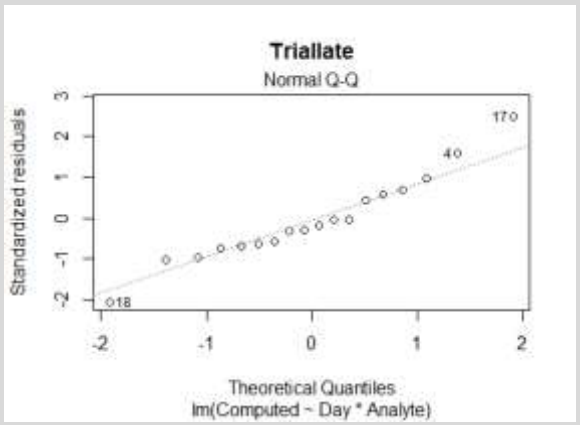
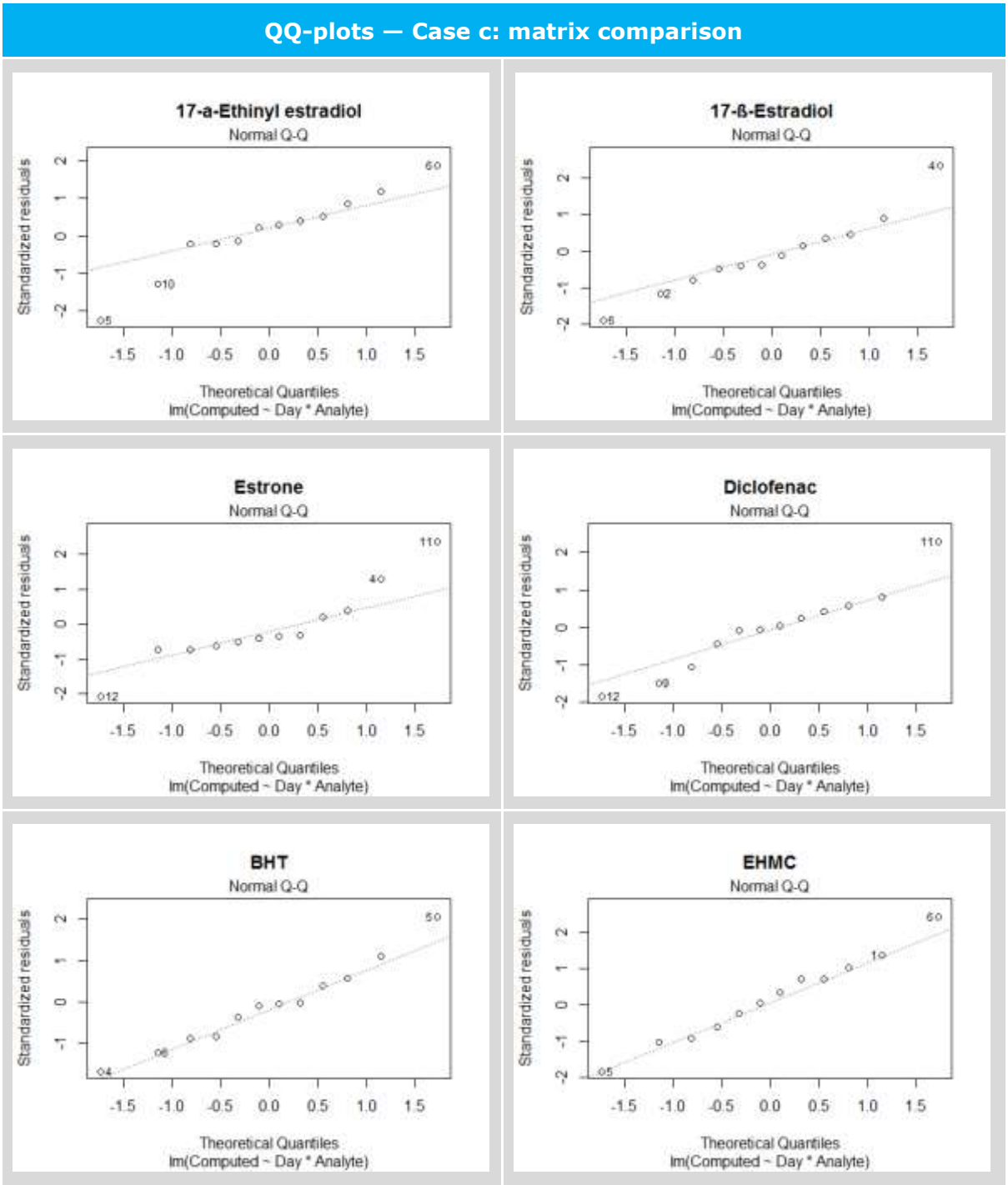
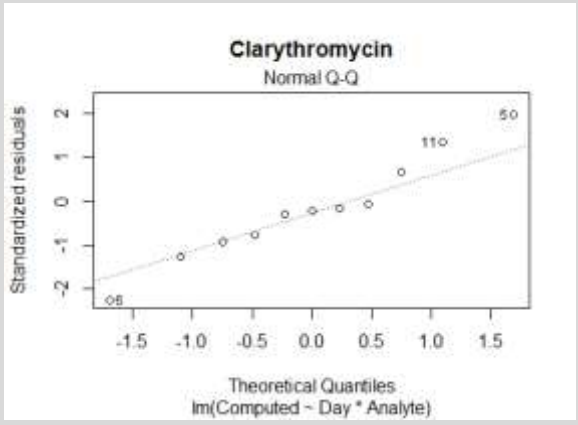
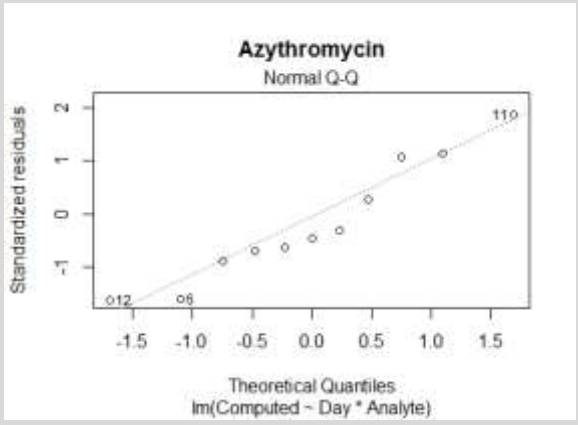


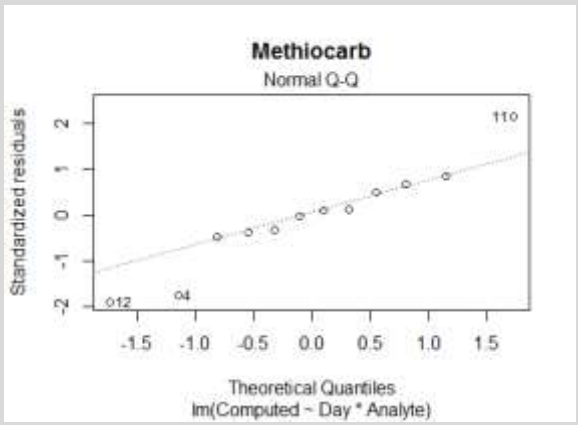
Table 36: R output of the normality test for the Matrix comparison

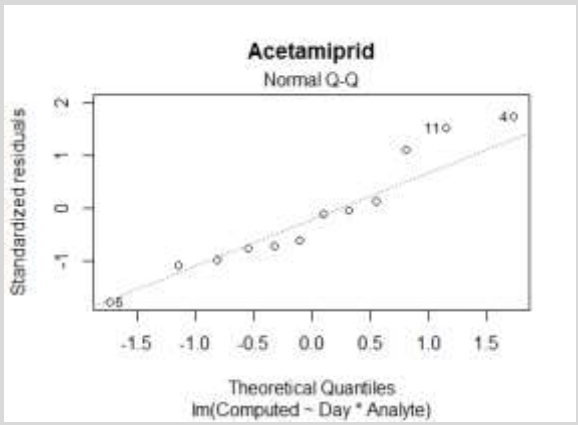


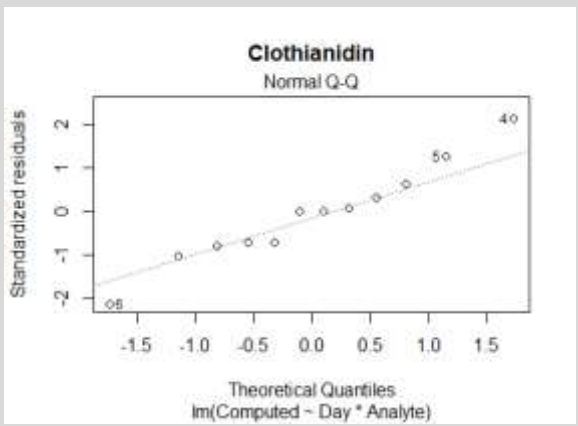
QQ-plots — Case c: matrix comparison

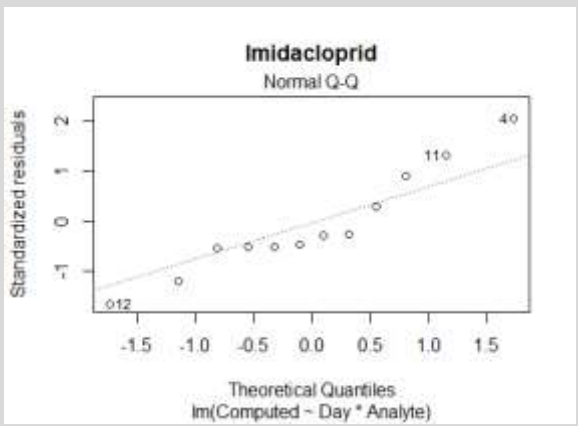




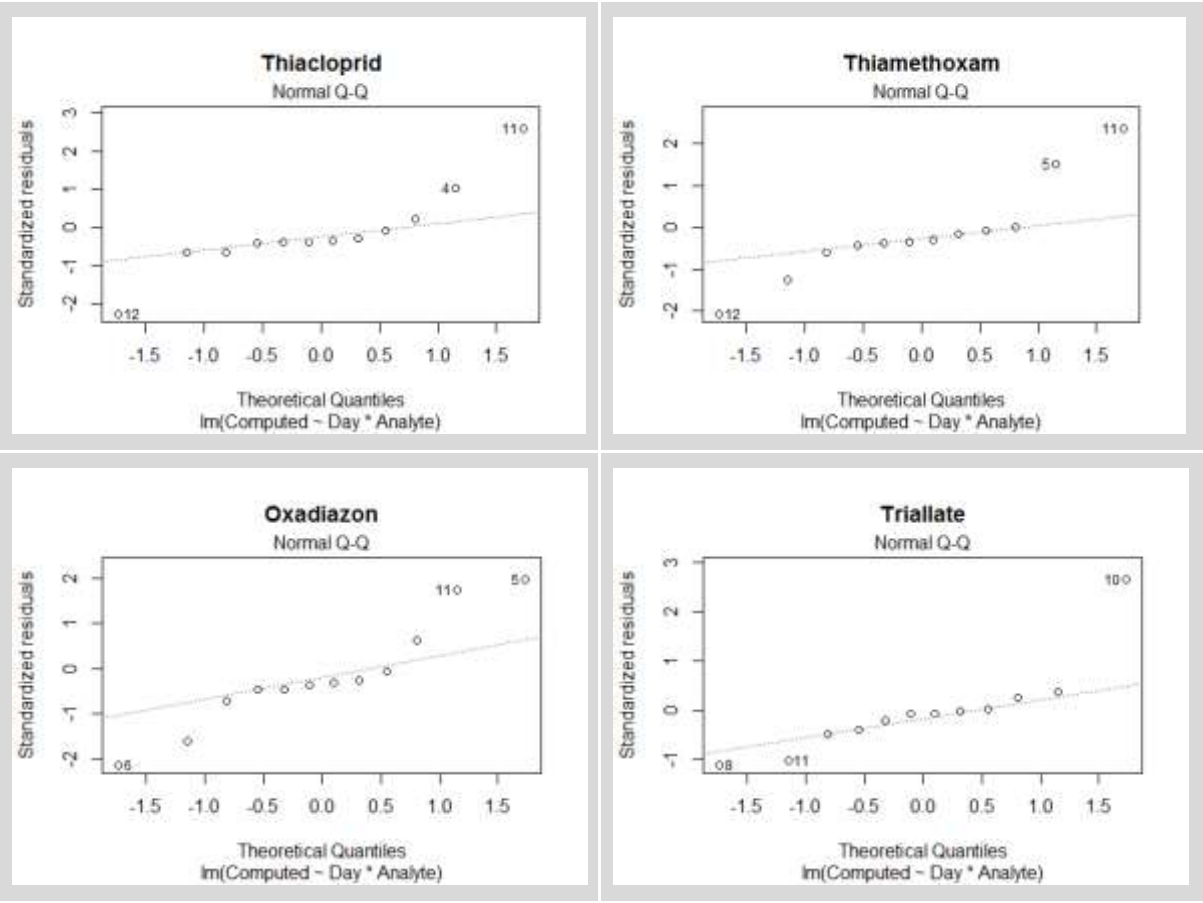








QQ-plots — Case c: matrix comparison



2.3. Homogeneity of variance

Levene's test is used to verify this assumption. The R code for the Levene's test is the following:

```
> leveneTest(Computed~Day, Data)
```

Full R output of Levene's test is given Tables 37, 38 and 39 for MilliQ water, surface water and matrix comparison, respectively.

Table 37: R output of the homogeneity of variance test for the MilliQ water

Chemical	R output — Case a: MilliQ water			
17-α-Ethinyl estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	6e-04	1
		25		
17-β-Estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	6e-04	1
		25		
Estrone	Levene's Test for Homogeneity of Variance (centre = median)			

Chemical	R output — Case a: MilliQ water			
		Df	F value	Pr(>F)
	group	4	6e-04	1
		25		
Diclofenac	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	0.0096	0.9998
		23		
BHT	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	9e-04	1
		25		
EHMC	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	0.005	0.9999
		25		
Clarythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0	0.9967
		8		
Azythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0	0.9973
		8		
Methiocarb	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	0.0279	0.9984
		22		
Acetamiprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	7e-04	1
		25		
Clothianidin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	7e-04	1
		25		
Imidacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	4e-04	1
		25		
Thiacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	5e-04	1
		25		
Thiamethoxam	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	8e-04	1
		25		

Chemical	R output — Case a: MilliQ water			
Oxadiazon	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	0.008	0.9999
		21		
Triallate	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	4	0.0059	0.9999
		25		

Table 38: R output of the homogeneity of variance test for the surface water

Chemical	R output – Case b: Surface water			
17-α-Ethinyl estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	0	1
		15		
17-β-Estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	3e-04	0.9997
		15		
Estrone	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	5e-04	0.9995
		15		
Diclofenac	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	4e-04	0.9996
		15		
BHT	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	3e-04	0.9997
		15		
EHMC	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	0.0115	0.9886
		15		
Clarythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	3e-04	0.9997
		15		
Azythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	4e-04	0.9996
		15		
Methiocarb	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)

Chemical	R output – Case b: Surface water			
	group	2	6e-04	0.9994
		15		
Acetamiprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	4e-04	0.9996
		15		
Clothianidin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	2e-04	0.9998
		15		
Imidacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	0.0021	0.998
		15		
Thiacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	4e-04	0.9996
		15		
Thiamethoxam	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	1e-04	0.9999
		15		
Oxadiazon	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	0.4396	0.6529
		14		
Triallate	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	2	0.004	0.996
		15		

Table 39: R output of the homogeneity of variance test for the matrix comparison

Chemical	R output – Case c: Matrix comparison			
17-α-Ethinyl estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	2e-04	0.9899
		10		
17-β-Estradiol	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	5e-04	0.9821
		10		
Estrone	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	3e-04	0.986
		10		

Chemical	R output – Case c: Matrix comparison			
Diclofenac	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.001	0.9757
		10		
BHT	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0012	0.9828
		10		
EHMC	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0195	0.8917
		10		
Clarythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0248	0.8784
		9		
Azythromycin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0252	0.8774
		9		
Methiocarb	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	1e-04	0.9907
		10		
Acetamiprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0016	0.9686
		10		
Clothianidin	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	8e-04	0.9786
		10		
Imidacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0	0.9993
		10		
Thiacloprid	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	3e-04	0.9868
		10		
Thiamethoxam	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	6e-04	0.9815
		10		
Oxadiazon	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	0.0016	0.9685
		10		

Chemical	R output – Case c: Matrix comparison			
Triallate	Levene's Test for Homogeneity of Variance (centre = median)			
		Df	F value	Pr(>F)
	group	1	2e-04	0.9899
		10		

3. ANCOVA results

3.1. R code explanation

For brevity, the complete R code explanation is given only for the Triallate in the MilliQ water. For the other compounds and for the surface water and matrix comparison cases, full R outputs are expressed in a tabular format.

In R, the ANCOVA model with five different slopes and five different intercepts (one per day) is specified using the following code:

```
> model_1<-lm(Computed~Day*Std, Data)
> summary(model)
```

The R output is the following (Triallate in MilliQ water):

Call:
lm(formula = Computed ~ Day * Std, data = Data)

Residuals:

Min	1Q	Median	3Q	Max
-53.011	-8.167	-0.100	12.167	40.245

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-7.726029	14.021113	-0.551	0.588
DayDay2	-10.838314	19.828849	-0.547	0.591
DayDay3	8.679657	19.828849	0.438	0.666
DayDay4	-4.979257	19.828849	-0.251	0.804
DayDay5	-19.675286	19.828849	-0.992	0.333
Std	1.017855	0.022207	45.834	<2e-16***
DayDay2:Std	0.002296	0.031406	0.073	0.942
DayDay3:Std	-0.01889	0.031406	-0.601	0.554
DayDay4:Std	0.011508	0.031406	0.366	0.718
DayDay5:Std	0.04547	0.031406	1.448	0.163

Signif. codes:	0 ‘***’	0.001 ‘**’	0.01 ‘*’	0.05 ‘.’	0.1 ‘ ’	1

Residual standard error:	25.01 on 20 degrees of freedom
Multiple R-squared:	0.9981
Adjusted R-squared:	0.9973
F-statistic:	1186 on 9 and 20 DF
p-value:	< 2.2e-16

The model estimated 10 parameters from the data (10 rows in the R output): five intercepts and five slopes. The first day (day was the unit used as factor) is used as a baseline against which to compare the other four days.

The coefficients -7.726029 (*Intercept*) and 1.017855 (*Std*) represent the intercept and the slope of the regression line for day 1. For the day 2, the intercept and the slope are given by the sum, respectively, of the first and second quantities (-7.726029 + -10.838314 = -18.564343) and the sum of the sixth and seventh quantities (1.017855 +0.002296 =1.020151). The other days' regression parameters can be computed in the same way by summing the proper rows.

The last column on the right indicates the parameter values which are significantly different from zero when compared with day 1. The table shows that intercepts (first five rows) and slopes (last five rows) do not differ significantly from day 1 at a level of significance of 95%. However, this model compares by a t-test, the slopes and the intercepts of different days, only with the slope and intercept for day 1.

To test the hypothesis of equal slopes of regression lines for several days, the complete model containing the interaction term must be compared with the model for which the parallelism hypothesis is considered valid. The model with equal slope is given by:

```
model_2 <- lm(Computed ~ Day + Std, Data)
```

and the comparison is obtained with the R code:

```
anova(model_1, model_2)
```

The output of the ANOVA command is:

```
Analysis of Variance Table

Model 1: Computed ~ Day * Std
Model 2: Computed ~ Day + Std
```

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)
1	20	12510				
2	24	15346	-4	-2836	1.1334	0.369

From the output, the p-value from the F test is higher than 0.05 (Pr=0.369) and the null hypothesis of equal slopes between the five regression lines is therefore accepted at 95 % level of confidence. At this point, it is possible to test the equality of the intercepts. This is done by comparing the previous model (equal slopes) with the model which assumes equal regression lines (equal slopes and equal intercepts).

```
Model_3 <- lm(Computed ~ Std, Data)
anova(model_2, model_3)
```

The output is:

```
Analysis of Variance Table

Model 1: Computed ~ Day + Std
Model 2: Computed ~ Std
```

	Res.Df	RSS Df	Df	Sum of Sq	F	Pr(>F)
1	24	15346				
2	28	15825	-4	-478.38	0.187	0.940

Based on the results, the hypothesis of equals regression lines (Pr>>0.05) is accepted at 95 % confidence level. This implies that the day at which the calibration curve is computed does not influence the output variable (concentration of the analyte).

The same results can be obtained by a summary R code which results in an ANOVA table with the summary parameters:

```
> model_B<-aov(Computed~Std*Day, Data)
> summary(model_B)
```

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Day	1	6675059	6675059	10671.236	<2e-16 ***
Std	4	478	120	0.191	0.940
Day:Std	4	2836	709	1.133	0.369
Residuals	20	12510	626		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The F values and the corresponding probability values for the interaction term (Day:Std) and for the intercept (Day), are the same as found in the previous computations taken separately. Again, this indicates that there is no significant difference between the slopes and the intercepts of the calibration curves, at a level of confidence of 95%.

This summary R code was used for all compound and results are given in the following section.

3.2. R outputs

This section reports the R output for the ANCOVA analysis in a tabular format.

ANCOVA results for the MilliQ water are given in Table 40.

ANCOVA results for the surface water are shown in Table 41

ANCOVA results for the matric comparison (MilliQ water vs. surface water) are listed in Table 42.

Table 40: ANCOVA output for the MilliQ water

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
17-α-Ethinyl estradiol	*Std.*	1	0.6928	0.6928	11033.834	<2e-
	Day	2	0.0000	0.0000	0.227	0.800
	Std:Da	2	0.0000	0.0000	0.115	0.892
	Residu	1	0.0008	0.0001		
17-β-Estradiol	*Std.*	1	34.17	34.17	2947.521	<2e-
	Day	4	0.00	0.00	0.000	1.000
	Std:Da	4	0.03	0.01	0.593	0.671
	Residu	2	0.23	0.01		
Estrone	*Std.*	1	34.54	34.54	3193.043	<2e-
	Day	4	0.00	0.00	0.000	1.000
	Std:Da	4	0.04	0.01	0.859	0.505
	Residu	2	0.22	0.01		
Diclofenac	*Std.*	1	21105	21105	3504.620	<2e-

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
	Day	4	0	0	0.001	1.000
	Std:Da	4	13	3	0.529	0.716
	Residu	1	108	6		
BHT	*Std.*	1	1.39e+8	1,39e+8	9349.931	<2e-
	Day	4	0	0	0.000	1.000
	Std:Da	4	102979	25745	1.729	0.183
	Residu	2	297885	14894		
EHMC	*Std.*	1	6.59e+8	6,59e+8	1542.451	<2e-
	Day	4	51818	12955	0.030	0.998
	Std:Da	4	3401002	850251	1.989	0.135
	Residu	2	8550328	427516		
Clarythromycin	*Std.*	1	39797	39797	1669.929	1.44e-
	Day	1	0	0	0.000	0.990
	Std:Da	1	0	0	0.004	0.954
	Residu	6	143	24		
Azythromycin	*Std.*	1	39250	39250	1313.927	2.9e-
	Day	1	0	0	0.001	0.973
	Std:Da	1	2	2	0.055	0.822
	Residu	6	179	30		
Methiocarb	*Std.*	1	1379.4	1379.4	2669.363	<2e-
	Day	4	0.0	0.0	0.000	1.000
	Std:Da	4	2.6	0.6	1.256	0.326
	Residu	1	8.8	0.5		
Acetamiprid	*Std.*	1	1071.1	1071.1	3065.138	<2e-
	Day	4	0.0	0.0	0.001	1.000
	Std:Da	4	0.2	0.1	0.147	0.962
	Residu	2	7.0	0.3		
Clothianidin	*Std.*	1	1082.5	1082.5	4292.329	<2e-
	Day	4	0.0	0.0	0.000	1.000
	Std:Da	4	0.1	0.0	0.093	0.984
	Residu	2	5.0	0.3		
Imidacloprid	*Std.*	1	1080.4	1080.4	2474.859	<2e-
	Day	4	0.0	0.0	0.000	1.000
	Std:Da	4	0.1	0.0	0.067	0.991
	Residu	2	8.7	0.4		
Thiacloprid	*Std.*	1	1075.9	1075.9	3225.518	<2e-
	Day	4	0.0	0.0	0.000	1.000
	Std:Da	4	0.1	0.0	0.099	0.982
	Residu	2	6.7	0.3		
Thiamethoxam	*Std.*	1	1099.7	1099.7	5326.621	<2e-
	Day	4	0.0	0.0	0.001	1.00
	Std:Da	4	0.2	0.1	0.292	0.88
	Residu	2	4.1	0.2		
Oxadiazon	*Std.*	1	103195	103195	2660.957	<2e-
	Day	4	0	0	0.000	1.000

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
	Std:Da	4	122	30	0.784	0.552
	Residu	1	620	39		
Triallate	*Std.*	4	478	120	0.191	0.940
	Day	1	6675059	6675059	10671.236	<2e-
	Std:Da	4	2836	709	1.133	0.369
	Residu	2	12510	626		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table 41: ANCOVA output for the surface water

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
17-α-Ethinyl estradiol	*Std.*	1	0.6928	0.6928	11033.834	<2e-
	Day	2	0.0000	0.0000	0.227	0.800
	Std:Da	2	0.0000	0.0000	0.115	0.892
	Residu	1	0.0008	0.0001		
17-β-Estradiol	*Std.*	1	21.016	21.016	6933.231	<2e-
	Day	2	0.000	0.000	0.001	0.999
	Std:Da	2	0.006	0.003	0.926	0.423
	Residu	1	0.036	0.003		
Estrone	*Std.*	1	21.070	21.070	7589.906	<2e-
	Day	2	0.000	0.000	0.001	0.999
	Std:Da	2	0.000	0.000	0.078	0.925
	Residu	1	0.033	0.003		
Diclofenac	*Std.*	1	13024	13024	2209.952	5.61e
	Day	2	0	0	0.001	0.999
	Std:Da	2	2	1	0.171	0.845
	Residu	1	71	6		
BHT	*Std.*	1	7.8e+07	7.8e+07	4989.278	<2e-
	Day	2	0	0	0.000	1.000
	Std:Da	2	57879	28939	1.835	0.202
	Residu	1	189232	15769		
EHMC	*Std.*	1	4.02e+08	4.02e+08	379.008	1.91e
	Day	2	30934	15467	0.015	0.986
	Std:Da	2	3460239	1730120	1.630	0.236
	Residu	1	12733329	1061111		
Clarythromycin	*Std.*	1	64284	64284	1469.963	6.38e
	Day	2	1	1	0.014	0.986
	Std:Da	2	36	18	0.412	0.671
	Residu	1	525	44		
Azythromycin	*Std.*	1	64929	64929	3475.074	3.75e
	Day	2	1	0	0.020	0.980
	Std:Da	2	6	3	0.172	0.844
	Residu	1	224	19		

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
Methiocarb	*Std.*	1	845.4	845.4	2229.055	5.33e
	Day	2	0.0	0.0	0.019	0.981
	Std:Da	2	0.5	0.3	0.712	0.510
	Residu	1	4.6	0.4		
Acetamiprid	*Std.*	1	669.1	669.1	4414.498	<2e-
	Day	2	0.0	0.0	0.001	0.999
	Std:Da	2	0.2	0.1	0.544	0.594
	Residu	1	1.8	0.2		
Clothianidin	*Std.*	1	692.2	692.2	1.05e+07	<2e-
	Day	2	0.0	0.0	0.021	0.979
	Std:Da	2	0.0	0.0	0.042	0.959
	Residu	1	0.8	0.1		
Imidacloprid	*Std.*	1	663.9	663.9	5854.999	<2e-
	Day	2	0.1	0.1	0.480	0.630
	Std:Da	2	0.4	0.2	1.543	0.253
	Residu	1	1.4	0.1		
Thiacloprid	*Std.*	1	662.2	662.2	3024.195	8.61e
	Day	2	0.0	0.0	0.001	0.999
	Std:Da	2	0.1	0.0	0.211	0.813
	Residu	1	2.6	0.2		
Thiamethoxam	*Std.*	1	654.5	654.5	2407.473	3.37e
	Day	2	0.0	0.0	0.000	1.000
	Std:Da	2	0.3	0.1	0.475	0.633
	Residu	1	3.3	0.3		
Oxadiazon	*Std.*	1	49054	49054	1368.322	6.8e-
	Day	2	1	0	0.007	0.993
	Std:Da	2	15	8	0.212	0.812
	Residu	1	394	36		
Triallate	*Std.*	1	3878151	3878151	2279.886	4.66e
	Day	2	0	0	0.000	1.000
	Std:Da	2	2600	1 300	0.764	0.487
	Residu	1	20412	1 701		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table 42: ANCOVA output for the matrix comparison

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
17-α-Ethinyl estradiol	*Std.*	1	0.4635	0.4635	2967.131	1.43e-
	Day	1	0.0000	0.0000	0.128	0.730
	Std:Da	1	0.0000	0.0000	0.064	0.806
	Residu	8	0.0012	0.0002		
17-β-Estradiol	*Std.*	1	13.925	13.925	5843.778	9.56e-
	Day	1	0.000	0.000	0.000	0.995
	Std:Da	1	0.003	0.003	1.076	0.330

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
	Residu	8	0.019	0.002		
Estrone	*Std.*	1	14.015	14.015	5218.700	1.5e-
	Day	1	0.000	0.000	0.002	0.970
	Std:Da	1	0.000	0.000	0.001	0.979
	Residu	8	0.021	0.003		
Diclofenac	*Std.*	1	9039	9039	3739.214	5.69e-
	Day	1	0	0	0.000	0.987
	Std:Da	1	0	0	0.035	0.857
	Residu	8	19	2		
BHT	*Std.*	1	5.2e+07	5.2e+07	6.326.330	6.96e-
	Day	1	0	0	0.000	1.000
	Std:Da	1	677	677	0.081	0.783
	Residu	8	66789	8349		
EHMC	*Std.*	1	2.6e+08	2.6e+08	790.074	2.77e-
	Day	1	23201	23201	0.070	0.798
	Std:Da	1	1120456	1120456	3.382	0.103
	Residu	8	2650450	331306		
Clarythromycin	*Std.*	1	42202	42202	2636.885	2.78e-
	Day	1	0	0	0.012	0.917
	Std:Da	1	5	5	0.326	0.586
	Residu	7	112	16		
Azythromycin	*Std.*	1	418.7	418.7	1483.855	2.07e-
	Day	1	0.0	0.0	0.018	0.897
	Std:Da	1	0.0	0.0	0.106	0.754
	Residu	7	2.0	0.3		
Methiocarb	*Std.*	1	571.0	571.0	1428.390	2.64e-
	Day	1	0.0	0.0	0.000	0.996
	Std:Da	1	0.6	0.6	1.567	0.246
	Residu	8	3.2	0.4		
Acetamiprid	*Std.*	1	442.0	442.0	2373.772	3.48e-
	Day	1	0.0	0.0	0.000	0.984
	Std:Da	1	0.1	0.1	0.585	0.466
	Residu	8	1.5	0.2		
Clothianidin	*Std.*	1	448.0	448.0	4991.4	1.79e-
	Day	1	0.0	0.0	0.0	0.993
	Std:Da	1	0.2	0.2	1.7	0.229
	Residu	8	0.7	0.1		
Imidacloprid	*Std.*	1	437.1	437.1	1583.147	1.75e-
	Day	1	0.0	0.0	0.000	0.997
	Std:Da	1	0.0	0.0	0.076	0.790
	Residu	8	2.2	0.3		
Thiacloprid	*Std.*	1	441.3	441.3	1771.310	1.12e-
	Day	1	0.0	0.0	0.001	0.978
	Std:Da	1	0.0	0.0	0.013	0.911
	Residu	8	2.0	0.2		

Chemical		D	Sum Sq	Mean Sq	F value	Pr(>F
Thiamethoxam	*Std.*	1	433.2	433.2	1461.782	2.41e-
	Day	1	0.0	0.0	0.001	0.974
	Std:Da	1	0.0	0.0	0.114	0.744
	Residu	8	2.4	0.3		
Oxadiazon	*Std.*	1	40106	40 106	724.802	3.9e-
	Day	1	0	0	0.000	0.998
	Std:Da	1	4	4	0.073	0.794
	Residu	8	443	55		
Triallate	*Std.*	1	2573223	2573223	4171.43	3.67e-
	Day	1	0	0	0.00	1.000
	Std:Da	1	290	290	0.47	0.512
	Residu	8	4935	617		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

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