Journal of Chromatography A, 1217 (2010) 6681–6691

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantification of endocrine disruptors and pesticides in water by gas chromatography–tandem mass spectrometry. Method validation using weighted linear regression schemes

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ARTICLE INFO

Article history:
Available online 6 May 2010

Keywords:
Endocrine disruptors
Pesticides
GC–MS
Water
Weighted linear regression schemes
Validation

ABSTRACT

A multi-residue methodology based on a solid phase extraction followed by gas chromatography–tandem mass spectrometry was developed for trace analysis of 32 compounds in water matrices, including estrogens and several pesticides from different chemical families, some of them with endocrine disrupting properties. Matrix standard calibration solutions were prepared by adding known amounts of the analytes to a residue-free sample to compensate matrix-induced chromatographic response enhancement observed for certain pesticides. Validation was done mainly according to the International Conference on Harmonisation recommendations, as well as some European and American validation guidelines with specifications for pesticides analysis and/or GC–MS methodology. As the assumption of homoscedasticity was not met for analytical data, weighted least squares linear regression procedure was applied as a simple and effective way to counteract the greater influence of the greater concentrations on the fitted regression line, improving accuracy at the lower end of the calibration curve. The method was considered validated for 31 compounds after consistent evaluation of the key analytical parameters: specificity, linearity, limit of detection and quantification, range, precision, accuracy, extraction efficiency, stability and robustness.

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1. Introduction

In the last decades, humans have introduced hundreds of new synthetic compounds into the nature, that might alter physiological functions causing health problems [1].

One of the topics in environmental toxicology that has received more attention from the scientific community and regulatory authorities worldwide is the issue of endocrine disruption [2,3] and numerous studies have been carried out concerning the possible harmful consequences of human and wildlife exposure [4].

Endocrine disrupting compounds (EDCs) include a wide variety of pollutants, namely substances of different sources of contamination: domestic (estrogens), industrial (plasticisers and heavy metals) and agricultural (some pesticides) [3,5]. Pesticides, even those which are not EDCs, occupy as well a unique position among the chemicals detected in the environment, since they are deliberately used [6].

Despite several countries have banned, in the last years, many pesticides with a recognized negative impact in human health and/or on ecosystems, in favour of more modern pesticide formulations, some of the old compounds remain the cheapest to produce and, for some purposes, highly effective. So, the dilemma of cost/efficacy vs. ecological impacts remains a contentious global issue. In addition, monitoring data for pesticides are generally poor in much of the world, especially in developing and underdeveloped countries, and despite their inclusion in the monitoring schedule of most developed nations, the cost of analysis and the necessity to sample at several times of the year (linked to atmo-

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doi:10.1016/j.chroma.2010.05.005

spheric conditions and periods of pesticide use) often preclude advancement of an extensive data set [7].

The aquatic environment is particularly susceptible to pollution [8] not only the surface waters but also groundwater's which contamination might be anticipated when the mitigation capacity of the soil system is exceeded [9,10].

As the detection of EDCs and pesticides in environmental matrices can have serious financial, human health and environmental consequences, numerous official environmental organizations and scientific groups have devoted themselves to the development of appropriate analytical methods for their determination. However, low concentrations in addition to the presence of complex aqueous matrices are two major difficulties associated with the detection and quantification of these environmental compounds. Therefore, sensitive and specific analytical procedures are required, as no detectible values are not evidence that the chemical is not present in concentrations that may be injurious to aquatic life and to human health. It is also generally accepted that a screening procedure must be confirmed by a second method based on a different analytical principle to decrease the probability of false-positive results and to provide additional information and assurance about the identity of the detected compounds.

One of the most critical steps involved in the determination of EDCs and pesticides in water is the pre-treatment of samples, which must include procedures for extraction, isolation and concentration of the analytes. Solid phase extraction (SPE) is a widely used methodology that offers an interesting alternative to liquid–liquid extraction providing a drastic reduction of the amounts of organic solvents. SPE coupled with gas chromatography/mass spectrometry (GC/MS) is commonly accepted as one of the most powerful techniques for the separation, identification and quantification of EDCs and pesticides, even in the nanogram range [11,12]. However, there are several factors involved in the success of the process and the profusion of methods for the same purpose indicate that optimization of the procedures has not always been performed [13–18].

Unequivocal analytical data require a specific set of validation criteria and method performance verification [19]. The importance of validation, at least of routine analytical methods, has been hardly overestimated. This is especially true in the context of quality management and accreditation, which have become matters of increased importance in analytical chemistry in recent years. Therefore, the present trend towards standardization of practices between countries has been at the genesis of the current need of international acceptation of analytical results and accurate validation of methods, which become increasingly important for ensuring a common level of quality.

The aim of the present work was the validation of a multi-residue method for detection and quantification of 32 compounds, including insecticides, herbicides and fungicides with a wide range of polarities, as well as two estrogens. The compounds were chosen among the list of persistent organic pollutants (POPs) of the Stockholm convention [20], the priority substances of Decision No. 2455/2001/EC [21], the final list of chemicals for tier 1 screening of the Endocrine Disruptor Screening Program of Environmental Protection Agency (EPA) [22], the Community Strategy for Endocrine Disruptors [3] and also according to Portuguese Decreto-Lei 506/99, Decreto-Lei 306/2007 and ERSAR [23] in order to comply with the European and Portuguese legislation on water quality.

The developed analytical method was implemented and validated following mainly the International Conference on Harmonisation (ICH) [24–26].

Official documents, based on ICH decisions [27], of regulatory authorities and major international bodies like the European Union [28,29], the Food and Drug Administration (FDA) [30], the Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS, [19,31,32], the EURACHEM group [33,34], the International Union of Pure and Applied Chemistry [35], the International Organization for Standardization (ISO) [36], the National Health Surveillance Agency (ANVISA) [37] and the National Institute of Metrology, Standardization and Industrial Quality (INMETRO) [38] served also as guidelines for the validation process. Moreover, several articles and reviews with definitions, procedures, parameters and strategies of validation, some of them applied to chromatographic-tandem mass spectrometry methodologies were also analyzed [39–45].

To ensure the reliability of the analytical method the following parameters were regarded as essential: specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), range, precision, accuracy, extraction efficiency, stability and robustness of the method.

As part of the assay validation a weighted least squares linear regression model (WLSLR) was used to obtain a calibration scheme as the assumption of homoscedasticity was not met for analytical data [29,30,39,46–48]. WLSLR is an efficient method that provides unbiased estimative for prediction, calibration and optimization when standard deviation of the data random errors are not constant across all levels of the explanatory variables. It works by incorporating extra nonnegative constants, or weights, associated with each data point, into the fitting criterion and the size of the weight indicates the precision of the information contained in the associated observation [49].

Optimization and validation of a multi-residue methodology based on a solid phase extraction followed by gas chromatography–tandem mass spectrometry for trace analysis of 32 compounds in water matrices was performed. Matrix-induced chromatographic response enhancement was avoided using matrix-standard calibration solutions and heteroscedasticity has been overtaken by a weighted least squares linear regression model application.

2. Materials and methods

2.1. Chemicals and reagents

All analytical standards with purity >98% were supplied by Sigma–Aldrich (Steinheim, Germany). Methanol and ethyl acetate were organic trace analysis grade SupraSolv and were supplied by Merck (Darmstadt, Germany). Acetonitrile was ChromaSolv grade from J.T. Baker (Deventer, Holland). Acetic acid (glacial) 100% was from Merck (Darmstadt, Germany).

Ultra-pure water (0.054 μS/cm) was obtained using a Milli-Q system from Millipore (Milford, MA, USA).

2.2. Standard solutions preparation

Individual stock standard solutions of 250 mg/L were prepared in methanol by exact weighing of the high-purity substances and accurate dilution. A mixture was then prepared, also in methanol, containing 1.5 mg/L of each individual compound.

Calibration standard solutions, with concentration levels ranging from 15 to 360 μg/L, were prepared by appropriate dilution of the 1.5 mg/L mixture with methanol in 10 mL volumetric flasks.

Matrix-standard calibration solutions (residue-free matrix spiked with standards) with concentration levels ranging from 15 to 360 μg/L were prepared by spiking 500 mL of water with different volumes of the 1.5 mg/L mixture just before extraction.

Stock standard solutions were stored in amber glass-stoppered flasks at 4 °C.

2.3. SPE procedure

Solid phase extraction was conducted in a SPE vacuum manifold system from Phenomenex (USA).

Table 1
Optimized GC–MS acquisition method parameters for 32 compounds and atrazine-d_5. Analytes, retention times (R_t), precursor ions and product ions for qualitative and quantitative analysis.

EDCs/pesticides		CAS	R_t (min)	Precursor ion (m/z)	MRM transitions, m/z	
					Identification	Quantification
1	Thiram	137-26-8	5.968	240	88 > 240; 120	88 > 120
2	EPTC	759-94-4	10.845	189	128 > 189; 160; 86	128 > 189
3	Folpet	133-07-3	12.386	296	76 > 295; 260; 130; 104	76 > 104
4	Phosmet	732-11-6	14.071	317	160 > 133; 104; 93; 77; 76	160 > 76
5	2,4-D	94-75-7	14.360	234	199 > 234; 175	199 > 234
6	Atrazine-desethyl	6190-65-4	14.637	187	172 > 187; 145; 104; 58	172 > 187
7	Terbuthylazine-desethyl	30125-63-4	14.840	201	186 > 145; 104; 68	186 > 145
8	Iprodione	36734-19-7	15.271	330	187 > 219; 124	187 > 124
9	Dimethoate	60-51-5	15.413	229	87 > 229; 125; 93	87 > 125
10	Atrazine-d_5		15.577	220	205 > 220; 178	220 > 205
11	Atrazine	1912-24-9	15.661	215	200 > 215; 58	200 > 215
12	Cyromazine	66215-27-8	15.744	166	151 > 166; 109; 69; 43	151 > 166
13	Terbuthylazine	5915-41-3	15.945	231	214 > 229; 216; 173	214 > 173
14	Pirimicarb	23103-98-2	16.633	238	166 > 238; 72	166 > 72
15	Alachlor	15972-60-8	17.383	269	45 > 269; 188; 160; 146	45 > 160
16	Metalaxyl	57837-19-1	17.565	279	206 > 279; 249; 160; 45	206 > 160
17	Linuron	330-55-2	18.135	248	61 > 248; 160; 46	61 > 248
18	S-Metolachlor	87392-12-9	18.303	283	162 > 238; 211	162 > 238
19	Aldrin	309-00-2	18.449	362	66 > 263	66 > 263
20	Thiamethoxam	153719-23-4	19.060	291	44 > 247; 212; 182; 132	44 > 212
21	Pendimethalin	40487-42-1	19.131	281	252 > 281;162; 119; 57	252 > 162
22	Cyprodinil	121552-61-2	19.166	225	224 > 225; 77	224 > 225
23	Tolyfluanid	731-27-1	19.348	347	137 > 346; 238; 181; 92	137 > 238
24	Fludioxonil	131341-86-1	20.466	248	248 > 182; 154; 127	248 > 154
25	Dieldrin	60-57-1	20.774	378	79 > 263	79 > 263
26	Endrin	72-20-8	21.220	378	81 > 345; 263; 67	81 > 67
27	o.p′-DDT	789-02-6	21.580	352	235 > 165	235 > 165
28	Fenehexamid	126833-17-8	22.298	302	97 > 301; 266; 179; 177; 55	97 > 301
29	Acetamiprid	135410-20-7	23.102	222	56 > 221; 166; 152; 126; 67	56 > 152
30	Methoxychlor	72-43-5	23.395	344	227 > 344	227 > 344
31	Estrone	53-16-7	24.728	270	270 > 185; 146	270 > 185
32	Estradiol	50-28-2	24.888	272	272 > 213; 172;160	272 > 160
33	Azoxystrobin	131860-33-8	30.872	403	344 > 403; 388; 372; 75	344 > 388

Some tests were run in order to optimize several factors affecting the extraction efficiency of the procedure, such as: cartridges, sample volume, flow rate, conditioning and elution solvents and pH adjustment.

SPE optimized conditions were as follows: 500 mL of water samples or matrix-standard calibration solutions was spiked with a methanolic solution of deuterated-atrazin standard at 360 μg/L. pH was adjusted at 3 with acetic acid (glacial) and 0.5% of methanol was added for improvement of SPE extraction.

Strata X cartridges (200 mg, 3 mL) from Phenomenex (Torrance, CA, USA) and LiChrolut EN RP-18 SPE cartridges (100 mg/200 mg, 6 mL) from Merck (Darmstadt, Germany) were tested. Extraction with SPE cartridges was then used with the subsequent scheme: (a) conditioning step, by the sequential addition of 7 mL of ethyl acetate, 7 mL of methanol and 7 mL of Milli-Q water at a flow rate of 1 mL/min; (b) loading step, by passing 500 mL of the sample through the cartridge at a flow of 5 mL/min; (c) washing step, by rinsing the cartridge with 5 mL water and dried by vacuum pressure during approximately 60 min; and (d) elution performed with 2× 2.5 mL of methanol and 2× 2.5 mL acetonitrile, at a flow of 1 mL/min.

After elution, the extracts were evaporated to dryness in a rotative evaporator (Buchi/Brinkman Rotavapor RE-111 & Water Bath B-461) and then re-suspended until a final volume of 500 μL in methanol and directly analyzed by GC/MS.

2.4. GC/MS analysis

Chromatographic analyses were carried out in a Shimadzu GCMS-QP2010 Gas Chromatograph Mass Spectrometer equipped with a fused-silica capillary column coated with 5% diphenyl-methylsiloxane, VF-5 MS (30 m × 0.25 mm ID, 0.25 μm film thickness) from Varian.

High-purity helium (99.9999%) at a constant flow rate of 1.5 mL/min was used as the carrier gas.

For injection an AOC-5000 auto injector was used. Injections (1 μL) were made in the splitless mode with a 1.0 min purge-off time and injector temperature set at 275 °C.

Samples were analyzed using the following oven temperature programme: initial temperature 60 °C (held for 2 min), increased by 10 °C/min to 200 °C (held for 1 min), increased again by 10 °C/min to 275 °C and held at this temperature for 10 min.

GC was directly interfaced to a Shimadzu QP 2010 quadrupole mass spectrometer with an interface temperature of 250 °C, and ionization by 70 eV electron impact. The transfer line was set at 275 °C and the source at 200 °C.

Positive fragment ions (m/z—ions mass/charge ratio) were analyzed over 43–500 m/z mass range in SCAN mode and in selected-ion monitoring (SIM) mode. Selected ions used for quantification are in Table 1.

As it was possible to select more than one ion, the identities of the peaks were confirmed through ratios of their respective abundances.

Comparison with comprehensive mass-spectral libraries (such as NIST, Wiley and special pesticide libraries as PEST and PESTAI) allowed an unequivocal identification of target compounds.

Instrument control and mass spectrometry data were managed by a personal computer running the *LabSolutions GCMS* software (2.50 SU3 version).

Validation parameters were obtained by introducing the respective formulas on a Microsoft Excel worksheet.

3. Theory

Appropriate application of analytical tools requires that the methods used are fit for their purpose and the instruments are operating correctly [19], so the analytical performance characteristics of the optimized method were studied and validated.

3.1. Matrix effect

From the standpoint of chromatographic methods validation, the requirements set by regulatory agencies are basically the same. However, when mass spectrometry is used as detection system combined with gas chromatography, some considerations must be made related to the matrix effect, since this may cause a withdrawal or increased efficiency of ionization and hence may experience a change in the sensitivity of the method [50]. The FDA [30] recommends that this parameter should be evaluated during methods validation to ensure that precision, selectivity and sensitivity are not affected.

Matrix-induced chromatographic response enhancement is a phenomenon that causes overestimation of the analytes concentration, or excessively high recovery results, that arise from higher detector responses observed when a substance is injected in a matrix-modified standard solution (matrix-standard) compared to an injection in pure solvent or matrix-free standard solution [51].

It is explained that matrix protects the analytes from adsorption or degradation during transfer from the injector to the column and thus a maximized amount of analyte reaches the detector, leading to a greater response. This theory provided reasonable explanation for recoveries considerably exceeding 100%, which were reported in several studies of different splitless inlet systems for some compounds [51–53].

One of the most reliable approaches is the use of matrix-matched calibration standards, i.e., standards with the same matrix composition as the analyzed samples [29,52,54]. Government laboratories in the United States as well as European guidelines have long used matrix-matched calibration standards for residue measurements whenever it is demonstrated that a combination of an analyte and matrix can result in a matrix-induced enhancement [52].

3.2. Specificity and selectivity

Specificity/selectivity can be assessed in several ways. The ICH [26] as well as the European Commission [29] and FDA [30] suggest that a simple way to assess the specificity is to demonstrate the lack of response in a blank matrix. This assessment is based on the thorough examination and comparison of different blank samples in the range of the expected retention times of the interesting chromatographic peaks [26,55,56]. As acceptance criteria the responses of interfering peaks at the retention time of the analytes should be less than 30% of the response of the limit of quantification (LOQ) standard [29].

A second way to assess the specificity/selectivity is the use of detectors such as mass spectrometers, which allow the comparison of mass spectra of chromatographic peaks with referenced standards and are often the most practical and least equivocal approach to confirmation purposes [29,31,57].

3.3. Calibration and linearity

3.3.1. Simple and weighted linear regression models—background

The choice of an appropriate calibration model is necessary for a reliable quantification. To properly define the relationship between concentration and response the ICH guidelines specified a minimum of 5 points for the study of the calibration line [26].

Least squares linear regression is the most commonly adopted model. However, the magnitude of the product-moment correlation coefficient (r), itself, is a poor indicator of linearity [48,58] as a significant proportion of errors at the lower end of the calibration line can coexist with acceptable correlation coefficients, errors which are underestimated in analyzing the dispersion of the regression parameters [59]. Therefore, the calculation of the random errors in the y-direction ($S_{y/x}$) as well as the standard deviations for the slope (S_b) and intercept (S_a) associated with the line is crucial to the study of linearity [48]. S_b and S_a can be used to estimate confidence limits for the slope and intercept, given by: $b \pm t_{(n-2)}S_b$ and $a \pm t_{(n-2)}S_a$, where the t-value is taken at 95% confidence level and $(n-2)$ degrees of freedom.

One of the common assumptions underlying most process modelling methods, including linear and nonlinear least squares regression, is that each data point provides equally precise information about the deterministic part of the total process variation. However, when there are dynamic ranges of concentration, the condition of homoscedasticity cannot possibly be met, i.e., the variance is not equal in all of the points of the calibration line. This assumption can be achieved by applying the F-test [39,48,60,61].

The study of homogeneity of variances makes part of the evaluation of the calibration line and can raise issues of acceptance that generally implies the decrease of the considered concentration range or the weighting of the linear regression [39,48,60].

The weighted linear regression model is now becoming rather more common despite their additional complexity in cases of heteroscedasticity [48] and it is desirable by some authors [39,42,62].

Unlike linear and nonlinear least squares regression, weighted least squares regression is not associated with a particular type of function used to describe the relationship between the process variables. Instead, weighted least squares reflect the behaviour of the random errors in the model. It works by incorporating extra non-negative constants, or weights, associated with each data point, into the fitting criterion. The size of the weight indicates the precision of the information contained in the associated observation [49].

3.3.2. Fundamentals of weighted linear regression

Given the evidence of heteroscedasticity, the linear weighted regression model is the simplest and the most effective way to harmonise the differences of variances of the line points. Taking into account the objective of WLSLR, appropriate weighting factors, w_i, can be calculated from the inverse of the variances ($s_i{}^{-2}$) [48]:

$$w_i = \frac{s_i^{-2}}{\sum_i s_i^{-2}/n} \tag{1}$$

The conversion of the linear regression equation in their weighted counterpart is carried out using the term w_i in a and b parameters calculation according to the following equations:

$$b_w = \frac{\sum_i w_i x_i y_i - n \cdot \bar{X}_w \cdot \bar{Y}_w}{\sum_i w_i x_i^2 - n \cdot \bar{X}_w^2} \tag{2}$$

$$a_w = \bar{Y}_w - b \cdot \bar{X}_w \tag{3}$$

In equations, $\bar{Y}_w$ and $\bar{X}_w$ represent the coordinates of the weighted centroid, through which the weighted regression line must pass. These coordinates are given as expected by $\bar{X}_w = \sum_i w_i x_i/n$ and $\bar{Y}_w = \sum_i w_i x_i/n$.

The weighted correlation coefficient (r_w) can be calculated by the formula:

$$r_w = \frac{\sum_i w_i \cdot \sum_i w_i x_i y_i - \sum_i w_i x_i \cdot \sum_i w_i y_i}{\sqrt{\sum_i w_i \cdot \sum_i w_i x_i^2 - \left(\sum_i w_i x_i\right)^2} \cdot \sqrt{\sum_i w_i \cdot \sum_i w_i x_i^2 - \left(\sum_i w_i y_i\right)^2}} \tag{4}$$

To calculate the concentration (x-value) corresponding to any instrumental signal of an analyte (y-value) it is still necessary to

calculate the error associated with the concentration estimation. As the slope and intercept have associated errors and the instrumental signal is also subject to random errors that should not be ignored, this determination is complex, and it often uses the following approximate formula for calculation of the overall error:

$$S_{x_{0w}} = \frac{S_{(y/x)w}}{b}\sqrt{\frac{1}{w_0} + \frac{1}{n} + \frac{(y_0 - \overline{y_w})^2}{b^2\sum_i (w_i x_i^2 - n\overline{x_w^2})}} \quad (5)$$

$$S_{(y/x)w} = \sqrt{\frac{\sum_i w_i (y_i - \hat{y}_i)^2}{n-2}} \quad (6)$$

In this equation, w_0 is a weighting appropriate to the value of y_0, from which the experimental value of x_o is calculated through the calibration curve, $\hat{y}_0$ values are the points on the calculated regression line corresponding to the individual x-values, n is the number of experimental points used to make the calibration curve, $S_{x_{0w}}$ is the estimated standard deviation of x_0, $\overline{x_w}$ and $\overline{y_w}$ are the mean of x and y values, respectively. The confidence limits can be calculated as $x_0 \pm t_{(n-2)}\, S_{x_{0w}}$ with $(n-2)$ degrees of freedom.

The uncertainty for the calibration curve (U) reflects the variability of the method and should be calculated for each of the standards according to the equation:

$$U = S_{x_{0w}}/x_0 \quad (7)$$

The effectiveness of the weighted regression can be assessed by calculating the percentage relative error (%RE), which compares the concentration (C_{exp}) obtained from the weighted and unweighted regression equations with the theoretical or nominal standard concentration (C_{nom}) [39]:

$$\%RE = \frac{C_{exp} - C_{nom}}{C_{nom}} \times 100 \quad (8)$$

The %RE sum, defined as the sum of absolute %RE values, is a sensitive indicator of the quality of fit.

The best w_i will be that which gives rise to a narrow horizontal band of randomly distributed %RE around the concentration axis and presents the least sum of the %RE across the whole concentration range [39].

3.4. Limit of detection and limit of quantification

One of the most common definition of limit of detection (LOD) assumed to be the lowest concentration of an analyte that can be reliably differentiated from the background noise but not necessarily quantified as an exact value [26] and can be calculated based on the calibration curve parameters [26,32,48].

The limit of quantification (LOQ) is defined, in turn, as the lowest amount of an analyte in a sample that can be quantified with acceptable precision and accuracy [26] and can also be calculated based on the calibration curve parameters [26,32,48].

For GC/MS analysis, the concentration measured in the appropriate matrix to which a suitable reference material has been added should be within ±20% of the expected value with a coefficient of variation not exceeding 20%. The imprecision of the analysis should be determined using a minimum of three measurements [31,63].

3.5. Range

The range is normally derived from linearity studies and is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure [26,31].

3.6. Precision and accuracy

The precision and accuracy are two of the main parameters in methods validation, as they determine their acceptance.

3.6.1. Precision

The precision of a method is usually expressed as relative standard deviation (RSD) or coefficient of variation (CV) of a series of measurements and was considered at two levels: repeatability and intermediate precision [26].

Repeatability or intra-day precision expresses the precision under the same operating conditions over a short interval of time. This study should not be confused with the instrumental precision, which is evaluated by a sequence of repeated injections of the same preparation.

Intermediate precision, commonly referred to as inter-day test or inter-assay precision, evaluate the influence of variations within the same laboratory: different days, different analysts, different equipments, etc. [26,31].

The ICH provides a minimum of 9 determinations for assessment of precision (3 concentration levels/3 replicates) [26], as both precision and bias can vary substantially over the calibration range, hence the need of the evaluation of at least three concentration levels (low, medium, high). Washington Conference as well as FDA and ANVISA required precision to be within 15% (RSD) except at the LOQ which can assume a value ±20% [30,37,63]. ICLS [31] and SANCO [29] defined a RSD not greater than 20% for the three levels.

3.6.2. Accuracy

The ICH defines accuracy as the degree of agreement between the value obtained in a test and a reference value accepted as true [26] and according to the Association of Official Analytical Chemists (AOAC International) and the International Organization for Standardization (ISO) the term accurately reflects the combination of systematic errors (bias) and random errors (precision) [64–67]. However, it must be mentioned that accuracy is often used to describe only the systematic error component, i.e., in the sense of bias [19,68] and it is common to perform the evaluation by addition of known amounts of a reference substance to the matrix in three different levels of concentration.

Statistically it can be evaluated by comparative analysis (t-test) [48,68]. Thus, for each concentration level, the bias can be obtained comparing the observed concentration that represents the average of the results obtained through the regression model equation of the calibration curve, and the theoretical or nominal concentration [43].

Bias is required to be within ±15% of the accepted true value, except at the LOQ where ±20% is accepted [30].

In addition, the accuracy can be further evaluated through recovery experiments and extraction efficiency. However, it is important to recognize that the analytes in the samples, when analyzed by GC–MS, may have different behaviours compared with spectral standards prepared in pure solvents and injected directly, as already mentioned in “Matrix effect” sub-chapter.

This reflects the need to apply the extraction methodology to the preparation of the calibration curve matrix-standards, which is important regarding the interpretation of recovery. As the analytical curve was obtained by fortification of blank samples, the recovery value turns out to reflect the precision of the different levels of concentration.

The FDA [30], ANVISA [37] and INMETRO [38] also suggest the use of recovery experiments as a measure of accuracy (recovery) and recommend, as ICH [26], the recovery calculation at three concentrations, low, medium and high (15, 90 and 360 ppb), three replications each.

The accuracy can be evaluated using a two-sided Student's *t*-test, with a 95% level of confidence and $n-1$ degrees of freedom, establishing as null hypothesis (H0): $\mu = 100\%$ and as alternative hypothesis (H1): $\mu \neq 100\%$. The experimental *t*-value (t_{exp}) is calculated by the equation [48,69]:

$$t_{\text{exp}} = \frac{(\bar{X}_{rec} - \mu)\sqrt{n}}{S_{rec}} \quad (9)$$

where $\bar{X}_{rec}$ is the average recovery of the method, μ is the expected value (100%), n the sample size and S_{rec} the standard deviation of recovery for each fortification level. The calculated value of t_{exp} is then compared with the tabulated *t*-value.

In addition, deuterated-atrazine (2-chloro-4-pentadeutero-ethylamino-6-isopropylamino-1,3,5-triazine or atrazine-d_5) was used as a quality control internal standard, so-called procedural or instrument internal standard, for process evaluation through recovery studies, being added in a constant amount to blanks, samples and calibration standards prior to extraction [31,42,70]. Recovery was calculated comparing the chromatographic peak area of blanks with those obtained in the presence of the compound under analysis.

As acceptance criteria set for these tests, the values should be reproducible and preferably with recoveries in the range of 70% and 120% [29,71].

3.7. Stability

The stability of an analyte must be evaluated during the process of their storage to determine whether the samples are properly preserved at the time of analysis [42,63] as there are several factors that can alter the stability, including: temperature, storage time, the concentration of analyte and matrix [29,72].

3.8. Robustness

Robustness is one of the parameters discussed in the recommendations of ICH [26], EU [29], and the CLSI [19,31], and is usually evaluated in the final stage of the method development/validation, in order to investigate the procedures/conditions that require strict control over its application in routine [68]. In the case of gas chromatography, examples of typical variations are: different columns and different extraction materials (different lots and/or suppliers) [26,31].

4. Results and discussion

4.1. GC–MS method optimization

Individual standards were injected in GC/MS in the full-scan mode. Technical parameters as injection conditions, flow and temperature gradients, were optimized for a better resolution of the chromatographic peaks. The analytes were then identified according to the technique described above (Section 2.4) by both their chromatographic characteristics as the retention time and through their specific fragmentation. Characteristic ion transitions and specific intensity ratios of the product ions were compared with library standards included at NIST, Wiley or PEST with an acceptance criterion of a match above a critical factor of 80%. A private library of our standards mass spectra was then created.

Subsequently, programs were developed in the SIM mode, based on the detection of selected ions for each analyte, with a significant increase in sensitivity (100–1000 times higher than that achieved with the full-scan), elimination of interfering compounds signals and lower limits of detection (Table 1). A multi-residue method was chosen as it allows the qualitative and quantitative monitoring of several analytes simultaneously.

4.2. Solid-phase extraction procedure

SPE procedure was assessed using ultrapure water spiked at 250 ppb with the compounds under study, except atrazine-desethyl, terbuthylazine-desethyl and dieldrin, which were included in the study some time later. The use of ultrapure water for extraction recoveries determination allowed the assessment of results avoiding signal suppression by co-extracted substances, identified as the main cause of ion inhibition [73].

Some tests were run in order to optimize factors that affect the extraction efficiency of the procedure, such as the cartridges (LiChrolut EN/RP-18 and Strata X), the sample volume (500, 1000 and 1500 mL) and flow-rate, the conditioning and elution solvents and pH adjustment of the water samples.

LiChrolut cartridges presented higher performance in the extraction procedure with greater retention capacity (data not shown). According to the results and literature, a volume of 500 mL was considered sufficient to detect compounds in concentrations at the sub-ppb, without inducing any breakthrough [74,75]. Additionally, it was observed that the efficiency of the extraction decreased when the pH increased, so pH was adjusted at 3.5. Ethyl acetate:methanol:water (7 mL:7 mL:7 mL) and methanol:acetonitrile (2.5 mL:2.5 mL) were shown to be satisfactory as conditioning and elution solvents, rather than dichloromethane:acetonitrile:water (7 mL:7 mL:7 mL) and methanol (5 mL), respectively. Methanol proved to be the best solvent for the final re-suspension of the extracts when compared to dichloromethane or a mixture of methanol:dichloromethane (1:1), with better chromatographic signals for almost analytes. The SPE procedure was already described in the Section 2.

4.3. Matrix effect and extraction efficiency

Matrix-induced effects during GC–MS determination, such as signal enhancement or suppression, were evaluated by comparison of the relative detector responses obtained from matrix-standard solutions and direct injection of methanolic solutions. Results showed significant matrix enhancement effects for almost all compounds, with the exception of aldrin (which showed a suppression of the absolute response with 30% recovery) and thiram that has a significantly reduced recovery, requiring the optimization of the procedure.

The results regarding the recovery rates of each compound in matrix-standard solutions, calculated considering the areas obtained by direct injection as 100%, are shown in Fig. 1.

To counterbalance the matrix effects, method validation was performed using standards prepared under the same experimental conditions applied for the samples, i.e., matrix-matched standards [29,51,52].

The use of a pre-treatment step more or less complex also made it essential the use of a quality control internal standard for assessing the effectiveness of the extraction technique and monitoring the recovery during sample analysis and atrazine-d_5 was chosen for that purpose (recovery results are presented in Section 4.4.4).

4.4. Validation of the method analytical parameters

To ensure that the optimized procedure was suitable for the application in routine analysis, the basic analytical performance parameters such as specificity and selectivity, linearity and linear range, limits of detection and quantification, precision, accuracy, trueness (recovery), stability, robustness as well as measurement uncertainties were determined and assessed.

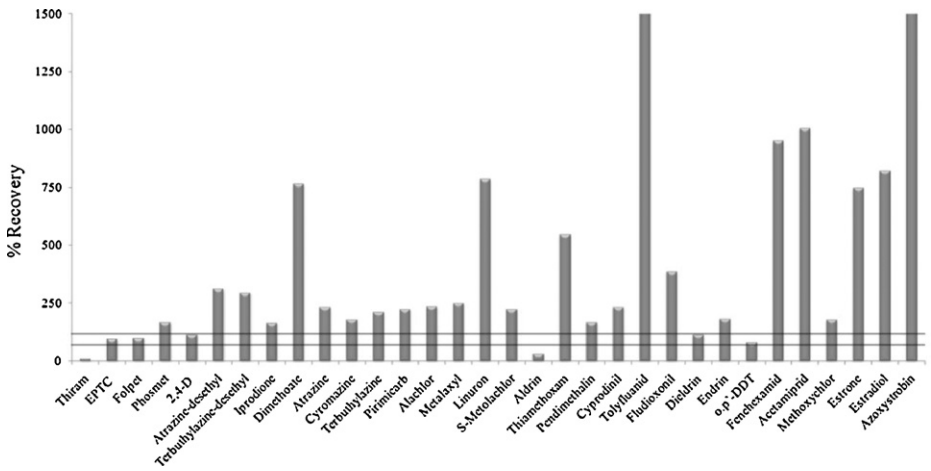

Fig. 1. Matrix-standard solutions recoveries (350 μg/L) calculated considering the areas obtained by direct injection as 100%. The dashed lines represent the range of acceptable recoveries of 70–120%.

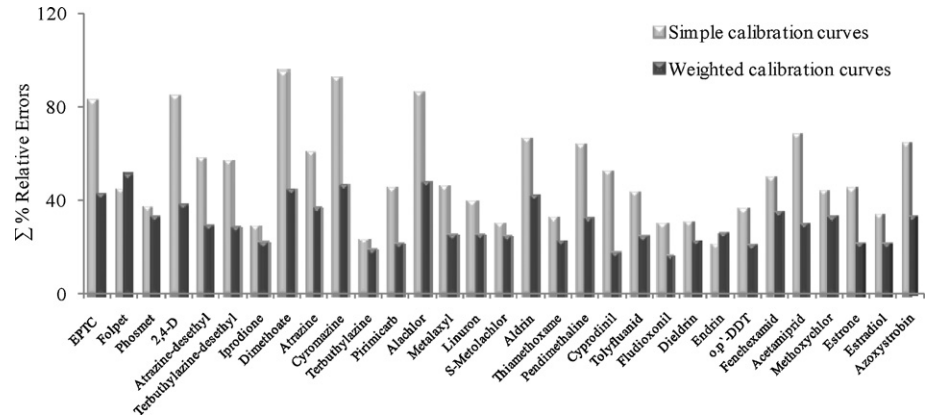

Fig. 2. Sums of relative errors (Σ%RE) for simple and weighted calibration curves.

4.4.1. Specificity and selectivity

For specificity/selectivity, which is essentially a qualitative assessment, analyses of matrix-blank samples (different samples of ultrapure and lab tap water) were performed to tested interferences using the proposed extraction procedure and chromatographic and spectroscopic conditions. The results were compared to those obtained with an aqueous solution of the analytes at concentrations near the limit of quantification. No significant interference has been detected in the retention time of the compounds.

Selectivity was also assessed by the comparison of the analytes mass spectra with spectra from libraries with a similarity ≥90% which gave the evidence that the proposed method has a selectivity/specificity in accordance with the standards set forth by the validation authorities.

4.4.2. Calibration curves: linearity and range

Calibration curves have been obtained in SIM mode and, as the assumption of homoscedasticity was not met, linearity was studied using a weighted least squares linear regression model in the concentration range between 15 and 360 μg/L, using six matrix-calibration standards (15, 45, 90, 150, 240 and 360 μg/L each analyte) prepared under the same conditions applicable to the samples and injected in triplicate. Each test was performed in at least three independent experiments. Detection has been performed according to the single molecular ions listed in Table 1.

The weighting factors applied were calculated using Eq. (1), and the sums of relative errors (Σ%RE) were used as quality indicators of adjustment in the assessment of weighted linear regression (Fig. 2).

An example of %RE plots for unweighted (model 1) and weighted (model 2) regressions across the concentration range is shown in Fig. 3.

As it can be seen, the unweighted model overestimates the concentrations in the lower range of the calibration curve, near the limit of quantification. The weighted model presents a best %RE distribution scatter, a lower sum of %RE and, finally, none of the studied compounds showed relative errors greater than the acceptable limits of 15% and 20% for the different calibration line standards and the first pattern, respectively. Similar results were obtained by Almeida et al. [39].

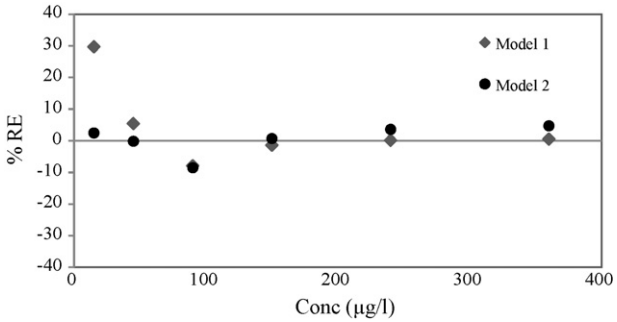

Fig. 3. Percentage of relative errors (%RE) vs. concentration obtained for model 1 ($w_i = 0$) and model 2 $\left(w_i = s_i^{-2}/\sum_i s_i^{-2}/n\right)$ for estrone.

Calibration graphs showed linearity for the concentration range of all target compounds. Regression parameters obtained after application of the weighting factors were calculated as described in Section 3.4 and are presented in Table 2. Calibration in the SIM mode was therefore performed using external standardization.

Good linear responses were achieved for each compound, presenting excellent correlation coefficients, equal to or higher than 0.995 for all compounds except for folpet, still above 0.99.

The percentages of $S_{(b)w}/b$ satisfy the validation condition of the calibration curves, with values less than 5% [48].

Good sensitivity (LOD), ranging from 1.60 to 28.80 ng/L, was found for the 31 compounds, under the optimized experimental conditions. Method limits of quantification (LOQ) were also in the ng/L range with values below 0.05 μg/L for 77.4% of the compounds and, in all cases, below the limit imposed by legislation (0.1 μg/L) [76].

The uncertainties associated with calibration curves (U) were calculated for the calibration standards and was noted, as expected, a marked decrease with the increasing of standards concentration, as can be seen for five randomly selected compounds (Fig. 4).

The uncertainties calculated for the first (maximum uncertainty—% U_{15ppb}) and final calibration standard (minimum uncertainty—% U_{360ppb}) is shown in Table 2.

The results suggest the great influence of the concentration and the nature of the compounds, which must be taken into account during the validation of the multi-residue method.

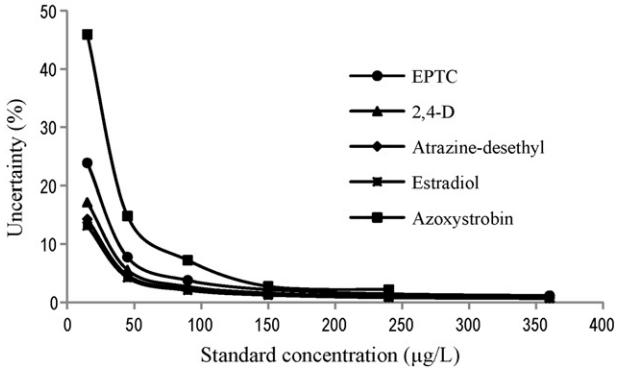

Fig. 4. Examples of the variation of global uncertainty with the calibration standard concentration.

4.4.3. Precision and accuracy

Precision and accuracy were determined by analysis of matrix-calibration standards according to the ICH and FDA recommendations. For repeatability there were performed 9 determinations in the same day (3 concentrations/3 replicates each). For intermediate precision there were performed 3 SPE-GC/MS independent analysis and 9 determinations, corresponding to the three levels of concentration, 1 determination per day in 3 days, were carried out. The results are shown in Table 3.

In order to evaluate the instrumental precision, $n = 5$ replicates of a standard mixture at 240 μg/L level of each pesticide were analyzed under optimum experimental conditions.

Regarding precision, the averages of the coefficients of variation were 11.47% for the inter-day assays and 6.93% for the intra-day tests. Fenhexamid was the only compound that presented values for the inter-day precision on the concentration of 15 and 360 ppb above 20% and 15%, respectively.

Concerning accuracy, bias values varied between −16.68% and 13.64% for the inter-day test and −15.91% and 17.76% for the intra-day test. All the compounds showed values within the proposed acceptance criteria, with the exception of linuron, fenhexamid and acetamiprid, with some unfavourable results only in intra-

Table 2
Method validation data. Calibration parameters and related uncertainties.

EDCs/pesticides	$b_w \pm t_{(n-2)} \cdot S_{(b)w}$ (95%)	$a_w \pm t_{(n-2)} \cdot S_{(a)w}$ (95%)	$S_{(y/x)w}$	$S_{(b)w}/b_w$%	r_w	LOD (ng/L)	LOQ (ng/L)	$\%U_{15ppb}$	$\%U_{360ppb}$
EPTC	143.57 ± 4.16	−781.11 ± 796.79	437.09	1.04	0.9965	9.10	30.40	23.87	1.13
Folpet	73.77 ± 5.03	1502.26 ± 999.45	460.83	2.14	0.9938	18.70	62.50	50.06	2.36
Phosmet	215.61 ± 10.88	257.38 ± 2161.83	996.80	1.59	0.9963	13.90	46.20	36.91	1.72
2,4-D	11.38 ± 0.24	−16.41 ± 45.19	24.79	0.75	0.9960	6.50	21.80	17.16	0.82
Atrazine-desethyl	155.17 ± 2.69	−884.61 ± 516.10	283.12	0.63	0.9987	5.50	18.20	14.28	0.67
Terbuthylazine-desethyl	204.23 ± 3.68	−336.09 ± 705.44	386.98	0.65	0.9995	5.70	18.90	14.81	0.69
Iprodione	40.11 ± 0.87	449.60 ± 173.41	79.96	0.68	0.9994	6.00	19.90	15.78	0.73
Dimethoate	154.23 ± 1.27	−1113.67 ± 242.28	132.91	0.30	0.9992	2.60	8.60	6.70	0.31
Atrazine	161.08 ± 3.09	−602.15 ± 592.67	325.12	0.69	0.9960	6.10	20.20	15.88	0.75
Cyromazine	22.80 ± 2.08	−210.07 ± 413.60	218.60	3.29	0.9966	28.80	95.90	76.87	3.79
Terbuthylazine	207.47 ± 1.05	−245.39 ± 200.61	110.05	0.18	0.9999	1.60	5.30	4.15	0.19
Pirimicarb	640.96 ± 11.40	−779.31 ± 2265.43	1044.56	0.56	0.9984	4.90	16.30	12.95	0.61
Alachlor	189.68 ± 2.02	1276.34 ± 387.33	212.48	0.38	0.9977	3.40	11.20	8.87	0.43
Metalaxyl	142.94 ± 9.19	−539.94 ± 1827.37	842.58	2.02	0.9968	17.70	58.90	46.92	2.22
Linuron	184.07 ± 3.19	−125.53 ± 611.02	335.19	0.62	0.9983	5.50	18.20	14.34	0.67
S-Metolachlor	178.01 ± 2.60	−834.52 ± 497.18	272.74	0.53	0.9984	4.60	15.30	12.00	0.56
Aldrin	19.91 ± 1.15	−114.13 ± 219.90	120.63	2.08	0.9988	18.20	60.60	47.60	2.28
Thiamethoxam	96.61 ± 1.97	−159.94 ± 391.89	180.69	0.64	0.9977	5.60	18.70	14.84	0.68
Pendimethalin	34.06 ± 1.01	236.39 ± 193.19	105.98	1.07	0.9982	9.30	31.10	24.40	1.15
Cyprodinil	886.28 ± 12.07	−2042.23 ± 2398.71	1106.02	0.43	0.9988	3.70	12.50	9.92	0.47
Tolyfluanid	110.48 ± 4.06	924.17 ± 806.44	371.84	1.15	0.9976	10.10	33.70	26.76	1.25
Fludioxonil	310.56 ± 4.90	−747.35 ± 974.07	449.13	0.50	0.9994	4.30	14.50	11.48	0.53
Dieldrin	133.93 ± 1.81	490.24 ± 345.93	189.77	0.49	0.9997	4.30	14.20	11.12	0.51
Endrin	23.66 ± 1.27	782.46 ± 242.52	133.04	1.93	0.9995	16.90	56.30	44.08	2.02
o.p′-DDT	64.67 ± 0.98	−135.30 ± 188.40	103.35	0.55	0.9997	4.80	16.00	12.51	0.58
Fenehexamid	30.22 ± 1.42	74.06 ± 282.55	130.28	1.48	0.9953	12.90	43.10	34.45	1.62
Acetamiprid	53.47 ± 3.13	−357.54 ± 621.68	286.65	1.84	0.9976	16.10	53.60	51.06	2.40
Methoxychlor	153.73 ± 4.71	216.13 ± 901.08	494.30	1.10	0.9980	9.60	32.20	25.16	1.15
Estrone	90.79 ± 1.88	−542.32 ± 359.99	197.48	0.75	0.9982	6.50	21.80	17.10	0.80
Estradiol	76.11 ± 1.21	−553.75 ± 230.97	126.70	0.57	0.9988	5.00	16.60	13.09	0.61
Azoxystrobin	56.07 ± 3.54	−370.69 ± 704.02	324.62	1.99	0.9972	17.40	57.90	45.96	2.17

b_w—weighted slope, a_w—weighted intercept, r_w—weighted correlation coefficient; $S(b)_w$ and $S(a)_w$—standard deviations of the weighted slope and weighted intercept, $S_{(y/x)w}$—standard deviation of y-residuals of weighted regression line, LOD—limit of detection; LOQ—limit of quantification; U—uncertainties associated with calibration curves.

Table 3
Intra-day, inter-day and instrumental precision (% CV) and accuracy (% Bias).

EDCs/pesticides	Inter-day						Intra-day						Instrumental precision %CV
	Precision (%CV)			Accuracy (%Bias)			Precision (%CV)			Accuracy (%Bias)			
Nominal conc. (μg/L)	15	90	360	15	90	360	15	90	360	15	90	360	240
EPTC	6.30	8.04	15.05	−8.80	5.90	−6.66	12.32	8.04	10.00	14.38	−13.78	−11.21	8.00
Folpet	5.92	11.11	9.74	2.52	6.32	−9.95	4.29	5.70	5.06	5.20	7.58	1.85	8.38
Phosmet	17.51	7.57	3.48	9.37	10.80	3.50	9.06	4.46	3.13	−15.91	10.55	7.19	3.86
2,4-D	13.06	11.76	12.89	−5.76	11.84	−13.60	12.60	4.19	8.14	4.47	6.06	−10.76	14.08
Atrazine-desethyl	16.81	10.82	13.19	−2.39	9.29	−10.13	14.64	4.70	6.05	3.42	−12.98	−11.65	14.36
Terbuthylazine-desethyl	12.99	4.75	14.20	−0.72	13.64	−13.37	10.27	4.75	5.65	6.56	5.19	−1.06	8.68
Iprodione	12.69	6.44	6.69	6.45	−0.21	−1.82	8.09	2.68	7.36	−12.58	−6.83	2.33	2.94
Dimethoate	14.74	13.17	9.46	−0.33	13.37	−0.81	3.49	2.64	2.23	−0.05	2.61	5.22	1.33
Atrazine	15.73	6.78	11.05	−2.01	6.14	−11.95	8.63	3.26	5.49	−4.86	−2.64	−14.37	5.70
Cyromazine	1.87	4.05	13.73	6.86	0.91	−10.00	0.58	10.09	2.11	2.75	10.09	−9.91	14.95
Terbuthylazine	7.52	1.70	14.23	0.03	4.04	−12.49	8.02	1.70	5.96	−3.13	−4.05	−0.33	3.67
Pirimicarb	12.78	11.08	9.89	−15.82	3.05	−5.74	13.79	7.07	8.22	−10.49	−8.74	5.71	5.88
Alachlor	5.46	6.22	5.73	−0.39	10.58	−12.75	5.46	6.43	5.71	−0.39	−9.08	15.53	3.60
Metalaxyl	16.38	8.14	13.16	−6.90	5.71	−14.50	3.13	6.06	8.12	−13.22	11.05	−4.66	3.66
Linuron	17.75	10.08	12.41	−6.25	10.25	−7.44	13.92	10.08	2.35	54.57	34.42	15.59	6.92
S-Metolachlor	14.78	0.93	10.02	−16.68	−3.86	−3.11	10.19	0.93	10.02	−13.07	−3.86	−3.11	8.39
Aldrin	5.82	15.54	9.68	−14.71	4.01	−13.13	5.16	5.22	4.75	5.21	5.61	−4.21	4.21
Thiamethoxam	12.63	7.18	9.26	5.34	3.83	−10.05	13.95	7.97	2.24	13.55	4.58	−13.15	10.21
Pendimethalin	19.34	5.77	8.73	−5.27	9.60	−11.24	3.76	7.19	3.61	17.76	−8.05	4.22	4.62
Cyprodinil	14.50	12.52	9.42	−0.27	7.67	−5.90	12.91	8.44	12.54	2.19	7.03	4.46	5.30
Tolyfluanid	18.26	6.96	12.47	6.83	8.72	−6.64	4.27	6.96	14.03	−11.24	−9.00	−0.71	13.21
Fludioxonil	15.70	9.60	11.62	−1.55	1.78	−3.15	7.02	4.42	1.17	14.85	6.95	−4.39	7.79
Dieldrin	15.27	14.88	15.00	−1.28	−10.15	0.06	5.07	2.56	4.12	−1.73	−9.44	7.38	3.06
Endrin	19.07	6.66	14.65	−10.02	−0.14	8.80	19.07	6.66	2.73	−5.89	−3.59	−10.95	13.38
o.p′-DDT	17.15	10.70	2.63	−0.38	11.31	−0.51	9.17	2.97	2.95	−7.80	−14.75	14.22	2.36
Fenehexamid	35.54	13.92	32.70	−6.87	−1.21	−7.96	5.97	9.59	6.48	1.41	54.67	19.54	10.23
Acetamiprid	8.44	13.59	7.94	−10.12	4.74	−5.27	5.55	7.33	13.97	58.65	3.93	−12.60	11.50
Methoxychlor	19.43	15.01	14.28	7.43	−4.65	1.70	18.30	5.82	4.58	3.62	−5.00	8.47	3.23
Estrone	4.05	12.25	8.38	−2.49	8.50	−11.81	6.22	3.02	7.18	−15.07	10.76	9.81	5.06
Estradiol	7.15	14.86	15.03	−2.76	11.08	−11.33	7.97	2.29	8.36	−10.43	−14.60	−6.49	14.14
Azoxystrobin	15.24	4.97	13.26	−4.74	9.62	−6.33	15.49	5.56	13.26	0.68	−5.94	10.33	12.29

day tests, suggesting that additional tests were need for these compounds.

The data obtained from the repeatability studies of the instrumental precision ranged between 1.33% and 14.95%, with a mean value of 7.58 and a standard deviation of 4.21 which was considered acceptable given the limit of 15% set by the chromatographic methods validation rules for intra-day precision.

4.4.4. Recovery

The experimental mean concentrations ($n \geq 3$) for three levels of calibration and related recoveries are shown in Table 4.

A paired comparison between the theoretical or nominal concentrations and experimental concentrations showed no statistical difference ($p > 0.05$) by applying the Student's *t*-test at 95% confidence level, which confirms the accuracy of the method [63].

The mean recovery of the method was 101.01% and the standard deviation of the mean recovery of each fortification level was 0.96%.

The statistical treatment of the recovery test was also performed by applying the Student's *t*-test, and the *t*-value calculated from experimental data was 1.81, inferior to the tabulated value 2.92, with 95% confidence and $n-1$ degrees freedom. It is accepted, therefore, the null hypothesis proposed.

Method recovery was also assessed by the recovery values obtained from the quality control internal standard (atrazine-d_5), whose dispersion is important to evaluate the extraction efficiency [70] (Fig. 5).

The mean recovery and standard deviation in a total of 29 tests was 90.74 ± 12.08% with a CV of 13.31%. The control limits established are set between 70% and 120%, with CV ≤ 20% according to SANCO [29].

4.4.5. Stability

Once the environmental waters were always been processed within 24 h after sampling, the stability studies

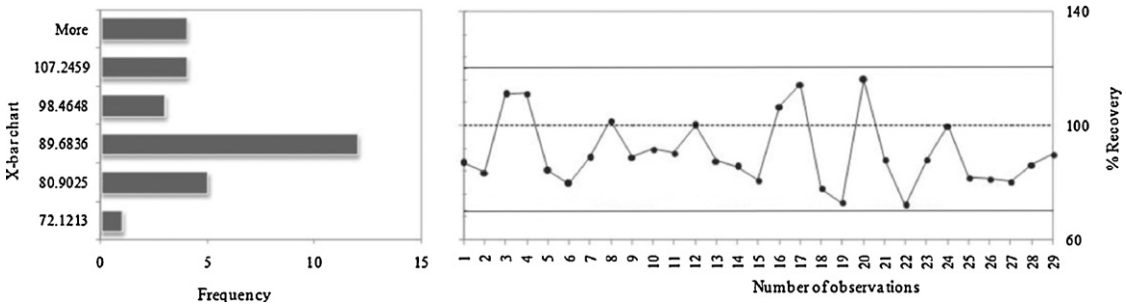

Fig. 5. Histogram and control chart of the percentage of recovery of atrazine-d_5 added to calibration standards and samples.

Table 4
Mean values of the experimental concentrations and recoveries for each concentration level ($n \geq 3$).

EDCs/pesticides	Exp. Conc. (μg/L)			% Recovery		
Nominal conc. (μg/L)	15	90	360	15	90	360
EPTC	15.66	102.40	368.00	104.39	113.78	102.22
Folpet	14.62	84.31	353.34	97.48	93.68	98.15
Phosmet	15.36	91.08	358.42	102.43	101.20	99.56
2,4-D	15.18	88.14	366.65	101.17	97.93	101.85
Atrazine-desethyl	15.08	87.44	369.62	100.54	97.15	102.67
Terbuthylazine-desethyl	15.11	85.33	363.82	100.72	94.81	101.06
Iprodione	15.40	90.19	355.84	102.66	100.21	98.84
Dimethoate	15.05	86.86	362.93	100.33	96.52	100.81
Atrazine	15.30	84.47	365.97	102.01	93.86	101.66
Cyromazine	15.75	89.18	358.95	105.00	99.09	99.71
Terbuthylazine	15.00	86.36	361.20	99.97	95.96	100.33
Pirimicarb	14.96	89.29	361.55	99.72	99.21	100.43
Alachlor	15.06	94.31	360.08	100.39	104.79	100.02
Metalaxyl	14.93	88.78	360.24	99.53	98.64	100.07
Linuron	15.94	93.57	358.16	106.25	103.96	99.49
S-Metolachlor	17.50	93.48	371.20	116.68	103.86	103.11
Aldrin	14.59	88.87	375.17	97.30	98.75	104.21
Thiamethoxam	14.82	90.57	355.26	98.77	100.63	98.68
Pendimethalin	15.79	94.10	365.16	105.27	104.55	101.43
Cyprodinil	15.04	91.29	361.66	100.27	101.43	100.46
Tolyfluanid	15.28	89.65	361.84	101.86	99.61	100.51
Fludioxonil	15.23	91.42	356.49	101.55	101.58	99.02
Dieldrin	15.21	95.39	359.78	101.38	105.99	99.94
Endrin	16.50	90.13	356.60	110.02	100.14	99.06
o.p′-DDT	15.06	94.48	361.83	100.38	104.98	100.51
Fenehexamid	16.03	91.09	357.80	106.87	101.21	99.39
Acetamiprid	14.37	90.24	360.53	95.79	100.27	100.15
Methoxychlor	15.22	94.50	354.12	101.50	105.00	98.37
Estrone	15.42	90.26	359.66	102.79	100.29	99.91
Estradiol	15.35	88.72	364.32	102.33	98.58	101.20
Azoxystrobin	15.03	89.27	353.77	100.19	99.19	98.27
Mean	15.32	90.49	361.29	102.11	100.54	100.36
Standard deviation	0.60	3.68	5.22	3.98	4.09	1.45

have not yet been carried out following the recommendations.

The stability of the calibration standards prepared in methanol and stored at 4 °C was evaluated by its periodic analysis for more than 3 months, with an averaged CV ≤ 15%. Stability was confirmed for all compounds except for folpet, whose degradation became evident 1 month after preparation.

4.4.6. Robustness

As temperatures and flow-rates were kept constant, robustness was assessed by testing two different columns from Varian (VF-5 MS 30 m × 0.25 mm × 0.25 μm) and a column from Teknokroma (TRB-5MS 30 m × 0.25 mm × 0.25 μm), without any observed discrepancy of the chromatographic data.

In addition, different lots of SPE cartridges (K91353212 and K91328612) and solvents were tested during the validation procedure and no differences were founded regarding the obtained results.

5. Conclusions

The results showed that the developed methodology SPE/GC–MS could be established as a suitable protocol for the simultaneous screening of ultra-trace levels of 31 EDCs and other pesticides in water.

The optimization of the extraction, purification, concentration and chromatographic methodologies, coupled with the high sensitivity and selectivity inherent to the detector, allowed the identification and quantification of the compounds of interest at concentrations below those reported in national and international legislation.

To avoid and minimize any ambiguity related to the matrix-induced enhancement effect, calibration curves for all quantification purposes were generated from matrix-matched standards and instrumental conditions in the SIM mode showed excellent linear responses for the studied analytes.

As the assumption of homoscedasticity was not satisfied, leading to improper estimation and inference in the statistical quantification model, a weighted least squares calibration procedure was applied revealing useful improvements in accuracy, particularly at the lower end of the range were percentage bias was considerably greater than the acceptable limits of ±20% when simple least squares regression was used.

Although weighted least squares regression is more complex and laborious than ordinary linear regression, involving the use of additional statistical tests and mathematical operations, it should be performed in order to obtain more realistic results and lower limits of quantification.

Good reproducibility and high sensitivity were obtained and the method was considered efficient, precise and accurate for all analytes in accordance with the suggested standards of acceptance, providing a good possibility of simultaneous screening for a large number of hazardous compounds in cases of suspected water poisoning.

Acknowledgement

One of the authors (A. Melo) wishes to thank FCT the grant SFRH/BD/41764/2007.

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