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Research Article

Recovery Assays: Determination of Methylparaben in Sediment Samples -

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ABSTRACT

Since were discovered by their antimicrobial activity, parabens have been widely used in many cosmetics, pharmaceuticals, personal care products and food, among others consumer products. These compounds, after human consumption, reach wastewater treatment plants, where are not efficiently removed, ending up in the environment, as well as by the direct discharge of detergents, soaps or products that may contain these compounds in their formulation. This concern has significantly boosted the number of publications in recent literature, although the number of papers on aqueous samples is still much higher compared to solid matrices, probably due to the complexity of these last. In this work, the accuracy of a new developed analytical method for the determination of emerging pollutants (Methylparaben (MeP)) in sediment samples has been carried out using recovery assays and evaluated by mean of the confidence ellipse. The sample treatment involve steps of lyophilization, solvent extraction and clean-up of the extracts with dispersive sorbents prior to liquid chromatography – tandem mass spectrometry analysis. Fortified blank samples for MeP at seven different analyte concentration levels (2.5, 12.5, 25, 50, 125, 250y 500 ng/g) were analyzed. Recovery calculations from fortified samples were performed from linear regression of the data found for each amount (level) of fortified analyte versus data added. Finally, the methodology to build the confidence ellipse, necessary operations required and the graphical representation, is applied in order to evaluate the acuracy of the method. Successful recoveries between 84 and 108% were achieved at all tested levels, although significantly different from 100% from a statistical point of view according with the confidence ellipse. However, these values are acceptable from the % practical point of view as contemplated in the recovery table of the AOAC.

Keywords: Validation; Acuracy; Recovery assays; Confidence Ellipse; Emerging Pollutants; Sediments

INTRODUCTION

Marine ecosystem tends to end up accumulating a huge variety of organic pollutants, primarily resulting from anthropogenic activities, through rivers, direct discharges, or atmospheric deposition. Over the past few decades a large number of previously unrecognized pollutants [1,2], called emerging contaminants, have been identified in this scenario. Many of these substances are especially relevant, in particular those with the capacity to disrupt the hormonal (endocrine) system of living organisms. Since were discovered by their antimicrobial activity, parabens have been widely used as bactericides, fungicides and preservatives agents in many cosmetics, pharmaceuticals, personal care products and food, among others consumer products. Although the toxicity of these compounds is very low, they present a weak estrogenic activity and are considered as endocrine disruptors, that is why they have been classified as emerging contaminants arousing a great interest in the scientific world [3,4]. These compounds, after human consumption, reach wastewater treatment plants, where are not efficiently removed, ending up in the environment, as well as by the direct discharge of detergents, soaps or products that may contain these compounds in their formulation. This concern has significantly boosted the number of publications in recent literature, although the number of papers on aqueous samples is still much higher compared to solid matrices, probably due to the complexity of these last. The development of new methodologies in solid waste matrices is key to estimate the persistence and risk of these compounds in the environment. The choice of analytical instrumentation and sample treatment should be based on the intended purpose and scope of the analytical method. The important parameters that may be evaluated during method development are accuracy, linearity, precision, sensibility and robustness [5-7]. This paper deals on the accuracy [8-12]. According to ISO 5725-1 [7], the general term "Accuracy" is used to describe the closeness of a measurement to the true value. When the term is applied to sets of measurements of the same measurand, it involves a component of random error and a component of systematic error. In this case trueness is the closeness of the mean of a set of measurement results to the actual (true) value and precision is the closeness of agreement among a set of results. Several methods of determining accuracy are available:

i) Use of certified reference materials

ii) Comparison of a proposed method with a reference method

iii) The use of recovery assays on samples and matrices

iv) Interlaboratory studies [13], which will not be considered here because in this paper we are dealing with internal quality control of accuracy.

It can also be inferred once precision, linearity and specificity have been established. In the field of environmental and pharmaceutical analysis, reference standards or alternative methods are usually not available. The recovery assays are then the tool of choice to check the accuracy [14-22]. Recovery calculations from matrices or fortified samples can be performed either from individual recoveries for each amount (level) of fortified analyte or from linear regression [23,24] of the data found versus data added:

$$y = x_{\text{found}} = a_0 + a_1 x_{\text{added}} \quad (1)$$

Where x_{found} and x_{added} refer to analyte concentrations found and fortified, respectively. The theory predicts a value of unity for the slope a_1 and a zero value for the intercept a_0 although the presence of systematic and random errors in the analytical process can produce deviations (bias) of this ideal situation.

Equations (2, a, b) provide the confidence intervals of the intercept, a_0 and of the slope, a_1 , of a straight line obtained by the least squares method in which the values of α_0 and α_1 represent the true values of the intercept and of the slope, which could be obtained by making an infinite number of measurements, and t is the tabulated Student's t for a significance level of $1-\alpha/2$ [24-28].

$$\begin{aligned} a_0 - t_{1-\alpha/2} s_{a_0} &\leq \alpha_0 \leq a_0 + t_{1-\alpha/2} s_{a_0} \\ a_1 - t_{1-\alpha/2} s_{a_1} &\leq \alpha_1 \leq a_1 + t_{1-\alpha/2} s_{a_1} \end{aligned} \quad (2, a, b)$$

It is possible to build a confidence rectangle drawing the limits of these intervals for the 95% confidence level in a diagram whose abscissa is the slope, and the ordinate, the intercept (or *vice versa*).

However, statistical tests on slope and intercept of a straight line obtained by linear regression ($t_a = a/s_a$; $t_b = \text{ABS}(b - 1)/s_b$), although frequently used by the workers, are not entirely reliable when the strong correlation existent between the slope and intercept are not taken into account [29-31]. The covariance between two variables is as

important as the variances and both contribute to the total analytical error significantly. That is to say, the confidence rectangle has to be substituted by the confidence ellipse (joint effect) that describes the behaviour of the system in a more rigorously way [23,24,32-38].

$$\text{cov}(a_0, a_1) = -\bar{X} \frac{s_{y/x}^2}{s_{xx}} \quad (3)$$

Instead of these individual tests, the confidence ellipse derived by Working and Hotelling [37] and adopted by Mandel and Linnig [38] is recommended. It is therefore preferable to calculate a set interval for these coefficients. The formula that describes this interval is an ellipse [23,24,37,38] centered on the point (a_1, a_0) .

$$n(a_0 - \alpha_0)^2 + 2(\sum x_i)(a_0 - \alpha_0)(a_1 - \alpha_1) + (\sum x_i^2)(a_1 - \alpha_1)^2 = 2s_{y/x}^2 F_{2,v,\alpha} \quad (4)$$

Here, $F(2, v, \alpha)$ is the tabulated F-distribution with a significance level of α and 2 and $(n - 2)$ are the degrees of freedom (n is the total number of samples), and $s_{y/x}^2$ is the regression variance.

$$s_{y/x}^2 = \frac{\sum (y_i - \hat{y}_i)^2}{n - 2} = \frac{S_{YY} - a_1^2 S_{XX}}{n - 2} \quad (5)$$

S_{YY} and S_{XX} are the corresponding sums of squares with respect to the means of y and x , respectively.

This ellipse is difficult to represent [38-42]. In order to simplify the calculations, a change of axes can be made, taking the values (A_0, A_1) , as the center of the ellipse, defined as

$$A_0 = a_0 - \alpha_0 \quad A_1 = a_1 - \alpha_1 \quad (6)$$

Eq. (4) becomes:

$$nA_0^2 + 2(\sum x_i)A_0A_1 + (\sum x_i^2)A_1^2 = 2s_{y/x}^2 F_{2,v,\alpha} \quad (7)$$

Taking into account that:

$$\bar{X} = \frac{\sum x_i}{n} \quad \overline{x^2} = \frac{\sum x_i^2}{n} \quad (8)$$

We get the following equation which describes the intersections of the ellipse with a line parallel to the x - axis.

$$A_0^2 + 2\bar{X}A_0A_1 + \overline{x^2}A_1^2 - 2\frac{s_{y/x}^2 F_{2,v,\alpha}}{n} = 0 \quad (9)$$

The two possible solutions after solving the eq. (9) are:

$$A_0 = -\bar{X}A_1 \pm \sqrt{\overline{x^2}A_1^2 - 2\frac{s_{y/x}^2 F_{2,v,\alpha}}{n}} = -\bar{X}A_1 \pm \sqrt{-A_1^2(\overline{x^2} - \bar{X}^2) + 2\frac{s_{y/x}^2 F_{2,v,\alpha}}{n}} \quad (10)$$

To have real solutions, the root discriminant must be greater than zero. Note that

$$\Delta = -A_1^2(\overline{x^2} - \bar{X}^2) + 2\frac{s_{y/x}^2 F_{2,v,\alpha}}{n} \quad (11)$$

$$\overline{x^2} - \bar{X}^2 = \frac{\sum x_i^2}{n} - \left(\frac{\sum x_i}{n}\right)^2 = \frac{1}{n} \left(\sum x_i^2 - \frac{(\sum x_i)^2}{n}\right) = \frac{1}{n} S_{xx} \quad (12)$$

and

$$\Delta = \frac{1}{n} (2s_{y/x}^2 F_{2,v,\alpha} - A_1^2 S_{xx}) \quad (13)$$

To build the ellipse, A_1 is varied in small increments in positive and negative directions, calculating the discriminant value. When this is positive, two real roots are obtained, getting the two branches of the curve. In order to make this diagram easier to use, the coordinates in the initial space (α_1, α_0) are now calculated by applying the change of variables in the reverse direction. In this regard, the study of the confidence ellipse is a very powerful and precise tool order to evaluate the accuracy of the method. In spite of the difficulties involved in representing the ellipse, it is preferable to use this joint confidence interval because it is much more efficient from the statistical point of view. To do this, one may use a spreadsheet like Excel and through a graphic visualization we can easily detect how reliable are the slope and the intercept of the regression line. Additionally, the procedure described in this work may be applied in a variety of areas of analytical importance such as calibration, recovery assays and comparison of methods. In this work, the evaluation of accuracy of a new developed analytical method [1] for the determination of emerging pollutants (Methylparaben (MeP)) in sediment samples has been carried out by mean of recovery assays. Recovery calculations from fortified samples are performed from linear regression of the data found for each amount (level) of fortified analyte versus data added and the methodology described above to build the confidence ellipse is applied in order to evaluate the accuracy of the method.

MATERIALS AND METHODS

Reagents

The compound studied MeP, was supplied by Dr. Ehrenstorfer GmbH (between 97 - 99.5% purity). The Internal Standard (IS) Ethylparaben - d_5 (EtP - d_5), was supplied by Cambridge isotope laboratories (MA, USA). The stock solution (1000 mg/L) was prepared in methanol and stored in a refrigerator at 4°C working solutions were prepared by diluting the stock standard solution in methanol. Acetonitrile, water and methanol all of HPLC quality purity, were supplied by Romil Ltd. (Barcelona, Spain). Ammonium acetate (reagent grade analysis), was supplied by Panreac (Barcelona, Spain). Water, methanol and Acetonitrile (AcN) (all of chromatographic analysis grade) were purchased from Romil Ltd. (Barcelona, Spain). Octadecyl functionalized silica (C18) was provided by Sigma - Aldrich (Steinheim, Germany). Ammonium acetate obtained from Panreac (Barcelona, Spain).

Sample collection

Marine sediments were hand collected by scuba divers at random locations in March 2016 along a transect (50m long and 7-13m deep) in the infralittoral zone of Marina del Este beach (Almuñecar, Southern Spain). Sediment samples were freeze-dried, homogenized, grounded into powder and frozen at - 20°C until analyses [1].

Preparation of matrix-matched calibration curves

Matrix-matched calibration standards were prepared at seven different analyte concentration levels (2.5, 12.5, 25, 50, 125, 250 and 500 ng/g de MeP). Working with matrix-matched standard in environmental samples could lead to representativeness problems, therefore a pool of different sediment samples were used. The mixtures were vortexed for 2 min and then left to stand for 24 h at 4°C in the dark before analysis. This allows the analyte to come into full contact with the sample.

Sample treatment

Aliquots of the samples (0.5 g) were weighed into 12 mL glass vials, containing 100 μ L of a methanol solution (250 ng/mL) of the IS (EtP - d₃). The samples were vortexed for homogenization twice in 7 mL of acetonitrile (2 min) and centrifuged for 10 min at 4050 \times g. In order to decrease the matrix co-extractives in the extract that could cause the matrix effect, a clean-up of the extract based on disperse solid phase extraction (d-SPE) was carried out. The supernatants obtained from each extraction step were combined and transferred to a 50 mL polypropylene conical tube containing 800 mg of C18 sorbent. The mixture was hand-shaken for 2 min and centrifuged for 5 min at 4050 \times g. Next, the solvent was evaporated to dryness at room temperature under a nitrogen stream and the extract was reconstituted in 0.25 mL of a mixture methanol: water (50:50 v/v), filtered through a 0.22 μ m nylon filter and a 20 - μ L aliquot was injected into the HPLC instrument.

High performance liquid chromatography – tandem mass spectrometry operating conditions

Chromatographic separation of MeP was performed using a HALO C-18 Rapid Resolution (50 \times 4.6 mm i.d., 2.7 μ m particle size) column. The compound was separated using a gradient mobile phase consisting of an aqueous buffer solution of acetic acid/ammonium acetate (pH 4.4) (solvent A) and methanol buffered with the same composition (solvent B). The gradient program was as follows: 0 - 14 min, linear gradient from 28 to 70% of solvent B, from 70% to 80% of solvent B in 5 min, and then increased to 100% in 6 min and held for 2 min. Flow rate was fixed at 0.6 mL min⁻¹. The injection volume was 20 μ L. The column temperature was maintained at 30°C. The HPLC system is coupled to a triple quadrupole mass spectrometer with ESI working in negative mode. The parameters selected for the spectrometer are: capillary voltage, 3000V; nebulizer pressure, 40psig; drying-gas flow rate, 9.0 L/min and drying-gas temperature, 355°C. The mode of operation of the spectrometer is MRM (Multiple Reaction Monitoring). Instrument control and data acquisition were carried out with MassHunter software (Agilent, USA). A previous optimization of the conditions of fragmentation was made using the Optimizer software. Negative mode was selected because it showed higher sensitivity for all compounds of interest. The two transitions, one for quantification and the other for confirmation, corresponding to the most abundant ion products were selected after the rupture of the precursor ion. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for product ions were fragmentation voltage and collision energy. The parameters selected to obtain optimum responses are presented in table 1.

RESULTS AND DISCUSSION

Quantification was carried out using matrix-matched calibration curves. Calibration curves were constructed by linear regression of the peak area ratio of the analyte and its corresponding internal standard against their respective concentrations. Figure 1 shows the found concentration versus the added concentration. The Linest; function in Excel provides us with the value of the parameters of the straight line. The values obtained for a_0 , a_1 and their standard deviations in the case of simple linear regression are shown in table 2.

Once the values of the intercept and slope, a_0 and a_1 , respectively are estimated, and before evaluating the recovery, a residual analysis should be undertaken [29-31] to check the validity of

the model. Certain assumptions fundamental to regression, such as independence of errors, homocedasticity (uniform or regular variance) and Gaussian distribution must be fulfilled. If the model represents the data properly, the residuals must be randomly distributed around the normal (Gaussian) predicted values or about the x values. In the secondary axis of figure1 is plotted the residual analysis, which shows absence of spurious data as well as an increase in the error with the concentration level. The methodology described in the introduction to build the confidence ellipse is treated in short in the following. Accuracy was calculated as the percentage of analyte extracted, quantified using matrix-matched calibration curves, in relation to the spiking level. Successful recoveries between 84 and 108% were achieved (Table 3). The theory predicts a value of unity for the slope a_1 and a zero value for the intercept a_0 . After application of the statistical test for the intercept and the slope, it can be concluded that the intercept is not significantly different from zero, since the value of zero falls in the range or confidence interval of this parameter ($t_{exp} = a_0/s(a_0) = 0.813 < t_{tab}$), while the slope is significantly different from one ($t_{exp} = (a_1 - 1) / s(a_1) = 6.924 > t_{tab}$), and hence the recovery is far from 100%. Nonetheless, statistical tests on the intercept and the slope of a straight line obtained by the least squares method are not completely reliable when performed independently. We must therefore proceed to obtain the confidence interval together, which requires the drawing of the confidence ellipse, better than the confidence rectangle corresponding to the individual tests. Point (1, 0) falls far enough away from both the

Table 1: Optimized parameters for the determination of MeP by QqQ – MS.

Compound	Precursor ion (m/z)	MRM 1 (quantification) (m/z)	MRM 2 (confirmation) (m/z)	Fragmentor (V)	Collision energy (V)
MeP	151.2	92.1	136.1	70	16

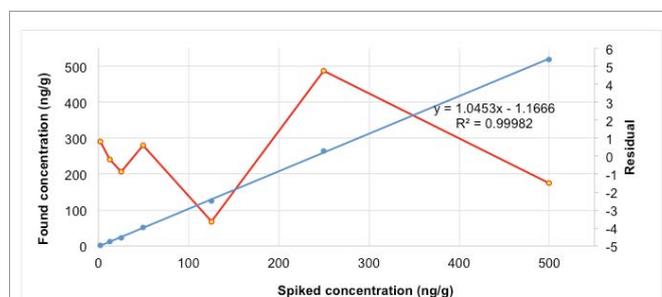


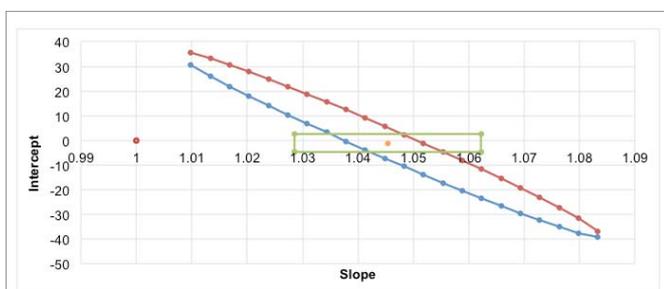
Figure 1: Calibration curve for recovery assays in the analysis of MeP in sediment samples

Table 2: Statistic parameters of the straight line, and confidence interval for the experimental study systems.

MeP calibration			
Straight line			
a_1	1.0457	1.1666	a_0
$S_{(a1)}$	0.0066	1.4275	$S_{(a0)}$
R^2	0.9992	5.0612	$S_{(y/x)}$
Confidence interval ($t_{(0.05; 6)}$)			
1.0284	< α_1 <		1.0621
- 4.8362	< α_0 <		2.5029

Table 3: Recovery assays of MeP in sediment samples.

Spiked concentration (ng/g)	Found concentration (ng/g)	Estimated found concentration (ng/g)	Residual	Recovery (%)
2.5	2.1	1.4	0.7	84.0
2.5	2.2	1.4	0.8	88.0
2.5	2.5	1.4	1.1	100.0
12.5	11.8	11.9	- 0.1	94.6
12.5	11.1	11.9	- 0.8	88.8
12.5	12.2	11.9	0.3	97.6
25.0	23.3	25.0	- 1.6	93.3
25.0	24.1	25.0	- 0.9	96.4
25.0	24.9	25.0	- 0.1	99.6
50.0	50.0	51.1	- 1.1	100.1
50.0	53.9	51.1	2.8	107.8
50.0	51.2	51.1	0.1	102.4
125.0	126.1	129.5	- 3.4	100.8
125.0	127.4	129.5	- 2.1	101.9
125.0	124.1	129.5	- 5.4	99.3
250.0	254.0	260.2	- 6.2	101.6
250.0	274.0	260.2	13.8	109.6
250.0	266.8	260.2	6.6	106.7
500.0	519.0	521.5	- 2.5	103.8
500.0	512.0	521.5	- 9.5	102.4
500.0	529.0	521.5	7.5	105.8

**Figure 2:** Confidence ellipse for recovery assays in the analysis of MeP in sediment samples

rectangle and the confidence ellipse (Figure 2). We can see how the study of the confidence ellipse is a very powerful and precise tool for graphic visualization of how reliable, are the slope and the intercept in the regression line. Nevertheless, once the recovery is computed, it should be checked for fulfilling the accuracy criteria according to the AOAC guidelines [43]. The AOAC manual for the peer verified methods program includes a table with estimated recovery data as a function of analyte concentration. The concentration should cover the range of concern and should particularly include one concentration close to the quantitation limit. The expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration. Notes that for trace analysis, e.g. MeP in sediment samples investigated (part per billion levels), recoveries about 50% are often the best that can be achieved. So, the recovery obtained from a practical point of view is acceptable, since it is within

the range of values allowed by the table of AOAC. The use of non-statistical criteria for judging accuracy based on recovery percentages as a function of analyte concentration has been previously reported [43,44] for many authors and frequently use in these circumstances.

CONCLUSION

The calculation of recoveries may be performed from linear regression analysis of estimated against spiked analyte concentration. The theory predicts a value of 1 for the slope and a value of 0 for the intercept. A very powerful and precise tool in these situations is the study of the confidence ellipse. Along this contribution the confidence ellipse has proven to be a powerful and precise tool for the evaluation of accuracy of a new developed analytical method for the determination of MeP in sediment samples. In spite of the difficulties to build it, through a graphic visualization we can easily detect how reliable are the slope and the intercept of the regression line. The recoveries obtained for MeP in sediment samples, of interest at the *ppb* level, ranged between 84 - 108%, significantly different from 100% from a statistical point of view although acceptable from the practical point of view contemplated by the AOAC table. The procedure described in this work may be applied in a variety of areas of analytical importance such as calibration, recovery assays and comparison of methods.

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