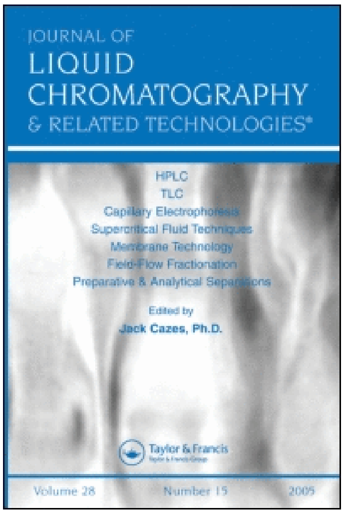

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Correction of the Matrix Effect in the Determination of Benzoylphenylurea Insecticides in Vegetables

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ABSTRACT

The matrix effect in the analysis of benzoylphenylurea insecticides, in four vegetables by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection, was investigated by applying the analysis of covariance (ANCOVA). Experimental results show that a matrix effect exists in the quantification of diflubenzuron and flufenoxuron in cucumber and flufenoxuron in green bean. In order to correct this effect, two different methods have been compared in the analysis of real samples: the first one was based on the use of calibration solutions prepared in blank matrix extracts for the quantification of the target

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analytes in the different matrices, and the second one consisted of the establishment of a correction function. The obtained results showed that the application of the correction function leads to results very similar to those obtained when the quantification was carried out using calibration curves prepared in blank matrix extract; the utility of this function is demonstrated to eliminate the matrix effect in the quantification of benzoylphenylurea insecticides in real samples with the advantages that this presents.

Key Words: Benzoylphenylurea insecticides; Photochemically induced fluorescence; HPLC; ANCOVA; Matrix effect; Vegetables.

INTRODUCTION

In the quantification of an unknown sample one of the most serious problems, which appears, when using a first-order calibration, is the presence of unexpected interferences in the matrix. In general, if this effect is unaccounted for in the calibration samples and, therefore, not modelled in the calibration phase, it produces erroneous results in the predicted concentration of the constituents.[1–3]

This effect can be due to different reasons,[4] which can be classified into three categories: (1) The presence of a blank due to solvent and/or reagents. The errors introduced do not vary with the amount of sample. (2) The presence of compounds in the sample that contribute to the analytical signal. In this case, the bias varies with the amount of sample. (3) The use of calibration curves that take no account of the matrix effect, i.e., an erroneous way to work.

The detection and correction of errors caused by matrix interferences have been extensively studied for a long time.[5–7] Matrix-induced enhancement is a phenomenon commonly found in the chromatographic analysis of pesticides in food[8–18] that has been noticed in the analysis of these contaminants by GC-FPD,[8,9] GC-ECD,[10] GC-NPD,[11] GC-MS,[12–15] and HPLC-MS.[16–18] Also, a matrix effect appeared in the analysis of pesticides in protective clothing pesticide applicators by GC-ECD[19] and in the analysis of pesticides in water by HPLC-MS.[20] In all cases, an enhancement of the analytical signal was reported, except in the last one, in which the salinity provided a dramatic decrease in the response for early eluting analytes. In addition, a signal suppression was found in the determination of benzoylphenylureas and pyrethroids in vegetables by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection.[21,22] Some of the factors that may cause sample matrix suppression or enhancement include: the nature of the pesticide, the nature of the matrix, the pesticide-to-matrix ratio, and the chromatographic system.[11]

Several methods may be taken to overcome or reduce the matrix suppression/enhancement effect, among which can be the following:

The addition of standards of the analyte to the sample (Standard Addition Method) is a fairly effective method, but their use requires a great amount of sample and consumes a long time due to the necessity of a calibration curve for analysing each sample. Therefore, U.S. federal regulatory agencies[23] proposed performing extensive clean-up of extracts to remove matrix components and reduce or eliminate the matrix effect. However, this may lead to the partial loss of some compounds, as well as an increase of labour, time, and cost. For example, Di Muccio and co-workers[24] developed a selective clean-up for the determination of carbendazim and thiabendazole in fruits and vegetables by HPLC with UV detection, and the complete clean-up takes 2 h.

Also, the use of internal standards is permissible for U.S. regulatory purposes,[23] but in the case of matrix suppression/enhancement, due to differences in the strength of the effect dependent on the pesticide, each pesticide would need its own internal standard, which are expensive, unavailable, and/or impractical in multiresidue analysis. In HPLC-MS-MS, the ionization source is highly susceptible to matrix effects[16–18] due to competition between matrix components and analyte ions in the sprayed solutions. Therefore, the application of internal standards has been especially useful, but in most cases may be insufficient for resolving the signal suppression effects when characterising complex matrix samples.

The use of standards in blank extracts (matrix-standard calibrations) is the option followed by many laboratories due to the ease of use and effectiveness of the approach.[25] To compensate for matrix-induced enhancement of recoveries, standard solutions in residue free sample extracts are used for chromatographic calibration.[8] In this way, several authors use matrix-matched standard for the quantification when high recoveries are found for pesticides in various food commodities.[12,26–30] So, improved accuracy of results achieved for a range of pesticides by the use of matrix-matched standards was documented by Johnson and co-workers.[13] However, its drawbacks can include the necessity of blank extracts and the extra labour potentially involved.

As an alternative to the daily use of blank extracts in the calibration stage, mathematical procedures, based on different principles, have been developed, such as multilinear regression (MLR) and methods of multivariate calibration (PCR and PLS).[31] The use of multivariate calibration methods requires the collection of a large number of standards for building the calibration set and, in some cases, it is necessary to include samples of the same nature as that of the unknown complex samples into the model. In this sense, Cuadros and co-workers[32] has proposed a simple methodology, consisting of the establishment of a correction function between the concentration of the sample obtained using calibration curves built with standards prepared in solvent and the concentration that would be obtained using calibration curves built with standards prepared in blank vegetable extracts. So, it would only be necessary to prepare a calibration curve in solvent and, applying the correction function calculated previously for each matrix, the real concentration would be obtained in each sample.

The aim of this work was to compare the results obtained when quantification is carried out using calibration curves prepared in blank extract of matrix with the results obtained using correction functions, in the analysis of real samples when an effect of the vegetable matrix is present on the signal of the pesticides of interest. These approaches were applied to the determination of five pesticides belonging to the benzoylphenylurea insecticides in different vegetable samples by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection.

EXPERIMENTAL

Chemicals and Solvents

Analytical standards (pestanal quality) of diflubenzuron (DFL), triflumuron (TRF), hexaflumuron (HF), lufenuron (LUF), and flufenoxuron (FLF), were obtained from Dr. Ehrënstorfer (Augsburg, Germany).

Analytical-reagent grade solvents, methanol (MeOH), acetonitrile (AcN), ethyl acetate, and anhydrous sodium sulphate for pesticide residue analysis were obtained from Scharlau (Barcelona, Spain). SPE cartridges aminopropyl-bonded silica 500 mg (Waters, Milford, MA) were used to clean up vegetable samples.

Mobile phases were filtered through a 0.45 µm cellulose acetate (water) or Teflon (MeOH) and degassed with helium prior to and during use. All standards and samples were filtered through Millipore membrane Teflon filters (0.45 µm particle size) before injection into the chromatographic column.

Distilled water, obtained from a Milli-Q water purification system Millipore (Bedford, MA), was used.

Instrumentation

The HPLC was a Waters (Milford, MA), composed of a Model 600 E multisolvent delivery system, a Rheodyne 7725i manual injector valve with a

400 µL sample loop, a Temperature Control System and a Model 474 scanning fluorescence detector. LC separations were performed with a Waters spherical silice-bases stationary phase 3.9 × 150 mm (4 µm particle size) column (Waters, Milford, MA).

The photochemical reaction was carried out in a post-column photochemical reactor (Softron GmbH, Gynkotek HPLC, Germering, Germany) fitted with a knitted open tube reactor coil (5 m × 1.6 mm e.d. and 0.3 mm i.d.) PTFE and a 4-W Xenon lamp.

A Digital Venturis FP 575 pentium personal computer using a Millennium 32 (Chromatography Manager, Waters, Milford, MA) software was used for acquisition and treatment of data.

A Model PT 2100 Polytron (Kinematica AG, Luzern, Switzerland) and a Model BV-401C blender (Fagor, Guipuzcoa, Spain) were used for blending the samples. A Model VV2000 LIF rotary vacuum evaporator (Heidolpf) thermostated by water circulation with a N-010 KN-18 vacuum pump (Telstar) was used to evaporate the extracts.

Extraction and Clean Up

Vegetable samples (50 g) were placed in a glass and homogenized with 75 mL ethyl acetate for 2 min. with Polytron. Then, 80 g of sodium sulphate were added and the mixture was homogenized for 1 min. The extract was filtered through a 12 cm Büchner funnel and washed with 2 successive 30 mL portions of ethyl acetate. The rinsings were added to the combined extraction fractions. The filtered liquid was collected in a 250 mL spherical flask and evaporated to dryness in a rotating vacuum evaporator with a water bath at 60 ± 1°C.

The residue obtained from the extract was redissolved in 5 mL dichloromethane. An aminopropyl-bonded silica SPE column was preconditioned with 1 mL dichloromethane and 1 mL of the sample extract was brought onto the SPE cartridge. The collection of the eluate started directly after applying the extract. The elution started with 3 mL dichloromethane, and this eluate was collected in the same 25 mL spherical flask. The total eluate was concentrated nearly to dryness in a rotating vacuum evaporator with a water bath at 60 ± 1°C and the remaining solvent was allowed to evaporate under a slight N_2 stream. The obtained residue was redissolved in 1 mL AcN : water 1 : 1 (v/v) and then filtered through an 0.45 µm Teflon filter.

Preparation of Both Matrix-Matched and Solvent-Based Standards

Standard solutions of pesticides (200 mg L^{-1}) were prepared by exactly weighing and dissolving the corresponding compounds in organic solvents. These standard solutions were stable for a period of at least 3 months. All solutions were protected against the light with aluminium foil and were stored in a refrigerator at 4°C. Lower concentration standards were prepared by serial dilution in solvent or in blank matrix extract.

Calibration solutions were prepared in triplicate containing increasing quantities of standards of the five benzoylphenylureas, whose final concentrations ranged from 0.1 to 1.0 mg L^{-1} of each pesticide in AcN : water 1 : 1 (v/v). Using pooled extracts of fresh samples, previously found to be blank, and extracted as described in the "Extraction and clean up" section, a set of matrix-matched standards was prepared for each commodity, also in triplicate.

Both sets of standards were prepared using the same stock solutions.

Preparation of Spiked Samples

For recovery determinations, samples (50 g) of finely chopped vegetables were spiked by addition of a standard stock solution (200 mg L^{-1}), at two concentration levels: 0.01 and 0.1 mg kg^{-1} (equivalent to 0.1 and 1.0 μg mL^{-1} in the final extract) for each pesticide. The spiked samples were allowed to stand for a few minutes before extraction to allow the spiked solution to penetrate the test material.

HPLC Procedure

The benzoylphenylurea insecticides were determined by analysing 400 μL of AcN : water 1 : 1 (v/v) sample solutions by HPLC with fluorimetric detection. The solvents were filtered daily before use through a 0.45 μm cellulose acetate (water) or politetrafluoroethylene (MeOH) and degassed with helium prior and during use. Samples were chromatographed isocratically with MeOH : water 80 : 20 (v/v), as mobile phase, for 18 min at a flow rate of 0.4 mL min^{-1}. The fluorimetric detection was performed at an excitation wavelength (λ_{ex}) of 330 nm and at an emission wavelength (λ_{em}) of 410 nm for all pesticides.

Estimation and Correction of Matrix Effect

Analysis of Covariance

The use of analysis of covariance (ANCOVA) for comparing calibration lines has been described in detail in the paper by Cuadros and co-workers,[32,33] so only a brief description will be given here.

To compare a group of regression curves by means of the analysis of the covariance, first, a test *F*, is applied, in order to check if the residual variances are homogeneous for all the calibration lines. Once checked, if it is fulfilled, the ANCOVA is applied, for which is calculated again, a parameter *F*, defined as:

$$F_{\text{cal}} = \frac{S_N{}^2}{S_D{}^2}$$

where

$$S_N{}^2 = \frac{SS^R - SS^F}{p-1} \qquad S_D{}^2 = \frac{SS^F}{v^F}$$

being $p =$ the number of slopes to compare

$$v^F = \sum_{i=1}^{p} v(i)$$

where $i = 1,2,\ldots,p$, being p the number of lines to consider and v the number of degrees of freedom of each straight line, being calculated as the number of standard of each straight line (n) minus 2 ($v = \mathrm{n} - 2$).

The result of this test can be interpreted as a function of the level of significance of the null hypothesis [*P*-value (%)], i.e., the probability that the null hypothesis is fulfilled instead of the alternative hypothesis, in such a way that if *P*-value (%) is higher than the value of the α (%) established (usually 5%), it can be concluded that significant differences do not exist among the slopes of the group of calibration curves.

If the slopes into their group differ significantly, it proceeds to carry out a comparison for couples, to check between which of them the differences are presented. For it, the test of Bonferroni is applied[1] considering all the possible couples of calibration curves that would come given by $K = p\ (p-1)/2$.

According to this test, the $t(b)$ obtained is compared with a t-tabulated considering a probability of α/K, being α usually 5%, and concluding that if t-tabulated is higher than t-calculated or if P-value (%) is higher than α/K (%), there is not significant difference between the slopes compared. This study should be applied to each of all the possible paired data.

Correction Function: Correction Coefficients

The correction function can be obtained from the regression lines prepared, using clean solvents ($R = a_S + b_S C_S$), (solvent calibration, SC), and prepared with the solvent containing co-extractives from the sample matrix ($R = a_M + b_M C_M$) (matrix calibration, MC), making equal both equations and obtaining the concentration C_M as a function of the concentration C_S as follows:

$$C_M = \frac{a_S - a_M}{b_M} + \frac{b_S}{b_M} C_S$$

which correspond to a straight line, where a_S y a_M, are the intercept of calibration curves in SC y MC, respectively and b_S y b_M, are the slopes of calibration curves in SC y MC, respectively.

The correction coefficients A and B are defined as the intercept and the slope, respectively, of this straight line. Thus, the correction function can be expressed as $C_M = A + BC_S$, which allows one to obtain the concentration C_M from the concentration C_S once one calculated the correction coefficients and established the correction function.

RESULTS AND DISCUSSION

Extraction of Pesticides from Vegetable Samples

Extraction and SPE clean up with different extractants (ethyl acetate, methanol, acetone, dichloromethane : methanol 9 : 1 and 1 : 1, dichloromethane : petroleum ether 1 : 1, and ethylacetate : methanol in different percentages), sorbents (aminopropyl-bonded silica, C_{18}, florisil and alumina), and eluents (dichloromethane and methanol) were tested to obtain interferents free extracts and acceptable recoveries for the five pesticides. Recoveries lower than 85% were obtained in all cases, except when extraction was carried out with ethyl acetate, using aminopropyl and dichloromethane in the clean up step. These conditions were, therefore, chosen for extraction and clean up. Figure 1 shows the HPLC-fluorescence chromatogram for a mixture of benzoylphenylureas

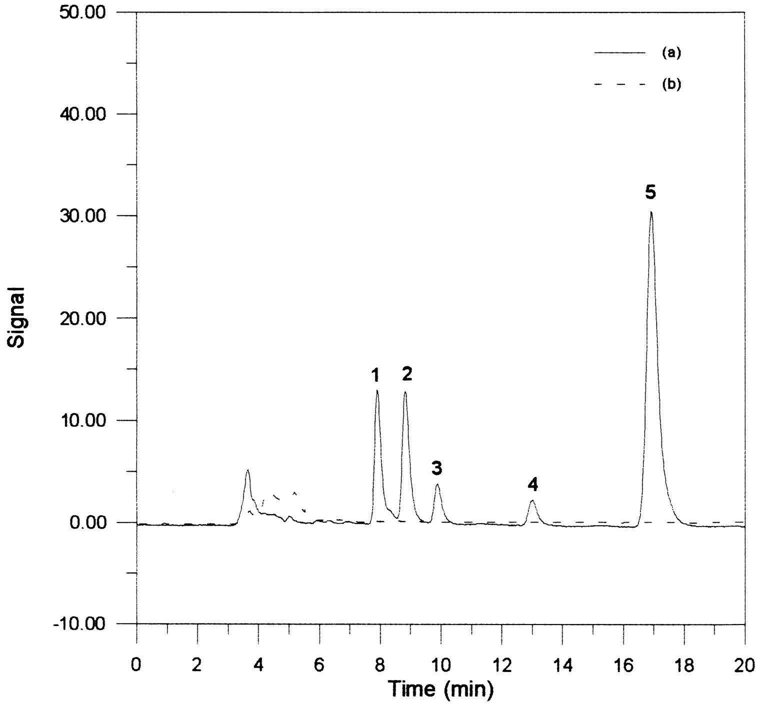

Figure 1. (a) Chromatogram corresponding to a standard of 0.4 mg L^{-1} of: (1) diflubenzuron, (2) triflumuron, (3) hexaflumuron, (4) lufenuron, and (5) flufenoxuron. (b) Chromatogram of a blank cucumber extract.

with 0.4 mg L^{-1} of each pesticide and a blank cucumber extract. It can be observed that no interferences appear at the retention times corresponding to those of the analytes.

Validation of the Analytical Method in Vegetable Samples

According to the golden rules of method validation[1] there are three important rules which must always be kept in mind: (i) validate the whole method, including the actual determination and the preparatory steps, (ii) validate over the entire range of concentrations and (iii) validate over the whole range of matrices. The analytical methodology for analysing the five benzoylphenylureas was developed in a previous work[21] and it was validated and applied for analysing the target pesticides in tomato. In that study it was shown the existence of an effect due to the matrix in the determination of DFL, TRF, HF, FLF in tomato, that was corrected carrying out the prediction with calibration curves prepared in blank extract of vegetable matrix. Matrix effect has been described by other authors as matrix and pesticide-dependent.[34] For that reason and following the third rule, the study of the behaviour of these pesticides has been carried out in four other representative matrices cultivated in greenhouses of the province of Almería (green bean, zucchini, cucumber and pepper). Each crop type represents a product class for which the application of the benzoylphenylurea insecticides is allowed in agriculture.

In this work, the characteristic parameters of the analytical method have been established, using solvent-based and matrix-matched standards for all vegetable matrices (Tables 1 and 2).

The limits of detection (LOD) for the benzoylphenylurea insecticides were calculated as the analyte concentration at which $S/N = 3$.[35] Limits of quantification (LOQ) were calculated as the amount that gives a previously defined precision ($RSD = 5\%$ in our case), according to the EURACHEM guidance.[36] LOD and LOQ were very similar for the different matrices, being those in blank matrix extract slightly higher than those obtained using standard solution prepared in solvent.

Once the characteristic parameters of the method were determined, the quantification of the five pesticides in spiked control samples of the four vegetables fortified at two levels of concentration (5 and $50\,\mu g\,kg^{-1}$) was carried out. For this purpose, calibration curves built with standard prepared in solvent and with standard prepared in blank vegetable extracts were used. The recovery percentages obtained are shown in Table 3, the RSD being calculated at two levels of concentration lower than 6% in all the cases. It can be observed, that the results obtained using both solvent-based and matrix-matched standard calibration curves were similar for all the pesticides, except for DFL and FLF in cucumber and FLF in green bean. In these three cases, the quantification carried out using the calibration curves built with standards prepared in blank vegetable extracts (80–105%) leads to better results than the quantification carried out using calibration curves prepared in solvent (79–130%). These results show the possible existence of an effect due to the matrix, and an attempt was made in order to statistically establish the presence of this effect.

Study of the Matrix Effect by Analysis of Covariance

With the aim of using ANCOVA[32,33] to detect matrix, calibration solutions were prepared three times, containing increasing quantities of standards of the five benzoylphenylureas, whose final concentrations ranged

Table 1. Limits of detection (LOD) and limits of quantification (LOQ) established in solvent-based and in matrix-matched standards.

	Solvent ($ng \cdot mL^{-1}$)		Cucumber ($ng \cdot mL^{-1}$)		Zucchini ($ng \cdot mL^{-1}$)		Green bean ($ng \cdot mL^{-1}$)		Pepper ($ng \cdot mL^{-1}$)	
Pesticide	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]
DFL	7	20	13	40	13	50	12	50	7	50
TRF	5	10	12	50	17	50	15	40	13	40
HF	10	30	23	50	25	40	20	50	14	40
LUF	12	30	14	50	25	50	15	50	24	50
FLF	3	10	9	25	15	30	10	25	8	25

[a] Based on the lowest concentration where the S/N ratio is estimated to be 3.

[b] Based on the lowest concentration where the RSD (%) is estimated to be less than 5%.

Table 2. Regression parameters established in solvent-based and in matrix-matched standards.

	Pesticide				
	DFL	TRF	HF	LUF	FLF
Solvent					
Linear range ($\mu g\,mL^{-1}$)	0.02–1.00	0.01–1.00	0.03–1.00	0.03–1.00	0.01–1.00
Regression equation	$y = 211{,}721x - 2{,}389$	$y = 173{,}049x + 7{,}186$	$y = 69{,}637x - 1{,}300$	$y = 50{,}041x - 362$	$y = 668{,}912x + 19{,}264$
Cucumber					
Linear range ($\mu g\,mL^{-1}$)	0.04–1.00	0.05–1.00	0.05–1.00	0.05–1.00	0.025–1.000
Regression equation	$y = 208{,}694x - 3{,}305$	$y = 174{,}508x + 7{,}517$	$y = 67{,}519x - 1{,}298$	$y = 52{,}943x - 421$	$y = 583{,}958x + 33{,}195$
Zucchini					
Linear range ($\mu g\,mL^{-1}$)	0.05–1.00	0.05–1.00	0.04–1.00	0.05–1.00	0.03–1.00
Regression equation	$y = 219{,}521x - 2{,}451$	$y = 180{,}743x + 7{,}308$	$y = 67{,}135x - 1{,}485$	$y = 49{,}616x - 408$	$y = 664{,}508x + 19{,}955$
Green bean					
Linear range ($\mu g\,mL^{-1}$)	0.05–1.00	0.04–1.00	0.05–1.00	0.05–1.00	0.025–1.000
Regression equation	$y = 215{,}728x - 2{,}485$	$y = 170{,}946x + 7{,}359$	$y = 63{,}552x - 1{,}268$	$y = 54{,}243x - 359$	$y = 641{,}034x + 25{,}206$
Pepper					
Linear range ($\mu g\,mL^{-1}$)	0.05–1.00	0.04–1.00	0.04–1.00	0.05–1.00	0.025–1.000
Regression equation	$y = 213{,}782x - 2{,}387$	$y = 174{,}417x + 7{,}348$	$y = 66{,}829x - 1{,}299$	$y = 53{,}422x - 358$	$y = 670{,}964x + 19{,}856$

Table 3. Recovery percentages and RSD (%) for samples fortified at two concentration levels (5 and 50 $\mu g\,kg^{-1}$) carrying out the quantification with calibration curves built with standard solutions prepared in solvent and in the corresponding vegetable matrix.

		Recovery (%)							
		Cucumber		Zucchini		Green bean		Pepper	
Pesticide	Fortification level ($\mu g \cdot kg^{-1}$)	Solvent	Matrix	Solvent	Matrix	Solvent	Matrix	Solvent	Matrix
DFL	5	130 (3.4)	105 (4.4)	97 (4.0)	94 (4.2)	104 (4.7)	103 (3.9)	101 (3.1)	100 (4.5)
	50	112 (3.1)	97 (3.5)	94 (3.3)	91 (3.8)	97 (3.5)	95 (3.3)	105 (3.4)	104 (3.8)
TRF	5	89 (4.8)	86 (3.7)	84 (5.1)	80 (4.2)	81 (4.9)	81 (4.4)	89 (4.8)	87 (4.1)
	50	86 (4.7)	85 (3.4)	80 (4.6)	82 (4.1)	85 (5.2)	86 (3.5)	87 (4.5)	86 (3.5)
HF	5	85 (5.9)	88 (5.6)	89 (6.2)	95 (6.0)	87 (5.7)	95 (5.9)	81 (6.1)	84 (5.8)
	50	81 (5.8)	84 (5.7)	86 (5.7)	89 (5.8)	84 (6.3)	92 (5.8)	80 (6.2)	83 (5.7)
LUF	5	108 (3.7)	103 (4.6)	103 (4.1)	105 (4.0)	99 (3.4)	91 (4.3)	105 (3.8)	98 (4.4)
	50	111 (3.3)	105 (3.8)	108 (4.0)	103 (3.7)	97 (3.6)	89 (3.5)	95 (3.1)	89 (3.9)
FLF	5	110 (3.1)	102 (3.7)	82 (2.9)	82 (4.0)	109 (3.5)	95 (3.9)	79 (2.6)	81 (3.6)
	50	82 (2.5)	91 (3.2)	85 (3.4)	85 (3.8)	78 (2.7)	89 (3.6)	88 (3.5)	88 (2.8)

Note: RSD (%) in parentheses; ($n = 3$).

from 0.1 to 1.0 mg L^{-1} of each pesticide in AcN : water 1 : 1 (v/v). Also, four series of solutions corresponding to the four vegetable matrices (cucumber, zucchini, green bean, and pepper), of the same concentration of that of the previous ones but prepared in extracts of the vegetable matrices free of pesticides, dissolved in AcN : water 1 : 1 (v/v), were prepared three times.

Using the area values obtained for the pesticides in the injections of the five series of calibration solutions, we proceeded to check if differences statistically significant existed among the three replicates of each series. First, it was proven that significant differences did not exist among the residual variances of the five groups of calibration curves, and subsequently, the comparison of the slopes of the groups of replied calibration curves, in SC and in each one of the MC, was carried out.

The values obtained for the level of significance of the null hypothesis in the studies are shown in Table 4. It can be observed that significant differences (P-value $<5\%$) exist between the slopes of the calibration curves prepared in solvent and blank extract of matrix, for DFL in cucumber and FLF in cucumber and green bean.

Calculation of the Correction Function

In order to take into account the intermediate precision in the calculation of the correction function, the previous study was repeated monthly, during three additional months, using the same vegetable matrices that previously had been frozen, since this is the habitual practice in the most of the pesticide residues laboratories.

Table 4. P-value (%) obtained in the comparison of slopes of calibration lines from SC and MC for benzoylphenylureas in the different fresh matrices.

Pesticide	Cucumber	Zucchini	Green bean	Pepper
DFL	4.82[a]	16.35	14.43	18.55
TRF	36.40	60.24	46.14	38.31
HF	88.05	64.66	34.18	54.04
LUF	28.82	88.71	20.05	94.69
FLF	0.37[a]	25.86	0.05[a]	23.79

[a] Significant differences exist between the slopes of the both calibration curves with a level of confidence of 95%. ($\alpha = 5\%$).

The obtained results showed that the matrix effect was presented in a random way. This fact can be due to the substances present in the vegetable matrix not being stable under the freezing conditions. Therefore, it could be thought that the freezing affects the vegetable matrix; therefore it does not seem advisable to freeze the vegetable matrix free of pesticides to be used to obtain the calibration curves.

For it, the study was repeated using fresh matrix of different origin every time. In Table 5 are shown the values of P-value (%) obtained for the five pesticides and the four matrices along the three months. It can be seen that the behaviour through the time is according to the one obtained the first month.

For those cases, in which, the existence of matrix effect was demonstrated, a correction function was calculated ($C_M = A + BC_S$). In Table 6 are shown the obtained correction coefficients for the pesticides and matrices for which the presence of matrix effect have been proved.

Table 5. P-value (%) obtained in the comparison of slopes of calibration lines from SC and MC for benzoylphenylureas in the different fresh matrices along three months.

	Pesticide				
	DFL	TRF	HF	LUF	FLF
Cucumber					
1 month	4.97[a]	18.90	43.48	36.91	2.34[a]
2 months	0.00[a]	10.95	18.78	27.12	0.00[a]
3 months	0.23[a]	12.43	15.34	25.67	0.54[a]
Zucchini					
1 month	13.13	42.73	53.68	86.38	34.34
2 months	14.00	76.52	69.13	21.51	49.57
3 months	12.35	56.73	40.56	34.89	56.37
Green bean					
1 month	15.41	35.32	31.62	18.47	0.00[a]
2 months	52.46	86.47	51.38	94.32	0.00[a]
3 months	32.98	67.23	45.92	89.36	0.00[a]
Pepper					
1 month	16.22	52.65	47.96	86.84	28.94
2 months	12.99	33.27	93.82	22.83	55.91
3 months	19.35	49.65	75.21	45.11	39.56

[a] Significant differences exist between the slopes of both the calibration curves with a level of confidence of 95% ($\alpha = 5\%$).

Table 6. Values of the coefficients *A* and *B* for the benzoylphenylureas with matrix effect.

	Cucumber		Green bean	
Pesticide	*A*	*B*	*A*	B
DFL	−0.0280	1.0146	—	—
FLF	−0.0241	1.1456	−0.0096	1.0443

Checking the Performance of the Correction Function

In order to prove that the calculated correction functions corrected the effect due to the presence of the vegetable matrix in the quantification of real samples fortified at two levels of concentrations (0.01 and 0.1 $\mathrm{mg\,kg^{-1}}$), the previously calculated functions were applied to the concentrations obtained in the recovery study carried out quantified with standards solutions prepared in solvent. The results obtained in this way were compared with those obtained when the quantification was carried out with calibration curves built with standard solutions prepared in the corresponding vegetable matrix, and both of them are shown in Table 7. It can be observed that recoveries obtained by both methodologies are similar. So, this proves the utility of these correction functions to eliminate the effect caused by the vegetable matrix, avoiding the use of calibration curves prepared in matrix extracts for the quantification of these pesticides in real samples, with the advantages that it supposes for the saving of time and cost.

Table 7. Recovery percentages (%) obtained using calibration curves built with standard solutions prepared in solvent and in blank matrix extracts and applying the correction function.

Pesticide (matrix)	Concentration ($\mu g \cdot kg^{-1}$)	Recovery (%)		
		Solvent	Matrix	Correction
DFL (Cucumber)	5	130.0	105.0	103.8
	50	112.0	97.0	97.6
FLF (Cucumber)	5	110.0	102.0	101.9
	50	82.0	91.0	91.5
FLF (Green bean)	5	109.0	95.0	94.8
	50	78.0	89.0	88.8

CONCLUSIONS

Analysis of covariance (ANCOVA) has been applied as a statistical tool to establish the presence of matrix effect in the analysis of five benzoylphenylurea insecticides in different vegetables.

A mathematical strategy has been applied to obtain a correction function that allows eliminating the matrix effect in the quantification of benzoylphenylureas in real samples, taking into account the intermediate precision.

This function has been used to correct the recoveries obtained in the study of recovery of the pesticides using calibration curves prepared in solvent, finding results that are comparable to those obtained quantified with calibration curves prepared in blank extracts of matrix.

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