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Photochemically induced fluorescence coupled to second-order multivariate calibration as analytical tool for determining imidacloprid in honeybees

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ABSTRACT

This paper presents a method for the determination of imidacloprid in honeybees based on the measurement of excitation-emission spectra of photo-induced fluorescence (PIF-EEMs) associated to unfolded partial least squares coupled to residual bilinearization (U-PLS/RBL) algorithm. As a preliminary step, matrix solid phase dispersion (MSPD) using C18 as dispersant, combined on-line with a solid phase extraction (SPE) clean-up on graphitized carbon-amino propyl silica phase was applied to diminish the interferences presents in samples. A previous study on the photochemical induction of fluorescence of imidacloprid in presence of bee matrix was included. The second order advantage achieved with RBL permitted the determination of imidacloprid in the presence of interferences present in samples (unexpected compounds of bees), which also shows photo-induced or native fluorescence. The LOD was 20 μg kg^{-1} (2.5 ng per bee; four bees treated), which is suitable for detecting imidacloprid at the oral LD50 for the insect. The predicted U-PLS/RBL concentrations compared favorably with those measured using high-performance liquid chromatography with diode array detection. The PIF-EEMs coupled to U-PLS/RBL was applied for the determination of imidacloprid in honeybees collected in field hives. The work demonstrates the feasibility of the determination of imidacloprid in a highly complex sample matrix as bee through photochemically induced fluorescence spectroscopy coupled to multivariate calibration.

1. Introduction

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine] belongs to the neonicotinoid insecticides, a new group of pesticides with properties that allow for their systemic distribution within plants after being absorbed by the leaves or roots. The major modes of application of these compounds are spraying and seed dressing, especially to control pests in crops, such as cereals, soybeans, corn and several fruits and vegetables. On the other hand, bees are the prominent and economically most important group of pollinators worldwide. The decline of pollinating species may lead to a parallel decrease of plant species or vice versa. More specifically, there is a great concern about the decline of honeybee (*Apis mellifera*) and the worldwide acute depopulation of hives called "Colony Collapse Disorder (CCD)", first named in 2007 [1]. In recent years it has been postulated that neonicotinoid pesticides could be a trigger of CCD. Some authors have done a wide overview on the effect of neonicotinoids on bees and their relation with CCD [2–4]. In the case of imidacloprid, honeybees that were feed with corn syrup containing this neonicotinoid showed symptoms consistent to CCD [5,6]; and field-realistic levels of imidacloprid reduce colony growth and queen production of bumble bee *Bombus terrestris*[7]. Thus, the use of these pesticides in agriculture has indubitable repercussions on the environment which has become a serious environmental concern.

The most relevant measures of exposure of bee to imidacloprid are the concentrations in bee-collected plant materials, such as pollen and bee products like bee bread, honey and beeswax, and in the bees themselves. The determination of pesticides in bees is difficult by the complexity of the matrix, the low levels of the analytes and the variety of interferents potentially present. Usually this entails the use of some sample pre-treatment as an essential part of the analytical process and a subsequent chromatographic determination [8,9]. Accordingly, the majority of the analyses of imidacloprid in bees involve an extraction followed by a clean-up step before a chromatographic determination using high-performance liquid chromatography with mass spectrometric (HPLC-MS) or tandem mass spectrometric (HPLC-MS/MS)

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detection [10–13]. The limits of detection reported ranged 0.2–4 μg kg^{-1}. Liquid chromatography with a diode array detector (HPLC-DAD) has also been used for the determination of imidacloprid in single bees with limits of detection of 7 ng per bee (approximately 50 μg kg^{-1}) [8] and 50 μg kg^{-1}[14]. On the other hand, the extraction and/or clean-up methods principally used to determine imidacloprid in bees are the solid-liquid extraction with acetonitrile, partition with hexane and a subsequent solid-phase extraction (SPE) with florisil [8] the QuEChERS method [8,12,13] and matrix solid phase dispersion (MSPD) [9,10].

Alternative methods based on the fluorimetry of a photoproduct of imidacloprid produced after the UV irradiation of an aqueous imidacloprid solution have been proposed for water analysis [15–18]. In aqueous media, imidacloprid does not exhibit native fluorescence; however, its irradiation with UV light results in a fluorescent signal [15]. However, the relevance of the fluorimetric methods has been limited by their lack of selectivity, especially when chemically similar compounds must be analyzed in a complex matrix. One approach to improve the analytical selectivity in this matrix would be the use of excitation-emission fluorescence measurements (three-way data set), in conjunction with different chemometric algorithms as parallel factor analysis (PARAFAC) or unfolded partial least square with residual bilinearization (U-PLS/RBL) to build a second-order calibration method. These methods permit the determination of the compounds of interest, without the use of chromatography, in a sample with overlapping spectral interferences that are not included in the calibration set (known as the second-order advantage) [19]. In a previous work we reported the use of photochemically induced fluorescence excitation-emission matrices (PIF-EEMs) coupled to PARAFAC and U-PLS/RBL for the determination of imidacloprid in water samples [20]. However, to our knowledge there are no available reports on the determination of imidacloprid in bee samples through photochemically induced fluorescence spectroscopy coupled to multivariate calibration. In this work U-PLS/RBL was applied to determine imidacloprid in bee samples using PIF-EEMs in presence of interferences from the matrix, associated to MSPD-SPE on graphitized carbon-amino propyl silica phase on-line as sample preparation step. At this point it is worth mentioning that bees is a very complex matrix, which represent an analytical challenge that we have solved presenting a new treatment of the sample and an additional study to assess whether the photo-induced fluorescence of imidacloprid is comparable in solvent and in matrix in order to validate the experimental conditions. The predicted U-PLS/RBL concentrations were compared with those obtained using high-performance liquid chromatography (HPLC) with UV–vis detection. The method was applied for the determination of imidacloprid in honeybees collected in field hives located in an area of great agricultural activity (maize and fruit trees) located in the central region of Chile.

2. Theory

2.1. UPLS/RBL

The U-PLS method is a variant of the classical partial least squares (PLS) that was proposed for second-order data where three-way data are unfolded into vectors before two-way PLS calibration. If the calibration was exact, the regression coefficients, v, could be employed to estimate the analyte concentrations in an unknown specimen using eq. (1),

$$y_u = t_u^T v \quad (1)$$

where t_u is the test sample score, which is obtained by projection of the vectorized (unfolded) data for the test sample $\mathbf{X}_u$ onto the space of the *A* latent factors, as indicated in eq. (2),

$$t_u = (\mathbf{W}^T\mathbf{P})^{-1}\mathbf{W}^T\, vec(\mathbf{X_u}) \quad (2)$$

where **P** and **W** are the matrix of loadings and weight loadings, respectively; vec(.) implies the vectorization operator and T the transposition operator.

When unexpected constituents occur in $\mathbf{X_u}$, the sample scores given by Eq. (1) are unsuitable for analyte prediction and the U-PLS method must be coupled to RBL to achieve the second-order advantage. RBL is a post-calibration procedure that is based on principal component analysis (PCA) to model the presence of unexpected constituents in a sample [21,22].

The RBL procedure consists maintaining the matrix of loadings **P** constant at the calibration values and varying t_u to minimize the norm of residual error. The standard deviation (s_{RBL}) of the residuals can be used as a measure of the goodness of fit (GOF) for the RBL procedure and to estimate the number of unexpected constituents according to Bortolato et al. [22]. In this approach the s_{RBL} is assumed to stabilize at a value compatible with the instrumental noise when the correct value of RBL components is reached [21,22].

3. Experimental

3.1. Reagents and solutions

Imidacloprid of high purity grade (99%) was obtained from Sigma-Aldrich (St. Louis. MO, USA). NaOH and Na_2HPO_4 were of analytical purity grade and obtained from Merck (Darmstadt, Germany). Hexane, dichloromethane, acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany). Supelclean® ENVI-Carb/aminopropyl-functionalized silica (500 mg/500 mg, 6cc) solid phase extraction (SPE) cartridges and Supelclean® ENVI-18 bulk packing for matrix solid phase dispersion (MSPD) were provided by Sigma-Aldrich (St. Louis. MO, USA).

Stock solutions of pure analyte (1000 μg mL^{-1}) and diluted solutions were prepared in acetonitrile. The stock solution was stored in amber vials at 4 °C in the dark. Under these conditions, the solution was stable for almost two months.

3.2. Apparatus and software

A Varian Cary-Eclipse luminescence spectrometer (Mulgrave, Australia) equipped with a xenon flash lamp was used to obtain excitation–emission fluorescent measurements. A Starna (Essex, England) quartz cell with a 700-μl inner volume and a 10×2-mm light path was used. The classic fluorescence spectra were recorded at λ_{exc} of 345 nm in the λ_{em} range of 365–700 nm every 2 nm at a scanning rate of 600 nm min^{-1} and 10 nm for emission slit. The EEMs were recorded in the λ_{exc} ranges of 220–400 nm every 5 nm and λ_{em} of 324–550 nm every 2 nm. The widths of the excitation and emission slits were 10 and 20 nm, respectively. The spectra were saved in ASCII format and transferred to a computer for subsequent manipulation. All chemometric computation and routines were implemented in Matlab v.7.6 (Mathworks, Natwick, MA). The routine for data pre-treatment used to eliminate Rayleigh and Raman scattering peaks from the EEMs was taken from Zepp et al., 2004 [23]. The routines used for PARAFAC [24] and U-PLS/RBL [25] are available on the internet. These algorithms were implemented using the graphical interface of the MVC2 toolbox, which is also available on the internet [26].

In order to visualize the photo-degradation of imidacloprid versus time of irradiation, the UV–vis spectra at different irradiation times were obtained. For this aim, the UV–vis absorbance spectra were recorded with an Agilet Cary 8453 spectrophotometer equipped with a photo-diode array, wavelength range from 200 to 500 nm with a 1 nm interval.

The HPLC-DAD analysis was carried out as reference method. The analyses were performed on a liquid chromatograph equipped with a Waters 600 HPLC pump, a Waters 996 diode array detector and a Waters 717 auto sampler (Milford, MA, USA). The column was an

Eclipse XDB C18 (150×4.6-mm ID, 5-μm particle size) from Agilent (Santa Clara, USA) the temperature was maintained at 35 °C during the analysis. The mobile phase was a mixture of acetonitrile (A) and water (B) at a flow rate of 1.3 mL min^{-1}. The following gradient program was used: 0–7 min linear gradient from 10 to 60% A; 7–10 min 60% A isocratic; 11–14 min linear gradient 60 to 10% A; 15–18 min 10% A isocratic. This program was optimized and used for the separation of imidacloprid, from the co-extracted compounds of the matrix. A volume of 20 μL was injected. Detection at 270 nm was used for the quantification of imidacloprid.

3.3. Sample collection

Honeybee samples used for spiking and validation studies were obtained during autumn of south hemisphere (April 2015) from hives localized in the experimental apiary on the laboratory located at the Instituto de Ciencias, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile. Honeybees collected in November 2015 from nine apiaries (one colony for each apiary) located in the vicinity of the towns of Rengo (3 apiaries; 34°28'41.38"S; 70°44'36.21"W), Chimbarongo (2 apiaries; 34°40'1.36"S; 71° 0'28.39"W), Codegua (2 apiaries, 33°59'55.24"S; 70°38'50.39"W) and Peumo (2 apiaries; 34°23'0.71"S; 71°10'58.96"W) in the O'Higgins region of Chile were also analyzed. Each apiary is seen as a flock and a single colony as an individual. 50 foraging bees were collected during the morning at the entrance to the hive. The insects were frozen and stored at −18 °C until the analysis.

3.4. MSPD-SPE of honeybees

Four bees (approximately 0.5 g) were placed into a mortar, 0.4 g of sodium acetate were added and gently blended with 1 g of Supelclean® ENVI-18 for 5 min using a pestle to obtain a semi-dry homogeneous mixture. During this procedure the sample was spraying with 0.2 ml of spiking solution in acetonitrile. This mixture was introduced into a Supelclean® ENVI-Carb/NH_2 cartridge and covered with a plug of silanized glass wood at the top. The prepared column was prewashed with 10 ml of hexane and the eluate was discarded. The analyte was eluted with 10 ml of a dichloromethane:methanol solution (90:10, v/v) that was allowed to elute dropwise by applying a slight vacuum. The eluent was collected and evaporated to dryness under a stream of nitrogen at 45 °C. The extract was reconstituted with 1.0 ml of 0.01 M phosphate buffer (pH 11.5)/acetonitrile (80:20) for irradiation with UV light or in 1.0 ml of acetonitrile for a HPLC analysis.

3.5. Photo-induced fluorescence and determination of imidacloprid

The UV irradiation was performed using a Vilbert Lourmat lamp (France, model VL-115.G) that operates a tube of 15 W with 254 nm as the spectral line (model T-15.C). A box with an internal coating of aluminum that permitted the maximum reflectance of UV light was placed over the lamp. Due to the short irradiation time applied to the samples, no cooling device was needed, and all experiments were performed at room temperature (25 °C). A 700-μl aliquot obtained after the extraction procedures described above or a phosphate buffer containing imidacloprid (calibration samples) were transferred to a quartz cell with a 700-μl inner volume. The cell was covered with its cap and placed 1 cm from the UV tube of the lamp and irradiated for 25 seconds. Subsequently, the cell was shaken gently by hand and placed in the luminescence spectrometer to obtain the EEMs.

3.6. Recording of UV–vis spectra during photo-degradation of imidacloprid

The UV-visible spectra were also recorded during the photoproduct formation in phosphate buffer (irradiation time ranged 0–90 seconds) and in presence of the matrix obtained after treatment of bees (irradiation time ranged 0–125 seconds). In order to record the spectra in one extract obtained after the treatment of the sample (total volume 1 ml) this was spiked with imidacloprid at 5 or 10 μg mL^{-1} and subjected to irradiation as described in our previous work [20]. Briefly, a 100-μl aliquot of the extract was aspirated into a 100-μl disposable glass micropipette (Brand, Wertheim, Germany). Then, one end of the micropipette was sealed with hematocrit sealing wax (Brand, Wertheim, Germany) and the other with Parafilms®. The micropipette was placed 1 cm from the UV tube of the lamp and irradiated. Subsequently, the Parafilm® was removed and the micropipette was cut on the side of the wax using a silica capillary column cutter. The sample was transferred to a quartz cell with a100-μl inner volume (Hellma, Müllheim, Baden, Germany) to obtain the UV-visible spectra.

3.7. Calibration and validation set samples

A calibration set of 9 samples in duplicate was prepared from the diluted solutions at concentrations of 0.02; 0.03; 0.04; 0.05; 0.10; 0.15; 0.20, 0.25 and 0.35 μg mL^{-1} of imidacloprid in phosphate buffer. With the aim of evaluating the predictive capacity of the calibration model in the presence of unexpected constituents, a validation set of 11 samples was prepared according to the sample preparation procedure for bee described above and spiked in the final step with different volumes of a diluted solution of imidacloprid. All the samples were irradiated, their EEMs were obtained and the data were subjected to second-order data analysis. Table 1 shows the concentration of the validation set.

3.8. Test sample set

To test the recovery and predictive capacity of the proposed method, a test set was prepared by spiking these samples with imidacloprid at the beginning of the process with the concentrations showed in Table 2 expressed in μg kg^{-1}. Subsequently, these samples were processed using the sample preparation procedure described above, irradiated and their EEMs were read and subjected to second-order data analysis. Additional recovery and precision experiments were performed by repeated analysis of blank samples (n=3) spiked with imidacloprid at LOQ, 2×LOQ and 4×LOQ levels. These test set was also analyzed using HPLC-DAD as a reference method for comparing purposes.

Table 1
Concentrations in μg mL^{-1} of imidacloprid in the validation set samples and predictions using U-PLS/RBL.

Sample	Added (μg mL^{-1})	U-PLS/RBL (μg mL^{-1})
V1	0.028	0.028
V2	0.035	0.030
V3	0.038	0.033
V4	0.045	0.048
V5	0.055	0.055
V6	0.060	0.062
V7	0.070	0.072
V8	0.080	0.072
V9	0.130	0.132
V10	0.165	0.172
V11	0.180	0.223
RMSEP (μg mL^{-1})[a]	–	0.013
REP (%)[b]	–	13

[a] Comparison with the added concentrations.
[b] Relative error of prediction, REP=100×RMSEP/c_{mean} where c_{mean} is the mean calibration concentration.

Table 2
Predictions of imidacloprid in the test set using U-PLS/RBL and values determined by HPLC-DAD as a reference method. Figures of merit obtained for the two methods.

Sample	Added ($\mu g\ kg^{-1}$)	HPLC-DAD ($\mu g\ kg^{-1}$)	U-PLS/RBL ($\mu g\ kg^{-1}$)
T1	56.0	non detected	44.0 (79)
T2	70.0	78.4 (111)[a]	88.0 (126)
T3	76.0	110.4 (145)	108.8 (142)
T4	90.0	96.0 (107)	84.0 (93)
T5	110	118.4 (107)	82.4 (75)
T6	120	116.0 (97)	132.0 (110)
T7	140	130.4 (93)	160.0 (114)
T8	260	194.4 (75)	214.4 (82)
T9	330	268.0 (81)	240.0 (73)
T10	360	384.0 (107)	372.0 (103)
T11	560	480.0 (86)	454.4 (81)
Mean recovery (%)	–	101	98
RMSEP ($\mu g\ kg^{-1}$) [b]	–	–	21
REP (%)	–	–	11
γ^{-1}[c]	–	13	6
LOD=3.3γ^{-1} ($\mu g\ kg^{-1}$)	–	42	20
LOD in ng per bee[d]	–	5.3	2.5

[a] () Values expressed in recoveries %.
[b] Comparison with values obtained by HPLC-DAD.
[c] Inverse of the analytical sensitivity (γ), γ^{-1}=sx/SENn where sx is the instrumental noise and SENn is the sensitivity. The sx and SENn values are averages of the values corresponding to 11 validation samples.
[d] LOD in ng per bee calculated considering four bees treated with a mean weight of 0.125 g.

4. Results and discussion

4.1. Preliminary studies on photochemical induction of fluorescence of imidacloprid

A solution of imidacloprid at 0.25 $\mu g\ mL^{-1}$ in phosphate buffer/acetonitrile (80:20) and in the bee extract obtained after the MSPD-SPE procedure were irradiated during 5–90 s and the fluorescence spectra were recorded at λ_{exc} of 345 nm in the λ_{em} range of 365–650 nm. As can be observed in Fig. 1A, the spectra of fluorescence of imidacloprid in phosphate buffer/acetonitrile show a maximal response at λ_{em} of 410. The curve of fluorescence recorded at this wavelength with respect to time exhibited an increase of the response (Fig. 1B), corresponding to the formation of the fluorescent product, and then, a decrease in the signal due to the photodegradation of this product. In phosphate buffer/acetonitrile the maximal signals were obtained in the 20–35 s range. However, in presence of the bee matrix the response showed a continuous increase to 60 seconds and then was constant. This continuous increasing in the fluorescent was attributed to fluorescent compounds presents in the matrix with similar emission spectra at λ_{exc} of 355 nm rather that of the fluorescent product of imidacloprid.

The UV-visible spectra recorded during the photoproduct formation, both in buffer (0–90 seconds) and in presence of the matrix obtained after treatment of bees (0 to 125 seconds), are presented in Fig. 2A and B, respectively. As is shown, the imidacloprid spectrum possess two absorbance maxima at 210 and 270 nm, and upon lighting the absorbance intensity at the second peak maxima is decreased. In addition, an increase in the absorbance with a maximum around 350 nm is observed. These changes in the absorbance spectra of imidacloprid can be attributed to the conversion to its degradation product(s). A similar behavior was observed in presence of the matrix; although in this case the spectra showed a slight distortion (Fig. 2 B).

According to these results 25 s was selected as the irradiation time. Furthermore, the stability of the photoproduct over time after the irradiation and a possible photo bleaching was evaluated by recording the fluorescence from 0 to 45 minutes every 5 minutes after the irradiation. The fluorescence intensities did not have significant variations in this period, which demonstrated its stability of the photoproduct over time and the absence of degradation during the measurement of EEM. Anyway, all the EEMs were obtained immediately after the irradiation of the samples. Fig. 3A–C shows the PIF-EEMs corresponding to a calibration sample containing imidacloprid at 0.25 $\mu g\ mL^{-1}$, a bee extract obtained after the MSPD-SPE procedure and a bee extract spiked with imidacloprid at 0.25 $\mu g\ mL^{-1}$.

As see in Fig. 3A, the imidacloprid photoproduct exhibits fluorescence with a maximum intensity at λ_{em} 410–415 nm and λ_{exc} 345–355 nm. The anomalous signal observed at excitation wavelengths lower than 250 nm in the calibration sample may be related to some impurities of the reagents used and/or light scattering. However, due to its position in the spectral range, these signals do not cause interference in the analysis. A fluorescence signal for the bee extract (Fig. 3B) is seen with excitation below 300 nm and emission ranged 324–360 nm and also a broad region with excitations between 260 and 360 nm, and emission ranged 370–500 nm. The first one can be arise from fluorescent amino acids, while the second one can be attributed to ATP, NADH and/or vitamins [27]. This emission region shows a meaningful overlapping with the imidacloprid photoproduct (Fig. 3C), so that the determination of the pesticide in the bee extracts requires the use of the second-order advantage achieved using the U-PLS/RBL algorithm.

4.2. Calibration and validation set analysis

The validation set was initially analyzed by PARAFAC. This algorithm was applied to the three-way data arrays constructed by combining the data matrices for each validation sample with those of the set of calibration samples. The model fits under the non-negativity constraint for loading of the excitation and emission mode and the

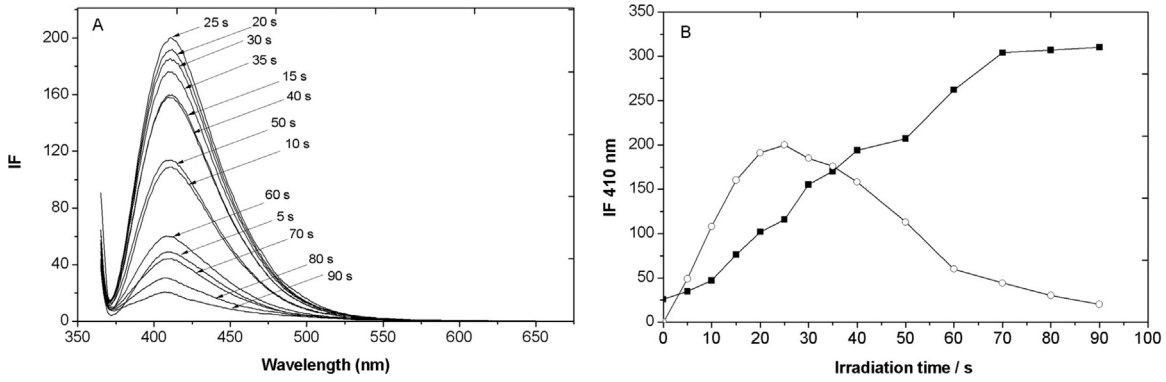

Fig. 1. Fluorescence spectra (λ_{exc} 345 nm) of a solution of imidacloprid at 0.25 $\mu g\ mL^{-1}$ in phosphate buffer/acetonitrile (80:20) obtained after irradiation during 5–90 s (A). Fluorescence recorded at 410 nm with respect to time for imidacloprid in buffer/acetonitrile (empty circles) and in the bee extract obtained after the MSPD-SPE procedure (full square) (B).

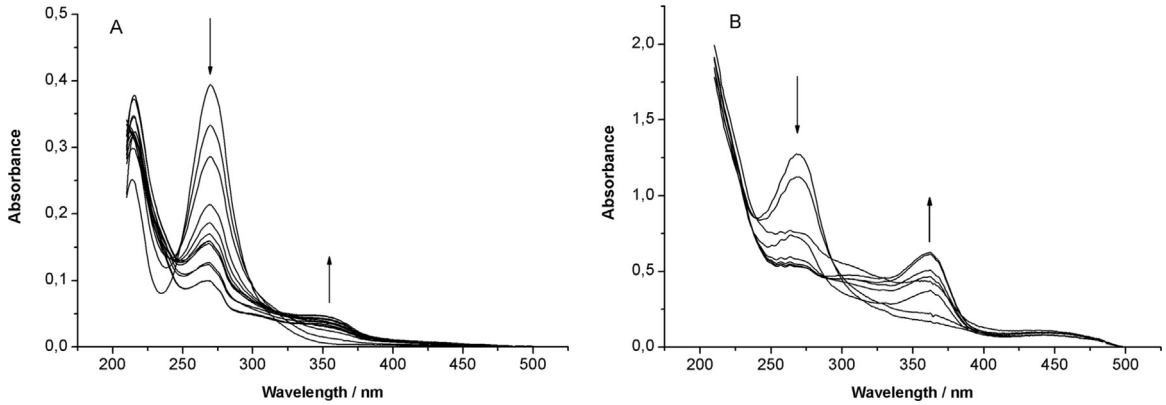

Fig. 2. UV-visible spectra of a solution of imidacloprid at 5 μg mL^{-1} in phosphate buffer/acetonitrile (80:20) obtained after irradiation (0–90 seconds) (A) and at 10 μg mL^{-1} in presence of the extract obtained after treatment of bees (0–125 seconds) (B).

initialization performed via a direct tri-linear decomposition (DTLD) of the three-way array. Two components were extracted for each validation samples with a core consistency of 90–100%; the first one corresponds to imidacloprid (by comparison with those for an irradiated standard solution) and the second one to the matrix retrieved as a unique fluorescent component. However, PARAFAC not predicted accurately the concentration of imidacloprid in the validation samples, particularly with overestimation in those samples whit added concentration lower or equal than 0.06 μg mL^{-1}. On the other hand, U-PLS was first applied to the EEMs data of calibration set unfolded into vectors. Later RBL factors were included to achieve the second order advantage in the analysis of the validation set. A reduced spectral range (280–380 nm for the λ_{exc} and 340–550 nm for the λ_{em}.) was chosen according to preliminary experiments. The selection of the optimum number of factors was performed using the cross-validation method described by Haaland and Thomas [28] over only the calibration set by calculating the ratio $F(A)=PRESS(A<A^*)/PRESS(A^*)$, where *PRESS* is the predicted error sum of squares, defined as $PRESS=\Sigma(y_{nominal}-y_{predicted})^2$; A is a trial number of factors; and A^* corresponds to the minimum *PRESS*. The number of optimum factors was selected as the number leading to a probability of less than 75% and F > 1. In this case, the resulting number of components was four. Unlike PARAFAC, these latent variables do not have any physical interpretation. While the latent variables should be close to the known or suspected number of chemically responsive constituent in the calibration set, instrumental variations from sample to sample, such as baseline drifts, nonlinearities, interactions, etc., may contribute with additional variables [29]. On the other hand, the number of optimum RBL factors for each validation sample, estimated according to the procedure described by Bortolato et al. [22], ranged one to four. The principal analytical characteristics for the determination of imidacloprid in the validation samples using U-PLS/RBL are shown in Table 1. This algorithm yielded good predictions with a relative error (REP) of 13%; considering the complexity of the matrix and the low concentrations of imidacloprid. U-PLS/RBL has been described to provide better figures of merit than their competitors because of their flexibility by transforming the original matrix structure of each sample data into a vector [21,30].

4.3. Recovery of the MSPD-SPE method and test set analysis

The high number of interference present in the bees makes this matrix highly complex, being mandatory the treatment of samples prior to analysis by fluorescence spectroscopy. Thus, the pre-treatment step based on MSPD-SPE was optimized to diminish these interferences and improve the sensitivity for the quantification of imidacloprid. The test set was subjected to the extraction procedure described previously and subsequently analyzed using HPLC-DAD under the optimized conditions. As example, Fig. 4A and B shows the chromatogram and contour plot of the PIF-EEM obtained for the test sample T5, respectively. On the other hand, in Table 2 are summarized the recoveries of imidacloprid in the test set after application of MSPD-SPE and HPLC-DAD analysis with a mean recovery of 101%.

The photochemically induced fluorescence EEM data were generated for the test set and analyzed using U-PLS/RBL. A great variability in the EEM obtained was observed, and the fluorescence intensity of the interference from the matrix was particularly high in some cases, being practically hidden the fluorescence of the imidaclopird photo-

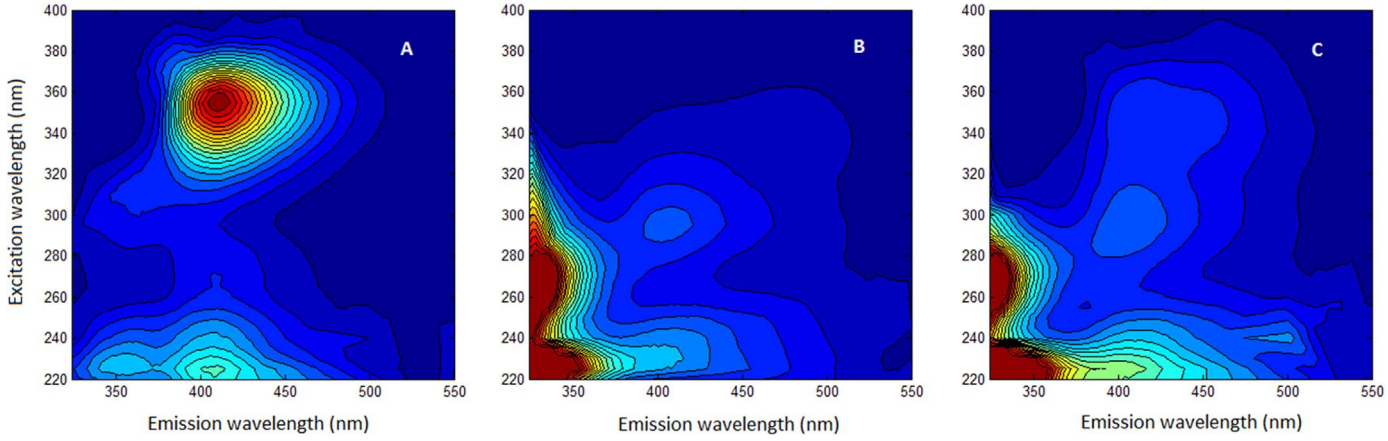

Fig. 3. Contour plots of PIF-EEM for a calibration sample where only imidacloprid is present at 0.25 μg mL^{-1} (A); a bee extract (B) and a bee extract spiked with imidacloprid at 0.25 μg mL^{-1} (C).

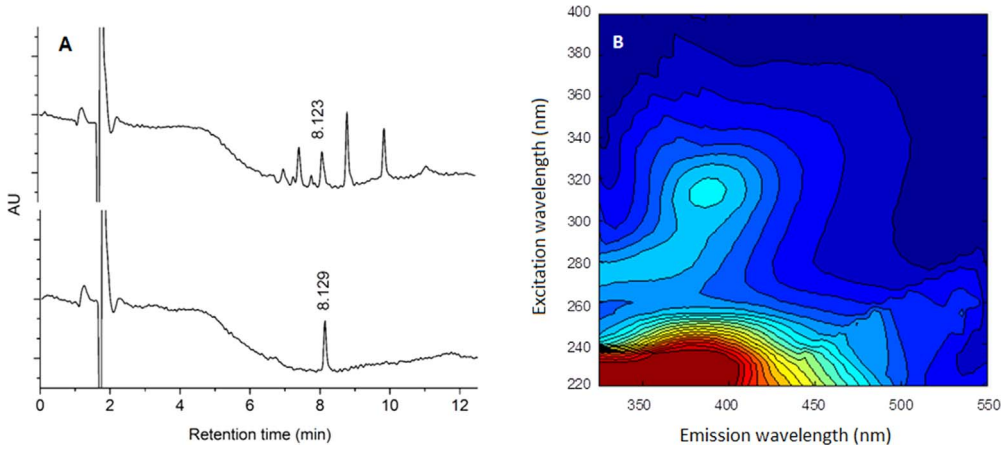

Fig. 4. HPLC-DAD chromatogram obtained for the test sample T5 and a standard of imidacloprid at 0.1 μg/ml (A); contour plots of PIF-EEM for the sample T5 (B).

product. As example the Fig. 4B displays the contour plot of the PIF-EEM for test sample T5 where the fluorescence of the imidacloprid photoproduct is totally overlapped with that of the matrix.

As for the validation samples, one to four RBL components were required for the analysis of the test samples. The prediction results corresponding to the application of U-PLS/RBL to the test samples subjected to the extraction procedure are listed in Table 2. In addition, Fig. 5 displays a comparison of these values with those obtained using HPLC as a reference method. A slight shot pattern was observed in the regression of the values obtained by HPLC-DAD vs. predicted by UPLS which could be associated with a factor not considered in the model U-PLS (unexplained background). However, there was not studentized residual greater than 2.5 (calculated by externally studentized residuals) in the residual versus predicted for the fitted line [31]. On the other hand, the theoretical (1,0) point was included within the elliptical joint region. Consequently, no significant differences between the concentrations predicted by the algorithm and the concentrations obtained from HPLC were observed. The root means square errors of prediction (RMSEP), which is a method for expressing the average error made in predicting the analyte concentration with respect to the reference value delivered using HPLC, was 21 μg kg^{-1}, corresponding to 11% relative error of prediction. On the other hand, the limit of detection of the proposed method was 20 μg kg^{-1} (2.5 ng per bee considering four bees treated with a mean weight of 0.125 g); which is suitable for detecting imidacloprid at the oral LD50 for the insect [2]. The LOD was obtained according to the approach reported by Allegrini and Olivieri (2014) [32], considering a value of uncertainty for the instrumental signal and the concentration of 2 and 0.001, respectively. Additional recovery and precision experiments were performed by repeated analysis (n=3) through MSPD-SPE, U-PLS/RBL and HPLC-DAD of spiked blank samples at LOQ, 2×LOQ and 4×LOQ levels (60, 120 and 240 μg kg^{-1}). Results are summarized in Table 3.The recovery for U-PLS/RBL ranged from 82 to 92% with RSD values lower or equal than 6.3%. The mean recovery at the two higher levels in μg kg^{-1} for U-PLS/RBL and HPLC-DAD were compared using a paired *t*-test, and no significant differences between the methods were observed (*p*-value > 0.05). Therefore, based on the obtained results, the proposed method using EEMs coupled with U-PLS/RBL is comparable with the reference HPLC-DAD method.

4.4. Analysis of samples from field hives

The method was applied in the analysis of bees collected from 9 apiaries in the region of O'Higgins in Chile. First, MSPD-SPE was applied to these samples, and subsequently U-PLS/RBL was used to process the PIF-EEMs three-way data and predict the imidacloprid concentrations. No detectable residues of the target pesticides were found in the 9 analyzed samples for the sampling campaign. In general, these samples showed PIF-EEMs with low fluorescence, indicating the effectiveness to eliminate interfering in the pretreatment of samples. These samples were also analyzed using HPLC-DAD obtaining clean chromatograms and confirming the absence of imidacloprid residues.

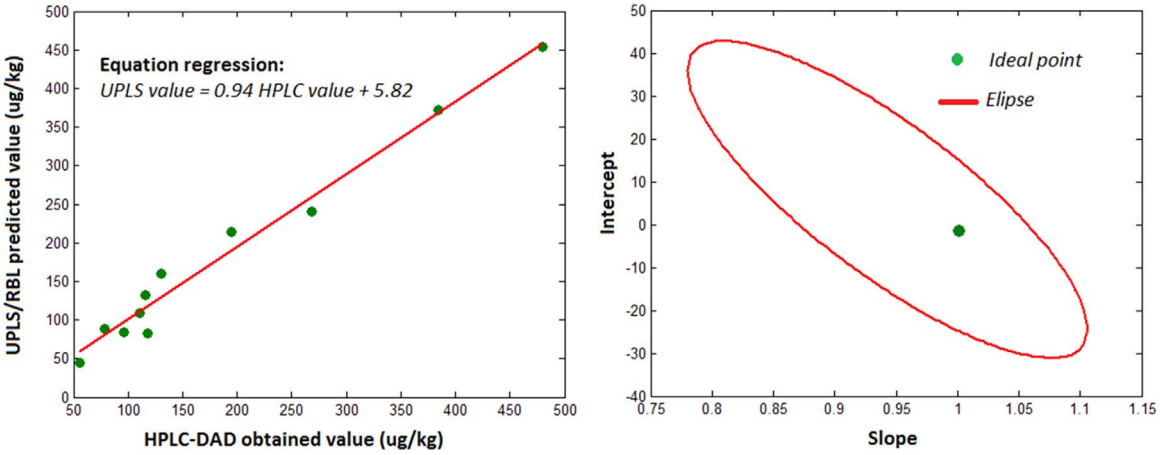

Fig. 5. Plots for imidacloprid predicted concentration with U-PLS/RBL in the test samples as a function of those obtained using HPLC and the corresponding elliptical joint region (at the 95% confidence level).

Table 3
Recovery and precision study for the imidacloprid determination in spiked bee samples (n=3) using MSPD-SPE associated to U-PLS/RBL or HPLC-DAD.

	MSPD-SPE/U-PLS/RBL			MSPD-SPE/HPLC-DAD			
Added (μg kg^{-1})	μg kg^{-1}	%	RSD	μg kg^{-1}	%	RSD	*p*-value[a]
60	49	82	2.4	–[b]	–	–	–
120	110	92	6.3	117	98	2.0	0.157
240	201	84	5.1	225	94	7.2	0.089

[a] *p*-value for a mean *t* test with (n_1+n_2–2) degrees of freedom and equal variances (*F* test).
[b] Spiked concentration lower than the limit of quantification of MSPD-SPE/HPLC-DAD method.

5. Conclusions

In alkaline aqueous medium, photo-degradation of imidacloprid is rapid and leads to the formation of a fluorescent photoproduct. The photochemically induced fluorescence of this compound coupled to U-PLS/RBL permitted the determination of imidacloprid in honeybees by achieving the second-order advantage. MSPD-SPE was applied as a sample preparation step to diminish the interferences and lower the limit of detection. Thus, although U-PLS/RBL has an outstanding prediction capability, it requires a complementary treatment of sample to face highly complex matrices. The values obtained after applying this extraction method and analysis using U-PLS/RBL do not differ significantly from those obtained using HPLC analysis. Thus, MSPD-SPE-PIF-EEMs, in conjunction with U-PLS/RBL, have been shown to be adequate for the control of the presence of imidacloprid in bees at the ng per bee level. In this sense, the work demonstrates the feasibility of the determination of imidacloprid in bee samples through photochemically induced fluorescence spectroscopy coupled to multivariate calibration.

Conflict of interest statement

The author does not have any conflict of interest to declare.

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