Original Paper

Solid-phase microextraction for the determination of benzoylureas in orange juice using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection

A solid-phase microextraction (SPME) method has been developed for the determination of six benzoylureas (diflubenzuron, triflumuron, hexaflumuron, teflubenzuron, lufenuron, and flufenoxuron) in natural orange juice based on the direct immersion mode of a 60 μm polydimethylsiloxane/divinylbenzene fiber. An orange juice was obtained from blended, homogenized, and diluted ecological natural orange juice samples. An aliquot of 3 mL of a spiked sample was extracted under optimum SPME conditions. The determination of benzoylureas was carried out using HPLC combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection. The limits of quantification obtained in matrix were within the range of 0.02 to 0.04 mg/kg and these limits are lower than the maximum residue levels established in Spanish regulations for all pesticides in this study. Recoveries in juice samples ranged between 85 and 110% and relative standard deviations between 1.8 and 7.4%.

Keywords: Benzoylureas / LC separation / Orange / Photochemically induced fluorescence / Solid phase microextraction

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1 Introduction

Many countries have given a high priority to the monitoring and control of pesticide residues on treated crops which are controlled by the maximum residue levels (MRLs) established by each country. For this purpose, methods have been developed and implemented as a part of regulatory programs, in order to detect residues which exceed MRLs.

The determination of pesticides in environmental matrices is usually accomplished by chromatographic techniques and involves many preliminary steps including sampling, extraction, and clean-up for interference removal. Current methods of analysis for aqueous or solid samples involve liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), solid phase extraction (SPE), and solid-phase microextraction (SPME).

Solid-phase microextraction is a current method used for the isolation and pre-concentration of the analytes in different matrices. SPME is significantly more rapid and simple than LLE and SPE. The need for solvents has been eliminated and only a small volume of sample is required.

SPME has been successfully coupled to LC by Chen and Pawliszyn in 1995 [1] in order to analyze a wide variety of compounds. Recently, several applications of SPME-HPLC have been found in the literature, such as polyaromatic hydrocarbons, alkyl-phenol ethoxylate, surfactants, proteins, pesticides, corticosteroids, etc. [2].

Benzoylureas (BUs) constitute an important group of pesticides with herbicide or insecticide activity. Due to their low toxicity to mammals and rapid degradation in soil and water, their commercial development and use in agricultural practice has increased. Therefore, rapid,
selective, and sensitive methods are required to determine these pesticides.

Direct gas chromatography is unreliable due to their thermal instability, high polarity, and low volatility, and complex derivatization processes would be required [3]. For this reason, HPLC with UV [4–6] and MS detection [7, 8] has been used to determine these BUs in some matrices. It was recently demonstrated that UV irradiation yields photochemical reactions in several classes of pesticides, whose photochemistry has been reviewed by Crosby [9] and Marcheterre [10], and more recently by Aaron [6]. BU insecticides are a group of non-fluorescent pesticides, which may be converted into fluorescent species by photochemical reactions [11, 12]. With respect to their photochemistry, only diflubenzuron has been studied by Coly et al. [6, 13], among several aromatic insecticides. An interesting property of photochemical reactions is that they can be easily implemented as detection system in a continuous system. Thus, post-column photochemical derivatization yields photochemical reactions (PIF) is coupled to LC with fluorescence detectors (FD). Martinez et al. [11, 12, 14–16] have used liquid chromatography with fluorescence detection for developing several methods for the determination of BUs in various matrices after PIF derivatization. Good results and appropriate sensitivity were obtained in all cases although additional off-line LLE or SPE steps are included in the above methods.

In this work we developed a proposed method for the analysis of the six benzoylurea derivatives diflubenzuron (DFL), triflumuron (TRF), hexaflumuron (HXF), tebufenuron (TFU), lufenuron (LUF), and flufenoxuron (FFU) in fresh orange juice using a sample preparation step involving SPME coupled to HPLC combined with photochemically induced fluorimetry and fluorescence detection for analysis of these pesticides.

2 Experimental

2.1 Chemicals and solvents

Analytical standards (pestanal quality) of diflubenzuron, triflumuron hexaflumuron, tebufenuron, lufenuron, and flufenoxuron were obtained from Riedel de Haën (Seelze, Germany).

Analytical reagent grade solvents, methanol (MeOH) and acetonitrile (ACN) for pesticide residue analysis were obtained from Merck (Darmstadt, Germany). Anhydrous sodium sulfate for pesticide residue analysis was obtained from Panreac (Barcelona, Spain). Ultra-pure water, obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA), was used. Ecological natural orange was obtained from a private orchard in Almeria (Spain).

The mobile phase was filtered through a 0.45-μm cellulose acetate (water) or PTFE (ACN) and degassed with helium prior to and during use.

2.2 Instrumentation

The SPME fiber assembly and SPME–HPLC interface were purchased from Supelco (Bellefonte, PA, USA). The SPME–HPLC Interface consists of a six-port injection valve and desorption chamber (chamber volume 60 μL) which replaces the injection loop of a six-port injection system. The SPE fibers, polydimethylsiloxane (PDMS 100 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB 60 μm), and carbowax/templated resin (CW/TPR 50 μm) were purchased from Supelco (Bellefonte, PA, USA).

The HPLC, gradient LC pump Model 600E, and a variable-wavelength scanning fluorescence detector Model 474 were from Waters (Mildford, MA, USA). Liquid chromatography separations were performed on a Gemini C18 150 × 3 mm (3 μm particle size) column from Phenomenex (USA).

The photochemical reaction was carried out in a post-column photochemical reactor (Softrom GmbH, Gynkoteck HPLC, Germering, Germany) fitted with a knitted open tube reactor coil (5 m × 1.66 mm od and 0.3 mm id) PTFE and a 4-W Xenon lamp. Recording of chromatograms and quantitative measurements of peak areas were performed with Millennium® 2 Software from Waters. A schematic diagram of the system is shown in Fig. 1.

2.3 Preparation of standards and spiked samples

Individual analytical standard solutions of pesticides (400 mg/L) were prepared by carefully weighing and dissolving the corresponding compounds in acetonitrile. Furthermore, the standard solutions were protected against light and stored at 4°C in a refrigerator. Under these conditions, they were stable for at least three months.

Working solutions were prepared daily by diluting an appropriate aliquot in Milli-Q water. Spiked juice samples were prepared by spiking appropriate amounts of the working solution with the diluted sample.

2.4 SPME procedure

The sample was prepared by weighing 0.5 g of freshly homogenized orange juice extract spiked with the desired amount of pesticide mixture diluted with Milli-Q water in a 10 mL volumetric flask. An aliquot of 3 mL of this solution was used for SPME extraction.

The fiber was conditioned before the initial application according to the instructions provided by the sup-
plier, and then it was conditioned in the interface with the mobile phase daily before use until it is free from any contaminants. For the SPME process, 4-mL vials were filled with 3-mL aliquots of aqueous samples of standard solutions, or real samples containing the mixture of pesticides and were sealed with hole caps and PTFE septa. Extraction was carried out through PTFE septa, which were punctured to permit passage of the fiber, while the depth of immersion was kept constant. The sample solution was stirred with a stirring bar controlled at 1100 rpm by a magnetic stirrer. The temperature of the solution was kept at 65 ± 2°C. The sample was extracted by direct immersion for a period of 40 min, all the experiments being performed in duplicate unless otherwise specified. After sample extraction, the SPME fiber was introduced into the desorption chamber previously filled with mobile phase. Inside the chamber, the fiber was soaked using ACN/water 50:50 (v/v) for 5 min before loading the analytes onto the column by switching the valve to the injection position. The mobile phase (ACN/water 45:55 v/v) circulated via the chamber containing the fiber for 5 min as the optimum time of desorption, the valve was then switched to load position, allowing conventional LC analysis in the HPLC system. To minimize the possibility of any analyte carry-over or contamination between extraction runs, the fiber was cleaned by flushing with several portions of acetonitrile, followed by portions of water making it ready for further extraction.

2.5 HPLC procedure
The SPME extracted samples were chromatographed by a programmed gradient with ACN/water as mobile phase for 33 min. The solvent program was as follows: initially 18 min isocratic with ACN/water 45:55 (v/v) at a flow rate of 0.4 mL/min; then 2 min linear gradient to flow rate of 0.5 mL/min; then 10 min isocratic ACN/water 45:55 at a flow rate of 0.5 mL/min (v/v), followed by an additional period of 3 min linear gradient to the initial conditions; finally 5 min under the initial conditions, allowing sufficient time before subsequent analysis runs. Fluorimetric detection was performed at an excitation wavelength (λex) of 330 nm and at an emission wavelength (λem) of 410 nm for all pesticides.

3 Results and discussion
3.1 HPLC analysis
After the extraction step with SPME, the analytes were desorbed inside the interface chamber and transferred to the HPLC column for separation and analysis. In order to achieve this, the composition and flow rate of the mobile phase were optimized. A series of aqueous ACN and MeOH gradient elution programs were evaluated in order to obtain maximum responses and selectivity with minimum broadening on the chromatograms. The highest fluorescence responses and the best separation in the shortest time were achieved using the ACN:water gradient program described in Section 2.5.

3.2 Fiber selection
In order to compare the effect of the fiber coating for the extraction of selected benzoylureas, several commercially available polymeric coatings such as polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 60 μm), and carbowax/templated resin (CW/TPR, 50 μm) were evaluated. The extraction ability of each fiber was assigned by peak area counting from each fiber under the same extraction conditions. From the results shown in Fig. 2 for the analysis of a standard aqueous solution of 200 μg/L of each pesticide (extraction experiments were carried out in duplicate), it is observed that PDMS/DVB showed a higher sensitivity towards diflubenzuron, hexaflumuron, and teflubenzuron. The same sensitivity was obtained using the three fibers for the lufenuron compound. PDMS/DVB and CW/TPR showed a similar sensitivity towards triflumuron. PDMS showed a higher sensitivity to flufenoxuron and a lower sensitivity to diflubenzuron, triflumuron, hexaflumuron, and teflubenzuron. The same sensitivity was obtained using the three fibers for the lufenuron compound. PDMS/DVB and CW/TPR showed a similar sensitivity towards triflumuron.
Liquid Chromatography

(CW/TPR, PDMS/DVB) and non-polar analytes are more attracted to the non-polar fiber (PDMS). Moreover, the nature of the analytes, molecular size, volatility, and porosity of the fiber coatings, play a role in the extraction efficiency. PDMS/DVB was selected as fiber for further investigation due to the fact that it shows a more favorable compromise recovery.

3.3 Dilution ratio

In order to obtain aqueous solutions from solid or high suspended matter content matrices such as vegetables or fruits, the desired amount of the matrix should be diluted with Milli-Q water. Diluting the sample in such cases improves the extraction efficiency, reduces the effect of matrix interferences, and, more importantly, protects the fiber coating from deterioration throughout the extraction process [17]. Dilution of the samples reduces the sensitivity. It is therefore important to find a compromise sample amount. In order to select the appropriate dilution percentage, different amounts of orange juice were diluted at different rates: 1:20, 1:15, 1:10, 1:5 (w/v) using 0.5, 0.75, 1 and 2 g of the matrix, respectively, in the final volume of 10 mL and spiked with the same amount of pesticides (final concentration 100 µg/L of each pesticide in all cases) and then evaluated by applying the proposed method. The results obtained comparing peak area counting showed an improvement in the extraction yield when the dilution ratio was increased. As a compromise between sensitivity and extraction efficiency, a dilution ratio of 1:20 w/v corresponding to 5% sample amount content was selected, due to better extraction efficiency.

3.4 Optimization of desorption process

Conditions affecting the desorption rate were optimized. Parameters included the selection of the desorption mode, soaking time, soaking solvent, and desorption time.

Analytes can be desorbed using either a dynamic mode or a static mode. In the dynamic mode, the analytes are desorbed on-line by a stream of HPLC mobile phase. In the static mode, the valve of the SPME assembly is switched so that the desorption chamber is off-line and the mobile phase is introduced and the analytes are desorbed from the fiber before the solvent is passed into the column. Extraction efficiencies of the six benzoylureas in the static desorption mode were higher than in the dynamic mode.

This indicates that the attraction between the fiber coating and the analytes was strong and desorption was slow. The static mode was used for desorption in this study. For a strong attraction between analytes and fiber, the static mode is recommended and has thus been used in other work [18, 19].

In the soaking period the fiber remains inside the desorption chamber before it is flushed with the desorption solvent. All analytes showed an increase in extraction efficiency when the soaking time was increased to 5 min. However, the maximum desorption for diflubenzuron was achieved at 7 min, and for triflumuron at 9 min. Five minutes was selected as soaking time in further experiments.

The desorption period refers to the period of time during which the fiber is washed by the desorption solvent (the mobile phase in this case), in the desorption chamber. The desorption period was evaluated up to 7 min. Results obtained in Fig. 3 show that the maximum desorption efficiency was obtained at 5 min for all analytes. A time of 5 min was therefore used as desorption time since good extraction and resolution were achieved.

Soaking solvent refers to the solvent which is used for desorbing the analytes from the fiber inside the chamber throughout the soaking period. Different mixtures of acetonitrile–water solvent were evaluated, the best results were obtained using ACN:water 50:50 v/v.
3.5 Optimization of extraction process

3.5.1 Extraction time profile

Figure 4 represents the performance of the PDMS/DVB fiber for a spiked mixture at 100 μg/L of diflubenzuron, triflumuron, hexaflumuron, teflubenzuron, lufenuron, and flufenoxuron in the range between 20 and 60 min. Extraction efficiencies below 20 min were low. From the results shown in the graph, several effects were observed in the analytes. Equilibrium was attained for lufenuron at 30 min, hexaflumuron and flufenoxuron at 40 min, and for triflumuron at 50 min. On the other hand, equilibrium was not attained for diflubenzuron and teflubenzuron up to 60 min (maximum studied time). The non-equilibrium increase in the extraction efficiency was significant for diflubenzuron on increasing the time of extraction but was lower in the case of teflubenzuron. Since the use of equilibrium time is not necessary if LODs are acceptable, working at times giving non-equilibrium extraction is possible. SPME under non-equilibrium conditions has been applied in the analysis of several organic compounds and pesticides in aqueous matrices with satisfactory results [20–22]. In our case, a time of 40 min was selected and it was sufficient to give satisfactory results with appropriate sensitivity, avoiding too lengthy extraction times.

3.5.2 Effect of extraction temperature

The effect of temperature on extraction is influenced by two factors: kinetics and thermodynamics. In the kinetic factor, a higher temperature increases the diffusion rate of the analytes, thus extraction efficiencies increase at a higher temperature. The two effects compete with each other and different analytes are affected in different ways [23]. From results obtained for the effect of temperature when analyzing a mixture at 100 μg/L of each analyte in Fig. 5, it is observed that an increase in the extraction efficiency was obtained on increasing the temperature to 80°C for all analytes except for diflubenzuron, where the maximum extraction efficiency was obtained at 65°C. Avoiding pre-concentration due to vaporization of the sample during extraction at higher temperatures, 65°C was selected as the extraction temperature.

3.5.2 Effect of stirring rate

The effect of stirring rate for the six benzoylureas was evaluated by analyzing samples at different stirring rates. From the results obtained in Fig. 6, it can be stated that an increase in extraction efficiencies was achieved when the stirring rate was increased for all analytes up to 1100 rpm (maximum studied stirring rate) without reaching equilibrium. The optimum selected stirring rate was 1100 rpm and was used for further experiments.

3.5.3 Effect of organic solvent

The effect of addition of organic solvent was studied by preparing a series of samples containing different concentrations of acetonitrile ranging from 0 to 20% (v/v) at a final concentration of 100 μg/L of each analyte. Extraction was performed on these samples using the previous conditions. The extraction efficiencies of benzoylurea analytes varied from one compound to another. A decrease in the extraction efficiency was obtained on the addition of ACN to the extracted sample for diflubenzuron and teflubenzuron. An increase was obtained up to equilibrium for triflumuron at ACN (5%, v/v) and for hexaflumuron at a percentage of ACN (10%, v/v). Both
Table 1. Analytical figures of merit obtained using Milli-Q water.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression equation</th>
<th>Linear range (μg/L)</th>
<th>( R^2 )</th>
<th>LOD( a) \ (μg/L)</th>
<th>LOQ( a) \ (μg/L)</th>
<th>LOQ( b) \ (μg/L)</th>
<th>Recovery( c) \ (%)</th>
<th>(RSD( c) \ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diflubenzuron</td>
<td>( Y = 11362X + 7606 )</td>
<td>0.75 – 150</td>
<td>0.9950</td>
<td>0.012</td>
<td>0.032</td>
<td>0.75</td>
<td>110 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Triflumuron</td>
<td>( Y = 16249X + 125486 )</td>
<td>0.5 – 150</td>
<td>0.9984</td>
<td>0.008</td>
<td>0.02</td>
<td>0.5</td>
<td>104 (6.1)</td>
<td></td>
</tr>
<tr>
<td>Hexaflumuron</td>
<td>( Y = 14046X + 141209 )</td>
<td>1.5 – 125</td>
<td>0.9953</td>
<td>0.005</td>
<td>0.01</td>
<td>1.5</td>
<td>96 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Teflubenzuron</td>
<td>( Y = 3384X + 7663 )</td>
<td>1.5 – 100</td>
<td>0.9984</td>
<td>0.05</td>
<td>0.13</td>
<td>1.5</td>
<td>102 (7.5)</td>
<td></td>
</tr>
<tr>
<td>Lufenuron</td>
<td>( Y = 8218X + 5304 )</td>
<td>1.5 – 100</td>
<td>0.9963</td>
<td>0.008</td>
<td>0.02</td>
<td>1.5</td>
<td>88 (6)</td>
<td></td>
</tr>
<tr>
<td>Flufenoxuron</td>
<td>( Y = 20287X + 35837 )</td>
<td>0.25 – 100</td>
<td>0.9973</td>
<td>0.003</td>
<td>0.01</td>
<td>0.25</td>
<td>101 (8.4)</td>
<td></td>
</tr>
</tbody>
</table>

\( a) \) IUPAC criterion.
\( b) \) EURACHEM criterion (RSD 10%).
\( c) \) Spiked at concentration level 50 μg/L, \( n = 6 \).

...lufenuron and flufenoxuron showed an increase in the extraction yield on increasing the ACN concentration without attaining equilibrium, even at a concentration of ACN 20% v/v (maximum concentration studied). The extractions of the analytes from the samples were carried out without addition of any organic solvent because of its negative effect on teflubenzuron and diflubenzuron which showed a lower extraction yield when using ACN.

### 3.5.4 Effect of ionic strength

The addition of salt (NaCl or Na₂SO₄) often improves recovery when conventional extraction methods are used [24]. In this study, the influence of Na₂SO₄ additive was evaluated. Different concentrations of Na₂SO₄ (up to 15% w/v) were used in analyzing a mixed solution of 100 μg/L of each pesticide. Different effects were observed on studying the effect of Na₂SO₄ on the extraction efficiency. From the results obtained it can be stated that equilibrium was obtained for diflubenzuron and triflumuron at a salt concentration of 2.5%, and for teflubenzuron at 5%. On the other hand, a decrease in extraction efficiency was obtained on adding Na₂SO₄ for hexaflumuron, lufenuron, and flufenoxuron. The addition of salt may lead to a decreased extraction when the compound solubility does not change and due to the interaction between the analyte molecules and the salt. The negative effect of salt additives has been observed for some groups of pesticides in other work [25, 26]. In this case, the extraction was performed without addition of salt during the extraction process.

### 3.5.5 pH effect

The influence of pH on the extraction efficiencies was investigated. A series of spiked samples were prepared and buffered with different pH values. On varying the pH value from 3 to 10 (the recommended range in instructions provided by the fiber supplier), no significant effect was observed on the extraction level and resolution. Thus the pH of the sample was not adjusted.

### 3.6 Effect of carry-over

In SPME techniques, a significant amount of the analytes often remains desorbed on the fiber after the desorption step. For evaluation of the effect of carry-over, a blank desorption experiment was performed after extraction of a spiked sample, containing the six benzoylurea pesticides at a concentration of 1 mg/kg. For the pesticides tested, the carry-over ranged between 1.5 and 5.6% and it was 1.5, 4.1, 4.6, 5.6, 5.3, and 5.5% for diflubenzuron triflumuron, hexaflumuron, teflubenzuron, lufenuron, and flufenoxuron, respectively. This carry-over was eliminated by flushing the fiber with portions of acetonitrile and portions of water.

### 3.7 Validation

The proposed analytical method was validated using the above conditions. The limits of detection, limits of quantifications, precision (RSDs), linearity, and recovery were studied. The analytical figures of merit, obtained under optimum conditions, are summarized in Table 1 for validation using spiked Milli-Q water solvent, and in Table 2 using spiked orange juice matrix. LODs and LOQs were calculated statistically [27] as 3.84 and 10 times, respectively, the standard deviation of the signal corresponding to 10 blank solutions divided by the slope of the calibration curve. The LOQs were also calculated, according to EURACHEM Guidance [28], as the lowest concentration of the analyte for which the RSD of the signal is equal to or less than a fixed percentage (10% in our case). According to these criteria, LOD and LOQ results showed values less than MRLs (MRLs: 1, 0.05, 0.5, 0.05, 0.3, and 0.3 mg/kg for diflubenzuron triflumuron, hexaflumuron, teflubenzuron, lufenuron, and flufenoxuron, respectively) established by Spanish regulations. The linear range was established for each pesticide and was between 1 and 125 μg/L, corresponding to 0.02 and 2.5 mg/kg with a good linear relationship (\( R^2 > 0.995 \)) in orange juice. The lower limit is the LOQ calculated according to the latter...
Matrix effects have been investigated by performing calibrations in both matrix-match and Milli-Q water-based standards of the same concentrations [29]. A suppression effect on the analytical signal due to the matrix was noted for all analytes in the matrix. For this reason, matrix-matched standards were used throughout for quantification.

In order to establish the accuracy and precision of the total method, six replicates of orange juice samples were spiked and analyzed using the proposed described method. Mean recovery percentages and RSD% were evaluated at two concentration levels and are shown in Table 3. In the results good precision was obtained for all analytes (RSD% <8%) and recoveries ranged between 85 and 110%, which are within the range expected for residue analysis [30].

### 3.8 Analysis of real samples

A total of five commercial orange samples were purchased from five different orchards in Almería (Spain) and analyzed by the proposed method. None of the benzoylureas were detected. Figure 7 shows two chromatograms corresponding to a commercial orange juice sample and the same sample spiked at 0.1 mg/kg. No peaks appear at the retention times of the analytes, and therefore, the proposed method is suitable for the analysis of the six benzoylureas in this study.

### 4 Concluding remarks

In this work, a new method is proposed for the analysis of six benzoylureas in orange juice samples. The proposed SPME–HPLC–PIF–FD method is simple and sensitive enough for the determination of diflubenzuron, triflumuron, hexaflumuron, teflubenzuron, lufenuron, and flufenoxuron in orange juice with LOD and LOQ values lower than the MRLs.

This method is less expensive than other extraction methods, avoids the use of extra amounts of organic solvents, and substantially reduces sample manipulation. It also enables use of the same fiber for more than 100 extraction runs when it is handled with care.

### 5 References