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Determination of benzoylureas in tomato by high-performance liquid chromatography using continuous on-line post-elution photoirradiation with fluorescence detection

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Abstract

A photochemically induced fluorescence post-column method, with HPLC separation and fluorescence detection, was developed for the determination of five naturally non-fluorescent benzoylurea insecticides: diflubenzuron, triflumuron, hexaflumuron, lufenuron and flufenoxuron. The applicability of the method to the determination of insecticides in spiked tomato was evaluated. Samples were extracted into ethyl acetate and further cleaned-up by solid-phase extraction using an aminopropyl-bonded silica cartridge. The interferences due to the matrix effect were eliminated using matrix matched standards. Linear dynamic ranges were established over more than two orders of magnitude. The limits of detection ranged from 5 to 21 ng ml^{-1} (or 0.5 and 2.1 μg kg^{-1} in the vegetable samples), with relative standard deviations lower than 5.0%, using blank tomato extract. Mean recoveries ranged from 79 to 102%. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Photochemically induced fluorescence; Pesticides; Benzoylureas

1. Introduction

Benzoylureas (BUs) constitute an important group of pesticides with herbicide or insecticide activity. These insecticides act by inhibition of chitin synthesis in the insect's cuticle [1]. Due to their low toxicity for mammals and rapid degradation in soil and water, their commercial development and use in agricultural practice has increased. Public concern of pesticide residues in food has been increasing in the last years and monitoring of pesticide residues is crucial for proper assessment of exposure to pesticide residues in food.

Because of their thermolability, BU insecticides are generally analysed by high-performance liquid chromatography (HPLC) using UV [2–5] or mass spectrometry [6,7] detectors. Only diflubenzuron (DFL) was determined by gas chromatography–electron-capture detection (GC–ECD), after derivatization with heptafluorobutyric anhydride [8,9].

An alternative to chromatographic techniques would be fluorimetric detection. This option is more sensitive and selective that most other detection systems [10]. Selectivity and sensitivity can be

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increased by photochemically induced fluorimetry (PIF). In the last decade, it has been demonstrated that a number of photoreactive compounds produced photochemical reaction after UV irradiation, leading to the formation of strongly fluorescent photoproducts. PIF is more recent than chemical derivatization and has been much less applied to quantitative analysis. PIF conversion has shown to be an efficient fluorophore-generating system for stationary media [11–13] as well as in flow injection analysis (FIA) [14,15] or HPLC post-column photoreaction [16–19]. A post-column photolysis fluorescence detector, combining HPLC and PIF was used to determine pesticides such as phenylcarbamates, phenylamides and phenylurea herbicides in ground water [16,17], different nitrogenous pesticides in groundwater [18] and niacin in foods [19].

The aim of the present work was to develop a HPLC method for determining diflubenzuron (DFL), flufenoxuron (FLF), triflumuron (TRF), hexaflumuron (HF) and lufenuron (LUF) insecticides, in tomato matrices, by continuous on-line post-column photoirradiation followed by fluorimetric detection.

2. Experimental

2.1. Chemicals and solvents

Analytical standards (pestanal quality) of DFL [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea], TRF [1-(2-chlorobenzoyl)-3-(4-trifluoro-methoxyphenyl) urea], HF {1-[3,5-dichloro-4(1,1,2,2-tetrafluoroetoxy)phenyl]-3-(2,6-difluorobenzoyl) urea}, LUF {(*RS*)-1-[2,5-dicloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl) urea} and FLF {1-[4-(2-chloro-α,α,α-trifluoro-*p*-tolyloxy)-2-fluorophenyl)-3-(2,6-difluorobenzoyl) urea}, were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Analytical-reagent grade solvents, methanol (MeOH), acetonitrile (ACN), dichloromethane, ethyl acetate, petroleum ether and anhydrous sodium sulfate for pesticide residue analysis were obtained from Scharlaw (Barcelona, Spain). Solid-phase extraction (SPE) cartridges of 500 mg aminopropyl-bonded silica (Waters, Milford, MA, USA) were used to clean up vegetable samples.

Mobile phases were filtered through a 0.45-μm cellulose acetate (water) or polytetrafluoroethylene (PTFE) (MeOH and ACN) and degassed with helium prior to and during use. All standards and samples were filtered through Millipore membrane PTFE 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, USA), was used.

2.2. Instrumentation

The HPLC system was a Waters, 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. Liquid chromatography (LC) separations were performed with a Waters spherical silica-based stationary phase 150×3.9 mm (4 μm particle size) column.

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

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

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

2.3. Preparation of standards and spiked samples

Standard solutions of pesticides (200 mg l^{-1}) were prepared by exactly weighing and dissolving the corresponding compounds in organic solvents. These standard solutions are stable for a period of at least 3 months. Dilutions were freshly prepared for the working solutions. All solutions were protected

against light with aluminium foil and were stored in a refrigerator at 4°C.

For recovery determinations, samples (50 g) of finely chopped vegetable were spiked by addition of a standard stock solution (200 mg l^{-1}), at two levels of concentration: 10 and 50 μg kg^{-1} (equivalent to 0.1 and 0.5 μg ml^{-1} in the final extract) for each of the pesticides.

The spiked samples were allowed to stand for a few minutes before extraction to allow the spike solution to penetrate the test material.

2.4. Procedure for determining the pesticide in vegetables

A sample (50 g) of vegetable was placed in a glass and homogenized with 75 ml ethyl acetate for 2 min with the Polytron instrument. Then, 80 g of sodium sulfate was added and the mixture was homogenized for 1 min. The extract was filtered through a 12 cm Büchner funnel and washed with two 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 a 0.45-μm PTFE filter.

Volumes of 400 μl of ACN–water (1:1, v/v) sample solutions were analysed by HPLC with fluorimetric detection. The solvents were filtered daily before use through a 0.45-μm cellulose acetate (water) or PTFE (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.

3. Results and discussion

Earlier [20], photolysis of the BUs was performed by PIF to allow a rapid screening with a view to photoinduced detection in LC. The strongest fluorescence signal occurred in aqueous mixtures with organic solvents such as MeOH, ethanol (EtOH) and 2-propan-ol (2-ProOH). The excitation and emission spectra were established for each BU insecticide, in order to find the λ_{ex} and λ_{em} maxima. No significant shift of the λ_{ex} and λ_{em} occurred upon changing the solvent polarity.

3.1. HPLC separation and fluorescence detection after photolysis

Fluorescence was measured at 410 nm for all pesticides, after excitation at 330 nm with a xenon source.

Mobile phase effect on responses and flow-rates through the C_{18} column and the reactor (residence times) was examined and adjusted to provide maximum responses and minimal broadening on the chromatograms. In this way, different MeOH–water and ACN–water mixtures were evaluated for fluorescence response and for use as mobile phases. The highest fluorescence responses and the best separation were achieved by using MeOH–water (80:20, v/v) as mobile phase. It was also found that the analytical response increases as the flow-rate decreases (Fig. 1) for all BUs, except for FLF. A flow-rate of 0.4 ml min^{-1} was chosen as a compromise solution between the residence time in the reactor and the band broadening on the chromatogram.

The effect of pH on the fluorescence intensity, as well as on the separation of peaks was tested by using different buffer solutions (C_T=0.01–0.1 M). No significant changes were found for pH values

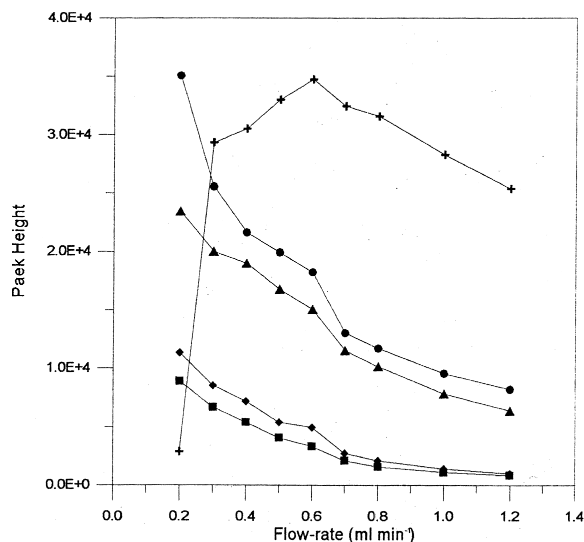

Fig. 1. Peak height response versus flow-rate for diflubenzuron (●), triflumuron (▲), hexaflumuron (♦), lufenuron (■) and flufenoxuron (+) after post-column photochemical reaction and fluorescence detection.

between 3 and 8. Therefore, no buffer was used in the mobile phase.

Fig. 2 shows the chromatogram for a mixture of BU insecticides under the optimum conditions (MeOH–water, 80:20, v/v at a flow-rate of 0.4 ml min^{-1}). In all cases, peak height was used as analytical signal in quantification.

3.2. Extraction

In order to develop a single multiresidue method to determine the above active ingredients in tomato, samples spiked at 100 μg kg^{-1} level were extracted using several solvents (ethyl acetate, methanol, acetone, dichloromethane–methanol, 9:1 and 1:1, dichloromethane–petroleum ether, 1:1, and ethyl acetate–methanol in different percentages). Then, an SPE clean up was tested using several sorbents (aminopropyl-bonded silica, C_{18}, florisil and alumnina) and different eluents (dichloromthane and methanol). 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 were, therefore, chosen for extraction and clean up.

Fig. 3a shows a HPLC–fluorescence chromatogram of a tomato blank extract without interferences,

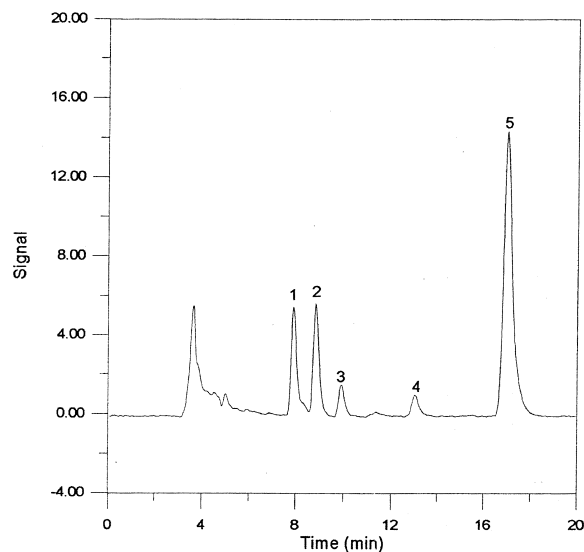

Fig. 2. Chromatogram corresponding to a standard of 0.4 μg ml^{-1} of: (1) diflubenzuron; (2) triflumuron; (3) hexaflumuron; (4) lufenuron and (5) flufenoxuron using a mobile phase of MeOH–water (80:20, v/v) at 0.4 ml min^{-1}.

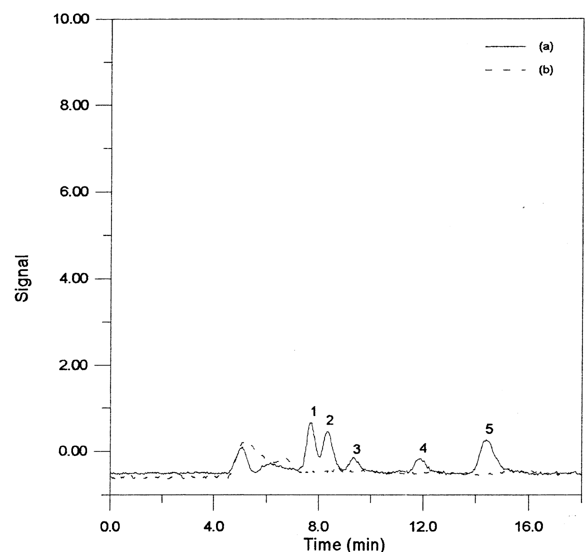

Fig. 3. (a) Chromatogram of a blank tomato extract. (b) Chromatogram of a tomato sample spiked with: diflubenzuron (15 ng ml^{-1}), triflumuron (14 ng ml^{-1}), hexaflumuron (18 ng ml^{-1}), lufenuron (22 ng ml^{-1}) and flufenoxuron (6 ng ml^{-1}).

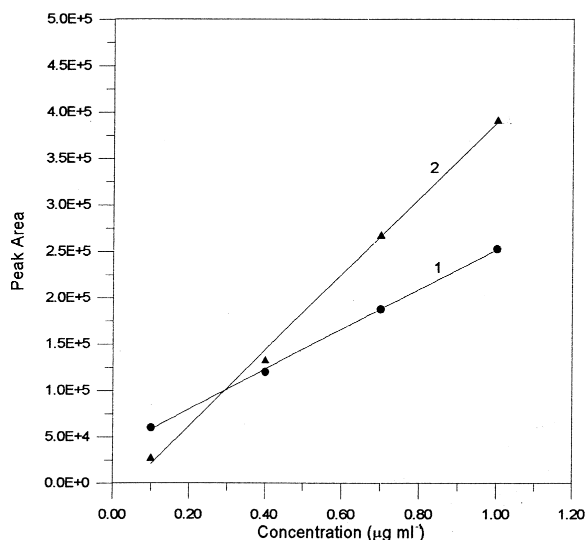

Fig. 4. Calibration curves of diflubenzuron in pure solvent (●) and tomato matrix (▲).

at the retention times corresponding to those of the analytes.

3.3. Analytical performance

Matrix effects have been reported by different authors in the determination of several classes of pesticides by GC–nitrogen–phosphorus detection (NPD) and GC–ECD [21,22], GC–mass spectrometry (MS) [23,24] and HPLC–MS [6,7]. Therefore, calibrations were performed by using both matrix-matched and solvent-based standards of the same concentration. An enhancement/suppression effect on the analytical signal due to the matrix, was noticed for all insecticides except for LUF, which was both compound- and concentration-dependent (Fig. 4).

For this reason, matrix-matched standards were used throughout for quantification and the analytical figures of merit were obtained in this way by measuring at $\lambda_{ex/em}$ of 330/410 nm for all compounds. In addition, those of FLF were obtained at $\lambda_{ex/em}$ of 286/357 nm because at these wavelengths the maximum response was obtained, with particularly low concentration values for linear range and limit of detection (LOD) (Table 1). However for our analytical purposes, we decided to determine FLF at the wavelengths of compromise (with linear range between 0.01 and 1.0 μg ml^{-1}) due to the concentration established in the MRL for FLF in tomato matrix is 50 μg kg^{-1} (corresponding to 0.5 μg ml^{-1} injected solutions), thus avoiding additional dilutions for real samples.

The LODs and limits of quantification (LOQs) for the BU insecticides were calculated statistically [25] on the values of the blank at the t_R of the analytes (six injections). The LOQs were also calculated as the lowest concentration where the relative standard deviation (RSD) is less than 5% [26]. The LOQ values were higher when the second criterion was used. We consider that the latter approach estimates more realistic values, in agreement with other authors [27].

The LODs calculated ranged from 5 to 21 ng ml^{-1} in injected solutions (corresponding to 0.5 and 2.1

Table 1
Analytical figures of merit for the determination of BU insecticides by using blank tomato matrix

Compound	Linear range (μg ml^{-1})	Regression equation	R^2	RSD* (%)	LOQ[a] (ng ml^{-1})	LOD[b] (ng ml^{-1})	LOQ[b] (ng ml^{-1})
Diflubenzuron	0.01–1.0	$y=215\,105x+37\,094$	0.999	2.36	50	14	46
Triflumuron	0.01–1.0	$y=200\,485x+10\,749$	0.999	3.72	50	13	43
Hexaflumuron	0.01–1.0	$y=55\,964x-699.6$	0.997	4.56	80	17	56
Lufenuron	0.01–1.0	$y=81\,049x-2449$	0.999	2.86	90	21	69
Flufenoxuron[c]	0.01–1.0	$y=1\,198\,246x+13\,408$	0.999	1.97	20	5	16
Flufenoxuron[d]	0.005–0.15	$y=32\,864\,320x+176\,435$	0.990	4.85	0.09	0.02	0.06

*10 injections of 0.1 μg ml^{-1}.
[a] Based on the lowest concentration where the RSD (%) is estimated to be less than 5%.
[b] Based on the values of the blank ($n=6$) at the t_R of the analytes.
[c] $\lambda_{ex/em}=330/410$ nm.
[d] $\lambda_{ex/em}=286/357$ nm.

μg kg^{-1} in the vegetable sample). Fig. 3b shows a chromatogram of a tomato sample spiked at concentration levels close to the LOD. The signals corresponding to the analytes are well defined vs. the matrix background and they allow detection of the pesticides.

In all cases, these LODs are significantly lower than those reported in the literature for the determination of the same insecticides by other techniques, such as GC–ECD [9] or HPLC–UV [2,4,5] and they are in the same order as those obtained by HPLC–atmospheric pressure chemical ionisation (APCI)–MS [6] or HPLC–APCI-MS with ionization polarity switching [7]. The LOQs are lower than the MRLs established in the European Union [28].

Tomato samples were spiked with 10 and 50 μg kg^{-1} of each BU, extracted and analysed as described in Section 2.4, to determine recoveries. The mean recovery percentages and the standard deviations associated, obtained using matrix matched standards for quantification, are given in Table 2. It can be seen that mean recoveries ranged from 79 to 109%, which are in the range expected for residue analysis.

3.4. Interferences

The influence of 30 pesticides on the determination of 0.1 μg ml^{-1} of BUs was studied by first testing 0.1 μg ml^{-1} of each interferent compound and if interference occurred, reducing the concentration progressively until interference ceased. The criterion for interference was an error in quantification of BUs higher than 5%.

The results obtained showed that only thiabendazole interferes, at a concentration level higher than 30 ng ml^{-1}.

3.5. Analysis of real samples

The proposed method was applied for analysing tomato samples in the "Residue Analysis Laboratory CUAM" in Almería, accredited according to EN 45001. Some of the BU insecticides were present in about 2% of the analysed samples, at levels lower than the MRLs. On the other hand, the roughness of this method has been proved over 1 year of use.

4. Conclusions

Post-column reaction detection allows the chemical structures of the analytes to be modified, allowing the development of unique analytical methods with excellent selectivity. Sensitivity is matrix and concentration dependent, requiring calibration with matrix-matched standards. Detection limits compare favourably with the ones obtained by HPLC–DAD and are in the same order as those obtained by HPLC–MS and lower than the MLR established in European countries.

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Table 2
Mean recoveries and standard deviations ($n=3$) from tomato samples spiked with 10 and 50 μg kg^{-1} of BU insecticides, calculated using the calibration graphs in blank tomato matrix for quantitation

Compound	Spiked samples (0.1 μg ml^{-1})	Spiked samples (0.5 μg ml^{-1})
Diflubenzuron	79 (1.5)	109 (5.9)
Triflumuron	90 (2.3)	79 (7.2)
Hexaflumuron	94 (2.8)	87 (7.8)
Lufenuron	99 (3.6)	98 (7.9)
Flufenoxuron	95 (2.1)	93 (13.7)

RSD (%) in parentheses ($n=3$).

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