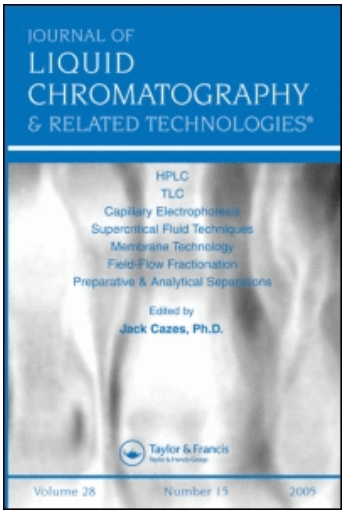

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HPLC/Diode-Array Method for the Determination of the Pesticide Diflubenzuron and Its Major Metabolites 2,6-Difluorobenzamide, 4-Chlorophenylurea, and 4-Chloroaniline in Forestry Matrices

E. Rodriguez[a]; Z. Gomez de Balugera[a]; M. A. Goicolea[b]; R. J. Barrio[a]
[a] Department of Chemical and Environmental Engineering School of Technical Engineering, University of the Basque Country, Vitoria-Gasteiz, Spain [b] Department of Analytxal Chemistry Faculty of Pharmacy, University of the Basque Country, Vitoria-Gasteiz, Spain

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HPLC/DIODE-ARRAY METHOD FOR THE DETERMINATION OF THE PESTICIDE DIFLUBENZURON AND ITS MAJOR METABOLITES 2,6-DIFLUOROBENZAMIDE, 4-CHLOROPHENYLUREA, AND 4-CHLORO-ANILINE IN FORESTRY MATRICES

E. Rodriguez,[1] Z. Gomez de Balugera,[1] M. A. Goicolea,[2] R. J. Barrio[2,*]

[1] Department of Chemical and Environmental Engineering
School of Technical Engineering
University of the Basque Country
Vitoria-Gasteiz, Spain

[2] Department of Analytical Chemistry
Faculty of Pharmacy
University of the Basque Country
Vitoria-Gasteiz, Spain

ABSTRACT

A method using liquid chromatography with diode-array detection, LC-DAD, has been developed for the determination of the pesticide diflubenzuron and some of its major metabolites, such as 2,6-difluorobenzamide, 4-chlorophenylurea, and 4-chloroaniline in pine-needles. Samples were extracted into acetonitrile and further cleaned-up through aminopropyl cartridge. LC was performed on a C_{18} column using acetonitrile-methanol-water (50:2:48) as mobile phase at 1 mL min^{-1} at room temperature.

The detection limits were lower than 0.6 ng mL^{-1} in all cases, except for 2,6-diflurobenzamide that was 9.4 ng mL^{-1}. Recoveries of the compounds from supplied pine-needle samples were higher than 83% with a relative standard deviation lower than 2.6%.

INTRODUCTION

Diflubenzuron, [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea], is a pesticide highly effective against several pests of insects.[1,2] It is an insect growth regulator that acts by inhibiting the synthesis of chitin cuticle and disrupting their normal growth and development processes. It is particularly toxic to the larval stages of some insects, effect apparently due to an ovicidal action.[3] This effect on eggs is also based on interferences with chitin deposition.[4,5]

All stages of insects known to form new cuticles are in principle susceptible to this pesticide. The inherent low toxicity for mammalians, birds, and fish and the clear selectivity with respect to many non target insects has encouraged its commercial development.[6,7]

Diflubenzuron is applied by aerial spray at a maximum operational dosage rate of 70 g AI/ha, using liquid WP-25 formulation (25% wettable powder in water) and it is estimated that only the 16% of the applied spray volume reached the forest floor.[8] It is known that the highest volume rate 10 l/ha provide high residues at the forest and that multiple applications of the wettable powder produces high and long-lasting residues on foliage and vegetation,[9] since diflubenzuron does not penetrate into the plant tissue.[10,11,12]

Various studies about the persistence and degradation of the pesticide in different media have been carried out. Thus, one established metabolite formed from diflubenzuron in water is p-chloroaniline, classified as a mutagen by the National Cancer Institute and the Cancer Assessment Group of E.P.A., although 2,6-difluorobenzamide and p-chlorophenylurea seem to be the major breakdown products of the primary process of the degradation of the pesticide in water.[13,14]

In addition, the photodegradation is also considered as an important process of degradation in the environment and 2,6-difluorobenzamide and 4-cholorophenylurea are the major products obtained upon irradiation.[15,16]

A number of authors have analyzed diflubenzuron and other pesticides with similar properties from different matrices. Thus, methods to determine this pesticide in milk,[17] soil, and water,[18,19] even from pine needles[20] and some fruits[21] have been reported.

Some authors have used GC/MS but the diflubenzuron is decomposed by the heat in some metabolites.[22] A thin layer chromatography method is also available to analyze this pesticide.[23] Some methods involving LC with UV detection[24,25] and others using LC-MS with thermospray ionization in the positive mode[26] or ionization technique of atmospheric pressure chemical ionization[27,28] have been used. Various LC methods are also available to analyze 4-chloroaniline using electrochemical and UV detection.[23,30,31]

Since no data are available about the simultaneous analysis of diflubenzuron and its major metabolites from forestry matrices, a LC-DAD method has been developed, as the most suitable analytical technique. This method will allow to establish the level of presence of this pesticide in the habitats where it is usually applied.

EXPERIMENTAL

Chemicals and Reagents

All the solvents used in this study were HPLC grade and tested for spectral purity: acetonitrile (Merck, Darmstadt, Germany) and methanol (Fluka, Buchs, Switzerland). Distilled water was obtained from a Milli-Ro and Milli-Q water purification system (Millipore, Milford, MA, USA).

All the solvents and samples were filtered through 0.22 mm Millipore membrane filters type GVWP and mobile phases were degassed by a Selecta (Barcelona, Spain) Ultrasounds System before utilization.

Diflubenzuron, 2,6-difluorobenzamide, 4-cholorophenylurea, and 4-chloroaniline standards were supplied by the laboratory of Dr. Ehrenstorfer GMBH (Augsburg, Germany) with a certified purity higher than 99,0%. Nifedipine used as internal standard was obtained from Sigma (St. Louis, MO, USA). The different cartridges (with 100 mg of sorbent) tested for the clean-up were from Waters (Waters Assoc., Milford, MA, USA).

Equipment

Hewlett-Packard (Palo Alto, CA, USA) model 1050 High-Performance Liquid Chromatograph (25 μL injection loop) fitted with a Diode-Array Detector (DAD) model 1040 and a Data Station Vectra 486/33 N were utilized to carry out the analysis. The column was a Spherisorb ODS2 (150 mm/4 mm ID, 5 mm particle size) from Tracer (Tracer Anal., Barcelona, Spain). A guard-column Tracer (1 cm/4 mm) was used. A Selecta model vibromatic 384 and a vacuum station Varian (Harbor City, C.A., USA) were used in the extraction procedure.

Preparation of the Standards and the Spiked Pine-Needle Samples

A set of calibration of 20 standards was prepared at concentrations from 5 to 10000 ng mL^{-1} in acetonitrile. Standard stock solutions were obtained by the dissolution of 10 mg of each chemical compound in 100 mL of acetonitrile and subsequent dilutions were made for their final preparation. All the standard solutions were stored in a refrigerator covered with aluminum-paper when not in use.

Spiked pine-needle samples for recovery determination were prepared by the addition of an appropriate amount of a standard stock solution to 3.0 g of pine needles, which were left to stand for 30 minutes before extraction to allow the spicked solution to impregnate the needles. All the experiments were carried out in triplicate.

Solid-Phase Extraction and Clean-Up Procedures

A 3 g sample of pine-needles was treated with 25 mL of acetonitrile, shaken in a capped centrifuge tube for 10 minutes and filtered by suction. Several cartridges were tested, all of them previously washed with 5 mL of acetonitrile for activation of the sorbent. Next, 1 mL of sample extract was passed through the cartridge by negative pressure in a Vac-Elut station and the elute subsequently injected into the LC system.

RESULTS AND DISCUSSION

Since diflubenzuron and its metabolites are not easily analyzed by standard GC methods, a LC technique was adopted for their analysis.

Table 1

Optimum Wavelengths and Retention Times

Compound	Optimum Wavelenth (nm)	Retention Time (s)
2,6-Difluorobenzamide	260	1.3
4-Chlorophenylurea	245	1.6
4-Chloroaniline	245	2.7
Diflubenzuron	260	8.0
Nifedipine	238	3.9

Mobile phase: acetonitrile-methanol-water (50:2:48). Flow rate: 1mL min^{-1}. Injected volume: 25μL.

The chromatographic behaviour of these compounds was studied, using as mobile phase, solvents which are generally used in liquid chromatography with UV detection: acetonitrile, methanol, water, or mixtures of them. The best separation was achieved when a mixture acetonitrile-methanol-water (50:2:48) was used. At a flow rate of 1.0 mL min^{-1}, using isocratic elution and room temperature, all the compounds were eluted in less than 10 minutes. The retention times of all of them are given in Table 1. Figure 1 shows a chromatogram in optimal conditions.

The use of a DAD detector gave advantages in acquiring uv-spectra on-line and monitoring signals at different wavelengths simultaneously. Optimum selectivity was attained by choosing the appropriate wavelengths for each compound. The study of absorption spectra of these compounds showed that they had two significant maxims. The secondary maximums were chosen in order to avoid possible interferences, since the most serious drawbacks of UV detectors arise from matrix and solvent interferences working below 205 nm. This is the case of 2,6-difluorobenzamide that showed interferences at the wavelength 204 nm. Even though its absorbance was quite smaller, this compound could also be determined at 260 nm, where the interferences are less significants. The absorption spectra are given in Figure 1 and the wavelengths that were selected can be observed in Table 1.

In order to verify the linearity of the response of the detector for each compound at the arranged wavelengths, standard solutions of them were prepared and 25 μL samples were injected and analyzed at previously described chromatographic conditions.

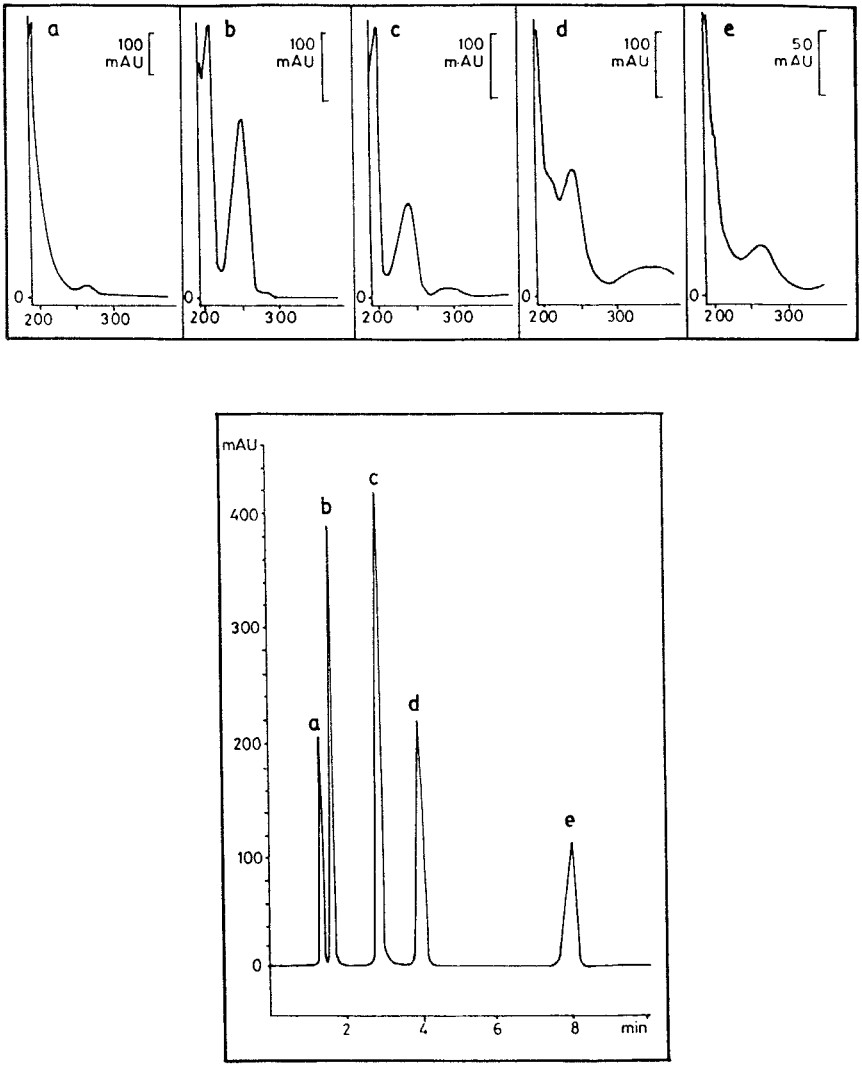

Figure 1. Signal plus spectra plot of: (a) 2,6-difluorobenzamide, (b) 4-chlorophenylurea, (c) 4-chloroaniline (d) nifedipine and (e) diflubenzuron. Standard concentration: 10 mg mL^{-1}. Chromatogram in optimum conditions: acetonitrile-methanol-water (50:2:48), flow rate 1.0 mL min^{-1}, injected volume 25 μL. Spectra range: 190-400 nm. Slit: 2 nm. Threshold: 0.1 mUA.

Table 2

Statistical Data for Calibration Graphs Detection and Quantification Limits

Compound	Slope	Intercept	r^2	DL (ng mL^{-1})	QL (ng mL^{-1})
2,6-Difluorobenzamide	0.0532	0.0004	0.9989	9.4	26.8
4-Chlorophenylurea	2.0325	0.0204	0.9993	0.3	7.3
4-Chloroaniline	1.6501	0.0113	0.9996	0.3	5.3
Diflubenzuron	0.9135	0.0110	0.9996	0.6	9.3

Table 3

Precision of the Method Study of Intra/Interdays and Retention-Time, RT, Repetitivity

Amount Range (ng mL^{-1})	Compound	Intraday* %RSD	Interday* %RSD	RT %RSD
60	2,6-Difluorobenzamide	4.5	2.7	0.6
	4-Chlorophenylurea	1.3	2.4	0.5
	4-Chloroaniline	2.8	2.6	1.8
	Diflubenzuron	4.5	4.9	1.1
300	2,6-Difluorobenzamide	1.2	1.4	0.5
	4-Chlorophenylurea	1.3	1.0	0.4
	4-Chloroaniline	1.0	1.6	2.9
	Diflubenzuron	0.9	1.7	0.9

* Intraday assay variance was calculated from the assay values of prepared standards on a single day (n=10). Interday, in alternative days on a 4-week period (n=15).

The calibration was achieved using the internal standard method. The selected internal standard was nifedipine, since its chromatographic behaviour was similar to diflubenzuron and its metabolites. Besides, it had a suitable retention time (3.9 min) and a secondary absorption maximum at 238 nm, which could be supposed not to interfere with signals of forestry matrices. The linear calibration plots correspond to the equation.

$$Area_{pesticide}/Area_{internal\ standard} = n + m\ C_{pesticide}/C_{internal\ standard}$$

where C is the concentration in ng mL^{-1}, m is the slope and n the intercept of the straight line.

A linear response was observed from 26.8, 7.3, 5.3 and 9.3 to 10000 ng mL^{-1} of 2,6-difluorobenzamide, 4-chlorophenylurea, 4-chloroaniline, and diflubenzuron injected respectively.

The detection limits, DL, (established as 3σ criterium) and the quantification limits, QL, (established as 7σ criterium) are shown in Table 2.

In order to check the precision of the method ten replicated injections of the standard solutions were carried out. These standards were 60 and 300 ng mL^{-1} in each compound and were analyzed at the prefixed analytical conditions. The relative standard deviations were lower than 1.4% at 300 ng mL^{-1} and lower than 4.5% at 60 ng mL^{-1} for all of them.

These same standards were also analyzed at intervals over a 4-week-period. The relative standard deviations in this case were acceptably good as well, as they can be seen in Table 3.

The deviation in retention time was lower than 1.2% for all the compounds excepting for 4-chloroaniline, which presented a more irregular chromatographic behaviour in relation to this parameter (Table 3). Thus, these results demonstrate the accuracy and precision of the method.

Application to Assay in Forestry Matrices

This analytical method was used to determine the residual diflubenzuron and its mentioned metabolites on pine-needles. Thus, the samples of pine-needles were supplied with 2.5 mg Kg^{-1} of each compound. This amount is estimated to be in the range of the expected concentrations in pine groves.

A study of the extraction method was carried out. The solvent, the shaking time, and the ratio between the pine-needle weight and the volume of the solvent were studied. The best results were obtained using acetonitrile as solvent in a ratio of 3 g of pine-needles to 25 mL solvent and shaking for 10 minutes.

Table 4

Average Recoveries of Spiked Samples*and Standard Deviations (n=5) in Different Solid-Phase Extraction Cartridges

	2,6-Difluor.	4-Chloroph.	4-Chloroa.	Difluben.
NH_2	101 ± 2.3	83.0 ± 2.3	98.8 ± 1.7	99.9 ± 2.6
Florisil	77.5 ± 2.2	5.5 ± 3.0	81.0 ± 0.5	55.5 ± 1.5
Diol	79.0 ± 7.5	60.0 ± 12.0	82.5 ± 3.8	83.0 ± 7.0
QMA	81.0 ± 1.8	15.3 ± 7.9	86.2 ± 2.1	74.0 ± 4.5
CN	93.5 ± 2.3	83.0 ± 2.7	95.7 ± 0.5	101.5 ± 2.6
C18	90.0 ± 2.0	87.0 ± 3.7	102.3 ± 4.5	87.8 ± 8.5

* Samples spiked with 2.5 mg Kg^{-1} of each compound and cleaned up through aminopropyl cartridge.

Subsequently, the clean-up stage was carried out by testing different solid-phase extraction cartridges, in order to determine the optimum extraction conditions. The reported extraction recoveries indicate that the best results were obtained with aminopropyl (-NH_2), which retained matrix interferences and eluted the studied compounds.

The recoveries of the supplied pine-needle samples were determined to estimate the effectiveness of the clean-up procedure (Table 4).

The chromatograms of the blank and a spiked sample are shown in Figure 2. Both of them were obtained using the proposed method of extraction and clean-up.

In order to test the accuracy of the extraction and clean-up procedures, peak purity studies were made. The spectra of each peak of the extracted samples were identified by comparing them with the spectra of the corresponding standards and later on, the peak purities were calculated for all of them.[32]

After correcting the background and smoothing the noise, each spectrum was compared with all spectra across the same peak and the calculated match factors were used to plot the spectral similarity curves. The threshold curves were used to demonstrate the significance of the match factor.[33]

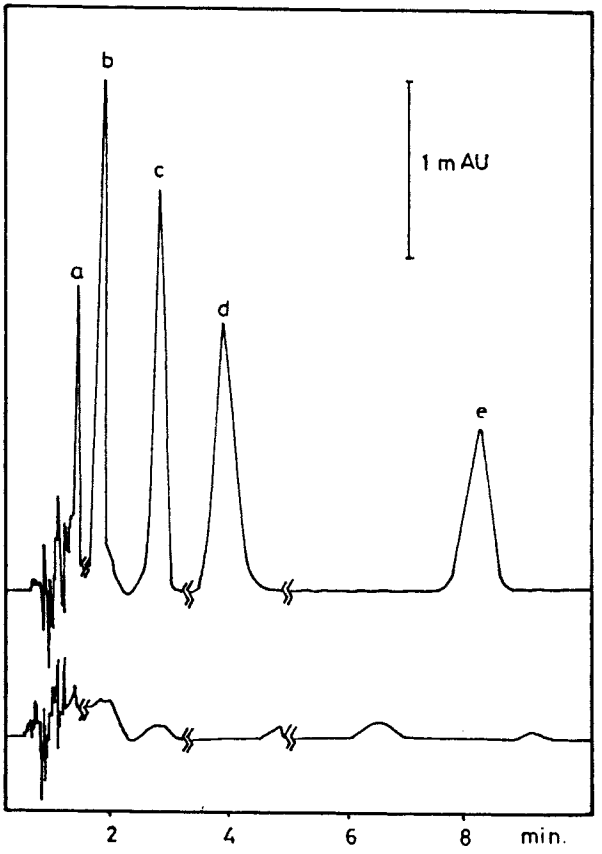

Figure 2. Chromatograms of the pine-needle blanks and a spicked standard: (a) and (e) 260 nm; (b) and (c) 245 nm; (d) 238 nm. Other conditions as in Figure 1.

In addition, the multiple absorbance ratios were used to generate the ratiograms. The ratios were plotted at all wavelengths relative to wavelength of maximum absorbance, giving a very quick overview of the purity of all peaks within the chromatogram. The ratiograms of each compound and their similarity and threshold curves are shown in Figure 3. The flat plot of each ratiogram proved the purity of the peaks. The similarity curves showed match factors higher than 950 in all cases (961, 995, 991 and 970 respectively). These values are inside the range of the automatch scores, what proved the suitability of the proposed methods of extraction and clean-up.

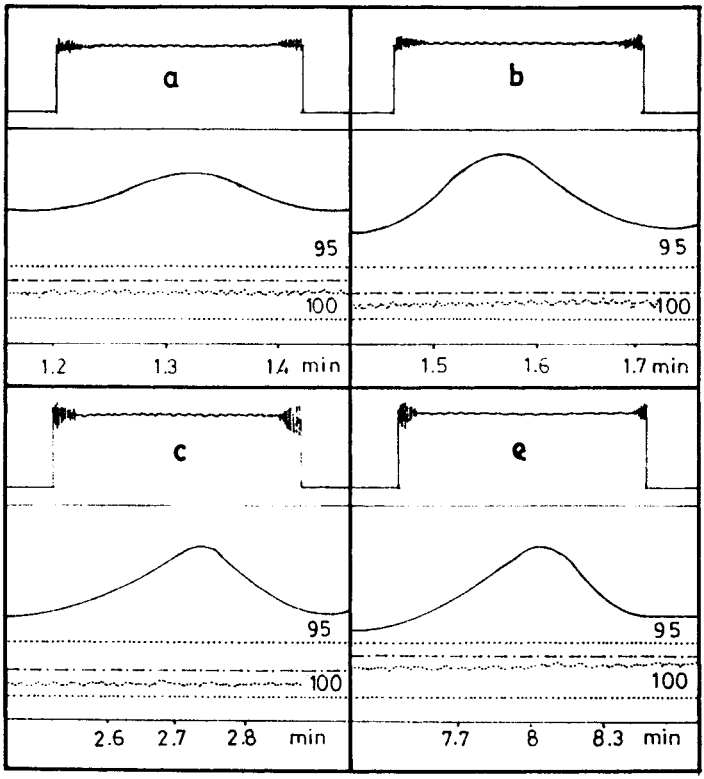

Figure 3. The ratiograms, similarity curves (·····) and threshold curves (·-·-) of (a) 2,6-difluorobenzamide, (b) 4-chlorophenylurea, (c) 4-chloroaniline and (e) diflubenzuron. Spiked samples, 2.5 mg Kg^{-1}.

The proposed analytical method allows determinations in pine-needles of 1.1, 0.3, 0.2, 0.4 mg Kg^{-1} in 2,6-difluorobenzamide, 4-chlorophenylurea, 4-chloroaniline, and diflubenzuron, respectively. Some experiments carried out in this laboratory suggest that a sensitivity 10 times higher could be reached by concentrating the extract to 2.5 mL, before passing it through the cartridge. A larger analysis time should be expected in that case.

This method, that has not been previously reported, was used to determine diflubenzuron and the mentioned metabolites in samples from pine groves after aerial applications with satisfactory results.

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