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Application of an internal surface reversed-phase column for the automated determination of flucycloxuron residues

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ABSTRACT

A liquid chromatographic column-switching system for the automated determination of flucycloxuron, a benzoylphenylurea pesticide, in crop and environmental matrices is described. The system consists of an internal surface reversed-phase (ISRP) column, a phenyl-bonded precolumn and an analytical reversed-phase (RP) C_{18} column. Sample extracts are evaporated to dryness and dissolved in the mobile phase of the ISRP column. An aliquot of this solution is injected into the column-switching system. Clean-up, with regard to removal of large molecules, is performed on the ISRP column. The flucycloxuron fraction from the ISRP column is concentrated on the phenyl-bonded precolumn. Additional clean-up can be performed by washing the precolumn. Finally, the compound is desorbed from the precolumn and separation and determination of the Z- and E-isomers of flucycloxuron are performed with the analytical RP-C_{18} column using UV detection at 254 nm. The total analysis time required is 40 min. The reproducibility of the method obtained with the column-switching system, expressed as relative standard deviation, varies between 3.7 and 10% for apple, strawberry, citrus and soil samples for flucycloxuron levels between 0.04 and 0.33 mg/kg. The system showed no loss of analytical performance after more than 300 analyses.

INTRODUCTION

Problems encountered in residue analysis are often caused by non-selective extraction procedures. Apart from the compound of interest, many matrix constituents are co-extracted and they can disturb the chromatographic determination and/or decrease the lifetime of a separation column. Sample extracts are, therefore, often subjected to a special clean-up step. Size-exclusion chromatography (SEC) is a widely used clean-up technique to remove high-molecular-mass components that could interfere with liquid or gas chromatographic determination of analytes, such as pesticides and other organic compounds, in environmental matrices [1–5].

Flucycloxuron is a benzoylphenylurea which has acaricidal and insecticidal properties. It is an experimental pesticide, to be used for protection of fruit and vegetable crops against a range of insects and mite species [6]. Extraction of flucycloxuron from these fruits and vegetables is performed by macerating the tissue with a suitable organic solvent.

Clean-up from matrix constituents is usually performed by liquid–liquid extraction or by adsorption chromatography using silica gel or Florisil.

Flucycloxuron is determined by high-performance liquid chromatography (HPLC) on a reversed-phase (RP) C_{18} column. The clean-up steps mentioned have the disadvantages that they are time consuming, they are not easy to automate and they always cause some loss of analyte, which unfavourably influences the reproducibility. We investigated on-line LC clean-up based on size-exclusion chromatography (SEC).

Bio-beads, an SEC packing material, can only be used for off-line clean-up procedures, as the mobile phase of this kind of column [gel permeation chromatography (GPC)] is not compatible with RP mobile phases [1,2]. Heart cutting is an alternative way to use a GPC column, with polystyrene–divinylbenzene (PS–DVB) as packing material and tetrahydrofuran as mobile phase coupled on-line with RP-HPLC. This method has been described for the analysis of malathion in tomatoes and lemonin in

grapefruit peel [4,5]. Williams *et al.* [7] developed an interface in order to couple non-aqueous SEC on-line with RP-HPLC. A GPC column based on PS–DVB, with tetrahydrofuran as mobile phase, was used. The eluate from this column, containing the analyte, was mixed with water and transferred via a switching valve to an analytical RP-C_{18} column. This method was described for the determination of 2,6-di-*tert.*-butyl-4-methylphenol in snacks and of dibutyl phthalate in chocolate at levels above 0.5 mg/kg. From initial experiments, we concluded that the clean-up performance of a PS–DVB column was not sufficient for our purposes, as the greater part of the matrix constituents had molecular masses that did not differ sufficiently from the molecular mass of flucycloxuron to obtain an acceptable separation. As flucycloxuron is almost insoluble in water, we started our study with a gel filration chromatographic (GFC) column packed with a cross-linked methacrylate for clean-up purposes in flucycloxuron analysis. This material could be used with a mobile phase containing up to 20% of organic modifier but we concluded that, apart from size exclusion, adsorption also occurred to such an extent that it was impossible to obtain an acceptable chromatographic behaviour.

Our efforts were more sucessful with another type of SEC, using an internal surface reversed-phase (ISRP) column which was introduced by Pinkerton and co-workers [8,9] in 1985. ISRP is a new concept for LC packing materials for bioanalytical purposes. The ISRP material has the ability to exclude large molecules, such as proteins, from the pores with negligible adsorption of these molecules on the external surface. Low-molecular-mass compounds, such as drugs, are separated with good capacity, selectivity and efficiency. Several methods have been described for the direct injection of biological matrices, such as serum and plasma, for the determination of drugs [10–14], in which proteins did not interfere with the chromatographic analysis. A review on the use of ISRP material was published by Pinkerton [15].

As fruit and vegetable extracts also contain relatively large molecules, we tried this type of packing as on-line LC clean-up material. We compared the results obtained with the ISRP column-switching system with the results obtained using standard methods, using liquid–liquid extraction and classical (low-pressure) column chromatography.

EXPERIMENTAL

Apparatus and materials

The chromatographic system consists of the following apparatus: a Model 231-401 diluter and programmable autosampler (Gilson, Villiers le Bel, France) fitted with a 100-μl loop; an HP1050 quaternary pump (Hewlett-Packard, Waldbronn, Germany), an HP1050 isocratic pump; three pneumatically operated Model 7010 valves (Rheodyne, Cotati, CA, USA); a Model 757 variable-wavelength UV detector (Applied Biosystems, Ramsey, NJ, USA); and an HP3396A integrating system (Hewlett-Packard). All three Rheodyne valves and two HP1050 pumps were controlled by the Gilson 231-401 autosampler. A Pinkerton ISRP GFF-S5-80 (5 μm) column (50 × 4.6 mm I.D.) was obtained from Regis (Morton Grove, TL, USA). A Zorbax ODS reversed-phase column (250 × 4.6 mm I.D.) was purchased from Chrompack (Middelburg, Netherlands). Precolumns filled with C_8 (10 μm), C_{18} (7 μm) and phenyl-bonded (5 μm) (all 30 × 4.6 mm I.D.) and PRP-1 (10 μm) (15 × 3.0 mm I.D.) materials were obtained from Brownlee Labs. (Santa Clara, CA, USA).

Acetonitrile and tetrahydrofuran used in preparing the mobile phases were of HPLC quality and were purchased from Baker (Deventer, Netherlands); ethanol was of pharmaceutical quality and distilled water was of HPLC quality. Solvents used for sample extraction (dichloromethane, acetonitrile and methanol) were of residue quality, obtained from Baker, or were purified by distillation. Light petroleum (b.p. 40–65°C) was obtained from Shell and purified by distillation. Florisil was obtained from Supelco (Bellefonte, PA, USA). Silica cartridges (500 mg) and alumina cartridges (neutral, 1000 mg) were obtained from Baker. An Ultra Turrax Model T 50 homogenizer (Janke & Kunkel, IKA Lab. Technology, Staufen, Germany) was used for grinding and extraction of samples. Fruit and soil samples were obtained from field trials. Flucycloxuron, (*E,Z*)-N-[({4-[({[(4-chlorophenyl)cyclopropylmethylene]amino}oxy)methyl]phenyl}amino)-carbonyl]-2,6-difluorobenzamide, obtained from our own resources, has the following structure:
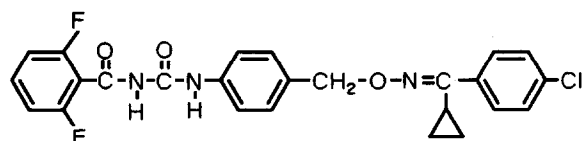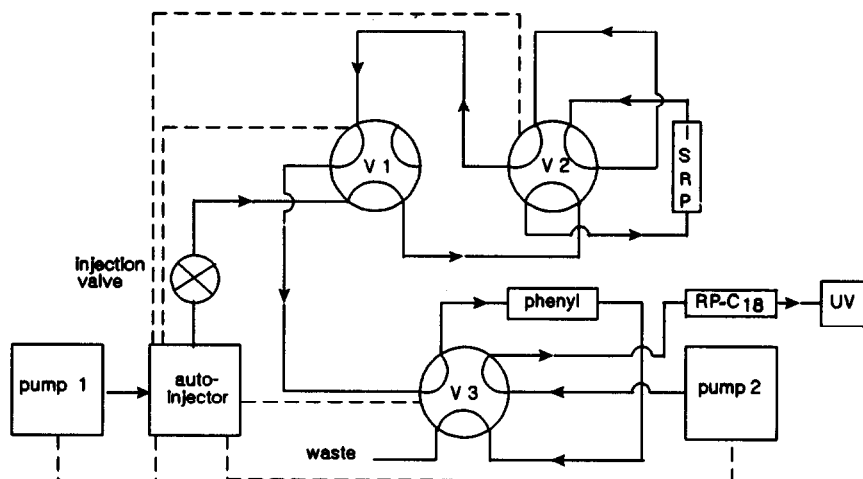
Fig. 1. Schematic representation of column-switching system.

Chromatographic system

The chromatographic system is presented in Fig. 1. Table I shows the schedule of the automated analysis; each valve position 1 corresponds to the position shown in Fig. 1.

In status A, the sample has been diluted with water in order to obtain a solution with the same composition as the ISRP mobile phase. Flucycloxuron cannot be stored in the mobile phase as the solubility during a longer period is insufficient. In

TABLE I

SCHEDULE OF THE AUTOMATED ANALYSIS OF SAMPLES FOR FLUCYCLOXURON USING ISRP AS ON-LINE CLEAN-UP PROCEDURE

Status	Time (min)	Pump 1					Pump 2	Description	Valve position		
		Flow-rate (ml/min)	Proportions (v/v)				Flow-rate (ml/min)		V_1	V_2	V_3
			Water	ACN[a]	THF[a]	EtOH[a]					
A	—	1.00	70	15	15	0	2.50	Sample dilution	1	1	1
B	0.00	1.00	70	15	15	0	0.00	Clean-up ISRP	1	1	2
B	7.00	1.00	70	15	15	0	0.00				
C	7.01	1.00	70	15	15	0	2.50	Preconcentration	1	1	1
C	12.99	1.00	70	15	15	0	2.50				
D	13.00	5.00	70	15	15	0	2.50	Wash precolumn	2	1	1
D	14.99	5.00	70	15	15	0	2.50	(clean-up)			
E	15.00	0.00	70	15	15	0	2.50	Desorption	1	1	2
E	17.00	0.00	70	15	15	0	2.50				
F	18.00	1.00	0	0	100	0	2.50	Back-flush ISRP	1	2	1
F	19.00	1.00	0	0	100	0	2.50				
F	20.00	0.00	70	15	15	0	2.50				
G	21.00	1.00	0	0	0	100	2.50	Wash precolumn	2	1	1
G	22.00	1.00	0	0	0	100	2.50				
G	23.00	0.00	70	15	15	0	2.50				
H	24.00	1.00	70	15	15	0	2.50	Ready for A	1	1	1

[a] ACN = Acetonitrile; THF = tetrahydrofuran; EtOH = ethanol.

status B, the ISRP eluate is directed to waste. In status C, the ISRP eluate is directed to the phenyl precolumn in order to preconcentrate flucycloxuron. In status D, an additional clean-up can be performed by washing the precolumn with the ISRP mobile phase. It is possible to increase the washing step up to a volume of *ca.* 35 ml (we determined the breakthrough volume to be 42 ml). After preconcentration and an additional clean-up step, flucycloxuron was eluted from the precolumn with acetonitrile–ethanol–water (50:15:35, pump 2) to the analytical column (status E). To prevent increasing back-pressure and decreasing ISRP performance, the ISRP column was back-flushed with tetrahydrofuran (status F). In status G, the precolumn was washed with ethanol and subsequently equilibrated with the mobile phase. The total procedure, including quantification by HPLC, is performed in 40 min.

Extraction procedures

Weighed amounts (50–100 g) of apple, strawberry, citrus and soil samples were extracted with 200–250 ml of dichloromethane (apple and strawberry), methanol (citrus) and acetonitrile (soil). For grinding, a top-drive mixer or Ultra Turrax homogenizer was used. For apple, strawberry and soil samples, sodium sulphate (50–100 g) was added to the samples to bind the water. After filtration, the solvent was removed by rotary evaporation. Clean-up from matrix constituents is performed, according to standard procedures, either by liquid–liquid partitioning (dichloromethane–water and acetonitrile–hexane) and adsorption chromatography on Florisil and alumina (citrus), by adsorption chromatography on silica (soil) or Florisil (apple and strawberry) only or by ISRP column switching. Final determination of the *Z*- and *E*-isomers of flucycloxuron is performed, in both instances, by chromatography on an RP-C$_{18}$ column with acetonitrile–water–ethanol (50:35:15) as mobile phase and using UV detection at 254 nm.

RESULTS AND DISCUSSION

ISRP chromatography

Before studying the clean-up performance of an ISRP column, we optimized the chromatography of flucycloxuron on the ISRP column (5 μm) (50 ×

4.6 mm I.D.). In the analysis of samples such as plasma and serum for drugs, the mobile phase may contain up to 20% (v/v) of any of four organic solvents: acetonitrile, isopropanol, methanol or tetrahydrofuran [16]. A combination of these solvents must not exceed the limit of 20% when blood serum or plasma samples are assayed. This upper level is applied, as serum albumin is denatured in mixtures containing over 20% of organic modifier. Denatured proteins cause clogging of the stationary phase. However, the ISRP stationary phase can withstand higher concentrations of organic solvents as Regis, the supplier of the ISRP columns, advises [11] flushing of the column with pure tetrahydrofuran in the case of increased back-pressure.

Optimization of the chromatography of flucycloxuron resulted in the mobile phase composition tetrahydrofuran–acetonitrile–water (15:15:70). The flow-rate of the mobile phase was 1.00 ml/min and the column temperature was 35°C. In this case, the mobile phase contains over 20% of organic modifier. However, this caused no problems as fruit and vegetable samples contain few proteins. As mentioned by Pinkerton and Koeplinger [17], the diol–Gly–Phe–Phe-bonded phase favours the retention of aromatic compounds and separates species primarily by a reversed-phase mechanism. We concluded that the retention behaviour of flucycloxuron did not fully correspond with that on reversed-phase material. When tetrahydrofuran was replaced with acetonitrile, the capacity factor did not increase as in reversed-phase chromatography, but remained almost constant. As flucycloxuron is a neutral component, this difference cannot be caused by the carboxylic group of the ISRP material, which is a weak cation exchanger.

ISRP clean-up

We prepared samples as described under *Extraction procedures*. Sample extracts were dissolved in the ISRP mobile phase and 100 μl of these solutions were, after filtration, injected directly on to the ISRP column. The outlet of the column was connected to a UV detector set at 254 nm. The chromatograms of apple, strawberry and soil samples showed that clean-up effects obtained with the ISRP column were good. The *Z*- and *E*-isomers of flucycloxuron were well separated from the bulk of the matrix constituents. After the injection of several samples,

we observed an increased back-pressure and decreased performance. This problem was overcome by back-flushing the ISRP column with tetrahydrofuran after each sample injection.

Preconcentration and HPLC analysis

The fraction in which flucycloxuron elutes from the ISRP column is too large to be transferred directly to the analytical RP-C_{18} column. A precolumn was used to preconcentrate the ISRP fraction in which flucycloxuron elutes and to perform an additional clean-up step. The choice of precolumns depended on the following criteria: the precolumn should not have any adverse effect on the chromatography of flucycloxuron on the RP-C_{18} column and the breakthrough volume should exceed 6 ml, as the ISRP fraction had a volume of 6 ml.

The breakthrough volumes were determined for precolumns (30 × 4.6 mm I.D.) packed with C_8 (10 μm), C_{18} (7 μm), phenyl-bonded (5 μm) and PRP-1 (10 μm) (15 × 3.0 mm I.D.) materials. The breakthrough volumes were determined by injecting 100 μl of a flucycloxuron solution (composition of ISRP mobile phase) containing *ca.* 1 μg/ml on to the precolumn. The precolumn was washed with a variable volume of the ISRP mobile phase, after which on-line desorption was carried out to the analytical RP-C_{18} column using acetonitrile–water–ethanol (50:35:15) as the mobile phase. The breakthrough volumes were between 30 and 50 ml and were sufficient in all instances. The phenyl-bonded (5 μm) precolumn (30 × 4.6 mm I.D.) with a breakthrough volume of 42 ml was selected for further experiments, as this column has the smallest particles and would contribute least to peak broadening on the analytical column.

Analytical data

Calibration solutions were used to study the repeatability, linearity and memory effects (alternate injections of solutions containing 50 μg/ml flucycloxuron and blank solutions) of the total system. Repeatability was studied by tenfold injection of three calibration solutions at levels of 1, 5 and 10 μg/ml [sum of *Z*- (30%) and *E*- (70%) isomers]. Relative standard deviations (R.S.D.) determined for the *Z*- and *E*-isomers ranged between 9.9% (for the lowest *Z*-isomer level, 0.3 μg/ml) and 3.4%. The average R.S.D. was 5%. Linearity was determined

by a twelve-point calibration graph at levels between 0.3 and 10 μg/ml (sum of *Z*- and *E*-isomers). The correlation coefficient was 0.9993 for both isomers. The memory effect was less than 0.3%.

Applications

Extracts of blank and treated samples were prepared according to the procedures outlined under *Extraction procedures*. Extracts were prepared to perform analysis in quintuplicate, according to both the standard and ISRP methods. In this way, we compared the clean-up performance obtained with the column-switching system with that of the standard method. Typical chromatograms, obtained from strawberry and citrus samples, are presented for both methods in Figs. 2 and 3.

Fig. 2a and b show the chromatograms of analyses of a strawberry blank and a strawberry sample (treated with flucycloxuron) analysed with the standard method and Fig. 2c and d show the chromatograms of the same samples analysed with the column-switching system. Fig. 2a shows a component that interferes with flucycloxuron determination at lower levels. It was difficult to remove this component using traditional clean-up procedures. However, using the ISRP system, this component was completely eliminated. We have therefore obtained a better clean-up procedure, without performing any off-line clean-up step, within a reduced analysis time.

Fig. 3a and b show the chromatograms of the analysis of a citrus blank and a citrus sample (treated with flucycloxuron), respectively, according to the standard method, and Fig. 3c and d show the chromatograms of the same samples analysed according to the ISRP method. As the extracts of citrus samples contained such a large amount of sample constituents that interfered with the HPLC determination of flucycloxuron, it was necessary to perform an extended clean-up procedure. Using the ISRP method, the off-line clean-up could be limited to one extra liquid–liquid extraction step. This resulted in a sharply reduced analysis time (1.25 h instead of 2.75 h/sample, calculated from a series of eight samples) in comparison with the standard method with the same clean-up effect. During optimization of the automated clean-up procedure, we observed that additional washing of the phenyl-bonded precolumn with a volume of 10 ml of the
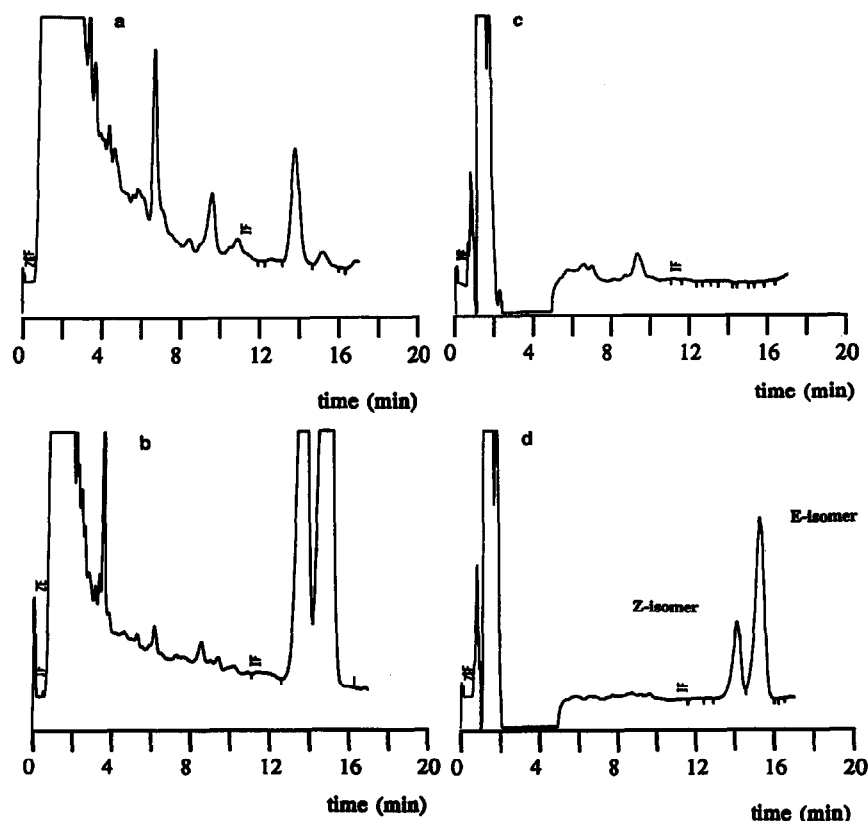
Fig. 2. Determination of flucycloxuron in strawberry samples. Amounts of 100 g of (a) blank sample and (b) a treated sample [ca. 0.5 mg/kg (Z- + E-isomers)] were analysed according to the standard method. An aliquot of the extract (100 ml from 250 ml) was taken and the solvent was evaporated. The residue was dissolved in a mixture of 3 ml of dichloromethane and 25 ml of light petroleum (b.p. 40–65°C) and submitted to Florisil clean-up; the final volume was 3.00 ml. A 100-μl volume was injected on to an RP-C_{18} column with acetonitrile–ethanol–water (50:15:35) as mobile phase. The same samples were analysed according to the ISRP method (c and d): 100 g of sample were extracted, the solvent was removed by evaporation and the residue was dissolved in 2.00 ml of acetonitrile–tetrahydrofuran (1:1). This solution was placed in the Gilson autosampler, where 300 μl were mixed with 700 μl of water. A 100-μl volume of this acetonitrile–tetrahydrofuran–water (15:15:70) mixture were injected into the column-switching system.

ISRP mobile phase resulted in a better clean-up performance. Two components with retention times almost equal to that of flucycloxuron were removed by this additional wash step of the phenyl-bonded precolumn.

The results of flucycloxuron analyses in several matrices are summarized in Table II. All analysis were performed in quintuplicate. For all samples, the amount of flucycloxuron residue determined by using the ISRP method was equal to or greater than that determined by the standard method. The R.S.D.s calculated from the five individual results of the ISRP method were satisfactory compared with the results of the standard method.

CONCLUSIONS

The ISRP material has excellent clean-up properties in residue analysis of flucycloxuron in crop and soil matrices. The combination of SEC and RP chromatography, which is characteristic for the ISRP material, has shown much better clean-up properties from matrix constituents than customary SEC techniques.

The column-switching system consists of an ISRP column, a phenyl-bonded precolumn and an RP-C_{18} analytical column. The clean-up is performed on the ISRP column. The fraction in which flucycloxuron elutes from the ISRP column is preconcen-
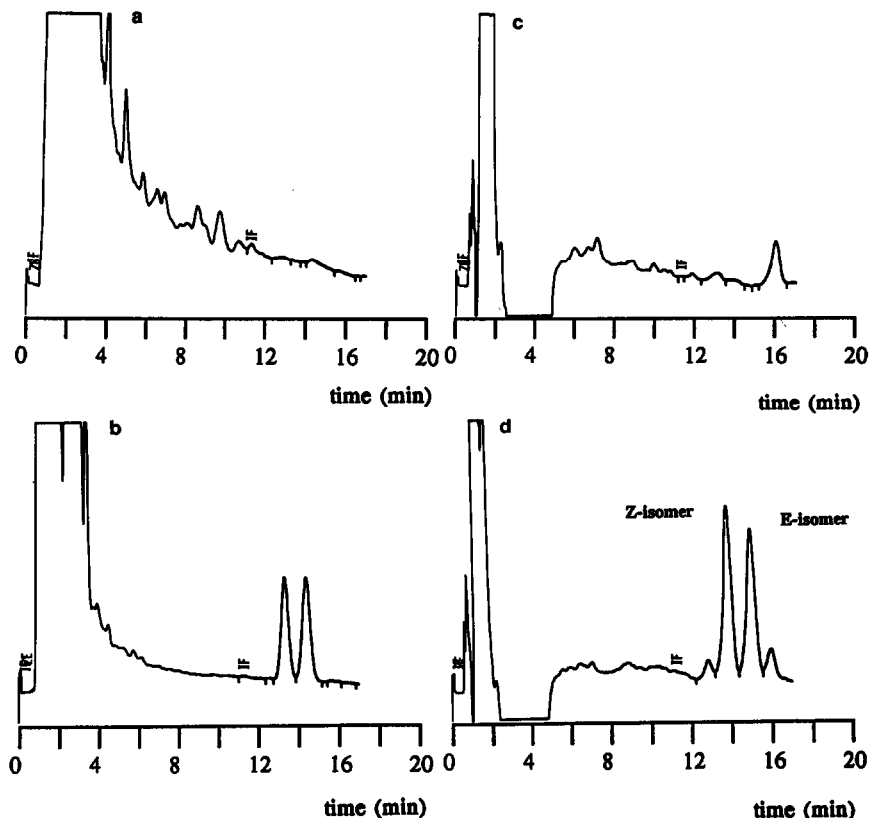
Fig. 3. Determination of flucycloxuron in citrus samples. Amounts of 100 g of (a) blank sample and (b) a treated sample [ca. 0.1 mg/kg (Z- + E-isomers)] were analysed according to the standard method. After evaporation of the methanol, the extract contained only water. An extra 150 ml of water were added and the flucycloxuron was extracted with three 50-ml portions of dichloromethane. The dichloromethane of the combined extract was evaporated and the residue was dissolved in 100 ml of hexane. The flucycloxuron was extracted with three 50-ml portions of acetonitrile. After evaporation of the acetonitrile, the residue was dissolved in a mixture of 3 ml of dichloromethane and 25 ml of light petroleum (b.p. 40–65°C) and submitted to Florisil and alumina chromatography. Finally, the residue was dissolved in 3.00 ml of the mobile phase and an aliquot of 100 μl was injected on to an RP-C_{18} column with aceto-nitrile–ethanol–water (50:35:15) as mobile phase. The same samples were analysed according to the ISRP method (c and d): 100 g of sample were extracted, the extract was reduced to contain only water by evaporation and then extracted with hexane. The residue was dissolved in a 2.00 ml of acetonitrile–tetrahydrofuran (1:1). This solution was placed in the Gilson autosampler, where 300 μl were mixed with 700 μl of water. A 100-μl volume of this acetonitrile–tetrahydrofuran–water (15:15:70) mixture was injected into the column-switching system.

TABLE II

COMPARISON OF STANDARD METHOD AND ISRP METHOD FOR RESIDUE ANALYSIS OF FLUCYCLOXURON

Type of sample	Standard method				ISRP method			
	Z-Isomer		E-Isomer		Z-Isomer		E-Isomer	
	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)
Clay soil	0.058	2.3	0.133	1.0	0.059	6.6	0.133	6.1
Apple	0.143	7.6	0.070	7.8	0.145	10.0	0.071	9.0
Strawberry	0.173	5.7	0.328	6.5	0.203	4.4	0.334	3.7
Citrus	0.058	20.3	0.050	15.8	0.068	9.1	0.057	9.8

trated on the phenyl-bonded precolumn. If desired, additional clean-up can be performed by washing the precolumn. After desorption from the precolumn, separation and determination of flucycloxuron are performed on an RP-C_{18} column with acetonitrile–ethanol–water (50:15:35) as mobile phase. The column-switching system is suitable for performing automated sample clean-up of apple, strawberry, citrus and soil samples for residue analysis of flucycloxuron. The total chromatographic analysis time is 40 min. The total analysis time is less than 1.5 h per sample, for all sample types tested, based on a series of eight samples.

Over 300 samples were analysed without a noticeably decreased performance of the ISRP column. Compared with standard methods, using off-line clean-up procedures, such as liquid–liquid extraction and classical column chromatography, the present method has the advantages of reduced loss of analyte (higher recovery), higher selectivity and considerable reduction in analysis time. The column-switching system may also be applicable for the determination of micro-organic pollutants in matrices such as soil. Another application could be automated off-line (perhaps even on-line) clean-up in GC analysis.

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