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Determination of the pesticide diflubenzuron in mushrooms by high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry

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Abstract

A method using high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry (HPLC–APCI-MS) has been developed and validated for the determination of the insecticide diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] in mushrooms. Samples were homogenised with acetone, extracted into dichloromethane–cyclohexane and further cleaned-up by size-exclusion chromatography (SEC). HPLC was performed on an ODS column with methanol–water at 1 ml/min. The limit of detection was 0.02 ng/μl (equivalent to 0.017 mg/kg in the crop). The calibration was linear over the range 0.025–1.0 ng/μl. Recovery of diflubenzuron from spiked mushrooms (0.06–0.58 mg/kg) was 85.5% with a relative standard deviation of 14.5% ($n = 56$).

1. Introduction

Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, M_r 310.0321, Fig. 1], first reported by Van Daalen et al. in 1972 [1], belongs to a group of insecticides which are effective as stomach and contact poisons and act by inhibition of chitin synthesis, thus interfering with the formation of the insect cuticle [2,3]. Therefore, all insect stages in which new cuticles are formed should be susceptible to diflubenzuron. It has a broad range of applications including use against the larvae of *Sciaridae* and *Phoridae* in mushrooms [4].

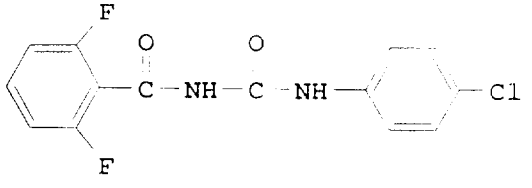

Fig. 1. Structure of diflubenzuron.

A number of analytical methods have previously been reported for the determination of diflubenzuron in various matrices. Although diflubenzuron is not itself amenable to GC, its thermal decomposition in the GC injector to form 4-chlorophenyl isocyanate, 4-chloroaniline, and 2,6-difluorobenzamide, has been used as the basis of a GC–MS method [5].

Methods involving HPLC with UV detection have been reported for various matrices includ-

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ing bolti fish (*Orechromis niloticus*) [6], forestry substrates [7], cabbage under sub-tropical conditions [8], a range of environmental samples including soil, sediment, agricultural crops, milk, eggs and animal tissues [9], and adult stable flies [10]. However, for the analysis of both chillies and plums, Wilkins has reported that a method based on multiple-wavelength UV detection was very prone to interferences [11].

An HPLC–MS method using thermospray (TSP) ionisation in the positive ion mode for the determination of diflubenzuron in foodstuffs was reported by Wilkins [11]. The base peak of the spectrum was the 2,6-difluorobenzamide ion (m/z 175) produced by loss of *p*-chlorophenyl isocyanate, and the protonated molecule (m/z 311) was present with an intensity of 80% of the base peak. Due to interferences at m/z 175 quantitation was based on the protonated molecule which resulted in a detection limit equivalent to 0.25 mg/kg of analyte in the crop.

Recently the HPLC–MS ionization technique of atmospheric pressure chemical ionisation (APCI), shown in several reports to be viable for the determination of various classes of pesticide residues [12,13], has become particularly attractive for routine application following the introduction of relatively low cost, dedicated instrumentation [14]. APCI is a gas-phase ion–molecule reaction process which leads to the ionisation of analyte molecules under atmospheric pressure conditions. The process is analogous to chemical ionisation but the reactant ions are produced from the effect of a corona discharge on a nebulised aerosol of solvent. Due to the atmospheric pressure conditions the high frequency of analyte/reactant ion collisions ensures a high sample ionisation efficiency. The ionisation is soft and results predominantly in protonated molecules $[M+H]^+$ in the positive ion mode or deprotonated molecules $[M-H]^-$ in the negative ion mode.

Our laboratory has previously carried out the analysis of diflubenzuron in mushrooms using a HPLC–UV method [15] which provides a detection limit of 0.10 mg/kg, limited by matrix-derived interferences. Although this detection limit is equivalent to the Codex maximum residue level (MRL) [16], higher sensitivity and a more specific technique for confirmation were desirable. We now report the application of HPLC–MS using APCI to the measurement of residues at lower concentrations.

2. Experimental

2.1. Materials

HPLC grade methanol and cyclohexane and glass-distilled grade acetone and hexane were obtained from Rathburn Chemicals (Walkerburn, UK). HPLC water was obtained from Fisons Scientific Equipment (Loughborough, UK) and pesticide grade dichloromethane from Merck (Leicestershire, UK). Granular AR anhydrous sodium sulphate was obtained from Fisons and was heated at 420°C for 4 h prior to use.

Polyethylene glycol (PEG) was a mixture of PEG 300, 600, and 1000 obtained from Koch Light (Haverhill, Suffolk, UK) and BDH (Dagenham, Essex, UK). Diflubenzuron standard was obtained from Promochem (Welwyn Garden City, UK) with a certified purity of 99%. Cultivated mushrooms were purchased from various retail outlets.

2.2. Preparation of standards and spiked samples

Two separate sets of calibration standards at concentrations of 0.025, 0.05, 0.10, 0.20, 0.50 and 1.0 ng/μl were prepared in (a) methanol–water (80:20) and (b) pooled extracted sample matrix (known to be blank).

Spiked mushrooms samples for recovery determination were prepared by the addition of an appropriate amount of a standard stock solution (7 μg/ml in hexane) to finely chopped mushrooms (30 g) which were left to stand for a few minutes before extraction to allow the spike solution to penetrate the mushrooms. Spiked

concentrations were 0.06, 0.12, 0.23 and 0.58 mg/kg.

2.3. Extraction and clean-up

Extraction of diflubenzuron was based on the method reported by Andersson and Palsheden [17]. Samples were homogenised with 100 ml of acetone for 3 min using a laboratory blender (Ultra-Turrax) and filtered under vacuum through a sintered glass funnel. The filtrate was transferred to a 500-ml separating funnel and shaken with 200 ml cyclohexane–dichloromethane (1:1). On standing two distinct layers were formed, with the lower layer containing water originating from the sample matrix. The lower layer was transferred to a second separating funnel and the upper organic layer was decanted through anhydrous sodium sulphate into a 500-ml round-bottom flask. The aqueous layer was further extracted with two successive 70-ml portions of dichloromethane. At each successive extraction the organic layer was decanted through anhydrous sodium sulphate and combined with the organic fraction from the first extraction. Using a rotary evaporator (controlled at 30°C and 300 mbar) the organic extract was concentrated to near dryness, transferred to a volumetric flask (5 ml) and made to volume with cyclohexane–dichloromethane (1:1). The extract was filtered through an Anachem (Luton, UK) 0.45-μm Nylon 66 syringe filter, and an aliquot (1 ml) injected on to a size-exclusion chromatography (SEC) system consisting of a glass column (500 × 10 mm I.D.) packed with Bio-Beads S-X3 (Bio-Rad, Hemel Hempstead, UK), a Gilson (Villiers-le-Bel, France) 232-401 automated sample processor and a Spectra-Physics (San-Jose, California) Isochrom HPLC pump. The mobile phase for SEC was cyclohexane–dichloromethane (1:1) at a flow-rate of 1 ml/min. (The dump time and collect time of 16 and 30 min respectively were determined by injecting extracts of mushrooms spiked with diflubenzuron and calculating the recoveries obtained.) The collected fraction was taken to very near dryness under a gentle stream of oxygen-free nitrogen. The cleaned up extract was transferred to a volumetric flask (5 ml) with methanol–water (80:20) and made up to volume.

2.4. HPLC

HPLC was performed using a Hichrom (Berkshire, UK) S5ODS2 (250 × 4.6 mm I.D.) column preceded by a S5ODS2 (10 × 4.6 mm I.D.) guard column. The mobile phase was methanol–water delivered at a flow-rate of 1 ml/min by a Spectra-Physics SP8800-20 pump, with a composition gradient consisting of 80% methanol for 8 min, a linear increase over 0.1 min to 95% methanol which was maintained for 9.9 min, a decrease to 80% methanol over 0.1 min and re-equilibration at 80% methanol for 7.9 min giving a total run time of 26 min. Both solvents were degassed by helium sparging prior to use and filtered through 10-μm inlet filters.

Partial-loop (50 μl) injections were made via a Gilson 231 XL autosampler fitted with a 100-μl loop on a Rheodyne (Cotati, CA, USA) 7125 injection valve. A solid-block column heater was used to maintain the column at a temperature of 45°C (Jones Chromatography, Mid Glamorgan, UK). Injections were made on to the HPLC column at 26.5 min intervals. Each sample or standard was analysed in duplicate.

2.5. Mass spectrometry

Mass spectra were obtained on a VG Platform "Classic" (Fisons Instruments, Altrincham, UK) bench-top mass spectrometer. Following preliminary evaluation of positive and negative ion modes (see below) all measurements were made using the latter. The instrument was initially tuned on background ions and mass calibrated in the positive ion mode on a mixture of PEG 300, 600 and 1000. Tuning parameters were then optimised in the negative ion mode on the deprotonated molecule (m/z 309) of diflubenzuron [10 ng/μl solution in methanol–water (80:20, v/v) injected directly into the mass spectrometer via a 500-μl Rheodyne loop]. Typical operating conditions were: corona 2.74 kV,

high voltage lens 0 kV, extraction 5 V, focus 10 V, source temperature 120°C, probe temperature 450°C, low mass resolution 14.0, high mass resolution 15.0, ion energy 0.7 V, ion energy ramp 0.0, multiplier 650.

Scanned acquisitions were made over the mass range 60–500 with a scan time of 1 s.

Single-ion monitoring (SIM) was performed at *m/z* 289.02, 291.03, 309.02, 310.03 and 311.04. The dwell time for each channel was 0.1 s, the interchannel delay was 0.02 s and the mass span was 1 mass unit. SIM data were collected during the first 10 min of the 26-min chromatographic run. The retention time of diflubenzuron was approximately 5.5 min. Quantitation was based on the area under the peak in the mass chromatogram of the deprotonated molecule (*m/z* 309).

3. Results

In preliminary experiments using full seanned acquisitions and loop injections containing 1 μg of diflubenzuron, both positive and negative ionisation modes were evaluated. No ions attributable to diflubenzuron were observed in the positive mode whilst the negative mode gave an intense $[M-H]^-$ ion (*m/z* 309) and consequently negative ionisation was used for all further studies.

As noted by Kawasaki et al. [18], in HPLC–APCI-MS the APCI probe temperature can have a large effect on sensitivity. Fig. 2 shows the effect of temperature on the peak area of the deprotonated molecule (*m/z* 309). The response clearly optimised at a probe temperature of 450°C which was used for subsequent measurements.

Fig. 3a shows the spectrum of diflubenzuron obtained at low extraction and focus voltages (5 and 10 V respectively) where $[M-H]^-$ was the base peak. Higher extraction and focus voltages (30 and 35 V respectively) caused increased fragmentation (Fig. 2b) and under these conditions the deprotonated difluorobenzamide fragment (*m/z* 156) was the base peak. Such "cone voltage" induced fragmentation provides the potential for generation of alternative confirmatory ions but at the expense of molecular ion sensitivity. To minimise fragmentation and thus maximise the sensitivity for the deprotonated molecule (*m/z* 309) on which quantitation was based, low extraction and focus voltages were used for quantitation experiments.

Fig. 4 shows the five ions monitored by SIM from a 0.10 ng/μl diflubenzuron standard (equivalent to 0.085 mg/kg in the matrix assuming a

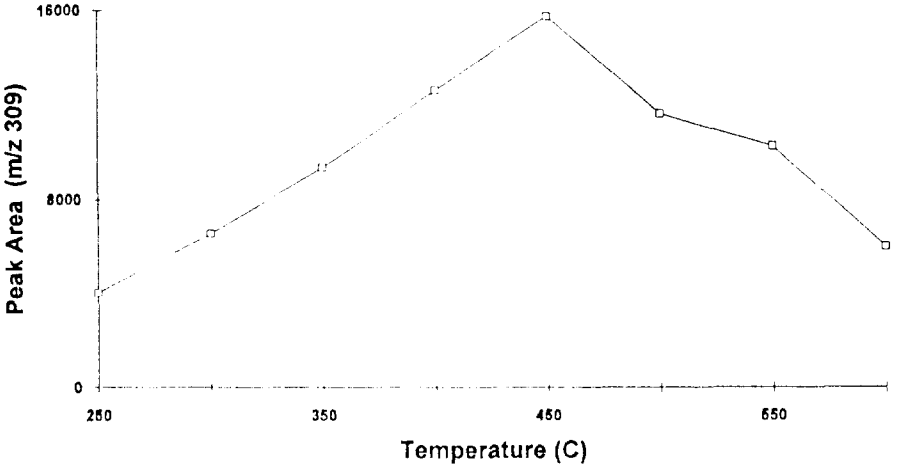

Fig. 2. Effect of the APCI probe temperature (°C) on peak area (arbitrary units) of the deprotonated diflubenzuron molecule (*m/z* 309). The peak area was measured on 10-μl loop injections of a 1 ng/μl standard.

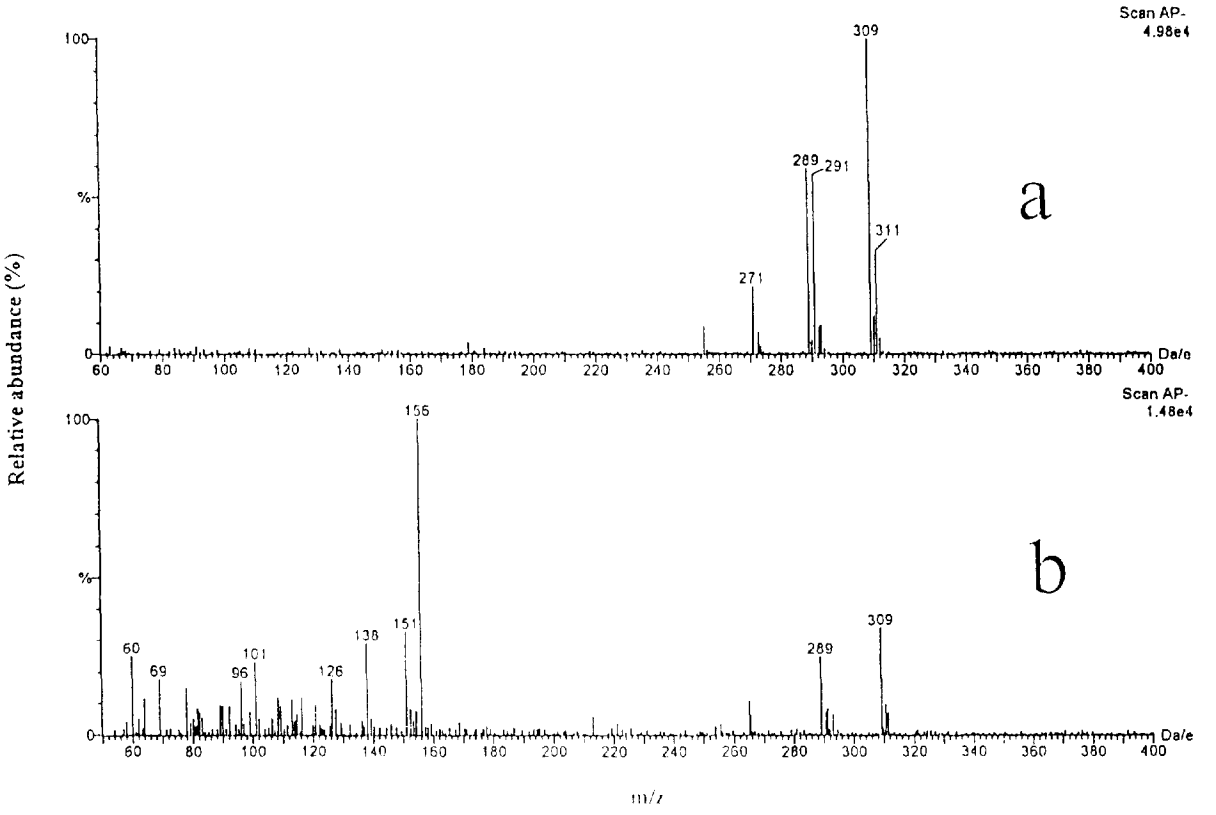

Fig. 3. Spectrum of diflubenzuron obtained at (a) low extraction and focus voltages (5 and 10 V, respectively), the base peak of which is the deprotonated molecule at *m/z* 309 and (b) higher extraction and focus voltages (30 and 35 V, respectively) the base peak of which is the deprotonated difluorobenzamide fragment at *m/z* 156.

100% recovery) prepared in the mobile phase. The isotope ratio observed for the deprotonated molecule was as expected for a compound with a single chlorine atom, with a ratio of 3 between the ions at *m/z* 309 and 311. For a total of 249 injections, including both standards and extracts, the relative standard deviation of the observed ratio (mean 2.97) was 9%, giving an indication of the measurement precision of the mass spectrometer.

Calibration curves for standards in methanol–water were found to be linear over the 0.025–1.0 ng/μl range (Fig. 5). Over a 5-week period, during which time the high voltage electrode and skimmer cone were cleaned several times, the mean correlation coefficient was 0.993 with a standard deviation of 0.020 ($n = 18$). However, the sensitivity on different occasions varied considerably, the extremes differing by a factor of 30. In the absence of an internal standard frequent recalibration was thus essential.

The calibration obtained when using standards prepared in matrix extracts was examined on two separate occasions and was also found to be linear (correlation coefficients of 0.994 and 0.988). Differences in slope were observed compared with calibrations using methanol–water solutions, but on one occasion the slope was greater (ratio 1.3) and on the other less (ratio 0.84) than the slope from the solvent standards. In view of this, and since the series compared were analysed consecutively and not interspersed, we believe the difference in slope reflects changes in instrument sensitivity rather than an effect attributable to the presence of co-extractives.

The use of methanol–water or matrix extract standards was found to influence precision. Thus

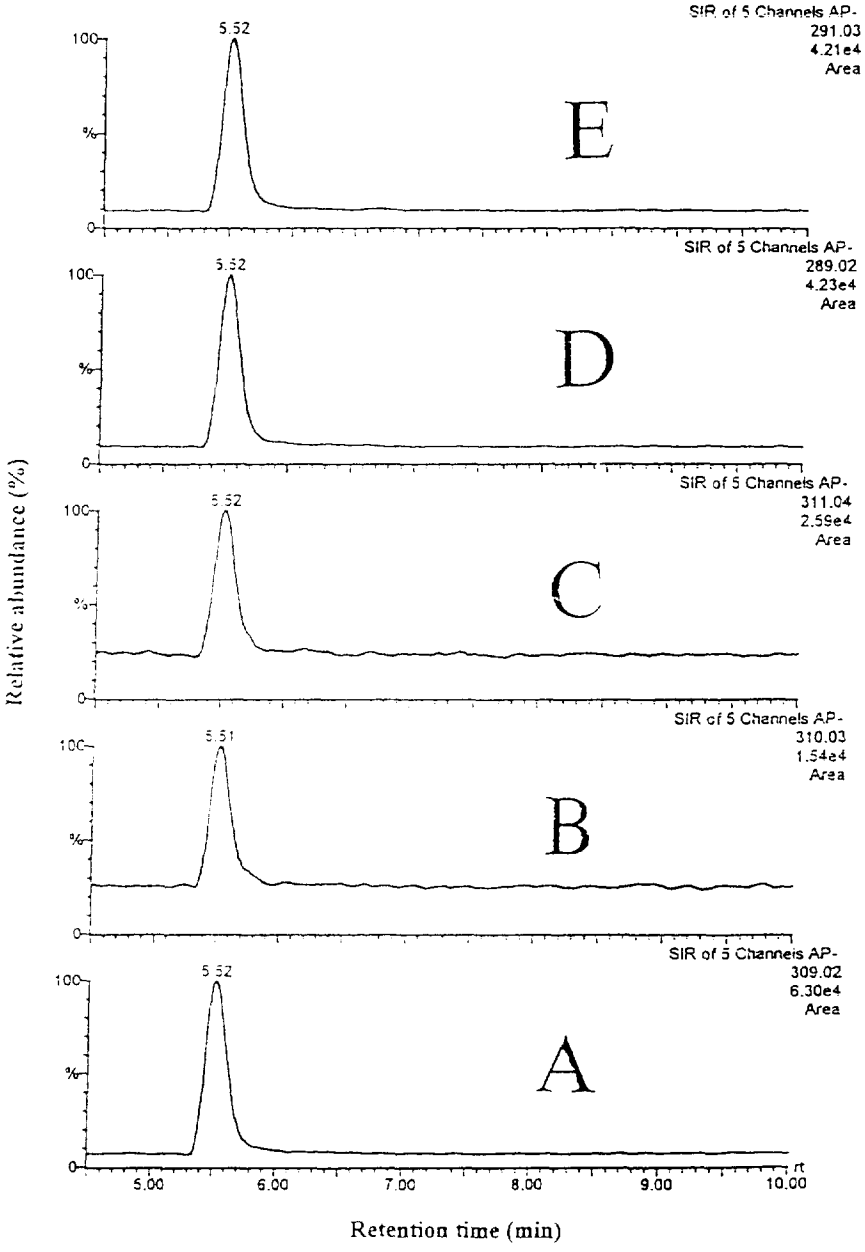

Fig. 4. The five ions monitored by SIM in a 0.10 ng/μl diflubenzuron standard prepared in methanol–water (80:20) mobile phase. (A) m/z 309, (B) m/z 310, (C) m/z 311, (D) m/z 289, (E) m/z 291.

the relative standard deviation at the 0.10 ng/μl level for standards prepared in solvent was 3% ($n = 9$) but for the same concentration in matrix extract was higher at 8% ($n = 9$). Consequently solvent-based standards were used throughout to quantitate the extracts.

Having established linearity, quantitation of extracts was routinely based on calibrations

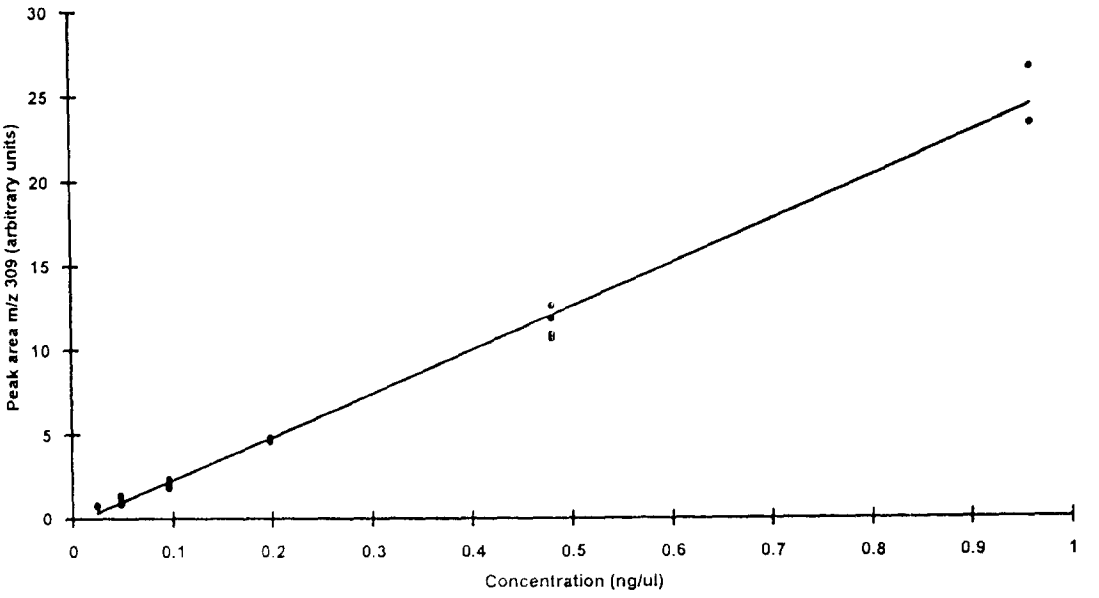

Fig. 5. Calibration of peak area (m/z 309) over the concentration range 0.025–1.0 ng/μl.

derived from standards in methanol–water at concentrations of 0.05, 0.20 and 0.50 ng/μl which were analysed before and after each batch of extracts (typically 10). The mean slope was used in calculations unless large deviations between the two sets of standards were observed, in which case the whole batch was re-analysed.

For quality control additional diflubenzuron standards (0.025–0.50 ng/μl in methanol–water) were also analysed at intervals. Over a 5-week period the measured concentrations had a mean of 96% of the theoretical value ($n = 35$), corresponding to a bias of −4%, and a relative standard deviation of 11%, thus demonstrating good accuracy and precision. The mean of results for standards prepared in blank extract was 92% of their theoretical value ($n = 20$), corresponding to a greater bias of −8%, and a relative standard deviation of 10%. Both sets of data were quantified from solvent standard calibrations and the accuracy of the results for the extract standards justifies the decision to use solvent based standards for quantitation.

Extrapolation based on a number of determinations of the 0.05 ng/μl standard prepared in blank extract gave a limit of detection (LOD) of 0.017 ng/μl [3 times the total standard deviation ($n = 14$)] which is equivalent to 0.0142 mg/kg in the crop with the extraction method employed and assuming 100% recovery. This theoretical LOD was consistent with the observed response for a 0.025 ng/μl extract standard.

Fig. 6 shows the channel for the diflubenzuron $[M - H]^-$ ion at m/z 309 for 0.06, 0.12, 0.23 and 0.58 mg/kg spiked extracts and for an extract blank.

The recovery of diflubenzuron spiked into mushrooms before extraction at concentrations from 0.06 mg/kg to 0.58 mg/kg was assessed in a series of seven replicate extraction batches (Table 1). The mean recovery was 85.5% with a relative standard deviation of 14.5% ($n = 56$). Allowing for recovery the average limit of detection of the method was 0.017 mg/kg in the crop.

4. Conclusions

This HPLC–APCI-MS method relies for quantitation on the deprotonated molecule of

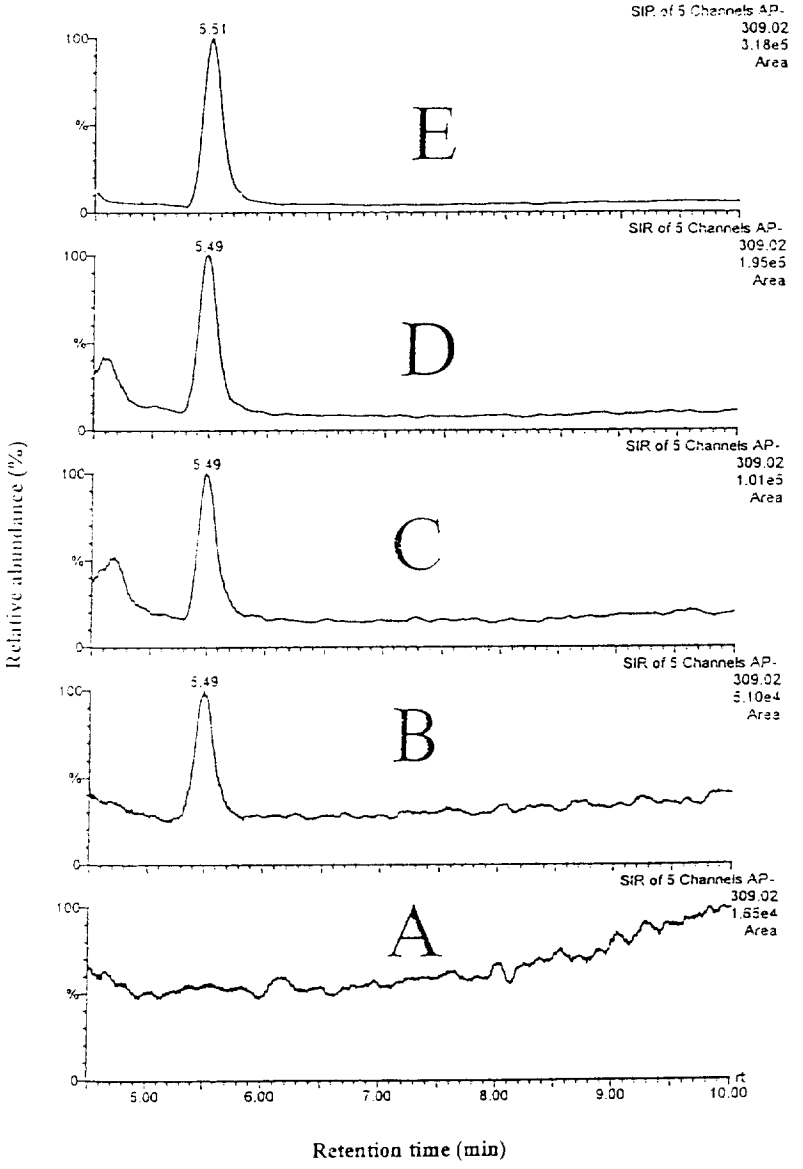

Fig. 6. The diflubenzuron [M − H]⁻ ion at m/z 309 for an extract blank and spiked extracts. (A) Extract blank, (B) 0.06 mg/kg spike, (C) 0.12 mg/kg spike, (D) 0.23 mg/kg spike, (E) 0.58 mg/kg spike.

diflubenzuron which is the base peak of the scanned spectrum. This has the advantage over previously described GC and TSP methods which rely on either fragment ions or non-base peak ions for quantitation. The limit of detection is an order of magnitude better than that obtained by HPLC–TSP-MS, and six times less than the MRL. Once optimised, tuning was found to be stable and data have been acquired for up to 72 h non-stop. Some changes in sensitivity were encountered over time but this was adequately compensated for by frequent calibration. For the determination of diflubenzuron in mushroom extracts this HPLC–APCI-MS method has proven robust with suitable accuracy and precision.

Table 1
Recovery of diflubenzuron from spiked mushrooms determined at four concentrations and in seven separate extraction batches

Spiking concentration (mg/kg)	Trial No.	Extraction batch							Between batch	
		1	2	3	4	5	6	7	Mean	R.S.D. (%)
0.06	1	78.4	107.0	98.5	77.9	76.8	86.6	85.3	88.8	10.7
	2	93.5	86.3	96.6	74.1	63.4	99.8	93.2	88.1	12.3
0.12	1	76.2	78.0	107.3	86.8	91.0	120.2	99.7	94.1	14.8
	2	72.7	80.0	115.9	79.5	84.5	102.7	91.3	89.6	14.0
0.23	1	53.0	75.0	88.6	60.4	88.3	91.8	103.1	79.6	16.7
	2	58.7	71.0	90.5	59.3	82.3	87.7	101.5	78.4	15.1
0.58	1	80.1	69.6	106.0	84.9	83.7	96.9	72.8	84.0	11.9
	2	80.5	65.9	106.0	82.7	77.6	94.0	74.6	82.2	12.2
Within-batch	Mean	74.1	79.1	101.2	75.7	81.0	97.5	90.2		
Within-batch	R.S.D. (%)	12.9	13.0	9.3	10.6	8.6	10.8	11.7		

A single extract of each spiking concentration was prepared in each batch and analysed in duplicate (Trial 1 or 2). The overall mean recovery was 85.5% with a relative standard deviation of 14.5%

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