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Determination of enantiomeric purity of paclobutrazol and fluazifop-P-butyl using a diode-laser-based polarimetric high-performance liquid chromatography detector

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ABSTRACT

Enantiomeric purities of resolved enantiomers of the agrochemicals paclobutrazol (I) and fluazifop-P-butyl (II) have been determined using a diode-laser-based polarimetric high-performance liquid chromatography detector. Reversed-phase achiral chromatography was used with polarimetric and absorbance detectors in series to measure optical rotation, α, and absorbance, A. In blind trials enantiomer mole fractions in unknowns, x_u, were calculated from a standard, x_s, and α/A values using the equation

$$(\alpha/A)_u/(\alpha/A)_s = (2x_u - 1)/(2x_s - 1).$$

The method always gave x_u within 1% of actual values. 95% confidence limits were roughly twice those from chiral chromatography and better than conventional polarimetry. The linear range of the polarimetric detector was 0.02 – 10 mg ml^{-1} for I and 0.1 – 10 mg ml^{-1} for II (20 μl injection). Limits of detection for I and II of 0.19 and 1.0 μg correlate with specific rotations of the compounds.

INTRODUCTION

With the advent of laser-based polarimetric detectors specially designed for high-performance liquid chromatography (HPLC)[1–4], the reduction of noise to microdegree levels in optical rotation (OR) measurements has allowed chiral molecules to be quantitated with detection limits in the 0.1–1-μg range. Applications of

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polarimetric detection in HPLC have been recently reviewed[5]. The use of absorbance and polarimetric detectors coupled in series to monitor peaks eluting from an achiral chromatography column has been shown to provide a simple method for determination of the enantiomeric purity of compounds for which a standard of known purity is available[2,6,7]. This technique was first applied to a permethrinic acid insecticide[6] and a detailed account of the precision and accuracy of the method has recently been given for the pharmaceuticals ephedrine hydrochloride and pseudoephedrine hydrochloride[8].

In the present paper the technique is applied to two agrochemicals, paclobutrazol and fluazifop-P-butyl, with particular reference to establishing 95% confidence limits and comparison with alternative procedures using chiral separation or polarimetry of bulk samples for quality control of these compounds which are used in enantiomerically pure form.

Paclobutrazol, (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol (I, Fig. 1), is one of a new class of plant growth regulators that affect both vegetative and reproductive components of fruit tree growth[9] and is a broad-spectrum growth retardant with a wide range of uses[10]. Paclobutrazol possesses two chiral centres, so four optical isomers may be separated. The 2*S*,3*S* enantiomer, the active ingredient in plant growth regulation, inhibits the biosynthesis of gibberellins, the plant hormones that primarily regulate the elongation of shoots[10] while the 2*R*,3*R* component has low plant-growth activity but high fungicidal activity[11]. The 2*R*,3*S* and 2*S*,3*R* diastereoisomers of paclobutrazol are potent inhibitors of plant sterol biosynthesis[12].

Fluazifop-butyl, butyl(*RS*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionate (II, Fig. 1), is the active ingredient of a highly selective systemic postemergence herbicide for use against annual and/or perennial grasses in cotton, soybean and other broad leaf crops. Compound II has one chiral centre and only the *R*-(+) form, termed fluazifop-P-butyl, is generally herbicidally active.

Because the enantiomers display large differences in the nature and degree of biological activity, analyses which quantitate the enantiomeric forms are of considerable importance in studies of activity, mode of action, translocation, metabolism and persistence of these agrochemicals in the environment[13]. Many methods have been developed for chiral analysis, the most useful being those based on chromatographic separation. Achiral chromatography may be carried out after chiral derivatisation of I[12] and II[14], but this is a lengthy process. Such procedures require the chiral reagent to be optically pure and the reaction to proceed to completion without racemisation at any stage[15]. Separations using a chiral stationary phase (CSP)[14] are generally taken to

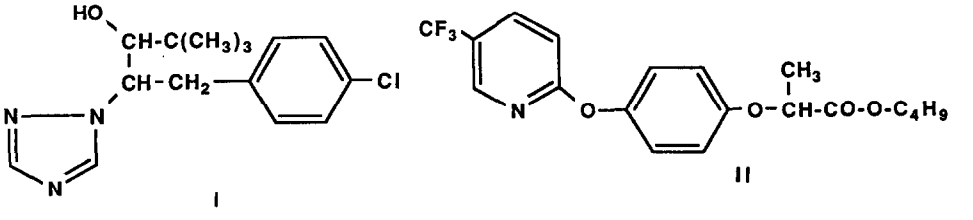

Fig. 1. Structural formulae of paclobutrazol (I) and fluazifop-butyl (II).

be the standard methods of analysis. Such analyses are not ideal for quality control because many CSPs are less robust and considerably more expensive than those for normal achiral chromatography.

EXPERIMENTAL

Reagents and chemicals

Experimental samples, supplied by ICI Agrochemicals, were fluazifop-P-butyl, the racemate fluazifop-butyl, and the 2*R*,3*R* and 2*S*,3*S* enantiomers of paclobutrazol. Solutions of all compounds were prepared immediately prior to use, and filtered through a 0.45-μm filter. The HPLC mobile phases were prepared using deionized water (Elgastat UHQ water purifier), methanol, ethanol, hexane (all HPLC grade) and trifluoroacetic acid (analytical-reagent grade).

Instrumentation

The HPLC system consisted of a ternary gradient pump (ACS, Model 352), an injection valve (Rheodyne 7152) with a 20-μl loop, a variable-wavelength UV detector (ACS 750/12) operating at 265 nm for paclobutrazol and 300 nm for fluazifop-P-butyl, and a polarimetric HPLC detector (ACS ChiraMonitor) which utilises a diode laser light source at 820 nm and a cell with pathlength 0.16 dm and volume 23 μl. The UV data were collected and analysed on an integrator (Trivector Trio), whilst a chart recorder (Chessell) recorded the output from the polarimetric detector. A polarimeter (Perkin-Elmer 141) with a 1-dm pathlength silica cell thermostatted at 20°C was used to measure the optical rotation of fluazifop-P-butyl and both enantiomers of paclobutrazol at the sodium D line (589 nm) and mercury arc wavelengths (579, 546, 436, 405, 365, 313 and 302 nm). A UV spectrophotometer (Shimadzu UV 260) was used to measure absorbance spectra.

Chromatographic conditions

In the analysis of paclobutrazol, a Chiralcel OC column (25 cm × 4.6 mm I.D.) was used for chiral separation with a hexane–ethanol (95:5) mobile phase at a flow-rate of 2.0 ml min^{-1}. The achiral determination used a C_{18} column (Hichrom, 10 cm × 4.6 mm I.D.) with a methanol–water (70:30) mobile phase at a flow-rate of 1.0 ml min^{-1}.

In the determination of fluazifop-P-butyl, the chiral column was a Pirkle type (CPIA-3, 25 cm × 4.6 mm I.D.) packed with covalent D-phenylglycine. A mixture of hexane–methanol–trifluoroacetic acid (1000:0.9:0.5) was used as the mobile phase at flow-rates of 1.0 or 2.0 ml min^{-1}. Conditions for the achiral analysis were the same as for paclobutrazol with the exception of the mobile phase composition (methanol–water, 75:25).

All chromatographic experiments were carried out at ambient temperature.

RESULTS AND DISCUSSION

Paclobutrazol

Chiral chromatography. Chiral chromatography was carried out as described in the experimental section to establish the enantiomeric purity of 2*R*,3*R* and 2*S*,3*S* samples of paclobutrazol. The enantiomer mole fractions determined from UV peak areas are given in Table I.

TABLE I

ENANTIOMERIC PURITY OF PACLOBUTRAZOL SAMPLES

Sample	*Enantiomer mole fractions*	
	(2R,3R)	*(2S,3S)*
2*R*,3*R*	0.97	0.03
2*S*,3*S*	0.074	0.926

(2*R*,3*R*)-Paclobutrazol elutes first, and because of peak tailing and lack of baseline resolution the small mole fraction of the 2*S*,3*S* enantiomer in the (2*R*,3*R*) paclobutrazol sample carries higher uncertainty than does the 2*R*,3*R* content of the 2*S*,3*S* sample. The (2*S*,3*S*)-paclobutrazol sample was consequently taken as the standard for enantiomeric purity determinations in subsequent work.

Polarimetry. The observed rotation, α, of a compound containing mole fractions of (+)- and (−)-enantiomers x and $(1 - x)$ respectively is given from Biot's law as

$$\alpha = [\alpha](2x - 1)cl \tag{1}$$

where c is the mass concentration, l the cell pathlength, and $[\alpha]$ the specific rotation of the (+)-enantiomer. For the racemate, with $x = 0.5$, $\alpha = 0$; for the pure (+)-form, with $x = 1$, $\alpha = [\alpha]cl$; for the pure (−)-form, with $x = 0$, $\alpha = -[\alpha]cl$.

Eqn. 1 was used to obtain specific rotations at the sodium D line and mercury arc wavelengths using the (2*S*,2*S*)-(−)-paclobutrazol reference standard for which x had been found from chiral chromatography as described in the previous section.

Extrapolation of $[\alpha]$ to the wavelength of the diode laser, 820 nm, was carried out using the Drude equation applicable to a material with one dominant chiral chromophore[16]

$$\lambda^2[\alpha]_\lambda = \lambda_0^2[\alpha]_\lambda + K \tag{2}$$

where λ is the wavelength of measurement and λ_0 (the wavelength of the dominant chiral chromophore) and K are constants. Fig. 2 shows the plot of $\lambda^2[\alpha]_\lambda$ *versus* $[\alpha]_\lambda$, from which $[\alpha]_{820}^{20}$ was deduced to be 65.6 ± 2.4° ml g^{-1} dm^{-1} and λ_0 = 217 ± 2 nm. Paclobutrazol has two chiral centres, and λ_0 coincides with the 220 nm UV peak maximum which may be assigned to a π–π^* transition on the triazole ring attached to the C2 chiral centre[17].

Achiral chromatography. A representative achiral chromatogram of (2*R*,3*R*)-paclobutrazol is shown in Fig. 3 and the calibration plot of the OR response, log (peak height) *versus* log(concentration) is given in Fig. 4. The linear range of the method is from 0.02 to 10 mg ml^{-1} (for a 20-μl injection) of (2*R*,3*R*)-paclobutrazol with correlation coefficient of 0.99998 and slope of 1.005 ± 0.005. The limit of detection of the technique is 9.5 μg ml^{-1} injected concentration (0.19 μg on column) calculated using the equation[18]:

$$\text{LOD} = 3s_{y/x}/b \tag{3}$$

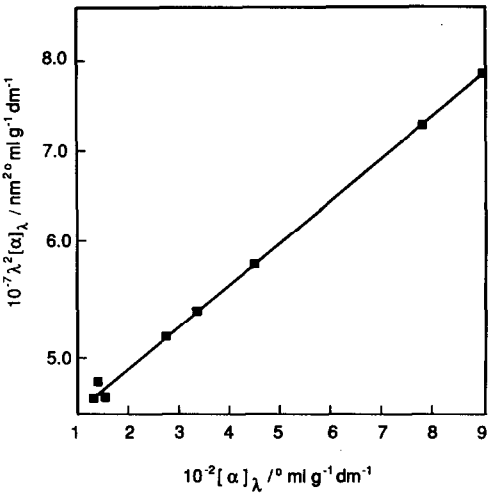

Fig. 2. Wavelength dependence of the optical rotation of (2*R*,3*R*)-paclobutrazol in methanol–water (70:30) measured at the sodium D line (589 nm) and mercury arc wavelengths (436, 405, 365, 313 and 302 nm) at 20°C.

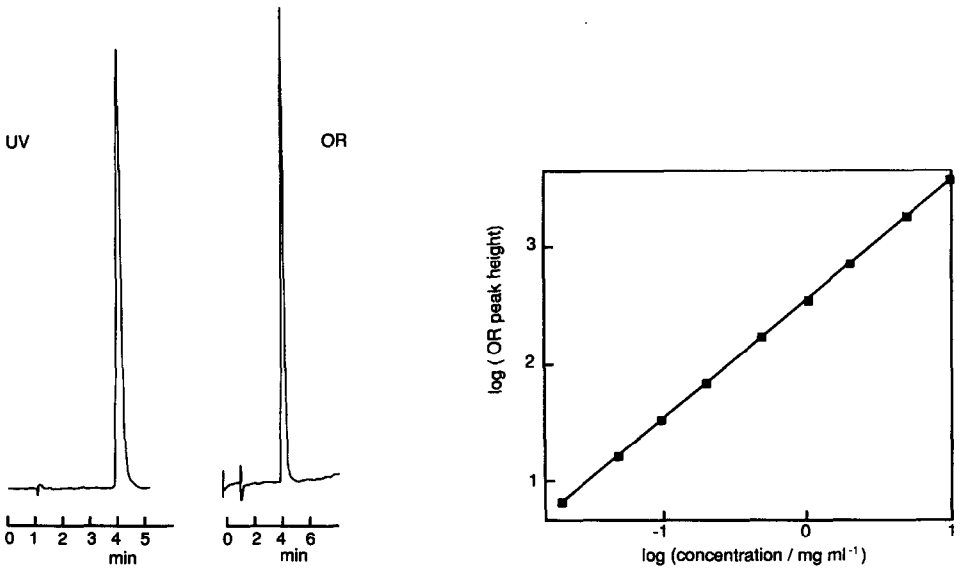

Fig. 3. Achiral chromatograms of (2*R*,3*R*)-paclobutrazol with dual optical rotation and UV absorbance detection. Column: Hichrom C_{18} (10 cm × 4.6 mm I.D.). Mobile phase: methanol–water (70:30). Flow-rate: 1.0 ml min^{-1}. Injection, 40 μg. ChiraMonitor attenuation 2, time constant 1 s and chart recorder 200 mV fsd.

Fig. 4. Linear response of the polarimetric detector. Plot of log(OR peak height) *versus* log(concentration/mg ml^{-1}) for (2*R*,3*R*)-paclobutrazol.

where $s_{y/x}$ is the root mean square (RMS) error on the data points and b the slope of the plot of OR peak height *versus* concentration using the lowest concentration points in Fig. 4. Eqn. 3 may be shown to follow[18] from the IUPAC recommendation[19] for the definition limit of detection $= 3\ s_B$, where s_B is the standard deviation on the blank signal.

Enantiomeric purity determination. From eqn. 1, the simplest method of determining the enantiomer mole fraction in a chemically-pure compound when an enantiomeric standard is available is to measure α/c values for unknown, u, and standard, s,

$$\frac{(\alpha/c)_u}{(\alpha/c)_s} = \frac{2x_u - 1}{2x_s - 1} \tag{4}$$

This is the procedure normally used when carrying out enantiomeric purity determination using conventional polarimetry.

Using the (2*S*,3*S*)-(−)-paclobutrazol as standard, with $x_s = 0.074$, x_u for the 2*R*,3*R*-(+)-sample was deduced from α/c ratios measured at 365 nm (the wavelength at which the highest sensitivity was obtained) to be 0.983 ± 0.010 (three replicate measurements).

An alternative procedure is to use achiral chromatography with polarimetric and spectrophotometric detectors in series, and calculate the enantiomeric purity from the ratio of the optical rotation and the absorbance (A) response, α/A, for unknown and standard samples[8],

$$\frac{(\alpha/A)_u}{(\alpha/A)_s} = \frac{2x_u - 1}{2x_s - 1} \tag{5}$$

This follows from eqn. 4 since A is directly proportional to c. As in previous sections the 2*S*,3*S* sample was used as standard, giving $x_u = 0.963 \pm 0.006$ (three measurements) for the (2*R*,3*R*)-(+)-paclobutrazol sample.

Table II compares the three methods for determination of enantiomeric purity. Mean values are given with their 95% confidence limits, calculated knowing the number of replicate measurements, n, and the t value for a confidence interval of 95%, t_{95} (ref. 18).

The achiral HPLC method with dual detectors is seen to give results in good agreement with chiral chromatography, and carries less uncertainty than does polarimetry on bulk samples. Perhaps of greatest importance when considering applications in quality control of agrochemicals is that the α/A method does not require chemically pure samples, and that it can be orders of magnitude more economical in material. To obtain the data in Table II, 40 μg was loaded for each HPLC injection whereas about 6 mg was required to fill the 6-ml polarimeter cell.

Fluazifop-P-Butyl

Chiral chromatography. Fig. 5 shows the chromatogram of fluazifop-P-butyl obtained using a chiral column under the conditions given in the experimental section. The mole fractions of each enantiomer in this sample and in the racemate

TABLE II

(2*R*,3*R*)-(+)-PACLOBUTRAZOL MOLE FRACTION DETERMINED BY THREE METHODS

Method	$\bar{x}_u \pm t_{95}s_{x_u}/\sqrt{n}$	*n*
(1) Chiral chromatography	0.97	
(2) Polarimetry	0.978 ± 0.010	3
(3) Achiral chromatography with OR and UV detectors	0.963 ± 0.006	3

fluazifop-butyl were calculated from the ratio of the peak areas of (+)- and (−)-forms. The results, given in Table III, establish the precision and accuracy of the method. It should be noted that the mole fractions for fluazifop-butyl agree within 95% confidence limits with those expected for the racemate.

Polarimetry. In the same way as for paclobutrazol, the optical rotations of fluazifop-P-butyl were measured at the sodium D line and mercury arc wavelengths (579, 546, 436, 405 and 365 nm) and $[\alpha]_\lambda$ calculated with eqn. 1. The $\lambda^2[\alpha]$ *versus* $[\alpha]$ plot (eqn. 2) gave $\lambda_0 = 237 \pm 5$ nm which corresponds to the 230 nm peak maximum in the UV spectrum, and $[\alpha]^{20}_{820} = 18.1 \pm 0.4°$ ml g^{-1} dm^{-1} (in methanol–water, 75:25).

Achiral chromatography. Fig. 6 gives an achiral chromatogram of fluazifop-P-butyl. Under the achiral conditions described in the experimental section, the linear range of the technique for the analysis of fluazifop-P-butyl was found to be 0.10–10 mg ml^{-1} with a correlation coefficient of 0.9989 for the log (OR peak height) *versus* log(concentration) plot (*cf.* Fig. 4). The slope and the RMS error of the plot of the OR height *versus* the concentration of II gave a detection limit of 1.0 μg using eqn. 3.

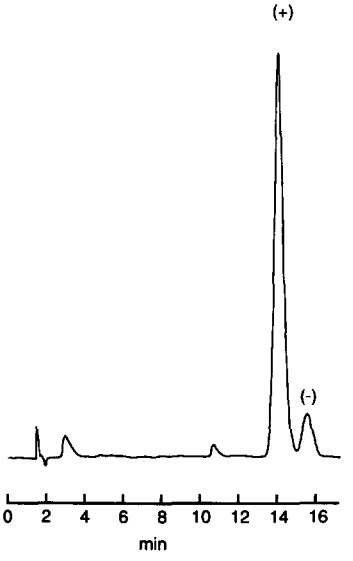

Fig. 5. Chiral chromatogram of fluazifop-P-butyl spiked with racemate fluazifop-butyl. Column: Pirkle CIPA-3 (25 cm × 4.6 mm I.D.). Mobile phase: hexane–methanol–trifluoroacetic acid (1000:0.9:0.5). Flow-rate: 1.0 ml min^{-1}. Total amount injected was 3.8 μg.

TABLE III

ENANTIOMERIC PURITY OF FLUAZIFOP-BUTYL SAMPLES FROM CHIRAL CHROMATOGRAPHY

Sample	*Enantiomer mole fraction* ($\bar{x}_u \pm t_{95}s_{x_u}/\sqrt{n}$)		n
	(+)-form	*(−)-form*	
Fluazifop-P-butyl	0.948 ± 0.005	0.052 ± 0.005	9
Fluazifop-butyl	0.501 ± 0.004	0.499 ± 0.004	4

The detection limits for I and II may be compared with the theoretical values obtained by considering the noise level on the detector. The RMS noise was measured to be 2.3 ± 0.6 microdegrees (1 s time constant). This is taken as the theoretical figure for the standard deviation in the blank signal, s_B (eqn. 3). Theoretical limits of detection of $3s_B$ are presented in Table IV. Eqn. 1 was used to convert from rotation to concentration, and the measured peak width to determine dilution between concentrat ions at injection and at peak maximum in the OR cell. There is good agreement between the theoretical and the observed LOD values, 0.19 μg for I and 1.0 μg for II injected on column (1-s detector time constant). It should be noted that the LOD is inversely proportional to the specific rotation and directly proportional to the peak width. The former factor contributes × 3.6 and the latter × 1.4 to the ratio $(LOD)_{II}/(LOD)_I = 5$.

Enantiomeric purity determination. In blind trials, mixtures of fluazifop-P-butyl (0.185 mg ml^{-1}) and the racemate fluazifop-butyl (0.304 mg ml^{-1}) were prepared to

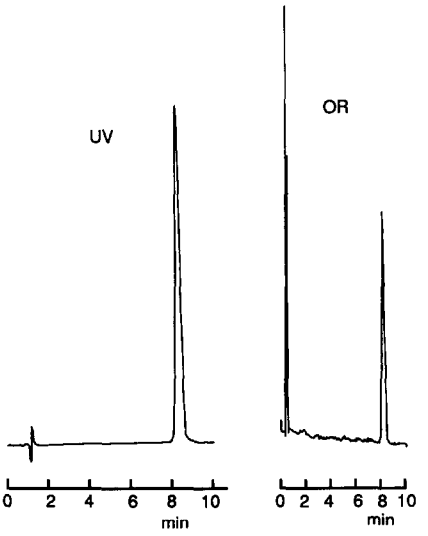

Fig. 6. Achiral chromatograms of fluazifop-P-butyl with dual optical rotation and UV absorbance detection. Column: Hichrom C_{18} (10 cm × 4.6 mm I.D.). Mobile phase: methanol–water (75:25). Flow-rate: 1.0 ml min^{-1}. Injection, 20 μg. ChiraMonitor attenuation 2, time constant 1 s and chart recorder 50 mV fsd.

TABLE IV

LIMITS OF DETECTION (LOD) FOR PACLOBUTRAZOL (I) AND FLUAZIFOP-P-BUTYL (II)

Time constant = 1 s.

	I	*II*
$[\alpha]_{820}/^{\circ}$ ml g^{-1} dm^{-1}	66	18
Half height peak width/ml	0.22	0.30
Dilution factor	12	16
Theoretical LOD/μg	0.15 ± 0.04	0.8 ± 0.2
Observed LOD/μg	0.19	1.0

give samples with x_u around 0.90. Assays were then carried out with both achiral chromatography and (for samples 5–7) chiral chromatography. For samples 1–4, solutions of the mixtures were prepared in the same solvent as the achiral mobile phase (methanol–water, 75:25). For samples 5–7, the mixture solutions were prepared in hexane–methanol (1000:0.9). As the different mobile phases used in chiral and achiral chromatographies were immiscible, the solution of each sample (Nos. 5–7) was divided into two portions. One of them was for direct chiral chromatographic assay. With the other portion for achiral chromatography, solvent was evaporated and methanol–water (75:25) added to redissolve the sample to a concentration of ~ 1 mg ml^{-1}. The enantiomer mole fractions of (+)-form in each of the mixture solutions were calculated from the ultraviolet peak ratio in chiral chromatography and from the α/A ratio (eqn. 5) in achiral chromatography. The results of the blind trials are shown in Table V. The quality of the achiral technique for enantiomeric purity determination is evident. Although the achiral chromatography carries a little more uncertainty than does the chiral chromatography, it is in good agreement with that from the chiral separation as well as with the actual mole fractions.

TABLE V

ENANTIOMER MOLE FRACTION OF FLUAZIFOP-P-BUTYL IN BLIND TRIALS

Sample	x_u[a] *(actual)*	$\bar{x}_u \pm t_{95}s_{x_u}/\sqrt{n}$ *(n)*	
		Chiral separation	*Achiral chromatography*
Standard		0.948 ± 0.005 (9)	
1	0.940		0.942 ± 0.016 (5)
2	0.931		0.938 ± 0.012 (5)
3	0.919		0.923 ± 0.008 (7)
4	0.910		0.901 ± 0.010 (7)
5	0.926	0.928 ± 0.004 (4)	0.916 ± 0.010 (6)
6	0.902	0.904 ± 0.008 (6)	0.902 ± 0.012 (8)
7	0.886	0.884 ± 0.007 (7)	0.880 ± 0.011 (6)

[a] The calculated uncertainty in all x_u values is 0.005, the dominant error being the enantiomer mole fraction of the standard.

CONCLUSION

Using a diode-laser-based polarimetric detector in series with a UV detector, the enantiomeric purities of paclobutrazol and fluazifop-P-butyl have been assayed and calculated from the α/A ratios of unknown and standard samples eluting from an achiral column. The accuracy of the technique has been confirmed by comparison with chiral separations on the same samples.

The precision using achiral chromatography is comparable to that of the chiral separations and the 95% confidence limits are better than those from conventional polarimetry. The achiral chromatography technique with dual OR/UV detection offers advantages for routine quality control of (i) using a normal achiral column instead of a less robust and more expensive chiral column, (ii) using μg of sample which need not be chemically pure, whereas conventional polarimetry requires mg amounts of chemically pure sample.

The detection limit of the technique is dependent on specific rotation and the quality of the chromatographic peak. Any increase in $[\alpha]$ or decrease in peak width will improve the limit of detection.

It is anticipated that this technique will provide a convenient routine analytical method for the enantiomeric purity determination of chiral agrochemicals and pharmaceuticals[8] with the precision dependent upon the availability of well-characterised standard samples and the number of replicate measurements taken.

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