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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STRATEGIES FOR THE DETERMINATION AND CONFIRMATION OF ANTICOAGULANT RODENTICIDE RESIDUES IN ANIMAL TISSUES

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SUMMARY

A comprehensive approach to the analysis of anticoagulant rodenticide residues in animal tissues based on high-performance liquid chromatography (HPLC) has been developed. Residues of warfarin, coumatetralyl, difenacoum, brodifacoum, bromadiolone, diphacinone and chlorophacinone were extracted with chloroform–acetone (1:1, v/v). Extracts were cleaned-up by an integrated gel permeation and adsorption chromatographic procedure which divided the rodenticides into two groups. Residues were then determined and confirmed using normal-phase, ion-pair and weak ion-exchange HPLC techniques. Ion-pair gradient separation resolved all seven rodenticides in a single chromatographic analysis. UV detection methods were employed for all seven rodenticides. Use of a diode array detection system permitted additional confirmation of residues down to 0.1 mg kg^{-1} by matching UV spectra and derivatives of spectra. Sensitive fluorescence detection was possible for the coumarin-based rodenticides but not for diphacinone and chlorophacinone. Post-column pH-switching fluorescence detection methods were shown to be superior to other methods of fluorescence detection of coumarin-based rodenticides. Recoveries from spiked liver tissue were around 90% at levels from 0.05 to 1 mg kg^{-1}. Detection limits of around 0.002 mg kg^{-1} for most rodenticides and of 0.01 mg kg^{-1} for warfarin could be achieved with animal tissue extracts.

INTRODUCTION

Anticoagulant rodenticides have been used internationally to control mice and rats for several decades. They have also been used in various parts of the world for the control of other vertebrate pests including grey squirrels in woodlands in Great Britain and the coypu in the U.S.A., and are currently being evaluated for use against other species[1]. Warfarin, the earliest of these rodenticides to be introduced, was developed as a therapeutic oral anticoagulant and is still of interest in the clinical chemistry field being used for the treatment of thromboembolic disorders[2]. Subsequently several other anticoagulant rodenticides including coumatetralyl, diphaci-

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none and chlorophacinone were marketed. Most of these early compounds were of comparable efficacy under field conditions although chlorophacinone was somewhat more active. Diphacinone has also been used therapeutically as a prothrombopenic agent[3]. The mode of action of these compounds was the same causing blood alterations leading to haemorrhaging as the ultimate cause of death, and all were antidotable with vitamin K_1. The recognition of anticoagulant resistance in rats[4] stimulated the development of a second generation of anticoagulant rodenticides, difenacoum, brodifacoum and bromadiolone, to which cross-resistant rodents were not immune. These newer rodenticides are much more toxic than the earlier generation having both acute and chronic toxic effects. The mode of action is the same as that of the earlier compounds and again all are antidotable with vitamin K_1.

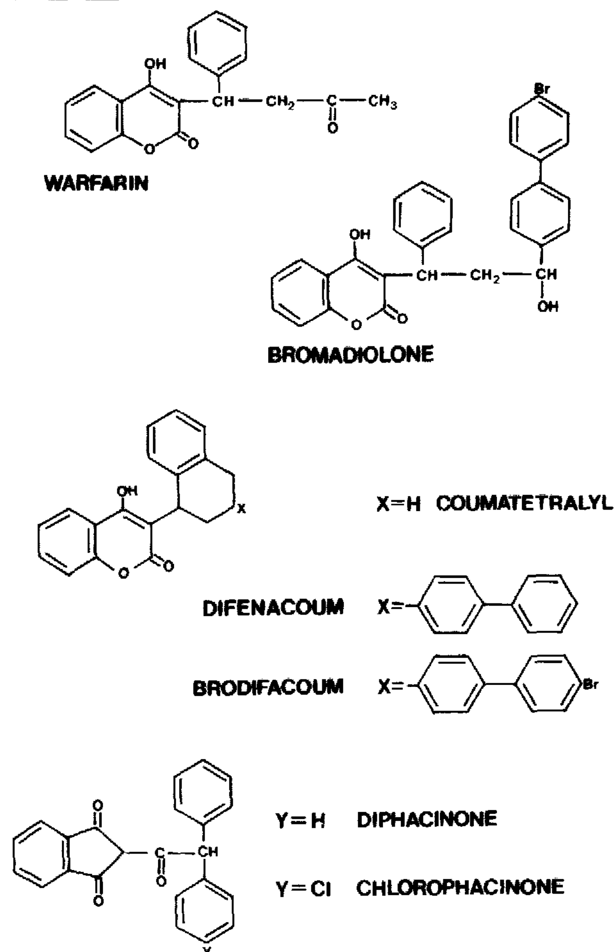
The chemical structures of all seven anticoagulant rodenticides currently used in Great Britain are shown in Table I. Formulations usually include concentrates, tracking powders and ready to use cereal-based baits. The use of this class of pesticide has led to a requirement for suitable analytical methods for the investigation of suspected poisonings in non-target species arising from direct ingestion of baits or caused by secondary poisoning through ingestion of poisoned rodents. Residues of anticoagulant rodenticides in the tissues of poisoned animals are usually very low, the bulk of the compounds being excreted before death. This together with limited quantities of animal tissues makes a multi-residue approach for analysis attractive. Additionally, savings in time and cost are likely to be gained and multi-residue methods have obvious advantages in cases where more than one rodenticide may be involved, or where reliable field information is unavailable.

Spectrophotometric, fluorimetric and thin-layer chromatographic (TLC) methods have been used for the determination of warfarin[5–11], coumatetralyl[12–14], diphacinone[15–18] and chlorophacinone[19–22] but usually these methods suffer from a lack of specificity and sensitivity and are not suitable for determining the low level residues that occur in animal tissues. Gas chromatographic (GC) methods have been successfully used for the analysis of warfarin[23–27] and coumatetralyl but reliable GC methods for the other coumarin-based rodenticides have not been reported. Two GC techniques[28–30] have been reported for the analysis of diphacinone and chlorophacinone but both are lengthy and can suffer from poor recoveries.

High-performance liquid chromatographic (HPLC) methods employing UV detection have been reported for residues of warfarin and coumatetralyl in baits[31,32] and biological material[33–37], for difenacoum and brodifacoum in baits[38–40] and animal tissues[41,42], and for diphacinone and chlorophacinone in baits[43–46] and animal tissues[47,48]. In addition HPLC methods utilising fluorescence detection have been reported for the coumarin-based anticoagulant rodenticides[49–52], including bromadiolone[53,54], in animal tissues. It is likely that HPLC will be the preferred technique for any multi-residue method for determining rodenticides. However low level residues and limited quantities of specimens may make unambiguous identification by off-line techniques, such as mass spectrometry, difficult to achieve; thus alternative methods of confirmation are desirable. This report describes the integration of techniques developed in this laboratory for the analysis of coumarin-based rodenticides[53,54] and of chlorophacinone[48] in animal tissues into a single analytical method, and its extension to include diphacinone, to provide a comprehensive multi-residue method for the determination and confirmation of residues of the seven anticoagulant rodenticides currently in use in Great Britain.

TABLE I

CHEMICAL STRUCTURES OF ANTICOAGULANT RODENTICIDES USED IN THIS STUDY



EXPERIMENTAL

Materials and apparatus

Glass distilled solvents and HPLC-grade hexane, dichloromethane, methanol and acetonitrile (Grade S) were supplied by Rathburn (Walkerburn, Tweeddale, U.K.). Bio-Beads SX-3 (200–400 mesh) were purchased from Bio-Rad Labs. (Watford, U.K.) and tetrabutylammonium phosphate from Sigma (Poole, U.K.). Sep-Pak silica cartridges and low UV PIC Reagent A, a commercially prepared tetrabutylammonium phosphate ion-pairing reagent buffered at pH 7.5, were obtained from Waters Assoc. (Hartford, U.K.). All other chemicals were supplied by BDH (Poole, U.K.). Analytical reference materials were obtained as follows: warfarin, diphacinone and coumatetralyl from the Laboratory of the Government Chemist (London, U.K.);

difenacoum and brodifacoum from Sorex (London, U.K.); bromadiolone from Rentokil (Kirkby, U.K.); and chlorophacinone from May & Baker (Romford, U.K.).

Tissue samples were homogenised with an Ultra-Turrax 18 N tissue disperser. Gel permeation chromatography (GPC) was done using an Altex glass column (500 × 25 mm I.D.) slurry packed with Bio-Beads SX-3 in hexane–chloroform–acetone (75:20:5, v/v/v) to a bed height of 375 mm. The gel was then compressed to a height of 340 mm using an adjustable plunger. The mobile phase was supplied by a Gilson Minipuls 2 peristaltic pump, with an isoversinic flow tube, which was controlled by a Frac-300 fraction collector (Sephadex). All other tubing was PTFE and where necessary Altex microplumbing components terminating in a tubing connector were used to couple the column drain tube to Sep-Pak cartridges. Samples were loaded onto the column via a rotary injection valve (Altex) fitted with a 5-ml sample loop.

Two HPLC systems were used in this study. System I consisted of a Spectra-Physics SP 8700 solvent delivery unit, a Rheodyne injection valve (20 μl) and a Perkin-Elmer LS-4 fluorescence detector. For applications involving post-column pH-switching a Varian post-column reagent pump and reaction coil cassette were incorporated into this system. The reaction coil was PTFE tubing (2 m × 0.3 mm I.D.). System II consisted of a Waters Assoc. ALC 200 liquid chromatograph with a Rheodyne injection valve (20 μl). For gradient separations an additional Model 6000 A pump and Model 660 solvent programmer were included in this system. Detection was either by the incorporated Model 440 fixed-wavelength UV absorbance detector or by a Hewlett-Packard 1040A diode array detection system. The HP1040A system was made up of a detector mainframe connected by an HP-IB interface bus to an HP-85 personal computer, HP82901M dual 5.25 in flexible disc drive and HP7470A plotter. HPLC columns (250 × 4.6 mm I.D.) were slurry packed, using a Haskel pneumatic amplifier pump, with propan-2-ol as the slurry medium and methanol as the packing medium. The column packing materials employed were Spherisorb silica (5 μm), Spherisorb ODS 1 (5 μm), ODS-Hypersil (5 μm) and APS-Hypersil (5 μm).

Extraction

Tissue samples (10 g) were chopped, dried by admixture with anhydrous sodium sulphate and homogenised in chloroform–acetone (1:1, v/v). The extract was filtered, the residual material re-extracted and the second extract added to the filter together with washings from the homogeniser. The combined filtrate was carefully evaporated to dryness at 35°C on a rotary evaporator and made up to 10 ml in hexane–chloroform–acetone (75:20:5, v/v/v).

Clean-up

The Bio-Bead SX-3 column was calibrated by applying individual reference solutions of rodenticides in hexane–chloroform–acetone (75:20:5, v/v/v) and then eluting with the same solvent mixture at 5 ml min^{-1}. Fractions (10 ml) were collected and monitored for the rodenticides. Elution volumes vary slightly from column to column; with the column used in this study all seven rodenticides eluted between 180 and 410 ml. Warfarin and coumatetralyl eluted between 180 and 260 ml, diphacinone between 200 and 350 ml, chlorophacinone between 210 and 380 ml, difenacoum between 240 and 360 ml, bromadiolone between 250 and 400 ml and brodifacoum between 280 and 410 ml.

Animal tissue extracts (5 ml) were applied to the GPC column and the first 170 ml of the eluate discarded. An on-line Sep-Pak silica cartridge was then coupled to the column drain tube and a further 240 ml of solvent passed through the combined GPC–Sep-Pak system, the effluent from the cartridge being collected. This fraction contained coumatetralyl, difenacoum, brodifacoum and warfarin. It was carefully evaporated to dryness at 35°C and redissolved in the appropriate solvent (1 ml) for HPLC analysis. The Sep-Pak cartridge was removed and washed with dichloromethane (7 ml) which was discarded. The cartridge was then eluted with 0.25% formic acid in dichloromethane (40 ml), the eluate carefully evaporated to dryness at 35°C and redissolved in solvent (1 ml) for HPLC analysis. This fraction contained bromadiolone, chlorophacinone and diphacinone.

HPLC

Normal-phase chromatography. Cleaned-up extracts were redissolved in dichloromethane (1 ml) and analysed on Spherisorb silica using two mobile phases; hexane–2% acetic acid in dichloromethane (65:35, v/v) was used for brodifacoum, difenacoum and coumatetralyl and hexane–2% acetic acid in dichloromethane (1:9, v/v) for warfarin and bromadiolone. Chloroform–*sec.*-butylamine (5:1, v/v) was used as a post-column reagent to make the column effluent slightly alkaline. The fluorescence of coumarin-based rodenticides was monitored at excitation and emission wavelengths of 310 nm and 390 nm, respectively.

Reversed-phase chromatography. Extracts were redissolved in methanol (1 ml) and analysed on Spherisorb ODS using gradient elution with methanol and water both containing 0.25% acetic acid[53]. A post-column reagent of 0.3 *M* borate buffer pH 10.4 was used to provide a column effluent of pH $\geq$8.0. The fluorimetric responses of coumarin-based rodenticides was monitored as described above.

Ion-pair chromatography. Extracts were redissolved in the HPLC mobile phase (1 ml). Solutions of reference materials were also diluted with the HPLC mobile phase. Analysis was carried out on ODS-Hypersil using mobile phases prepared from a 0.005 *M* solution of tetrabutylammonium phosphate in methanol and an aqueous solution of PIC Reagent A. Both isocratic and gradient elution methods were employed. The elution of coumarin-based rodenticides was monitored by fluorescence detection[54]. All rodenticides were monitored by UV detection using fixed-wavelength detection at 254 nm or 280 nm as appropriate, or using simultaneous multi-wavelength detection with the diode array system. Suitable pilot wavelengths were selected for peak detection by the HP1040A and apex and baseline spectra (205–400 nm) of recognised peaks automatically stored.

Ion-exchange chromatography. Extracts were redissolved in acetonitrile (1 ml) and analysed on the weak ion-exchange material APS-Hypersil, a chemically bonded amino-phase. The mobile phase employed was acetonitrile–0.04 *M* Tris–HCl buffer pH 7.5 (75:25, v/v) at a flow-rate of 1.1 ml min^{-1}. UV detection was as described above.

All HPLC mobile phases were degassed by continuous sparging with helium. The elution orders of the rodenticides in the four chromatographic modes described are given in Table II.

RESULTS AND DISCUSSION

Extraction and clean-up

Chloroform–acetone (1:1, v/v) was used as the extraction solvent in the work reported here. An earlier extraction study[53] had shown that residues of coumarin-based anticoagulant rodenticides were extracted efficiently with this mixture, recoveries from spiked liver tissue samples were ≥90% after GPC clean-up. Subsequently the same extraction solvent was used for chlorophacinone[48] where again recoveries from spiked liver tissue were around 90% after a combined GPC–adsorption clean-up.

Preliminary experiments using the combined Bio-Bead–Sep-Pak clean-up procedure used previously for chlorophacinone[48] indicated that diphacinone and bromadiolone, like chlorophacinone were retained by the silica cartridge. The other four coumarin-based rodenticides appeared in the fraction that passed through the cartridge. Recovery experiments with reference samples of coumatetralyl, warfarin and bromadiolone confirmed that there was no retention at all of the first two compounds

TABLE II

RELATIVE ELUTION ORDERS FOR HPLC OF ANTICOAGULANT RODENTICIDES IN ORDER OF INCREASING ELUTION VOLUME

Normal-phase: Spherisorb silica; hexane–dichloromethane–acetic acid. Reversed-phase: Spherisorb ODS; methanol–water–acetic acid. Ion-pair: ODS-Hypersil; methanol–phosphate buffer–TBA^+ pH 7.5. Ion-exchange: APS-Hypersil; acetonitrile–Tris–HCl buffer pH 7.5.

Normal-phase	*Reversed-phase*	*Ion-pair*	*Ion-exchange*
Brodifacoum	Warfarin	Warfarin	Brodifacoum
Difenacoum	Coumatetralyl	Coumatetralyl	Difenacoum
Coumatetralyl	Bromadiolone	Diphacinone	Bromadiolone
Warfarin	Difenacoum	Chlorophacinone	Chlorophacinone
Bromadiolone	Brodifacoum	Bromadiolone	Warfarin
		Difenacoum	Diphacinone
		Brodifacoum	Coumatetralyl

TABLE III

RECOVERY OF RODENTICIDES FROM SPIKED LIVER TISSUE

At each level three separate samples of liver tissue were fortified with a single rodenticide prior to extraction. Residues were extracted, cleaned-up and determined by normal-phase HPLC or ion-pair HPLC as described in the text.

Rodenticide	*Percentage recovery (mean ± S.D.)*		
	Fortification level (mg kg^{-1})		
	1	*0.2*	*0.05*
Warfarin	97 ± 3.2	98 ± 2.6	95 ± 4.2
Bromadiolone	89 ± 2.0	91 ± 5.7	86 ± 3.4
Chlorophacinone	90 ± 3.3	91 ± 1.3	86 ± 2.6
Diphacinone	89 ± 3.5	89 ± 3.0	87 ± 4.8

by the cartridge whilst the latter compound was quantitatively retained. Recovery experiments with spiked animal tissues were carried out with warfarin, bromadiolone, diphacinone and chlorophacinone. As warfarin is significantly more polar than the other rodenticides collected in the fraction that passed through the Sep-Pak cartridge it was used to represent the compounds eluted in that fraction.

Liver tissue was chosen for recovery experiments since residues are usually greater than in any other tissue making it the most useful specimen for the diagnosis of anticoagulant poisoning. Percentage recoveries were determined using canine or goose liver samples spiked with individual rodenticides at levels of 0.05, 0.2 and 1 mg kg^{-1}. After extraction, samples were cleaned-up by the combined GPC-Sep-Pak procedure and the results are shown in Table III. In general the recoveries were consistently high with mean values around 90% or greater. Recoveries of bromadiolone, chlorophacinone and diphacinone were slightly lower at the minimum fortification level.

GPC using Bio-Beads SX-3 has been successfully employed elsewhere for the clean-up of pesticides[55,56] including rodenticides[42,53] and is well suited for a multi-residue method where it is necessary to screen for a group of compounds. The removal of lipid material was particularly important in this study as three of the chromatographic modes involved the use of polar/aqueous mobile phases. The simple insertion of a silica Sep-Pak cartridge into the GPC system facilitated the determination of low level rodenticide residues using UV detection. The eluting solvent used with the Bio-Bead column was that used previously[48,53,54]. A reduction in polarity of this eluting solvent may lead to retention of other rodenticides on the Sep-Pak cartridge with the possibility of selective elution and cleaner final extracts, however, the clean-up achieved with real samples has been satisfactory and investigation in this direction has not been justified. Alternatively if further clean-up should be required an additional off-line Sep-Pak procedure such as that used by Koubek *et al.*[42] could be employed.

HPLC determination

Normal-phase chromatography. Detailed comment on the use of the post-column pH-switching technique utilising fluorescence detection has been made elsewhere[53]. It offers sensitive and selective analysis of coumarin-based rodenticides and includes the possibility of some degree of confirmation by repeating the analysis in the absence of the post-column reagent. This chromatographic mode is the only one reported here that resolved the isomers of difenacoum and of brodifacoum. The separation of the isomers is of limited value in the type of work undertaken by this laboratory although the presence of two isomers could be considered as additional evidence of identification. However there can be considerable variation in the isomeric ratios in tissue samples and in many instances of difenacoum poisoning only a single isomer was detected. Examples of the use of this technique for extracts cleaned-up by the combined GPC–adsorption method are shown in Figs. 1 and 2.

Reversed-phase chromatography. An ion-suppression gradient separation of coumarin-based rodenticides with post-column pH-switching to enhance fluorescence detection has been described earlier[53]. This method is complementary to the method described above and includes a similar degree of confirmation by re-analysing extracts in the absence of the post-column reagent.

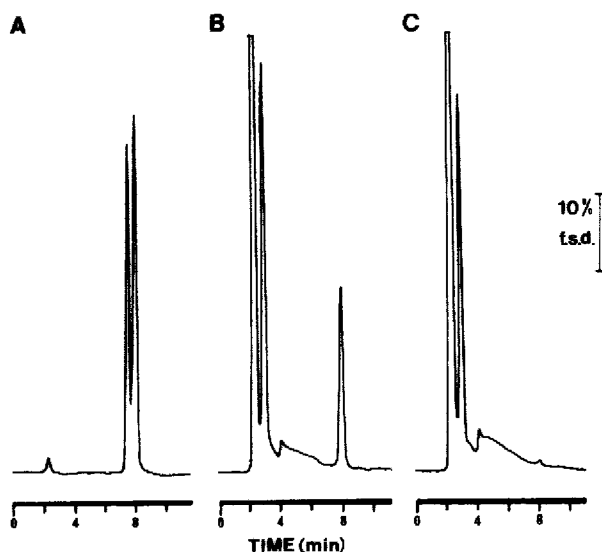


Fig. 1. Normal-phase HPLC of difenacoum on Spherisorb silica (5 μm). Eluent, dichloromethane acidified with 2% acetic acid–hexane (35:65, v/v); flow-rate 1.5 ml min^{-1}; post-column reagent, chloroform–*sec.*-butylamine (5:1, v/v); post-column reagent flow-rate 0.25 ml min^{-1}; fluorescence detection, × 5; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Difenacoum standard (14 ng). (B) Extract from chicken muscle tissue containing difenacoum (≡ 0.035 mg kg^{-1}) as the *trans*-isomer. (C) Same chicken muscle extract chromatographed in the absence of the post-column reagent.

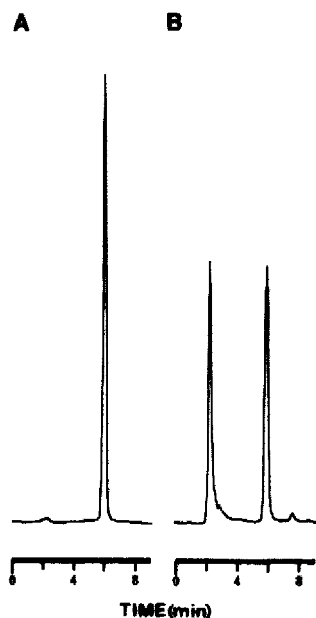


Fig. 2. Normal-phase HPLC of warfarin on Spherisorb silica (5 μm). Eluent, dichloromethane acidified with 2% acetic acid–hexane (90:10, v/v); flow-rate 1.5 ml min^{-1}; post-column reagent, chloroform–*sec.*-butylamine (5:1, v/v); post-column reagent flow-rate 0.7 ml min^{-1}; fluorescence detection, × 2.5; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Warfarin standard (50 ng). (B) Extract from canine liver containing warfarin (≡ 0.82 mg kg^{-1}) final volume of extract 3 ml.

Mundy and Machin[51] reported the use of fluorescence detection in a method based on high-performance exclusion chromatography but coumatetralyl, difenacoum and brodifacoum were unresolved. Kieboom and Rammell[49] used fluorescence detection for the HPLC analysis of brodifacoum but were limited to a quenched fluorescence because of an acidified mobile phase[8]. Subsequently the sensitivity of their reversed-phase method was considerably improved[52] by replacing the carboxylic acid in the mobile phase with an acidic phosphate buffer and using an excitation wavelength of 210 nm instead of 280 nm. This improved method for brodifacoum was compared with a reversed-phase pH-switching technique using the same HPLC conditions in an experiment summarised in Table IV. The sensitivity achieved by excitation at 210 nm was equally matched by using the pH-switching method with excitation at 310 nm, but the latter had a signal-to-noise ratio about an order of magnitude better. Use of lower excitation wavelengths with this pH-switching method increased the response from brodifacoum. At an excitation wavelength of 255 nm the response was almost double that gained at 310 nm, whilst at 210 nm the response was almost ten times greater. However at 210 nm the signal to noise ratio was again poor and the magnitude of the signals rapidly exceeded the operational limits of the detector.

Ion-pair chromatography. While adsorption[38,41,42] and reversed-phase[39,49,51,52] techniques for the analysis of coumarin-based rodenticides have been well documented in the literature little use has been made of ion-pairing methods, with the exception of a method for the analysis of warfarin in concentrates[32]. The use of ion-pair chromatography, with the tetrabutylammonium cation (TBA^+) as counter-ion, to permit the sensitive fluorescence detection of coumarin-based rodenticides has been described elsewhere[54]. The TBA^+ counter-ion has also been used in methods for the analysis of diphacinone in formulations[46] and baits[45], for chlorophacinone in baits[43] and by this laboratory for chlorophacinone residues in animal tissues[48]. Difficulties in obtaining satisfactory chromatography of chlorophacinone and diphacinone in other HPLC modes have been noted[45,48,57], so it is clear that reversed-phase ion-pairing is the most viable HPLC technique for the complete multi-residue analysis of anticoagulant rodenticides. It was particularly useful because

TABLE IV

INFLUENCE OF MOBILE PHASE pH AND OF EXCITATION WAVELENGTH ON THE FLUORIMETRIC RESPONSE OF BRODIFACOUM IN HPLC

Brodifacoum (80 ng) was chromatographed on Spherisorb ODS using methanol–0.005 *M* phosphate buffer pH 3.0 (85:15, v/v) at 1.5 ml min^{-1}. Fluorimeter excitation and emission monochromator slits were set at 10 nm and all emissions were monitored at 390 nm. Where necessary 0.015 *M* sodium hydroxide was used as the post-column reagent to give a mobile phase pH ≥ 8. Fluorimetric responses are quoted in arbitrary units in terms of peak height.

	Excitation wavelength (nm)	*Response*
No post-column pH-switching	310	18
	210	206
With post-column pH-switching	310	204
	255	370
	210	1970

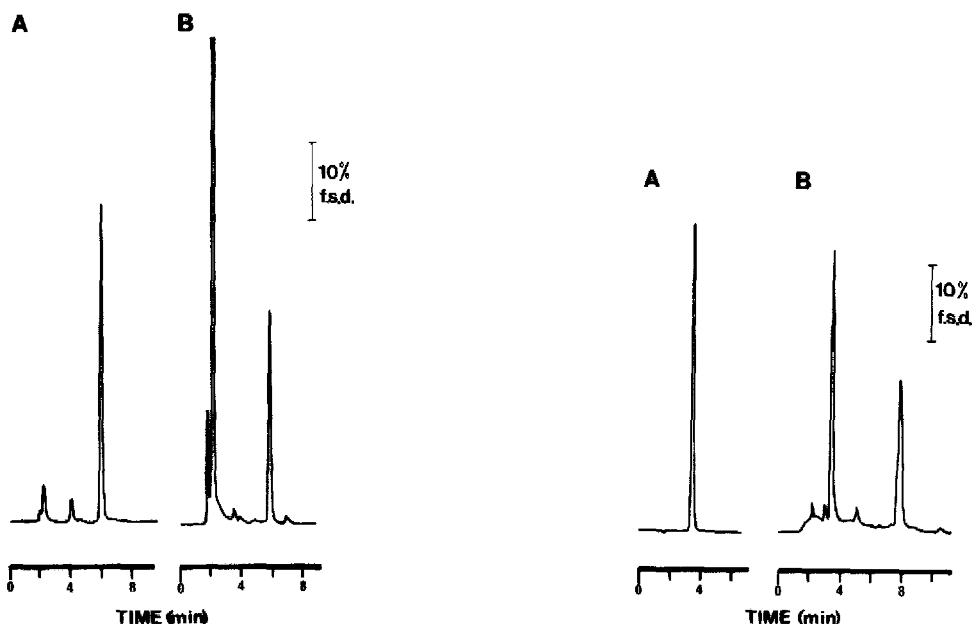


Fig. 3. Ion-pair HPLC of bromadiolone on ODS-Hypersil (5 μm). Mobile phase, methanol–water, containing 0.005 *M* tetrabutylammonium phosphate and PIC reagent A, respectively, (74:26, v/v); flow-rate, 1.5 ml min^{-1}; fluorescence detection, $\times$ 3; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Bromadiolone standard (18 ng). (B) Extract from pheasant liver tissue containing bromadiolone ($\equiv$ 0.57 mg kg^{-1}) final volume of extract 5 ml.

Fig. 4. Ion-pair HPLC of difenacoum on ODS-Hypersil (5 μm). Mobile phase, methanol–water, containing 0.005 *M* tetrabutylammonium phosphate and PIC A reagent, respectively, (78:22, v/v); flow-rate 1.5 ml min^{-1}; fluorescence detection, $\times$ 2.5; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Difenacoum standard (3.8 ng). (B) Extract from chicken muscle tissue containing difenacoum ($\equiv$ 0.031 mg kg^{-1}).

the pH of the mobile phase allowed the unquenched native fluorescence of coumarin-based rodenticides to be monitored. Examples of this technique with extracts cleaned-up by the combined GPC–adsorption procedure are shown in Figs. 3 and 4.

TLC methods for diphacinone[13,15] have made use of a natural fluorescence, however attempts to exploit this for HPLC detection have not proved successful. Under ion-pairing conditions with excitation at 285 nm a weak fluorescence signal was monitored at 510 nm from chlorophacinone and diphacinone. The sensitivity and signal-to-noise ratio were very poor in comparison to UV detection rendering the latter more suitable for low level residue monitoring. Diphacinone has a very characteristic UV absorption spectrum which is virtually identical to that of chlorophacinone so that the UV detection parameters used earlier for chlorophacinone[48] were applicable to it. These were simultaneous multi-wavelength monitoring by the diode array detector at 224, 285, 313 and 325 nm, with 285 nm as the pilot wavelength for peak detection, or fixed-wavelength UV detection at 280 nm.

The use of UV detection, particularly via the diode array detector, for the coumarin-based rodenticides has also been examined. Fixed-wavelength detection at 280 nm was most suitable for the analysis of warfarin and coumatetralyl while greater sensitivity was achieved at 254 nm with difenacoum, brodifacoum and bromadiolone.

TABLE V

INFLUENCE OF MOBILE PHASE ORGANIC MODIFIER CONCENTRATION ON THE CAPACITY FACTORS OF ANTICOAGULANT RODENTICIDES IN ION-PAIR HPLC

Column: ODS-Hypersil (250 × 4.6 mm I.D.), mobile phases made up from 0.005 *M* tetrabutylammonium phosphate *in methanol and an aqueous solution of PIC* Reagent A.

	Percentage methanol in mobile phase									
	80	*78*	*75*	*74*	*72*	*70*	*68*	*65*	*58*	*55*
Warfarin	–	–	–	–	–	–	–	0.52	1.33	1.89
Coumatetralyl	–	–	0.28	–	–	–	–	1.0	2.44	3.56
Diphacinone	–	–	0.58	0.71	0.86	1.03	–	1.67	–	–
Chlorophacinone	–	0.63	1.0	1.18	1.45	1.94	2.63	3.74	–	–
Bromadiolone	–	1.03	1.74	2.05	2.89	–	–	–	–	–
Difenacoum	1.11	1.67	2.83	–	4.44	–	–	–	–	–
Brodifacoum	2.11	3.33	5.67	–	–	–	–	–	–	–

Pilot wavelengths of 310 nm for warfarin and coumatetralyl and a wavelength in the range of 254–265 nm for the remaining coumarin-based rodenticides were used with the diode array system.

Ion-pair chromatography was usually employed after screening extracts with one of the pH-switching techniques described earlier and hence was used for confirmation of specific coumarin-based rodenticides or for the primary detection of chlorophacinone and diphacinone. Accordingly isocratic conditions were normally suitable for these purposes. Retention data for the seven rodenticides are tabulated in Table V and applications of this technique are shown in Figs. 5 and 6.

An ion-pair gradient separation of coumarin-based rodenticides, using fluorescence detection, was demonstrated previously[54]. Gradient elution with UV detection was examined in this study. A similar program to that used earlier provided

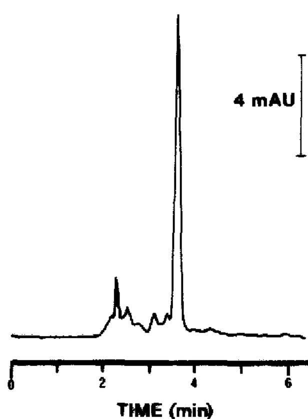


Fig. 5. Ion-pair HPLC of warfarin on ODS-Hypersil (5 μm). Mobile phase, methanol–water, containing 0.005 *M* tetrabutylammonium phosphate and PIC reagent A, respectively (58:42, v/v); flow-rate, 1.3 ml min^{-1}; UV detection at 310 nm, bandwidth 4 nm, 0.02 AUFS using the diode array detector. Sample extract from canine liver tissue containing warfarin (≡ 0.82 mg kg^{-1}).

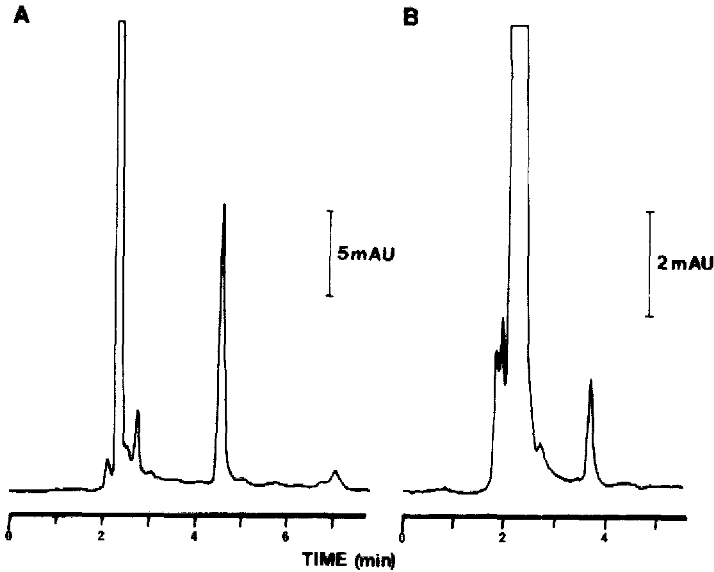


Fig. 6. Ion-pair HPLC of difenacoum and bromadiolone on ODS-Hypersil. Mobile phase, as in Fig. 4; flow-rate, 1.1 ml min^{-1}; UV detection with the diode array detector. (A) Extract from chicken liver tissue containing difenacoum ($\equiv$ 0.36 mg kg^{-1}) monitored at 254 nm, bandwidth 4 nm, 0.03 AUFS. (B) Extract from feline liver tissue containing bromadiolone ($\equiv$ 0.11 mg kg^{-1}) monitored at 260 nm, bandwidth 4 nm, 0.01 AUFS.

complete resolution of all seven rodenticides within 8 min (Fig. 7). The HP1040A system was used with either time-programmed wavelength changes or in a total absorbance mode as suggested by Clark *et al.*[58]. For the latter a pilot wavelength of 280 nm was used with a band width of 80 nm. Wavelength programming offered the greatest responses but the signal-to-noise ratio appeared slightly poorer than in the total absorbance chromatogram. In both cases significant baseline drift was observed at very low residue levels which may well make isocratic separations a better option when maximum sensitivity is required for a particular rodenticide.

Ion-exchange chromatography. The lack of success in finding a satisfactory HPLC technique to enable confirmation of chlorophacinone and diphacinone residues determined by ion-pair HPLC prompted a re-evaluation of the use of an amino-phase. Addison[47] had used LiChrosorb NH_2 with an acetonitrile–water mobile phase for the determination of chlorophacinone residues in mouse tissue but previous attempts to use this method with another amino-phase, APS-Hypersil, were unsuccessful[48].

Further experiments with APS-Hypersil, a weak ion-exchange material, using acetonitrile and aqueous buffers have indicated that satisfactory chromatographic behaviour can be achieved if the mobile phase is buffered to a slightly alkaline pH. A mobile phase of acetonitrile–0.04 *M* Tris–HCl buffer pH 7.5 (75:25, v/v) was used for the confirmation of chlorophacinone and diphacinone residues. This system was also found to be suitable for chromatographing coumarin-based rodenticides, but use of the isocratic conditions stated did not permit complete resolution of all seven rodenticides. Difenacoum and brodifacoum peaks were not totally resolved, bro-

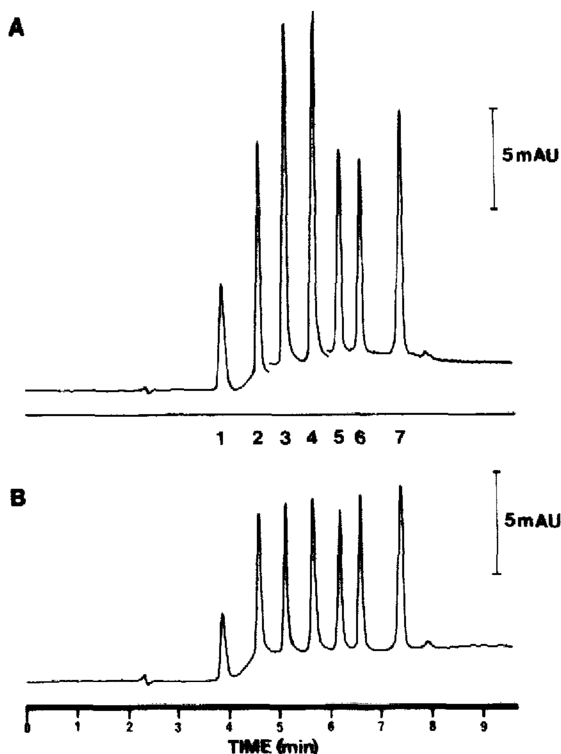


Fig. 7. Ion-pair gradient separation of anticoagulant rodenticides on ODS-Hypersil. Mobile phase components, methanol–water containing 0.005 *M* tetrabutylammonium phosphate and PIC reagent A, respectively; I, 52:48 (v/v); II, 95:5 (v/v). Gradient: 20% II to 90% II over 8 min, curve 4 Waters Model 660 solvent programmer; flow-rate 1.2 ml min^{-1}. UV detection with the diode array detector. (A) Wavelength programming, peaks 1 and 2 at 310 nm, peaks 3 and 4 at 285 nm, peaks 5–7 at 265 nm, bandwidth in each case 4 nm, 0.025 AUFS. (B) Total absorbance chromatogram monitored at 280 nm, bandwidth 80 nm, 0.025 AUFS. Reference standards: 1 = warfarin 33.6 ng; 2 = coumatetralyl 32.3 ng; 3 = diphacinone 31.4 ng; 4 = chlorophacinone 33.6 ng; 5 = bromadiolone 35.8 ng; 6 = difenacoum 30.1 ng; 7 = brodifacoum 32 ng. Standards correspond to a residue level of approximately 0.3 mg kg^{-1}.

madiolone was poorly resolved from difenacoum, and diphacinone was not completely resolved from warfarin. However the technique had merit as a confirmation mode where a single rodenticide was to be analysed. Some applications of this technique with animal tissue extracts are shown in Fig. 8 and a summary of UV data is presented in Table VI.

In this chromatographic mode increasing the proportion of the organic modifier in the mobile phase had the effect of increasing the retention of all of the rodenticides. The relationships between capacity factors (k') and acetonitrile concentration are shown in Fig. 9. The non-linear behaviour exhibited by warfarin probably resulted from decreases in the apparent pH of the mobile phase as the proportion of buffer was decreased. Use of the same buffer at a slightly higher pH significantly increased the retention of warfarin at acetonitrile concentrations greater than 80%, while retention of the other rodenticides was slightly decreased. It is not clear why this effect was only observed with warfarin and not with the other coumarin-based rodenticides.

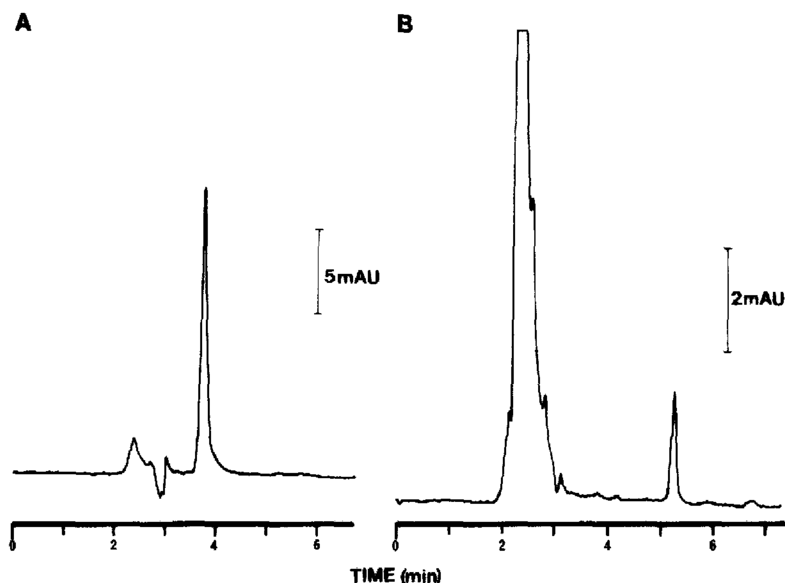


Fig. 8. Ion-exchange HPLC of chlorophacinone and warfarin on APS-Hypersil (5 μm). Mobile phase, acetonitrile–0.04 *M* Tris–HCl buffer pH 7.5 (75:25, v/v); flow-rate, 1.1 ml min^{-1}; UV detection with diode array detector. (A) Extract from canine liver tissue containing chlorophacinone ($\equiv$ 0.72 mg kg^{-1}) monitored at 285 nm, bandwidth 4 nm, 0.03 AUFS. (B) Extract from chicken liver tissue containing warfarin ($\equiv$ 0.12 mg kg^{-1}) monitored at 310 nm, bandwidth 4 nm, 0.01 AUFS.

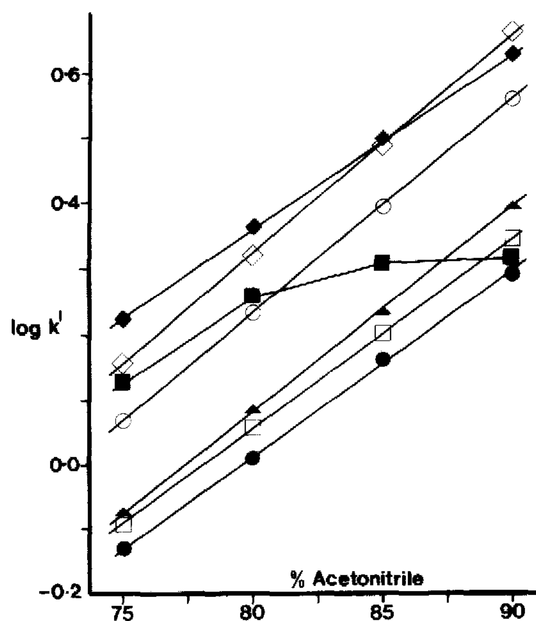


Fig. 9. Influence of organic modifier (acetonitrile) concentration on the capacity factors (k') of anticoagulant rodenticides on APS-Hypersil. Mobile phases prepared from acetonitrile and 0.04 *M* Tris–HCl buffer pH 7.5; flow-rate, 1.1 ml min^{-1}. ●, Brodifacoum; □, difenacoum; ▲, bromadiolone; ○, chlorophacinone; ■, warfarin; ◇, diphacinone; ◆, coumatetralyl.

TABLE VI

SUMMARY OF UV RESPONSES (mAU) OBTAINED FROM ANTICOAGULANT RODENTICIDES BY FIXED-WAVELENGTH AND DIODE ARRAY DETECTION

Responses were determined as peak heights derived from HPLC of rodenticides on APS-Hypersil using acetonitrile–0.04 *M* Tris–HCl buffer pH 7.5 (75:25, v/v), 1.1 ml min^{-1}. Bandwidths for diode array detector were all 4 nm.

	Wavelength (nm)								
	210	*224*	*254*	*254**	*260*	*265*	*280**	*285*	*310*
Brodifacoum (100 ng)	81.5	26.9	27.4	50.5	29.3	31.5	28.0	18.9	13.7
Difenacoum (94 ng)	76.4	25.1	26.0	48.5	24.3	–	15.5	10.1	12.5
Bromadiolone (112 ng)	61.7	20.6	25.1	41.0	26.0	–	24.5	14.1	11.1
Chlorophacinone (108 ng)	–	31.0	9.0	19.2	5.7	–	30.2	25.7	12.3
Warfarin (105 ng)	45.1	–	–	10.3	–	–	10.8	8.6	12.9
Coumatetralyl (112 ng)	66.2	24.7	7.2	10.5	3.3	–	12.3	10.3	18.7

* Fixed-wavelength detection.

After prolonged but intermittent use the performance of the column deteriorated particularly for chlorophacinone and diphacinone. However washing the column with acetonitrile–0.01 *M* phosphate buffer pH 3 (70:30, v/v) and the subsequent use of acetonitrile–0.04 *M* Tris–HCl buffer pH 8.4 (75:25, v/v) as mobile phase largely restored its chromatographic performance. The non-acidic mobile phases employed for this form of ion-exchange chromatography also make the fluorescence detection of coumarin-based rodenticides possible.

Confirmation. The absolute identification of low-level residues in animal tissues can be difficult to achieve because of the small amounts involved, and many laboratories do not have ready access to sophisticated techniques such as mass spectrometry. Alternatively the multi-chromatographic procedures described above may be

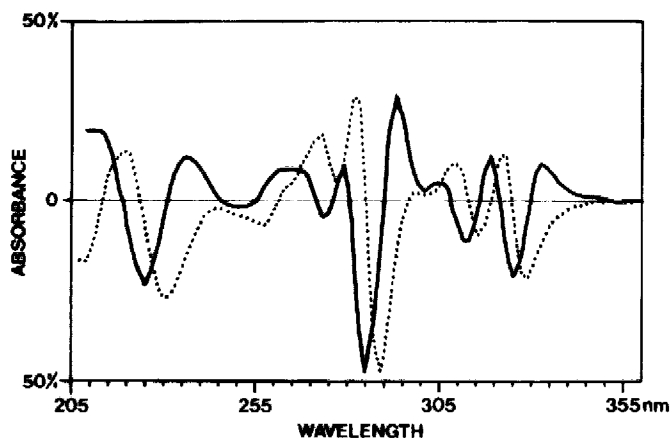


Fig. 10. Normalised first and second derivatives of UV absorption spectrum of chlorophacinone (highest absorbance set at 50%) obtained from ion-pair HPLC of a reference standard (105 ng) on ODS-Hypersil with a mobile phase containing 75% methanol. (· · · · · · · ·), First derivative, absorbance 0.0043 AU at 283 nm. (——), Second derivative, absorbance 0.017 AU at 293 nm.

used to provide confirmation of identification and also to verify actual residue levels. Ion-pair HPLC and ion-exchange HPLC with either UV or, preferably, sequential UV and fluorescence detection appear to be well suited as primary detection modes for multi-residue work covering all seven rodenticides. For convenient screening purposes in this laboratory the normal-phase pH-switching technique was most frequently used as the primary detection mode for coumarin-based rodenticides. Cleaned-up extracts were firstly redissolved in the volatile dichloromethane before being transferred into the more polar solvents used for the other chromatographic methods. Before proceeding extracts were re-chromatographed in the absence of the post-column reagent. Ion-pair HPLC was then employed in the second stage for confirmation of any coumarin-based rodenticides, using both fluorescence and UV detection, and for the primary detection of chlorophacinone and diphacinone by UV detection. Isocratic conditions were normally satisfactory for these purposes. Finally, in a third stage, ion-exchange chromatography on APS-Hypersil was used with UV detection for the confirmation of any of the seven rodenticides found in the first two stages.

Further on-line confirmation of residues detected by the diode array system was gained by overlaying and matching normalised UV spectra of sample peaks with those obtained from the peak of the appropriate reference standard. Residues of all seven rodenticides could be confirmed in this manner at a level of 0.1 mg kg^{-1}. Below this limit simultaneous multi-wavelength monitoring yielded some measure of confirmation. Later additions to the software for the HP1040A system permitted the overlay and matching of normalised first or second derivative spectra. This procedure was particularly useful for confirming chlorophacinone and diphacinone which yielded very characteristic derivative spectra (Fig. 10), although the limit for application was higher than that achieved by matching original spectra.

Using fluorescence monitoring practical detection limits in animal tissue extracts ranged from 0.002 mg kg^{-1} for coumatetralyl, difenacoum and brodifacoum, to 0.008 mg kg^{-1} for bromadiolone and 0.01 mg kg^{-1} for warfarin. With UV monitoring detection limits of around 0.003 mg kg^{-1} for difenacoum, brodifacoum, bromadiolone, chlorophacinone and diphacinone, of 0.008 mg kg^{-1} for coumatetralyl and 0.01 mg kg^{-1} for warfarin could be achieved.

Fluorescence detection with either the reversed-phase pH-switching method or the ion-exchange method offered further possibilities of confirmation of coumarin-based rodenticides. Reversed-phase systems with acidic buffers could also be employed with UV detection of these compounds but these did not appear to offer any advantage over the use of the ion-pair technique, unless slight differences in selectivity can be exploited to overcome any difficulties experienced with interferences from co-extracted material. Similarly other normal-phase materials such as diol and cyano-phases may offer advantages in selectivity over silica for particular samples. Additionally UV monitoring could be employed as an alternative detection mode for normal-phase separations.

CONCLUSIONS

HPLC can provide a complete analysis of the seven anticoagulant rodenticides used in Great Britain. Multi-residue clean-up with good recoveries can be achieved

by a combined GPC–adsorption method. The use of three chromatographic modes allows the identification and confirmation of residues of these compounds in animal tissues. UV detection methods are applicable to all seven compounds and confirmation can be provided by comparison of absorption spectra and derivative spectra. Fluorescence monitoring can be used for coumarin-based rodenticides. Post-column pH-switching techniques are superior to methods combining acidic mobile phases with fluorescence detection at a lower excitation wavelength. Ion-pair gradient separation can resolve all seven rodenticides within 8 min. Routine detection limits range from 0.002 to 0.01 mg kg^{-1}.

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