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ANALYSIS OF THE ANTI-COCCIDIAL DRUG, HALOFUGINONE, IN CHICKEN TISSUE AND CHICKEN FEED USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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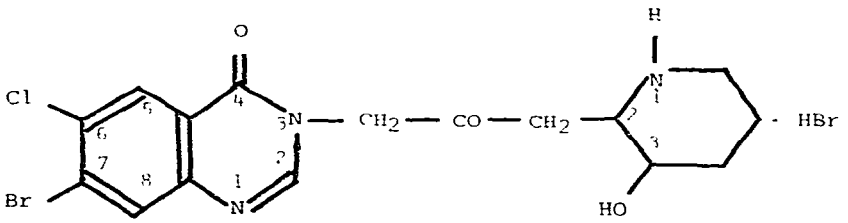
SUMMARY

Methods are described for the analysis of the anti-coccidial drug, halofuginone, in chicken tissue at concentrations as low as 1 ppb (0.001 ppm) and in chicken feed at a concentration of 3 ppm, using high-performance liquid chromatography. The tissue analysis involves: enzymatic release of the halofuginone followed by ethyl acetate extraction under basic conditions, partition into ammonium acetate buffer, concentration using Sep-pak™ C_{18} cartridge. The feed analysis involves: ethyl acetate extraction under basic conditions, partition into hydrochloric acid, concentration using XAD-2 column chromatography. Both methods use high-performance liquid chromatography with ultraviolet detection for the final analysis. Precision and accuracy data for both methods are given.

INTRODUCTION

Halofuginone (DL-*trans*-7-bromo-6-chloro-3-{3-(3-hydroxy-2-piperidyl) acetonyl}-4(3H)-quinazolinone hydrobromide) (I) is an anti-coccidial drug proposed for incorporation in poultry feed to permit continuous administration to broiler chickens. For the purposes of registration, sensitive and precise methods were required for the analysis of halofuginone in chicken tissues, particularly liver and kidney, and in chicken feed at the inclusion concentration of 3 ppm. A previous paper[1] from this laboratory has described a high-performance liquid chromatography (HPLC) method for analysing feed at 3 ppm. To eliminate the interference



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caused by matrix co-extractives from some feeds, modification of the HPLC conditions of this method was necessary. This paper describes the modification, with precision and accuracy data generated over 2 years, and the extension of the method to the analysis of chicken tissues at concentrations as low at 1 ppb (0.001 ppm).

EXPERIMENTAL

Apparatus

HPLC. A Waters Assoc. (Hartford, Great Britain) M-6000A pump fitted with a μBondapak C_{18} (30 cm × 3.9 mm I.D.) pre-packed column (Waters Assoc.) was used. Injection was achieved by means of a WISP 710A automatic sample injector (Waters Assoc.). The eluate from the column was monitored using a LC–UV variable-wavelength UV detector (Pye-Unicam, Cambridge, Great Britain). Output from the detector was measured on either a Spectra-Physics SP4100 computing integrator (St. Albans, Great Britain) or W + W series 1100 chart recorder (Basle, Switzerland).

General. Sample maceration was achieved using a Polytron model PCU-2 macerator (Kinematica, Switzerland). Glass columns (30 × 1 cm I.D.) with sintered filters were used for macroreticular resin separations. A Beckman Model 3500 digital meter with combination electrodes was used for pH measurements. Centrifugation was accomplished using a MSE "GF-8" centrifuge (Crawley, Great Britain) capable of accepting 200-ml glass centrifuge bottles. Büchi "Rotavapor R" rotary film evaporators (RFE; Orme Scientific, Middleton, Great Britain) were used for reduced pressure evaporations.

Materials

Analytical-grade halofuginone (micronised) was obtained from Roussel Uclaf (Paris, France). The macroreticular resin, Amberlite XAD-2 standard grade (BDH, Poole, Great Britain), was freed from chloride ions by water washing and purified by overnight Soxhlet extraction with methanol. The purified resin was stored, under methanol, in stoppered glass bottles. Immediately prior to use, a slurry of the purified resin (10 g), in methanol, was transferred to a glass chromatography column and, after draining of the methanol, washed thoroughly with glass distilled water. Sep-pak™ C_{18} cartridges (Waters Assoc.) were pre-washed sequentially with methanol (2 ml) and glass-distilled water (5 ml). All chemicals were analytical-reagent grade and all solvents HPLC grade. The enzyme preparation, Trypsin, was laboratory-reagent grade (Fisons Scientific, Loughborough, Great Britain). Unmedicated (control) poultry diet was prepared at Huntingdon Research Centre to a fixed commercial recipe and control and treated chicken tissues were obtained from the Department of Animal Science at Huntingdon Research Centre.

Acetate buffer (0.25 *M*, pH 4.3) was prepared by dissolving ammonium acetate (19.4 g) and glacial acetic acid (30 ml) in glass-distilled water and diluting to 1 l, with glass-distilled water. Acetate buffer (0.125 *M*, pH 4.3) was prepared by diluting 500 ml of the 0.25 *M*, pH 4.3 acetate buffer to 1 l, with glass-distilled water.

Chromatography

The HPLC mobile phase consisted of acetonitrile–acetate buffer (0.25 *M*)–water (5:3:12, v/v/v), re-adjusted to pH 4.3 with glacial acetic acid after mixing.

Sample injection volume was 40 μl for feed analysis and 100 μl for tissue analysis. Chromatography was carried out at ambient temperature (23 ± 3°C) using a mobile phase flow-rate of 2 ml/min. The eluate from the HPLC column was monitored at a wavelength of 243 nm. Chart recordings were made at a chart speed of 0.5 cm/min using a detector sensitivity of 0–0.04 a.u.f.s. (W + W) or 0–1 volt unattenuated (SP4100). Under these conditions the retention volume of halofuginone was 14 ml.

Procedures

Finished feeds (approximately 3 ppm halofuginone). A representative sample of feed (10 g) was macerated with sodium carbonate solution (10 ml; 10% w/v) and ethyl acetate (100 ml). The phases were separated by centrifugation and the ethyl acetate phase decanted. The residue was re-extracted with ethyl acetate (100 ml) and the combined ethyl acetate extracts washed with salt-saturated sodium carbonate solution (50 ml; 5%, w/v). The ethyl acetate phase was separated, extracted with hydrochloric acid (2 × 50 ml, 0.1 *M*) and the combined hydrochloric acid extracts washed, by gentle agitation, with ethyl acetate (10 ml). Residual ethyl acetate was removed from the acid solution (RFE at 40°C, 5 min). The acid solution was passed through an Amberlite XAD-2 macroreticular resin column prepared as under *Materials* and the column washed with hydrochloric acid (20 ml, 0.1 *M*). Any residual acid solution was removed from the column using low-pressure compressed air. The column was equilibrated with methanol for 10 min and eluted with methanol (100 ml). The solvent was evaporated (RFE at 40°C) and the residue dissolved in HPLC mobile phase (10 ml), filtered through a Whatman GF/F paper and analysed by HPLC.

Tissues. Trypsin (0.5 g) and water (10 ml) were added to a representative sample (20 g) of homogenised tissue, mixed, and the pH adjusted to 8 ± 1 by the dropwise addition of sodium carbonate solution (10%, w/v). The mixture was incubated for 3 h (water bath, 40°C), allowed to cool to ambient temperature and macerated for 3 min with sodium carbonate solution (10 ml; 10%, w/v) and ethyl acetate (100 ml). After centrifuging, the organic phase was decanted, the residue re-extracted with ethyl acetate (100 ml) and the combined extracts washed with salt-saturated sodium carbonate solution (50 ml; 5%, w/v).

The organic layer was extracted with ammonium acetate buffer (2 × 50 ml, 0.125 *M*) and the combined aqueous extracts washed, by gentle agitation, with ethyl acetate (10 ml). Residual ethyl acetate was removed from the aqueous solution (RFE at 40°C, 5 min). The solution was transferred quantitatively to a volumetric flask (100 ml) and diluted to volume with acetate buffer (0.125 *M*). An aliquot (10 ml) of the solution, after filtration through a Whatman GF/F paper, was passed through a pre-washed Sep-pak C_{18} cartridge. This was repeated with a second aliquot (10 ml) and the cartridge washed with glass-distilled water (3 ml). Halofuginone was eluted from the cartridge with methanol (5 ml), the eluate collected in a tapered test tube (10 ml) and evaporated to dryness using a stream of dry nitrogen. The residue was dissolved in HPLC mobile phase (200 μl) and analysed by HPLC.

Standard solutions

Halofuginone (50 mg) was dissolved in acetate buffer (250 ml, 0.25 *M*) to produce a stock standard solution. Portions of the stock standard solution were diluted with HPLC mobile phase to provide calibration solutions in the range 0–5 μg halofuginone/ml.

TABLE I

PRECISION AND ACCURACY (FINISHED FEEDS)

The following procedural recovery data has been generated by three analysts over a 2-year period.

Concentration (nominal)	3 ppm
Number of determinations	85
Mean recovery	92.7%
Standard deviation	±4.2%

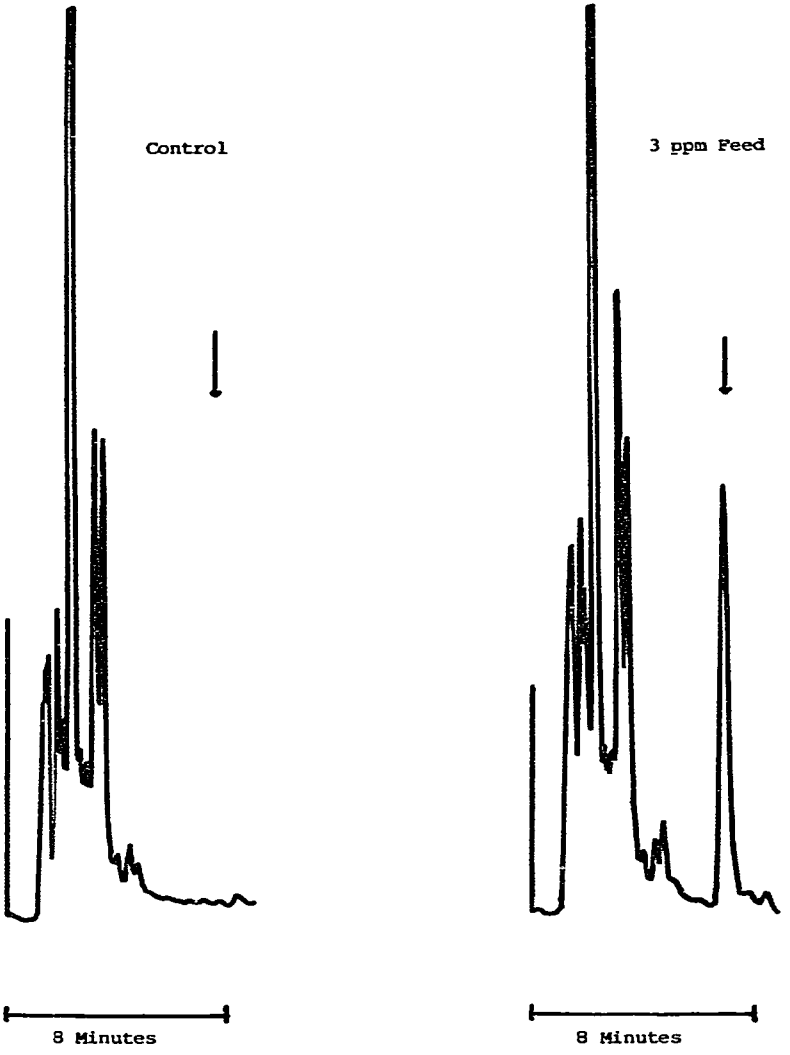

Fig. 1. Chromatograms obtained for extracts from control feed and feed containing halofuginone at 3 ppm.

Procedural recoveries

Finished feed. The stock standard halofuginone solution was diluted to a concentration of approximately 10 μg halofuginone/ml with glass-distilled water and portions (3 ml) of this solution used to fortify control feed (10 g) to produce halofuginone concentrations of 3 ppm. The mixture was analysed immediately as described under *Procedures.*

Tissues. The stock standard halofuginone solution was diluted with glass-distilled water to a concentration such that the addition of 100 μl of the solution to control homogenised tissue (20 g) gave halofuginone concentrations in the range 0.015–1.0 ppm in the tissue. The mixture was analysed as described under *Procedures.*

Calibration and calculation

Calibration curves of chromatographic peak height or peak area *versus* concentration of halofuginone (μg/ml) were constructed using the data obtained by injections of aliquots of the standard solutions prepared as described under *Standard solutions.*

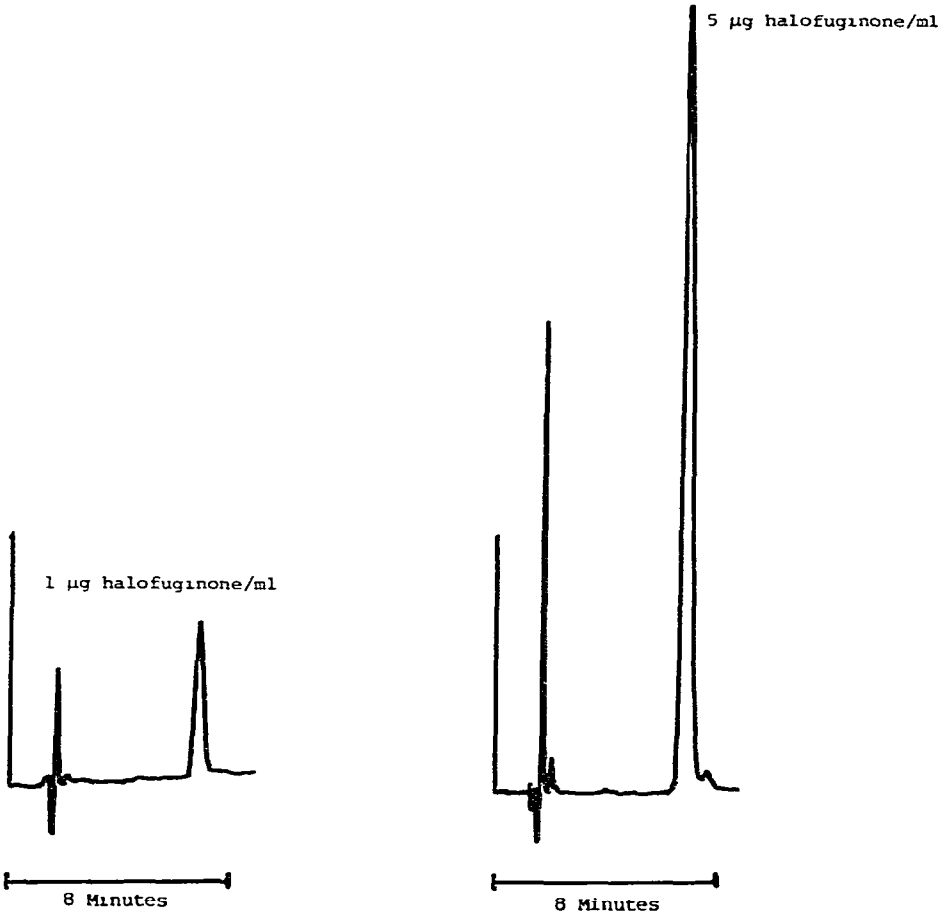

Fig. 2. Chromatograms obtained for calibration standards of halofuginone.

Concentrations of halofuginone in samples were determined by measurement of the chromatographic peak height or peak area at the characteristic retention volume for halofuginone and, after interpolation to the appropriate calibration curve, application of the necessary dilution factors.

RESULTS AND DISCUSSION

HPLC was found to be superior to gas–liquid chromatography for the analysis of halofuginone at 3 ppm in finished feed[1]. Using the HPLC method detailed in this paper we have analysed approximately 500 commercial finished feeds from many different feed compounders without interference from feed co-extractives. During this

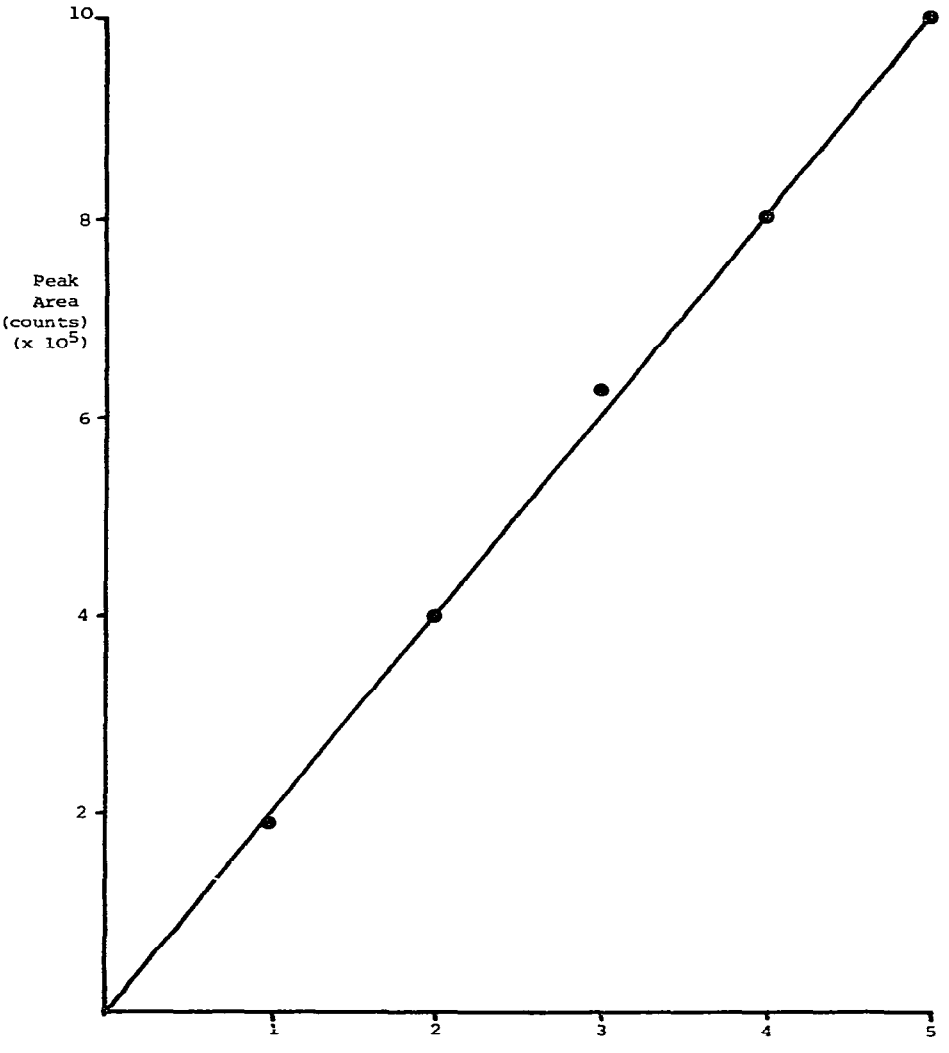

Fig. 3. Calibration curve.

TABLE II

PRECISION AND ACCURACY (TISSUES)

The following procedural recovery data illustrates the precision and accuracy of the method used for analysing tissues.

Tissue	*Fortification range (ppm)*	*No. of determinations*	*Mean recovery (%)*	*Recovery range (%)*
Liver	0.015–1.03	18	81.0	68–93*
Kidney	0.015–1.03	17	84.3	69–100
Muscle	0.03	7	98.7	74–117
Skin and fat	0.03	10	87.3	72–104

* One recovery was obtained at 124% (0.03 ppm).

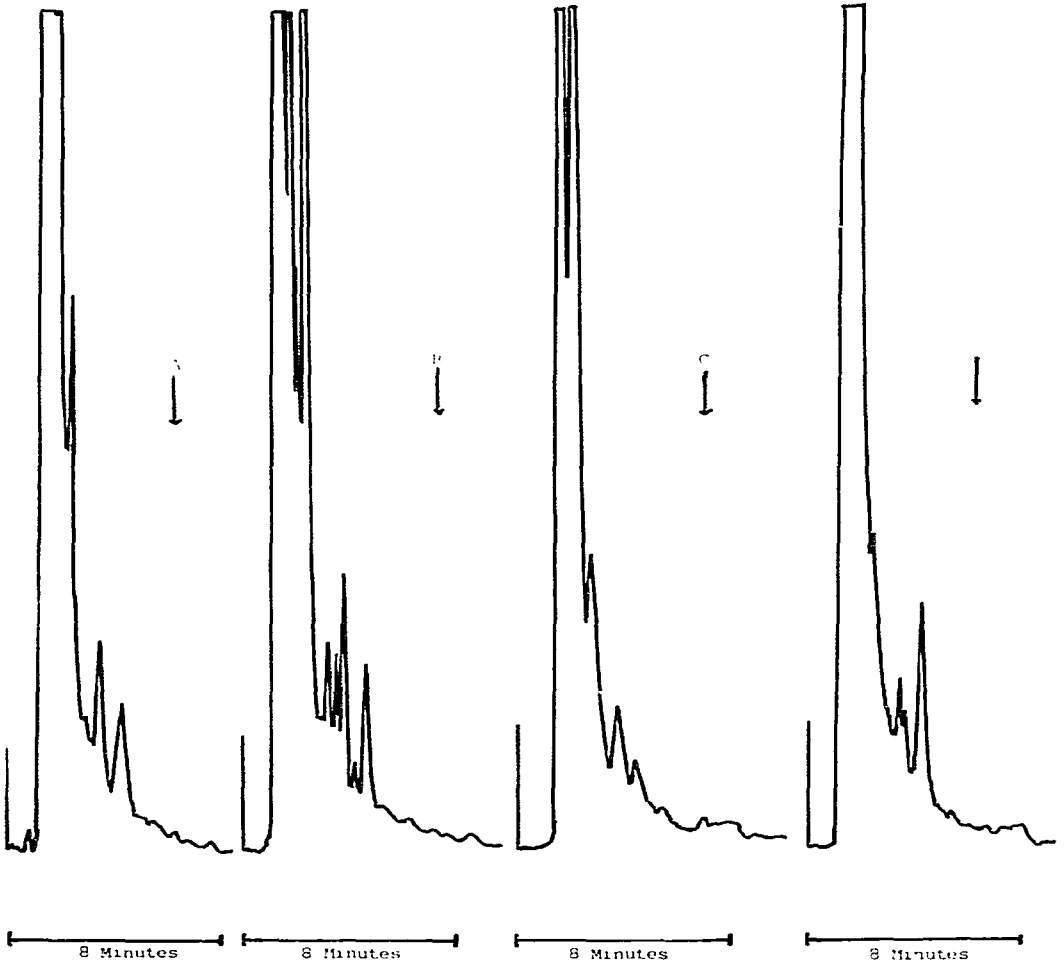

Fig. 4. Chromatograms obtained for extracts from control chicken tissues. Control tissue extracts: liver (A), kidney (B), muscle (C), and skin and fat (D).

time a total of 85 procedural recoveries have been undertaken, by three different analysts. The precision and accuracy data are shown in Table I, indicating the method to be both precise and accurate. Typical chromatography for the feed analysis is shown in Fig. 1, with typical calibration chromatograms and calibration curve shown in Figs. 2 and 3, respectively. The method has proved to be both very selective, so eliminating interference from other compounded animal products, and stability indicating. Analysis of small-scale (1–7 kg) mixes of halofuginone with poultry feed and radioactive experiments have shown that the procedural recovery data, although generated by aqueous addition of halofuginone to feed, is a good reflection of the accuracy of the method.

The HPLC method for analysing halofuginone in feed was modified to develop a procedure capable of detecting halofuginone in tissues at concentrations as low as 0.001 ppm (1 ppb). To reduce solvent volumes, the decision was taken to replace the XAD-2 resin column with a Sep-pak C_{18} cartridge. Previous work had suggested that

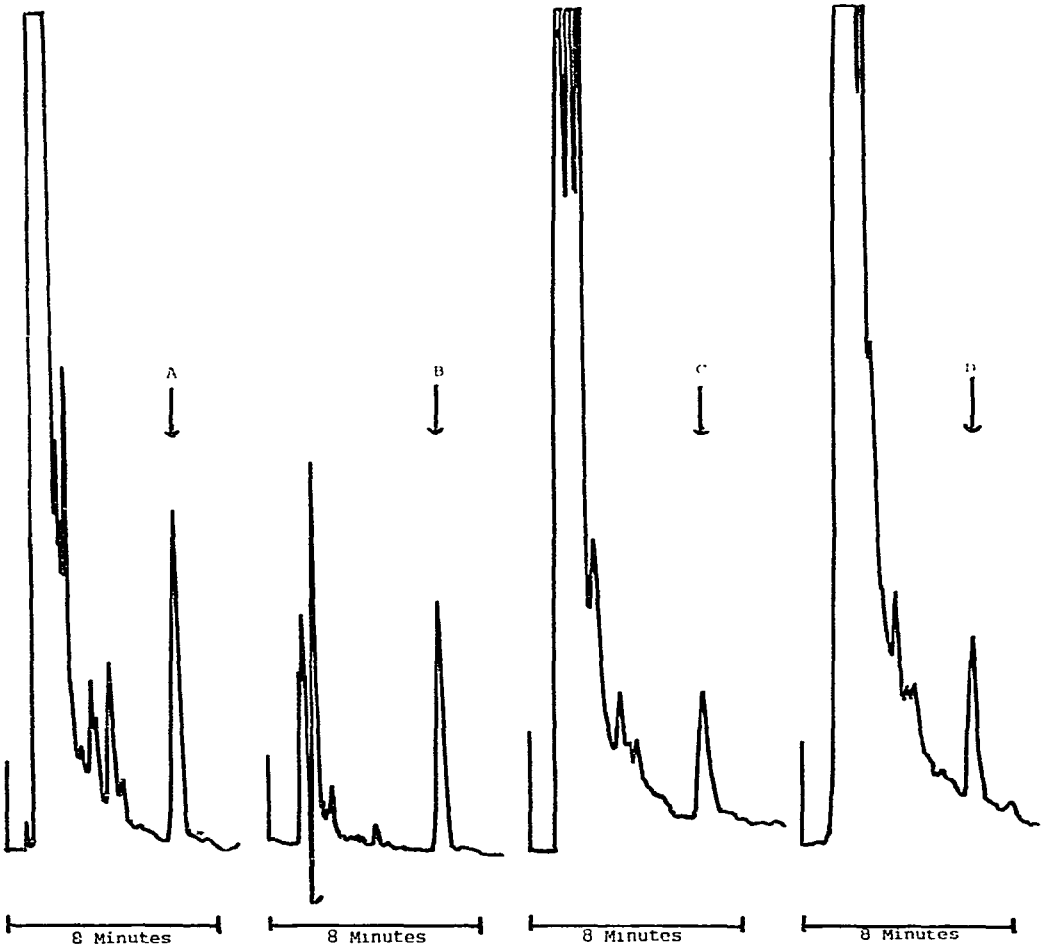

Fig. 5. Chromatograms obtained for extracts from control chicken tissues fortified with halofuginone. A, Liver (0.1 ppm halofuginone); B, kidney (1 ppm); C, muscle (0.03 ppm); D, skin and fat (0.03 ppm).

the use of 0.1 *M* hydrochloric acid resulted in breakdown of the Sep-pak C_{18} support. Therefore, the hydrochloric acid, used to extract halofuginone from the ethyl acetate, was replaced with ammonium acetate buffer. An aliquot of the buffer solution was passed through a Sep-pak C_{18} cartridge, enabling collection of the eluate in a small tapered glass tube and the ability to concentrate to a final mobile phase volume of 200 μl to achieve the required sensitivity. The use of Sep-pak C_{18} for feed analysis is also possible, and precision and accuracy data are presently being generated using this modification. It was also necessary to use the enzyme, trypsin, to release halofuginone from the tissues.

The precision and accuracy data of the method used for analysing tissues are given in Table II with typical chromatography, obtained from control tissues and tissues fortified with halofuginone, shown in Figs. 4 and 5 respectively. The accuracy of the method was not significantly different over the concentration range of the procedural recoveries or for different tissues, although recoveries were generally higher from muscle. The lowest concentration of halofuginone at which procedural recoveries were undertaken was 0.015 ppm, although the limit of sensitivity, defined as the concentration of halofuginone producing a peak with a height three times instrument noise, was estimated at 0.001 ppm (1 ppb).

This method has now been used successfully to analyse tissues from chickens dosed for 14 days with radioactive halofuginone, in order to determine the target tissue and the ratio of halofuginone to total radioactive residues.

ACKNOWLEDGEMENT

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1 A. Anderson, D. H. Christopher and R. N. Woodhouse, *J. Chromatogr.*, 168 (1979) 471–480.