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

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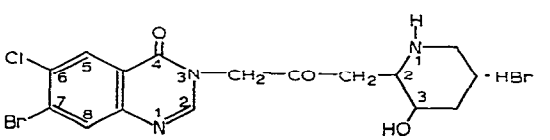
SUMMARY

Methods are described for the analysis of the anti-coccidial drug, halofuginone, at concentrations of 3 ppm in chicken feed, using gas–liquid chromatography and high-performance liquid chromatography. Both methods are based on ethyl acetate extraction, partition into hydrochloric acid and purification and concentration using XAD-2 column chromatography. The precision and accuracy of both methods is 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, it became essential to develop a sensitive, and precise, method of analysis for halofuginone in chicken feed, at the inclusion concentration of 3 ppm, and in concentrate at 6000 ppm. This paper describes and compares a gas–liquid chromatographic (GLC) and a high-performance liquid chromatographic (HPLC) method of analysis for halofuginone in finished feed (3 ppm). Application of the HPLC procedure to the analysis of mineral supplement (300 ppm halofuginone) and concentrate (6000 ppm halofuginone) is also described.



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EXPERIMENTAL

Apparatus

GLC. A Hewlett-Packard 5710A gas–liquid chromatograph, fitted with a ^{63}Ni electron-capture detector, was used. Glass columns (90 cm × 3 mm I.D.) were packed with 0.5% (w/w) FFAP on Diatomite CLQ (80–100 mesh; J.J.'s Chromatography, King's Lynn, Great Britain). The output from the detector was measured using a Honeywell Electronik 194 chart recorder.

HPLC. A Siemens S100 high-performance liquid chromatograph fitted with a Partisil™ 10 ODS (250 mm × 4.6 mm I.D. × 6.35 mm O.D.) prepacked column (Whatman, Maidstone, Great Britain) was used. Injection was achieved by means of a Rheodyne syringe loading injection valve Model 7120 (Rheodyne, Berkeley, Calif., U.S.A.) fitted with a 100-μl loop. The eluate from the column was monitored by means of a Zeiss PM2DLC variable wavelength UV detector fitted with an 8-μl flow cuvette, path length 10 mm. Output from the detector was measured on a Siemens Kompensograph III chart recorder.

General. The UV spectra of halofuginone were recorded on a Unicam SP1800 double beam spectrophotometer (Pye Unicam, Cambridge, Great Britain) using 10-mm path length quartz cells. Sample maceration was achieved using Ultra-Turrax, Model TP18/2N homogenisers. Glass columns, 30 cm × 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 adjustments. Centrifugation was accomplished using an MSE "super medium" centrifuge capable of accepting 200-ml glass centrifuge bottles. Bucchi Rotavapor R rotary film evaporators (RFE; Orme Scientific, Middleton, Great Britain), were used for all evaporations.

Materials

Analytical grade halofuginone (micronised) was obtained from Roussel Uclaf (Paris, France). The macroreticular resin, Amberlite XAD-2 standard grade (BDH Chemicals, Poole, Great Britain), was freed from chloride ions, by water washing, and purified by Soxhlet extraction with methanol for 8 h. 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 distilled water.

Acetate buffer was prepared by adding glacial acetic acid (30 ml/l) to ammonium acetate and dilution to produce a 0.25 *M* solution. All other chemicals used were of analytical-reagent grade. Unmedicated (control) poultry diet, "Chickmash", was obtained from Flowers and Sons (Ramsey, Great Britain). Mineral supplement was obtained from Coopers (Witham, Great Britain). Halofuginone concentrate (6000 ppm halofuginone nominal, in Blanc OMYA) was supplied by Roussel Uclaf.

Chromatography

GLC. The GLC column oven was operated isothermally at 235° with the injection port and detector at 250 and 300°, respectively. The carrier gas, argon–methane (95:5), flow-rate was 60 ml/min. Sample injection volume was 5 μl. Strip chart recordings were made at a chart speed of 12 in./h using a detector attenuation of 64. Under these conditions the retention time of halofuginone was approximately 8 min.

HPLC. The HPLC mobile phase consisted of methanol–acetate buffer (70:30, v/v). Sample injection volume was 100 μl. Chromatography was carried out, at ambient temperature, using a solvent flow-rate of 1 ml/min. The eluate from the column was monitored at a wavelength of 243 nm. Strip chart recordings were made at a chart speed of 120 mm/h using a detector attenuation of 0.04 a.u.f.s. Under these conditions the retention time of halofuginone was approximately 7 min.

Procedures

Finished feeds (approximately 3 ppm halofuginone). A representative sample of feed (10 g) was macerated with sodium carbonate solution (10%, w/v; 10 ml) 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). The combined ethyl acetate extracts were washed with sodium carbonate solution (5%, w/v; 50 ml), previously saturated with sodium chloride. The ethyl acetate phase was separated, extracted with 0.1 *M* hydrochloric acid (2 × 50 ml) and the combined hydrochloric acid extracts backwashed with ethyl acetate (10 ml). Any remaining ethyl acetate was removed from the acid solution (RFE at 38°). The acid solution was passed through an Amberlite XAD-2 macroreticular resin column (prepared as under *Materials*) and the column washed with 0.1 *M* hydrochloric acid (20 ml). 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 38°) to residual moisture, acetonitrile (10 ml) added, and the evaporation continued to dryness. The resultant residue was dissolved in either methanol (25 ml) for HPLC, or acetonitrile (25 ml) for GLC (halofuginone concentration typically 1.2 μg/ml).

Mineral supplements (approximately 300 ppm halofuginone). A representative sample (10 g) of supplement was macerated with methanol (200 ml) for 15 min. The solution was filtered and the residue washed with methanol. The filtrate and washings were collected in a volumetric flask (250 ml) and diluted to volume with methanol. For HPLC analysis, this solution was diluted ten-fold using methanol (halofuginone concentration typically 1.2 μg/ml).

Concentrates (approximately 6000 ppm halofuginone). A representative sample (1 g) of concentrate was dissolved in acetate buffer (approximately 100 ml). When effervescence had ceased, the solution was filtered and the filtrate collected in a volumetric flask (250 ml). The residue was washed with acetate buffer, the washings added to the volumetric flask and the volume adjusted to 250 ml with acetate buffer. For HPLC analysis, the solution was diluted ten or twenty fold with methanol (halofuginone concentration, typically 1.2 to 2.4 μg/ml).

Standard solutions

GLC. Halofuginone (50 mg) was dissolved in acetate buffer (250 ml) and an aliquot further diluted with distilled water to provide a concentration of halofuginone of 10 μg/ml.

To produce standard solutions, suitable for injection on to the GLC, portions of the 10 μl/ml standard (3 ml) were added to 10% sodium carbonate solution (10 ml) and processed as described under *Finished feeds (approximately 3 ppm halofuginone).* The final acetonitrile extract was suitably diluted to obtain concentrations of

halofuginone (as the hydrochloride) in the range 0–2 μg/ml. Extracts of some feeds were found to enhance the response of the GLC electron-capture detector to halofuginone. Under these circumstances, the final standard solutions were prepared in control diet extracts.

HPLC. Halofuginone (50 mg) was dissolved in acetate buffer (250 ml) and aliquots of the resulting solution diluted with methanol to provide concentrations of halofuginone in the range of 0–3 μg/ml.

Procedural recoveries

Aliquots (typically 1–3 ml) of a standard solution of halofuginone in acetate buffer were added to representative samples of control feed (10 g), feed supplement (10 g), or calcium carbonate* (1 g) to produce concentrations of 3 ppm, 300 ppm and 6.000 ppm, respectively. The resulting mixtures were allowed to remain at room temperature for 30 min before analysing as described under *Procedures.*

Calibration and calculation

Calibration curves of chromatographic peak height *versus* concentration of halofuginone were constructed using the data obtained by injection of aliquots of the standard solutions prepared as described under *Standard solutions.*

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

RESULTS AND DISCUSSION

The GLC method of analysis was adapted from a procedure developed jointly in our laboratories and in the Laboratoire Municipal (Bordeaux, France)[1]. One of the major modifications was the use of macroreticular resin[2] which permitted further purification and concentration of the extracts without additional operations on the labile free base form of halofuginone.

Typical chromatograms, obtained when this modified method was applied to finished feed, are shown in Fig. 1, with a calibration curve illustrated graphically in Fig 2. The precision and accuracy of the method is indicated in Table I.

Although suitable for routine quality control, the GLC procedure has the disadvantage that occasional enhancement of detector response by feed extracts necessitates the availability of control feed. Moreover, a polar molecule of high molecular weight, such as halofuginone (495.6 as the hydrobromide), would not be expected to elute easily from a GLC column, even at an oven temperature of 235°, and there is evidence to suggest that the compound eluting is an on-column decomposition product of halofuginone. Because of the possibility that the same decomposition product could also be formed, as a result of storage of halofuginone in chicken feed, a stability-indicating HPLC method was developed.

Typical HPLC chromatograms are shown in Fig. 3, with a calibration curve illustrated in Fig. 4, and the precision and accuracy of the method indicated in Table

* Simulating Blanc OMYA.

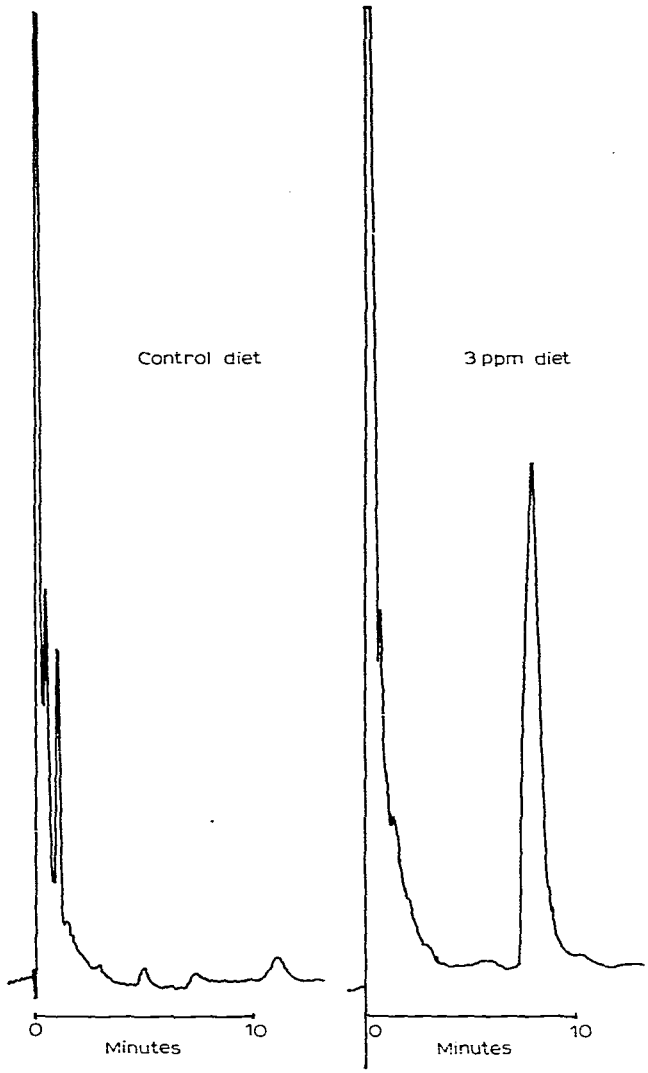


Fig. 1. Chromatograms obtained, using GLC, for extracts from control feed and feed containing halofuginone at 3 ppm.

TABLE I

PRECISION AND ACCURACY (FINISHED FEEDS)

The following procedural recovery data illustrate the precision and accuracy of the HPLC and GLC methods as applied to finished feed.

	HPLC	*GLC*
Concentration (nominal)	3 ppm	3 ppm
Number of determinations	19	15
Mean recovery	95.6%	98.7%
Standard deviation	±7.0%	±12.4%

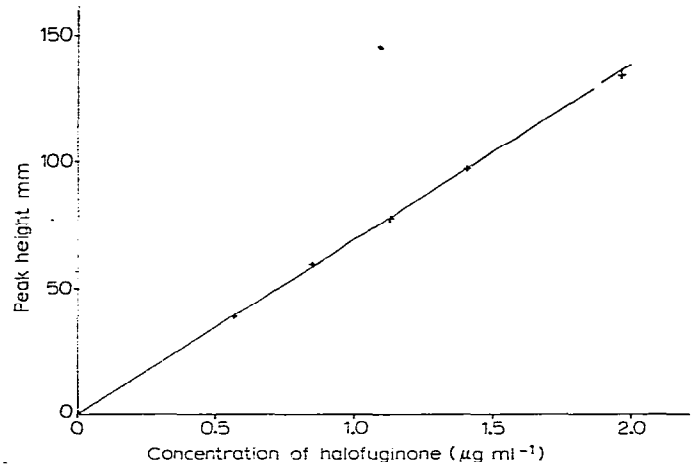
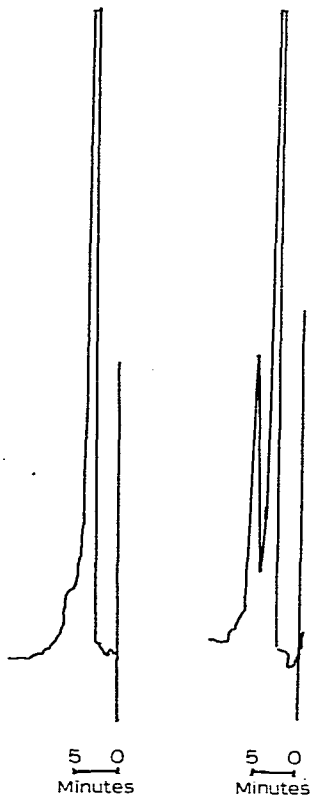


Fig. 2. Calibration curve obtained using GLC.



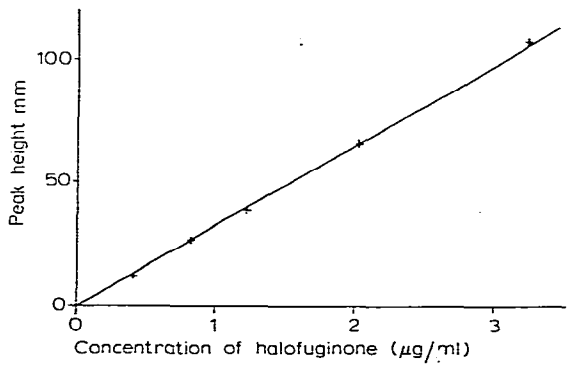


Fig. 3. Chromatograms obtained, using HPLC, for extracts from control feed and feed containing halofuginone at 3 ppm.

Fig. 4. Calibration curve obtained using HPLC.

TABLE II

PRECISION AND ACCURACY (MINERAL SUPPLEMENT AND CONCENTRATE)

The following procedural recovery data illustrates the precision and accuracy of HPLC method as applied to mineral supplement and concentrate.

	Mineral supplement	*Concentrate*
Concentration	300 ppm	6.000 ppm
Number of determinations	10	8
Mean recovery	91.6%	95.0%
Standard deviation	±2.08%	±1.7%

I. The method was extended to mineral supplement and concentrate and the relevant chromatographic data are shown in Fig. 5, with precision and accuracy in Table II. The retention times of possible degradation products and the *cis* isomer of halofuginone are shown in Fig. 6. None of these components has the same retention time as

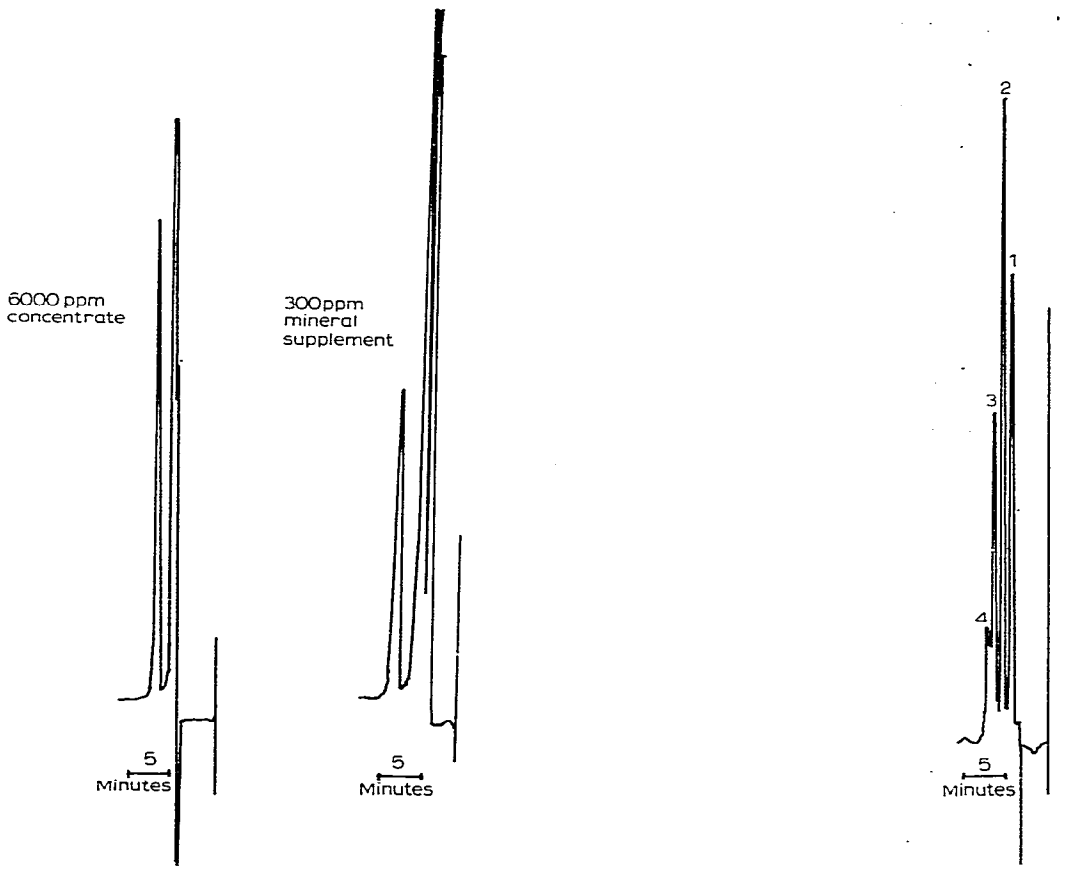


Fig. 5. Chromatograms obtained, using HPLC, for extracts from feed supplement and concentrate.

Fig. 6. Separation using HPLC, of possible degradation products of halofuginone. Peaks: 1 = 5-chloro-4-bromo-2-aminobenzoic acid + 2-hydroxy-6-chloro-7-bromo-quinazolinone; 2 = 6-chloro-7-bromo-quinazolinone; 3 = *trans*-halofuginone; 4 = *cis*-halofuginone.

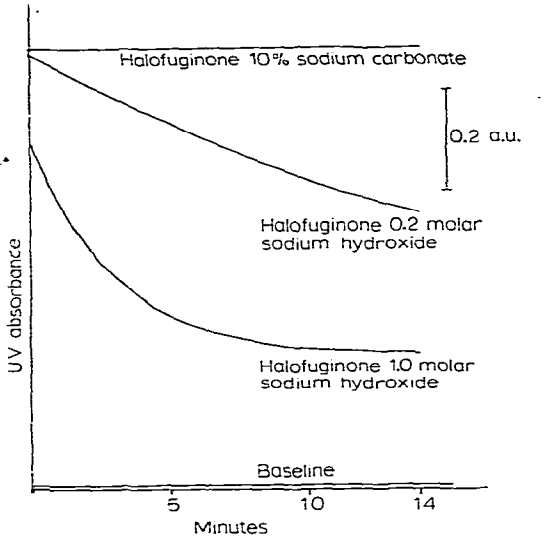


Fig. 7. Rate of degradation of halofuginone in alkaline solution.

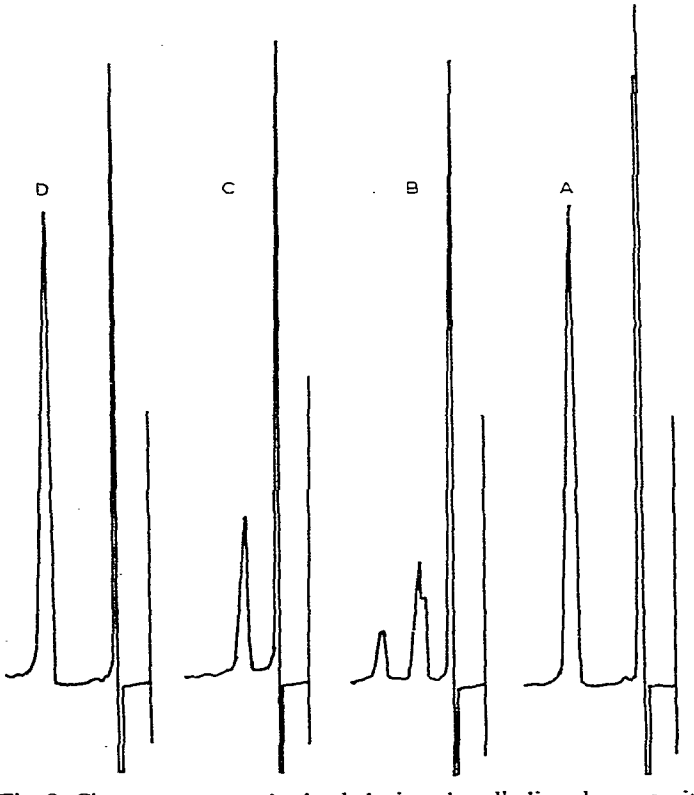


Fig. 8. Chromatograms obtained during the alkaline decomposition of halofuginone. Conditions: methanol–acetate buffer (50:50, v/v). A = 0 min; B = 10 min 1.0 *M* sodium hydroxide; C = 30 min 1.0 *M* sodium hydroxide; D = 25 min 10% (w/v) sodium carbonate.

halofuginone, although reduction of the methanol content of the mobile phase is necessary to achieve complete separation of cis and trans halofuginone. It was shown[3] that the major product of strong alkaline treatment is 5-chloro-4-bromo-2-amino-benzoic acid and Figs. 7 and 8 illustrate how rapid this decomposition is in 1.0 and 0.2 *M* sodium hydroxide. Since the extraction of halofuginone from feed is accomplished under mildly alkaline conditions the stability of the compound was investigated in sodium carbonate solution (0.94 *M*). No decomposition was evident over 5 h at laboratory temperature. During routine analysis, halofuginone remains in mild alkaline solution for no more than 15 min.

Both the GLC and HPLC methods of analysis have been used routinely in this laboratory to analyse samples of chicken feed containing halofuginone. The GLC method has proved to be less precise than the HPLC method and for this, and the other reasons detailed above, we have preferred to use HPLC. The HPLC method has

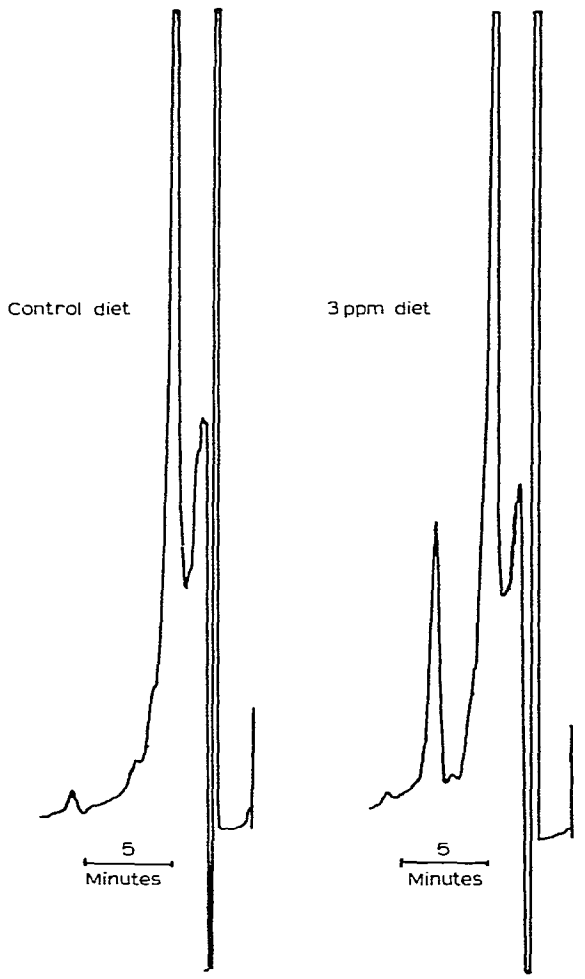


Fig. 9. Separation of halofuginone in feed extracts using a μBondapak C_{18} column. Conditions: methanol–0.125 *M* acetate buffer (60:40, v/v), 1.5 ml/min.

proved versatile and has been used to analyse halofuginone in a variety of chicken feeds. Diets from Argentina, South Africa, Bulgaria, Mexico, Denmark, France, Holland, Germany and Ireland have been analysed in this laboratory. The method has also proved sufficiently sensitive for the analysis of experimental rodent and dog diets containing halofuginone at a concentration of 0.4 ppm and 1.25 ppm, respectively.

The HPLC method has the additional advantage that, because of the excellent purification procedure, it is easily adapted to a variety of reversed-phase HPLC and ion-exchange columns. The separation of halofuginone in feed extracts has been demonstrated using LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.)[4], Partisil 10 SCX (Whatman) and μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.). This laboratory is now routinely analysing halofuginone using a μBondapak C_{18} column and Fig. 9 illustrates typical chromatograms obtained.

ACKNOWLEDGEMENT

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