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Note

Determination of halofuginone in poultry feeds by high-performance liquid chromatography

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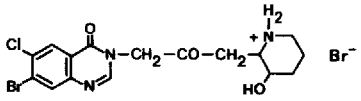
Halofuginone hydrobromide (I) {(±)-*trans*-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperyl)acetonyl]-4(3*H*)-quinazoline hydrobromide} is an anticoccidial drug added to poultry feeds at a concentration of 3 mg/kg of active compound.



For registration purposes, sensitive, specific and reliable methods are required for the determination of halofuginone in chicken tissues and feeds. In terms of selectivity, the method must be able to separate both isomers of halofuginone, as the *cis* compound is biologically inactive and may be formed during the preparation of the feed via biotransformation or during the analytical procedure. An efficient chromatographic resolution is necessary for accurate quantification of *trans*-halofuginone, which is the only active isomer.

The first method to be developed used gas chromatography (GC) for the quantitative step[1]. Investigations by GC–mass spectrometry (GC–MS) carried out in our laboratory clearly show that the GC peak was not in fact halofuginone but a compound coming from its thermal degradation in the injection port of the chromatograph. Therefore, both *trans* and *cis* isomers lead to the same GC peak.

To correct for this lack of selectivity, a high-performance liquid chromatographic (HPLC) method was developed that was able to separate both isomers[2,3]. Although very accurate, these methods described by Anderson and co-workers[2,3] appeared to be very difficult to apply for routine control and great discrepancies were observed between inter-laboratory assays. The aim of this study was to simplify the whole method and to avoid any extraction of halofuginone as a free base, as it can be transformed into its *cis* isomer under alkaline conditions such as those employed by Anderson and co-workers[2,3] during the extraction step.

Clean-up on disposable cartridges is replaced by an automated purification procedure that involves a multi-column chromatographic (MC-HPLC) procedure[4,5] with on-line UV detection. The overall method greatly reduces sample handling and saves time during control analysis; further, it can be applied without any modification to both commercial forms of halofuginone in poultry feed (coated and uncoated compound).

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EXPERIMENTAL

Materials

Acetic acid, ammonium acetate and hydrochloric acid were of analytical-reagent grade (Merck or Riedel-de Haën). Acetonitrile was of HPLC far-UV grade (LiChrosolv; Merck). Distilled water was purified through a Milli-Q system (Waters Assoc.).

Analytical-reagent grade halofuginone hydrobromide (batch 112R, No. 6L1321) was used to prepare standard solutions. Gelatin-coated halofuginone was sieved, and the 50–80-μm fraction was used for recovery studies on unmedicated feed samples. The halofuginone content of this fraction was 7.2% (w/w).

Acetate buffer (0.5 *M*; pH = 4.5) was prepared by dissolving ammonium acetate (38.6 g) and glacial acetic acid (60 ml) in 1 l of distilled water; other acetate buffers were obtained by subsequent dilution of this solution.

Sample extracts were filtered on Millex HV (0.45-μm) (Millipore) before HPLC.

Standard solutions

Halofuginone hydrobromide (about 3 mg) is accurately weighed into a 10-ml volumetric flask and sonicated in 0.05 *M* acetate buffer. This stock solution, after dilution with 0.05 *M* acetate buffer in the range 25–265 ng/ml, is used as a calibration solution for HPLC. When stored at 4°C protected from light it may be used for over 1 week without detectable degradation. This stock solution is also used for recovery studies from spiked samples of the uncoated material.

Extraction solvent A

Ammonium acetate (27 g) is dissolved in 530 ml of 4 *M* hydrochloric acid and the solution is diluted to 1 l with distilled water.

Columns and mobile phases

Three different mobile phases of increasing eluting strength are used: S_1, acetonitrile–water (18:82); S'_1, acetonitrile–0.05 *M* acetate buffer (pH 4.4) (20:80); and acetonitrile–0.15 *M* acetate buffer (pH 4.4) (25:75). Solvents S_1 and S'_1 are used on the pre-column at a flow-rate of 2 ml/min. Solvent S_2 is used as the analytical mobile phase at a flow-rate of 3 ml/min without creating any excessive back-pressure (1500 p.s.i.). After a set of runs, the flow-rate is decreased automatically to 0.2 ml/min. This procedure avoids any drastic and daily changes of solvent on the analytical column during the rinsing step; this precaution increases significantly the stability of the whole HPLC system, which always remained in buffered solvents.

The columns and pre-columns are fitted with PTFE sieves at the top and stainless-steel frits at the bottom. The sieves are easily changed. Their use instead of frits ensures a more efficient filtration of the sample. They avoid any column plugging, especially when biological samples containing proteins are run.

For the same reasons, large-sized particles (25–40 μm) are used in the pre-columns (5 cm × 4.6 mm I.D.). They are easily hand-packed with dry stationary phase (LiChroprep RP-2; Merck).

The analytical column (25 cm length) is packed with a C_{18} reversed phase

(μBondapak, 10 μm, from Waters Assoc., or Nucleosil, 10 μm, C_{18} from Macherey, Nagel & Co., which seems to be more stable under our analytical conditions).

Apparatus

Sample extractions are performed directly into 150-ml glass centrifuge bottles (Mistral 3000; MSE Scientific Instruments). The water-bath fitted with a magnetic stirrer is maintained at 55°C during extraction (Heildolph 94361; Bioblock Scientific).

The HPLC equipment consists of two M45 high-pressure pumps operating at constant flow-rate, a Wisp 710 autosampler and an M730 data integrator, all from Waters Assoc. The on-line sample clean-up is performed on a column-switching device including two pneumatic valves (Rheodyne): (i) a three-port, low-pressure valve, Model 5701, for the selection of different HPLC solvents used in the purification process; and (ii) a six-port high-pressure valve, Model 7000, for connection of both the pre-column and the analytical column during the transfer step. All the modules are under control of the M720 processor (Waters Assoc.). UV detection is performed at 243 nm ($5 \cdot 10^{-3}$ a.u.f.s.) on a 757 Spectroflow (Kratos). A scheme of the apparatus is shown in Fig. 1.

Extraction procedure

Sub-samples (20 g) are weighed from a bulk medicated feed (300–500 g) previously mixed by manual shaking. Extractions are performed under acidic conditions directly in the centrifuge bottles by adding 80 ml of solvent A. The use of large bottles (150 ml) instead of centrifuge tubes avoids any sample loss during the transfer steps in different vessels. Up to four samples are run simultaneously and maintained under magnetic stirring at 55°C for 5 min, then they are centrifuged for 2 min at 2900 *g*. The supernatant is poured into 250-ml volumetric flasks. This extraction is repeated twice under the same conditions. The extracts are combined and the volume is adjusted to 250 ml with distilled water before the automated clean-up and HPLC analysis.

Multi-dimensional HPLC clean-up

Since Huber *et al.*[4] extended the use of column-switching chromatography to HPLC, many examples of trace enrichment and sample clean-up have been developed[5]. The clean-up procedure developed here is the simplest that can be used in column-switching technology. The main actions of the cleaning procedure and HPLC analysis are summarized in Fig. 1.

A 4-ml volume of the acidic feed extracts (4 ml) is flushed through disposable filters (Millex HV, 0.45 μm) and an aliquot of 200 μl is injected on to the pre-column (P). The first solvent (S_1) is selected in order to wash out most of the co-extracts to waste, and to concentrate halofuginone on top of the pre-column, which is filled with RP-2 adsorbent (purification step in Fig. 1 and chromatogram B in Fig. 2). Switching of both pneumatic valves enables solvent S_1' to elute halofuginone from the pre-column to the C_{18} analytical column (transfer step in Fig. 1).

The purification efficiency and valve timing involved in this heart-cutting method are easily determined by coupling the pre-column to the UV detector. The time programme for the valves, which depends mainly on the dead volume of the whole apparatus, is as follows: clean-up step, up to 6 min; transfer step, from 6 to

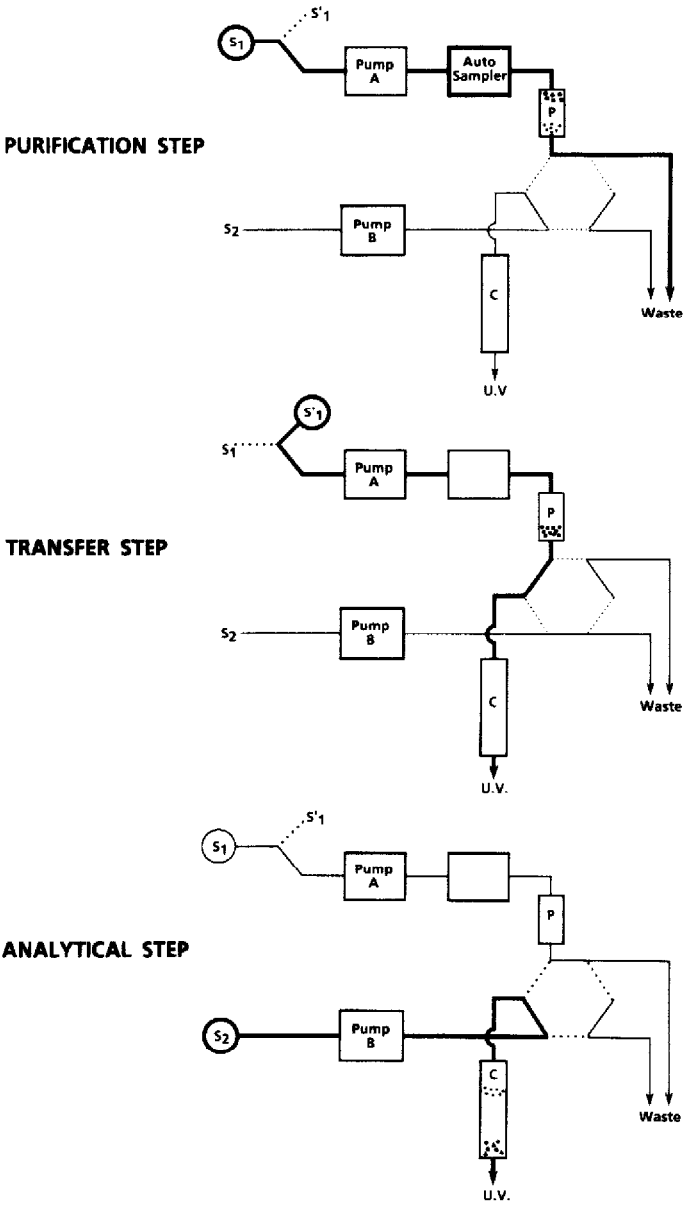


Fig. 1. Main steps of the on-line purification procedure used for halofuginone feed extracts: P = RP-2 pre-column; C = C_{18} analytical column; S_1, S'_1 and S_2 = solvents of increasing eluting strength.

8 min; a slight difference can be observed from one pre-column to another, depending on the packing efficiency; equilibration of the pre-column for the next run takes place from 7 to 20 min.

As an example, the chromatograms in Fig. 2 show the fraction of the sample matrix that is flushed on to the analytical column (C) during the transfer step.

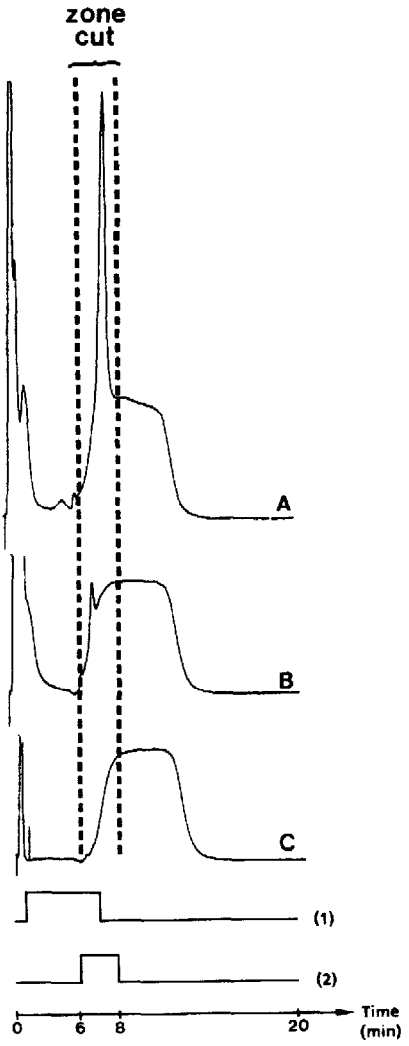


Fig. 2. UV chromatograms from direct injection on RP-2 pre-column. (A) Spiked feed extract (10 μg of halofuginone injected); (B) control feed extract; (C) solvent change on pre-column. (1) Timing of valve 1 during solvent change $S_1 \rightarrow S'_1$; (2) timing of valve 2 during transfer step.

The few interfering compounds that are co-eluted with halofuginone from the RP-2 phase are easily separated from the analyte on the C_{18} column during the analytical step.

When combined with trace enrichment, this clean-up has been applied with success to various matrices. Large sample volumes (up to 2 ml) allows low-level determinations of halofuginone in biological samples. By selecting appropriate solvents and stationary phases, detrimental band broadening during the last analytical step can be avoided. In the present instance, up to 4 ml of solvent S_1 are transferred to the C_{18} analytical column without any loss of efficiency.

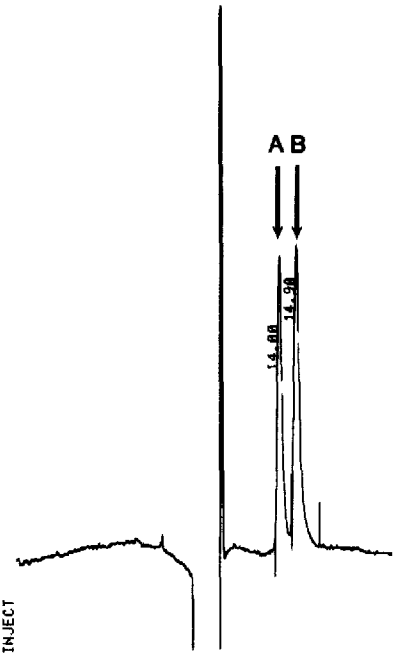


Fig. 3. MC-HPLC analysis of halofuginone (A) and its *cis* isomer (B).

As an example, Fig. 3 represents the chromatographic analysis by MC-HPLC of a mixture of halofuginonee (peak A) and its *cis* isomer (peak B). The excellent resolution between the two compounds allows the specific determination of halofuginone. To avoid overestimated data, such a resolution must be maintained throughout the entire routine analysis and must always be checked to ensure accuracy and specificity of the final results. During the analytical step, the pre-column is equilibrated to the starting conditions for the next run.

Calibration graphs obtained from standard solutions exhibit highly reproducible and linear responses. Graphs obtained with direct or multi-column injections of standard (up to 265 ng/ml) are obtained. No difference is observed between these two chromatographic modes as long as solutions are prepared in acetate buffer or in the solvent used for sample extraction. The recovery from the zone-cut on the pre-column is 100% in both instances.

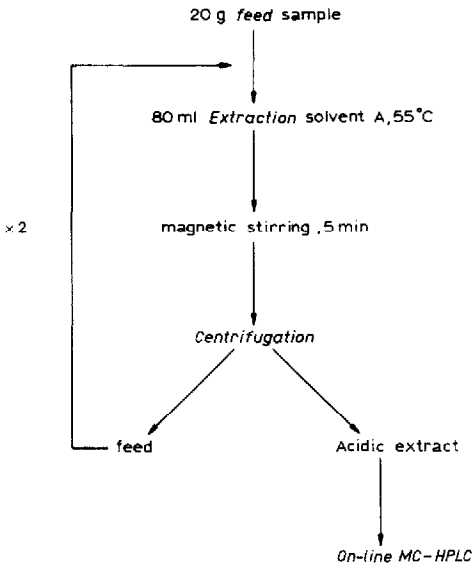
On the other hand, if the same dilutions are performed in the presence of feed extracts a slight decrease (3.5%) in efficiency is observed during the transfer step. To take into account this feed effect during the on-line purification step, concentrations are determined from a calibration graph obtained with halofuginone standard solutions diluted in blank feed extracts.

RESULTS AND DISCUSSION

The method commonly used in halofuginone assays[2,3,6] gives very reliable results but is time consuming (about 90 min per sample) and difficult to simplify. Our

aim was to obtain similar results using a method that may possibly be fully automated later. The extraction technique described in this paper reduces the sample handling time by half, and provides feed extracts that can be analysed directly through on-line MC-HPLC clean-up.

The scheme of the overall analytical procedure is summarized below:



This new method presents two major improvements over the previous method, based on off-line stages. First, the complexity of the extraction procedure has been greatly reduced; second, the use of an automatic sample clean-up by column switching eliminates some of the drawbacks encountered in the off-line liquid–solid purification procedure, such as unreliable results observed on disposable cartridges due to batch-to-batch variations, lack of reproducibility in the recovery of the analyte and time-consuming manipulations.

The stability of halofuginone under the strongly acidic conditions of the extraction was tested by mass spectrometry using direct introduction. A fit of 90% was obtained in a library search between the spectra of standard halofuginone and the sample maintained under extraction conditions. Some additional fragments observed in the extracted standard are due to solvent impurities, as shown in Fig. 4.

Chromatograms

Typical chromatograms obtained from feed extract analysis are shown in Fig. 5 to illustrate the specificity of the method. The observed retention time of halofuginone depends mainly on the timing of the operating valves and on the total volume transferred from the pre-column to the analytical column. The total analysis time, including equilibration to the starting conditions, is 20 min per sample. For calibration purposes, a standard solution is run every sixth injection; this allows the stability of the HPLC system to be checked continuously.

About 50 analyses are performed on the same pre-column without any sig-

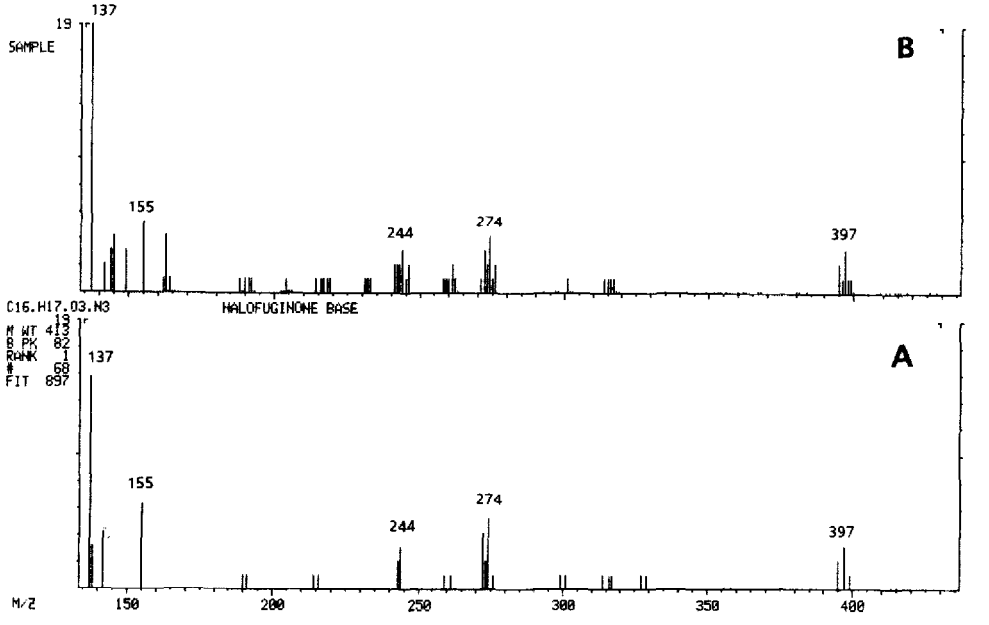


Fig. 4. Comparison of mass spectra of halofuginone (A) before and (B) after acid extraction.

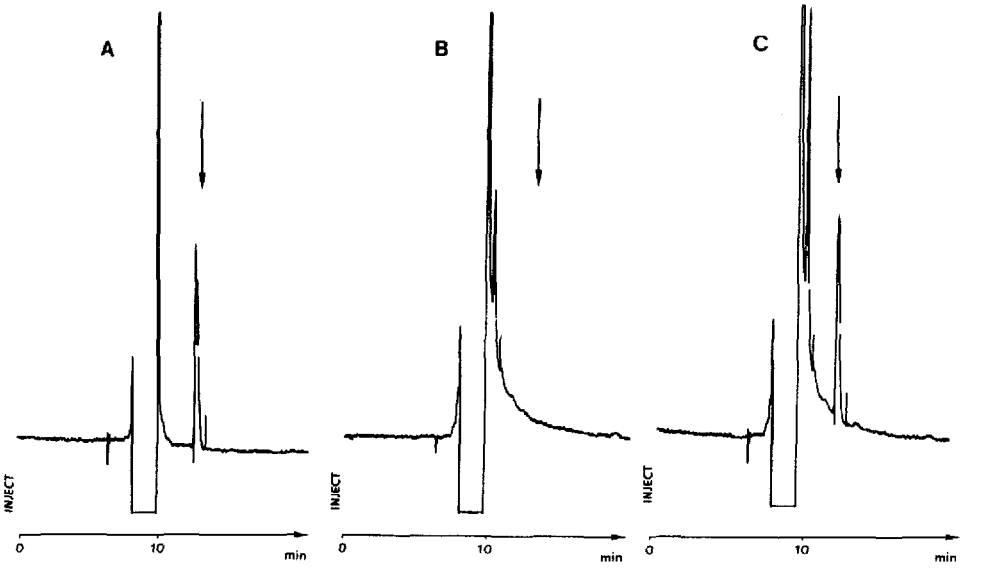


Fig. 5. Typical chromatograms from MC-HPLC clean-up of halofuginone. (A) Standard, 29 ng; (B) control feed; (C) spiked feed, 3 ppm.

TABLE I

VARIATION OF UV RESPONSE FACTOR OF HALOFUGINONE DURING ROUTINE CONTROL ANALYSIS

Run No.	*Mean response factor (area/ng injected)*	*R.S.D. (%)*	*Decrease in response factor (%)*
1–5	5776 ± 58	1.00	–
25–30	5692 ± 56	1.00	1.40
45–50	5546 ± 56	1.00	4.00
60–65	5530 ± 65	1.20	4.30
75–80	4058 ± 435	11.00	30.00

nificant decrease in the transfer efficiency as determined by injecting a standard solution prepared with feed extract medium. The relative standard deviation (R.S.D.) of response factor of halofuginone is used to check the reliability of the whole analytical system under such conditions. The results are summarized in Table I.

A slight decrease in the response factor (about 4%) is observed after the first 40 injections but it remains constant up to the 60th injection. The R.S.D. is less than 1.5%. As a consequence, if one runs a standard solution every ten feed sample analysis to account for the slight variation of the response factor, a highly precise and reproducible determination of halofuginone in feeds will be obtained on the same pre-column for more than 50 samples.

Unreliable results are observed after 70 injections of feed extract, probably owing to the instability of the C_2 bonded phase in an acidic medium. As a consequence, the pre-column must be changed. The use of a more stable polymeric phase is currently under investigation.

Recovery study

As it cannot be assumed that no errors occur during the production of the medicated feed or during sampling, recovery studies must be undertaken. They are performed by fortifying a control feed with a known amount of coated or uncoated halofuginone. To avoid overestimated results, the samples are allowed to stand overnight at room temperature before extraction is performed.

For comparison purposes and despite the fact that the solubilization step of gelatin is useless for the uncoated compound, both coated and uncoated halofuginone are analysed under the same conditions. As calibration solutions are diluted in the control feed extract, the results are corrected for the sample effect during the purification procedure.

Recoveries, including the extraction and purification procedures, are reported in Table II; the overall precision better is than 3%. These results are similar to those published by Anderson *et al.*[6] in an internal report. It can be seen that, whichever extraction method is used, the recovery is higher for coated halofuginone; this may be due to irreversible adsorption of the uncoated analyte on the feed components.

Stability study

As numerous assays are performed within a day, feed extracts may wait in the

TABLE II

COMPARISON OF RECOVERIES OBTAINED FROM TWO DIFFERENT ANALYTICAL PROCEDURES

Procedure	*Recovery (%)*	
	Uncoated halofuginone	*Coated halofuginone*
H.R.C. method*	87.4 ± 2.8 ($n=8$)	90.6 ± 6.2 ($n=8$)
R.U. method**	83.4 ± 1.4 ($n = 7$)	89.2 ± 2.8 ($n = 6$)

* H.R.C. = Huntingdon Research Center.
** R.U. = Roussef Uelof Research Center.

autosampler for a long time before analysis takes place. It seems important to check the stability of halofuginone under such drastic conditions (strongly acidic medium, light and high temperature).

Stability control was performed by analysing in duplicate the extracts obtained from the recovery study. Statistically there is no difference between the two sets of results: 86.6% ± 1.5% at T_0 and 89.5% ± 3% at T_0 ± 48 h, and no degradation occurs after days in the autosampler. This may allow one to carry out first the extraction step for all the samples to be analysed and then the purification and quantitation steps by the MC-HPLC procedure. Further, a given extract can be reprocessed if any doubtful data are observed.

We used this new method to determine coated halofuginone in various poultry feeds and tried to establish the influence of additives or the formulation process on the final results. So far no differences have been found between mash and pellet feeds and the final results seem to be independent of fat content.

CONCLUSION

The aim of this study was to develop a rapid and reliable method for the determination of halofuginone in poultry feed which could be used for routine quality control. Compared with previously used methods, the extraction and sample clean-up procedures are modified. Desorption from feed components is effected in a strongly acidic medium and gives accurate results for both coated and uncoated halofuginone; a study is currently in progress on others commercial forms.

An attempt will be made to automate fully the overall procedure by using robotics. As this will need the sample size to be reduced, the accuracy will depend mainly on the reliability of the sampling procedure, which will be defined. Regarding the analytical part of this method, the automated HPLC purification is a real improvement over the disposable cartridges that were previously used.

Taking into account the high sensitivity of the UV detector, an on-line concentration step was useless for feed assays (detection limit, 3 ng injected). Nevertheless, this step was included in the method developed for the trace analysis (5–50 ppb range) of halofuginone residues in biological samples (plasma, milk etc.). In such a

case, large sample volumes are injected (up to 2 ml), after dilution or protein precipitation. As no major modification was involved for these applications, this provides evidence of the flexibility of this automatic sample clean-up.

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