II

(Acts whose publication is not obligatory)

COMMISSION

ELEVENTH COMMISSION DIRECTIVE 93/70/EEC
of 28 July 1993
establishing Community analysis methods for official control of feedingstuffs

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs (1), as last amended by Regulation (EEC) No 3768/85 (2), and in particular Article 2 thereof,

Whereas Directive 70/373/EEC requires official controls on feedingstuffs for the purpose of checking compliance with requirements arising under quality and composition provisions laid down by law, regulation or administrative action, to be carried out using Community sampling and analysis methods;

Whereas a Community analysis method for the additive halofuginone should be established for use in checking compliance with the conditions for its use in animal nutrition;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE:

Article 1

Member States shall require that analyses for halofuginone content for the purposes of official checks on feedingstuffs be made using the method described in the Annex hereto.

Article 2

Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by 30 June 1994. They shall immediately inform the Commission thereof.

When Member States adopt these provisions, these shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by Member States.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 28 July 1993.

For the Commission
René STEICHEN
Member of the Commission

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 362, 31. 12. 1985, p. 8.

ANNEX

DETERMINATION OF HALOFUGINONE

DL-trans-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)acetonyl]-quinazolin-4-(3H)-one hydrobromide

1. **Purpose and scope**

The method is for the determination of halofuginone in feedingstuffs. The lower limit of determination is 1 mg/kg.

2. **Principle**

After treatment with hot water, halofuginone is extracted as the free base into ethyl acetate and subsequently partitioned as the hydrochloride into an aqueous acid solution. The extract is purified by ion-exchange chromatography. The content of halofuginone is determined by reserved-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. **Reagents**

3.1. Acetonitrile, HPLC grade

3.2. Amberlite XAD-2 resin

3.3. Ammonium acetate

3.4. Ethyl acetate

3.5. Acetic acid, glacial

3.6. Halofuginone standard substance (DL-trans-7-brome-6-chloro-3-[3-hydroxy-2-piperidyl)acetonyl] quinazoline-4-(3H)-one hydrobromide, E 764)

3.6.1. Halofuginone stock standard solution, 100 µg/ml

Weight to the nearest 0,1 mg, 50 mg of halofuginone (3.6) in a 500 ml graduated flask, dissolve in ammonium acetate buffer solution (3.18), make up to the mark with the buffer solution and mix. This solution is stable for three weeks at 5 °C if stored in the dark.

3.6.2. Calibration solutions

Into a series of 100 ml graduated flasks transfer 1,0, 2,0, 3,0, 4,0 and 6,0 ml of the stock standard solution (3.6.1). Make up to the mark with mobile phase (3.21) and mix. These solutions have concentrations of 1,0, 2,0, 3,0, 4,0 and 6,0 µg/ml of halofuginone respectively. These solutions must be freshly prepared before use.

3.7. Hydrochloric acid (ρ_{20} approximately 1,16 g/ml).

3.8. Methanol

3.9. Silver nitrate

3.10. Sodium ascorbate

3.11. Sodium carbonate

3.12. Sodium chloride

3.13. EDTA (ehtylenediaminetetraacetic acid, disodium salt)

3.14. Water, HPLC grade

3.15. Sodium carbonate solution, θ = 10 g/100 ml

3.16. Sodium chloride-saturated sodium carbonate solution, θ = 5 g/100 ml

Dissolve 50 g of sodium carbonate (3.11) in water, dilute to 1 l and add sodium chloride (3.12) until the solution is saturated.

3.17. Hydrochloric acid, approximately 0,1 mol/l

Dilute 10 ml of HCl (3.7) with water to 1 l.

3.18. Ammonium acetate buffer solution, approximately 0,25

Dissolve 19,3 g of ammonium acetate (3.3) and 30 ml of acetic acid (3.5) in water (3.14) and dilute to 1 l.

3.19. Amberlite XAD-2 resin preparation

Wash an appropriate quantity of Amberlite (3.2) with water until all chloride ions have been removed, as indicated by a silver nitrate (3.20) test performed on the discarded aqueous phase. Then wash the resin with 50 ml of methanol (3.8), discard the methanol and store the resin under fresh methanol.

3.20. Silver nitrate solution, approximately 0,1 mol/l

Dissolve 0,17 g of silver nitrate (3.9) in 10 ml of water.

3.21. HPLC Mobile phase

Mix 500 ml of acetonitrile (3.1) with 300 ml of ammonium acetate buffer solution (3.18) and 1 200 ml of water (3.14). Adjust the pH to 4,3 using acetic acid (3.5). Filter through a 0,22 µm filter (4.8) and degas the solution (e.g. by ultrasonification for 10 minutes). This solution is stable for one month, if stored in the dark in a closed container.

4. Apparatus

4.1. Ultrasonic bath

4.2. Rotary film evaporator

4.3. Centrifuge

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector

4.4.1. Liquid chromatographic column, 300 mm × 4 mm, C 18, 10 µm packaging, or an equivalent column

4.5. Glass column (300 mm × 10 mm) fitted with a sintered-glass filter and a stopcock

4.6. Glass-fibre filters, diameter 150 mm

4.7. Membrane filters, 0,45 µm

4.8. Membrane filters, 0,22 µm

5. Procedure

Note: Halofuginone as the free base is unstable in alkaline and ethyl acetate solutions. It should not remain in ethyl acetate for more than 30 minutes.

5.1. General

5.1.1. A blank feed should be analysed to check that neither halofuginone nor interfering substances are present.

5.1.2. A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of halofuginone, similar to that present in the sample. To fortify at a level of 3 mg/kg, add 300 µl of the stock standard solution (3.6.1) to 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note: for the purpose of this method, the blank feed should be similar in type to that of the sample and on analysis halofuginone should not be detected.

5.2. Extraction

Weigh to the nearest 0,1 g, 10 g of the prepared sample, into a 200 ml centrifuge tube, add 0,5 g of sodium ascorbate (3.10), 0,5 g of EDTA (3.13) and 20 ml of water and mix. Place the tube for 5 minutes in a water bath (80 °C). After cooling down to room temperature, add 20 ml of sodium carbonate solution (3.15) and mix. Add immediately 100 ml of ethyl acetate (3.4) and shake vigorously by hand for 15 seconds. Then place the tube for three minutes in the ultrasonic bath (4.1) and loosen the stopper. Centrifuge for two minutes and decant the ethyl acetate phase through a glass fibre filter (4.6), into a 500 ml separating funnel. Repeat the extraction of te sample with a second portion of 100 ml of ethyl acetate. Wash the combined extracts for one minute with 50 ml of sodium chloride-saturated sodium carbonate solution (3.16) and discard the aqueous layer.

Extract the organic layer for 1 min with 50 ml of hydrochloric acid (3.17). Run the lower acid layer into a 250 ml separating funnel. Re-extract the organic layer for 1,5 minutes with a further 50 ml of hydrochlorid acid and combine with the first extract. Wash the combined acid extracts by swirling for approximately 10 seconds with 10 ml of ethyl acetate (3.4).

Quantitatively transfer the aqueous layer into a 250 ml round-bottomed flask and discard the organic phase. Evaporate all the remaining ethyl acetate from the acid solution using a rotary film evaporator (2.4). The temperature of the water bath should not exceed 40 °C. Under a vacuum of approximately 25 mbar all of the residual ethyl acetate will be removed within 5 minutes at 38 °C.

5.3. Clean up

5.3.1. Preparation of the Amberlite column

An XAD-2 column is prepared for each sample extract. Transfer 10 g of prepared Amberlite (3.19) into a glass column (4.5) with methanol (3.8). Add a small plug of glass-wool to the top of the resin bed. Drain the methanol from the column and wash the resin with 100 ml of water, stopping the flow as the liquid reaches the top of the resin bed. Allow the column to equilibrate or 10 minutes before use. Never allow the column to run dry.

5.3.2. Sample clean up

Transfer the extract (5.2) quantitatively to the top of the prepared Amberlite column (5.3.1) and elute, discarding the eluate. The rate of elution should not exceed 20 ml/min. Rinse the round-bottomed flask with 20 ml of hydrochlorid acid (3.17) and use this to wash the resin column. Blow through any remaining acid solution with a stream of air. Discard the washings. Add 100 ml of methanol (3.8) to the column and allow 5 to 10 ml to elute, collecting the eluate in a 250 ml round-bottomed flask. Leave the remaining methanol for 10 minutes to equilibrate with the resin and continue the elution at a rate not exceeding 20 ml/min, collecting the eluate in the same round-bottomed flask. Evaporate the methanol on the rotary film evaporator (4.2), the temperature of the waterbath should not exceed 40 °C. Transfer the residue quantitatively into a 10 ml calibrated flask using the mobile phase (3.21). Make up to the mark with mobile phase and mix. An aliquot is filtered through a membrane filter (4.7). Reserver this solution for the HPLC determination (5.4)

5.4. HPLC determination

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results.

Liquid chromatographic column (4.4.1)

HPLC Mobile phase (3.21)

Flow rate : 1,5 to 2 ml/min.

Detection wavelength : 243 nm

Injection volume : 40 to 100 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.6.2) containing 3,0 µg/ml several times, until constant peak heights (or areas) and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.6.2) several times and measure the peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights or areas of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.4.3. Sample solution

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the halofuginone peaks.

6. Calculation of results

Determine the concentration of the sample solution in µg/ml, from the mean height (area) of the halofuginone peaks of the sample solution by reference to the calibration graph (5.4.2).

The content of halofuginone w (mg/kg) of the sample is given by the following formula :

$$w = \frac{c \times 10}{m}$$

in which :

— c : halofuginone concentration of the sample solution in µg/ml,

— m : mass of the test portion in grams.

7. Validation of the results

7.1. Identity

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.6.2) containing 6,0 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of a calibration solution (3.6.2). The amount of added halofuginone should be similar to the estimated amount of halofuginone found in the sample extract.

Only the height of the halofuginone peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within ± 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria :

(a) the wavelength of maximum asbsorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within ± 2 nm ;

(b) between 225 and 300 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 to 100 % or relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte ;

(c) between 225 and 300 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. Repeatability

The difference between results of two parallel determinations carried out on the same sample must not exceed 0,5 mg/kg for halofuginone contents up to 3 mg/kg.

7.3. Recovery

For the fortified blank sample the recovery should be at least 80 %.

8. Results of a collaborative study

A collaborative study (1) was arranged in which three samples were analysed by eight laboratories.

Results

	Sample A (Blank) on receipt	Sample B (Meal)		Sample C (Pellets)	
		on receipt	after two months	on receipt	after two months
Mean (1)	n.d.	2,80	2,42	2,89	2,45
S_R	—	0,45	0,43	0,40	0,42
CV_R	—	16	18	14	17
rec.		86	74	88	75

(1): units in mg/kg.
n.d.: not detected.
S_R: standard deviation of reproducibility.
CV_R: coefficient of variation (%).
rec.: recovery (%).

(1) *The Analyst* 108, 1983, pp. 1252 to 1256.