INTERNATIONAL STANDARD

ISO 14797

First edition
1999-03-15

Animal feeding stuffs — Determination of furazolidone content — Method using high-performance liquid chromatography

Aliments des animaux — Dosage de la furazolidone — Méthode par chromatographie liquide à haute performance

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 14797 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Annexes A to C of this International Standard are for information only.

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Printed in Switzerland

Animal feeding stuffs — Determination of furazolidone content — Method using high-performance liquid chromatography

1 Scope

This International Standard specifies a high-performance liquid chromatographic (HPLC) method for the determination of the furazolidone content of premixtures and animal feeding stuffs.

The method is applicable to animal feeding stuffs with a furazolidone content of 25 mg/kg to 5 000 mg/kg and to premixtures with a mass fraction of furazolidone of up to 20 % [formerly written as 20 % (m/m)].

NOTE 1 For animal feeding stuffs, the furazolidone content is expressed in milligrams per kilogram; for premixtures, as a mass fraction in percent [% (m/m)].

NOTE 2 Furazolidone is a chemotherapeuticum belonging to the group of nitrofuranes. Nitrofuranes are bacteriostatic or bactericidal against Gram-positive and Gram-negative microorganisms and against some moulds and protozoa.

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2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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ISO 6498:1998, *Animal feeding stuffs — Preparation of test sample*.

3 Principle

Furazolidone is extracted from the sample with a mixture of acetonitrile and methanol. Animal feeds are pre-wetted with water. The extract of animal feeds is purified through a short aluminum oxide column and subsequently diluted with water. The extract of premixtures is directly diluted with a mixture of water, acetonitrile and methanol. The final extract is analysed by reverse-phase HPLC with UV detection at a wavelength of 365 nm (see references [1] to [3]).

4 Reagents

Use only reagents of recognized analytical grade.

4.1 Water, demineralized or deionized, with resistivity of at least 10 MΩ·cm, or water of at least equivalent purity.

4.2 Extraction solvent: mixture of acetonitrile and methanol (1:1 by volume).

Combine equal volumes of acetonitrile and methanol. Mix well and allow to adjust to room temperature before use.

4.3 Dilution solvent: mixture of extraction solvent (4.2) and water (4.1) (35:65 by volume).

Mix 350 ml of extraction solvent (4.2) with 650 ml of water (4.1).

4.4 Acetic acid (CH_3CO_2H), volume fraction of 10 % [10 % (V/V)].

Dilute 10 ml of glacial acetic acid to 100 ml with water (4.1).

4.5 Sodium acetate buffer solution, $c(CH_3CO_2Na)$ = 0,01 mol/l, pH = 6,0.

Weigh 0,82 g of sodium acetate into a 1 000 ml one-mark volumetric flask. Dissolve in 700 ml of water. Adjust the pH to 6,0 with acetic acid (4.4). Dilute to the mark with water and mix.

4.6 Mobile phase for HPLC.

Combine 800 ml of sodium acetate buffer solution (4.5) and 200 ml of acetonitrile and mix. Filter the eluent through a 0,22 µm filter using a solvent filtration system (5.2), and degas for 10 min in an ultrasonic bath (5.3) before use.

4.7 Furazolidone standard material: *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone; CAS number 67-45-8 according to Chemical Abstracts Registry.

WARNING — Because of the sensitivity of furazolidone to light, conduct all operations in the absence of daylight or artificial white light. Avoid inhalation of and exposure to the toxic furazolidone standard material and solutions thereof. Work in a fumehood when handling the solvents and solutions. Wear safety glasses and protective clothing.

4.8 Furazolidone stock solution (approximately 250 µg/ml).

Weigh 25 mg ± 1 mg of furazolidone (4.7) to the nearest 0,1 mg into a 100 ml one-mark volumetric flask. Dissolve in extraction solvent (4.2), dilute to the mark and mix. Calculate the concentration taking into account the purity of the standard material. Prepare fresh every month. Store in the dark at 0 °C to 8 °C.

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4.9 Furazolidone working solutions (approximately 5 µg/ml and 12,5 µg/ml).

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Pipette 2,0 ml and 5,0 ml respectively of the furazolidone stock solution (4.8) into separate 100 ml one-mark volumetric flasks. Add 65 ml of water, dilute to the mark with extraction solvent (4.2) and mix. Prepare fresh for each series of samples.

4.10 Neutral aluminum oxide, activity 1.

NOTE 0 % to 1 % of water is necessary for total de-activation.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 pH-meter.

5.2 Solvent filtration system, all-glass apparatus suitable for 0,22 µm filters.

5.3 Ultrasonic bath.

5.4 Rotary shaker, horizontal rotation, rotation frequency 250 min^{-1} to 300 min^{-1}.

5.5 Glass microfibre filter, of diameter 15 cm.

5.6 Glass wool.

5.7 Glass column for chromatography, of length 30 cm, internal diameter 10 mm, restricted at the end and fitted with a wad of glass wool (5.6).

5.8 Filtration system, equipped with poly(vinylidene difluoride) (PVDF) filters or polytetrafluorethylene (PTFE) filters of pore size 0,45 µm.

5.9 HPLC system, comprising the following.

5.9.1 Pump, pulse free, capable of maintaining a volume flow rate of 0,1 ml/min to 2,0 ml/min.

5.9.2 Injection system with loop suitable for 20 µl to 50 µl injections.

5.9.3 UV detector, suitable for measurements at a wavelength of 365 nm.

If available, a diode array detector may be used for confirmation purposes.

5.9.4 Recorder.

5.9.5 Guard column: silica bonded C_{18} packing with particle size 37 µm to 100 µm, of length 20 mm, internal diameter 3,9 mm, or a guard column of equivalent quality.

5.9.6 Analytical column: silica bonded C_{18} packing with particle size 5 µm, of length 200 mm, internal diameter 3,0 mm, or an analytical column of equivalent quality.

For furazolidone a capacity factor (K') of at least 1,0 shall be obtained.

NOTE The capacity factor is defined as:

$$K' = \frac{t_R}{t_0} - 1$$

where

K' is the capacity factor;

t_R is the retention time, in minutes, of furazolidone;

t_0 is the retention time, in minutes, of the unretained furazolidone peak.

5.10 Disposable syringe, of capacity 5 ml.

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6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [4].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

Grind the laboratory sample (usually 500 g) so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

8 Procedure

8.1 General

In conjunction with the analysis of the test sample (or a series of test samples), analyse a blank sample, a spiked blank sample and, if available, a reference sample.

NOTE Blank samples are homogenates of comparable feeds with a furazolidone content of less than 10 mg/kg. Spiked blank samples are blank feed samples to which furazolidone is added. Blank samples and reference samples can be kept for a year, if stored at a temperature of 0 °C to 8 °C.

The analysis should be repeated when the recovery is lower than 94 % or higher than 106 %.

8.2 Preparation of a spiked sample

The furazolidone content of the spiked sample should be approximately equal to the expected furazolidone content of the sample. For a spiked sample with a furazolidone content of 250 mg/kg, use the following procedure.

Pipette 5,0 ml of the furazolidone stock solution (4.8) into a 250 ml conical flask. Under a flow of nitrogen, evaporate to a volume of approximately 0,5 ml and add 5 g of blank feed. Mix thoroughly and allow to stand for at least 10 min before proceeding with the extraction (8.3).

8.3 Extraction

8.3.1 Feeding stuffs with a furazolidone content of 25 mg/kg to 2 500 mg/kg

Weigh 5,0 g of the prepared test sample to the nearest 0,05 g in a 250 ml conical flask. Add 15,0 ml of water, mix and allow to stand for 5 min. Add 35,0 ml of extraction solvent (4.2), stopper and shake vigorously for 30 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5) and use the filtrate for column chromatography according to 8.4.

8.3.2 Feeding stuffs with a furazolidone content of 2 500 mg/kg to 5 000 mg/kg

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Weigh 5,0 g of the prepared test sample to the nearest 0,1 g in a 250 ml conical flask. Add 30,0 ml of water, mix and allow to stand for 5 min. Add 70,0 ml of extraction solvent (4.2), stopper and shake vigorously for 30 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5) and use the filtrate for column chromatography according to 8.4.

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8.3.3 Premixtures with a mass fraction of furazolidone of 0,5 % to 7 % [0,5 % (*m*/*m*) to 7 % (*m*/*m*)]

Weigh 1,0 g of the prepared test sample to the nearest 0,01 g in a 250 ml conical flask. Add 100,0 ml of extraction solvent (4.2), stopper and shake vigorously for 30 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5).

Dilute the fitrate with dilution solvent (4.3) to obtain a final solution with a furazolidone content between 5 µg/ml and 10 µg/ml. The dilution factor is f.

Mix well and filter the solution using the filtration system (5.8). Use the filtrate for HPLC analysis according to 8.5.

NOTE The required dilution factor (f) can be estimated by using the equation:

$$f_{\mathrm{e}} = \frac{m \cdot w_{\mathrm{exp}}}{V \cdot \rho_{\mathrm{r}}}$$

where

- f_{e} is the estimated required dilution factor of the sample extract;
- m is the mass, in grams, of the test portion;
- w_{exp} is the expected furazolidone content, in milligrams per kilogram, of the sample;
- ρ_{r} is the required furazolidone content, in micrograms per millilitre, of the final solution;
- V is the total volume, in millilitres, of extraction solvent added to the test portion (see also note in 8.5.2).

8.3.4 Premixtures with a mass fraction of furazolidone of 7 % to 10 % [7 % (*m*/*m*) to 10 % (*m*/*m*)]

Weigh 1,0 g of the prepared test sample to the nearest 0,01 g in a 500 ml conical flask. Add 200,0 ml of extraction solvent (4.2), stopper and shake vigorously for 30 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5).

Dilute the fitrate with dilution solvent (4.3) to obtain a final solution with a furazolidone content between 5 µg/ml and 10 µg/ml. The dilution factor is f.

Mix well and filter the solution using the filtration system (5.8). Use the filtrate for HPLC analysis according to 8.5.

NOTE See the note in 8.3.3 for the calculation of the dilution factor.

8.3.5 Premixtures with a mass fraction of furazolidone of 10 % to 20 % [10 % (m/m) to 20 % (m/m)]

Weigh 0,5 g of the prepared test sample to the nearest 5 mg in a 500 ml conical flask. Add 200,0 ml of extraction solvent (4.2), stopper and shake vigorously for 30 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5).

Dilute the fitrate with dilution solvent (4.3) to obtain a final solution with a furazolidone content between 5 µg/ml and 10 µg/ml. The dilution factor is f.

Mix well and filter the solution using the filtration system (5.8). Use the filtrate for HPLC analysis according to 8.5.

NOTE See the note in 8.3.3 for the calculation of the dilution factor.

8.4 Column chromatography

For each sample extract, dry-pack a glass column (5.7), fitted at the bottom with a plug of glass wool (5.6), with 4 g of aluminium oxide (4.10). Apply 20 ml of extract, prepared according to 8.3.1 or 8.3.2, to the column and discard the first 4 ml of eluate. Collect the following 8 ml of eluate in a small graduated cylinder.

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Pipette 5,0 ml of eluate in a 5 ml one-mark volumetric flask and dilute to the mark with water. Mix well.

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If necessary, dilute the solution with dilution solvent (4.3) to obtain a final solution with a furazolidone content between 5 µg/ml and 10 µg/ml. The dilution factor is f.

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Filter the diluted solutions using the filtration system (5.8) and use the filtrate for HPLC analysis according to 8.5.

8.5 HPLC analysis

8.5.1 HPLC conditions

Use the following conditions:

— mobile phase (4.6) volume flow rate: 0,6 ml/min;

— injection volume: 20 µl;

— wavelength: 365 nm;

— recorder: 10 mV;

— chart speed: 0,5 cm/min.

8.5.2 Procedure

8.5.2.1 Inject the furazolidone working solutions (4.9) until a stable baseline and reproducible peak heights or peak areas are obtained. For peak heights or peak areas, the difference between the highest and the lowest result should be less than 5 % of the mean result of three consecutive injections.

The furazolidone peak shall be symmetrical ($f_{as} < 2$).

NOTE f_{as} is the width on the tail side of the perpendicular line of the peak, divided by the width on the front side of the perpendicular line of the peak, both measured at 10 % of the height of the peak.

There shall be a proportional relation (within 5 %) between the concentration and peak heights of the two furazolidone working solutions. If a deviation of more than 5 % is found, new furazolidone working solutions shall be prepared.

Inject the extracts of the blank sample and the spiked blank sample. If the peak of furazolidone is not symmetrical or not fully separated from the feed matrix peaks, it is necessary to use another HPLC column or to adjust the chromatographic conditions by an increase or decrease of the aqueous content of the mobile phase (4.6).

Consecutively inject furazolidone working solutions (4.9), five sample extracts and furazolidone working solutions (4.9). Repeat this sequence, if necessary, for the other sample extracts in the series.

The observed peak heights or peak areas for the furazolidone working solutions should be within a margin of 5 % of the results of the furazolidone working solutions injected before.

An example of a chromatogram is presented in annex A. From the chromatogram for furazolidone, values of K' of 2,1 can be calculated (see also note in 5.9.6).

8.5.2.2 If the furazolidone content of a premixture is obviously lower than the expected content (taking into account the tolerance), it is recommended to repeat the analysis with an additional 50 ml of extraction solvent (4.2) applied in 8.3.3, 8.3.4 or 8.3.5.

If the new result is more than 15 % (m/m) higher than the original value, the analysis should be repeated with another additional 50 ml of extraction solvent (4.2). This addition should be repeated until the difference in results of consecutive determinations is less than 15 % (m/m).

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9 Confirmation

9.1 General

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If the identity of the substance causing the peak in the chromatogram is in doubt, based on the peak shape or on the result obtained, the identity of the determined analyte may be confirmed by either applying co-chromatography or a diode array detector. In the first case, proceed in accordance with 9.2; in the latter case, proceed in accordance with 9.3.

9.2 Co-chromatography

Prepare a spiked sample extract by adding an appropriate amount of furazolidone working solution (4.9) to the sample extract. The amount of furazolidone added shall be approximately equal to the estimated amount of furazolidone in the sample extract.

Inject the sample extract, furazolidone working solution (4.9) and spiked sample extract. Only the peak in the chromatogram presumed to be the analyte peak should intensify, should increase in height proportionally to the spiking level, and should increase in peak width at half height by no more than ± 10 % of the original width.

Proceed in accordance with clause 10.

9.3 Diode array detector

9.3.1 Conditions

The conditions are as specified in 8.5.1 but use a diode array detector instead of a UV detector with the following parameters:

Parameter	Setting
sample wavelength	365 nm
sample bandwidth	4 nm (i.e. wavelength of 365 nm ± 2 nm)
reference wavelength	450 nm
reference bandwidth	100 nm
spectrum range	225 nm to 400 nm
spectrum	baseline, apex, upslope and downslope inflection points

9.3.2 Procedure

Allow the system to stabilize. Inject the 5 µg/ml furazolidone working solution (4.9), suspected sample extracts and again the 5 µg/ml furazolidone working solution (4.9). Record the spectra at the baseline, upslope and downslope inflection points and peak apex. Store all data.

9.3.3 Evaluation

Plot in one figure the normalized difference spectra (sample - baseline) of the sample peak, recorded at the apex and at the upslope and downslope inflection points. Plot in one figure the normalized spectra of the sample peak and of the furazolidone working solution peak, recorded at the apex.

9.3.4 Confirmation criteria

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The identity of the analyte is confirmed if the following criteria are satisfied.

a) The retention time of the sample peak shall be equal (± 5 %) to the retention time of the standard peak. If in doubt, standard addition (standard material added to the sample) shall be performed.

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b) Assess the purity of the sample peak on the basis of the conformity of the difference spectra, recorded at apex and at upslope and downslope inflection points. At each wavelength the relative absorption shall be equal (to within 15 %) for all spectra.

c) Above a wavelength of 220 nm, the difference spectra of the sample and standard peaks recorded at the peak apex shall not be visually different for those parts of the spectra with a relative absorption of at least 10 %. This criterion is met when the same maxima are present within a margin determined by the resolution of the detection system (typically 2 nm to 4 nm). At no observed point shall the deviation between the two spectra exceed 15 % of the absorbance of the standard analyte at that particular wavelength.

10 Calculation of results

10.1 General

Calculate the furazolidone content of the sample extract by comparing the peak height or peak area of the sample extract chromatogram with the mean of the peak heights or peak areas of the furazolidone working solution injected before and after the sample extract. Use the results obtained with the furazolidone working solution with the best matching furazolidone content.

10.2 Feeding stuffs with a furazolidone content of 25 mg/kg to 5 000 mg/kg

Calculate the furazolidone content of feeding stuff samples (w_f) by the equation:

$$w_\mathrm{f} = \frac{h}{h_\mathrm{s}} \times \rho_\mathrm{s} \times \frac{V}{m} \times f$$