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

Aliments des animaux — Détermination de la teneur en carbadox — 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.

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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.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 14939 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Annexes A and B of this International Standard are for information only.

Animal feeding stuffs — Determination of carbadox 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 carbadox content in premixtures and animal feeding stuffs.

The method is applicable to animal feeding stuffs with a mass fraction of carbadox of 0,5 mg/kg (limit of quantification) to 100 mg/kg, and to premixtures with a mass fraction of carbadox up to 10 %.

The lower limit of detection is 0,1 mg/kg.

NOTE 1 For animal feeding stuffs the mass fraction of carbadox is expressed in milligrams per kilogram, and for premixtures as a percentage by mass.

NOTE 2 Carbadox is a chemotherapeuticum belonging to the quinoxaline group. Carbadox is used as a growth-promoting feed additive for piglets.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of IEC and ISO maintain registers of currently valid International Standards.

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

3 Principle

Carbadox is extracted from the sample with a mixture of acetonitrile and methanol. Animal feeds are prewetted with water. The extract of animal feeds is purified through a short aluminium oxide column. 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]).

The presence of dimetridazole, nitrofurazone or sulfadimidine sodium can interfere with the determination of carbadox.

Alternatively, carbadox may be determined after post-column derivatization with sodium hydroxide with detection at a wavelength of 420 nm.

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) (70:30 by volume).

Mix 70 ml of extraction solvent (4.2) with 30 ml of water (4.1).

4.4 Acetic acid, volume fraction, $w(CH_3CO_2H)$ = 10 %.

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

4.5 Sodium acetate solution, $c(C_2H_3NaO_2)$ = 0,01 mol/l, pH = 6,0.

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

4.6 Mobile phase for HPLC.

Combine 825 ml of sodium acetate solution (4.5) and 175 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 Carbadox standard material, 3-(2-quinoxalinyl methylene) carbazic acid methy ester *N*, *N′* -dioxide (CAS number 6804-07-5).

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

4.8 Carbadox stock solution (approximately 100 µg/ml).

Weigh 10 mg ± 1 mg of carbadox (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.

4.9 Carbadox working solutions (approximately 2 µg/ml and 10 µg/ml).

Pipette 1,0 ml and 5,0 ml of the carbadox stock solution (4.8) into separate 50 ml one-mark volumetric flasks. Dilute to the mark with dilution solvent (4.3) and mix. Prepare fresh for each series of samples.

4.10 Carbadox working solutions (approximately 0,4 µg/ml and 2 µg/ml).

Pipette 1,0 ml of the carbadox stock solution (4.8) into a 50 ml one-mark volumetric flask, dilute to the mark with mobile phase (4.6) and mix. Pipette 10 ml of this solution (2 µg/ml) into a 50 ml one-mark volumetric flask, dilute to the mark with mobile phase (4.6) and mix. Prepare fresh for each series of samples.

4.11 Dimetridazole standard material, 1,2-dimethyl-5-nitro-1*H*-imidazole (CAS number 551-92-8).

WARNING — Because of the sensitivity of dimetridazole to light, conduct all operations in the absence of daylight or artificial white light. Avoid inhalation of and exposure to the toxic dimetridazole standard

material and solutions thereof. Work in a fume cupboard when handling the solvents and solutions. Wear safety glasses and protective clothing.

4.12 Dimetridazole stock solution (approximately 100 µg/ml).

Weigh 10 mg ± 1 mg of dimetridazole (4.11), to the nearest 0,1 mg, into a 100 ml one-mark volumetric flask. Dilute to the mark with methanol 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.

4.13 Dimetridazole working solution (approximately 20 µg/ml).

Pipette 2,0 ml of the dimetridazole stock solution (4.12) into a 10 ml one-mark volumetric flask. Dilute to the mark with water and mix. Prepare fresh for each series of samples.

4.14 Sulfadimidine standard material, sodium salt of 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzene sulfonamide (CAS number 1981-58-4).

WARNING — Avoid inhalation of and exposure to the toxic sulfadimidine standard material and solutions thereof. Work in a fume cupboard when handling the solvents and solutions. Wear safety glasses and protective clothing.

4.15 Sulfadimidine stock solution (approximately 200 µg/ml).

Weigh 10 mg ± 1 mg of sulfadimidine standard material (4.14), to the nearest 0,1 mg, into a 50 ml one-mark volumetric flask. Dilute to the mark with methanol 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.

4.16 Sulfadimidine working solution (approximately 20 µg/ml).

Pipette 1,0 ml of sulfadimidine stock solution (4.15) into a 10 ml one-mark volumetric flask. Dilute to the mark with water and mix. Prepare fresh for each series of samples.

4.17 Nitrofurazone standard material, 5-nitro-2-furaldehyde semicarbazone (CAS number 59-87-0).

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

4.18 Nitrofurazone stock solution (approximately 100 µg/ml).

Weigh 10 mg ± 1 mg of nitrofurazone (4.17), to the nearest 0,1 mg, into a 100 ml one-mark volumetric flask. Dilute to the mark with methanol 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.

4.19 Nitrofurazone working solution (approximately 20 µg/ml).

Pipette 2,0 ml of nitrofurazone stock solution (4.18) into a 10 ml one-mark volumetric flask. Dilute to the mark with water and mix. Prepare fresh for each series of samples.

4.20 Neutral aluminium oxide, activity 1.

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

4.21 Sodium hydroxide solution, c(NaOH) = 0,5 mol/l.

Weigh 20 g of sodium hydroxide into a 1 litre one-mark volumetric flask and dissolve in 10 ml of water. Dilute to the mark with water and mix.

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, diameter 15 cm.

5.6 Glass wool.

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

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

5.9 Water bath, capable of being heated to 50 °C, or **heating module**, equipped with a supply of nitrogen.

5.10 HPLC system, comprising the following.

5.10.1 Pump, pulse free, capable of maintaining a volume flow rate of 0,5 ml/min to 1,5 ml/min.

5.10.2 Injection system, with loop suitable for 20 µl to 100 µl injections.

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

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

5.10.4 Recorder.

5.10.5 Guard column: silica-bonded C_{18} packing with particle size of *ca*. 30 µm, length 20 mm, internal diameter 3,9 mm, or a guard column of equivalent quality.

5.10.6 Analytical column.

For mass fractions of carbadox less than 10 mg/kg (feeding stuffs), use silica-bonded C_{18} packing with particle size 5 µm, length 200 mm, internal diameter 3,0 mm, or an analytical column of equivalent quality.

For mass fractions of carbadox greater than or equal to 10 mg/kg (feeding stuffs and premixtures), use silica-bonded C_{18} packing with particle size 5 µm, length 300 mm, internal diameter 3,0 mm, or an analytical column of equivalent quality.

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

The capacity factor is defined as:

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

where

K' is the capacity factor;

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

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

5.10.7 Peristaltic pump (for post-column derivatization).

5.10.8 Spiral reaction coil (for post-column derivatization), polytetrafluorethylene (PTFE), length 2 m, internal diameter 0,5 mm.

5.10.9 UV/Vis detector, suitable for measurements at a wavelength of 420 nm (for post-column derivatization).

5.11 Disposable syringe, of capacity 5 ml.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [5].

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 and a spiked blank sample. If available, a reference sample may be analysed to check the performance of the method.

Annex A shows a flow chart of the procedure.

For blank samples, use homogenates of comparable feeds with a mass fraction of carbadox of less than 0,1 mg/kg. For spiked blank samples, use blank feed samples to which carbadox is added. Blank samples and reference samples may be kept for a year if stored at a temperature of 0 °C to 8 °C.

The analysis should be repeated if the recovery is lower than 91 % or higher than 103 % for mass fractions of carbadox of 50 mg/kg.

8.2 Preparation of spiked sample

The mass fraction of carbadox in the spiked sample should be approximately equal to that expected in the test sample. Prepare a spiked sample containing 50 mg/kg of carbadox as follows.

Pipette 5,0 ml of the 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 10 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 containing 0,1 mg/kg to 10 mg/kg of carbadox

Weigh 10,0 g of the prepared test sample to the nearest 0,1 g in a 250 ml conical flask. Add 50,0 ml of extraction solvent (4.4), 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 containing 10 mg/kg to 100 mg/kg of carbadox

Weigh 5,0 g of the prepared test sample to the nearest 0,1 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), then 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.

8.3.3 Premixtures containing up to 2,0 % of carbadox

Weigh 1,0 g of the prepared test sample, to the nearest 0,01 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), then 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 filtrate with dilution solvent (4.3) to obtain a final solution with a mass fraction of carbadox 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.

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

$$f_e = \frac{m \cdot w_e}{V \cdot \rho_r}$$

where

f_e is the estimated required dilution factor of the sample extract;

m is the mass, in grams, of the test portion;

w_e is the expected mass fraction of carbadox, in milligrams per kilogram, in the sample;

ρ_r is the required concentration of carbadox, in micrograms per millilitre, in the final solution;

V is the total volume, in millilitres, of extraction solvent added to the test portion (see also 8.5.2.3).

8.3.4 Premixtures containing 2 % to 10 % of carbadox

Weigh 0,5 g of the prepared test sample, to the nearest 5 mg, in a 250 ml conical flask. Add 45,0 ml of water, mix and allow to stand for 5 min. Add 105,0 ml of extraction solvent (4.2), stopper and mix. Place the flask in an ultrasonic bath (5.3) for 15 min. Shake vigorously for 15 min on the rotary shaker (5.4). Filter the solution through a glass microfibre filter (5.5).

Dilute the filtrate with dilution solvent (4.3) to obtain a final solution with a mass fraction of carbadox 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 in accordance with 8.5.

NOTE See 8.3.3 for the calculation of an estimated dilution factor.

8.4 Column chromatography

8.4.1 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 (4.20). Apply 15 ml of extract, prepared according to 8.3.1 or 8.3.2, to the column and discard the first 2 ml of eluate.

For samples containing 0,1 mg/kg to 10 mg/kg of carbadox, proceed in accordance with 8.4.2.

For samples containing 10 mg/kg to 100 mg/kg of carbadox, proceed in accordance with 8.4.3.

8.4.2 Collect 6 ml of eluate in a small graduated cylinder. Pipette 4 ml of the eluate into a calibrated tube and evaporate the solvent to near dryness, using the water bath or heating module (5.9) at 40 °C to 50 °C, under a gentle stream of nitrogen. Dilute with 2 ml of mobile phase (4.6). Mix in an ultrasonic bath (5.3) and filter the solution using the filtration system (5.8). Use the filtrate for HPLC analysis according to 8.5.

8.4.3 Collect 4 ml of eluate in a small graduated cylinder and filter the solution using the filtration system (5.8). Use the filtrate for HPLC analysis according to 8.5.

8.5 HPLC analysis

8.5.1 HPLC conditions

See Table 1.

Table 1

Parameter	Setting for mass fractions of carbadox up to 10 mg/kg	Setting for mass fractions of carbadox ⩾10 mg/kg
Analytical column	ChromSpher C_{18}[a]	µBondapak C_{18}[a]
Mobile phase volume flow rate	0,6 ml/min	1,5 ml/min
Injection volume	50 µl	20 µl
Wavelength	365 nm	365 nm
Sensitivity (indicative)	0,005 AUFS to 0,04 AUFS	0,02 AUFS to 0,08 AUFS
Recorder	10 mV	10 mV
Chart speed	1,0 cm/min	1,0 cm/min

[a] ChromSpher and µBonapak are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

The conditions given in Table 1 are only indicative, because in practice settings will be related to the column and detector used.