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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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Case postale 56 • CH-1211 Geneva 20  
Tel. + 41 22 749 01 11  
Fax + 41 22 749 09 47  
E-mail [copyright@iso.ch](mailto:copyright@iso.ch)  
Web [www.iso.ch](http://www.iso.ch)

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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.

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# 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 M $\Omega$ -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,  $w(\text{CH}_3\text{CO}_2\text{H}) = 10\%$ .

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

**4.5 Sodium acetate solution**,  $c(\text{C}_2\text{H}_3\text{NaO}_2) = 0,01$  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  $\mu\text{m}$  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-quinoxalinylnyl 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  $\mu\text{g/ml}$ ).

Weigh 10 mg  $\pm$  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  $^\circ\text{C}$  to 8  $^\circ\text{C}$ .

**4.9 Carbadox working solutions** (approximately 2  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$ ).

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  $\mu\text{g/ml}$  and 2  $\mu\text{g/ml}$ ).

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  $\mu\text{g/ml}$ ) 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, $c(\text{NaOH}) = 0,5 \text{ mol/l}$ .

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<sup>-1</sup> to 300 min<sup>-1</sup>.

**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<sub>18</sub> 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<sub>18</sub> 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<sub>18</sub> 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;

$\rho_r$  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 $\geq$ 10 mg/kg
Analytical column	ChromSpher C <sub>18</sub> <sup>a</sup>	$\mu$ Bondapak C <sub>18</sub> <sup>a</sup>
Mobile phase volume flow rate	0,6 ml/min	1,5 ml/min
Injection volume	50 $\mu$ l	20 $\mu$ l
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

<sup>a</sup> ChromSpher and  $\mu$ Bonapak 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.

## 8.5.2 Procedure

**8.5.2.1** Inject the carbadox working solutions (4.9) into the chromatogram 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.

In the analysis of premixtures and feeding stuffs containing 10 mg/kg or more of carbadox, use the carbadox working solutions (4.9).

In the analysis of feeding stuffs containing less than 10 mg/kg of carbadox, use the lower-concentration carbadox working solutions (4.10).

The carbadox 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 peak height.

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

Inject the extracts of the blank sample, the spiked blank sample and the working solutions of dimetridazole (4.13), sulfadimidine (4.16) and nitrofurazone (4.19). If the carbadox peak is not symmetrical or not fully separated from the feed matrix peaks or the peaks of the injected standard solutions, it is necessary to use another HPLC column or to adjust the chromatographic conditions by an increase or decrease in the aqueous content of the mobile phase (4.6).

Consecutively inject the carbadox working solutions (4.9 or 4.10), five sample extracts and again the carbadox working solutions. Repeat this sequence, if necessary, for the other sample extracts in the series.

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

**8.5.2.3** If the mass fraction of carbadox in a premixture is quite obviously lower than expected (taking into account the tolerance), it is recommended to repeat the analysis applying in steps 8.3.3 or 8.3.4 an additional 50 ml of an extraction solvent consisting of 35 ml of extraction solvent (4.2) and 15,0 ml of water.

If the newly obtained mass fraction of carbadox is more than 15 % higher than the original value, the analysis should be repeated with another additional 50 ml of the described extraction solvent. This addition should be repeated until the difference in results of consecutive determinations of the mass fraction of carbadox is less than 15 %.

## 9 Confirmation

### 9.1 General

If the identity of the substance causing the peak in the chromatogram is doubtful 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. Alternatively, the presence of carbadox may be confirmed by applying post-column derivatization with sodium hydroxide solution (see 9.4).

### 9.2 Co-chromatography

Prepare a spiked sample extract by adding an appropriate amount of carbadox working solution (4.10) to the sample extract. The amount of carbadox added shall be approximately equal to the estimated amount of carbadox in the sample extract.

Inject the sample extract, carbadox working solution (4.10) 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  $\pm 10\%$  of the original width.

Proceed in accordance with clause 10.

### 9.3 Diode array detector

#### 9.3.1 Conditions

The conditions are as described in 8.5.1, however a diode array detector is used instead of a UV detector. See Table 2.

Table 2

Parameter	Setting
Sample wavelength	365 nm
Sample bandwidth	4 nm (i.e. wavelength of 365 nm $\pm$ 2 nm)
Reference wavelength	450 nm
Reference bandwidth	100 nm
Spectrum range	225 nm to 400 nm
Spectrum	baseline, apex, up-slope and down-slope inflection points

#### 9.3.2 Procedure

Allow the system to stabilize. Inject the 2  $\mu\text{g/ml}$  carbadox working solution (4.10), suspected sample extracts and again the 2  $\mu\text{g/ml}$  carbadox working solution (4.10). Record the spectra at the baseline, up-slope and down-slope 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 up-slope and down-slope inflection points. Plot in one figure the normalized spectra of the sample peak and of the carbadox working solution peak, recorded at the apex.

#### 9.3.4 Confirmation criteria

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

- The retention time of the sample peak shall be equal ( $\pm 5\%$ ) to the retention time of the standard peak. In case of doubt, standard addition (standard material added to the sample) shall be performed.
- Assess the purity of the sample peak on the basis of the conformity of the difference spectra, recorded at the apex and at up-slope and down-slope inflection points. At each wavelength the relative absorption shall be equal (within 15 %) for all spectra.
- 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.

**9.4 Post-column derivatization**

**9.4.1 General**

Additional evidence for the presence of carbadox, especially in samples containing less than 5 mg/kg, may be found by applying a post-column derivatization which is specific for carbadox. In that case proceed as follows.

Using a T-joint and a peristaltic pump (5.10.7), after the analytical column and before the reaction coil (5.10.8), add sodium hydroxide solution (4.23) to the mobile phase. The absorption maximum of carbadox will shift to 420 nm.

**9.4.2 Conditions**

See Table 3.

**Table 3**

Parameter	Setting
Reagent volume flow rate	0,23 ml/min
Wavelength	420 nm
Injection volume	100 µl

**10 Calculation of results**

**10.1 General**

Calculate the mass fraction of carbadox in the sample by comparing the peak height or peak area of the sample extract with the mean of the peak heights or peak areas of the carbadox working solution injected before and after the sample. Use the results obtained with the carbadox working solution with the best matching mass fraction of carbadox.

**10.2 Feeding stuffs containing 0,1 mg/kg to 10 mg/kg of carbadox**

Calculate the mass fraction of carbadox ( $w_c$ ) in the feeding stuff sample by the equation:

$$w_c = \frac{h}{h_s} \cdot \rho_s \cdot \frac{V}{2m}$$

where

$w_c$  is the mass fraction of carbadox, in milligrams per kilogram, in the test sample;

$h$  is the peak height, in length units, obtained for the sample extract;

$h_s$  is the peak height, in length units, obtained for the carbadox working solution;

$\rho_s$  is the concentration of carbadox, in micrograms per millilitre, in the carbadox working solution;

$V$  is the total volume, in millilitres, of extraction solvent added to the test portion;

$m$  is the mass, in grams, of the test portion.

Alternatively, the peak area may be used in the calculation instead of the peak height and a linear regression model may be used to calculate the carbadox content in the sample extracts.

Round the result to the nearest 0,1 mg/kg.

### 10.3 Feeding stuffs containing 10 mg/kg to 100 mg/kg of carbadox

Calculate the mass fraction of carbadox ( $w_c$ ) in the feeding stuff sample by the equation:

$$w_c = \frac{h}{h_s} \cdot \rho_s \cdot \frac{V}{m}$$

where the symbols are as in 10.2.

Alternatively, the peak area may be used in the calculation instead of the peak height and a linear regression model may be used to calculate the carbadox content in the sample extracts.

Round the result to the nearest 1 mg/kg.

### 10.4 Premixtures containing up to 10 % of carbadox

Calculate the mass fraction of carbadox ( $w_{cp}$ ) in the premixture sample by the equation:

$$w_{cp} = \frac{h}{h_s} \cdot \rho_s \cdot \frac{V}{m} \cdot f \times 10^{-4} f_u$$

where

$w_{cp}$  is the mass fraction of carbadox in the premixture sample, expressed as a percentage;

$w_s$  is the concentration of carbadox, in micrograms per millilitre, in the carbadox working solution;

$f$  is the dilution factor of the sample extract (see 8.3.3 or 8.3.4);

$f_u$  is the unit correction factor ( $f_u = 1 \text{ kg} \cdot \text{mg}^{-1} \cdot \%$ ).

The other symbols are as in 10.2.

Alternatively, the peak area may be used in the calculation instead of the peak height and a linear regression model may be used to calculate the carbadox concentration in sample extracts.

Round the result to the nearest 0,01 %.

## 11 Precision

### 11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method, including a note on the validity of the precision figures, are given in annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

### 11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed:

- 10 % of the mean of the two test results for mass fractions of carbadox up to 50 mg/kg;
- 14 % of the mean of the two test results for mass fractions of carbadox between 1 % and 10 %.

### 11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed:

- a) 25 % of the mean of the two test results for mass fractions of carbadox up to 50 mg/kg;
- b) 18 % of the mean of the two test results for mass fractions of carbadox between 1 % and 10 %.

### 12 Test report

The test report shall specify:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this International Standard;
- d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test results;
- e) the test result obtained, or the two test results obtained if the repeatability has been checked.

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## Annex A (informative)

### Flow chart

