

Stofnaam	Carbadox
Type methode	HPLC
Te onderzoeken in	Diervoeders
Minimum bepaalbaarheidsgrens	2 mg/kg
Herhaalbaarheid	RSD _r (relatieve standaarddeviatie) voor diervoeders op de volgende niveaus: 2.5 mg/kg: 5.0% 10 mg/kg 3.2%
Reproduceerbaarheid	RSD _R voor diervoeders op de volgende niveaus: 2.5 mg/kg: 23% 10 mg/kg: 14%
Categorie	A
Titel	Determination of low level contents of carbadox in feeding stuffs by High Performance Liquid Chromatography CANFAS/SMT4-CT98-2216/ final method carbadox/ 2002-10-08

BEPALING VAN CARBADOX

1. Purpose and scope

This method is for the determination of low contents of carbadox in feedingstuffs. The limit of determination (=quantification) is 2 mg/kg. The limit of detection is 0,2 mg/kg.

2. Principle

The sample is equilibrated with water and extracted with methanol-acetonitrile. For feedingstuffs, an aliquot portion of the filtered extract is subjected to clean-up on an aluminium oxide column. The content of carbadox is determined by reversed-phase high performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1 Methanol

3.2 Acetonitrile, HPLC grade

3.3 Acetic acid, w = 100 %

3.4 Sodium acetate, CH₃COONa

3.5 Aluminium oxide: neutral, activity grade I, 70 – 230 mesh or 0,063 – 0,2 mm ASTM, e.g. Merck, Art. 1077

3.6 Methanol-acetonitrile 1 + 1 (v + v):
Mix 500 ml of methanol (3.1) with 500 ml of acetonitrile (3.2).

3.7 Acetic acid σ = 10 %:
Dilute 10 ml of acetic acid (3.3) to 100 ml with water.

3.8 Water, HPLC grade

3.9 Acetate buffer solution c = 0.01 mol/l, pH 6.0:
Dissolve 0.82 g of sodium acetate (3.4) in 700 ml of water (3.8) and adjust the pH to 6.0 with acetic acid (3.3 or 3.7). Transfer to a 1000 ml graduated flask, make up to the mark with water (3.8) and mix

3.10 Mobile phase for HPLC:
Mix 825 ml of acetate buffer solution (3.9) with 175 ml of acetonitrile (3.2). Degas the solution (e.g. by ultrasonification for 10 minutes).

3.11 Standard substance
Pure carbadox: Methyl-3-(2-quinoxalinylmethylene)carbazate - N¹, N⁴-dioxide, E 850

3.12 Carbadox stock standard solution, 100 μ g/ml (see Note 5. Procedure);
Weigh to the nearest 0.1 mg, 25 mg of carbadox standard substance (3.11) into a 250 ml graduated flask. Dissolve in methanol-acetonitrile (3.5) by ultrasonification (4.7). After ultrasonic treatment bring the solution to room temperature, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flask with aluminium foil or use amber glassware and store in a refrigerator. At this temperature of $\leq 4^{\circ}\text{C}$ the solution is stable for 1 month.

3.13 Calibration solutions

Transfer 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 ml of the stock standard solution (3.12) into a series of 100 ml calibrated flasks. Add 30 ml of water, make up to the mark with methanol-acetonitrile (3.6) and mix. Wrap the flask with aluminium foil. These solutions correspond to 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/ml of carbadox respectively. Calibration solutions must be freshly prepared before use.

4. Apparatus

4.1 Laboratory shaker or magnetic stirrer

4.2 Folded Filter (MN 619 G 1/4 or equivalent)

4.3 Glass column (length 300 to 400 mm, internal diameter approximately 10 mm) with sintered glass frit and draw-off valve.

Note: a glass column fitted with a stopcock or a glass column with a tapered end may also be used; in this case, a small glass-wool plug is inserted into the lower end and it is tamped down using a glass rod

4.4. HPLC equipment with injection system, suitable for injection volumes of 20 – 100 µl

4.4.1 Liquid chromatographic column: 250 mm x 4 mm, C18, 5 µm packing or equivalent e.g. Spherisorb ODS 1, 5 mm, 250 x 4 mm or Spherisorb S10 ODS-1, 10 mm, 250 x 4 mm

4.4.2. UV detector with variable wavelength adjustment or diode array detector operating in the range of 225 to 400nm

4.5. Ultrasonic bath

4.6 Membrane filter, 0.45 µm

5. Procedure

Note: Carbadox is light-sensitive. Carry out all procedures under subdued light or use amber glassware or glassware wrapped with aluminium foil.

5.1 General

5.1.1. Blank feed

For the performance of the recovery test (5.1.2) a blank feed should be analysed to check that neither carbadox nor interfering substances are present. The blank feed should be similar in type to that of the sample and on analysis carbadox or interfering substances should not be detected.

5.1.2. Recovery test

A recovery test should be carried out by analysing the blank feed (5.1.1), which has been fortified by the addition of a quantity of carbadox, similar to that present in the sample. To fortify at a level of 5 mg/kg, transfer 0.5 ml of the stock standard solution (3.12) to a 200 ml conical flask. Add 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, prepare two independent laboratory sample aliquots (A and B) of the feed to be examined. Spike one of them (A), before extraction with a quantity of carbadox, similar to that already present in the sample. Both samples are analysed. Calculate the analyte content in sample A and B and calculate the recovery by subtraction.

5.2. Extraction

Weigh to the nearest 0.01 g approximately 10 g of the sample and transfer to a 200 ml conical flask. Add 15.0 ml of water, mix, and equilibrate for 5 min. Add 35.0 ml of methanol-acetonitrile (3.6), stopper and shake for 30 min on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a folded filter (4.2). Retain this solution for the purification step (5.3).

5.3. Purification

5.3.1. Preparation of the aluminium oxide column

Weigh 4 g of aluminium oxide (3.5) and transfer it to the glass column (4.3).

5.3.2. Sample purification

Apply 15 ml of the filtered extract (5.2) to the aluminium oxide column and discard the first 2 ml of eluate. Collect the next 5 ml and filter an aliquot through a 0.45 µm filter (4.6).

Proceed to 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 (gradient elution is not recommended because this may lead to interfering peaks):

Liquid chromatographic column (4.4.1): 250 mm x 4 mm, C18, 5 µm packing or equivalent (e.g. Spherisorb ODS 1, 5 mm, 50 x 4 mm or Spherisorb S10 ODS-1, 10 mm, 250 x 4 mm)

Mobile phase (3.10):	Mixture of acetate buffer solution (3.9) and acetonitrile (3.2), 825 + 175 (v+v)
Flow rate:	1.5 - 2 ml/min
Detection wavelength:	365 nm
Injection volume:	20 µl

Check the stability of the chromatographic system, injecting the calibration solution (3.13) containing 5.0 µg/ml several times, until constant peak heights (areas) and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.13) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg/ml as the abscisses.

5.4.3. Sample solution

Inject the sample extract and determine the peak height (area) of the carbadox peaks.

6. Calculation of the results

From the height (area) of the carbadox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.4.2).

6.1. Feedingstuffs

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

$$w = \frac{c \times 50}{m} \quad [\text{mg/kg}]$$

in which:

c carbadox concentration of the sample extract (5.3.2) in µg/ml,
m mass of the test portion in g