SLOVENSKI STANDARD
oSIST prEN 16930:2016

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Krma: Metode vzorčenja in analize - Določevanje karbadoksa in olakvindoksa s tekočinsko kromatografijo visoke ločljivosti - Ultravijolična (UV) detekcija (HPLC/UV)

Animal feeding stuffs: Methods of sampling and analysis - Determination of carbadox and olaquindox by high performance liquid chromatography - UV detection (HPLC/UV)

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung von Carbadox und Olaquindox mittels Hochleistungsflüssigkeitschromatographie mit UV-Detektion (HPLC/UV)

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Détermination des teneurs en carbadox et olaquindox par chromatographique liquide à haute performance avec détection UV (CLHP/UV)

Ta slovenski standard je istoveten z: **prEN 16930**

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Animal feeding stuffs: Methods of sampling and analysis - Determination of carbadox and olaquindox by high performance liquid chromatography - UV detection (HPLC/UV)

Bestimmung von Carbadox und Olaquindox in Konzentrationen unterhalb von Zusatzstoffen in Mischfuttermitteln mittels HPLC-UV

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If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Page

European foreword

This document (prEN 16930:2015) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

WARNING — The use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This European Standard specifies a high performance liquid chromatographic – UV detection (HPLC-UV) method for the simultaneous determination of two growth promoters Carbadox and Olaquindox contents in compound feeds and raw materials at levels ranging from the limit of quantification to 100 mg kg-1.

The limit of quantification of the method has been demonstrated to be better than 3 mg kg-1 for olaquindox and 4 mg kg-1 for carbadox.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6498, *Animal feeding stuffs — Guidelines for sample preparation*

3 Principle

The two growth promoters are extracted from the sample with a mixture of methanol and water 1:1, v:v. The extract of animal feeds is purified through a short open glass aluminium oxide column or by an alumina Solid Phase Extraction (SPE).

The final extract is analysed by reversed phase HPLC with UV detection at a wavelength of 375 nm. Alternatively, for confirmation purposes, a Diode Array Detector (DAD) can be used.

The presence of furazolidone can interfere with the determination of carbadox.

If a DAD is used, many other veterinary drugs or feed additives can be detected (ronidazole, meticlorpindol, nitrofurazone, dimetridazol, furaltadon, sulphonamides...), but their signals do not interfere with the target ones.

In some special feeds, matrix interfering peaks may be present, very close to the carbadox peak.

4 Reagents and materials

WARNING

a) This method requires the use of solutions of Carbadox and Olaquindox. These substances were used as growth-promoting feed additives for piglets. Carbadox and Olaquindox are chemotherapeutics belonging to the quinoxaline group. They are suspected to be teratogen and mutagen. Avoid inhalation of and exposure to the toxic standard materials and solutions.

b) Use adequate protection of materials: wear safety glasses, protective clothing and avoid skin contact.

c) These 2 growth promoters are subject to light degradation. Protect analytical work adequately from daylight, and keep standard solutions protected from light by using amber glassware, amber vials or aluminium foil.

Use only reagents recognized as analytical grade at least unless otherwise stated.

4.1 Water, demineralized or deionized or at least equivalent

4.2 Methanol

4.3 Acetonitrile

4.4 Glacial acetic acid, minimum purity 96 %

4.5 Ammonium acetate water free salt, $CH_3CO_2NH_4$

4.6 Extraction solvent: methanol water mixture (1:1 v:v)

Combine equal volumes of methanol (4.2) and water (4.1). Mix well.

NOTE Only for 8.1 purposes, it is possible to combine equal volume of technical methanol (4.14) and water (4.1). Mix well.

4.7 Ammonium acetate buffer, 25 mM, pH = 4,35

Weigh 2,0 ± 0,1 g of ammonium acetate (4.5) into a 1 000 ml volumetric flask. Dissolve in 900 ml of water (4.1). Add 3,0 ml of glacial acetic acid (4.4). Adjust (5.1) the pH to pH = 4,35 with acetic acid (4.4), if necessary. Dilute to the mark with water (4.1) and mix well.

4.8 Mobile phase for HPLC: acetonitrile:buffer mixture (10:90; v:v)

Transfer 100 ml of acetonitrile (4.3) into a 1 000 ml volumetric flask. Dilute to the mark with ammonium acetate buffer (4.7). Filter the eluent through a 0,45 µm cellulose acetate membrane filter (5.9) using a solvent filtration system (5.2). If necessary, perform degassing for 5 min in an ultrasonic bath (5.3).

NOTE This mobile phase is suitable for the three recommended columns in 5.11.8. If another column is used, optimization of the mobile phase composition may be necessary before performing real analysis (see 8.4.1).

4.9 Carbadox dissolving solvent: methanol:acetonitrile mixture (1:1; v:v)

Combine equal volumes of methanol (4.2) and acetonitrile (4.3). Mix well.

4.10 Reference standards

Guaranteed purity is required for each lot of reference standard.

4.10.1 Carbadox, 3-(2-quinoxalinyl methylene) carbazic acid methyl ester N,N'-dioxide

4.10.2 Olaquindox, (2-[N-2'-(hydroxyethyl) carbamoyl] 3–methyl quinoxaline) N,N' dioxide)

4.11 Standard solutions

Protect all standard solutions from daily light.

4.11.1 Carbadox stock standard solution ca. 100 µg ml^{-1}

Weigh 25 mg of carbadox (4.10.1), to the nearest 0,1 mg, into a 250 ml amber volumetric flask. Dissolve in 200 ml of mixture (4.9). Mix well and place the flask in an ultrasonic bath (5.3) until total dissolution. Allow to cool down to room temperature, dilute to the mark with mixture (4.9) and mix well. Calculate the accurate concentration taking into account the purity of the reference standard (4.10.1).

Prepare fresh every month. Store in the dark at 0 °C to 8 °C.

4.11.2 Olaquindox stock standard solution ca. 250 µg ml^{-1}

Weigh 50 mg of olaquindox (4.10.2), to the nearest 0,1 mg, into a 200 ml amber volumetric flask. Dissolve in about 190 ml of water (4.1). Mix well and place the flask in an ultrasonic bath (5.3) until total dissolution. Allow to cool down to room temperature. Dilute to the mark with water (4.1) and mix

well. Calculate the accurate concentration taking into account the purity of the reference standard (4.10.2).

Prepare fresh every month. Store in the dark at 0 °C to 8 °C.

4.11.3 Calibrations solutions

4.11.3.1 Carbadox/olaquindox calibration solution ca. 10 µg ml^{-1}

Pipette 5 ml of the carbadox stock standard solution (4.11.1) and 2 ml of the olaquindox stock standard solution (4.11.2) in a 50 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.11.3.2 Carbadox/olaquindox calibration solution ca. 5 µg ml^{-1}

Pipette 5 ml of the carbadox stock standard solution (4.11.1) and 2 ml of the olaquindox stock standard solution (4.11.2) in a 100 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.11.3.3 Carbadox/olaquindox calibration solution ca. 2,5 µg ml^{-1}

Pipette 5 ml of the carbadox stock standard solution (4.11.1) and 2 ml of the olaquindox stock standard solution (4.11.2) in a 200 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.11.3.4 Carbadox/olaquindox calibration solution ca. 1 µg ml^{-1}

Pipette 5 ml of the Carbadox/olaquindox calibration solution ca. 10 µg ml^{-1} (4.11.3.1) in a 50 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.11.3.5 Carbadox/olaquindox calibration solution ca. 0,5 µg ml^{-1}

Pipette 5 ml of the Carbadox/olaquindox calibration solution ca. 5 µg ml^{-1} (4.11.3.2) in a 50 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.11.3.6 Carbadox/olaquindox calibration solution ca. 0,25 µg ml^{-1}

Pipette 5 ml of the Carbadox/olaquindox calibration solution ca. 2,5 µg ml^{-1} (4.11.3.3) in a 50 ml volumetric flask. Dilute to the mark with the extraction solvent (4.5) and mix well.

Prepare fresh for each series of samples.

4.12 Neutral aluminium oxide, Brockmann activity I

4.13 Neutral aluminium oxide SPE cartridge, 2 cm^3, 1 850 mg

4.14 Technical methanol

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 pH meter

5.2 Solvent filtration system, suitable for 0,45 µm membrane filters

5.3 Ultrasonic bath

5.4 Rotary on mechanical shaker

5.5 Centrifuge

5.6 Glass wool

5.7 Glass column for chromatography, length 200 nm to 400 nm; internal diameter 10 mm, restricted at the end and fitted with a wad of glass wool (5.6) or equivalent column

5.8 Filter papers or glass microfibre filter

5.9 Cellulose acetate membrane filters of pore size 0,45 µm

5.10 PVDF syringe filters of pore size 0,45 µm and adaptable syringes

5.11 HPLC system

5.11.1 pump, capable of maintaining a volume flow rate of 0,4 ml min^{-1} to 1,5 ml min^{-1}

5.11.2 Column heater set at 30 °C

5.11.3 Injection system, with a loop suitable for 10 µl to 50 µl injections

5.11.4 UV detector, suitable for measurements at a wavelength of 375 nm

5.11.5 Diode array detector for confirmation purposes

5.11.6 Recorder or data acquisition system

5.11.7 Guard column, silica bounded C_8

5.11.8 LC analytical column, 250 × 3 mm, 5 µm pore size, packed with Lichrosorb C_8 or Lichrospher C_8 or Lichrospher C_{18} or equivalent column.

NOTE During the method validation, these recommended LC columns have proved to be fit for purpose, especially with samples containing interfering peaks close to the carbadox zone.

5.11.9 Refrigerated autosampler set between 0 °C and 8 °C

6 Sampling

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

Sampling is not part of the method specified in this European standard. A recommended sampling method is given in Commission Regulation (EU) No 691/2013 [1].

7 Preparation of test sample

7.1 General

Prepare the test sample in accordance with EN ISO 6498.

7.2 Laboratory sample

Grind the laboratory sample (usually 50 g) so that is passes completely through a sieve with 1 mm apertures. Mix thoroughly.

7.3 Test sample

The test sample consists of a representative and homogenized aliquot of the ground laboratory sample of at least 20 g.

7.4 Test portion

Accurately weigh 10,0 g to the nearest 0,1 g of the thoroughly mixed test sample into a 250 ml conical flask. Note down the mass expressed in g. Submit it to the analysis procedure (8).

8 Procedure

8.1 Extraction of feeding stuffs containing 0,5 mg kg^{-1} to 100 mg kg^{-1} of growth promoters

Add 100,0 ml of extraction solvent (4.5) to the test portion (7.4), stopper and shake vigorously for 1 h or 2 h on the rotary shaker (5.4).

8.2 Filtration

Filter the solution obtained in 8.1 through a paper or glass micro fibre filter (5.8).

Alternatively centrifugation (5.5) is possible, if filtration is too long, for example.

Use the filtrate or the supernatant for the purification step.

8.3 Purification

Apply 8.3.1 **or** 8.3.2.

8.3.1 Open glass clean up

For each sample extract, dry-pack a glass column (5.7), fitted at the end with a plug of glass wool (5.6) with 5 g of aluminium oxide (4.12). Load 20 ml of extract (8.2) on the column and discard the first 5 ml of eluate. Collect the next 5 ml. Filtrate these 5 ml through a PVDF syringe filter (5.10). Use this filtrate for HPLC determination.

8.3.2 SPE clean up

Load 8 ml of extract (8.2), on a SPE cartridge (4.13) and discard the first 1,5 ml of eluate. Collect the next 2 ml. Filtrate these 2 ml through a PVDF syringe filter (5.10). Use this filtrate for HPLC determination.

8.4 HPLC analysis

8.4.1 Analytical conditions

The following conditions are provided for guidance. Other conditions may be used provided they yield to equivalent results.

8.4.1.1 HPLC column: as in 5.11.8.

8.4.1.2 guard column: as in 5.11.7.

8.4.1.3 mobile phase: as in 4.8, flow rate: 0,5 ml min^{-1}.

8.4.1.4 injection volume: 20 µl-50 µl.

8.4.1.5 column temperature: 30 °C.

8.4.1.6 detection wavelength: 375 nm.

Using these conditions the retention times should approximately be 4,0 min to 6,0 min and 18,0 min to 22,0 min for olaquindox and carbadox respectively with respective capacity factors of 3 and 13

8.4.1.7 system set up

Check the stability of the HPLC system by injecting several times one of the calibration solutions (4.11.3.1; 4.11.3.2; 4.11.3.3) until constant peak heights and retention times are achieved.

NOTE Furazoline has approximately the same retention time than carbadox. In case of doubt, confirm the result. See 8.5.1 or 8.5.2.

In few particular feeds, a matrix interfering peak is present and has approximately the same retention time than carbadox. Using the described LC parameters, the interfering peak is eluted just before the carbadox peak. Apex should however be sufficiently separated. In case of doubt, confirm the result. See 8.5.1 or 8.5.2.

8.4.2 External calibration curve

Inject each calibration solution (4.11.3) at the beginning and at the end of a sample series.

Plot each of the individual measured peak height or peak area (y-axis) versus the respective concentration of carbadox or olaquindox (x-axis) in µg ml^{-1}.

8.4.3 Sample extracts

Inject several times (the determined area or peak height should be stable) the sample extract obtained in (8.3). The injection volume should be the same than the one selected for the calibration solutions. Determine the mean peak height (or peak area) for the carbadox and/or olaquindox signals.

8.5 System suitability – Confirmation

The identity of the target analyte(s) can be confirmed by co-chromatography or by using a DAD.

8.5.1 Co-chromatography

Prepare a spiked sample extract by adding an appropriate amount of one of the carbadox/olaquindox calibration solutions (4.11.3) to the sample extract. The amount of carbadox or olaquindox added shall be approximately equal to the estimated amount of the corresponding growth promoter detected in the sample extract.