SLOVENSKI STANDARD
oSIST prEN 16162:2011

01-februar-2011

Krma - Določevanje dekokvinata s HPLC s fluorescentno detekcijo

Animal feeding stuffs - Determination of Decoquinate by HPLC with fluorescence detection

Futtermittel - Bestimmung von Decoquinat mit Hochleistungs-Flüssigchromatographie (HPLC) und Fluoreszenzdetektion

Aliments des animaux - Dosage du décoquinate par CLHP avec détection par fluorescence

Ta slovenski standard je istoveten z: **prEN 16162**

ICS:

65.120	Krmila	Animal feeding stuffs

oSIST prEN 16162:2011 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 16162

October 2010

ICS 65.120

English Version

Animal feeding stuffs - Determination of Decoquinate by HPLC with fluorescence detection

Aliments des animaux - Dosage du décoquinate par CLHP avec détection par fluorescence

Futtermittel - Bestimmung von Decoquinat mit Hochleistungs-Flüssigchromatographie (HPLC) und Fluoreszenzdetektion

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 327.

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.

This draft European Standard was established by CEN in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Warning : This document is not a European Standard. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a European Standard.

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

Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. prEN 16162:2010: E

Contents

Page

Foreword

This document (prEN 16162:2010) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

Introduction

This European Standard has been developed to quantify Decoquinate in feedingstuff to enable the European Commission to control their content of animal feed products. However, that method can be used to evaluate the cross contamination from medicated feed to feedstuff.

1 Scope

This European Standard describes the determination of decoquinate. This high-performance liquid chromatographic (HPLC) method with a fluorescence detection is applicable to the quantification of decoquinate content in complete -, complementary compound feeds, supplements, premixture and feed additives.

The limit of detection is around 0,1 or 0,3 mg/kg and the limit of quantification is around 0,5 mg/kg. These limits were validated during the collaborative study [11], from results on the blank feed. Lower limits of detection or quantification could be reached but an in-house validation is then requested.

2 Normative references

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

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

3 Principle

Decoquinate is extracted from samples with a solution of 1% calcium chloride in methanol using mechanical shaking or stirring for 60 minutes. After centrifugation or filtration, an aliquot is, if necessary, diluted with the extraction solvent and analysed by reversed phase HPLC with fluorescence detection. Positive trace level samples should be confirmed by HPLC analysis using an alternate excitation wavelength.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralised water or water of equivalent purity.

WARNING — This method requires the handling of hazardous substances. It is recommended to use various regulations for potentially hazardous chemicals. Organisational, technical and personal safety has to be observed.

4.1 Methanol, HPLC grade

4.2 Methanol, technical grade

4.3 Calcium chloride anhydrous or Calcium chloride dehydrate, each >99% purity

4.4 HPLC dilution solution

Dissolve a mass of calcium salt (4.3) equivalent to 10 g of calcium chloride anhydrous in methanol (4.1). Mix well and make up to 1000 ml.

4.5 Extraction solvent

Dissolve a mass of calcium salt (4.3) equivalent to 10 g of calcium chloride anhydrous in technical methanol (4.2). Mix well and make up to 1 000 ml.

4.6 Decoquinate reference standard with guaranteed purity

Pure reference standard is available by Alpharma® supplier

Purity should be certified by a certificate of analysis.

4.6.1 **Decoquinate stock standard solution**, approximately 300 µg/ml

Accurately weigh, to the nearest 0,1 mg, 30 mg of decoquinate reference standard (4.6) into a 100 ml volumetric flask and dissolve in dilution solution (4.4). Use ultrasonic bath if necessary to aid dissolution. Calculate the exact concentration taking into account the purity of the standard material (4.6), given in the certificate.

Prepare fresh monthly. Store in the dark at 0°C to 10°C.

4.6.2 HPLC standard solutions

All solutions described under that clause, are prepared fresh daily.

4.6.2.1 **Intermediate standard solution**, approximately 6 µg/ml

Transfer by pipette, 2,0 ml of stock standard (4.6.1) into a 100 ml volumetric flask, dilute to volume with dilution solution (4.4). Check that intermediate solution for each series of analysis. The absorbance density of intermediate solution (4.6.2.1) can be evaluated at 265 nm, with dilution solvent (4.4) as reference for optical density measurement. In these conditions, the absorbance range is between 0,67 and 0,73.

4.6.2.2 **HPLC calibration standard solutions**, approximately 0,15, 0,30, 0,60 and 1,2 µg/ml

Prepare 4 concentrations of HPLC standard solutions as it is explained in table 1.
Transfer by pipette the required volume of intermediate standard (4.6.2.1) into volumetric flasks, and make to volume with dilution solution (4.4). Mix well.

Table 1 — preparation of calibration standard solutions

Standard	Parts of intermediate (4.6.2.1) solution, in ml	Volumetric flask, in ml	Dilution Factor	≅µg/ml
A	5	200	40	0,15
B	5	100	20	0,30
C	10	100	10	0,60
D	10	50	5	1,20

Evaluate precisely each exact concentration by using the exact concentration of the stock solution (4.6.1)

4.7 HPLC Mobile Phase

Dissolve a mass of calcium salt (4.3) equivalent to 10 g calcium chloride anhydrous into 1l of a solvent mixture of methanol (4.1)/water in proportion of 825/175 (v/v). Filter under vacuum (5.2) before use.

HPLC shutdown solution

Prepare a methanol (4.1)/water solution in proportion of 85/15 (v/v) to flush the HPLC column and equipment at the end of each day of analysis.

5 Apparatus

Usual laboratory apparatus, in particular, the following.

5.1 Mechanical shaker or magnetic stirrer

5.2 Solvent filtration system, suitable for 0,45 µm PTFE filter or equivalent

5.3 Centrifuge and centrifuge tube (50 ml)

5.4 HPLC system consisting of the following

5.4.1 Pump, pulse free, flow capacity of 0,2 ml/min to 5 mL/min

5.4.2 Injection system, manual or autosampler, with loop suitable for 10 to 50 µl injection volumes

5.4.3 Analytical C18 column like ACE® or Luna® or Symmetry® ; 5µm ; 4.6 * 250 mm or equivalent

5.4.4 Fluorescence detector suitable for measurement using 330 nm and 260 nm excitation wavelengths and 390 nm emission wavelength

5.4.5 Integrator or computer data system

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage. Sampling is not part of the method specified in this European Standard. A recommended sampling procedure is given in ISO 6497 [1].

7 Sample preparation

All sample feeds are grinded before extraction as recommended in guideline EN ISO 6498. Premixtures and concentrates (feed additives) are not obligatory grinded.

8 Procedure

Preferably, duplicate analysis is performed. Portions of samples are weighed into amber conical flasks.

8.1 Extraction of feeds (decoquinate content between 10 to 500 mg/kg)

NOTE For milk-replacer give a particular attention in adding extraction solvent. Small or large lumps could be formed and quantitative results will be compromised. The extraction solvent addition should be done slowly under rotative shaking of the conical flask. To dissolve lumps, ultrasonic system, during approximately five minutes, is very helpful.

Accurately weigh, to the nearest 0,1 g , 10,0 g of feed in a 250 ml conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 hour on apparatus (5.1). Let settle down particules about 10 minutes. Transfer an aliquot of the nearly clear overstanding extraction solution. For uncleared extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge. Dilute the clear extract with dilution solution (4.4) to obtain a concentration between 0,3 and 0,6 µg/ml. Filter on membrane filter (5.2) the diluted solution. Inject onto HPLC system (5.4).

8.2 Extraction of complementary feeds, premixtures and feed additives (decoquinate content higher than 500 mg/kg)

Accurately weigh, to the nearest 0,01 g, in function of guarantees, 0,50 to 2,00 g of sample into a 250 ml conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 hour on equipment (5.1). Let settle down particules

about 10 minutes. Transfer an aliquot of the nearly clear overstanding extraction solution. For uncleared extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge. Dilute with dilution solution (4.4) to obtain a concentration between 0,30 and 0,6 µg/ml. Filter on membrane filter and inject onto HPLC system (5.4).

NOTE For a decoquinate guarantee of 1 000 mg/kg, weigh 2 g of test portion and dilute 1 ml of clear extract in a 100 ml volumetric flask. For feed additives guaranteed at 6%, weigh 0,5g and dilute of a factor 1/400.

8.3 Extraction of trace feeds (decoquinate content lower than 10 mg/kg)

Accurately weigh, to the nearest 0,1 g, 10,0 g of feed in a 250 ml conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 hour on apparatus (5.1). Let settle down particules about 10 minutes. Transfer an aliquot of the nearly clear overstanding extraction solution. For uncleared extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge. That extract, without dilution, is filtered on membrane filter and injected onto HPLC system (5.4).

8.4 Quality control spiked feeds

Accurately weigh, to the nearest 0,1 g, 10,0 g of quality control blank feed in a 250 ml amber conical flask.

8.4.1 Blank Feed to spike at 30 mg/kg

Add 1 ml of stock solution (4.6.1). Wait for 15 minutes. Then add 100 ml of the extraction solution (4.5). Proceed as described in (8.1). The recovery must be from 80 to 110%.

8.4.2 Blank feed to spike at 9 mg/kg

Add 15 ml of intermediate solution (4.6.2.1). Wait for 15 minutes. Then add 85 ml of the extraction solution (4.5). Proceed as described in (8.3). The recovery must be from 80 to 120%.

8.5 HPLC parameters

This HPLC parameters are given for guidance. Other parameters should be applicable (column, flow rate adapted to HPLC columns and optimum response of the fluorescence detector, …)

Column: analytical ACE C18 column; 5µm; 4.6 * 250 mm

Column Temperature: ambient or 30°C

Mobile phase (4.7): MeOH/water 825/175 (v/v) with 1% of CaCl2

Injection Volume: 20 µl

Flow rate: 0,5 ml/min

Excitation wavelength: 330 nm

Emission wavelength: 390 nm

Equilibrate the system by running mobile phase before beginning the sequence. Check the stability of the HPLC system by injecting several times one of the calibration solutions (4.6.2.2), until constant peak heights (areas) and retention times are achieved.

With these HPLC conditions, the decoquinate retention time is within 14 minutes and 18 minutes (capacity factor = 5).

After each day of analysis, flush all the HPLC system with shutdown solution.

8.6 Standards' injections and calibration curve

The linearity system has been tested between 0,015 µg/ml to 1,5µg/ml

Inject the HPLC standard solutions on HPLC system at the beginning and the end of a samples' sequence.

Plot calibration graphs using the peak areas of the calibration solutions as the ordinates and the corresponding concentrations, in µg/ml, of decoquinate, as the abscissas.

Use a linear regression as mathematic model (y=ax+b).

8.7 Sample extracts

Inject several times, if necessary, the sample extract obtained in (8.1), (8.2), (8.3) or (8.4), using the same injection volume as taken with calibration solutions. Determine the mean area of decoquinate peaks.

8.8 Confirmation procedure

In cases of doubts on the decoquinate peak identification, in sample solution or for trace levels, a confirmatory excitation wavelength at 260 nm must be applied. The HPLC standards solutions and sample solutions will be re-injected in these confirmatory conditions at 260 nm. Others LC parameters stay identical as described in (8.5).

9 Calculations

Report the area of unknown sample on the calibration curve and evaluate the concentration C of the injected solution.

The decoquinate amount A, in the sample, in mg/kg, is obtained by application of the formula:

$$A = \left(C * DF * V\right) / m \qquad (1)$$

where

C is the decoquinate concentration of the sample extract, in µg/ml;

DF is the dilution factor;

V is the total volume, in ml, of extraction solvent added to the test portion (100 ml);

m is the mass, in g, of the test portion (10 g for feeds).

Round the result to the nearest:

— 0,1 mg/kg for feedingstuffs containing 0,5 to 10 mg/kg of decoquinate

— 1 mg/kg for feedingstuffs containing 10 to 100 mg/kg of decoquinate

— 10 mg/kg for feedingstuffs containing 100 to 1 000 mg/kg of decoquinate

— 100 mg/kg for premixtures containing 1 000 to 10 000 mg/kg (= 1%) of decoquinate

— 0,1% for feed additives containing more than 1% of decoquinate

10 Precision

10.1 Limit of Detection and Limit of Quantification

Detection Limit L_D = 0,1 to 0,3 mg/kg

Quantification Limit L_Q = 0,3 to 1 mg/kg

10.2 Interlaboratory test

A international collaborative study was conducted in 2009, with 28 laboratories (5 from North america and 23 from EU). In total 28 laboratories delivered results for 7 blind duplicate materials (MAT A1-A7) and 24 laboratories for 4 additional blind duplicate materials (MAT B1-B2 and MAT C1-C2). However, after checking the rigorous application of the protocol in the collaborative trial, only 27 were considered valid by the organising laboratory (SCL L-35). Statistics were performed by JRC-IRMM, in accordance with ISO 5725 [10] and all details are given in the JRC-IRMM report [11]. See details in annex A.

10.3 Repeatability

The relative difference, between two independent single test results, obtained with 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 the cases, exceed approximately 6% of the mean value.

$$r = 1{,}92 * 2{,}8/100 * A \approx 6/100 * A \quad (2)$$

where

r is the repeatability in mg/kg;

A is the Decoquinate amount in mg/kg.

See table A.2 of annex A.

10.4 Reproducibility

The relative difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5% of the cases, exceed approximately 18% of the mean value.

$$R = 6{,}27 * 2{,}8/100 * A \approx 18/100 * A \quad (3)$$

where

R is the reproducibility in mg/kg;

A is the Decoquinate amount in mg/kg.

See table A.2 and figure A.2 of annex A.

11 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;