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Animal feeding stuffs — Determination of decoquinate by HPLC with fluorescence detection

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/10, Animal feeding stuffs.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Aliments des animaux - Détermination du décoquinate par Chromatographie Liquide Haute Performance avec détection fluorimétrique

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

This European Standard was approved by CEN on 4 February 2012.

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Foreword

This document (EN 16162:2012) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by September 2012, and conflicting national standards shall be withdrawn at the latest by September 2012.

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Introduction

This European Standard has been developed to quantify decoquinate in feeding stuffs to enable the European Commission to control the content of animal feed products. However, this method can also be used to evaluate the cross contamination from medicated feed to feedstuff.

1 Scope

This European Standard specifies a method for 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 and complementary compound feeds, medicated feeds, semi-liquid feeds, premixtures and feed additives.

The method was fully validated from LOQ to 60 000 mg/kg on different matrices during an international collaborative study [11], especially on complete compound feeds for poultry, at trace contamination level of 3 mg/kg and at European authorized level of 20 mg/kg to 40 mg/kg [12].

The limit of detection is between 0,1 mg/kg and 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 a single laboratory validation is then requested.

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.

prEN ISO 6498, *Animal feeding stuffs — Guidelines for sample preparation (ISO/DIS 6498)*

3 Principle

Decoquinate is extracted from samples with a solution of 1 % calcium chloride in methanol using mechanical shaking or stirring for 60 min. 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 dihydrate, 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 1 000 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 standards.

4.6.1 Decoquinate powder reference standard with guaranteed purity.

Purity shall be certified by a certificate of analysis.

NOTE Pure reference standard is available at e.g. Alpharma®.

4.6.2 Decoquinate stock standard solution, approximately 300 µg/ml.

Accurately weigh, to the nearest 0,1 mg, 30 mg of decoquinate reference standard (4.6.1) 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 (4.6.1), given in the certificate. Prepare fresh monthly. Store in the dark at 0 °C to 10 °C.

4.6.3 HPLC standard solutions.

4.6.3.1 Intermediate standard solution, approximately 6 µg/ml.

Transfer by pipette 2,0 ml of stock standard solution (4.6.2) 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 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. That intermediate solution is prepared fresh daily.

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

Prepare 4 concentrations of HPLC standard solutions as it is explained in Table 1 (standards A/B/C/D). Transfer by pipette the required volume of intermediate standard (4.6.3.1) 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 (4.6.3.1) 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 (4.6.2).

All solutions described here (standards A/B/C/D) are prepared fresh daily.

4.7 HPLC Mobile Phase.

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

NOTE HPLC shutdown solution.

Prepare a methanol (4.1) / water solution in proportion by volume of 85/15 without calcium salt 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 µl to 50 µl injection volumes.

5.4.3 Analytical C18 column like ACE® or Luna® or Symmetry® or Restek Ultra®; 5 µm; 4.6 mm x 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 EN ISO 6497 [1].

7 Sample preparation

All feed samples, with the exception of premixtures and concentrates (feed additives), are ground extraction as recommended in the guidelines prEN ISO 6498.

8 Procedure

8.1 General

Preferably, duplicate analysis is performed.

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

NOTE For milk-replacer particular attention should be given to the addition of extraction solvent. Small or large lumps could be formed and quantitative results will be compromised. The extraction solvent addition should be done slowly under rotate shaking of the conical flask. To dissolve lumps, ultrasonic system, during approximately 5 min, is very helpful.

Accurately weigh, to the nearest 0,1 g, 10,0 g of feed in a 250 ml amber conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 h on apparatus (5.1). Allow contents of flask to settle particles for about 10 min. Dilute an aliquot of the almost clear supernatant extract with dilution solution (4.4) to obtain a concentration between 0,3 µg/ml and 0,6 µg/ml. Filter the diluted solution on membrane filter (5.2) and inject it onto HPLC system (5.4). For cloudy extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge before dilution, membrane filtration and HPLC injection.

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

Accurately weigh, to the nearest 0,01 g, depending on the declared concentration of decoquinate in the sample, 0,50 g to 2,00 g of sample into a 250 ml amber conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 h on equipment (5.1). Allow contents of flask to settle particles for 10 min. Dilute an aliquot of the almost clear supernatant extract with dilution solution (4.4) to obtain a concentration between 0,3 µg/ml and 0,6 µg/ml. Filter the diluted solution on membrane filter (5.2) and inject it onto HPLC system (5.4). For cloudy extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge before dilution, membrane filtration and HPLC injection.

NOTE For a decoquinate declaration 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 declared at 6 %, weigh 0,5 g and dilute by a factor of 1/400.

8.4 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 amber conical flask. Add 100 ml of the extraction solution (4.5). Shake or stir for 1 h on apparatus (5.1). Allow contents of flask to settle particles for 10 min.. Dilute an aliquot of the almost clear supernatant extract with dilution solution (4.4) to obtain a concentration between 0,3 µg/ml and 0,6 µg/ml. Filter the diluted solution on membrane filter (5.2) and inject it onto HPLC system (5.4). For cloudy extracts, if necessary, transfer at least 40 ml of the extract into 50 ml centrifuge tube (5.3) and centrifuge before dilution, membrane filtration and HPLC injection.

8.5 Quality control spiked feeds

8.5.1 Blank Feed to spike at 30 mg/kg

Accurately weigh, to the nearest 0,1 g, 10,0 g of quality control blank feed in a 250 ml amber conical flask. Add 1 ml of stock solution (4.6.2). Wait for 15 min. Then add 100 ml of the extraction solution (4.5). Proceed as described in 8.2. The recovery shall be from 80 % to 110 %.

8.5.2 Blank feed to spike at 9 mg/kg

Accurately weigh, to the nearest 0,1 g, 10,0 g of quality control blank feed in a 250 ml amber conical flask. Add 15 ml of intermediate solution (4.6.3.1). Wait for 15 min. Then add 85 ml of the extraction solution (4.5). Proceed as described in 8.4. The recovery shall be from 80 % to 120 %.

8.6 HPLC parameters

These 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…).

— Analytical column: C18 column; 5 µm; 4.6 mm x 250 mm as described in 5.4.3;

— Column Temperature: ambient or 30 °C;

— Mobile phase (4.7): MeOH/water in proportion by volume of 825/175 with 1 % of $CaCl_2$;

— Injection Volume: 20 µl;

— Flow rate: 0,5 ml/min;

— Excitation wavelength: 330 nm;

— Emission wavelength: 390 nm.

Equilibrate the system by running a mobile phase before beginning the sequence. Check the stability of the HPLC system by injecting one of the calibration solutions (4.6.3.2) several times until consistent peak heights (areas) and retention times are achieved.

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

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

8.7 Standards' injections and calibration curve

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

8.8 Sample extracts

Inject several times, if necessary, the sample extract obtained in 8.2, 8.3, 8.4 or 8.5, using the same injection volume as taken with calibration solutions. Determine the mean area of decoquinate peaks.

8.9 Confirmation procedure

In cases of doubts on the decoquinate peak identification, in sample extracts, particularly at trace levels, a confirmatory excitation wavelength at 260 nm shall be applied. Using this excitation wavelength, re-inject the HPLC standard solutions and sample extracts, while keeping all other LC parameter the same as described in 8.6.

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:

$$\mathrm{A} = \left(C \times DF \times V\right) / m \qquad (1)$$

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 feeding stuffs containing 0,5 mg/kg to 10 mg/kg of decoquinate;

— 1 mg/kg for feeding stuffs containing 10 mg/kg to 100 mg/kg of decoquinate;

— 10 mg/kg for feeding stuffs containing 100 mg/kg to 1 000 mg/kg of decoquinate;

— 100 mg/kg for premixtures containing 1 000 mg/kg 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 mg/kg to 0,3 mg/kg

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

10.2 Interlaboratory test

An international collaborative study was conducted in 2009, with 28 laboratories (5 laboratories from North America and 23 laboratories 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 laboratories were considered valid by the organising laboratory (SCL L-35). Statistics were performed by JRC-IRMM, in accordance with ISO 5725 (all parts) [2] 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.

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

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 no more than 5 % of the cases, exceed approximately 18 % of the mean value.

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

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 the following information:

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 European Standard;

d) all operating details not specified in this European Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result obtained;

f) if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Results inter-laboratory study

A full collaborative study was organised by SCL L35, on behalf of CEN TC327 and EU Commission, during summer 2009. The statistical evaluation was performed by JRC IRMM, in Geel, Belgium.

The design of the study and the target performance characteristics for the evaluation of the HPLC-FLD method in detecting decoquinate in feed samples were selected according to internationally accepted guidelines for method validation [10]. The quantitative results submitted by the laboratories were used to estimate average and standard deviations under repeatability and reproducibility conditions by applying the analysis of variance approach as recommended in ISO guidelines [2].

In total, 28 laboratories were selected from the training period to take part in the validation period. All 28 laboratories delivered results. One laboratory did not strictly follow the proposed protocol and was therefore disqualified by the organising laboratory SCL L35. However, the results of that rejected lab are given in Annex C as additional information, especially for the purpose of robustness. The results obtained in the study were used to evaluate the suitability of the method for the determination of decoquinate in feeding stuffs for official control purposes.

The participants to the validation trial received the 14 ground samples (7 samples in blind duplicate) from different feeding stuffs type (feed additive, premixture, poultry feed, blank feed and lamb feed) to be compulsory analysed, 8 optional samples (4 additional samples in blind duplicate = samples B1, B2, C1, C2 ; ground –, milk replacer and bovine feed – and in pelleted form). The target concentrations of decoquinate, obtained from firms, in the test samples are displayed in Table A.1.

Table A.1 — Materials' nature and decoquinate targeted concentrations

Material	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**B1**	**B2**	**C1**	**C2**
Feeding stuff type	MCF	MCF	FA	CF	CF	B	PM	MCF	MCF	MCF	MCF
species	Ruminant (Vegetable support)	Lamb	Deccox	Poultry	Poultry	Poultry	Poultry and/or Ruminant	Cattle	Ruminant (lactoprotein support)	Ruminant (Vegetable support)	Lamb
Target concentration mg/kg	1 000	54	60 000	3	30	0	6 000	240	1 000	1 000	54

NOTE 1: Materials A (1 to 7) and B (1,2) are ground.

NOTE 2: Materials C1 and C2 are the pelleted forms of MAT A1 and A2 respectively.

NOTE 3: FA: feed additive (Deccox® 60 g/kg); PM: premixture; MCF: medicated compound feed; CF: complete compound feed; B: blank compound feed.

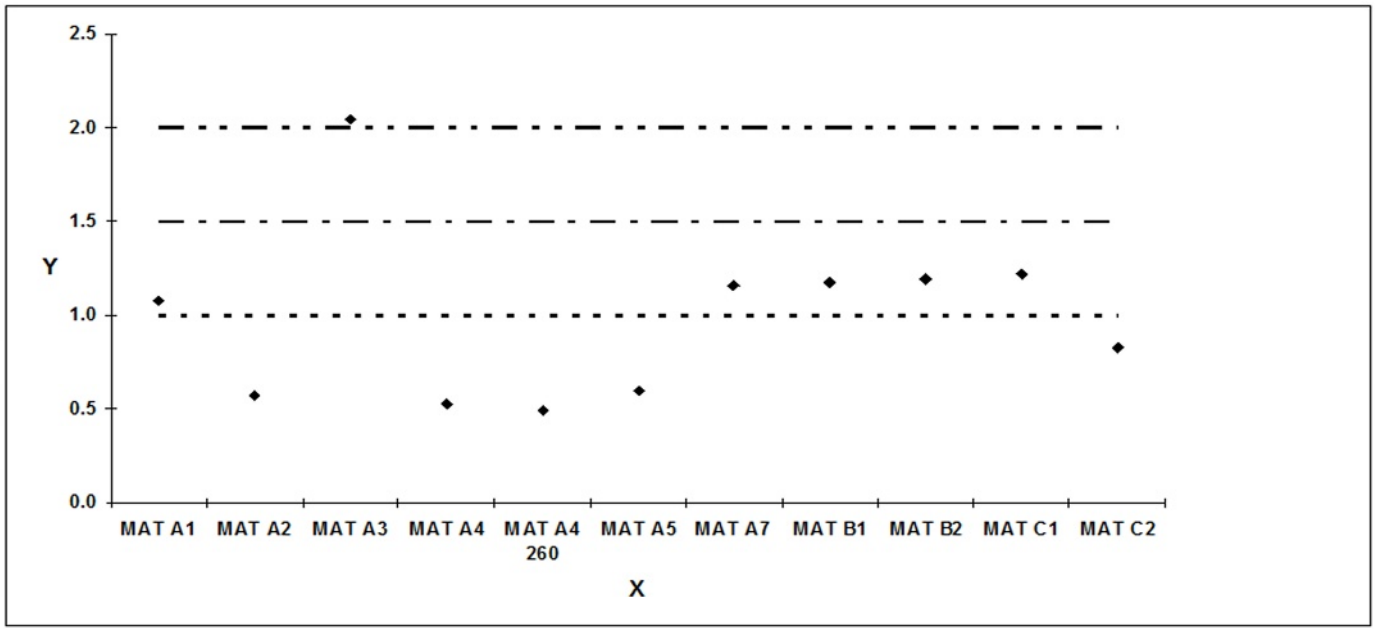
NOTE 4: Target concentrations are as guaranteed by the producers on the labelling.

Table A.2 — Results of the collaborative study after statistical evaluation [11]

	Number of laboratories	Target (mg/kg)	Av (mg/kg)	$S_{(r)}$ (mg/kg)	$RSD_{(r)}$ (%)	$S_{(R)}$ (mg/kg)	$RSD_{(R)}$ (%)	HOR
MAT A1	28 (4)	1 000	913	14	2	56	6	1,08
MAT A2	28 (3)	54,00	57,71	0,79	1,37	2,86	4,95	0,57
MAT A3	28 (4)	60 000	57 926	1 110	2	3 640	6	2,05
MAT A4	28 (5)	3,00	2,69	0,06	2,17	0,20	7,31	0,53
MAT A4 (260 nm)	28 (5)	3,00	2,71	0,06	2,33	0,18	6,77	0,49
MAT A5	28 (2)	30	26,82	0,71	2,63	1,57	5,86	0,60
MAT A7	28 (9)	6 000	6 188	153	2	307	5	1,15
MAT B1	24 (4)	240	218	8	4	18	8	1,18
MAT B2	24 (1)	1 000	912	32	3	63	7	1,20
MAT C1	24 (3)	1 000	867	24	3	61	7	1,22
MAT C2	24 (3)	54,00	57,10	1,03	1,81	4,08	7,15	0,82

NOTE 1 Target: target concentration are as guaranteed by the producers on the labelling; Av: average concentration; $S_{(r)}$: within-laboratory standard deviation (repeatability); $RSD_{(r)}$: relative within-laboratory standard deviation (repeatability); $S_{(R)}$: between-laboratory standard deviation (reproducibility); $RSD_{(R)}$: relative between-laboratory standard deviation; HOR: HORRAT value for reproducibility.

NOTE 2 The number between brackets, in the second column, indicates the number of laboratories identified as outliers due to deviation from the protocol or/and by statistical tests.



Key

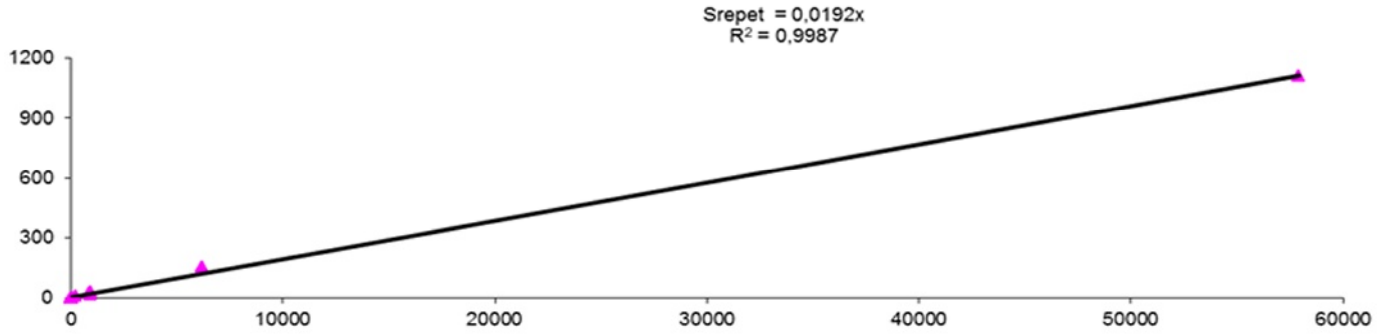
Axes: the X- axis displays the material code (Note: MAT A4 260 is for MAT A4 at the confirmation wavelength of 260 nm in comparison with MAT A4 at 330 nm), the Y-axis displays the HORRAT value.

decoquinate (◆), target limit (— — —), acceptance limit (— · — · —), rejection limit (— · · —).

Figure A.1 — Graphical representation of HORRAT values

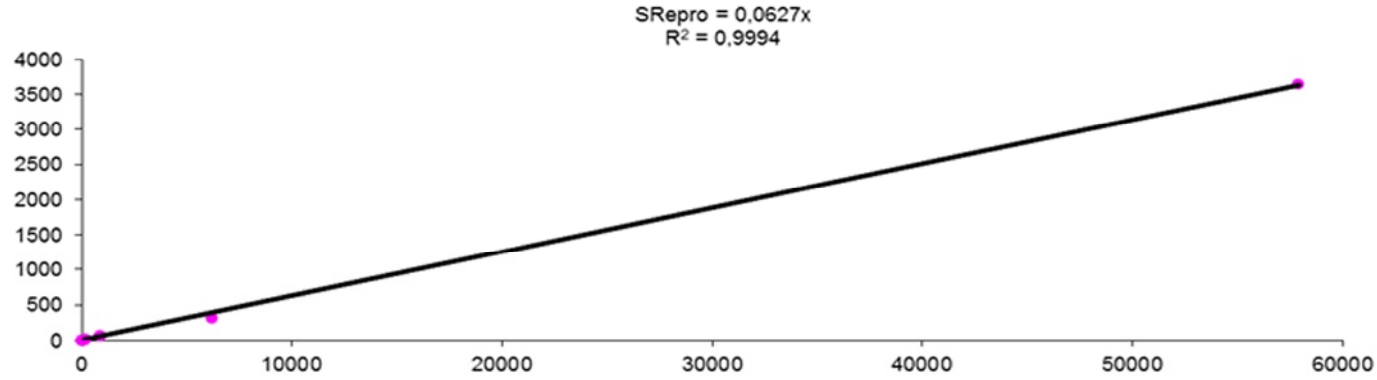
As highlighted from the graph, for the 10 duplicate materials and for the sample analysed using the confirmatory parameters, the HORRAT value was always below 1,5 except for the evaluation of decoquinate in MAT A3.

Material A3 is the feed additive itself where the decoquinate concentration is very high (6 %), hence reaching the limits of the Hortwitz equation. The phenomenon is well known for high concentrated material. Indeed, the concentration of the analyte being very high, the RSD(R)Horwitz is around 3. However, laboratory reproducibility of the method is very good with a RSD(R) at 6 % for MAT A3 hence leading to a HORRAT value of 2,05. Taking into account both the high concentration and the very good reproducibility, it can still be concluded that the method can also be considered as validated for MAT A3.



Key: X-axis = decoquinate content in mg/kg; Y-axis = Srepet in mg/kg

Figure A.2 — Graphical representations of repeatability versus decoquinate content in mg/kg



Key: X-axis = decoquinate content in mg/kg; Y-axis = SRepro in mg/kg

Figure A.3 — Graphical representations of reproducibility versus decoquinate content in mg/kg

Annex B
(informative)

Additional recovery results

B.1 Recoveries from the familiarization phase

During the familiarization phase of the final collaborative study, in February 2009, 2 blanks (milk replacer for testing an additional sonic bath step during extraction procedure and poultry complete compound feed) were distributed to the 28 candidates. They had to spike the 2 blanks at 6 mg/kg and 30 mg/kg respectively. For the lowest spike, they also had to use routine and confirmatory fluorimetric parameters (see 8.9). Raw data is given hereunder in Table B.1.

Table B.1 — Raw data obtained on 2 blank feeds, at 2 levels

	Calf blank milk replacer ; spike at 6 mg/kg		**Poultry blank feed ; spike at 30 mg/k**
	330 nm routine wavelength	260 nm confirmatory wavelength	330 nm routine wavelength
Lab1	90,6	91,0	94,4
Lab2	109,1	106,5	100,5
Lab3	103,4	103,3	107,4
Lab4	95,3	95,6	100,4
Lab5	113,8	103,1	107,8
Lab6	86,7	85,9	78,7
Lab7	101,6	100,8	99,5
Lab8	123,8	104,1	-
Lab9	105,2	97,4	104,5
Lab10	98,7	100,3	105,9
Lab11	102,8	104,8	97,2
Lab12	105,5	111,7	110,2
Lab13	105,8	108,2	103,9
Lab14	100,3	101,4	100,4
Lab15	101,6	100,7	104,5
Lab16	103,6	104,9	103,0
Lab17	112,8	107,4	132,8
Lab18	100,7	100,4	97,8
Lab19	105,6	102,8	99,1
Lab20	105,3	103,3	98,5
Lab21	96,1	100,4	94.0
Lab22	95,8	104,3	85,1
Lab23	94,5	93,7	98,8
Lab24	89,0	128,5	97,4
Lab25	103,9	105,0	98,8
Lab26	91,3	91,1	97,9
Lab27	100,7	99,4	99,7
Lab28	100,8	101,1	94,1
mean	**101,6**	**102,0**	**100,7**
sd	7,9	7,6	9,3
mini	86,7	85,9	78,7
maxi	123,8	128,5	132,8

B.2 Pre-trial; recovery test on a milk-replacer

During a transferability test of the method in 2007, 5 European laboratories performed different spikes on a blank milk-replacer, a reputed difficult matrix, with an additional sonic bath step during the extraction procedure. Recoveries, in %, are given in Table B.2.

Table B.2 — Recoveries obtained by 5 EU laboratories on a blank milk replacer

	spike at 3 mg/kg		spike at 9 mg/kg		spike at 30 mg/kg		spike at 150 mg/kg	
lab1	107,0	106,7	no tested	no tested	106,6	106,6	no tested	no tested
lab2	no tested	no tested	81,2	81,2	102,5	96,8	88,4	87,5
lab3	no tested	no tested	93,3	91,8	86,0	91,0	no tested	no tested
lab4	no tested	no tested	88,7	87,8	94,9	100,0	no tested	no tested
lab5	no tested	no tested	93,0	93,0	93,7	91,5	no tested	no tested
n =	2		8		10		2	
Mean	106,9		88,8		97,0		87,9	

B.3 Recoveries from the single-laboratory validation

During the single-laboratory validation in 2006, several recovery experiments were performed on 4 blanks (poultry, pig, cattle and milk replacer).

Table B.3 sums up the recovery results, in %, obtained on these 4 blank feeds, analysed on 6 replicates per day, on 2 independent days and spiked at 2 levels (9 mg/kg and 30 mg/kg). Mean recoveries reported are obtained after discarding 6 Dixon outliers.

Table B.3 — Recovery average, 2 independent days, 4 matrices, 2 spike levels

	Species	Pig			Poultry			Cattle			Milk replacer		
Spike level	Series	1	2	Global series	1	2	Global series	1	2	Global series	1	2	Global series
9 mg/kg	Recovery	96,8	103,3	100,1	109,8	91,8	100,8	101,9	101,5	101,7	97,8	101,7	99,9
30 mg/kg	Recovery	99,2	103,8	100,9	108,0	96,2	101,6	99,5	100,4	99,4	102,5	92,9	97,4

From all the results (n= 90), the global average of recovery is 100,1 %.

Annex C
(informative)

Additional results for robustness purposes

C.1 Robustness in terms of excitation wavelength, during the final collaborative study

During the final collaborative study [11], one laboratory, by error, performed analysis at an excitation wavelength of 260 nm instead of 330 nm, on four samples. The results are in accordance with those obtained by the others labs. These results are given in Table C.1, for ruggedness purposes.

Table C.1 — Results obtained with the confirmatory wavelength at 260 nm instead of the routine wavelength at 330 nm

Material	A1	A2	A3	A4
Test Portion Weight (g)	3,085 6	15,173 8	1,514 4	14,869 1
Theorical Test Portion Weight (g)	2,000 0	10,000 0	0,5000	10,000 0
Practical injection volume used insteaf of 20 µl	15	15	10	15
Decoquinate content (mg/kg) at 260 nm	850	60,14	53 542	2,78
Decoquinate content from study (mg/kg) at 330 nm	913	57,71	57 926	2,71

C.2 Comparison between ground and unground samples during the final collaborative study

Materials A1-A2 ground versus materials C1-C2 unground in pellet form.

Materials A1 and A2 were fully prepared and ground by the organizing laboratory and dispatched to the 28 laboratories. Same exact materials C1 and C2 were also dispatched to the 28 laboratories but maintained in the pellet form. They have to be ground by participants, according to their own sample preparation procedure.

The results obtained for the pelleted MAT C1 and MAT C2 are comparable to those obtained for the corresponding ground MAT A1 and MAT A2, hence indicating that the grinding step of the protocol when performed by the laboratories, has no significant influence on the precision and the accuracy of the method although the relative standard deviation on repeatability and on reproducibility turned to be slightly higher for the pelleted materials. That appears logical and reflects the differences between laboratories' sample preparation.

Table C.2 — Comparative results on 2 feed samples, ground and unground

	Number of laboratories	Target (mg/kg)	Av (mg/kg)	$S_{(r)}$ (mg/kg)	$RSD_{(r)}$ (%)	$S_{(R)}$ (mg/kg/)	$RSD_{(R)}$ (%)	HOR
MAT A1 ground	28 (4)	1 000	913	14	2	56	6	1,08
MAT C1 pelleted	24 (3)	1 000	867	24	3	61	7	1,22
MAT A2 ground	28 (3)	54,00	57,71	0,79	1,37	2,86	4,95	0,57
MAT C2 pelleted	24 (3)	54,00	57,10	1,03	1,81	4,08	7,15	0,82

NOTE 1 Target: target concentration as guaranteed by the producers on the labelling; Av: average concentration; $S_{(r)}$: within-laboratory standard deviation (repeatability); $RSD_{(r)}$: relative within-laboratory standard deviation (repeatability); $S_{(R)}$: between-laboratory standard deviation (reproducibility); $RSD_{(R)}$: relative between-laboratory standard deviation; HOR: HORRAT value for reproducibility.

NOTE 2 The number between brackets, in the second column, indicates the number of laboratories identified as outliers due to deviation from the protocol or/and by statistical tests.

C.3 Robustness from one laboratory during the final collaborative study

During the final collaborative study [11], one laboratory was rejected because it did not strictly follow the protocol. It adapted some sample test portions, injection volumes and dilution factors. However, the results from this laboratory are in accordance with those obtained by the others labs. These results are given in Table C.3, for ruggedness purposes.

Table C.3 — Comparative results, after modifications of the protocol

Material	A1		A2		A3		A4		A5		A6		A7		B1		B2		C1		C2	
Practical Test Portion Weight (g)	3,086	3,187	15,174	15,179	1,514	1,510	14,869	14,266	14,451	14,581	14,715	15,103	3,117	3,069	14,905	14,807	2,924	3,111	3,049	3,036	14,026	14,049
Theorical Test Portion Weight (g)	2,000	2,000	10,000	10,000	0,500	0,500	10,000	10,000	10,000	10,000	10,000	10,000	0,500	0,500	10,000	10,000	2,000	2,000	2,000	2,000	10,000	10,000
Practical dilution factor	25	25	10	10	500	500	1	1	10	10	1	1	500	500	50	50	25	25	25	25	10	10
Theorical dilution factor	25	25	10	10	500	500	1	1	10	10	1	1	50	50	50	50	25	25	25	25	10	10
Practical injection volume used instead of 20 µl	15	15	15	15	10	10	15	15	15	15	15	15	10	10	15	15	15	15	15	15	15	15
Decoquinate content (mg/kg)	850	838	60,06	59,41	53 663	53 859	2,80	2,83	29,49	28,84	ND	ND	5 640	5 700	205	199	819	822	800	770	49,45	51,18
Mean content (mg/kg)	844		59,74		53 761		2,82		29,17		ND		5 670		202		821		785		50,32	
Decoquinate content obtained from study [11] (mg/kg)	913		57,71		57 926		2,69		26,82		ND		6 188		218		912		867		57,10	

Annex D
(informative)

HPLC parameters and chromatogram examples

D.1 General

HPLC parameters are those described in (8.6). The column used was a Restek Ultra ® C18, 250 mm x 4,6 mm, 5 µm particle size; the oven temperature was set at 30 °C and the injection volume was 20 µl.

D.2 Chromatogram example: Calibration point at 0,6 µg/ml

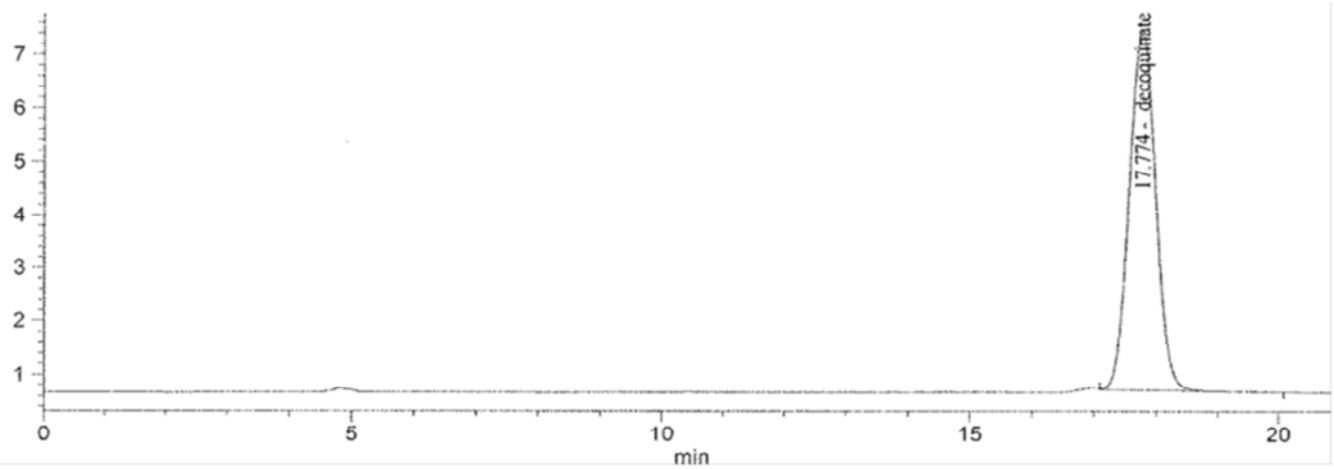


Figure D.1

D.3 Chromatogram example: Commercial Lamb feed containing around 50 mg/kg of decoquinate

Reference excitation wavelength at 330 nm

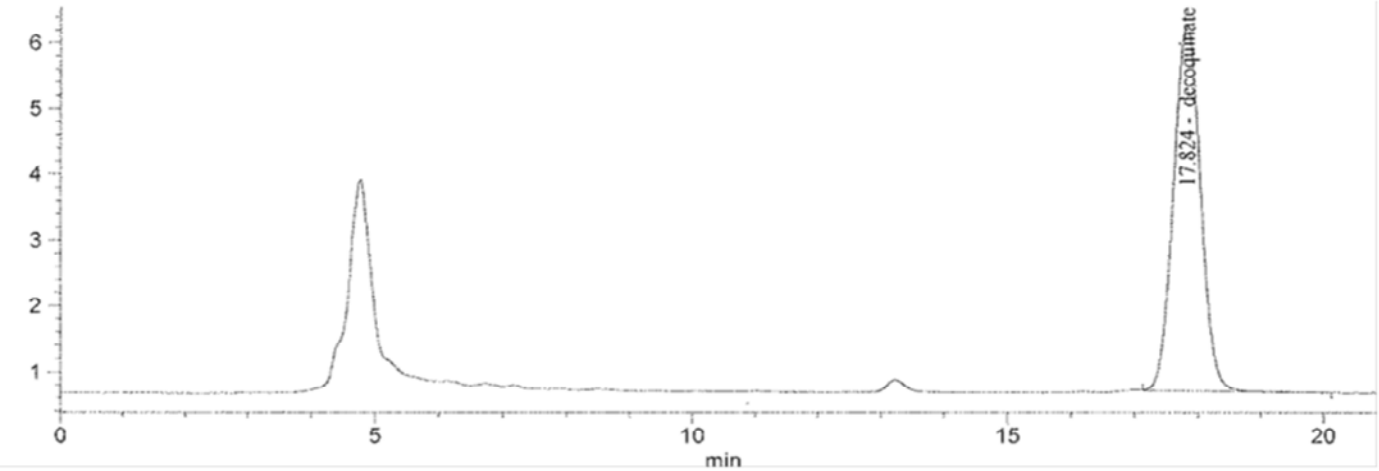


Figure D.2

D.4 Chromatogram example: same commercial Lamb feed, as in D.3, containing around 50 mg/kg of decoquinate

Confirmatory excitation wavelength at 260 nm

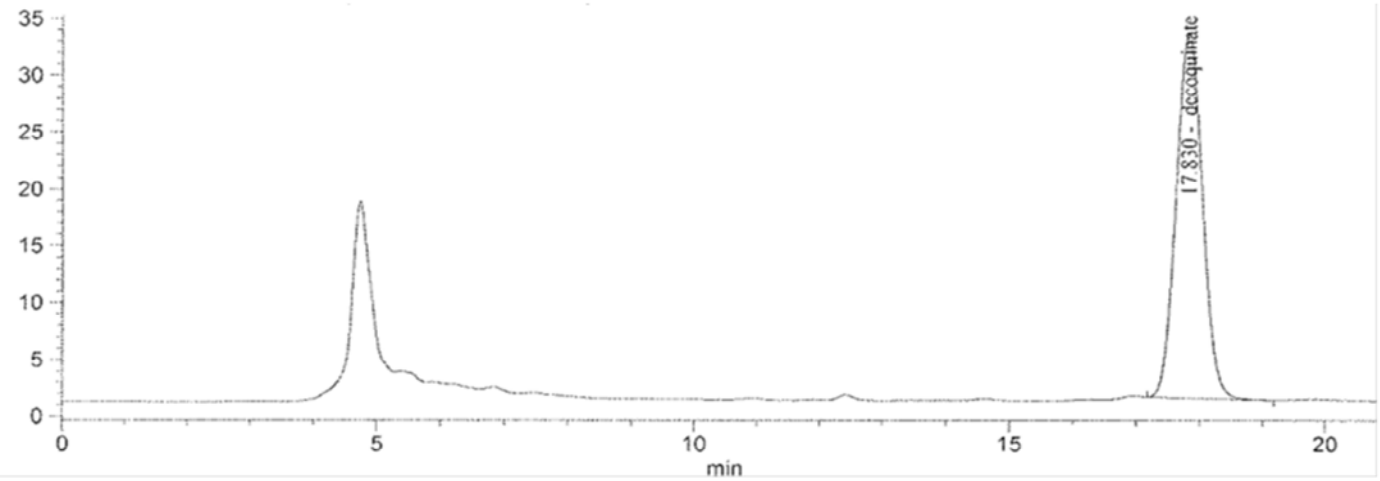


Figure D.3

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