EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

D08/FSQ/CVH/GS/D(2007)9556

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2006-134 FAD-2006-0023
Name of Additive:	Clinacox® 0.5%
Active Substance(s):	Diclazuril
Rapporteur Laboratory:	RIKILT – Institute of Food Safety, The Netherlands
Report prepared by:	Wim M.J. Beek Jacob de Jong
Report checked by:	Giuseppe Simone (CRL-FAA) 16/04/07
Report approved by:	Christoph von Holst (CRL-FAA)
Date:	20/04/07

EXECUTIVE SUMMARY

Clinacox® 0.5% is a feed additive authorised as coccidiostat according to Directive 70/527/EEC. Clinacox® 0.5% contains 0.5% w/w diclazuril as active substance. The proposed inclusion rate of the active substance in feedingstuff for chickens and turkeys for fattening is 1 mg/kg, corresponding to 200 mg Clinacox® 0.5% per kg feed. The recommended withdrawal period is 5 days.

In the current application a modification of the authorisation is sought for establishing Maximum Residue Levels (MRLs) of diclazuril in tissues of all poultry species after application of Clinacox® 0.5%. The proposed MRLs are: 3000 µg/kg in liver, 2000 µg/kg in kidney, 1000 µg/kg in skin with fat and 500 µg/kg in muscle.

Diclazuril is the established marker residue, after application of Clinacox® 0.5%.

For determination of the marker residue diclazuril in *animal tissues* several methods have been proposed by the applicant. The procedures are based on high performance liquid chromatography (HPLC), gas chromatography (GC) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In our opinion the LC-MS/MS procedure is satisfactory validated for chicken (broiler) tissues, but in principle also applicable to turkey tissues. The obtained validation results carried out at 100 µg/kg of diclazuril in tissue show a relative within-laboratory standard deviation for reproducibility ranging from 3.7 % to 8.3 % depending on the specific tissue (muscle, liver, kidney, fat/skin). The limit of quantification (LOQ) is 5 µg/kg and therefore well below the target MRLs. According to the validation results the LC-MS/MS method can be considered suitable as a quantitative method. The suitability as a confirmatory method for official control is not fully established, since the applied mass spectrometry parameters do not fulfil the identification criteria as established by Commission Decision 2002/657/EC. However, most probably the LC-MS/MS method can fulfil the required confirmation criteria for diclazuril in the frame of official control when using the confirmatory mass spectrometry conditions proposed by another but similar LC-MS/MS method (Mortier, L et al. (2005) Analytica Chimica Acta 529, 229-234), although this method was only described for poultry meat and not for liver, kidney and fat/skin.

Besides, in order to establish the suitability for official control, it is strongly recommended that the method will be checked by a second independent laboratory.

KEYWORDS

Clinacox® 0.5%, diclazuril, coccidiostat, feed additive, poultry tissues.

BACKGROUND

Clinacox® 0.5% is a feed additive authorised as coccidiostat according to Directive 70/527/EEC. The additive Clinacox 0.5% is a product in powder form, which contains diclazuril ($C_{17}H_9Cl_3N_4O_2$) as the active substance. The formulation is intended for use in broilers, replacement pullets and turkeys for fattening at a diclazuril content of 1 mg/kg feed.

In the current application a modification of the authorisation is sought for establishing Maximum Residue Limits (MRLs).

The applicant proposes the following MRLs for diclazuril in all poultry species [1], retaining parent diclazuril as the marker residue, which are 3000 µg/kg in liver, 2000 µg/kg in kidney, 1000 µg/kg in skin with fat and 500 µg/kg in muscle.

A dossier has been provided for the establishment of MRLs in poultry tissues. This evaluation report is specifically based on the information of the dossier Clinacox 0.5% submitted for authorisation. The evaluation concerns the tissue analytical methods included in Section IV "Studies concerning the safety of the additive" of the dossier.

TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with Clinacox® 0.5% dossier (EFSA-Q-2006-134) and their suitability to be used for official controls were evaluated.

EVALUATION

Description of the qualitative and quantitative analytical methods for determining the marker residue(s) of the active substance in target tissues and animal products.

The evaluation under this point follows the requirements listed in Commission Directive 2001/79/EC and Commission Decision 2002/657/EC [2].

A variety of residue assay methods have been developed and validated to analyse diclazuril residues in poultry tissues. Diclazuril has been identified as the marker residue. The analytical methods are evaluated below.

HPLC-UV method [3]: An HPLC-UV method is proposed for the routine monitoring of residues of diclazuril in tissues and plasma of chickens and rabbits.

Description of the method

Diclazuril (R64433: 2,6-dichloro-α-[4-chlorophenyl)-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin-2(3H)-yl)benzeneacetonitrile) and its internal standard (R 62370: 4-chloro-α-[2-chloro-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin-2(3H)-yl)phenyl]-α-3-(trifluoromethyl)benzeneacetonitrile) are extracted from the aqueous tissues homogenates by heptane-isoamyl alcohol or ethyl acetate. After evaporation of the crude extracts, further clean-up is accomplished by hexane/acetonitrile partition. The analytes are finally analyzed by reversed –phase HPLC with UV-monitoring at 278 nm.

Validation of the method

Accuracy and reproducibility: The accuracy and within-lab reproducibility of the HPLC method for the determination of diclazuril was tested in rabbit tissue, namely muscle, liver, and kidney.

The results are summarised in Table 1.

Table 1: Accuracy and within-lab reproducibility in rabbit tissues

Diclazuril added (µg/ml)	**Diclazuril found (µg/ml)**			**Mean ± S.D.**	**% C.V.**	**% R.E.**
	liver	**kidney**	**muscle**			
0.100	0.101	0.106	0.106	0.104 ± 0.003	2.8	+ 4.3
0.200	0.191	0.181	0.196	0.189 ± 0.008	4.0	- 5.3
0.400	0.386	0.376	0.399	0.387 ± 0.012	3.0	- 3.3
1.00	0.983	0.920	0.1.02	0.974 ± 0.051	5.2	- 2.6
2.00	1.98	1.95	1.98	1.97 ± 0.02	0.9	- 1.5
4.00	4.02	4.14	3.98	4.05 ± 0.08	2.1	+ 1.2
10.0	10.4	10.9	9.97	10.4 ± 0.5	4.5	+ 4.2
Whole range					3.2	± 3.2

C.V. = coefficient of variation R.E. = relative error from nominal value S.D = standard deviation

Limit of quantification (LOQ): The LOQ, defined as the concentration that can be determined with a C.V. and R.E. of less than 10%, was about 0.10 µg/g for tissues.

Summary and conclusions:

The developed procedure is able to determine residues of diclazuril in tissues (muscle, liver, kidney). The method has been validated for tissues of rabbits. The procedure proved to be not applicable to determine diclazuril in fat (of rabbits). Regarding validation acceptable results were found for accuracy, within-laboratory reproducibility and limit of quantification. Other validation parameters such as specificity, precision, limit of detection, susceptibility of interferences are not presented. The used spiking range of 0.1 – 10.0 µg/ml to rabbit tissues is not fully clarified and the corresponding concentration expressed as µg/kg is not given. In addition, validation data with respect to tissues of chicken and turkey have not been provided.

Consequently, it has to be concluded that the applicability of the LC-UV procedure to determine diclazuril in tissues of chicken and turkeys (muscle, kidney, liver, fat+skin) has not been demonstrated by experiments.

GC method [4], [5]: Gas chromatographic methods with thermionic detection are proposed for the determination of residues of diclazuril in chicken liver.

Description of the method

After addition of sodium phosphate buffer to liver, the homogenate is extracted with ethyl acetate. The ethyl acetate phase is evaporated and the residue is reconstituted in methanol. The methanol-phase is diluted with water, and further clean-up is done by solid phase extraction (SPE) on a SPE-C18 cartridge. After elution with methanol an internal standard (R 62646: 2,6 dichloro-α-[4-chlorophenyl)-4-(4,5-dihydro-3,5-dixox-1,2,4-triazin-2(3H)-yl- α – methylbenzeneacetonitrile) has been added to the eluate. The mixture is evaporated to dryness, and reconstituted in methanol. To this phase triethylamine and pentafluorobenzylbromide is added, heated (to derivatize) for 45 minutes at 55 ^{o}C, and analysed by gas chromatography on a capillary column (coated with DB-1301) and a thermionic detector.

Validation of the method

The method is linear from 25 to 500 µg/kg diclazuril in liver tissue. Average recovery for the method is 77.7%. The method has a precision (within-lab reproducibility, expressed as % coefficient of variation) of 13.74% in the sample matrix. The limit of detection was equivalent to 20 µg/kg. The method was found to be specific for quantitation of diclazuril in chicken liver and no interferences were observed.

Summary and conclusions:

The developed procedure is able to determine residues of diclazuril in chicken livers. The method seems to be applicable across the range 25 to 500 µg/kg. However, the underlying

data that proofs this applicability range has not been given and therefore the suitability of the method could not be evaluated.

HPLC-UV method [6]: An HPLC-UV method is proposed for the determination of residues of diclazuril in poultry liver.

Description of the method

After extraction with acetonitrile, the extract is defatted on a SPE C-18 cartridge. Other matrix components which may interfere with the HPLC analysis are washed from a second SPE C-18 cartridge with 40% tetrahydrofuran in water prior to elution of the diclazuril for analysis.

Validation of the method

The method is described and shown to be valid to quantify diclazuril in poultry liver (chicken and turkey) in the range of 45 – 1000 µg/kg. Average recovery is 88.2% for turkey liver, with a precision (within-laboratory reproducibility, expressed as % coefficient of variation) of 6.2%, and 84% for chicken liver, with a precision of 5.1%. Since no major differences were observed between turkey and chicken liver, the method had an overall recovery of 86.2% and a precision of 6.2% or better.

For both matrices, the limit of detection is 3 µg/kg and the limit of quantification is 6 µg/kg. No interferences (near the chromatographic retention time of diclazuril) have been observed from turkey and chicken liver samples obtained from eight different sources.

Summary and conclusions:

The developed procedure is able to determine residues of diclazuril in chicken and turkey livers. The method is applicable across the range 45 to 1000 µg/kg. The validation is supported with underlying summary tables.

LC-MS/MS method [7] : An LC-MS/MS method is proposed for the routine monitoring of residues of diclazuril in tissues of chickens.

Description of the method

Diclazuril and its internal standard (R 62370: 4-chloro-α-[2-chloro-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin-2(3H)-yl)phenyl]-α-3-(trifluoromethyl)benzeneacetonitrile) are extracted from tissue (muscle, liver, kidney, skin/fat) homogenate by ammonium hydroxide and phosphate-buffered-saline buffer. After centrifugation the supernatant is mixed with acetonitrile. The solution is centrifuged and the supernatant is transferred into a tube. Afterwards the sample is evaporated to dryness, and redissolved in reconstitution solution. An aliquot of this solution is injected into an LC-MS/MS system.

Diclazuril and the internal standard are measured using reverse phase HPLC with tandem mass spectrometric detection in negative ion mode, monitoring the ion transition of diclazuril (406.9>335.9 amu) and internal standard (452.9>354.9 amu). Diclazuril is quantified in the samples by comparing the peak area ratios calculated from the measured areas of the target analyte and the internal standard with the corresponding ratios obtained from calibration standards, using a weighted least square linear regression line.

Validation of the method

Specificity/susceptibility to interference: Specificity was examined by analyzing tissue samples (muscle, liver, kidney, skin/fat) without and with spiked diclazuril. The chromatograms showed that diclazuril could be detected separately from endogenous compounds.

Accuracy/Repeatability/Within laboratory reproducibility: Repeatability and accuracy was tested for each tissue type by analysis at four different concentrations (n = 6 for each concentration). The percentage relative standard deviation (RSD %) and the percentage relative deviation from the nominal value (RD %) were calculated. The results are summarised in Table 2.

Table 2: Accuracy and repeatability

Matrix	**Concentration added µg/kg**	**Mean measured concentration µg/kg**	**RSD (%)**	**RD (%)**
Muscle	500	485.1	3.5	-3.0
	100	97.4	2.5	-2.6
	10	10.5	8.9	5.0
	5	4.97	10.5	-0.6
Liver	800	834.1	7.7	4.3
	100	104.3	7.4	4.3
	10	10.5	9.0	5.0
	5	5.50	2.3	10.0
Kidney	800	788.4	5.1	-1.5
	100	102.3	4.1	2.3
	10	10.4	10.2	4.0
	5	4.69	8.3	-6.2
Skin/fat	500	456.1	4.0	-8.8
	100	95.3	10.1	-4.7
	10	10.4	7.1	4.0
	5	5.03	7.1	0.6

RSD = relative standard deviation (coefficient of variation)

RD = percentage relative deviation from nominal value

The within-laboratory reproducibility was determined for each tissue type at one concentration (n = 6 for quality control samples (QC-M)) at three different occasions. The coefficient of variation as well as the percentage relative deviation from the nominal value was calculated for each occasion and additionally over all three occasions. The results are summarised in Table 3.

Table 3: within laboratory reproducibility

Matrix	Concentration added µg/kg	Mean measured concentration µg/kg	RSD (%)	RD (%)
Muscle	100	95.7	3.7	-4.3
Liver	100	102.8	8.3	2.8
Kidney	100	101.0	6.6	1.0
Fat / skin	100	95.4	6.8	-4.6

RSD = relative standard deviation (coefficient of variation)

RD = percentage relative deviation from nominal value

Limit of detection (LOD): The LOD was determined for each tissue type by analysing 20 blank samples. The LOD was calculated by the standard deviation (SD) of response and the slope of the standard curve (S) according to the formula LOD=3.3 (SD/S) The LOD determined for each chicken tissue type was 0.177 µg/kg for muscle, 0.202 µg/kg for liver, 0.203 µg/kg for kidney and 0.285 µg/kg for fat/skin.

Limit of quantification (LOQ): A limit of quantification (LOQ) was defined as the lowest concentration of the standard curve, i.e. 5 µg/kg.

Assay linearity: The linear range is 5 to 500 µg diclazuril/kg for muscle and skin/fat, and 5 to 800 µg diclazuril/kg for liver and kidney. The correlation coefficient values found for these ranges are acceptable (>0.990).

Stability: The stability of diclazuril and the internal standard in stock solution has been guaranteed for up to 4 weeks when stored at 2 -8 ^{o}C and up to 6 h when stored at room temperature.

The stability of diclazuril in tissue was investigated using quality control samples of two different concentrations (muscle and fat/skin: 10 and 500 µg/kg; liver and kidney: 10 and 800 µg/kg) during storage at -20 ^{o}C for 5 weeks, for 6 h at room temperature and during three thaw/freeze cycles. Results showed that diclazuril is stable in muscle, liver, kidney, and fat/skin tissue samples during storage at -20 ^{o}C for at least 5 weeks, for up to 6 h at room temperature and after three thaw/freeze cycles.

Diclazuril is stable in the final extract up to 24 hours storage in the refrigerator and than 24 h during waiting time in the auto samplers.

Summary and conclusions:

A well described LC-MS/MS method for the determination of residues of diclazuril in chicken tissues has been proposed. The method is validated across a range of 5 – 500 µg/kg

(muscle and fat/skin) and 5 – 800 µg/kg (liver and kidney). The validations on accuracy, precision, applicability range and stability are acceptable and meet the demands of international recognised guidelines. The detection is based on mass spectrometry (MS/MS techniques) and is specific with regard to possible interferences.

The procedure is validated at concentration levels below or equal to the proposed MRLs. Nevertheless, to our opinion these results can also be found when validated across the range of 0.5 – 2xMRL as required in Commission Decision 2002/657/EC [2]. The procedure has not been validated for turkey tissues. Nevertheless, in our opinion the same validation results can be expected for turkey tissues. The method can be considered as suitable for use as a quantitative method. The method uses only one ion-transition (407>336) instead of the minimum required number for confirmation of two ion-transitions, according to Directive 2002/657/EC.

Another but similar LC-MS/MS method described by Mortier et al. [9] has been successfully tested for confirmatory purposes in poultry meat but not in poultry liver, kidney and fat/skin. Since the mass spectrometry detection is based on two transitions (407>336 and 405>334) thereby leading to four identification points, the method fulfils the identification criteria specified in Commission Decision 2002/657/EC [2]. The sample preparation selected by Mortier et al. [9] is only slightly different from the above described method. Therefore it is plausible that the proposed LC-MS/MS method [7] may also be regarded as suitable for confirmatory purposes when utilising the mass spectrometry parameters suggested by Mortier et al. [9].

GC-ECD method [8]: A GC-ECD method is proposed for the determination of residues of diclazuril in tissues and plasma of chickens.

Description of the method

Muscle, liver and kidney samples are homogenised with water (1:4 w/v) and spiked with internal standard (R 62646). Diclazuril and the internal standard are then extracted from tissue homogenate with ethyl acetate. The organic layer is evaporated under nitrogen at 60 ^{o}C, redissolved in acetonitrile-water. The aqueous layer is than washed with hexane and extracted with ethyl acetate. The organic layer is evaporated to dryness and submitted to a derivatisation procedure.

Fat/skin samples are homogenised in 0.1 M citric acid (1:9 w/v) and spiked with the same internal standard. Diclazuril and the internal standard are extracted from tissue homogenate with ethyl acetate. The organic layer is evaporated under nitrogen at 60 ^{o}C and redissolved in acetonitrile-water. The aqueous layer is than washed twice with hexane, followed by adding distilled water to the aqueous layer. The acetonitrile-water layer is finally extracted with ethyl acetate. The organic layer is evaporated to dryness and submitted to a derivatisation procedure.

Derivatisation: The dried extraction residues were dissolved in diazomethane solution, and allowed to react for 5 min. at room temperature and evaporated to dryness.

Gas chromatography: The methylated extracted residues are dissolved in 1 ml toluene and aliquots are directly injected into the gas chromatograph, equipped with a column packed with 3% OV-17 on 80-100 mesh Supelcoport.

Calibration: Standard curves are constructed by plotting, for each investigated tissue, the peak-area and peak-height ratios (diclazuril / internal standard) against the diclazuril standard concentrations. Linear regression analysis from these curves is used to determine the diclazuril residue concentration.

Validation of the method

Accuracy and reproducibility: The accuracy and between-assay reproducibility of the GC method for the determination of diclazuril was tested in plasma and tissue.
The results are summarised in Table 4.

Table 4: Accuracy and between-assay reproducibility in chicken tissues

Diclazuril added (µg/kg)	**Diclazuril found (µg/kg)**				**Mean ± S.D.**	**% C.V.**	**% R.E.**
	liver	**pectoral muscle**	**femoral muscle**	**fat/skin**			
10	10.9	10.6	10.9	10.2	10.5 ± 0.5	4.5	- 4.8
20	19.2	18.4	18.4	20.6	19.2 ± 0.9	4.7	- 4.1
50	47.1	51.0	48.3	47.0	49.4 ± 2.8	5.8	- 1.2
100	97.9	101	99.7	98.8	101 ± 3	3.2	+ 0.7
200	199	-	208	207	201 ± 9	4.4	+ 0.4
500	526	-	-	-	499 ± 39	7.8	- 0.3
Whole range						5.1	± 1.9

S.D = standard deviation C.V = coefficient of variation R.E. = relative error from nominal value

Limit of quantification (LOQ): The limit of quantification is not defined.

Summary and conclusions:

The GC procedure is able to determine residues of diclazuril in tissues (plasma, muscle, liver, kidney). The method has been validated for tissues of broilers. Acceptable validation results were found for accuracy and reproducibility, whereas other validation parameters such as specificity, precision, limit of detection, limit of quantification and susceptibility of interferences are not presented.

Consequently, it has to be concluded that the applicability of the GC procedure to determine diclazuril in tissues of chickens has not been fully demonstrated by experiments. The presence of interfering substances can not be excluded.

CONCLUSIONS AND RECOMMENDATIONS

Various HPLC and GC methods have been proposed for the determination of residues of diclazuril in tissues. The LC-MS/MS procedure [7] described by Globig et al (2006) is the most satisfactory one. The procedure has been validated to detect residues in muscle and fat/skin tissues at concentrations between 5 and 500 µg/kg and in liver and kidney tissues between 5 and 800 µg/kg. The procedure is validated at concentration levels below or equal to the proposed MRLs. Nevertheless, to our opinion these results can also be found when validated across the range of 0.5 – 2xMRL (according to Commission Directive 2002/657/EC concerning the performance of analytical methods and the interpretation of results). The method is validated for chicken (broiler) tissues. The procedure is not validated for turkey tissues. Nevertheless, in our opinion the same validation results can be expected for turkey tissues.

According to the validation results the method can be considered as suitable for use as quantitative method for the determination of diclazuril in tissues of broilers. The suitability as a confirmatory method for official control is not fully established. However, most probably the LC-MS/MS method can fulfil the criteria for confirmation for diclazuril as described in Directive 2002/657/EC when using the confirmatory conditions of Mortier et al. [9] although this method was only described for poultry meat and not for liver, kidney and fat/skin.

Besides, in order to establish the suitability for official control, it is strongly recommended that the method is checked by a second independent laboratory.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS method).

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of Clinacox® 0.5% have been sent to the Community Reference Laboratory for feed additives authorisation on FAD-2006-0023. The dossier has been made available to the CRL by EFSA.

REFERENCES

The dossier provided by the applicant is divided into various documents structured according to the Annex of Commission Directive 2001/79/EC, containing the following files:

[1] Annex III – Proposal of Register entry

[2] COMMISSION DECISION 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities L 221/8-36

[3] Technical dossier, Section IV, ref. 15. Woestenburghs R., Lorreyne W., Heykants J. (1988). Determination of diclazuril in plasma and animal tissues by high-performance liquid chromatography. Report number: V6865/1, Jansen Animal Health B.V.B.A.

[4] Technical dossier, Section IV, ref. 16. Corbin T.S. (1990). Determination of diclazuril in tissues by gas chromatographic analysis. Report numbers: V7467/1, Jansen Animal Health B.V.B.A.

[5] Technical dossier, Section IV, ref. 17. Corbin T.S. (1990). Determination of diclazuril in tissues by gas chromatographic analysis. Report numbers: V7468/1, Jansen Animal Health B.V.B.A.

[6] Technical dossier, Section IV, ref. 18. Pfotenhauer T.A., Iyer, K.S., McRoberts R.C.(1991). Description and Validation of an HPLC Method for Diclazuril in Poultry Liver. Report number: MR-4975, Pitman-Moore R&D Division.

[7] Technical dossier, Section IV, ref. 19. Globig S., Scheidel B., Schüssler C. (2006). Pre-study validation of determination of diclazuril in tissue samples (muscle, liver, kidney, skin/fat) of chicken. Harlan Bioservice Study-No.: 20-16-0175-05.

[8] Technical dossier, Section IV, ref. 20. Woestenborghs R. (1988). Validation of the analytical method for the determination of Diclazuril in broiler plasma and tissues. Report No.: V 6889/2 Janssen Research Foundation, R.

[9] Mortier, L., Daeseleire, E., Van Peteghem, C.. (2005). Determination of the coccidiostat diclazuril in poultry feed and meat by liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 529, 229-234.

RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was RIKILT – Institute of Food Safety, Wageningen, the Netherlands. Responsible persons of RIKILT for the evaluation are Jacob de Jong and Wim Beek.

ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Laboratori Agroalimentari, Department d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Spain.
- Thüringer Landesanstalt für Landwirtschaft , Jena, Germany
- National Veterinary Research Institute, Puławy, Poland
- Landwirtschaftliches Untersuchngs- und Forschungsanstalt (LUFA), Speyer, Germany