Projectnr.: 71.316.24
Development and Validation of HPLC-methods for the official control of Coccidiostatics and Antibiotics used as Feed AdditiveS (SMT4-CT98-2216)

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FINAL REPORT

Report 2002.007 December 2002

CANFAS - 2nd collaborative study for the determination of nicarbazin in a premixture by HPLC

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SUMMARY

This report describes the results of a 2nd collaborative study of an HPLC method for the coccidiostat nicarbazin in one premixture. The collaborative study forms part of the EU-project "Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS-SMT4-CT98-2216).

The results of the first collaborative study showed that the reproducibility of the method was not satisfactory for the premixture (the HORRAT ratio for the premixture was too high). During the evaluation meeting it was decided that a new small collaborative study had to be organised with a modified method in which the sample weight and the extraction volume are doubled compared to the original method. In the modified method the calibration method is also more strict described stating that the concentration of the premixture extract should be in the middle of the calibration curve.

The principle of both the original and the modified method is as follows: samples are extracted by heating in a waterbath, mechanical shaking and sonoration using an acetonitrile/methanol mixture. The mixture is transferred in a volumetric flask. After settlement of the solids, an aliquot is filtered and assayed using a reverse-phase isocratic method, which measures the 4,4'dinitrocarbanilide (DNC) moiety at a wavelength of 350 nm.

For the collaborative study 1 premixture with declared content of 0,75 % nicarbazin was prepared. The premixture was sent to the participants as a single sample. The participants were asked to analyse the premixture in duplicate.

Results were reported by 17 laboratories. Statistical evaluation was performed according to ISO 5725.

During the first collaborative study satisfactory results for recovery, blind blank feed and feedingstuffs were obtained. The results of the second collaborative study show that with the modified method for premixtures acceptable results are obtained for repeatability.

Statistical evaluation of the data with regard to reproducibility shows that it is very important to follow the method strictly. If only the results are taken into account of the laboratories that applied the method in sufficient detail, a Horrat ratio < 2 is obtained. It can be concluded that the reproducibility of the modified method is satisfactory, provided that it is followed strictly. The following points seem to be of special importance:

- Weight of the premixture (1 g)
- Range of the calibration curve (1 - 10 mg/kg)
- Quality (correlation coefficient) of the calibration curve

The final method can be recommended for adoption as an official method and, together with the results of the collaborative studies, it will be sent to the European Commission (CEMA), CEN and ISO.

1 INTRODUCTION

Within the framework of the EU-project "Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS-SMT4-CT98-2216), a method was developed for nicarbazin. Nicarbazin is a coccidiostat which is registered for broiler feeds with contents ranging between 40 - 50 mg/kg or 100 - 125 mg/kg.
The method was developed and validated by RIKILT, Wageningen, The Netherlands (see report H.J. Keukens, Development of Liquid Chromatographic Methods for the Determination of Nicarbazin in concentrates, premixtures and broiler feed, 01-11-1999). Subsequently, the method for feeds and premixtures was subjected to between-lab validation by the Universität Hamburg, Institut für Angewandte Botanik, Germany (see report H.-A. Putzka, 17-01-2000) and Istituto Superiore di Sanità, Roma, Italy (see report G. Brambilla, 26-01-2000) with satisfactory results (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).
Prior to the first collaborative study, a kick-off meeting was organised (Brussels, 13-14/6/2000) and participating laboratories were given the opportunity to familiarise themselves with the method, using feed samples with stated contents of nicarbazin. Also prior to the production of the materials for the collaborative study, separate batches of the materials were produced for stability testing, indicating that nicarbazin is stable at room temperature for 4 months.
The results of the first collaborative study (see report "CANFAS - Collaborative study for the determination of nicarbazin in feedingstuffs and premixtures by HPLC", J.J.M. Driessen, Y.P. van Adrichem, M.J.H. Tomassen and J. de Jong, RIKILT-Report 2002.012) showed that the reproducibility of the method was not satisfactory for the premixture (the HORRAT ratio for the premixture was too high). During the evaluation meeting it was decided that a new small collaborative study had to be organised with a modified method.
The modifications of the method are:

- the sample weight is increased to 1 gram (double compared to original method).
- the extraction volume is increased to 200 ml (double compared to original method).
- the calibration method is more strictly described, stating that the concentration of the premixture extract should be in the middle of the calibration curve
- excessive dilution is avoided (higher concentrations of calibration curve)

The handling of the premixture prior to the subsampling (mixing) is described in the method.
For the collaborative study 1 premixture with declared content of 0,75 % nicarbazin was prepared. The premixture was sent to the participants as a single sample. The participants were asked to analyse the premixture in duplicate.
Before the sample was shipped, the between-sample homogeneity was checked with satisfactory results (see par. 3.1.2).
Apart from the samples, a letter with instructions, reporting forms, etc. was sent to the participants (see Appendix 1).
This report describes the results of the collaborative study.

2 PARTICIPANTS

The following laboratories/persons participated in the collaborative study.

- CCL-Nutricontrol, Veghel, The Netherlands; R. Margry, J.G.P. van der Palen
- IEEB, Bordeaux, France; J.P. Antalick, T. Gron
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, C. Saldanha
- Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy; G. Brambilla, C. Cartoni, M. Fiori.
- Istituto Zooprofilattico Sperimentale della Lombardia e dellémilia Ronagna, Reparto Chimico, Brescia, Italy; E. Faggionato, A. Baiguera
- Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy; C. Testa, N. Rubattu, A. Serra
- Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy; G. Biancotto, B. Allegretta
- Istituto Zooprofillatico Sperimentale delle regioni Lazio e Toscana, Roma, Italy; A. Ubaldi, A. di Lullo.
- Laboratorio Nacional de Sanidad y Produccion Animal - M.A.P.A., Santa Fe, Spain; R. Checa-Moreno, A. Ariza-Avidad
- Laboratory of the Government Chemist, Teddington, United Kingdom; J. Cowles
- LUFA-ITL Kiel, Kiel, Germany; H. Wehage, H. Graepel
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf
- Pre-Mervo Kwaliteitsdienst, Utrecht, The Netherlands; C. Schreuder, C.J.J. van Wijk
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Lekens, R. van Sandt.
- RIKILT, Wageningen, The Netherlands; H.J. van der Kamp, H.C.H. Kleijnen
- Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany; A. Thalmann, K. Wagner
- Universität Hohenheim, Landesanstalt für Landwirtschaftliche Chemie, Stuttgart, Germany; K. Schwadorf, A. Eschle

3 MATERIALS

3.1 Samples for collaborative study

3.1.1 Sample composition

Specifications of the premixture, which was produced for the collaborative study, are given in Table 1.

Table 1: Specifications of the samples

Type of feed / premixture	Declared content	Unit	Subcontractor	Date of production
Premixture for broiler feed	0.75	%	Trouw Nutrition, Putten (NL)	September 2001

The premixture was based on inorganic support material and contained regular contents of vitamins, minerals and trace elements. The composition of the premixture is enclosed in Appendix 2.
The composition of the premixture was the same as the product used in the first collaborative trial and in the stability trial (see Report on homogeneity and stability studies of samples for the collaborative studies for nicarbazin, J.J.M. Driessen and J. de Jong, RIKILT, Wageningen, NL, 12/10/2000).

The premixture was prepared in a quantity of about 3 kg by Trouw Nutrition, Putten, the Netherlands. TNO-Voeding, Zeist, the Netherlands, performed the subsampling with an automatic sample device that resulted in about 30 PE bottles containing about 100 g of premixture each. The bottles were stored at room temperature prior to forwarding them to the participants.

3.1.2 Sample homogeneity

The homogeneity of the samples was studied by RIKILT by random selection of 10 subsamples, applying the HPLC-method developed in CANFAS (see Annex 1 of Appendix 1).
The results of the homogeneity determinations of the premixture are attached in Appendix 2. Table 2 gives a summary of these results.

Table 2: Results of homogeneity tests for nicarbazin in the premixture

Results / Product	Declared content (%)	Measured content (%)	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Premixture	0,75	0,73	6,2	5,2

According to the Project Plan the CV's for homogeneity should not exceed 2 times the CV's for repeatability ($CV_{hom} \leq 2\ CV_r$). Based on previous results of within-lab validation (see Second Annual Report CANFAS, J. de Jong, 12-08-2000) the maximum limit for CV_{hom} was set to 7 % for the premixture.
The between- and within-sample CV's fulfil this requirement. Thus, it is concluded that the samples are sufficiently homogeneous.

3.1.3 Sample logistics

The sample codes are given in Appendix 3. The premixture was sent as a single sample and was labelled as such. The samples were sent to the participants by courier service on 13 December 2001 together with a letter with instructions (Appendix 1). During transport no special precautions were taken with regards to the temperature of the samples.

3.2 Reference standard

The reference standard was supplied by Mr. S. Ready, Eli Lilly, Liverpool (UK). The specifications of the reference standard (Lot Nr. X47623) are described in Appendix 4. The participants were instructed by e-mail to set the purity of the reference standard at 100 % (See appendix 1)

4 METHODS

4.1 Method of analysis

The method of analysis is included as annex 1 to Appendix 1. The participants were instructed to use this method without any modifications. A mistake was discovered in the formula for calculation of the nicarbazin content in premixtures. The participants were informed about the mistake and the correct formula was send to them by e-mail (see Appendix 1).

4.1.1. HPLC-conditions

Various types of HPLC-columns were used (the column which is recommended in the method is a Nova-Pak C-18 300 x 3,9 mm with a particle size between 5 and 10 µm).
The mobile phase described in the method is Acetonitrile-Water 65/35. Two laboratories used a different mobile phase.
The HPLC conditions (Column and mobile phase) used by the participants are shown in Table 3.

4.2 Method for statistical evaluation

Statistical evaluation was performed according to ISO 5725 Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (First edition, 1994-12-15).
The scrutinity of results for consistency and outliers was checked by

a) Graphical consistency techniques: Mandel's h plot for between-laboratory variability, Mandel's k plot for within-laboratory variability
b) Numerical outlier techniques: Cochran's test of the within-laboratory variability, Grubbs' test (single and double) for between-laboratory variability

Whenever necessary and appropriate, laboratories which showed consistently high within-cell variation and/or extreme cell means across many levels and/or Cochran or Grubbs' outliers were contacted to try to ascertain the cause of the discrepant behaviour.
The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility of the method. The HORRAT ratios are given in Table 5. The HORRAT ratios should be lower than 2 (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744).

Table 3: HPLC-conditions

Partner	Column	Mobile phase
13	Not reported	Not reported
15	Inertsil ODS-2; 5 µm; 250 mm	As described in the method
16	Waters Spherisorb ODS 1; 5 µm; 250 x 4 mm	As described in the method
19	Chromspher C18; 200 mm x 3,0 mm	As described in the method
20	Alltima C18; 5 µm; 250 x 4,6 mm	As described in the method
21	Supelcosil LC18; 5 µm; 250x4,6 mm with Supelguard LC18 (20x4,6 mm)	Acetonitrile - ammonium acetate buffer (0,01 M, pH 4,6) Gradient elution
23	Not reported	Not reported
24	Waters C18; 5 µm; 250 mm x 4,6 mm	As described in the method
25	Lichrocart Lichrospher 100 RP18; 5 µm; 250 mm	Acetonitril 80 % /water = 70:30 (v/v)
26	Luna C18 (2); 5 µm; 250 x 4,6 mm	As described in the method
29	Nova Pak C18; 4µm; 250 x 4,6 mm	As described in the method
30	Kromasil; 150x4,6 mm	As described in the method
31	Bondapak C18; 300mm x 3,9 mm	As described in the method
33	As described in the method	As described in the method
35	Merck C18; 5 µm; 250 mm	As described in the method
38	Lichrospher; 5 µm; 250 x 4,6 mm	As described in the method
39	Lichrospher RP18; 5 µm; 125 x 3 mm (Merck 51232)	As described in the method

5 RESULTS

For each participant the reported results for the samples, the completed questionnaire and representative chromatograms are annexed in Appendix 5.

5.1 Statistical evaluation

The results reported by the participants are given in Table 4. Figure 1 demonstrates the Mandel h and k plots of these results.
According to the Project Plan, the rsd_r-values should be ≤ 10 %. The rsd_r value is 5,0 % and consequently it can be concluded that the repeatability is satisfactory.
The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744). The HORRAT ratio is given in Table 5. The HORRAT ratio should be lower than 2. A HORRAT ratio of 3,21 is obtained.
In the evaluation meeting it was concluded that it should be possible to obtain a rsd_R (reproducibility) of approximately 7 %. The rsd_R obtained is 13,4 %.
In order to exclude that the high rsd_R and HORRAT ratio is caused by the fact that some laboratories did not strictly follow the method all the participants were asked to send the following information:

- Weight, extraction volume and additional dilution factor for the premix
- Areas/heights and concentration of the premix sample extract
- Preparation of the stock standard solution(s), incl. weight and volume
- Characteristics of the calibration curves, incl. areas / heights, correlation coefficients, etc.

Laboratory 23 responded that they could not recover their raw data. A summary of the raw data of the other labs is given in Table 6.
The results of the evaluation of the information from the laboratories is as follows:

- Lab 13 weighed 0,5 g of the premixture and extracted with 100 ml extraction solvent. While the increase of the weight from 0,5 to 1 g was an important issue in the improvement of the method (see chapter 1, Introduction), the results of lab 13 cannot be taken into account.
- Lab 15 followed the method in sufficient detail.
- Lab 16 diluted the extracts 20 times and measured concentrations in the final extracts of 1,675 and 1,7605 µg/ml. Although this concentration is rather low compared to what is described in the procedure (ca. 5 µg/ml, see par. 6.2.1 of the procedure), the results can be taken into account because of the perfect linearity of the calibration curve, also in the lower range.
- Lab 19 prepared a more concentrated stock standard solution, containing 296,8 µg/ml nicarbazin, instead of a stock standard solution of 100 µg/ml (see par. 6.4.1.1 of the procedure) and did not check the solubility according to par. 9.2 of the procedure. While the dissolution of the nicarbazin is a very critical step in the method, the test on solubility should have been performed to give proper evidence of dissolution. Moreover, this lab also applied a 1-point calibration. Consequently, the results cannot be taken into account.

- Lab 20 dissolved the stock standard solution in DMF instead of acetonitrile - methanol (500 + 500 V / V). This procedure was also followed by lab 20 in the first collaborative study and at that occasion it was shown that the use of DMF leads to acceptable results (see report "CANFAS - Collaborative study for the determination of nicarbazin in feedingstuffs and premixtures by HPLC", J.J.M. Driessen, Y.P. van Adrichem, M.J.H. Tomassen and J. de Jong, RIKILT-Report 2002.012). Consequently, the results of lab 20 can be taken into account.
- Lab 21 applied two different dilution steps for the 2 duplicates, viz. 1 : 25 and 1 : 5, leading to concentrations in the final extracts of 1,48 and 7,45 µg/ml resp. Both results can be taken into account because of the perfect linearity of the calibration curve, also in the lower range.
- Lab 24 followed the method in sufficient detail.
- Lab 25 followed the method in sufficient detail.
- Lab 26 followed the method in sufficient detail.
- Lab 29 applied a dilution of a factor 50, leading to concentrations in the final extracts of 1,030 and 1,005 µg/ml. Moreover, the correlation coefficient of the calibration curve of this lab (0,98035) was unacceptably low. For these reasons, the results of lab 29 cannot be taken into account.
- Lab 30, like lab 19, prepared a more concentrated stock standard solution of 207 µg/ml. This lab was contacted and asked about the results of the check on solubility, see par. 9.2 of the procedure. Lab 30 replied that they had not performed the check on solubility but that they did not expect any problems because they had applied heating and sonoration and had not observed any problems. They also argued that the final concentration is lower than in the extract of a premixture containing 2,5 % nicarbazin. However, while the dissolution of the nicarbazin is a very critical step in the method, the test on solubility should have been performed to give proper evidence of dissolution and so the results cannot be taken into account.
- Lab 31 followed the method in sufficient detail.
- Lab 33 weighed 2 g of the premixture and extracted with 100 ml extraction solvent. According to the procedure 1 g premixture should be extracted with 200 ml extraction solvent (see par. 6.2.1). It is not clear whether the method can be successfully applied when the ratio of premixture to extraction volume is increased 4-fold. There is a clear risk of incomplete extraction and so, the results of lab 33 cannot be taken into account.
- Lab 35 diluted the extracts 25 times and measured concentrations in the final extracts of 1,51 and 1,39 µg/ml. This lab prepared a 3-point calibration curve (0, 4,36 and 10,90 µg/ml). While the calibration curve has a perfect linearity, the results can be taken into account.
- Lab 38 weighed 0,5 g of the premixture and extracted with 100 ml extraction solvent. Besides, lab 38 used a calibration curve of 0,5 – 5,4 µg/ml (in stead of 1 - 10 µg/ml, see par. 6.4.1.2 of the procedure) as was used for the first collaborative study. While the increase of the weight from 0,5 to 1 g and the increase of the concentrations of the calibration solutions were important issues in the improvement of the method (see chapter 1, Introduction) the results of lab 38 cannot be taken into account.

- Lab 39 diluted the extracts 25 times and measured concentrations in the final extracts of 1,691 and 1,551 µg/ml. The results can be taken into account because of the perfect linearity of the calibration curve, also in the lower range.

According to the evaluation of the raw data, the results of 10 laboratories can be taken into account. The results of the statistical evaluation are given in Table 7. Figure 2 shows the new Mandel h and k plots.
The repeatability (rsd_r = 5,7 %) is satisfactory. The new value for the rsd_R is 8,6 % with a corresponding HORRAT ratio of 2,04 %. The rsd_R includes a contribution from the between-sample heterogeneity (see Table 2). This contribution S(hetero) can be calculated by the following formula:

$$S^2 \text{ (hetero)} = 0{,}5 \times (S^2(\text{between-sample})_{hom} - S^2(\text{within-sample})_{hom})$$

Correction of the rsd_R for the between-sample heterogeneity by means of the following formula

$$S^2_{R,\ corrected} = S^2_{R,\ uncorrected} - S^2(\text{hetero})$$

yields a final value for the rsd_R (corrected) of 8,2 % with a corresponding HORRAT ratio of 1,95 (see Table 8). Consequently it can be concluded that the reproducibility of the modified method is satisfactory.
The improvement of the reproducibility clearly shows that it is important to apply the new method strictly. Especially the following points seem to be very critical:
- Weight of 1 g of the premixture (in 200 ml) instead of 0,5 g (in 100 ml)
- Shift of the calibration curve and sample extract to higher concentrations (1-10 µg/ml instead of 1-5 µg/ml)
- Quality of the calibration curve.

On the other hand the preparation of the stock standard solution seems to be less critical because statistical evaluation of the results of 12 labs (labs 19 and 30 that prepared a more concentrated stock standard solution and the 10 labs that applied the method in sufficient detail) yields slightly better results for repeatability (rsd_r = 5,6 %) and reproducibility (rsd_R = 7,9 %, not corrected for between-sample homogeneity) than for 10 labs. However, no final conclusions can be drawn about this point. While the preparation of the stock standard solution has been identified as a critical factor in previous parts of the project, the results of labs 19 and 30 should not be taken into account.

Table 4: Nicarbazin in a premixture for broiler feed, results of all participants

	Result (mg/kg)	
Lab	NIC 7500 mg/kg	
13	8829	9148
15	6908	8317
16	6706	6572
19	7043	7350
20	7534	7554
21	7450	7500
23	7300	7100
24	6600	7000
25	7950	7950
26	6387	6416
29	10050	10300
30	7580	6960
31	7740	7235
33	8100	8700
35	7486	6768
38	6441	5886
39	8430	7664

Summary of all results

Number of all participating labs	17
m (mg/kg)	7557
rsd_r (%)	5,0
rsd_R (%)	13,4

Table 5: Horrat ratios of the Nicarbazin collaborative study (results of all participants)

Mean (mg/kg)	Predicted rsd_R	Established rsd_R	Horrat[1]	Conclusion
7557	4,17	13,4	3,21	Reproducibility NOT OK

[1] = Horrat is the ratio between the established rsd_R and the predicted rsd_R

Table 6: Overview of the raw data supplied by the participants of the collaborative study

	Sample preparation			Premix sample extract		Stock standard and standard solutions					
Partner	Weight premix (g)	Extraction volume	Additional dilution factor	Height/area Sample	Concentration Sample (µg/ml)	Stock	Range standards	Height/area standards	R^2	Calculation[1]	Conclusion method compliance
13	**0,5**	**100**	10	1,6535 1,7133	± 4,4 (C) ± 4,6 (C)	10,0 mg in 100 ml	1-2-3-4-5-10	0,3463 – 0,7997 – 1,1178 – 1,4995 – 1,8727 – 3,7658	0,9996 (C)	OK	**Not OK**
15	1,000 1,000	200 200	10 10	196,0 235,8	3,454 4,159	10,07 mg in 100 ml	1-2-3-4-5-10	58 – 116 – 168 – 227 – 285 - 565	0,9999	OK	OK
16	0,9991 1,0716	200 200	20 20	140834 150182	1,675 1,7605	11,16 mg in 100 ml	1,16 - 3,48 – 5,80 -11,60	95587 - 290936 - 488782 - 979283	0,9999	OK	OK
19	1,2865 1,2550	250 250	5 5	1680999 1711655	7,25 7,38	**29,68** mg in **100** ml	**8,904**	**2065016**	Not applicable	OK	**Not OK**
20	1,0077 1,0178	200 200	5 5	737,6 756,9	7,59 7,69	10,3 mg in 10 ml DMF	1 - 2 - 3 - 4 - 5 - 10	107 - 204 - 303 -403 - 488 - 1010	0,9997	OK	OK
21	1,00 1,00	200 200	5 25	608,6 120,6	7,45 1,48	10,0 mg in 100 ml	1 – 2 - 4 – 5 - 10	81,09 – 162,44 – 326,92 – 409,23 – 816,32	1,000	OK	OK
24	1	200	10	800,9 860,8	3,3 3,5	11,6 mg in 100 ml	1,16 - 2,32 - 5,80 - 11,6	277 – 554 - 1425 - 2852	0,9999	OK	OK

[1] = calculation of the results by means of the right formula

(C) = calculated by authors

	Sample preparation			Premix sample extract		Stock standard and standard solutions					
Partner	Weight premix (g)	Extraction volume	Additional dilution factor	Height/area Sample	Concentration Sample (µg/ml)	Stock	Range standards	Height/area standards	R^2	Calculation[1]	Conclusion method compliance
25	1	200	5	171065 181287 171699 180875	7,73 8,18 7,75 8,16	10 mg in 100 ml	2,5 – 5,0 – 7,5 – 10,0	5309 – 112061 – 164821 - 221866	0,9998	OK	OK
26	1,076 1,127	200 200	10 10	274072 277847	3,5123 3,5612	9,9 mg in 100 ml	0,99 - 1,98 - 2,97 - 3,96 - 4,95 - 9,90	77754 - 154745 - 228425 - 314811 - 386860 - 765993	0,9998	OK	OK
29	1,0	200	50	11403 10937	**1,030** **1,005**	Not reported	0,5 – 1 – 2 – 3 – 5	6450 – 11592 – 23414 – 44948 - 89828	**0,9804**	OK	**Not OK**
30	0,9806 1,1165	200 200	5 5	617 765	6,82 (C) 8,46 (C)	**20,7** mg in 100 ml	1,0 - 2,07 - 4,14 - 5,18 - 10,35	98 - 189 - 380 - 474 - 929	0,9999	OK	**Not OK**
31	1,00	200	5	653621 610916	7,74 (C) 7,23 (C)	9,98 mg in 100 ml	0,998 - 1,996 - 2,994 - 3,992 - 4,990 - 9,980	82472 – 168898 – 255014 – 336999 – 423332 - 847853	0,9999 (C)	OK	OK
33	**2,0**	**100**	20	254968 277008	?	No data	Conform method, no data	10: 318643	0,9999	?	**Not OK**
35	1,0065 1,0278	200	25	144650 133508	1,51 1,39	11 mg in 100 ml	0 - 4,36 – 10,90	0 – 421600 - 1044799	0,9999	OK	OK
38	**0,5031** **0,5023**	**100** **100**	25 25	5,27 4,77	1,30 1,18	**5,42 mg in 50 ml**	**0,54 - 1,08 – 2,16 – 3,24 - 5,40**	2,2, - 4,6 - 8,6 - 13 - 22,5	0,9987	OK	**Not OK**
39	1	200	25	391249 259101	1,691 1,551	10,7 mg in 100 ml	1,07 - 2,14 - 3,21 - 4,28 - 5,25 - 10,7	248862 - 491924 - 740078 - 984405 - 1230148 - 2453851	1,0000	OK	OK

[1] = calculation of the results by means of the right formula

(C) = calculated by authors

Table 7: Nicarbazin in a premixture for broiler feed; results after elimination of the laboratories that did not follow the method in sufficient detail or did not send raw data.

	Result (mg/kg)	
Lab	NIC 7500 mg/kg	
15	6908	8317
16	6706	6572
20	7534	7554
21	7450	7500
24	6600	7000
25	7950	7950
26	6387	6416
31	7740	7235
35	7486	6768
39	8430	7664

Summary of all valid results

Number of valid labs*	10
M (mg/kg)	7308
rsd_r (%)	5,7
rsd_R (%)	8,6

* Laboratories 13, 19, 29, 30, 33 and 38 did not follow the method in sufficient detail; lab 23 did not send raw data. Results of these laboratories are not taken into account.

Table 8: Horrat ratios of the Nicarbazin collaborative study after elimination of laboratories that did not follow the method in sufficient detail and the lab that did not send raw data

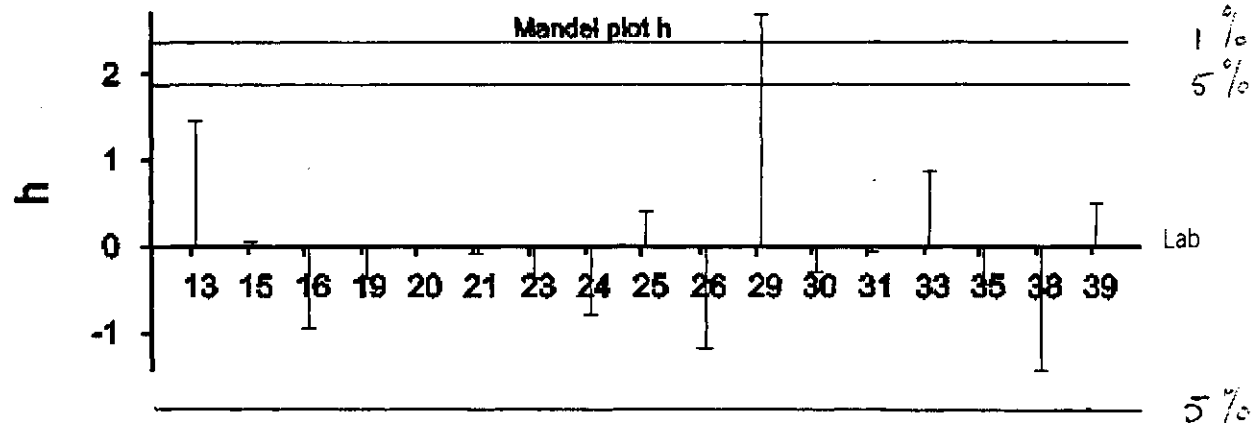
Mean [1] (mg/kg)	Predicted rsd_R	Established [2] rsd_R	Horrat[3]	Conclusion
7308	4,193	8,2	1,95	Reproducibility OK

[1] = Laboratories 13, 19, 29, 30, 33 and 38 did not follow the method in sufficient detail; lab 23 did not send raw data. Results of these laboratories (italic figures) are not taken into account.

[2] = Corrected for the contribution of between-sample heterogeneity

[3] = Horrat is the ratio between the established rsd_R and the predicted rsd_R

Figure 1: Mandel h and k plots of the results reported by the participants (all results)



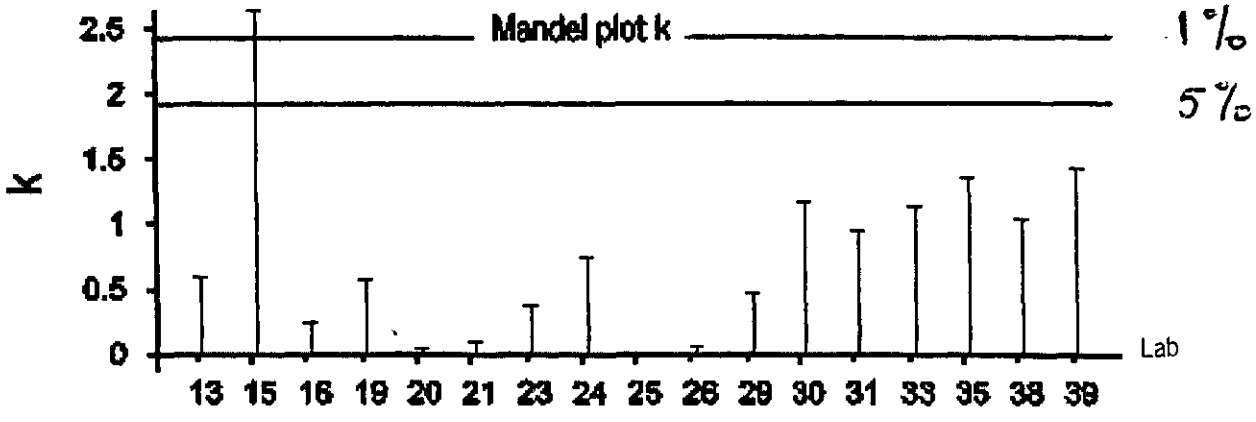
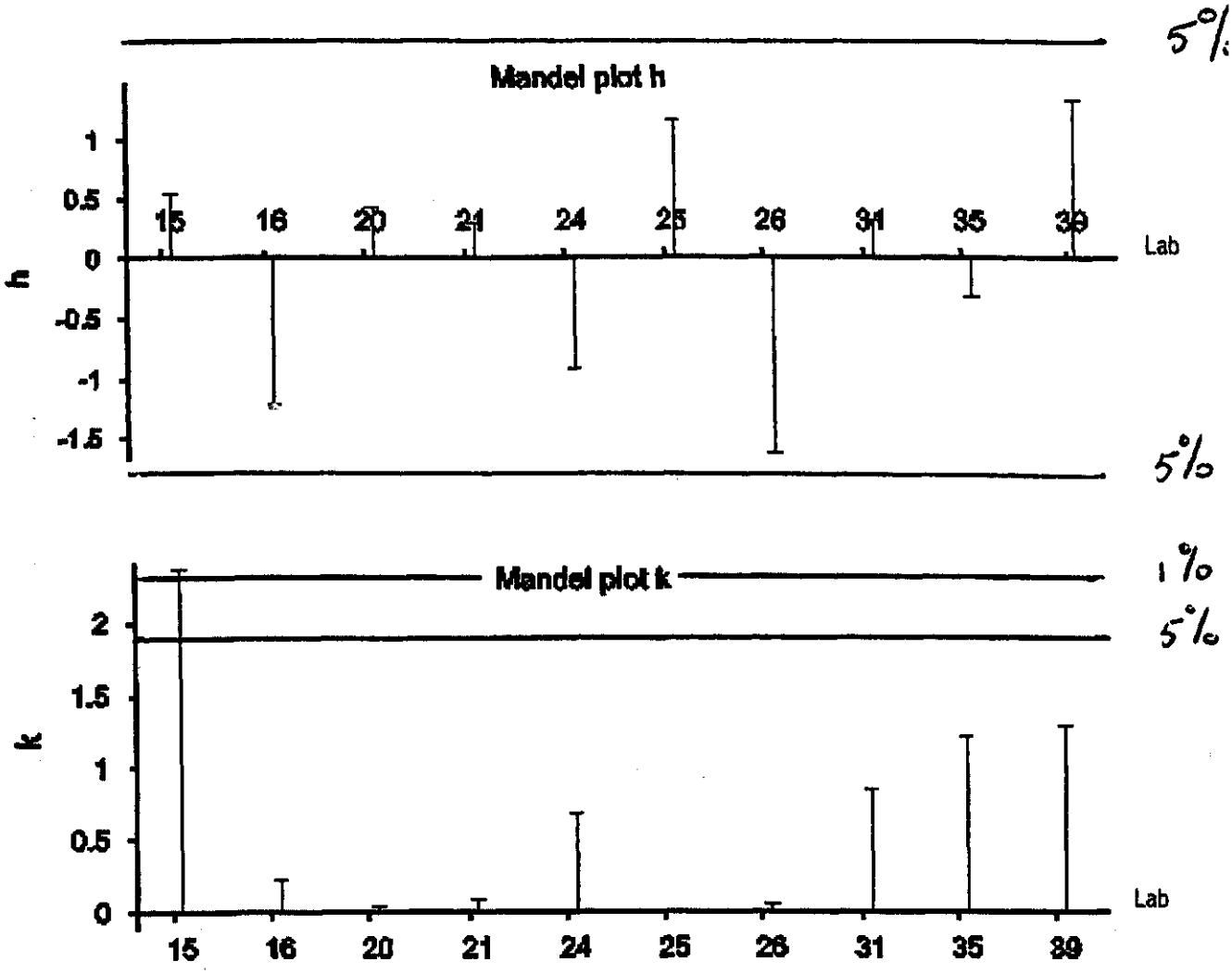


Figure 2: Mandel h and k plots of the results reported by the participants after elimination of laboratories 13, 19, 29, 30, 33 and 38 that did not follow the method in sufficient detail and lab 23 that did not send raw data



5.2 Recoveries

Table 9: Recoveries

Partner	Spiking level (mg/kg)	Recovery 1 in %	recovery 2 in %	recovery average in %
13	Not reported	100		100
15	100	98	98	98
16	116	99	100	100
19	75	93		93
20	200	100	100	100
21	100	98	100	99
23	Not reported	Not reported		
24	100	96	108	102
25	2,5 - 5 - 7,5 - 10 - 12,5	85	85	85
26	100	98		98
29	100	101	101	101
30	Not reported	Not reported		
31	100	97		97
33	125	101		101
35	20000	100	101	101
38	86,4	89	91	90
39	25000	101	102	102

Mostly, recoveries were close to 100 %. The lowest recovery reported was 85 % (lab 25). The results correspond to the results of within- and between-lab validation of the method (task 1 and 2 of the project) where recoveries >85 % were measured.

5.3 Remarks

Table 10: Remarks made by the partners

Partner	Remarks
13	Not reported
15	No remarks
16	No remarks
19	Weight of sample was 1,25 g in 250 ml extraction solution
20	Nicarbazin stock solution in DMF
21	No remarks
23	Not reported
24	No remarks
25	No remarks
26	We experienced no problems with the method. The extract of the premix was diluted 10 and 20 times, because the level was unknown and to try and ensure that we did not have to repeat the work because the response was outside the range of the top calibrant standard.
29	We used a folded filter to filter the whole extract and then proceeded the microfiltration step.
30	No remarks
31	I think the use and the purity of the reference standard has to be mentioned in the method. Nicarbazin is analysed as dinitrocarbanilide (DNC). On the reference standard profile (Ely Lilly) is mentioned "69,9 % DNC", while in practice nicarbazin is calculated following the method. We calculated with a purity of 100 %, because it is assumed that nicarbazin is added to the sample with the same purity as the reference standard.
33	No remarks
35	No remarks
38	No remarks
39	No remarks

6 CONCLUSIONS

The results of the second collaborative study show that with the modified method for premixtures acceptable results are obtained for repeatability (rsdr). Evaluation of the experimental conditions applied by the laboratories showed that a number of laboratories did not follow the method in sufficient detail. Statistical evaluation of the data shows that this is very important: if the results of all laboratories are taken into account the reproducibility is unsatisfactory (rsd_R of 13,4 %; Horrat ratio of 3,21). If only the results are taken into account of the laboratories that applied the method in sufficient detail, an rsd_R of 8,2 % and a Horrat ratio of 1,95 is obtained.
The draft report, containing the evaluation of which laboratories applied the method in sufficient detail, was sent to the participants and the scientific officer for comments. No comments were received before the deadline of one month.
Consequently, it can be concluded that the reproducibility of the modified method is satisfactory and that the modified method is suitable for premixtures, provided that it is followed strictly. The following points seem to be of special importance:

- Weight of the premixture (1 g)
- Range of the calibration curve (1 - 10 mg/kg)
- Quality (correlation coefficient) of the calibration curve

From the results of the first collaborative study it was already concluded that the repeatability and reproducibility of the method for feedingstuffs was acceptable. The results obtained for the recovery and for the blind blank samples were also satisfactory.
The final method can be recommended for adoption as an official method and together with the results of the collaborative studies it will be sent to the European Commission (CEMA), CEN and ISO.

ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Eli Lilly and Company, Mr. S. Ready, is thanked for supplying the nicarbazin reference standard.
Dr. H. van de Voet, Biometris, Wageningen University and Research Centre, is thanked for statistical advice.

APPENDIX 1

Letter with instructions, sent with the samples (with four annexes) and e-mails with additional information

cc. Jong, Driessen

Participants CANFAS collaborative study Nicarbazin

DATE
13 December 2001

SUBJECT
CANFAS collaborative
nicarbazin (71316.24)

ENCLOSURE(S)
4

OUR REFERENCE
01/0030595

HANDLED BY
Ing. J.J.M. Driessen

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E-MAIL
j.j.m.driessen@rikilt.w

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CHAMBER OF COMMERCE REGISTR
09098104 te Arnhem

THE INTERNET
www.rikilt.wageningen

Dear colleague,

As agreed at the CANFAS evaluation meeting June 19th, 2001 at Tervuren a second round of collaborative study for nicarbazin in a premixture has to be organised. We appreciate your willingness to participate very much. Together with this letter you will find:

- 1 sample of premixture labeled with the text "additive: NICARBAZIN" and with a sample code. The sample contains nicarbazin in the range between 0.5 and 3%.
- the modified method of analysis (annex 1). By participation you agree with application of this method!
- the reporting form (annex 2). This form will also be send to you by E-mail as an Excel 5.0 file. We strongly prefer to get the results back in electronic form by E-mail; you are asked to use the e-mail address mentioned in the right margin of this letter.
- instructions for handling (storage) of the samples (annex 3).
- a questionnaire (annex 4). We kindly ask you to give us information about the experimental conditions, recoveries, etc.. On this form you can also give your remarks about the method.

The sample must be analysed in *duplicate*.
For recovery purposes we ask you to use a sample from your own collection.
Because the reference standard that was sent to you in May 2000 by mr. Towell (Eli Lilly) has expired you receive together with the premixture a new Eli Lilly reference standard of nicarbazin, lot number X47623, that has to be used at the analyses.

The **deadline** for reporting the results is **January 25, 2002**.

We wish you and your colleagues the best with the collaborative study. If you have any questions, do not hesitate to contact us.

Kind regards,

dr. J. de Jong
CANFAS co-ordinator

ing. J.J.M. Driessen
co-ordinator CANFAS collaborative studies

QUALIFIED BY STERLAB

Annex 1 - The modified method of analysis

RIKILT

CANFAS/NIC/03102001/H.KEUKENS

Animal feeding stuffs and premixtures - Determination of NICARBAZIN - High-performance liquid chromatographic method.

1 SCOPE

This operating procedure specifies a method for the determination of the nicarbazin content in animal feeding stuffs and premixtures (maximum concentration 2.5 % nicarbazin) using high performance liquid chromatography. The limit of quantitation (LOD) determined in the pre-validation study was 20 mg/kg.

2 PRINCIPLE

Samples are extracted by heating in a waterbath, mechanical shaking and sonoration using an acetonitrile/methanol mixture. For feeding stuffs, also water is added. The mixture is transferred in a volumetric flask. After settlement of the solids, an aliquot is filtered and assayed using a reverse-phase isocratic method which measures the 4,4'dinitrocarbanilide (DNC) moiety at a wavelength of 350 nm.

3 REAGENTS

Use only reagents of recognised analytical grade. Use water complying with at least grade 3 in accordance with ISO 3696.

3.1 Acetonitrile, HPLC grade

3.2 Methanol, HPLC grade

3.3 Extraction solvent. Mix 500 ml of acetonitrile (3.1) with 500 ml of methanol (3.2). Mix well using a magnetic stir plate and stir bar.

3.4 Eluent for liquid chromatography. Mix 650 ml acetonitrile (3.1) with 350 ml of purified water. Mix well using a magnetic stir plate and stir bar and degas (e.g. with helium) before use.

3.5 Nicarbazin reference standard.

4 APPARATUS

Using laboratory apparatus and, in particular, the following:

4.1 High performance liquid chromatography system consisting of the following:

4.1.1 An autosampler or manual injector set to inject a volume of 20 µl.

4.1.2 A pump set to deliver a constant eluent flow rate of 1,0 ml/min

Annex 1 - The modified method of analysis

RIKILT

4.1.3 A column, length 300 mm, internal diameter 3.9 mm, packed with a stationary phase consisting of C-18 material. The particle size should not be smaller than 5 µm and not greater than 10 µm. (A Nova-Pak column is recommended, but also other columns can be used providing that a satisfactory separation of DNC is achieved).

4.1.4 A detector allowing the measurement of absorbance of UV light at a wavelength of 350 nm, with integrator/recorder.

4.2 Mechanical shaker (e.g. Gyratory shaker, wrist action shaker)

4.3 Micro filters for sample filtration, 0.2 - 0.5 µm

4.4 Mill to prepare laboratory samples with a maximum particle size of 1 mm

4.5 Ultrasonic bath

4.6 Waterbath, 50 °C

4.7 Disposable centrifuge tubes of 50 ml with a screw cap

5 PREPARATION OF THE SAMPLES

5.1 Test samples
The milling and mixing of compound feed samples prior to assay is obligatory. Grind feed samples through a mill (4.4) equipped with a 1 mm screen. After milling, mix the entire sample thoroughly. Store the sample at room temperature in subdued light. Premix samples are not milled.

5.1.1 Mixing of the test samples before weighing
The container should be filled to a maximum of 50 % of the total volume. Bring the container in a horizontal position and rotate bottom and top of the container in circles moving the container up and down along the virtual centre of the container for 30 seconds. Put the container in an upright position and wait a few seconds for settlement of the generated dust.

5.2 Spiked feed samples; 100 mg/kg
Transfer 2.5 ml of the stock standard solution (6.4.1.1) in the sample tube or flask. Evaporate to a small volume (less than 0.5 ml) with a gentle stream of nitrogen, add 2.5 g blank feed, mix thoroughly and wait 10 minutes before starting the extraction procedure by adding water for swelling (see 6.2.2).

6 PROCEDURE

6.1 General
Complete each assay within one working day.

6.2 Extraction

6.2.1 Premixtures

Weigh to the nearest 0.001 g, approximately 1.0 g of the test sample directly into a wide neck volumetric flask of 200 ml.

Add 80 ml of extraction solvent (3.3), close the flask and mix manually by swirling.

Annex 1 - The modified method of analysis

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Put the flasks in a waterbath of 50 °C for 15 minutes with intermediate swirling at 8 minutes.

Mix thoroughly 15 minutes using a mechanical means (4.2).

Put the flasks in an ultrasonic bath (4.5) and sonorate for 15 minutes.

Cool down to room-temperature, adjust to volume with HPLC eluent (3.4) and mix.

Allow sample solids to settle (minimum 30 minutes).

If additional dilutions are required, dilute the samples with HPLC eluent (3.4) to a final nicarbazin concentration of ca 5 µg/ml.

Filter an aliquot of the final dilution through a micro filter (4.3) for analysis by HPLC.

6.2.2 Animal feeding stuffs

Weigh to the nearest 0.01 g, approximately 2.5 g (see remark 9.1) of the test sample into a 50 ml disposable centrifuge tube (4.7) or directly into a wide neck volumetric flask of 100 ml.

Add 5 ml of water. Take care that the whole sample is wetted.

Wait at least 10 minutes.

Add 35 ml of extraction solvent (3.3), close the tube or flask and mix manually by swirling.

Put the tubes or flasks in a waterbath of 50 °C (4.6) for 15 minutes with intermediate swirling at 8 minutes.

Mix thoroughly 15 minutes using a mechanical means (4.2).

Put the tubes or flasks in an ultrasonic bath (4.5) and sonorate for 15 minutes.

Transfer the sample extract if necessary quantitatively in a 100 ml volumetric flask with HPLC eluent (3.4), adjust to volume and mix.

If additional dilutions are required, dilute the samples with HPLC eluent (3.4) to a final nicarbazin concentration which falls within the standard curve levels.

Filter an aliquot of the final dilution through a micro filter (4.3) for analysis by HPLC.

6.3 Determination

6.3.1 Inject 20 µl of the sample extract on to the column of the liquid chromatograph (4.1) and measure the area/height of the DNC peak.

6.3.2 Determine the nicarbazin concentration of the extract by reference to the mean of a calibration curve prepared as described in 6.4 and analysed before and after the sample extracts.

Annex 1 - The modified method of analysis

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6.4 Calibration

6.4.1 Preparation of nicarbazin standard solutions

6.4.1.1 Nicarbazin stock standard solution, 100 µg/ml
Dissolve 10 mg, weighed to the nearest 0.1 mg, of nicarbazin reference standard (3.5) in 100 ml extraction solvent (3.3). To aid with dissolution, sonoration for approximately 5 minutes is recommended. Mix well. This solution is stable for 24 hours when stored in subdued light at ambient or refrigerated storage conditions (see remark 9.2).

6.4.1.2 Nicarbazin working standard solutions for feedingstuffs containing 50-250 mg/kg nicarbazin and for premixtures
Prepare a range of calibration working standards containing 0, 1, 2, 3, 4, 5 and 10 µg/ml nicarbazin by diluting the stock standard solution (6.4.1.1) with HPLC eluent (3.4). Working standards must be prepared daily.

6.4.1.3 Nicarbazin working standard solutions for feedingstuffs containing 20-50 mg/kg nicarbazin
Prepare a range of calibration working standards containing 0; 0.25; 0.5; 1; 2 and 2.5 µg/ml nicarbazin by diluting the stock standard solution (6.4.1.1) with HPLC eluent (3.4). Working standards must be prepared daily.

7 EXPERSSION OF RESULTS

Calculate the nicarbazin content of the test sample by the equation:

$$W_E = \frac{100 \times c}{M} \times f$$

Where:

W_E is the numerical value for the nicarbazin content of the test sample in mg/kg

C is the numerical value of the nicarbazin concentration of the sample extract in µg/ml

M is the numerical value of the mass of the test sample, in g

F is the dilution factor introduced to prepare final sample extracts fitting with the standard curve levels

8 RECOVERY

The recovery obtained for compound feeds should be higher than 90 % at spike levels between 20 and 200 mg/kg.

9 REMARKS

9.1 Homogeneity
For relatively inhomogeneous compound feed samples, the weighed sample amount should be increased to 10 gram with simultaneous up-scaling of the volume of extraction solvent used.

Annex 1 - The modified method of analysis

RIKILT

9.2 Solubility

The solubility of the nicarbazin reference standard in extraction solvent is critical. The nicarbazin concentrations in the prepared stock solutions must be monitored by use of a cuvet spectrophotometer as follows. Prepare a solution of 10 µg/ml by diluting the prepared stock standard solution (6.4.1.1) with acetonitrile. Record a UV-Vis spectrum between 220 and 450 nm using a mixture of methanol/acetonitrile (5:95 v/v) as a reference solution. The maximum absorbance measured between 340 and 350 nm should be within a margin of +/- 5 % of the default value. The default value should be established in your own laboratory by preparing a stock standard solution in duplicate and monitoring the UV-Vis spectra as described above. The default value is the mean result of the duplicates.

9.3 Method characteristics

Precision, repeatability and reproducibility data will be included in the final version of the method description that will be prepared after completion of the collaborative study.

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis:

Analyte: NICARBAZIN

Product: Premixture

Sample code	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 3 - Instructions for handling of the premixture sample

1. Storage

Store the sample at room temperature until analysis

2. Milling (see par. 5.1)

- The sample must not be milled

3. Mixing of the test sample before weighing (see par. 5.1)

Bring the container in a horizontal position and rotate bottom and top of the container in circles moving the container up and down along the virtual centre of the container for 30 seconds.
Put the container in an upright position and wait a few seconds for settlement of the generated dust.

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: .....

Dilution factor of the sample:

- Premixture: .....

Chromatographic conditions:

- Column:
  - As described in the method
  - Other: .....
- Mobile phase:
  - As described in the method
  - Other: .....
- Flow-rate: ..... ml/min
- Injection volume: .....µl
- Retention time of nicarbazin: ..... min

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: single duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg

Remarks /Comments (if necessary, continue on another page) : .....

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.....

Please complete this questionnaire and return it together with representative chromatograms to:

Ing. J.J.M. Driessen
RIKILT
P.O. Box 230
6700 AE Wageningen
The Netherlands
Fax +31-317-417717

Thank you for your cooperation !

-----Oorspronkelijk bericht-----

Van: Driessen, ing. J.J.M.
Verzonden: vrijdag 18 januari 2002 11:43
Aan: Antalick, J; Biancotto, Giancarlo; Brambilla, Gianfranco; Checa-Moreno, Ramon; Cowles, John; Faggionato, Elena; Felgueiras, Ilidia; Fontaine, André; Haustraete, Karel; Henk van der Kamp; Margry, Rob; Nunes Costa, José; Schreuder, Cor; Schwadorf, Klaus; Testa, Cecilia; Thalmann, Alfred; Ubaldi, Alessandro; Van Schalm, Klaas; Wehage, Hubert
CC: Jong, Dr. J. de
Onderwerp: Additional information 2nd coll. study Nicarbazin
Urgentie: Hoog

Dear participant,
Our covering letter 30595 of December 13th, 2001 concerning the second interlaboratory study for nicarbazin in a premixture lacks an instruction for the calculation of the results with respect to the reference standard. **In the calculations the reference standard should be regarded as 100% pure.**

Those who sent already their results: please take note of this information; when the results have to be reconsidered send me please the corrected results.

We apologize for this inconvenience and for the confusion it might have caused.

Best regards,
Jaap Driessen
Coordinator CANFAS collaborative study

Date: Mon, 07 Jan 2002 12:20:28 +0100
From: "Tomassen, ing. M.J.H." <M.J.H.Faassen-Tomassen@RIKILT.DLO.NL>
Subject: CANFAS - collaborative study Nicarbazin
To: "'rob.margry@ccl-nutricontrol.nl'" <rob.margry@ccl-nutricontrol.nl>,
"'jp.antalick@ieeb.fr'" <jp.antalick@ieeb.fr>,
"'llidia.Felgueiras@mail2.ineti.pt'" <llidia.Felgueiras@mail2.ineti.pt>,
"'g.brambi@iss.it'" <g.brambi@iss.it>,
"'efaggionato@bs.izs.it'" <efaggionato@bs.izs.it>,
"'aubaldi@rm.izs.it'" <aubaldi@rm.izs.it>,
"'ctesta@sardegna.izs.it'" <ctesta@sardegna.izs.it>,
"'biancobina@izsvenezie.it'" <biancobina@izsvenezie.it>,
"'bioquim@moebius.es'" <bioquim@moebius.es>,
"'jose.costa@lniv.min-agricultura.pt'" <jose.costa@lniv.min-agricultura.pt>,
"'thalmann@lufa.bwl.de'" <thalmann@lufa.bwl.de>,
"'wehage@lufa-itl.de'" <wehage@lufa-itl.de>,
"'Klaas.van.Schalm@nutreco.com'" <Klaas.van.Schalm@nutreco.com>,
"'CSchreuder@premervo.nl'" <CSchreuder@premervo.nl>,
"'karel.haustraete@cmlag.fgov.be'" <karel.haustraete@cmlag.fgov.be>,
"'schwadorf@lachemie.uni-hohenheim.de'" <schwadorf@lachemie.uni-hohenheim.de>,
"Kamp, H.J. van der" <H.J.vanderKamp@RIKILT.DLO.NL>
Cc: "'J.dejong@rikilt.wag-ur'" <J.dejong@rikilt.wag-ur>,
"'jrc@lgc.co.uk'" <jrc@lgc.co.uk>

Dear participant,

Thanks to John Cowles we found out an error in the calculation of the nicarbazin content of the test sample (annex 1, the modified method of analysis, page 4, paragraph 7).

The formula given is fine for animal feeds where the volume of extractant is 100 ml, but for premixes the factor has to be 200, because the volume of extractant is 200 ml.

so, the fomula has to be: WE = 200 x c x f / M

For those who have already sent in their results: Will you please check if you used the correct factor (200).
Will you please confirm if you used factor 200.
If you used the wrong factor, will you please send the new results calculated with factor 200?
Please send the results to Jaap Driessen.

Thanks in advance,

Kind regards,
Marinka Tomassen.

APPENDIX 2

Composition and homogeneity of the premixture

CANFAS, 2nd coll. Study. Najaar 2001

Samenstelling premix met nicarbazin (0,75%)

ontv 28/09

3 kg + 90 g Canijam

Inclusion percentage : 0.250%

Calculated analysis:

Name	Per kg premix		Per kg end product	
Bushelweight	+1.211	kg	+0.003	kg
Vitamin A	+3600000.000	IE	+9000.000	IE
Vitamin D3	+800000.000	IE	+2000.000	IE
Vitamin E	+4800.000	IE	+12.000	IE
Vitamin K3	+400.000	mg	+1.000	mg
Vitamin B1	+200.000	mg	+0.500	mg
Vitamin B2	+1600.000	mg	+4.000	mg
Pantothenic acid	+4000.000	mg	+10.000	mg
Niacin	+8001.000	mg	+20.003	mg
Biotin	+12000.000	mcg	+30.000	mcg
Vitamin B12	+6000.000	mcg	+15.000	mcg
Folic acid	+600.000	mg	+1.500	mg
Vitamin B6	+1000.000	mg	+2.500	mg
Cholin Chlorid	+120001.000	mg	+300.003	mg
Iron	+14001.000	mg	+35.003	mg
Copper	+4000.000	mg	+10.000	mg
Zinc	+20001.000	mg	+50.003	mg
Manganese	+28000.000	mg	+70.000	mg
Cobalt	+200.000	mg	+0.500	mg
Iodine	+320.000	mg	+0.800	mg
Selenium	+80.100	mg	+0.200	mg
Cd Max.	+1.504	mg	+0.004	mg
Pb Max.	+39.049	mg	+0.098	mg
Apocarothenester	+800.000	mg	+2.000	mg
Antiox E310,320,321	+20000.000	mg	+50.000	mg
Crude protein	+80.538	g	+0.201	g
Crude fat	+0.265	g	+0.001	g
Crude fiber	+19.183	g	+0.048	g
Ash	+759.000	g	+1.898	g
Moisture content	+14.798	g	+0.037	g
Lysin	+0.012	g	+0.000	g
Methionin	+0.005	g	+0.000	g
Methionin+Cystin	+0.011	g	+0.000	g
Threonin	+0.010	g	+0.000	g
Tryptophan	+0.004	g	+0.000	g
Isoleucin	+0.010	g	+0.000	g
Starch Ewers	+16.622	g	+0.042	g
Sugar	+0.097	g	+0.000	g
Sugar/Starch	+16.727	g	+0.042	g
Calcium Total	+226.463	g	+0.566	g
Phosphorus Total	+0.021	g	+0.000	g
Potassium Total	+0.031	g	+0.000	g
Sodium Total	+1.049	g	+0.003	g
Chlorid Total	+30.688	g	+0.077	g
oP'97 plv	+0.006	g	+0.000	g
oP'97 leg	+0.006	g	+0.000	g
OE slk	+2.640	kcal	+0.007	kcal
OE leg	+3.783	kcal	+0.009	kcal
V.LysPLV	-0.358	g	-0.001	g
V.MetPLV	-0.143	g	+0.000	g
V.M+CPLV	-0.432	g	-0.001	g
V.ThrPLV	-0.360	g	-0.001	g
V.TryPLV	-0.070	g	+0.000	g
Liters	+1.033	l/kg	+0.003	l/kg

HOUDBAARHEID: zie etiket of geleidedocument.
VERWERKING: zie FARMIX-instruktie "Veilig werken met voormengsels."

nicarbazine 7,5 g

TROUW NUTRITION
a nutreco company

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Homogeneity test 2nd collaborative study

Additive : **Nicarbazin**
Product : **Premixture: 0.75%**

Date of determination : October 9th, 2001

Sample	Content (%)	Duplicate average %
314201-a	0,735	0,74
314201-b	0,746	
314211-a	0,698	0,73
314211-b	0,755	
314219-a	0,779	0,73
314219-b	0,681	
314204-a	0,680	0,70
314204-b	0,713	
314216-a	0,619	0,64
314216-b	0,659	
314221-a	0,701	0,71
314221-b	0,711	
314215-a	0,744	0,73
314215-b	0,716	
314228-a	0,672	0,70
314228-b	0,726	
314222-a	0,752	0,71
314222-b	0,662	
314206-a	0,723	0,74
314206-b	0,764	

Homogeneity **OK**		
Crtiterion : $CV_{between}$ = < 7%		
Average	**0,73**	
SD (between samples)	**0,046**	
CV (between samples)	**6,2%**	Result Grubb's test
Grubb's test, single lower	**2,143**	**no outlier**
Grubb's test, single upper	**0,989**	**no outlier**
Grubb's test, double lower	**0,2330**	**no outliers**
Grubb's test, double upper	**0,7284**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,038**
CV (within samples)	(CV (%))	**5,2**

APPENDIX 3

Sample codes

Sample codes supplied to the participants in the nicarbazin collaborative study 2nd round

Lab	Sample
13	134229
15	154225
16	164223
19	194232
20	204218
21	214213
23	234214
24	244208
25	254220
26	264217
29	294226
30	304224
31	314212
33	334203
35	354230
38	384202
39	394210

APPENDIX 4

Nicarbazin reference standard profile

CONFIDENTIAL
DISTRIBUTION TO LILLY PERSONNEL ONLY

ELI LILLY AND COMPANY
PRODUCT DEVELOPMENT OPERATIONS DIVISION

REFERENCE STANDARD PROFILE

* Effective Date: October 29, 2001
* Expiry Date: October 28, 2004
* Supersedes Date: October 9, 1998

Compound: 093760
Revision: 11

Name: Nicarbazin
Lot Number: X47623
Defined Potency: 69.6% 4,4'-Dinitrocarbanilide on an "as is" basis; 27.0% 4,6-Dimethyl-2-pyrimidinol on an "as is" basis

* Handling: Refer to current MSDS for handling and caution information.
Storage: Tightly closed amber glass bottle at room temperature, 15 to 30 C.

* Evolution: Lot X47623 was reevaluated in September 2001.

	Tests	Results
(x)	HPLC Assay (QA322A)	69.6% 4,4'-dinitrocarbanilide, 98.2% of theory vs. previous nicarbazin standard; RSD=0.51% (n=6)
(x)	HPLC Assay (Method AM-AA-CA-J063-AB-755)	27.1% 4,6-dimethyl-2-pyrimidinol, 93.1% of theory, vs. previous nicarbazin standard; RSD=2.36% (n=4)
	HPLC Scan, High/Low (Conditions of Method B00271)	0.80% total related substances detected (n=3)
	*HPLC Scan, High/Low (Conditions of Method B05511)	0.35% total related substances detected (n=3)
	*HPLC Scan, High/Low (Conditions of Method AM-AA-CA-J063-AB-755)	1.07% total related substances detected (n=3)
	Elemental Analysis	C: Theory=53.52%; Found=52.90% H: Theory=4.26%; Found=4.21% N: Theory=19.71%; Found=19.88% O: Theory=22.51%; Found=20.70% (n=1)
(x)	*X-ray Pattern (USP 24, 941)	Pattern compares favorably to the previous standard pattern for this lot; material is crystalline (n=1)
(x)	*IR Spectrum (USP 24, 197K)	The FT-IR spectrum compares favorably with that of the previous spectrum for this lot (n=1)

(x)	1H NMR Spectrum (Method RP1, DMSO-d6)	The 1H spectrum is consistent with the structure and compares favorably to previous spectra (n=1)
	Mass Spectrum	Spectrum compares favorably to previous spectrum (n=1)
	DTA (Method RP7)	The thermograms show an endotherm at 270 C followed by an exotherm at 292 C (n=3)
	TGA (Method RP9)	The thermogram shows no weight loss until 134.6 C where a loss begins which results in a continuous loss through decomposition (n=1)
	Water, KF (GP0032)	0.12 % (n=2)
	*LOD (Method B00272)	1.19 % (n=2)
	Residue on Ignition (QA322A)	0.07 % (n=1)

Note: (x) indicates the standard material is approved for use as a reference for the test.

4,4'dinitrocarbanilide is compound 015595.
4,6-dimethyl-2-pyrimidinol is compound 023948.

* Revised September 13, 2001

Beverly J. Krabel

Revision 11

APPENDIX 5

Table with results and chromatograms

of partner 13

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: **Task 4 COLLABORATIVE STUDY - 2nd round**

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis:

Analyte: **NICARBAZIN**

Product: **Premixture**

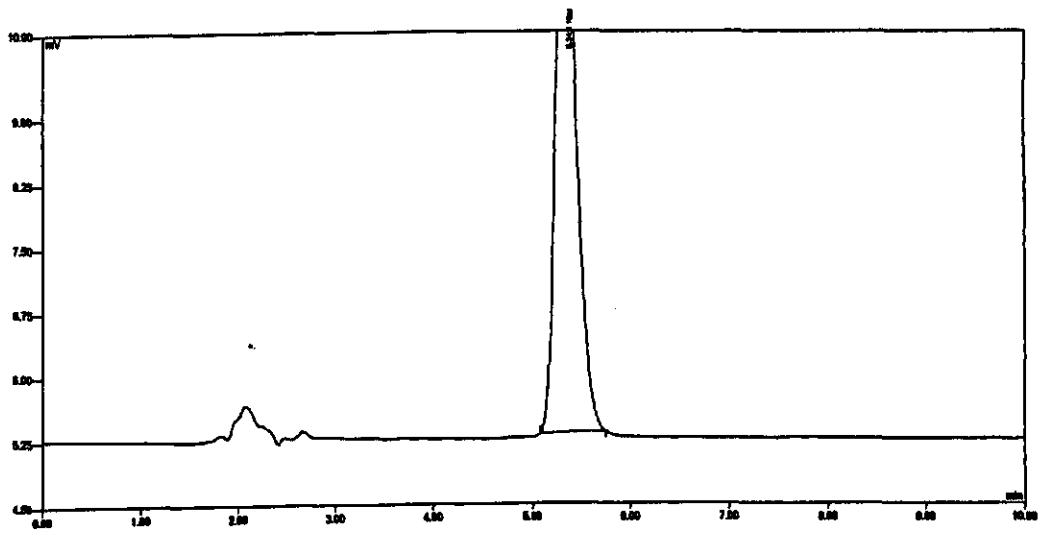
Sample code	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
134229		8829	9148

KromaSystem 2000

Channel 2

2

Program File ..... NICAR .....
Worksheet ..... NICARB .....
Peak Table ..... NICAR .....
Parameter Table .. NICAR .....
Report File ....
Document File ....



No.	PNo	Ret.Time min	Type	Name	Area mV*min	Amount	Rel.Ar %
1	1	5.34	MOD		1.6880e+000	1.6158e+004	100.00
					1.6880e+000	1.6158e+004	100.00

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 15

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 25,01,2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
154225	6908	8317

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: ....24-25 JANUARY 2002 ✗

Dilution factor of the sample:

- Premixture: ...1g in 200 ml, then 1.000 ml (SAMPLE) + 6.00 ml (65 ACN / 35 H2O) . D.F. = 2000 ✗

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ...Lucrisil ODS-2 5 µm x 250 mm...
- Mobile phase:
  - ☐ As described in the method
  - ☐ Other: .....
- Flow-rate: ...1.0..... ml/min
- Injection volume: ...20....µl
- Retention time of nicarbazin: 4.3 min

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

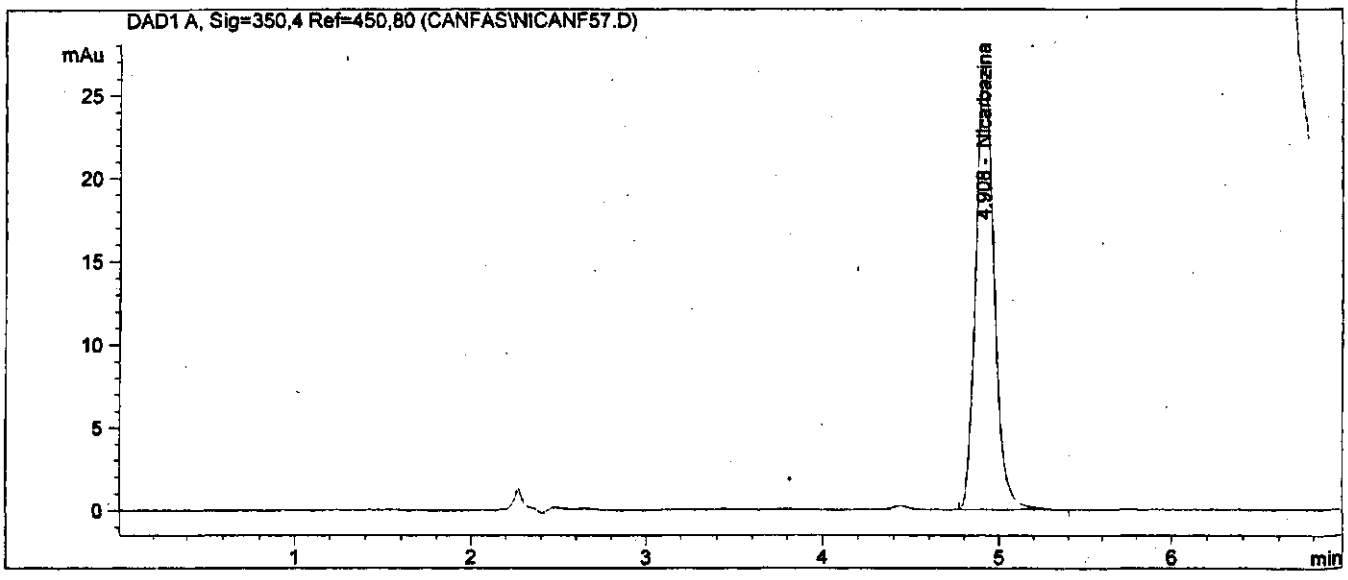
- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 98.2 % and 98.9 %
- Spiking level: ...100... mg/kg

Code 152225

15

Injection Date : 1/25/02 11:54:56 AM Seq. Line : 7
Sample Name : premix 1 Vial : 6
Inj : 2
Inj Volume : 20 µl

Acq. Method : (
Last changed : 1/25/02 10:52:55 AM k
Analysis Method : C
Last changed : 1/25/02 12:24:43 :
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : Friday, January 25, 1902 12:09:11 PM
Multiplier : 1.0000
Dilution : 2.000e3
Uncalibrated Peaks : not reported

Signal 1: DAD1 A, Sig=350,4 Ref=450,80
Results obtained with enhanced integrator!

RetTime [min]	Type	Area [mAu*s]	Amt/Area	Amount [ng/ul]	Grp	Name
4.908	BB	195.77600	1.76255e-2	6901.29895		

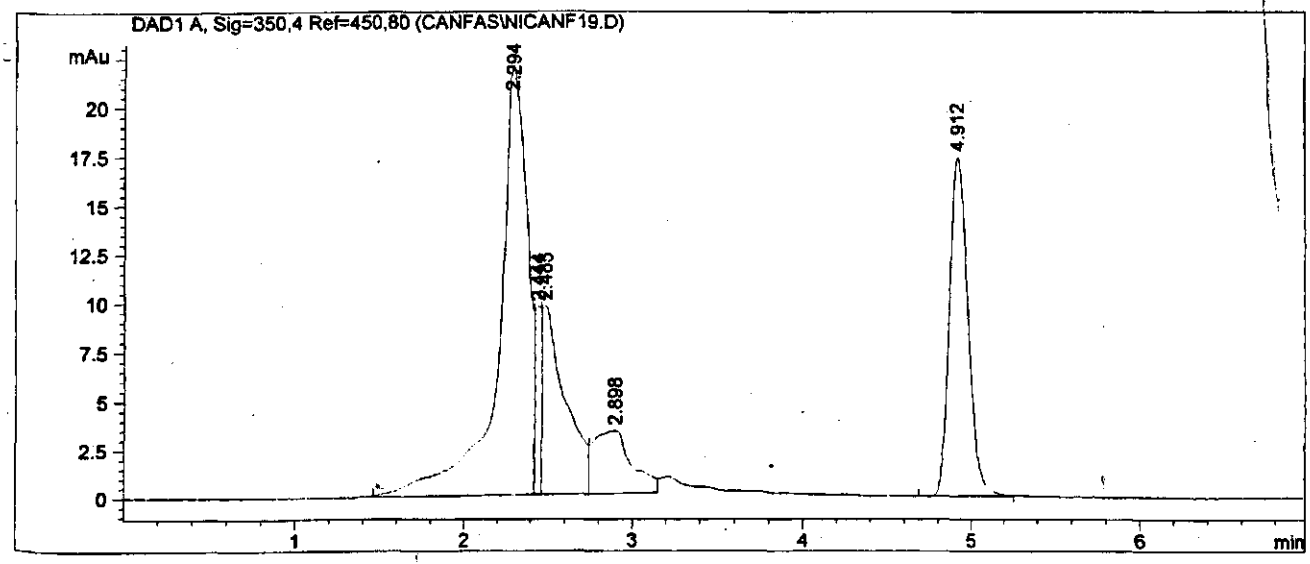
Totals : 6901.29895 mg/Kg

Data File C:\HPCHEM\1\DATA\CANFAS\nicanf19.D Sample Name: aggiunta 100 ug/Kg

15

Injection Date : 1/18/02 1:15:08 PM Seq. Line : 12
Sample Name : Vial : 11
Acq. Operator : -- Inj : 1
Inj Volume : 20 µl

Sequence File :
Acq. Method :
Last changed : 1/18/02 10:19:35 AM by
Analysis Method :
Last changed : 1/24/02 10:02:39 AM by --



External Standard Report

Sorted By : Signal
Calib. Data Modified : Thursday, January 24, 1902 10:02:39 AM
Multiplier : 1.0000
Dilution : 1.0000
Uncalibrated Peaks : not reported

Signal 1: DAD1 A, Sig=350,4 Ref=450,80
Results obtained with enhanced integrator!

RetTime [min]	Type	Area [mAu*s]	Amt/Area	Amount [ng/µl]	Grp	Name
4.912	PB	137.14476	1.78909e-2	2.45364		

Totals : 2.45364 $\times \frac{100}{25}$ = 98.1 ug/Kg => 98.1%

*** End of Report ***

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 16

CANFAS COLLABORATIVE STUDIES - 2nd round - December 2001

ADDITIVE: NICARBAZIN

Annex 4 - Questionnaire

Laboratory:

Contact person:

Person(s) doing analysis of this collaborative study:

Date(s) of analysis: December 20., 2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - x Other: Waters-Spherisorb ODS1, 5 µm, 250 x 4 mm
- Mobile phase:
  - x As described in the method
  - ☐ Other:

  .....
- Flow-rate: 1 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: about 4 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 99.4 %
- Single / duplicate determinations: ☐ single x duplicate
- If duplicate, please give both percentages: 99.02 and 99.73 %
- Spiking level: 116 mg/kg

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: Dec. 20, 2001

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
164223	6706	6572

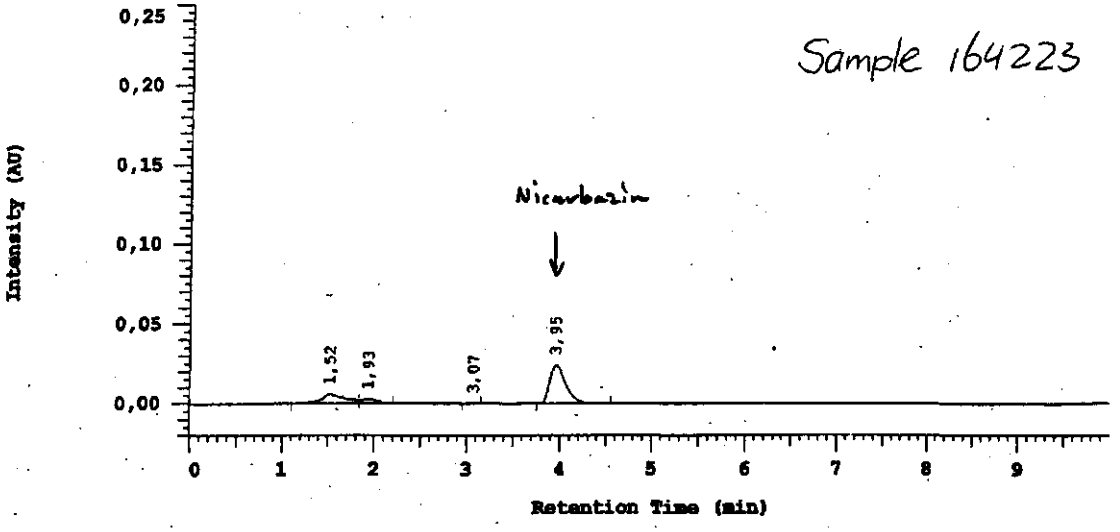
D-7000 HPLC System Manager Report

Analyzed: 20.12.01 14:34
Reported: 20.12.01 16:14
Processed: 20.12.01 16:14

Data Path:
Processing Method: 1
System(acquisition): Sys 1
Application:
Sample Name:
Injection from this vial: 1 of 2
Sample Description:

Series:0001
Vial Number: 9
Vial Type: UNK
Volume: 20,0 ul

Chrom Type: HPLC Channel : 1



Acquisition Method:
Column Type: WATERS. ODS1 5,0µm
250x4,0
Pump A Type: L-7100
Solvent A: AcCN:H2O (65:35)
Method Description:

Chrom Type: HPLC Channel : 1

Peak Quantitation: HEIGHT
Calculation Method: EXT-STD

No.	RT	Area	Height	Conc 1 ug/mL	Name
4	3,95	140834	11917	1,671	Nicarbacin
		140834	11917	1,671	

Peak rejection level: 0

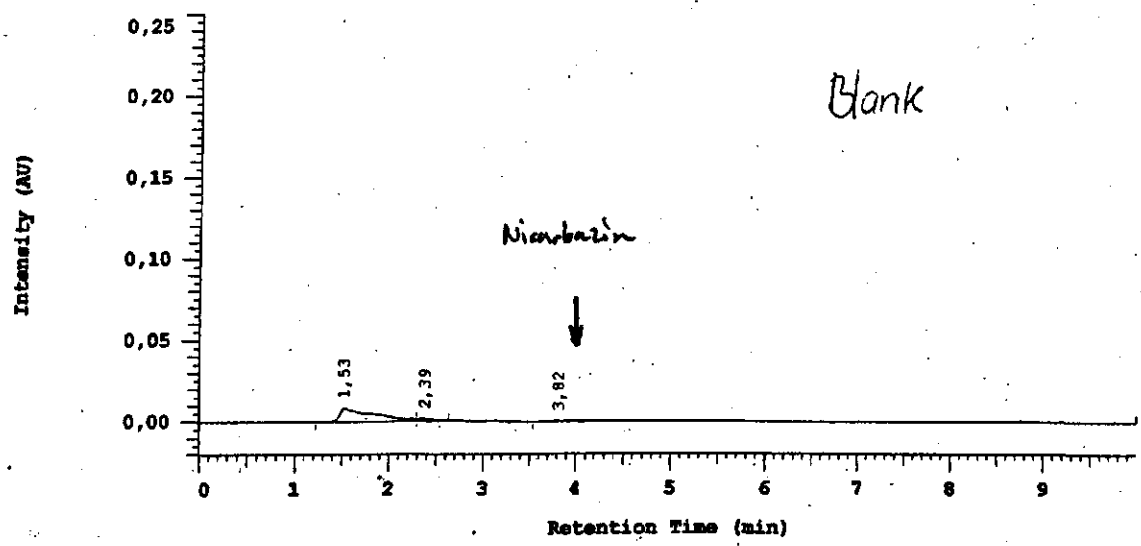
D-7000 HPLC System Manager Report

Analyzed: 20.12.01 13:30
Reported: 20.12.01 16:05
Processed: 20.12.01 16:05

Data Path:
Processing Method:
System(acquisition): Svs 1
Application:
Sample Name:
Injection from this vial: 2 of 2
Sample Description:

Series:0001
Vial Number: 6
Vial Type: UNK
Volume: 20,0 ul

Chrom Type: HPLC Channel : 1



Acquisition Method: N
Column Type: WATERS. ODS1 5,0µm
250x4,0
Pump A Type: L-7100
Solvent A: AcCN:H2O (65:35)
Method Description:

Chrom Type: HPLC Channel : 1

Peak Quantitation: HEIGHT
Calculation Method: EXT-STD

No.	RT	Area	Height	Conc 1 ug/mL	Name
3	3,82	15125	127	0,013	Nicarbacin
		15125	127	0,013	

Peak rejection level: 0

expected retention time : 3.95-4.00 min

-16

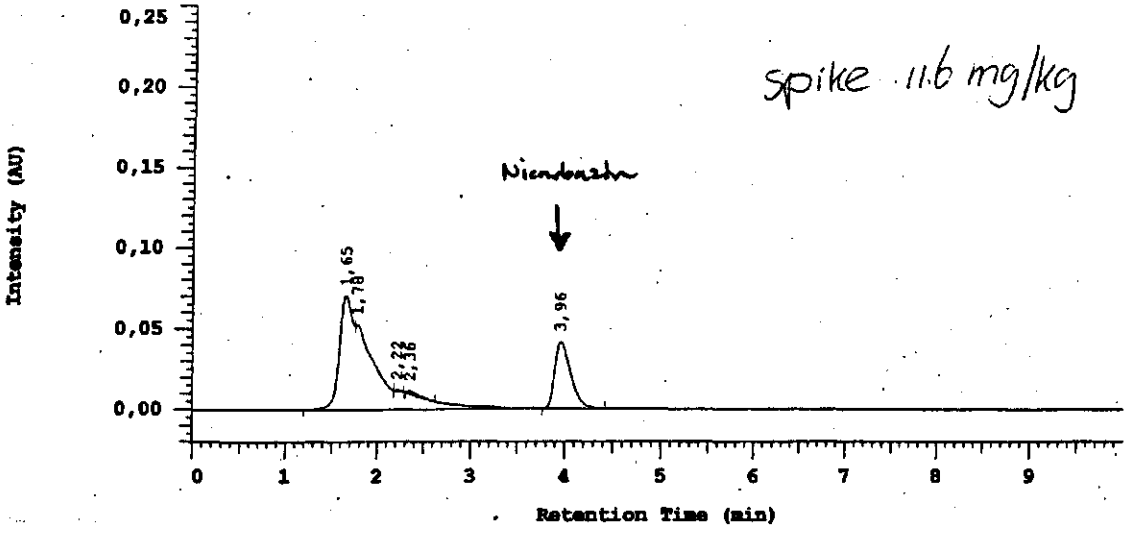
D-7000 HPLC System Manager Report

Analyzed: 20.12.01 13:43
Reported: 20.12.01 16:06
Processed: 20.12.01 16:05

Data Path:
Processing Method:
System(acquisition):
Application:
Sample Name:
Injection from this vial: 1 of 2
Sample Description:

Series:0001
Vial Number: 7
Vial Type: UNK
Volume: 20,0 ul

Chrom Type: HPLC Channel : 1



Acquisition Method:
Column Type: WATERS. ODS1 5,0µm
250x4,0
Pump A Type: L-7100
Solvent A: AcCN:H2O (65:35)
Method Description:

Chrom Type: HPLC Channel : 1

Peak Quantitation: HEIGHT
Calculation Method: EXT-STD

No.	RT	Area	Height	Conc 1 ug/mL	Name
5	3,96	241078	20487	2,874	Nicarbacin
		241078	20487	2,874	

Peak rejection level: 0

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 19

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 23-01-2002

Analyte: NICARBAZIN

Product: Premixture

Sample code	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
194232		7043	7350

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: ....

Date(s) of analysis: .....21.12.01.....

Dilution factor of the sample:

- Premixture: .....10.....5.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: .....Chromsphere C18.....250.mm...3.0.mm.10.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....0.5..... ml/min
- Injection volume: ...50....µl
- Retention time of nicarbazin: ..3.8.. min

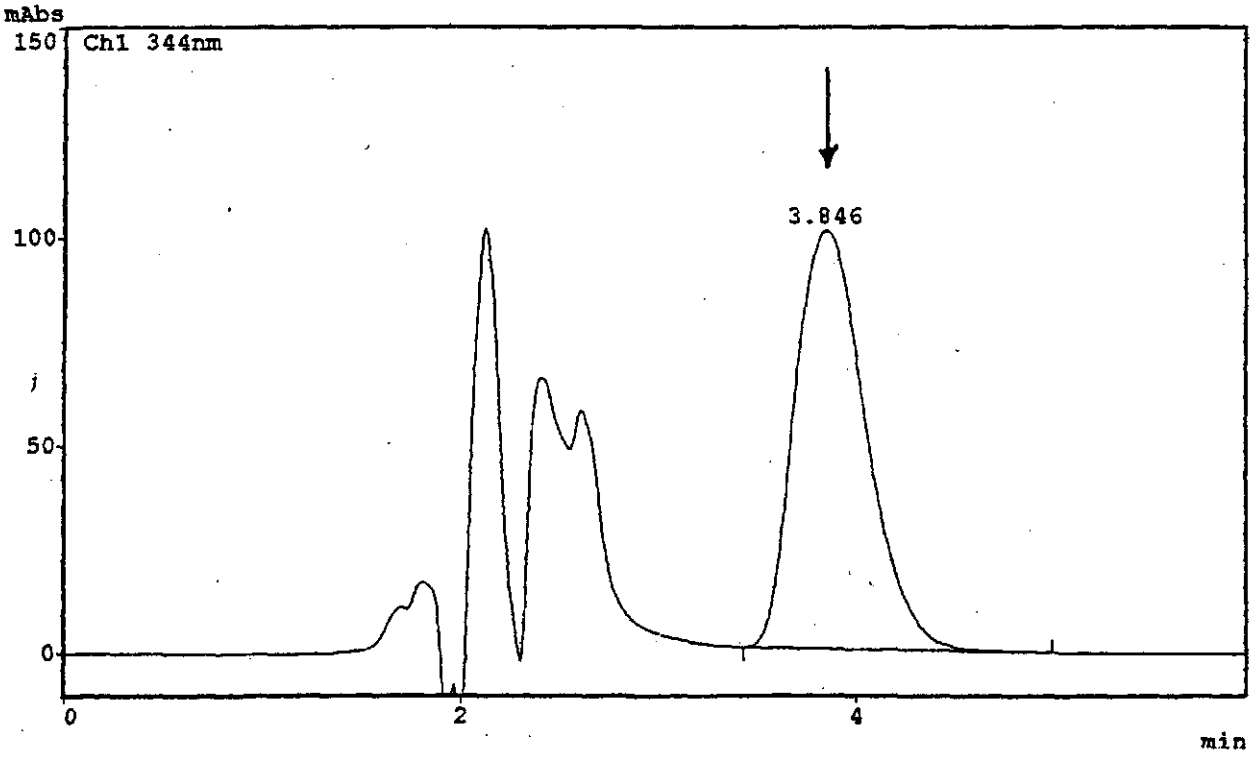
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..93.. %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ...75.... mg/kg


mAbs
150
Ch1 344nm
3.846
100
50
0
0
2
4
min

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 20

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 23-01-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
204218	7534,4	7554,4

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 – NICARBAZIN

Annex 4 – Questionnaire

Laboratory:

Contact person:

Date of analysis: 23/01/2002

Dilution factor of the sample:

- Premixture: 1:5

Chromatographic conditions:

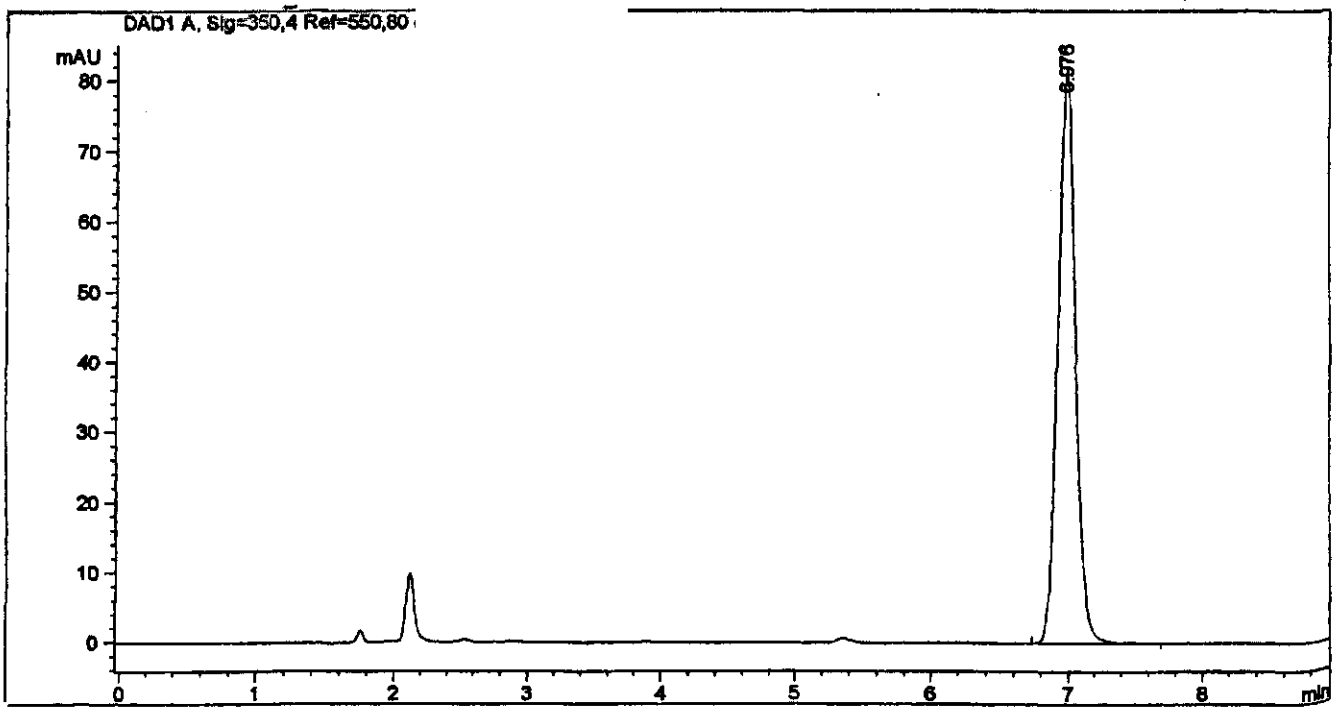
- Column:
  - □As described in the method
  - ✓Other: Alltima C18 250x4.6 mm, 5 µm
- Mobile phase:
  - ✓As described in the method
  - □Other
- Flow rate: 1.0 ml/min
- Injection volume: 20 µl
- Retention time of nicarbazin: min

Chromatograms: Please include representative chromatograms of:

- **Premixture**
- **Nicarbazin working standard solution**

Recovery results:

- Percentage recovery: 100.02 %
- Duplicate determination: 99.92 % and 100.13 %
- Spiking level: 200 mg/Kg



External Standard Report

Sorted By : Signal
Calib. Data Modified : Wednesday, January 23, 2002 12:48:18 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=350,4 Ref=550,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/mL]	Grp	Name
6.976	BB	737.61462	1.02932e-2	7.59243		

Totals : 7.59243

Results obtained with enhanced integrator!

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 21

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 17-01-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
214213	7450	7500

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory:

Contact person: .....

Date(s) of analysis: 17/01/2002

Dilution factor of the sample:

- Premixture: 1:5 (dilution with eluent for liquid chromatography)

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Supelcosil LC-18 (25cm x 4,6mm ID) + SUPELGUARD LC-18 (2cm x 4,6mm ID 1.5)
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: CH_3CN – Ammonium Acetate Buffer 0,017 pH 4,6 (with Acetic Ac
- Flow-rate: 1,2 ml/min GRADIENT ELUTION
- Injection volume: 10 µl
- Retention time of nicarbazin: 17.4 min

Chromatograms: Please include representative chromatograms of:

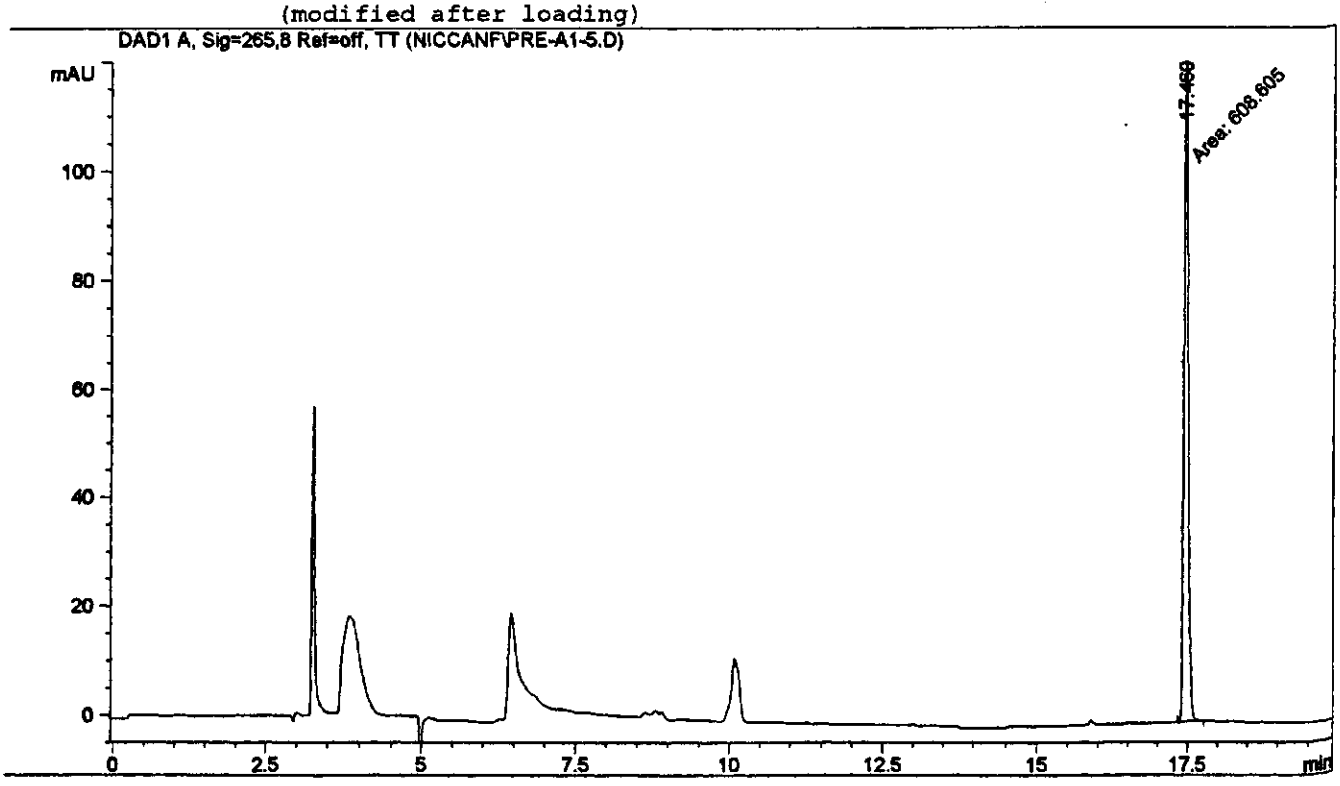
- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: 98,8 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 98,0 % and 99,6 %
- Spiking level: 100 mg/kg

```
Injection Date  : 17/01/2002 15.11.34          Seq. Line :   3
Sample Name     : PREMIX-A-1:5                  Location : Vial 6
Acq. Operator   :                                   Inj :   1
                                                Inj Volume : 10 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 20 µl
Acq. Method     :
Last changed    : 17/01/2002 15.19.20
                  (modified after loading)
Analysis Method :
Last changed    : 18/01/2002 9.14.51
                  (modified after loading)
```



External Standard Report

```
Sorted By             :      Signal
Calib. Data Modified  :      18/01/2002 9.07.31
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 A, Sig=265,8 Ref=off, TT
```

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng inj.]	Grp	Name
17.460	MM	608.60498	2.44886e-1	149.03880		NICA-nicarbazina
Totals :				149.03880		

Results obtained with enhanced integrator!

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 23

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name: D-24116 Kiel

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 21-01-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
234214	7300	7100

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 24

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 23rd Jan 2002

Analyte: NICARBAZIN

Product: Premixture

Sample code	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
244208		6600	7000

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person: ...

Date(s) of analysis: ....27th January 2002....

Dilution factor of the sample:

- Premixture: ....2000....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ...Waters Xterra C18 (250 x 4.6) mm 5µ....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: ....
- Flow-rate: ....1.... ml/min
- Injection volume: ...50....µl
- Retention time of nicarbazin: ..8.. min

Chromatograms: Please include representative chromatograms of:

- Premixture

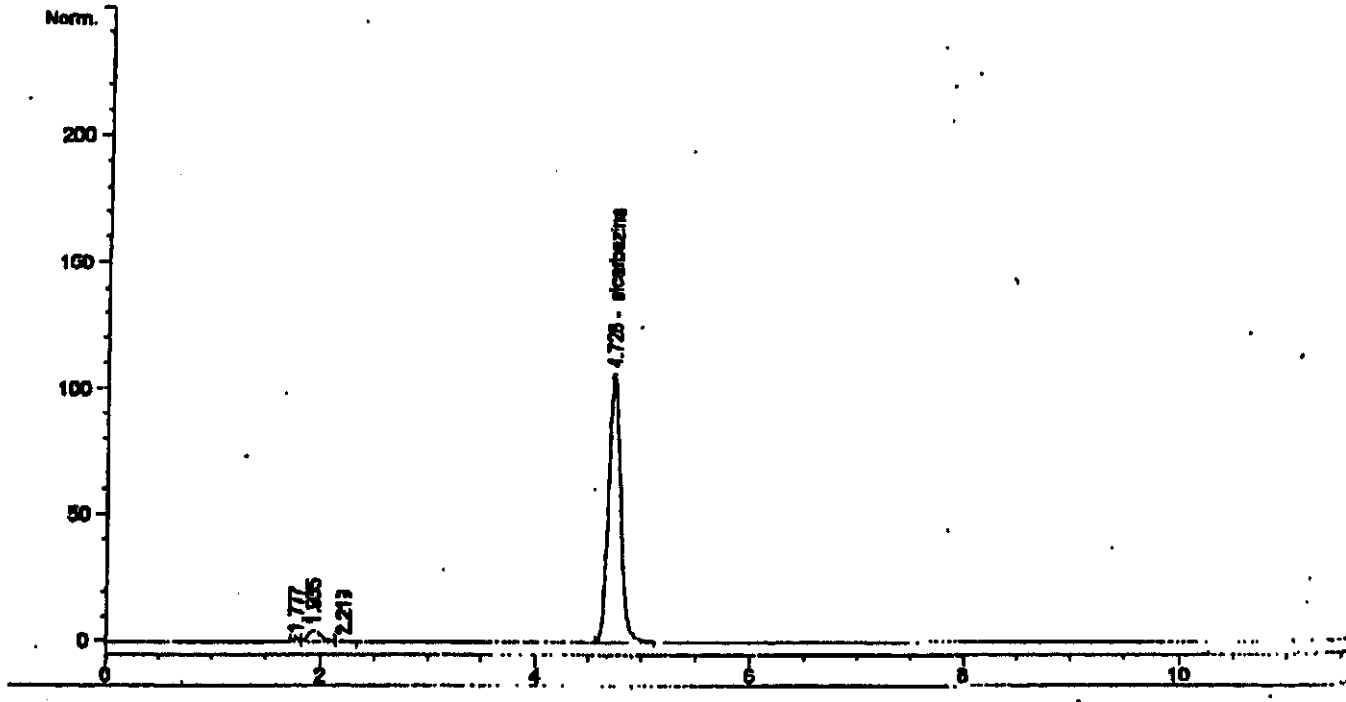
Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..102.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..96.. % and ..108.. %
- Spiking level: ..100... mg/kg

24

Injection Date :
Sample Name :
Acq. Operator :
Method :
Last changed :



External Standard Report Sample No. 244208

Sorted By : Signal
Calib. Data Modified : 23/01/02 15.07.57
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=350,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
4.728	BB	860.79669	4.09351e-3	3.52360		nicarbazina
Totals :				3.52368		

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 25

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 00-01-1900

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 mg/kg	Result 2 mg/kg
254220	7950	7950

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 23/4/2002

Dilution factor of the sample:

- Premixture: 1/50

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: LICHROCART 250-4 LICHROSPHER 100 RP 18 (5 µm)
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: H_2O/ACN 80% 30/70 v/v
- Flow-rate: 1 ml/min
- Injection volume: 50 µl
- Retention time of nicarbazin: 6,35 min

Chromatograms: Please include representative chromatograms of:

- Premixture

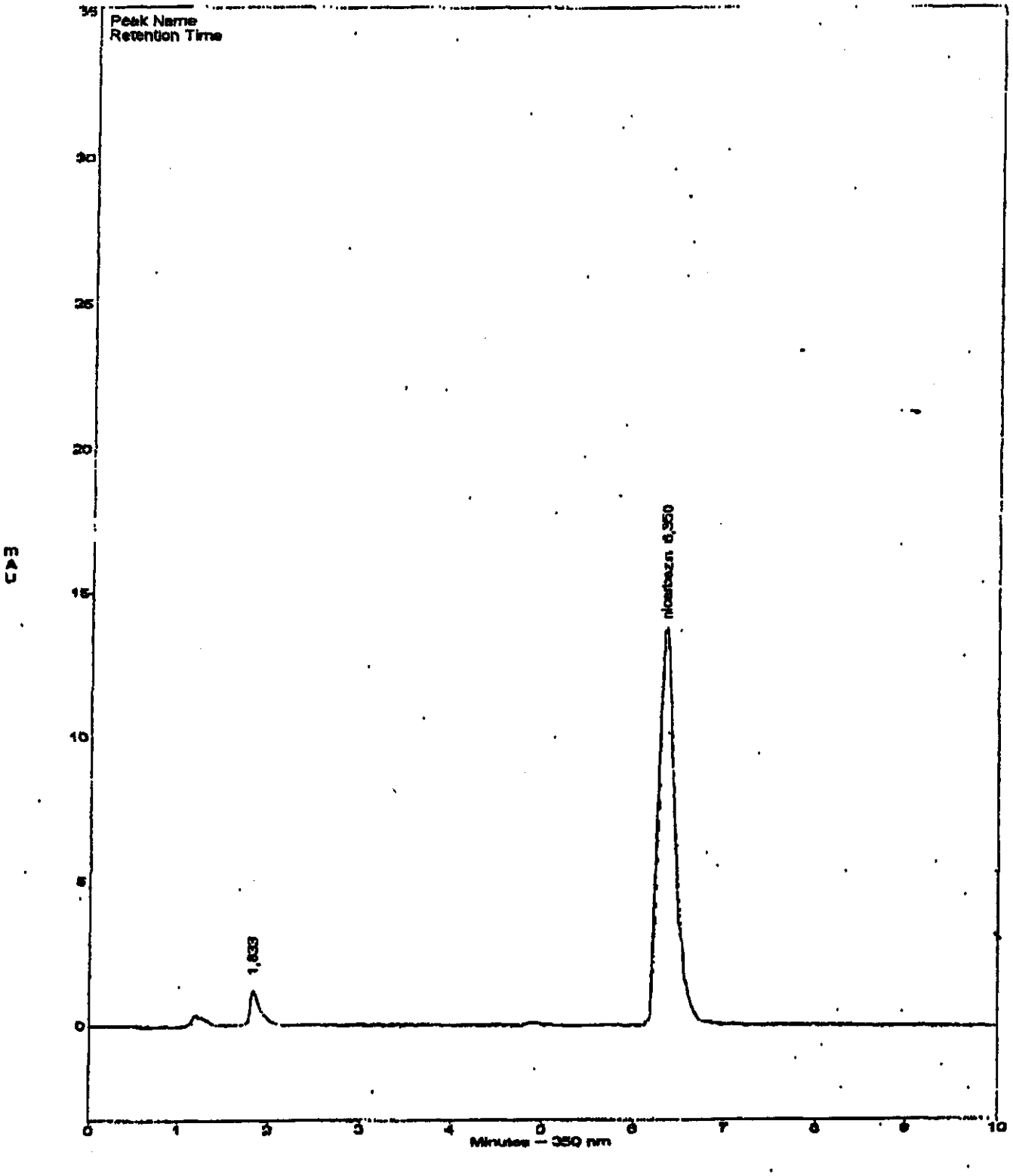
Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: 85 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 85 % and 85 %
- Spiking level: ..... mg/kg

2,5 - 5 - 7,5 - 10 - 12,5

25


Peak Name
Retention Time
35
30
25
20
15
10
5
0
mAU
1,833
nisinbazin 6,350
0
1
2
3
4
5
6
7
8
9
10
Minutes — 350 nm

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 26

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 3-1-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
264217	6387	6416

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person: .....

Date(s) of analysis: 03/01/2002

Dilution factor of the sample:

- Premixture: Injected at 10 and 20 times dilution

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: LUNA C18 (2) 5µm 250 mm x 4.6 mm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 20 µl
- Retention time of nicarbazin: 6.7 min

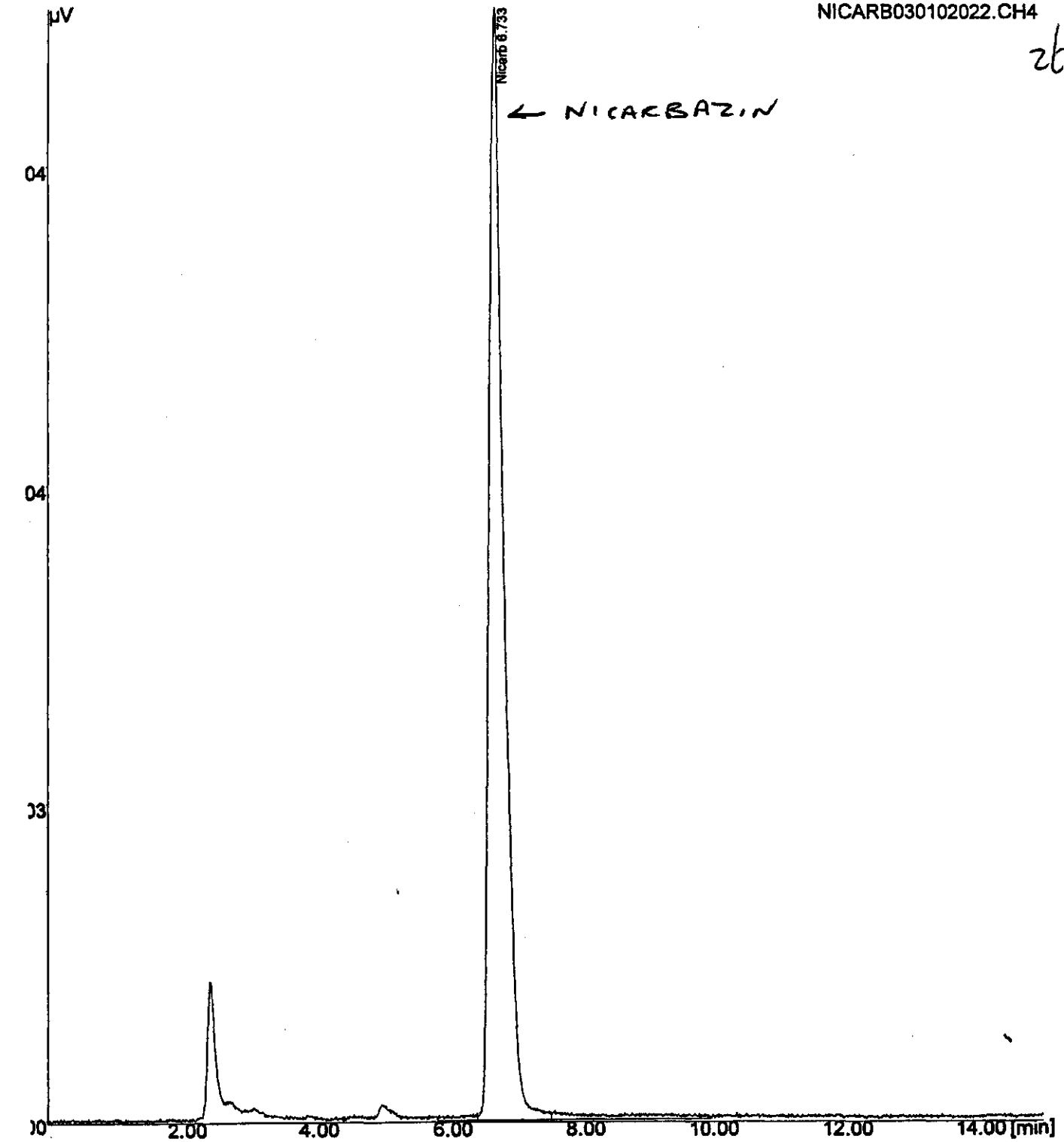
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: 98 %
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 100 mg/kg


µV
NICARB030102022.CH4
Nicarb 8.733
← NICARBAZIN
04
04
03
00
2.00
4.00
6.00
8.00
10.00
12.00
14.00 [min]

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 29

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 14.01.02

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
294226	10050	10300

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person: . .....

Date(s) of analysis .....

Dilution factor of the sample:

- Premixture: .....50.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: ..Nova-Pak C18, 4,6x250 mm, 4 µm.....
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1..... ml/min
- Injection volume: ..20....µl
- Retention time of nicarbazin: 4,62 min

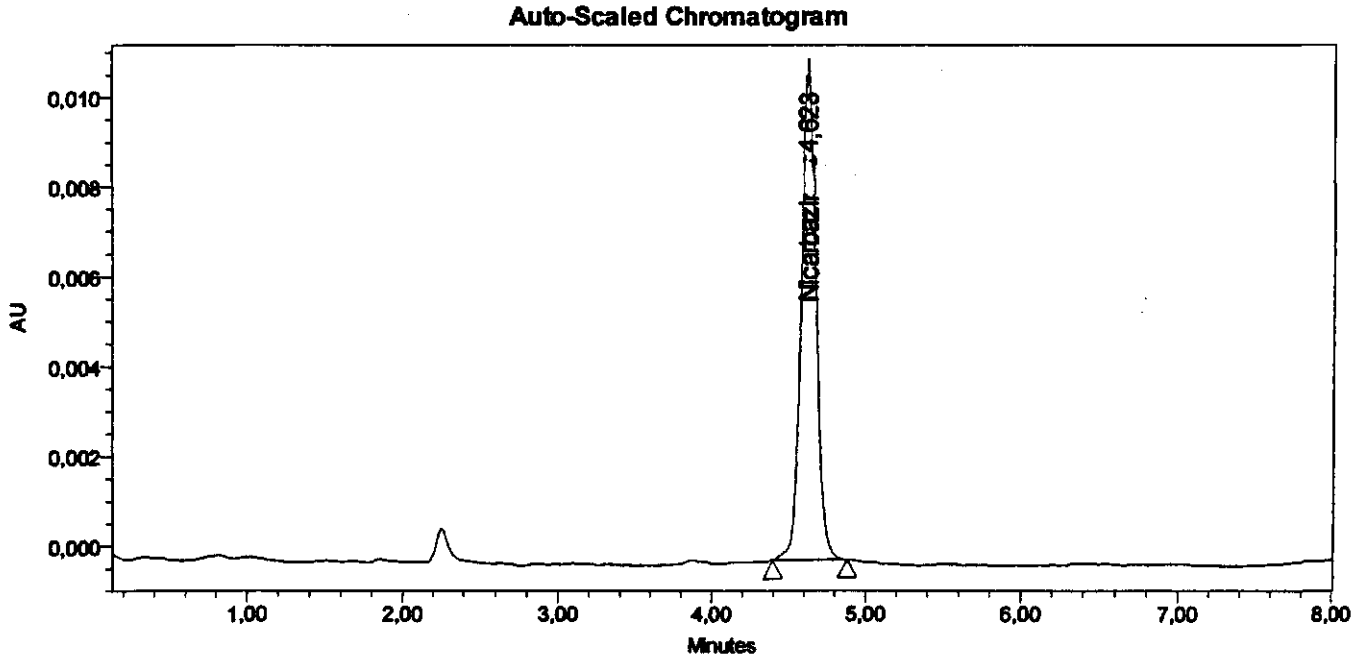
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: 100,7 % and 100,8 %
- Spiking level: ...100 mg/kg



Peak Results

RT	Area	Height	Amount	Units
4,623	73356	10937	1,005	ug/ml

10050 mg/kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 30

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 08-02-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
304224	7580	6960

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: ....

Date(s) of analysis: ....07/01/02....

Dilution factor of the sample:

- Premixture: ....N....200....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ....Kromasil....150 x 4.6 mm....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ...1.0..... ml/min
- Injection volume: 20...µl
- Retention time of nicarbazin: 5.4 min

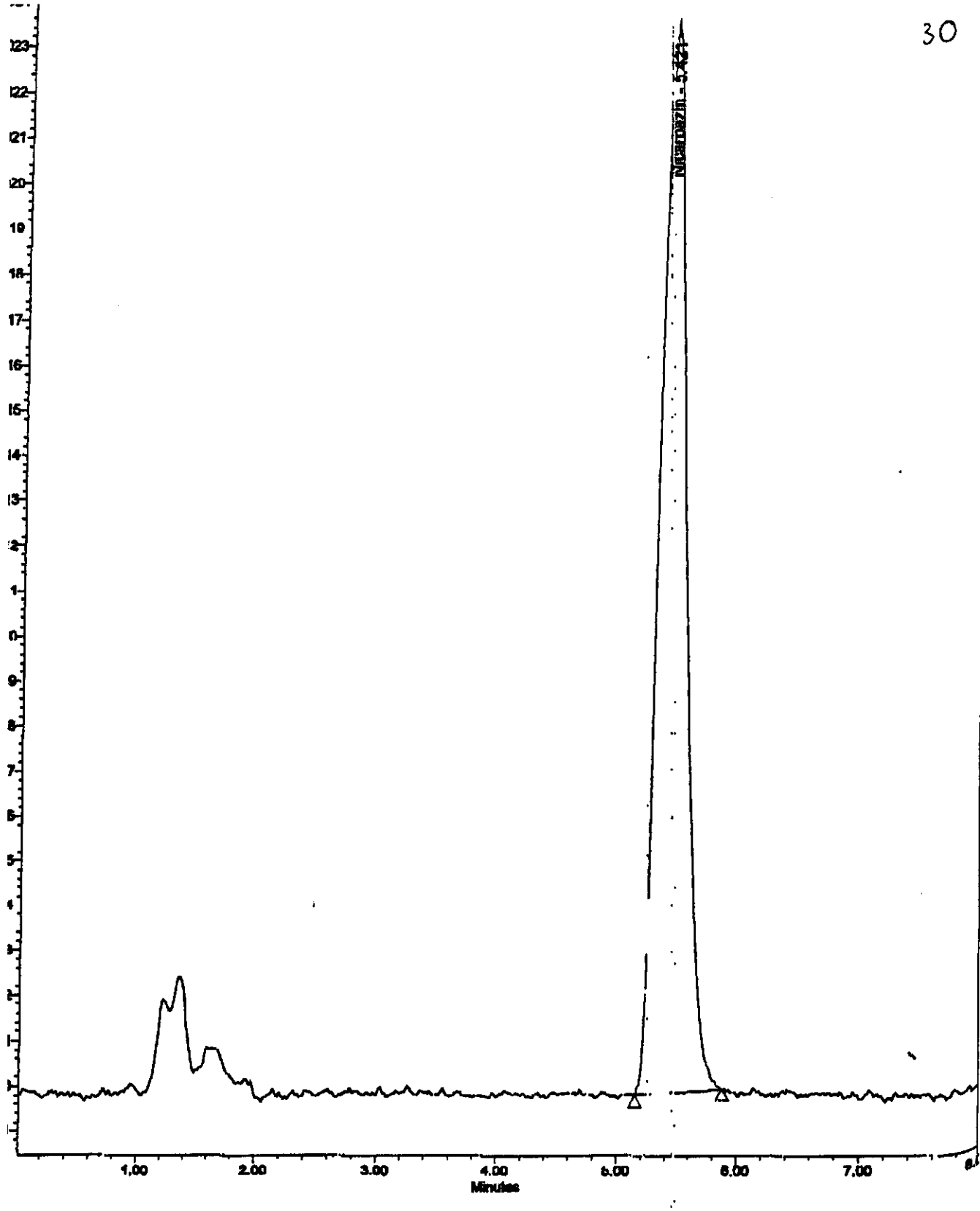
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg



premixture 304224

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 31

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 07-01-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
314212	7740	7235

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: .....07.-01.-2002.....

Dilution factor of the sample:

- Premixture: .....5.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ..Bondapack C18....;...300 x 3,9....;.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1..... ml/min
- Injection volume: ..20....µl
- Retention time of nicarbazin: .5,2. min

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: 96,7 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ....100.. mg/kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 33

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 18-12-2001

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
334203	8100	8700

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .. ..

Contact person: .....

Date(s) of analysis: .....12/12/2001.....

Dilution factor of the sample:

- Premixture: .....2g./100.ml.....2.ml/40.ml.....

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ...0,6..... ml/min
- Injection volume: ..20.....µl
- Retention time of nicarbazin: 7,1... min

Chromatograms: Please include representative chromatograms of:

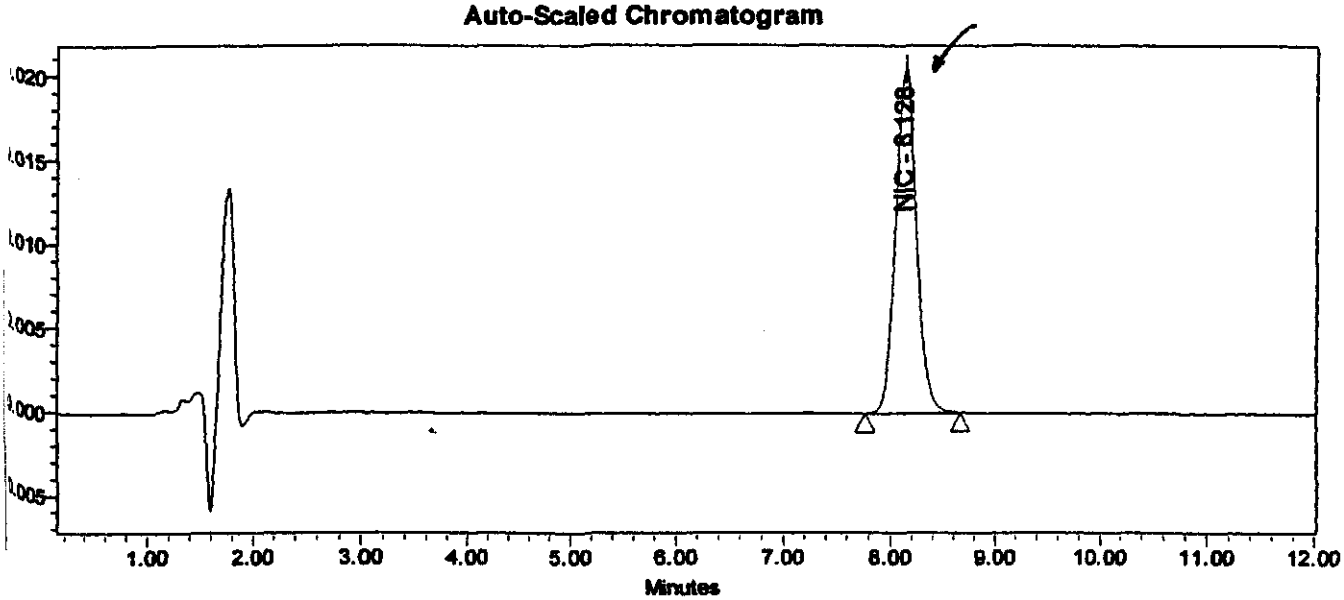
- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: ..101.. %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..125.... mg/kg

Auto-Scaled Chromatogram



Peak Results

	SampleName	Name	RT	Area	Height	Amount	Units
1	7221/01	NIC	8.128	277473	20546	0.874	mg/kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 35

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 03-01-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
354230	7486	6768

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - Questionnaire

Laboratory: .....; .....

Contact person: ....

Date(s) of analysis: .....3.1.1.02.....

Dilution factor of the sample:

- Premixture: .....25..X.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ..Merck...C8...250 mm...5 µm.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1..0..... ml/min
- Injection volume: ..20.....µl
- Retention time of nicarbazin: 4,4 min

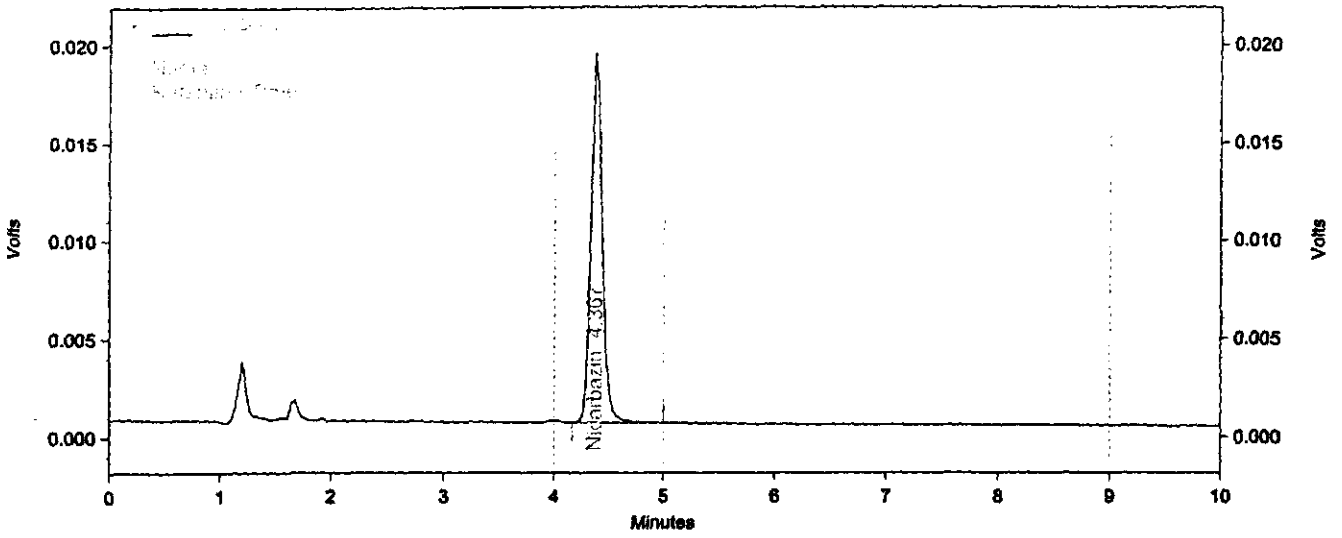
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: 100,4 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ~~98~~ % and ~~100~~ %
- Spiking level: 200000 mg/kg 99.5 101.2


0.020
0.015
0.010
0.005
0.000
Volts
Nicarbazin 4.307
0
1
2
3
4
5
6
7
8
9
10
Minutes

CANFAS COLLABORATIVE STUDIES - Nicarbazin

Annex 4 – Questionnaire

Laboratory:

Contact Person:

Date(s) of analysis: **23/01/2002**

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - **X** Other: **Lichrospher 250 x 4 mm 5 µm**
- Mobile phase:
  - ☐ As described in the method
  - **X** Other: **Isocratic AcCN/Water (65:35)**
- Flow-rate: **1,0** ml/min
- Injection volume: **10** µl
- Retention time of nicarbazin: **9** min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank samples

Please indicate the nicarbazin peak with an arrow

Recovery results:

- Percentage recovery: **90 %**
- Single / duplicate determinations: ☐ single **X** duplicate
- If duplicate, please give both percentages: **89%** and **91%**
- Speaking level: **86.4** mg/Kg (2 mL 108 ppm NCBZ in 2.5 g feedingstuff)

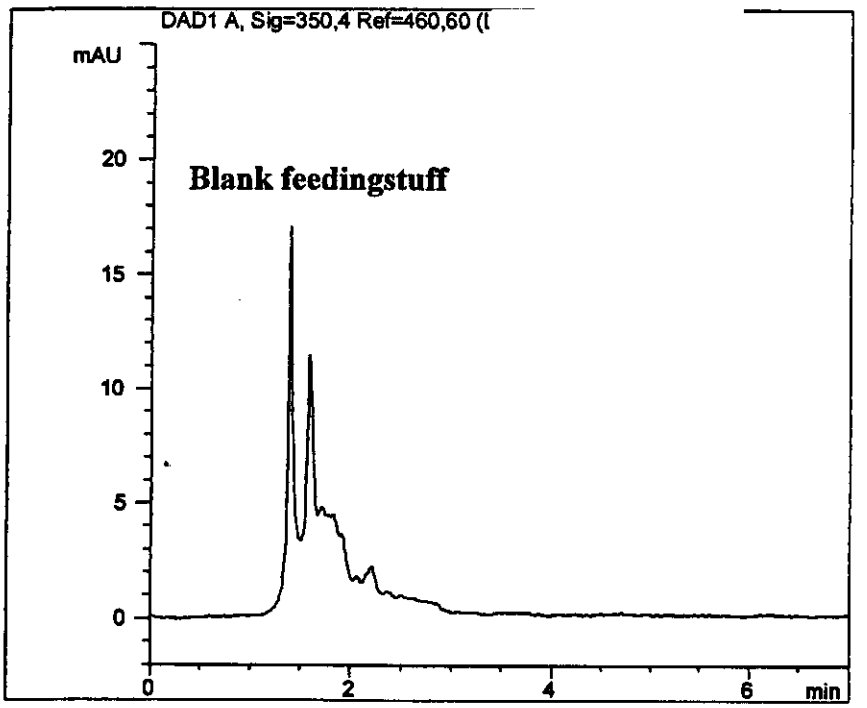
CANFAS COLLABORATIVE STUDIES - NICARBAZIN

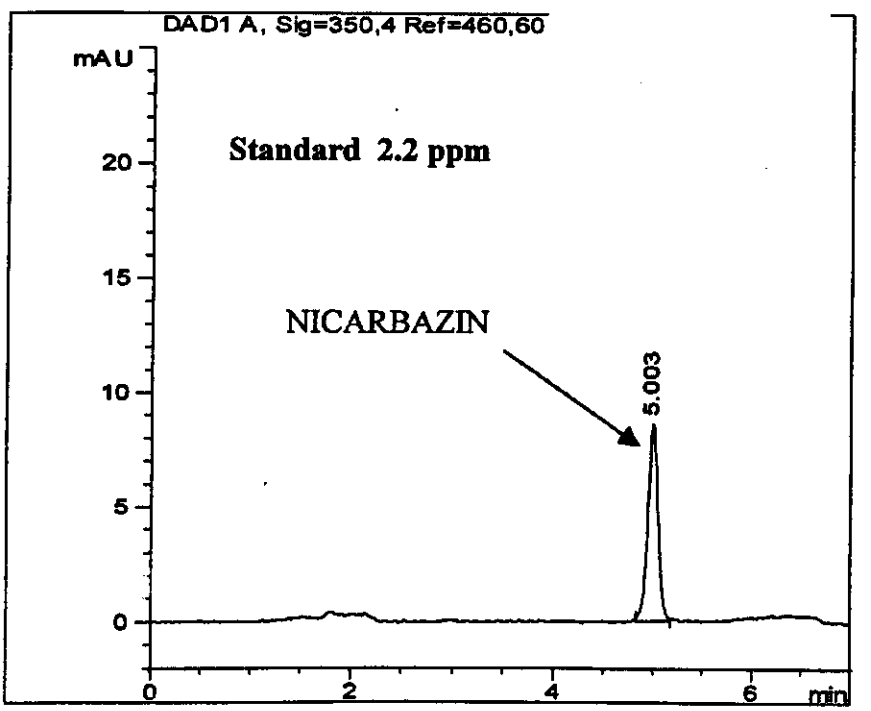
Remarks / Comments (if necessary, continue on another page):

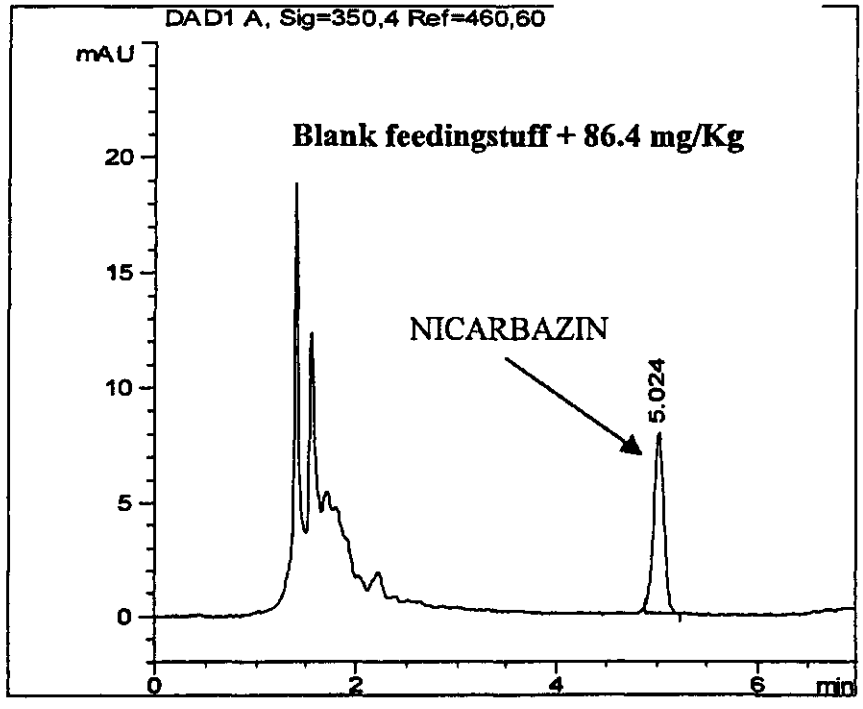
Detection limit: **4 mg/Kg**

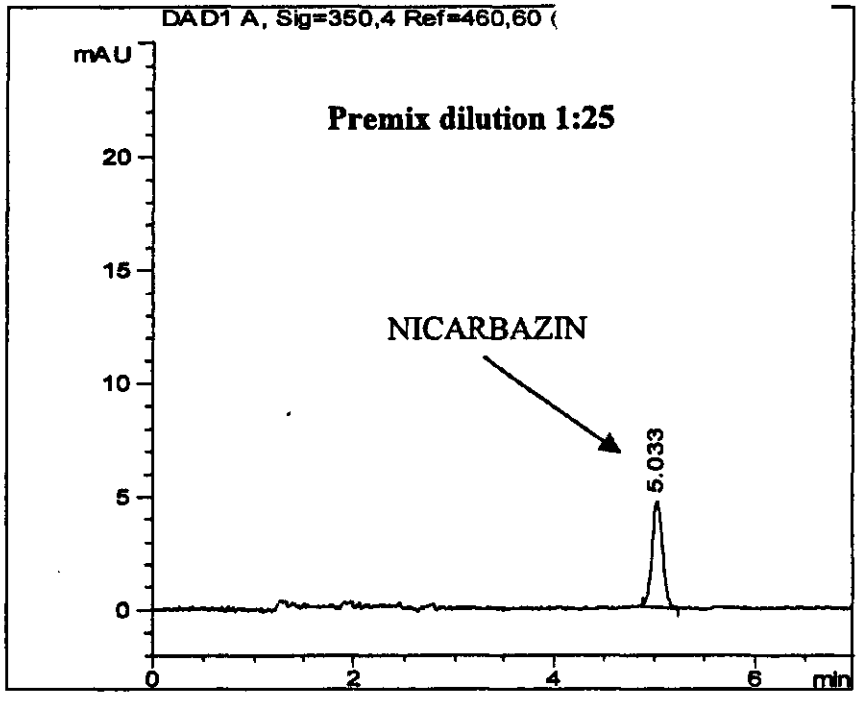
Determination Limit: **8 mg/Kg**

Representative chromatograms:






DAD1 A, Sig=350,4 Ref=460,60
mAU
Blank feedingstuff + 86.4 mg/Kg
NICARBAZIN
5.024
20
15
10
5
0
0
2
4
6
min


DAD1 A, Sig=350,4 Ref=460,60
mAU
Premix dilution 1:25
NICARBAZIN
5.033
20
15
10
5
0
0
2
4
6
min

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 39

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 14-1-2002

Analyte: NICARBAZIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
394210	8430	7664

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NICARBAZIN

Annex 4 - *Questionnaire*

Laboratory: ....

Contact person: ....

Date(s) of analysis: .....14-1-2002.....

Dilution factor of the sample:

- Premixture: .....0.5000g (VOLUMETRIC FLASK OF 200 ml + 25 g)

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ■ Other: ...125 x 3 mm, LICHROSPHER RP-18, 5 µm, MERCK 51232
- Mobile phase:
  - ■ As described in the method
  - ☐ Other: .....
- Flow-rate: ....0.5..... ml/min
- Injection volume: ..2.5....µl
- Retention time of nicarbazin: 3.4 min

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the nicarbazin peak with an arrow

Recovery results:

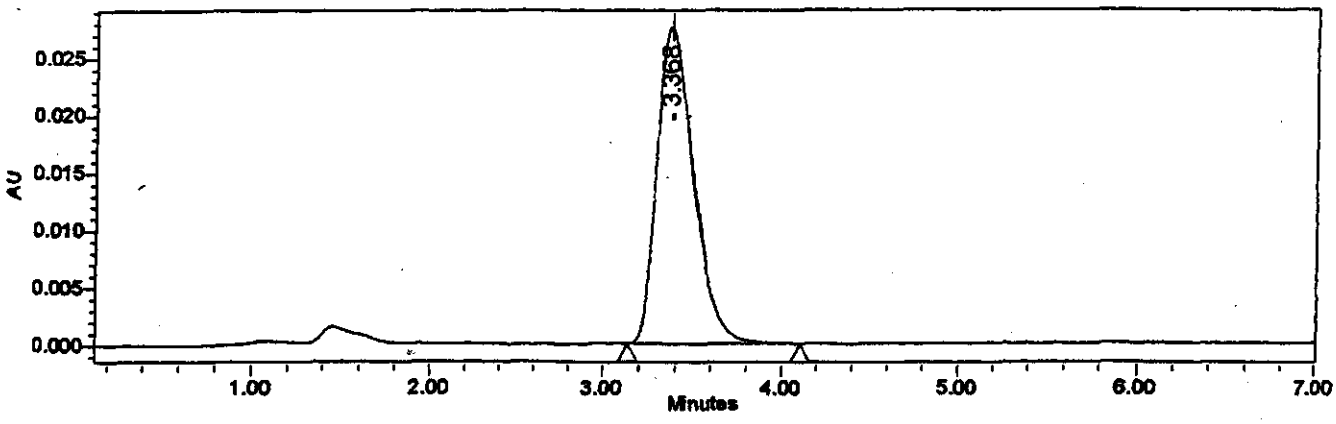
- Percentage recovery: 101. %
- Single / duplicate determinations: ☐ single ■ duplicate
- If duplicate, please give both percentages: 101. % and 102 %
- Spiking level: 25000 mg/kg

Current Date 1/14/02

Sample Information

SampleName	20011218.106 1	Sample Type	Unknown
Vial	11	Date Acquired	1/14/02 1:26:10 PM
Injection	1	Acq Method Set	
Injection Volume	25.00 ul	Processing Method	
Channel	996	Date Processed	1/14/02 4:28:23 PM
Run Time	7.0 Minutes		

Auto-Scaled Chromatogram



	Name	RT	Area	Height	Amount	Units
1		3.368	391249	27666	8430.202	mg/kg

	Name	RT	Purity1 Angle	Purity1 Threshold	Match1 Angle	Match1 Threshold	Match1 Spect. Name	Match1 Lib. Name
1	r	3.368	0.424	1.623	1.247	1.147