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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

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CANFAS - 2nd Collaborative study for the determination of narasin 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 narasin 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 method can be successfully applied to feeds with narasin contents in the range of 20-120 mg/kg, but 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 for premixtures a new small collaborative study had to be organised with a modified method.

The principle of both the original and the modified method is as follows: Narasin is extracted using a mixture of methanol and phosphate buffer (90+10) with mechanical shaking. After dilution with mobile phase and filtration through a membrane filter, narasin is determined by reverse phase HPLC using post column derivatisation with dimethylaminobenzaldehyde (DMAB) in a solution containing sulphuric acid and detection at 600 nm.

The declared content of narasin in the premixture that was prepared for the 2nd collaborative study was 1%. One single premixture was sent to the participants. The participants were asked to analyse the premixture in duplicate. Results were reported by 12 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 (rsd$_r$) and reproducibility (Horrat ratio <2).

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 narasin. Narasin is a coccidiostat which is registered for broiler feeds at contents of 40 - 50 or 60 - 70 mg/kg.
The method for feeds and premixtures was developed and validated by by LUFA - Augustenberg, Karlsruhe, Germany (see Final report on development and validation of a HPLC-method to determine narasin in feedstuffs, A. Thalmann, 29-10-1999). Subsequently, the method for feeds and premixtures was subjected to between-lab validation by the Danish Plant Directorate, Lyngby, Denmark (see report A. Pløger, 23-11-1999) and the State Laboratory, Dublin, Ireland (see report P. Shearan, January 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 narasin. Also prior to the production of the materials for the collaborative study, separate batches of the materials were produced for stability testing, indicating that narasin is stable in feeds and premixtures at room temperature for 4 months.
The results of the first collaborative study ("CANFAS - Collaborative study for the determination of narasin in feedingstuffs and premixtures by HPLC", J.J.M. Driessen, M.J.H. Tomassen, J. de Jong, RIKILT report 2002-015) 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 organised after the 1st collaborative study it was decided that a new small collaborative study had to be organised with a modified method.
The declared content of narasin in the premixture that was prepared for the collaborative study was 1%. One single sample was sent to the participants. The participants were asked to analyse the sample in duplicate.
Before the sample was shipped, the between- and within-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). Later on, an e-mail was sent to the partners with additional information. In this e-mail the participants were instructed not to take note of the moisture content of the standard and not to look at the factors D and I of narasin (see Appendix I).
This report describes the results of the 2nd collaborative study.

2 PARTICIPANTS

The following laboratories/persons participated in the collaborative study.

- Danish Plant Directorate, Lyngby, Denmark; A. Pløger, L. Junggreen
- IEEB, Bordeaux, France; J.P. Antalick, C. Fiette
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, R. Novo
- Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy; G. Biancotto, B. Allegretta
- Laboratory of the Government Chemist, Teddington, United Kingdom; J. Cowles
- LUFA-ITL Kiel, Kiel, Germany; H. Wehage, Kollwitz
- Masterlab, Putten, The Netherlands; K. van Schalm, B. Wolters
- Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland; R. Muhonen, T.Heikkinen
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Lekens, A. Voets
- 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
- State Laboratory Dublin, Ireland; P. Shearan

3 MATERIALS

3.1 Sample 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 premixture

Type of feed	Declared content	Units	Subcontractor	Date of production
Premixture	1	%	Trouw-Nutrition, Putten (NL)	October 2001

The premixture was based on inorganic material and contained regular contents of vitamins, minerals and trace elements. The composition of the premixture is enclosed in Appendix 2.
For logistic reasons, the premixture was not prepared by SDS-Trouw, Witham (UK), as in the first round of collaborative studies and in the stability trial (see report on homogeneity and stability of narasin in broiler feeds and premix, A. Thalmann, LUFA-Augustenberg, 27/06/2000), but by Trouw Nutrition, Putten, the Netherlands. Although the composition of the premixture is not exactly the same, both premixtures are based on inorganic material (ca. 50%) and for this reason the stability of narasin is sufficiently guaranteed.

The premixture was prepared in a quantity of about 3 kg. 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 LUFA Augustenberg by random selection of 10 subsamples per premixture, 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 narasin in the premixture

Results / Product	Declared content	Measured content	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Premixture	1 %	1,06 %	3,1	3,2

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 10 %. The between-sample CV fulfils these requirements. Thus, it is concluded that the premixture is sufficiently homogeneous.

3.1.3 Sample logistics

The premixture was dispatched in PE bottles, each containing approximately 100 grams. The codes of the samples are given in Appendix 3. The samples were sent to the participants by courier service from RIKILT on December 13, 2001. 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 Eli Lilly and Company. The purity of the reference standard (Lot Nr. RS 0302) is 963 mg microbiological activity per mg on an "as is" basis. The certificate of analysis is described in Appendix 4. In the covering letter sent with the samples and reference standard, the participants were instructed to take note of the microbiological potency and the moisture content of the standard. (see Appendix 1). Later on, by sending an e-mail to the participants, they were instructed not to take note of the moisture content (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 that this method has to be used without any modifications.

4.1.1. HPLC-conditions

Various types of HPLC-columns were used (the column which is recommended in the method is a Hypersil ODS C18, 250 x 4 mm Shandon, with a particle size of 4 µm).
The mobile phase described in the method is a mixture of 900 ml methanol and 100 ml phosphate buffer pH 4. As far as reported, all laboratories used this 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 4. 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
11	As described in the method	As described in the method
13	As described in the method	As described in the method
23	Not reported	Not reported
24	Spherisorb C18; 5 µm; 250x4,6 mm	As described in the method
26	Waters Spherisorb ODS-2; 5 µm; 250x4,6 mm	As described in the method
29	Nova-Pak C18; 4 µm; 4,6x250 mm	As described in the method
30	Kromasil 150 x 4,6 mm	As described in the method
31	As described in the method	As described in the method
33	As described in the method	As described in the method
35	Chromspher C18; 200x3,0 mm	As described in the method
37	Hypersil BDS C18; 5 µm; 250x4,6 mm	As described in the method
41	As described in the method	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

Originally laboratory 33 reported results that were not in agreement with the results of the other participants and that deviated much from the theoretical narasin concentration. The reported results from lab 33 were 5800 and 5800 mg of narasin/kg. Due to the magnitude of the deviations it was most likely that the results would cause outliers on both levels. Lab 33 was contacted to try to ascertain the cause of the discrepant behaviour. The lab recovered a mistake in the calculation of the results and asked to replace them by 12000 and 12000 mg/kg. Based on the explanation mentioned above it was decided to accept the new results.

The results reported by the participants are given in Table 5. Figure 1 demonstrates the Mandel h and k plots of these results.
Statistical analysis shows that the results do not contain Cochran or Grubb's outliers or stragglers. The resulting values for the statistical parameters (mean, relative standard deviation for repeatability and reproducibility) are given in Table 5. According to the Project Plan, the rsd_r-values should be ≤ 10 %. This criterion is met 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 4. The HORRAT ratio is lower than 2. Consequently it can be concluded that the reproducibility of the method is satisfactory for premixtures.

Table 4: Horrat ratios of the Narasin collaborative study

Mean (mg/kg)	Predicted rsd_R	Established rsd_R	Horrat[1]	Conclusion
10714	3,959	6,158	1,56	Reproducibility OK

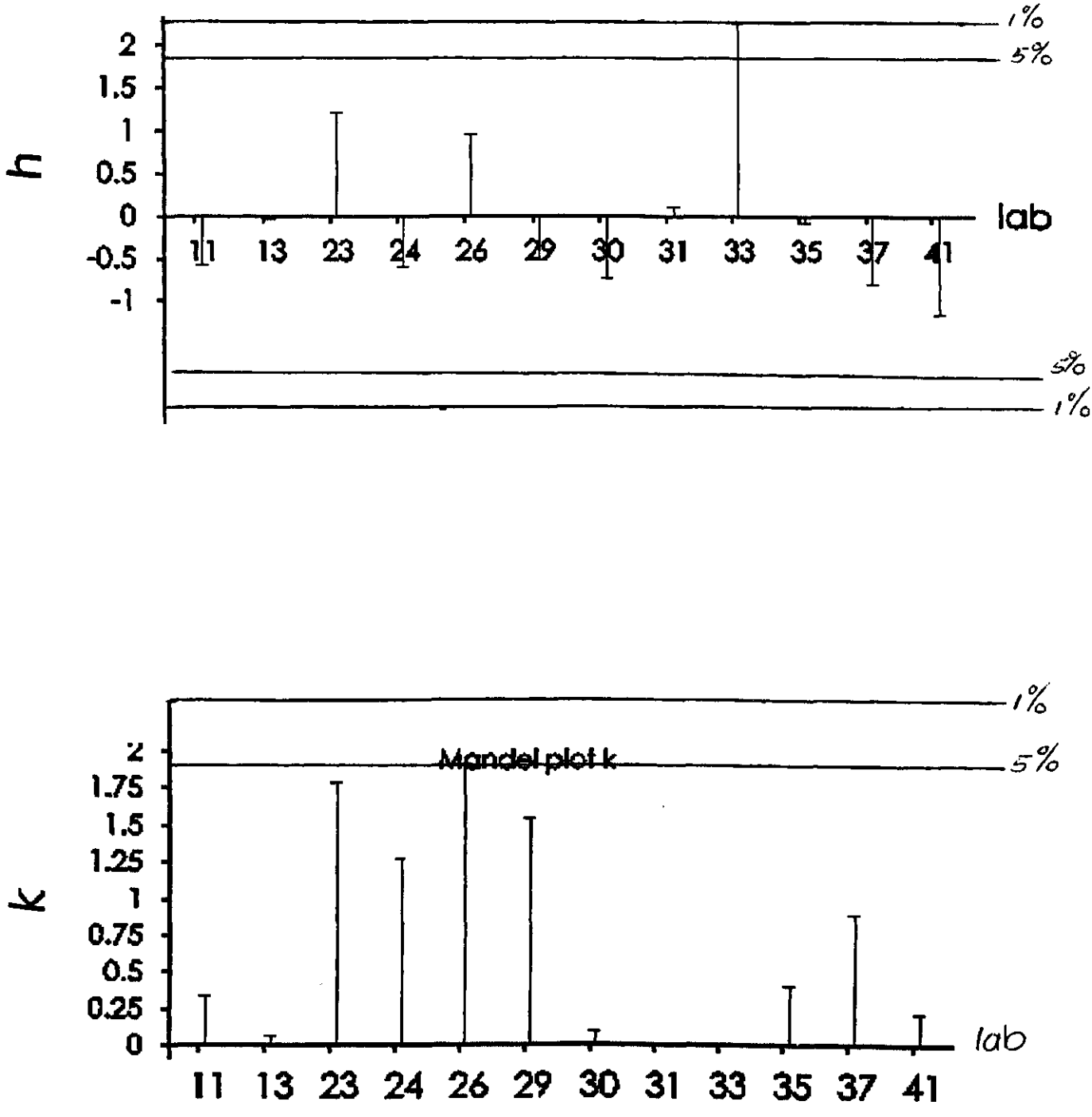
[1] Horrat is the ratio between the established rsd_R and the predicted rsd_R

Table 5: Results reported by the participants

		Result (mg/kg)	
Lab	Sample	NAR 10000 mg/kg	
11	113106	10267	10495
13	133123	10667	10713
23	233121	12000	10800
24	243117	10801	9950
26	263124	11887	10612
29	293107	10935	9902
30	303118	10320	10260
31	313114	10779	10778
33	333103	12000	12000
35	353122	10812	10539
37	373115	10560	9960
41	413116	9976	10122

Summary of all results	
number of labs	12
m (mg/kg)	10714
rsd_r (%)	4,415
rsd_R (%)	6,158

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



5.2 Recoveries

Table 6: Recoveries

Partner	Spiking level (mg/kg)	Recovery 1 in %	Recovery 2 in %	recovery average in %
11	50	90	112	101
13	Not reported	Not reported	Not reported	Not reported
23	Not reported	Not reported	Not reported	Not reported
24	50	96		96
26	200	99		99
29	50	102	99	101
30	Not reported	Not reported	Not reported	Not reported
31		102		102
33	50	103		103
35	1000	106	105	106
37	50	89	90	90
41	100	98	101	100

The laboratories reported recoveries of 89% or higher. In task 1 and 2 of the project (within- and between-lab validation) recoveries of 86% and higher were measured.

5.3 Remarks

Table 7: Remarks made by the partners

Partner	Remarks
11	No remarks.
13	No remarks.
23	Not reported
24	The pump for the reagent of the derivatisation had technical problems.
26	We injected the premixtures at 50 and 100 times dilutions 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	No remarks.
30	No remarks.
31	We calculated with a microbiological activity of 963 mcg/mg. The moisture content of 2.13% was not taken into account.
33	No remarks.
35	No remarks.
37	No remarks.
41	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 (rsd$_r$) and reproducibility (Horrat ratio <2). So, it can be concluded that the modified method is suitable for premixtures.
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

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Eli Lilly and Company, Mr. S. Ready, is thanked for supplying the narasin 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 five annexes) and e-mail with additional information

Participants CANFAS collaborative study Narasin

Dear colleague,

As agreed at the CANFAS evaluation meeting June 20th, 2001 at Tervuren a second round of collaborative study for narasin 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: NARASIN" and with a sample code. The sample contains narasin 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.
- a second questionnaire (annex 5) regarding the factors of narasin

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 2000 by mr. Towell (Eli Lilly) has expired you receive together with the premixture a new Eli Lilly reference standard of narasin, lot number RS0302, that has to be used at the analyses. Please take note of the microbiological potency and the moisture content of this standard (see Annex 5).

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

DATE
13 December 2001

SUBJECT
CANFAS collaborative
narasin (71316.24)

ENCLOSURE(S)
4

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Annex 1 - the modified method of analysis

Determination of Narasin with High Performance Liquid Chromatography (HPLC)

1 Scope

The method serves for the quantitative determination of Narasin sodium in feedstuffs, premixtures and concentrates. The limit of determination is 20 mg/kg, the limit of detection 1 mg/kg.

2 Principle

Narasin is extracted using a mixture of methanol and phosphate buffer (90+10) with mechanical shaking. After dilution and filtration through a membrane filter narasin is determined by reverse phase HPLC using post column derivatisation with dimethylaminobenzaldehyde in a solution containing sulphuric acid and detection at 600 nm.

3 Reagents

3.1 Methanol - HPLC grade

3.2 di-potassiumhydrogenphosphate, waterfree

3.3 di-potassiumhydrogenphosphate solution, c (K_2HPO_4) = 0.05 mol/l water

3.4 Potassiumdihydrogenphosphate, waterfree

3.5 Potassiumdihydrogenphosphate solution, c (KH_2PO_4) = 0.01 mol/l water

3.6 1,5-dimethylhexylamine (6-methyl-2-heptylamine, $C_8H_{19}N$)

3.7 Ortho-phosphoric acid, w (H_3PO_4) = 85 %

3.8 Sulphuric acid, w (H_2SO_4) = 95-97 %

3.9 4-(dimethylamino)-benzaldehyde (DMAB, $C_9H_{11}NO$)

3.10 Extraction solvent: 900 ml methanol (3.1) are mixed with 100 ml di-potassiumhydrogenphosphate solution (3.3).

3.11 Phosphate buffer: To 500 ml solution of potassiumdihydrogenphosphate (3.5) 3.0 ml o-phosphoric acid (3.7) and 10.0 ml 1,5-dimethylhexylamine (3.6) are added. The pH is adjusted to 4.0 with o-phosphoric acid, and the solution is made up to 1000 ml with water.

3.12 Mobile phase: 900 ml methanol (3.1) are mixed with 100 ml phosphate buffer (3.11). The solution is degassed prior to use in an ultrasonic bath (8.3) during 15 min.

3.13 Methanol-sulphuric acid: 40 ml sulphuric acid (3.8) are given cautiously while stirring to 950 ml methanol (3.1). The solution is degassed prior to use in an ultrasonic bath (8.3) during 15 min.

3.14 DMAB-solution: 60.0 g dimethylaminobenzaldehyde (3.9) are solved in 950 ml methanol (3.1). The solution is degassed prior to use in an ultrasonic bath (8.3) during 15 min.

3.15 Narasin-sodium reference standard (monocarboxylic acid-polyether-sodium salt, $C_{43}H_{71}NaO_{11}$) with defined microbiological activity and factor composition.

3.16 Narasin-stock solution, 250 µg/ml
An amount of Narasin-sodium (3.15) equivalent to 25.00 mg microbiological activity is solved in 100 ml methanol (3.1). The solution is stable for 4 weeks if kept at >0 - < 10 °C.

4 Apparatus

4.1 HPLC-system consisting of:

4.1.1 Pump - pulse free, flow capacity 0.1-2.0 ml/min

4.1.2 Injection system, manual or autosampler with loop suitable for 100 µl injections

4.1.3 Post-column reactor (double pump or two single pumps) with mixing chamber, reaction coil of inert material (f.e. Teflon or Peek) for operation at **93 °C**, 7.0 m with 0.33 mm ID and water bath or reactor oven for operation at **93 °C**

4.1.4 VIS-detector, variable wavelength, suitable for measurements at the wavelength of 600 nm

4.1.5 Analytical column - 4 µm C18 Hypersil ODS, 250 x 4 mm f.e. Shandon or equivalent (8.2)

4.2 Magnetic stirrer or mechanical shaker

4.3 Ultrasonic water bath

4.4 Membrane filter of Teflon, pore diameter 0.45 µm

4.5 Commercially available equipment

5 Procedure

5.1 General

5.1.1 Blank feed

For the performance of the recovery test (5.1.2) a blank feed should be analysed to check that neither Narasin nor interfering substances are present. The blank feed should be similar in type to that of the sample and Narasin or interfering substances should not be detected.

5.1.2 Recovery test

A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of Narasin, similar to that present in the sample. To fortify at a level of 50 mg/kg transfer 4 ml of the stock solution (3.16) to a conical flask and evaporate the solution to approximately 0.5 ml. Add 20 g of the blank feed, mix thoroughly and leave for 10 minutes mixing again several times before processing with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of Narasin similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery can be calculated by subtraction.

5.1.3 Mixing of the test sample before weighing

The container should be filled to a maximum of 50 % of its total volume. Bring the container in a horizontal position and rotate bottom and top in circles moving it up and down along its virtual centre for about 30 seconds. Put the container in an upright position and wait a few seconds to let the generated dust settle.

5.2 Extraction

5.2.1 **Premixtures**

5.0 g are weighed into a 500-ml-Erlenmeyer flask, 200 ml extraction solvent (3.10) added, treated 5 min in the Ultrasonic water bath and stirred on a magnetic stirrer or shaken on a mechanical shaker (4.2) for 1 h. Let settle the coarse particles. The extract is diluted subsequently with mobile phase (3.12) to 4.0 µg/ml and filtered through a membrane filter (4.4).

5.2.2 **Final feeds**

20.0 g are weighed into a 250-ml-Erlenmeyer flask, 100 ml extraction solvent (3.10) added, treated 5 min in the Ultrasonic water bath and stirred on a magnetic stirrer or shaken on a mechanical shaker (4.2) **for 1 h.** Let settle the coarse particles. The extract is diluted subsequently to 1.0 µg/ml with mobile phase (3.12) and filtered through a membrane filter (4.4).

5.3 HPLC procedure

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

Narasin is separated on a reversed phase column (4.1.5), detected and its concentration measured after post-column reaction (4.1.3) with a UV-Detector (4.1.4) at 600 nm.

A aliquot of the sample solution (5.2), f.e. 100 µl is injected on the separation column and eluted with the mobile phase (3.12). The mean heights of the peaks resp. the areas of several injections of the calibration solutions (5.4.2) are measured.

HPLC-conditions

Column (4.1.5)	Hypersil ODS, 250 x 4 mm, 5 µm
Mobile phase (3.12)	Mixture of 900 ml methanol (3.1) + 100 ml phosphate buffer (3.11)
Flow rate of mobile phase	0.7 ml/min
Flow rate of methanol-sulphuric acid-mixture (3.13)	0.4 ml/min
Flow rate of DMAB-solution (3.14)	0.4 ml/min
Temperature of the post-column reaction	**93 °C**
VIS-Detector after post-column reaction	600 nm
Volume of injections	100 µl
Calculation	Height or area of peak

5.4 Calibration curve

5.4.1 Preparation of the working standard solution: **10** ml the stock solution (3.16) are diluted with the extraction solvent (3.10) to **100** ml. The concentration of narasin-sodium is w = 25 µg/ml. The solution is stable for 4 weeks if kept at >0 - < 10 °C.

5.4.2.1 Preparation of the calibration solution for concentrations 200 mg/kg: 5.0, 10.0, 20.0 and 40.0 ml of the working standard solution (5.4.1) are pipetted into a 100-ml-volumetric flask each, filled up with mobile phase (3.12) and mixed. The concentration of narasin-sodium corresponds to = 1.25; 2.50; 5.00, and 10.00 µg/ml.

The calibration solutions have to be prepared daily.

5.4.2.2 Preparation of the calibration solution for concentrations 200 mg/kg: 1.0, 2.0, 4.0 and 8.0 ml of the working standard solution (5.4.1) are pipetted into a 100-ml-

volumetric flask each, filled up with mobile phase (3.12) and mixed. The concentration of narasin-sodium corresponds to = 0.25, 0.50, 1.00 and 2.00 µg/ml.

The calibration solutions have to be prepared daily

5.4.3 Preparation of the calibration curve

100 µl each of the calibration solutions (5.4.2) are injected and the mean height or area of the peaks of several injections measured. Under the above conditions the retention time of narasin is approximately 19 min.

6 Calculation

The concentration of narasin-sodium is calculated in mg/kg microbiological activity from the mean height or area of the peak of factor A in sample solution (5.3) and the calibration curve (5.4.3) based on the assumption that the relation of microbiological activity to content of factor A is the same in the feed additive and in the standard.

The content w in the sample is calculated from the concentration received respecting weigh and dilution by means of the following formula:

$$w = \frac{V * b * F}{E} \quad \text{mg/kg.}$$

V = volume of extractant in ml **(200 ml for premixtures (see 5.2.1)** and 100 ml for final feeds (see 5.2.2))
b = concentration of the sample solution in µg/ml microbiological activity of narasin-sodium
E = weigh of the sample in g
F = factor of dilution

7 Statistics

(Will follow)

8 Remarks

8.1 Extraction

Due to the addition of di-potassiumhydrogenphosphate to the extractant solvent with most of the samples it is possible to let stand the extracts over night **at room temperature performing dilution and chromatography the following day.** Since it may occur - especially in premixtures and mineral feeds - that there is a slight breakdown of narasin the analysis has to be repeated with shaking of the extract for not more than 1 hour before chromatography.

In a few feedstuffs it was observed that unknown compounds interfered with the retention time and peak shape in chromatograms when low concentrations (< 20 mg/kg) of narasin were present. To overcome this difficulty 10 g of Alumina 90 (Merck 1.01097 or equivalent) were added to the weigh.

If interfering pharmaceutical agents are present the following procedure **may be** applied:

Weigh 20.0 g sample into 250 ml Erlenmeyer flask. Add 100 ml hexane, stopper and shake for at least one hour on a wrist-action shaker. Filter sample solutions through 42 Whatman filter or equivalent into 125-ml-Erlenmeyer flask. Pipet 20.0 ml of extract and evaporate to dryness on the nitrogen evaporator. Dissolve the residue in 20.0 ml of extraction solvent. Introduce this solution into a prepared column with 10 g Alumina 90. Filter a portion of the eluate before proceeding to the HPLC analysis.

8.2 Separation material

Baseline separation between narasin factor A and salinomycin must be obtained. Hypersil ODS 5 mm in a 250 x 4 mm steel column has been proven as the best one. It is possible to separate narasin from other polyether antibiotics and to get the peaks of the 4 main factors. Inertsil and Purospher can be recommended if there is doubt whether narasin is separated from other compounds. The retention times are longer than with Hypersil.

8.3 Protection against corrosion

All fittings, which come in contact to the methanol-sulphuric acid-mixture (3.13), should be made from Teflon, Peek or comparable material.

8.4 Post-column reaction

If only one pump for the post-column reaction is available the reagents 3.13 and 3.14 may be mixed. Since DMAB undergoes quick auto-oxidation resulting in darkening of the solution this has to be kept protected from light in an ice bath and has to be used within 24 h.

9 Literature

BLANCHFLOWER, W.J., RICE, D.A and HAMILTON, J.T.G., 1985: Simultaneous high-performance liquid chromatographic determination of monensin, salinomycin and narasin in feeds using post-column derivation. Analyst, 110, 1283-1287

CAMPBELL, H., 1989: HPLC analysis of monensin, salinomycin and narasin in feeds, using post-column derivation. Antibiotic and drug workshop, 103rd annual international meeting of the AOAC, St. Louis, USA

JOHANNSEN, F. H, 1990: Zur Analytik von Wirkstoffen. 1. Bestimmung von Ionophor-Antibiotika und Sulfonamiden in Futtermitteln und Lebensmitteln mittels Hochleistungsflüssigkeitschromatographie und Post-column-reaction. VDLUFA Kongressband, S. 487

JOHANNSEN, F. H., 1991: Zur Analytik von Wirkstoffen. 2. Bestimmung von Ionophor-Antibiotika in Futtermitteln und Lebensmitteln mittels Hochleistungsflüssigkeitschromatographie und Post-column-reaction. Agribiol. Res. 44, 79

SCHÜEP, W. and STEINER, K., 1988: Determination of Lasalocid (sodium) in complete feeds and premixes with HPLC on reversed phase; in "Analytical methods for vitamins and carotenoids in feeds"; Hrsg. KELLER, H. E.; Department of vitamin research and development. ROCHE, Basel

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: NARASIN

Product: Premixture

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

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 3 - Instructions for handling of the premixture sample

1. Storage

Store the sample at room temperature until analysis. Protect the samples from light.

2. Milling (see par. 5.1)

The sample must not be milled!!

3. Mixing of the test samples 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 - NARASIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

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

Chromatographic conditions:

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

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) 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 !

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 5 - Questionnaire 2 (narasin factors), page 1

Eli Lilly reference standard profile for Narasin

Effective date:	March 29, 2001
Expiry date:	March 28, 2003
Lot number:	RS0302
Defined potency:	963 mcg microbiological activity per mg on an 'as is' basis; 85.4% factor A, 1.9% factor D and 0.7% factor I on an 'as is' basis.
Handling:	Refer to current MSDS for handling and caution information.
Storage:	125 mg quantities in heat sealed amber glass ampoules with argon overlay at freezer temperature, -10 to –25 degrees C.
Water:	2.13%

Annex 5, page 2 - Questionnaire 2 (narasin factors)

Laboratory: .....

Contact person: .....

Sample code: .....(please complete one annex for each sample)

Weigh standard:

Microbiological activity standard:

Moisture content standard:

Concentration of the stock standard solution (in microbiological activity):

Dilution standard:

Weigh sample:

Dilution sample:

The concentration of narasin is calculated from the peak of factor A (see paragraph 6 of the method). However, we ask you to give information on the area of the peaks of the factors D and I as well (the peaks of factors D and I are indicated in the chromatogram attached). If you cannot detect the peaks of these factors in the standards or samples, please indicate with ND (= non detected).

Factor A retention time (min)	
Standard[a]	
Sample	
Factor A peak height or area	Height / area[b]
Standard [.../(µg/ml)][c]	
Sample	
Content determined via factor A	
Factor D + I peak area (combined)	Area
Standard [.../(µg/ml)][d]	
Sample	

[a]give the range of retention times for the calibration solutions
[b]indicate if you measured peak height or area
[c]give the mean peak height / area for the calibration solution containing 1 µg/ml, as derived from the calibration curves
[d]give the mean peak area for the calibration solution containing 1 µg/ml, as derived from the calibration curves

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 11:45
Aan: Antalick, J; Biancotto, Giancarlo; Cowles, John; Felgueiras, Ilidia; Fontaine, André; Haustraete, Karel; Henk van der Kamp; Muhonen, Raija; Nunes Costa, José; Pløger, Annette; Shearan, Paula; Thalmann, Alfred; Van Schalm, Klaas; Wehage, Hubert
CC: Jong, Dr. J. de
Onderwerp: Additional information 2nd coll. study Narasin
Urgentie: Hoog

Dear participant,
With our covering letter 30596 of December 13th, 2001 concerning the 2nd collaborative study for narasin in a premixture we asked you to take note of the moisture content of the reference standard. **This is a mistake!** In agreement with the first ringtest the moisture content of the newly supplied standard **should not taken into account!!**

A second remark concerns annex 5 regarding the factors for narasin. From the first collaborative study it is concluded that calculations only should be based on factor A. This means that **only page 1 of annex 5 is of interest**; so do not pay attention to pages 2 and 3.

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

APPENDIX 2

Composition and homogeneity of the premixture

Recept: 3 27-560.2
Fx UNIVERSEEL-MIX 1 % Versie: 2
Ingangsdatum:14-03-00

Kleur: Bruin Geur: Neutraal
Verpakking: 20.00 KG
Poedervormig Voormengsel voor Voeders voor: Diverse Diersoorten

Inmengingspercentage: 1.000%

+ 1% Narasin

Berekende analyse:

Naam	Per kg Premix		Per kg Eindvoer	
Stortgewicht	+0.756	kg	+0.008	kg
*Vitamine A	+1000000.000	IE	+10000.000	IE
*Vitamine D3	+200000.000	IE	+2000.000	IE
*Vitamine E	+2001.000	IE	+20.010	IE
Vitamine K3	+200.500	mg	+2.005	mg
Vitamine B1	+150.000	mg	+1.500	mg
Vitamine B2	+400.000	mg	+4.000	mg
d-Pantotheenzuur	+1200.000	mg	+12.000	mg
Niacine	+2501.000	mg	+25.010	mg
Biotine	+5000.000	mcg	+50.000	mcg
Vitamine B12	+2000.000	mcg	+20.000	mcg
Foliumzuur	+50.000	mg	+0.500	mg
Vitamine B6	+200.000	mg	+2.000	mg
Choline Chloride	+20001.000	mg	+200.010	mg
*IJzer	+7500.000	mg	+75.000	mg
*Koper	+1000.000	mg	+10.000	mg
*Zink	+5500.000	mg	+55.000	mg
*Mangaan	+6001.000	mg	+60.010	mg
*Cobalt	+20.000	mg	+0.200	mg
*Jodium	+80.000	mg	+0.800	mg
*Selenium	+30.000	mg	+0.300	mg
Cd Tot.	+0.517	mg	+0.005	mg
Pb Tot.	+16.845	mg	+0.168	mg
Ruw Eiwit	+93.537	g	+0.935	g
Ruw Vet	+20.586	g	+0.206	g
Ruwe Celstof	+47.282	g	+0.473	g
As	+460.000	g	+4.600	g
Vocht	+68.881	g	+0.689	g
Lysine	+3.208	g	+0.032	g
Methionine	+1.253	g	+0.013	g
Meth+Cys	+2.958	g	+0.030	g
Threonine	+2.657	g	+0.027	g
Tryptophaan	+1.103	g	+0.011	g
Isoleucine	+2.557	g	+0.026	g
Zetmeel Ewers	+117.496	g	+1.175	g
Suiker	+25.065	g	+0.251	g
Suiker/Zetmeel	+142.561	g	+1.426	g
Calcium totaal	+105.859	g	+1.059	g
Fosfor totaal	+4.512	g	+0.045	g
Magnesium totaal	+25.359	g	+0.254	g
Kalium totaal	+6.560	g	+0.066	g
Natrium totaal	+1.021	g	+0.010	g
Chloor totaal	+5.236	g	+0.052	g
oP'97 plv	+1.203	g	+0.012	g
oP'97 leg	+1.203	g	+0.012	g
OE slk	+681.759	kcal	+6.818	kcal
OE leg	+979.527	kcal	+9.795	kcal
OE plv	+960.979	kcal	+9.610	kcal
V.LysPLV	+2.082	g	+0.021	g
V.MetPLV	+0.863	g	+0.009	g
V.M+CPLV	+1.938	g	+0.019	g
V.ThrPLV	+1.581	g	+0.016	g
V.TryPLV	+0.808	g	+0.008	g
Besch.P	+1.053	g	+0.011	g
b.P.plv	+1.053	g	+0.011	g
b.P.leg	+1.053	g	+0.011	g
VEM	+419.081		+4.191	
VEVI	+439.634		+4.396	
DVE	+21.536	g	+0.215	g

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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 : Narasin
Product : Premixture: 1.0%

Date of determination : November 21th, 2001

Sample	Content (%)	Duplicate average %
133101-a	1,0130	1,04
133101-b	1,0644	
133105-a	1,0255	1,05
133105-b	1,0735	
133112-a	1,0775	1,06
133112-b	1,0364	
133119-a	1,0955	1,12
133119-b	1,1365	
133125-a	1,0877	1,06
133125-b	1,0285	
133126-a	1,0345	1,08
133126-b	1,1166	
133127-a	1,0703	1,07
133127-b	1,0620	
133128-a	1,0485	1,07
133128-b	1,0907	
133129-a	1,0216	1,03
133129-b	1,0371	
133130-a	1,0520	1,07
133130-b	1,0961	

Homogeneity OK Crtiterion : $CV_{between}$ = < 7%		
Average	**1,06**	
SD (between samples)	**0,033**	
CV (between samples)	**3,1%**	Result Grubb's test
Grubb's test, single lower	**1,190**	**no outlier**
Grubb's test, single upper	**2,143**	**no outlier**
Grubb's test, double lower	**0,5693**	**no outliers**
Grubb's test, double upper	**0,3262**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,034**
CV (within samples)	(CV (%))	**3,2**

APPENDIX 3

Sample codes

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

Lab	premixture 1%
11	113106
13	133123
23	233121
24	243117
26	263124
29	293107
30	303118
31	313114
33	333103
35	353122
37	373115
41	413116

APPENDIX 4

Narasin reference standard profile

CONFIDENTIAL
DISTRIBUTION TO LILLY PERSONNEL ONLY

ELI LILLY AND COMPANY
PRODUCT DEVELOPMENT OPERATIONS DIVISION

REFERENCE STANDARD PROFILE

* Effective Date: March 29, 2001
* Expiry Date: March 28, 2003
* Supersedes Date: November 2, 2000

Compound: 079891
Revision: 22

Name: Narasin
Lot Number: RS0302
Defined Potency: 963 mcg microbiological activity per mg on an 'as is' basis; 85.4% factor A, 1.9% factor D, and 0.7% factor I on an 'as is' basis (See Note).

Handling: Refer to current MSDS for handling and caution information.
Storage: 125 mg quantities in heat sealed amber glass ampoules with argon overlay at freezer temperature, -10 to -25 C.

* Evolution: Lot RS0302 (bulk lot H93-NXK-394235678 was reevaluated in March 2001.

	Tests	Results
(x)	*HPLC Assay (Method B01795)	949 mcg/mg on an 'as is' basis versus lot RS0306 (n=18)
	*HPLC Factor Composition, Factor A (Greenfield Ntbk. NB-NBK-1879)	85.0% vs. narasin factor A reference standard lot RS0306 (n=21)
	*HPLC Factor Composition, Factor B (Greenfield Ntbk. NB-NBK-1879)	None detected vs. narasin factor B reference standard lot RS0297 (n=21)
	*HPLC Factor Composition, Factor D (Greenfield Ntbk. NB-NBK-1879)	2.2% vs. narasin factor I reference standard lot RS0305 (n=21)
	*HPLC Factor Composition, Factor I (Greenfield Ntbk. NB-NBK-1879)	0.6% vs. narasin factor I reference standard lot RS0305 (n=21)
	HPLC Factor Composition, Unknown (Greenfield Ntbk. NB-NBK-1503)	0.4% vs. narasin factor I reference standard lot RS0305 (n=128)
(x)	Microbiological Assay (Method B00175, pH 5.2)	913 mcg per mg vs. narasin factor A reference standard lot RS0306 (n=62)
	Cation Screen, Ion Chromatography (Method RP29)	Li+, NH4(+), K(+), Mg(2+), and Ca(2+) were not detected (n=1)
	Anion Screen, Ion Chromatography (Method RP35)	F-, NO2(-), NO3(2-), Br(-), PO4(3-), and SO4(2-) were not detected (n=1)

	Metal Screen, ICP (Method RP40)	Al, B, Ba, Bi, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Pb, Pd, Pt, Sb, Si, Sn, V, Zn, and Zr were not observed at the 0.08% level; Ca detected but was much less than 0.08% (n=1)
(x)	*X-ray Pattern (Method RP18)	Pattern compares favorably to the previous pattern of this lot; material is crystalline (n=1)
	1H NMR Spectrum	Spectrum is consistent with the structure and has been filed for future reference (n=1)
	13C NMR Spectrum	Spectrum is consistent with the structure and has been filed for future reference (n=1)
	TGA (Method RP9)	The thermograms show a weight loss beginning at 25 C which results in a 2.0% loss at 113 C (n=8)
	Water, KF (Method RP19)	2.13 % (n=72)
	Fatty Acids, GC	3.9 % (n=1)

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

The microbiological potency was determined from the overall average of the initial HPLC and autoturb assays performed by GL757, GL791, and CL44C (n=188, Greenfield Ntbk. NB-NBK-1503)

* Revised March 21, 2001

Beverly J. Krabel

Revision 22

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 11

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: 04-02-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
113106	10267	10495

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnair

Laboratory: .....
Contact person: .....

Date(s) of analysis: 4-02-02

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....0.7..... ml/min
- Injection volume: ..100.....µl

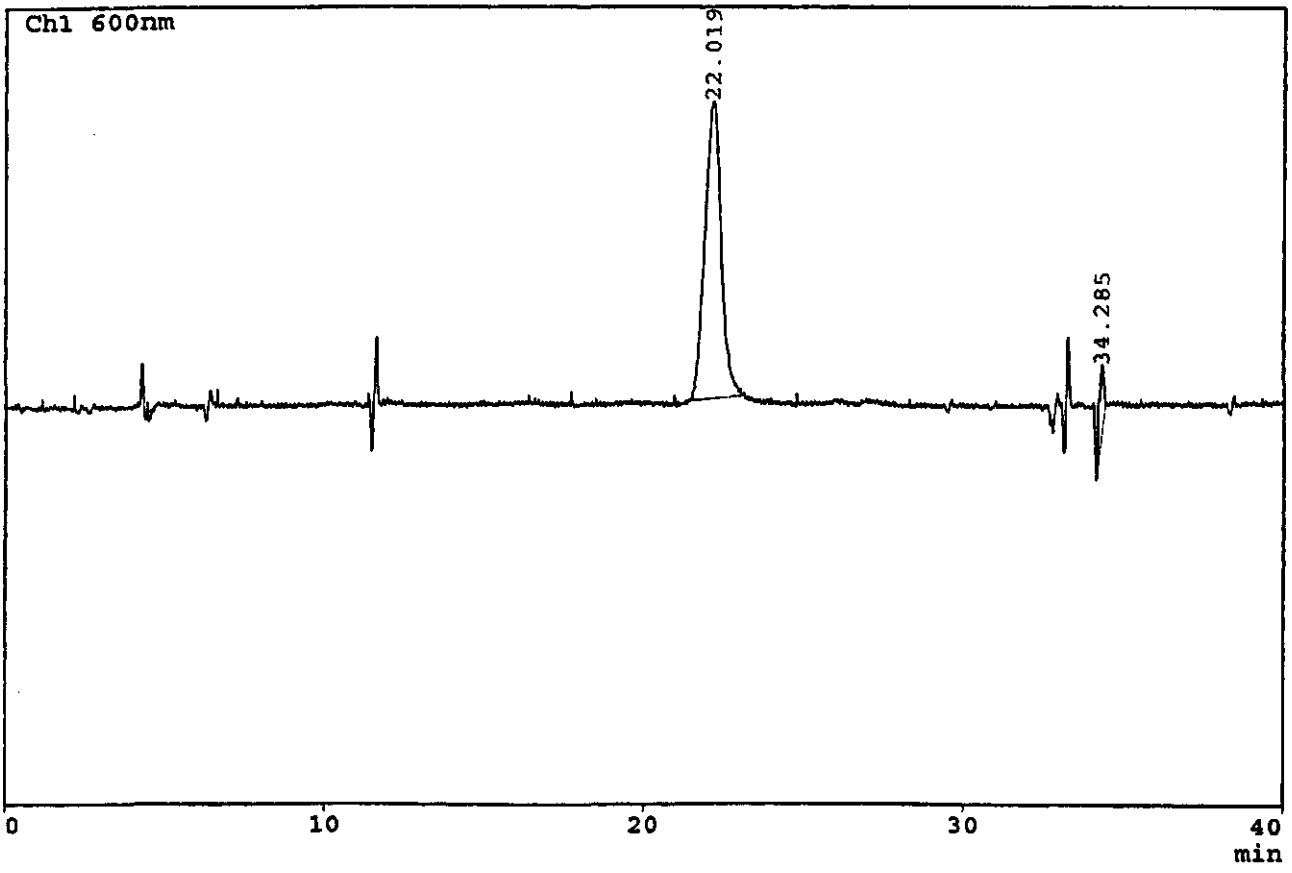
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 101... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..90.. % and 112.. %
- Spiking level: ....50..... mg/kg



ak Report ***

hNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN	IDNO	CONC
1	22.019	53050	S	0.9984(0.9587)	0.9990(0.9722)	1	10603.3477
		53050					10603.3477

APPENDIX 5

Table with results, questionnaire (page 1) 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: 03-01-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
133123	10667	10713

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person: .....

Date(s) of analysis: 03-01-02

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,7 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Premixture

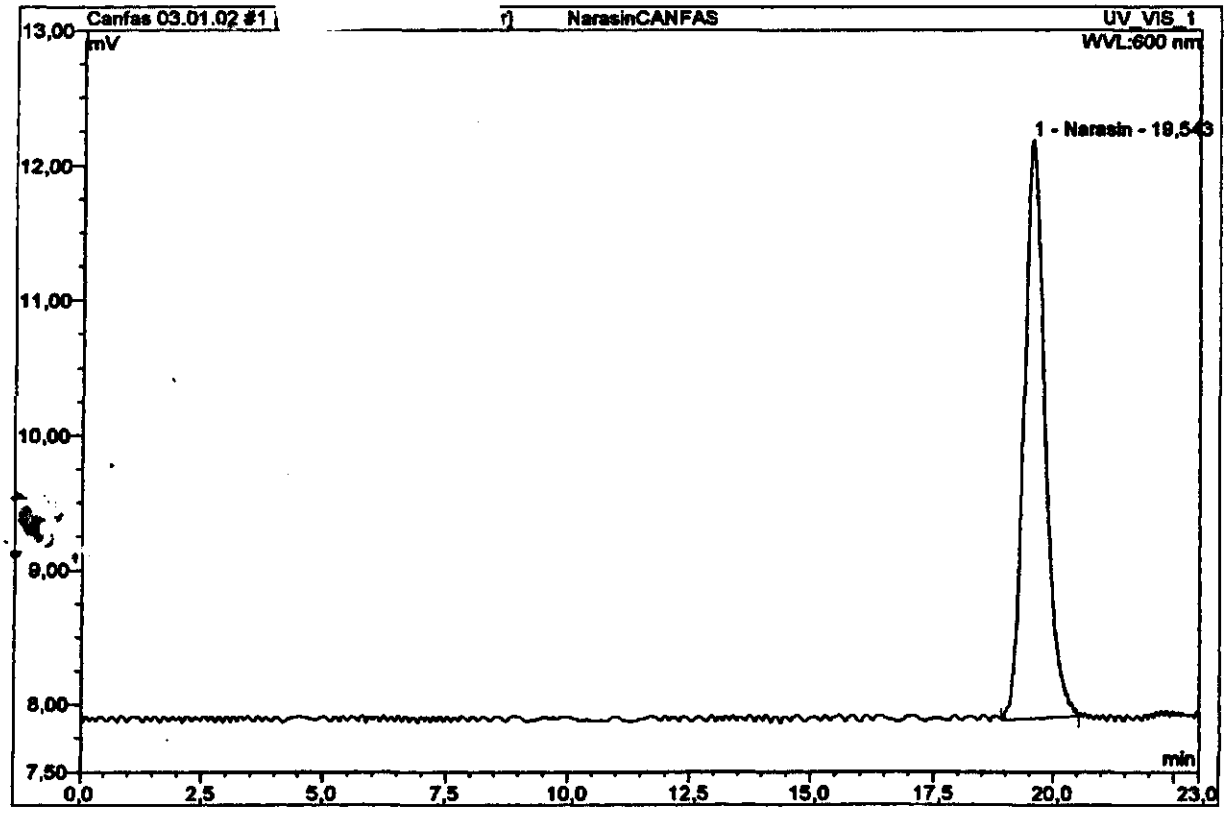
Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

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

1 NarasinCANFAS

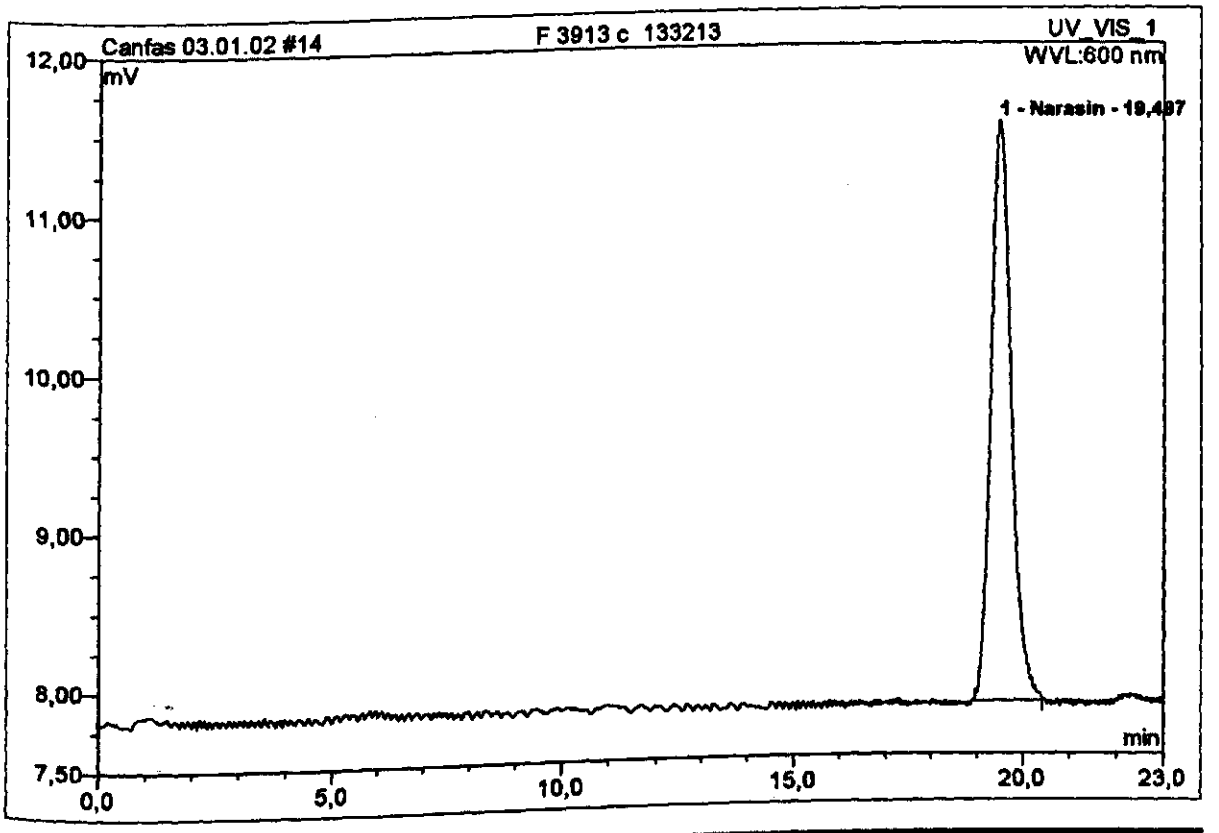
Sample Name:	**NarasinCANFAS**	*Injection Volume:*	**100,0**
Vial Number:	**1**	*Channel:*	**UV_VIS_1**
Sample Type:	[redacted]	*Wavelength:*	**600**
Control Program:	**NarasinCANFAS**	*Bandwidth:*	**n.a.**
Quantif. Method:		*Dilution Factor:*	**1,0000**
Recording Time:	**3.1.02 10:48**	*Sample Weight:*	**1,0000**
Run Time (min):	**23,00**	*Sample Amount:*	[redacted]000



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount mg/kg	Type
1	19,54	Narasin	4,302	2,215	100,00	496,953	BMB*
Total:			4,302	2,215	100,00	496,953	

14 F 3913 c 133213

Sample Name:	F 3913 c [redacted]	*Injection Volume:*	100,0
Vial Number:	6	*Channel:*	UV_VIS_1
Sample Type:	unknown	*Wavelength:*	600
Control Program:	NarasinCANFAS	*Bandwidth:*	n.a.
Quantif. Method:	--	*Dilution Factor:*	1,0000
Recording Time:	3.1.02 16:01	*Sample Weight:*	400,0000
Run Time (min):	23,00	*Sample Amount:*	1,0000



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount %	Type
1	19,49	Narasin	3,685	1,888	100,00	1,064	BMB
Total:			3,685	1,888	100,00	1,064	

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:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 07-01-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
233121	12000	10800

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: 22nd Jan 2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
243117	10801	9950

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 22nd January 2002

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: Spherisorb C18 5µm (250 x 4.6) mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0.9 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 96 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 50 mg/kg

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: 9-1-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
263124	11887	10612

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person: .....

Date(s) of analysis: 7-9/1/02

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: WATERS SPHERISORB ODS-2 5µm 250mm x 4.6mm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 100 µl

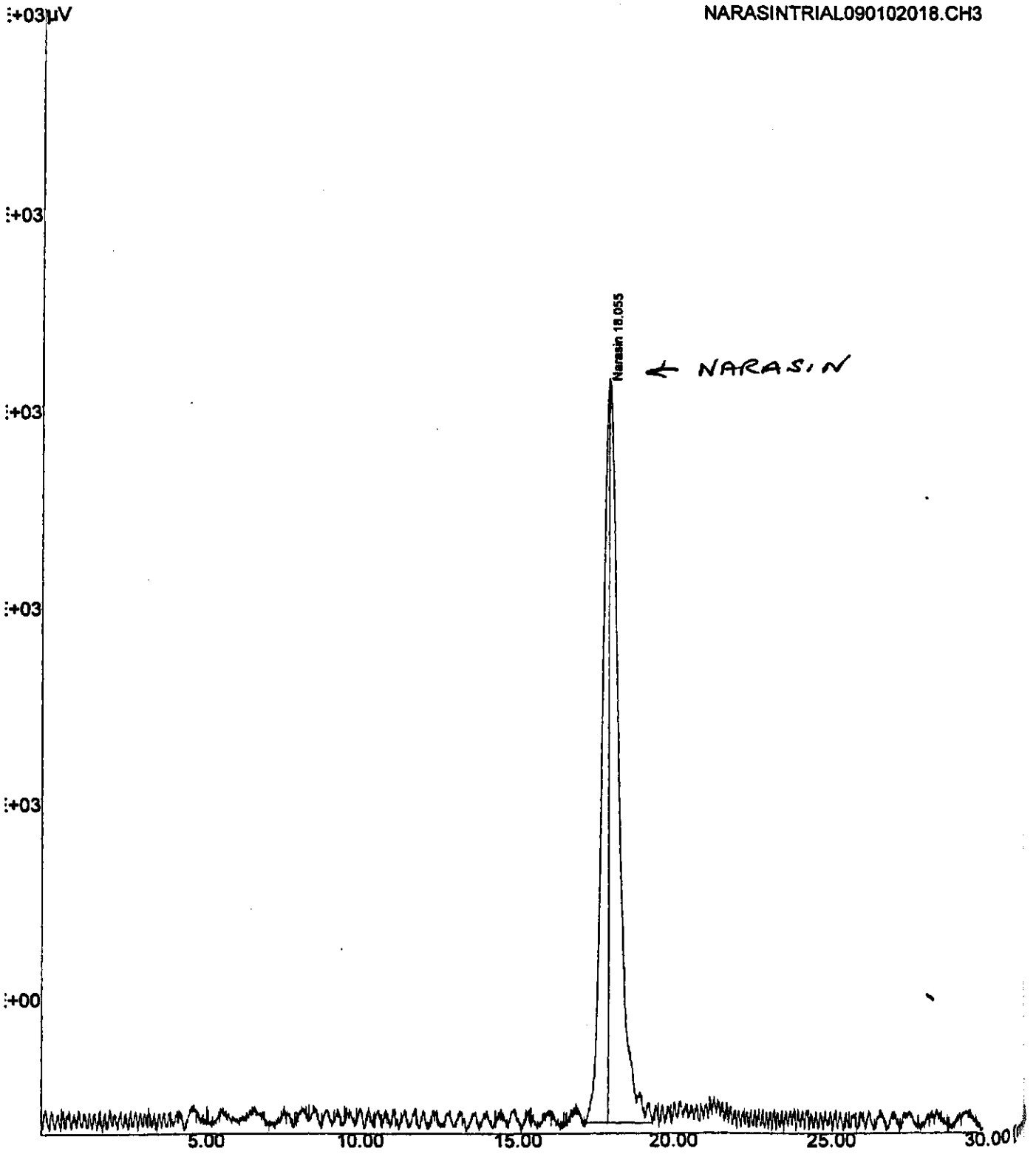
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 99 %
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 200 mg/kg


NARASINTRIAL090102018.CH3
µV
+03
+03
+03
+03
+03
+00
Narasin 18.055
NARASIN
5.00
10.00
15.00
20.00
25.00
30.00

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: 18.01.2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
293107	10935	9902

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

.....

.....

Date(s) of analysis: .....18....January 2002.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: .....Nova-Pak C18 4,6×250mm 4µm.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....0,9..... ml/min
- Injection volume: ...100...µl

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

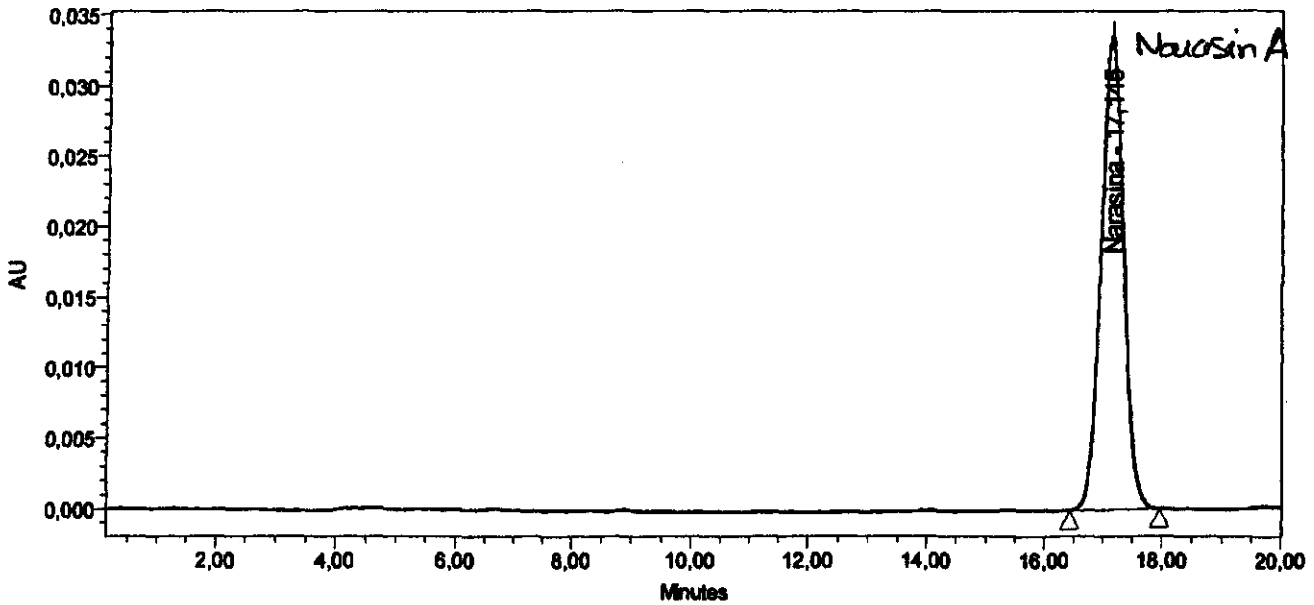
- Percentage recovery: 100,7 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 102,3% and 99,1 %
- Spiking level: ...50... mg/kg

Project Name: Narasin_CANFAS

SAMPLE INFORMATION

Sample Name:	Premixture 293107 I dil2/50	Processing Method:	Nar 18_01_2002
Vial:	35	Run Time:	20,0 Minutes
Injection #:	1	Proc. Chnl. Descr.:	PDA 600,0 nm
Injection Volume:	100,00 ul		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Narasina	17,145	904316	33574	10,935	ug/ml

Result : mg/kg narasin

$$C = \frac{V \times A \times D}{w}$$

A - amount (ug/ml)
D - dilution factor
w - weight
V - volume

A = 10,935 µg/ml
D = 25
w = 5g
V = 200 ml

C 10935 mg/kg narasin

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: 21-01-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
303118	10320	10260

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: ...

Date(s) of analysis: ....21/01/02.....

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: ...0,8..... ml/min
- Injection volume: 100...µl

Chromatograms: Please include representative chromatograms of:

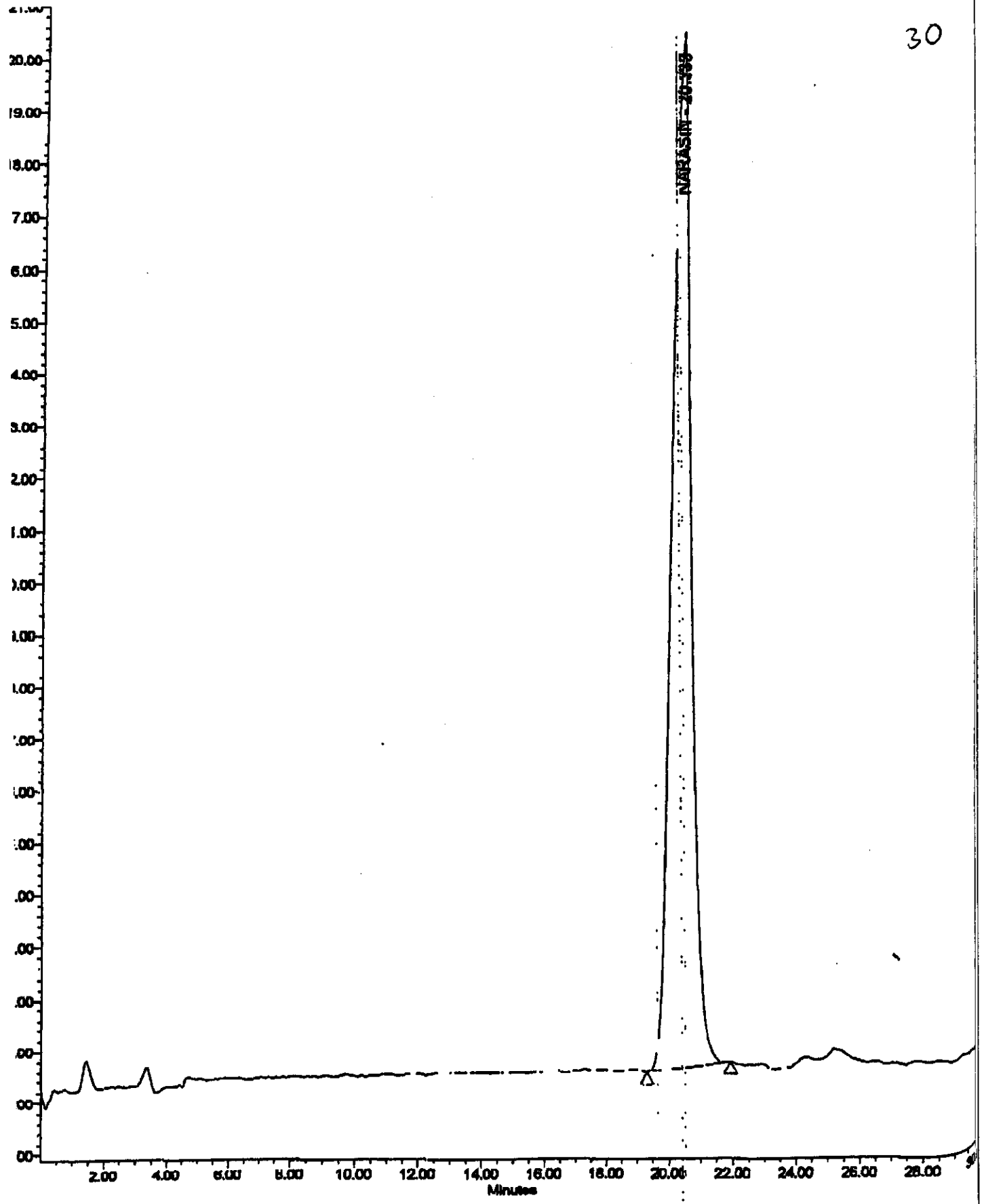
- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5A with an arrow

Recovery results:

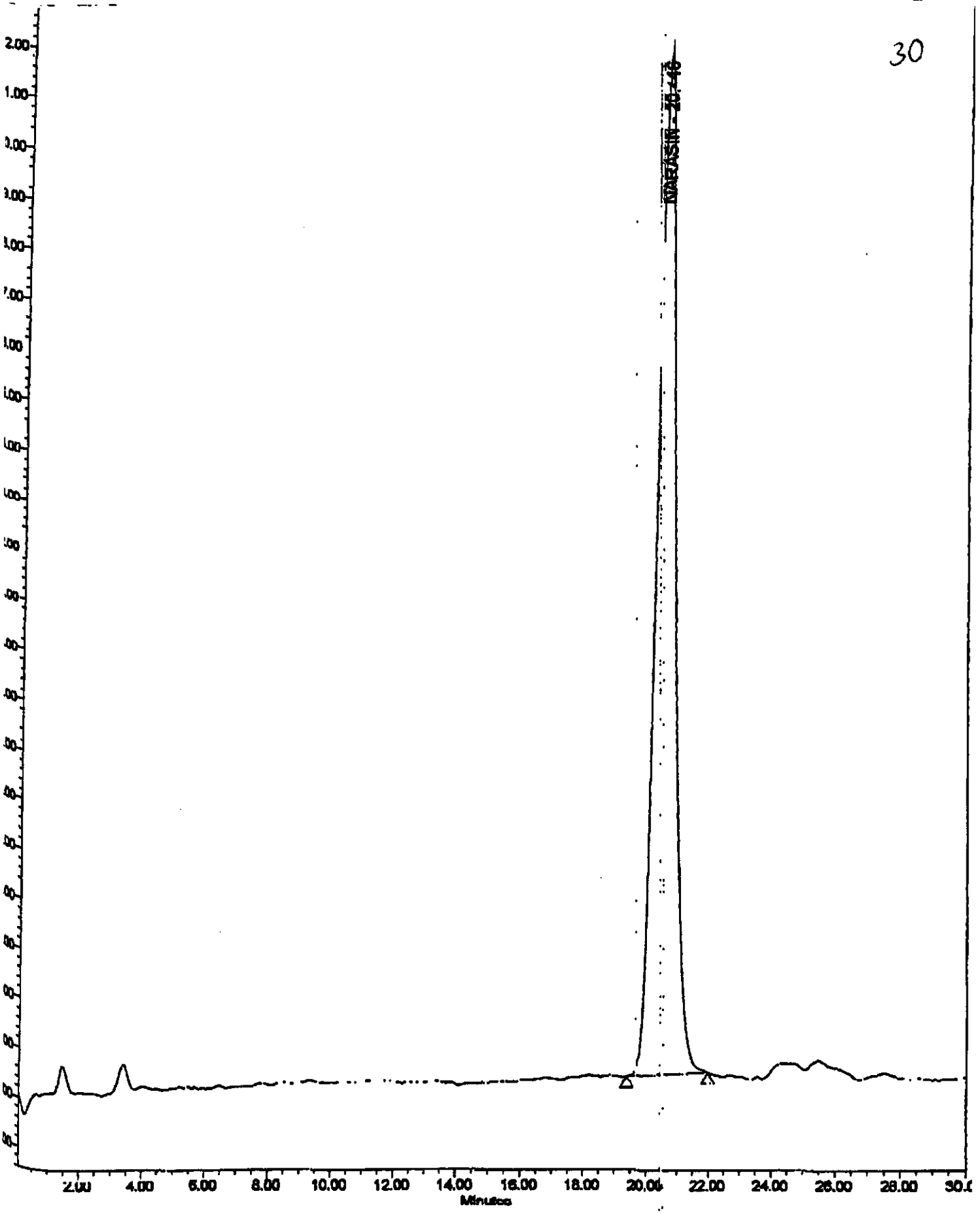
- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg

30



Narasin 25,04 µg/mL

30



premixture

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: 03-01-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
313114	10779	10778

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - *Questionnaire*

.....

.....

Date(s) of analysis: ....03-01-2002.....

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....0,7..... ml/min
- Injection volume: ...100..µl

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

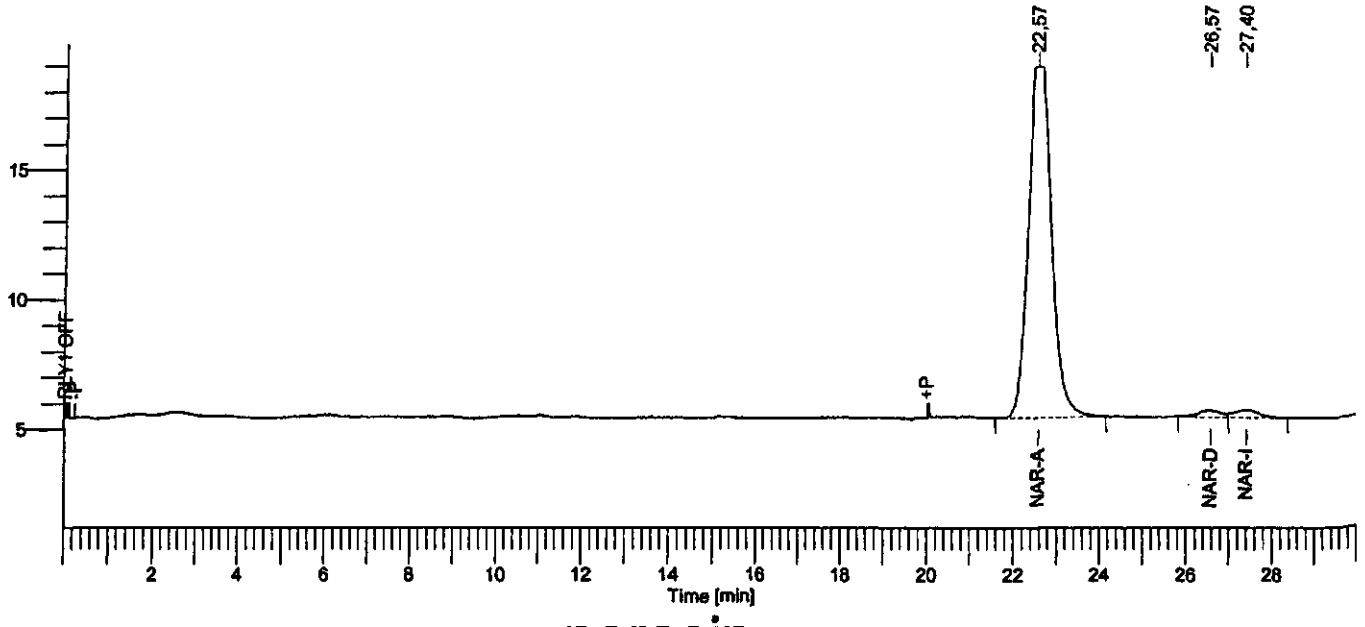
Recovery results:

- Percentage recovery: 101,9%
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg

ftware Version : 6.1.2.0.1:D19
mple Name :
trument Name :
ck/Vial : 0/0
mple Amount : 1,000000
cle : 9

simple

Date : 15-1-02 14:00:09
Data Acquisition Time : 3-1-02 15:27:04
Channel : A
Operator :
Dilution Factor : 1,000000



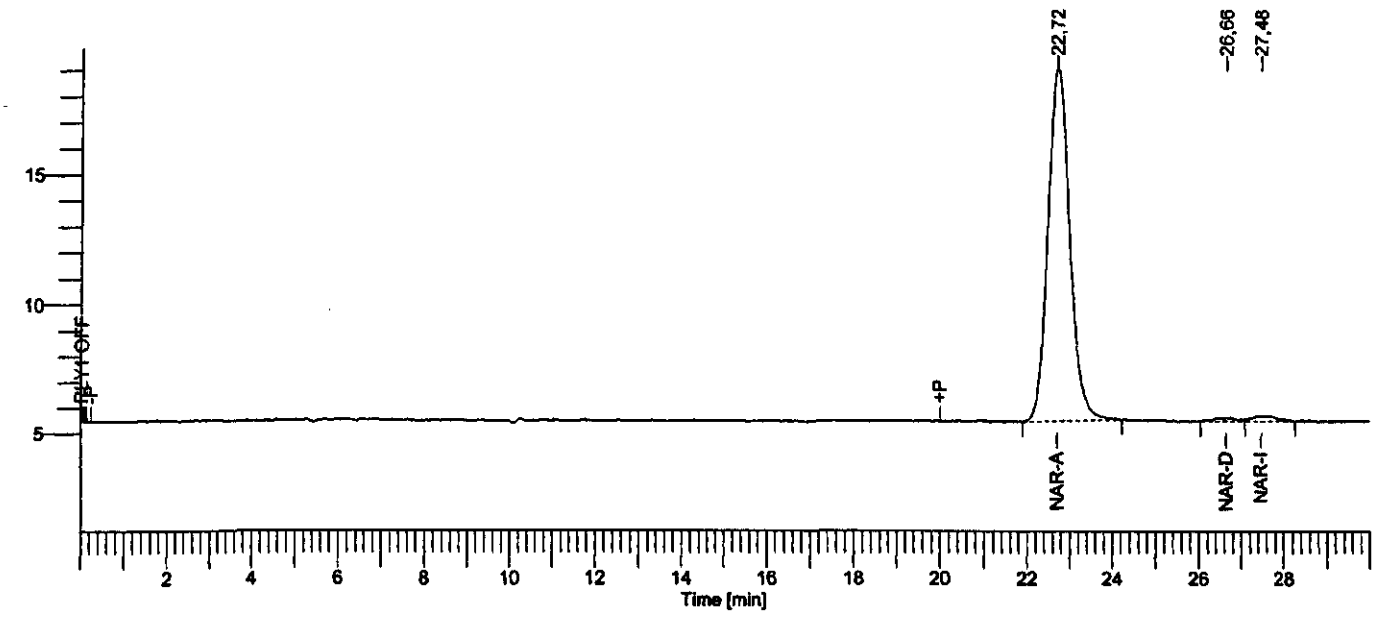
narasin

ak !	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	22,57	nar-A	521936,00	15033,18	95,86	95,86	BB	34,7189
2	26,57	nar-D	10338,47	267,49	1,90	1,90	BV	38,6502
3	27,40	Nar-I	12183,53	294,87	2,24	2,24	VB	41,3181
			544458,00	15595,53	100,00	100,00		

sing Component Report
mponent Expected Retention (Calibration File)

components were found

ftware Version	: 6.1.2.0.1:D19	Date	: 15-1-02 14:07:58
mple Name	: std 10.00 µg/ml	Data Acquisition Time	: 3-1-02 12:38:50
trument Name	: HPLC-1	Channel	: A
ck/Vial	: 0/0	Operator	:
mple Amount	: 1,000000	Dilution Factor	: 1,000000
cle	: 4		



narasin

ak t	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	22,72	nar-A	484728,00	13712,84	97,09	97,09	BB	35,3485
2	26,66	nar-D	5738,49	156,14	1,15	1,15	BV	36,7511
3	27,48	Nar-I	8768,51	224,48	1,76	1,76	VB	39,0609
			499235,00	14093,47	100,00	100,00		

ssing Component Report
mponent Expected Retention (Calibration File)

components were found

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: 21-12-2001

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
333103	12000	12000

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: .. ..

Contact person: ... .

Date(s) of analysis: ....21/11/01.....

Chromatographic conditions:

- Column:
  - [X] As described in the method
  - [ ] Other: .....
- Mobile phase:
  - [X] As described in the method
  - [ ] Other: .....
- Flow-rate: ...2,7..... ml/min
- Injection volume: .50.....µl

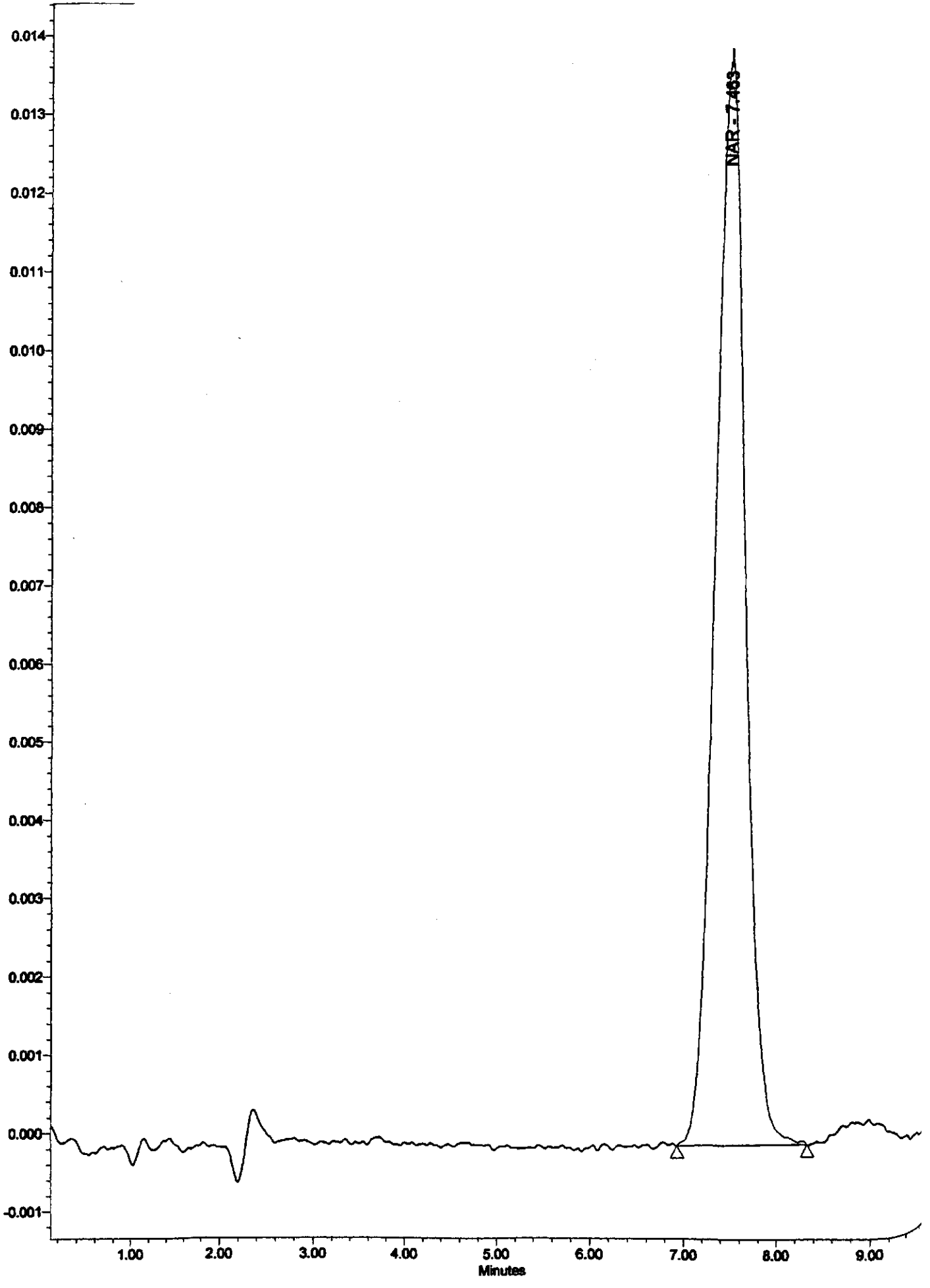
Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 103. %
- Single / duplicate determinations: [X] single [ ] duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: .5.0..... mg/kg


NAR - 7.463
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Minutes

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: 23-01-2002

Analyte: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
353122	10812	10539

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .. ..

Date(s) of analysis: 23 januari 2002

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ChromSpher 100 3mm 2X
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,7 ml/min
- Injection volume: 50 µl

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: ~~0,5~~ 105,9 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 1000 mg/kg 106,4 105,4

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 37

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: NARASIN

Product: Premixture

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
373115	10560	9960

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory: ....

Contact person:

Date(s) of analysis: 22/1/02

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: HYPERSIL 5Am BDS C18 (25cm x 4.6mm)
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 0.7 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 89.7 %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 89. % and 90. %
- Spiking level: 50 mg/kg

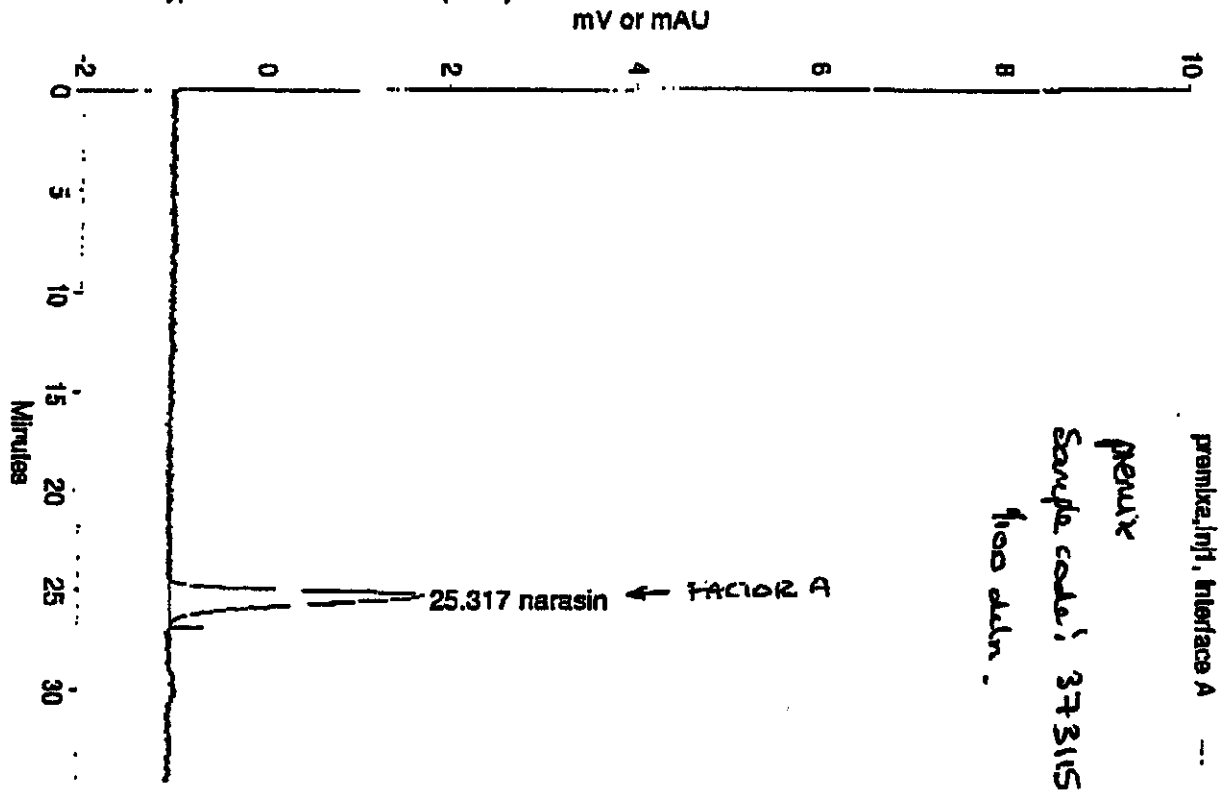
Name: premixa
Description: PremixA (1/100)
Type: Sample

Vial: A08

Acquisition Log
Column Pressure: N/A
Noise (microAU): 2e+001
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): -3e+001

Calculation Type: External Standard (Area)



APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 41

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: 21-01-2002

Analyte: **NARASIN**

Product: **Premixture**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
413116	9976	10122

CANFAS COLLABORATIVE STUDIES DECEMBER 2001 - NARASIN

Annex 4 - Questionnaire

Laboratory:

Contact person: .

Date(s) of analysis: 21.01.2002

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0.7 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

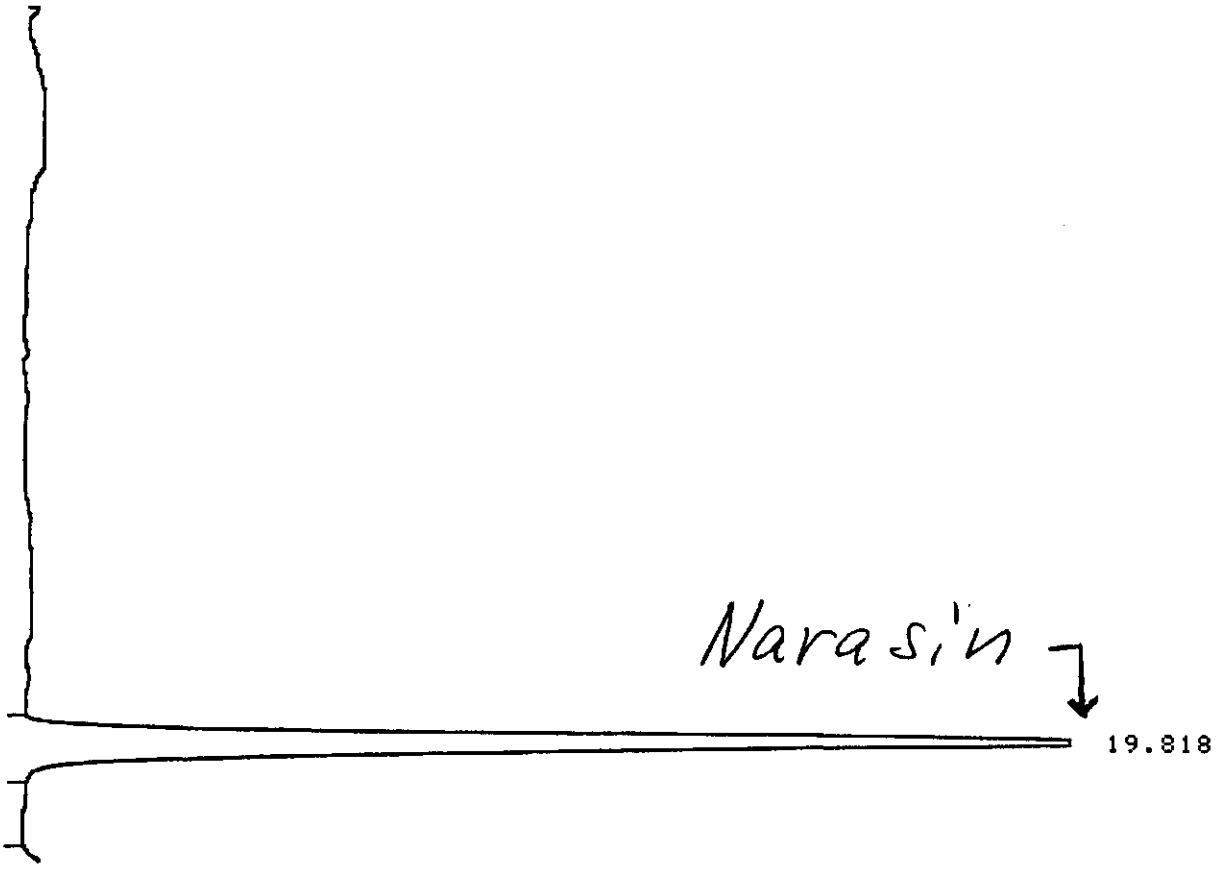
- Premixture

Please indicate the narasin factor A peak (and the factor D / I peak, see Annex 5) with an arrow

Recovery results:

- Percentage recovery: 99.3 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 97.8 % and 100.7 %
- Spiking level: 100 mg/kg

START 1
START 1



CHROMATOGRAM 10 MEMORIZED

CHROMATOPAC C-R3A FILE 0
SAMPLE NO 0 METHOD 3021
REPORT NO 7726

PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
1	19.818	8891			100	
		276511ar				
	TOTAL	8891			100	

SAMPLE# FROM	TO	VOL	REP	FILE#	RUN.T (min)
70	70	100	2	0	22