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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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CANFAS - Collaborative study for the determination of narasin in feedingstuffs and premixtures by HPLC

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SUMMARY

This report describes the results of a collaborative study of an HPLC method for the coccidiostat narasin in five broiler feeds and 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 principle of the method is as follows: 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 (DMAB) in a solution containing sulphuric acid and detection at 600 nm.

The samples which were prepared for the collaborative study were 4 broiler feeds with declared narasin contents of 20, 45, 70 and 120 mg/kg, 1 blank broiler feed and 1 premixture with declared content of 1,2 % narasin. The feed samples were sent to the participants as blind duplicates, the premixture as a single sample. The participants were asked to do a single determination per sample for the feed samples and to analyse the premixture in duplicate. Results were reported by 13 laboratories. Statistical evaluation was performed according to ISO 5725.

The results of the collaborative study were evaluated in a meeting attended by the participants. It can be concluded that for feedingstuffs the repeatability and reproducibility of the method is acceptable. The results obtained for the recovery and for the blind blank sample are also satisfactory. The overall conclusion is that for feedingstuffs the performance of the method is satisfactory.

For the premixture the rsd_R (18,1 %) is far too high. According to the panel, a value of approx. 7 % for the rsd_R of the premixture should be attainable. It was decided that for premixtures a new small-scale collaborative study will be organised (ca. 10 laboratories) with a modified method.

Only a few laboratories have detected the factors D+I in some or all samples or standard solutions. This is a sound justification of the choice made in the method to quantify the narasin content in the samples on the basis of the factor A peak alone.

Two laboratories used vanillin for post-column derivatisation. The results do not differ significantly from the results with DMAB (dimethylaminobenzaldehyde). The method description for feedingstuffs will be improved in several aspects. These modifications will not negatively affect the performance of the method.

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 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 samples which were prepared for the collaborative study were 4 broiler feeds with declared narasin contents of 20, 45, 70 and 120 mg/kg, 1 blank feed and 1 premixture with declared content of 1,2 % narasin. The feeds with 20 and 120 mg narasin per kg have been included in order to assure that the method is applicable for contents 2 times lower and 2 times higher than the permitted content.
The feed samples were sent to the participants as blind duplicates, the premixture as a single sample. The participants were asked to do a single determination per sample for the feed samples and to analyse the premixture in duplicate. Before these samples were 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.

- Danish Plant Directorate, Lyngby, Denmark; A. Pløger, A. Kraemer-Peterson
- 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, D. Berto, V. Capuzzo.
- Laboratory of the Government Chemist, Teddington, United Kingdom; G. Merson, J. Cowles, F. Lee.
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.
- LUFA-ITL Kiel, Kiel, Germany; F.H. Johannsen, 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. Bral, R. van San.
- 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, R. Reilly

3 MATERIALS

3.1 Samples for collaborative study

3.1.1 Sample composition

Specifications of the samples, which were produced for the collaborative study, are given in Table 1.

Table 1: Specifications of the samples

Type of sample	Declared content	Units	Subcontractor	Date of production
Broiler feed	20	mg/kg	SDS-Trouw, Witham (UK)	05/12/2000
Broiler feed	45	mg/kg	SDS-Trouw, Witham (UK)	05/12/2000
Broiler feed	70	mg/kg	SDS-Trouw, Witham (UK)	05/12/2000
Broiler feed	120	mg/kg	SDS-Trouw, Witham (UK)	05/12/2000
Premixture	1,2	%	SDS-Trouw, Witham (UK)	04/09/2000

Mixing was completed at Special Diet Services (SDS) small-scale mixing facility.

The main composition of the four feeds is given in Table 2.

Table 2: Main composition of the four feeds

Product / Ingredient	Broiler feed
Crude protein (%)	18,3
Crude fat (%)	5 or 10*
Starch (%)	45,2
Crude fibre (%)	4,1
Crude ash (%)	6,5
Moisture (%)	8,7

* see text

The basic feed material contained 2,6% of crude fat. Fat was added up to 5% for the feeds with 20 and 70 mg narasin per kg and up to 10% for the other two feeds containing 45 and 120 mg narasin per kg. The percentages of crude protein, starch, crude fibre and crude ash are calculated for the basic feed.

The premixture was based on inorganic feed material and contained regular contents of vitamins, minerals and trace elements. The complete composition of the feeds and the premixture is stored in the files of the co-ordinator (confidential).
The composition of the feeds and the premixture was the same as the composition of the products which were produced by SDS-Trouw in September 1999 for stability testing (see Report on homogeneity and stability of narasin, in broiler feeds and premix, A. Thalmann, LUFA-Augustenberg, 27/06/2000).

The feed products have been prepared in a quantity of 13 kg each. The 13 kg sack was laid horizontally to allow removal of about 40 aliquots of 200 – 250 grams from the middle of the contents using a large plastic scoop. Each sample was taken as a single aliquot and transferred to a foil-lined paper sack which was then heat-sealed. The sacks were stored at room temperature prior to shipping to the participants.

Next to the above mentioned samples which contained narasin, a blind blank feed was sent to the participants as well as a blank feed labelled "blank feed for narasin recovery purposes" (see Appendix 1). Both blank feeds concerned a broiler feed containing 2 mg/kg of virginiamycin (see the corresponding report) produced by IPC-Dier. This feed was analysed at LUFA Augustenberg prior to the collaborative studies and was found to contain no detectable amounts of narasin or interfering substances.

3.1.2 Sample homogeneity

The homogeneity of the samples was studied by LUFA Augustenberg by random selection of 10 subsamples per feed or premixture, applying the HPLC-method developed in CANFAS (see Annex 1 of Appendix 1).
The results of the homogeneity determinations of the individual feeds are attached in Appendix 2. Table 3 gives a summary of these results.

Table 3: Results of homogeneity tests for narasin in four broiler feeds and one premixture

Results / Product	Declared content	Measured content	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Broiler feed	20 mg/kg	18,91 mg/kg	4,51	not determined
Broiler feed	45 mg/kg	41,56 mg/kg	2,84	n.d.
Broiler feed	70 mg/kg	64,75 mg/kg	2,98	n.d.
Broiler feed	120 mg/kg	114,76 mg/kg	1,04	n.d.
Premixture	1,2 %	1,20 %	4,7	3,9

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 %. All between-sample CV's fulfil these requirements. Although for the feeds the within-sample CV was not determined it can reasonably be assumed that the within-sample variation is smaller than the between-sample variation. Thus, it is concluded that the samples are sufficiently homogeneous.

3.1.3 Sample logistics

The feed samples were sent as blind duplicates. The premixture was dispatched in foil-lined paper sacks each containing approximately 110 grams. The codes of the feed samples are given in Appendix 3. The samples were sent to the participants by courier service from Eli Lilly between February 12 and February 14, 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 together with the samples. 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. The participants were instructed to take note of the microbiological potency and the moisture content of the standard. (see Appendix 1). Later on (March 12, 2001) the participants were instructed by e-mail not to take note of the moisture content and to use the defined potency stated above.

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. One laboratory used a different mobile phase.
The HPLC conditions (Column and mobile phase) used by the participants are shown in Table 4.

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 4: 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, 250x4,6 mm, 5 µm	As described in the method
26	ODS-3, 10 µm	As described in the method
29	Nova-Pak C18, 4,6x250 mm, 4 µm	As described in the method
30	Kromasil C18, 150x4,6 mm	As described in the method
31	As described in the method	As described in the method
32	Waters Spherisorb S5 ODS-2, 250x4,6 mm, 5 µm	Methanol : phosphate buffer = 97:3 (v/v)
33	Hypersil ODS, 3 mm, 15 cm	As described in the method
35	Chromspher C18, 200x3,0 mm	As described in the method
37	Hypersil BDS C18, 250x4,6 mm, 5 µm	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

The results reported by the participants are given in Table 6. Figure 1 demonstrates the Mandel h and k plots of these results.
Statistical analysis of the results shows that lab 26 is a Cochran outlier for the 45 mg/kg sample. The resulting values for the statistical parameters (mean, relative standard deviations for repeatability and reproducibility) are given in Table 6. According to the Project Plan, the rsd_r-values should be ≤ 10 %. For all samples 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 ratios are given in Table 5. The HORRAT ratios should be lower than 2. For the four feed samples this criterion is met and established rsd_R-values are in line with values predicted by the Horwitz equation. Consequently it can be concluded that the reproducibility of the method is satisfactory for the feed samples. For the premixture the HORRAT ratio is much higher than 2.

Table 5: Horrat ratios of the Narasin collaborative study

Mean after elimination of outliers[1] (mg/kg)	Predicted rsd_R	Established rsd_R	Horrat[2]	Conclusion
18,12	10,345	9,154	0,88	Reproducibility OK
41,97	9,117	7,284	0,80	Reproducibility OK
64,62	8,543	6,786	0,79	Reproducibility OK
110,29	7,883	6,086	0,77	Reproducibility OK
10603	3,965	18,09	4,56	Reproducibility NOT OK

[1] = lab 26/sample 45ppm

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

Lab 24 reported a value for the premixture which is about twice as low as the values reported by many other laboratories and which is recognised as a Grubbs' lower straggler. In order to exclude the possibility of a calculation error (e.g. a wrong dilution factor) this lab was contacted and replied that they were not able to find any mistake. Consequently this result is retained in the statistical evaluation.
The Mandel h plot (see Figure 1) shows that lab 32 reports low results for all feed levels. This lab reported a recovery (82 %) that is lower than the mean recoveries of the other laboratories which are all 90 % or higher (see par. 5.3). Lab 32 was contacted to try to ascertain the cause of the

discrepant behaviour. Lab 32 indicated that the only possible reason could be the instability of the DMAB reagent. No problems were encountered with vanillin. While the recovery value of lab 32 is not a Grubbs' outlier (see par. 5.3) the results were not discarded from statistical evaluation.
In the evaluation meeting the reason(s) why the HORRAT ratio for the premixture is too high were discussed. One possible reason could be that the premixture was already produced on 4 September 2000. Decisions were made about how to proceed. The following options were regarded:

- Redo the collaborative study for the premixture after modifications in the method.
- Conclude that the method is not suitable for premixtures.
- Accept the relatively high CV_R for the premixture.

The results of the discussions in the evaluation meeting are described in Chapter 6 of this report.

Table 6. Narasin in four broiler feeds and one premixture

	Result (mg/kg)									
Sample	NAR 20 mg/kg		NAR 45 mg/kg		NAR 70 mg/kg		NAR 120 mg/kg		NAR 12000 mg/kg	
Lab										
11	18,5	20,6	42,9	43,1	61,9	67,5	102,6	111,6	10708	11402
13	17,9	18,5	42,2	43,6	64,9	66,4	112,6	114,1	11402	11520
23	18,3	19,3	44,8	45,4	64,1	69,1	117,0	119,0	10420	10500
24	16,4	20,1	41,1	41,6	63,3	67,9	112,9	113,6	5244$^{Gls/Gdls}$	6634$^{Gls/Gdls}$
26	17,9	20,4	*33,6*^{Co}	*42,5*^{Co}	63,1	64,7	103,7	103,8	8212	10063
29	15,9	17,0	44,3	45,9	65,5	68,1	114,4	119,5	12800	13060
30	16,0	20,0	42,0	42,0	64,0	70,0	114,0	116,0	11600	12600
31	17,7	18,0	40,4	41,2	64,1	66,4	109,3	111,9	11646	12102
32	15,2	15,6	34,9	35,9	52,43$^{Gls/Gdls}$	56,69$^{Gls/Gdls}$	93,5	97,7	10041	10451
33	16,4	17,0	38,7	39,7	59,3^{Gdls}	59,7G^{dls}	105,5	105,9	8474^{Gdls}	8501^{Gdls}
35	18,0	19,0	42,0	44,0	67,0	70,0	108,0	116,0	12036	12583
37	17,3	19,4	38,6	40,7	60,1	64,8	102,0	114,6	10039	11214
41	20,4	20,5	45,2	47,1	69,5	69,7	113,6	114,7	11007	11420

number of labs	13	12	13	13	13
m (mg/kg)	18,12	41,97	64,62	110,30	10603
rsd_r (%)	7,570	2,165	4,069	3,403	5,596
rsd_R (%)	9,154	7,284	6,786	6,086	18,09

Remark : Italic printed results are not taken into account in the statistical evaluation!

Key to symbols:

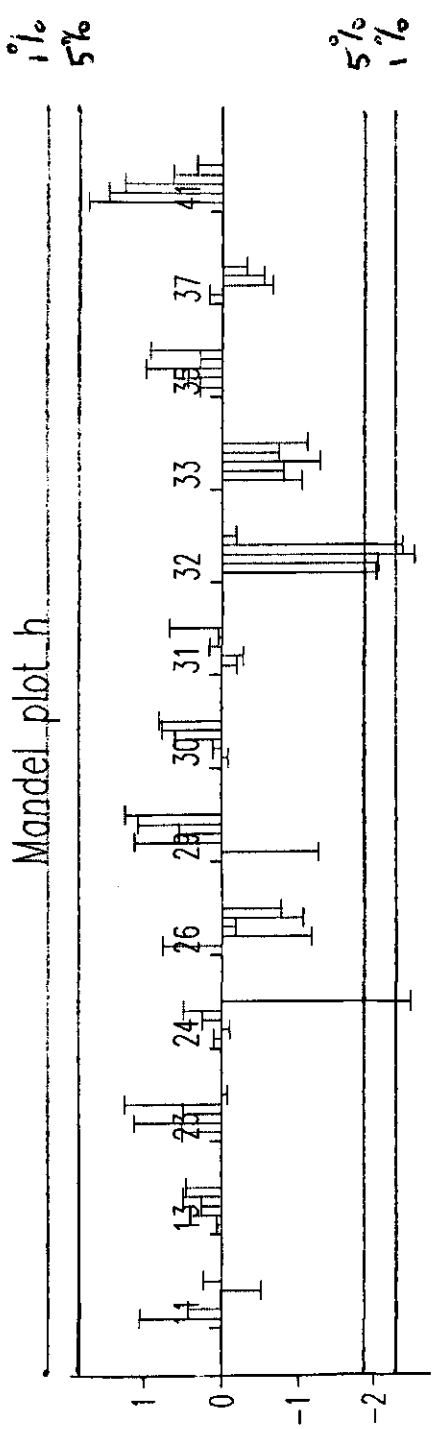
result^{Co} = Cochran outlier

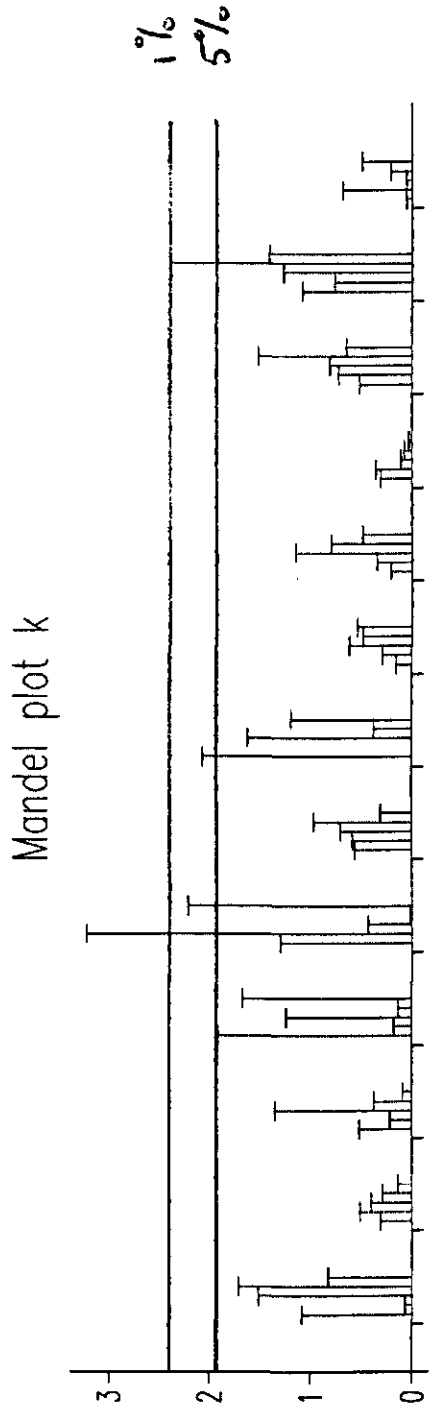
result^{Gls} = Grubb's lower straggler

result^{Gdls} = Grubb's double lower straggler

Table 6: Results reported by the participants

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





5.2 Blank samples

Table 7: Reported results of the participants for the blank samples

Partner	Blank sample 1 (mg/kg)		Blank sample 2 (mg/kg)	
11	ND		ND	
13	<0,5		<0,5	
23	<2		<2	
24	0		0	
26	0,6		2,8	
29	0		0	
30	<5		<5	
31	0		0	
32*	Negative	Negative	Negative	Negative
33	Not found		Not found	
35	<1		<1	
37	ND		ND	
41	0		0	

* Participant 32 performed the analyses in duplicate

One lab (nr. 26) detected small signals in the blind blank samples above the limit of quantification, which was estimated at 0,5 mg/kg by lab 26. The other laboratories did not detect signals in the blind blank samples.

5.3 Recoveries

Table 8: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	recovery average in %
11	106	97	102
13	97	97	97
23	Not reported	Not reported	Not reported
24	94 (level 12,5 mg/kg)		94*
26	91	92	92
29	101		101
30	98		98
31	99	102	101
32	82	82	82
33	98		98
35	94	95	95
37	89	90	90
41	106	103	104

* Laboratory 24 also reported a recovery of 77% at a level of 5 mg/kg. This value is not taken into account because this level is too low.

Only lab 32 reported recoveries lower than 90%, viz. 82%. In task 1 and 2 of the project (within- and between-lab validation) recoveries of 86% and higher were measured. According to ISO 5725 the mean recovery of lab 32 (82%) is not a Grubbs' outlier.

5.4 Remarks

Table 9: Remarks made by the partners

Partner	Remarks
11	The meaning of 8.1 - Let stand over night - is not clear.
13	No remarks
23	Not reported
24	The available post-column reactor was equipped with a single pump; the coil was 3 m long; reaction temperature was fixed at 95 °C. Reactant flow rate was 0,4 ml/min.
26	1) Paragraph 4.1.3 is a little confusing because it refers to two temperatures. The reference to 95°C would be better amended to 90°C. 2) Paragraph 5.2 needs some editing. It is not very often that an analyst knows the level of the drug additive in a feedingstuff. It would be better if a definite weight was specified and the concentration determined by subsequent dilutions of the extract. 3) We found it necessary to have a significant length of tubing between the end of the reaction coil and the detector. This was to allow the mobile phase, which is predominantly methanol, to cool down and minimise the risk of bubbles entering the detector flow cell. This is the same problem as was encountered with the maduramycin trial. Even with this tubing present, which was between 2 and 3 metres in length we still experienced the occasional electronic spike which we have assigned to a bubble entering the flow cell. 4) Paragraph 5.2 we do not consider it good practice to recommend taking such small test portions of feed for this type of analysis. This will increase the uncertainty and imprecision of the analytical method significantly depending on the homogeneity of the sample. If a 20g test portion is taken these parameters can be reduced. 5) It is our opinion that the calibrant range described in paragraph 5.4.2 needs a complete revision. The organisers advised us that the concentration of narasin in the samples was in the range 10 mg/kg to 150 mg/kg. This meant that by following the extraction procedure as written and by taking a 20 g test portion of sample the concentration of narasin in the final extract was 2.0µg/ml. This concentration is equivalent to the level of the top calibrant standard. This means that we would have had to have injected all the samples extracts twice or at worse we would have had to re-extract them a second time because the run time for this study was some 24 hours and the method gives no indication of the extract stability. We in fact produced a linear calibration curve between 0.4 µg/ml and 12.25 µg/ml and with the equipment that we used believe that 0.4 µg/ml is the lowest standard that we could realistically work with. 6) We found the sample extraction procedure generally simple and easy to follow. 7) We, in fact, used the option to pump the post column reagent separately (i.e. we used three pumps).

Partner	Remarks
29	- We repeated the analysis, as we discovered that our DMAB reagent wasn't very good, and we used a new one. So the standards had a greater area and a better correlation. - Only samples 293384 and 293489 were not injected again as they were the blind blank duplicates of feed samples sent. - The dilution factors applied this time were decided, as we knew now which samples were the duplicates and their contents.
30	1) Usually for narasin determination we use vanillin reaction and not buffered mobile phase. The response is twice better. 2) Concentrations of calibration solutions are too low and peak area small. This increases the error of area measurement.
31	Calibration curve adjusted to: 0,5 - 1,0 - 2,5 - 5,0 - 10,0 µg/ml. All samples have been diluted by a factor of 3 before injection.
32	The reagents 3.13 (methanol + sulphuric acid) and 3.14 (DMAB solution) were mixed before HPLC analysis. The flow rate of the mixture for post column derivatisation was 0,8 ml/min.
33	No remarks
35	We used one reagent pump at flow 0,8 ml/min. The flow of the mobile phase was 0,7 ml/min
37	I have presented a number of combinations of results to you (in the order in which the extracts were analysed) A) The sample extracts were analysed initially as blind samples (unknown) and therefore run undiluted vs. the calibration curve as outlined in method (the spike of 50 µg/kg was diluted within calibration range and the premixture was also diluted). As most of the extracts contain narasin (outside the calibration range) - Are they valid data? 2 approaches were taken: B) Prepare a calibration curve 10 fold greater and run extracts undiluted. C) Dilute extracts 10 fold within calibration range as outlined in method. Remarks - Introducing dilutions introduces possible further errors. - Introducing dilutions introduces loss of sensitivity with respect to factor D/I. NOTE: after consultation of the co-ordinator it was agreed to use the results obtained with option C in the statistical evaluation because option C follows the method most strictly.
41	No remarks

5.5 Narasin factors D+I

Participants were asked to supply information about detection of the D and I factors in narasin, see Annex 5 of appendix 1.
The results are summarised in Table 10.

Table 10: Results for factors D+I

Lab nr.	Information on factors D+I
11	ND*
13	ND
23	Not reported
24	ND
26	ND
29	Only detected in the standard solution of 25 µg/ml
30	ND
31	ND
32	Not detectable in most of the samples
33	Detected in standard, premixture, and feed samples 333478, 333392, 333396, 333485, 333497 Not detected in feed samples 333381, 333491, 333451, 333476, 333459
35	ND
37	Only in undiluted extracts factors D+I appeared in samples >60 mg/kg (see chromatograms); factors are not noted in 1 µg/ml standard solution.
41	D+I detected in all positive samples

* ND means that factors D+I are not detectable in the samples and the standard solutions.

Only a few laboratories have detected the factors D+I in some or all samples or standard solutions. This is a sound justification of the choice made in the method to quantify the narasin content in the samples on the basis of the factor A peak alone.

5.6 Special Request 1: post-column derivatisation with vanillin

The following partners performed the post-column derivatisation with vanillin:

- Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany; A. Thalmann, K. Wagner
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.
- Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland; R. Muhonen, T.Heikkinen

5.6.1 HPLC conditions

Table 11: HPLC conditions

Partner	HPLC column	Mobile phase
LUFA, Augustenberg, Germany	Not reported	Not reported
LNIV, Lisbon, Portugal	Same as normal method	Same as normal method
Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland	Same as normal method	Same as normal method

5.6.2 Recoveries

Table 12: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Average recovery in %
LUFA, Augustenberg, Germany*	Not reported		
LNIV, Lisbon, Portugal	100	100	100
Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland	81		81

* recovery compared with normal extraction procedure

5.6.3 Results of the samples

Table 13: Results of the samples that were derivatised with vanillin

Partner	LUFA Augustenberg, Germany	LNIV, Lisbon, Portugal		Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland
Sample content (mg/kg)	Results (mg/kg)	Result 1 (mg/kg)	Result 2 (mg/kg)	Results (mg/kg)
0	Not reported	Negative	Negative	0
0	Not reported	Negative	Negative	0
20	Not reported	18,74	18,58	19,4
20	Not reported	18,17	18,34	18,6
45	Not reported	41,35	40,4	43,1
45	Not reported	43,44	43,21	47,1
70	Not reported	68,73	68,82	72,4
70	Not reported	63,95	62,2	75,7
120	Not reported	114,83	115,28	118,7
120	Not reported	108,39	107,21	124,8
Premixture	Not reported	10932,8	11013,55	10.962 - 11.164

The values do not differ significantly from the mean values obtained with DMAB (see par. 5.1)

5.6.4 Remarks

Table 14: Remarks made by the partners

Partner	Remarks
LUFA, Augustenberg, Germany	area monensin equal to monensin with DMAB area narasin < 30% less than with DMAB salinomycine <30% less than with DMAB Vanillin solution stable ~ 1 day
LNIV, Lisbon, Portugal	No remarks
Plant Production Inspection Centre Agricultural Chemistry Department	Reactor temperature was 90°C

5.7 Special request 2: extraction overnight

The following partners performed the extraction overnight:

- Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany; A. Thalmann, K. Wagner
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.

5.7.1 HPLC conditions

Table 15: HPLC conditions

Partner	HPLC column	Mobile phase
LUFA, Augustenberg, Germany	Not reported	Not reported
LNIV, Lisbon, Portugal	Same as normal method	Same as normal method

5.7.2 Recoveries

Table 16: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Average recovery in %
LUFA, Augustenberg, Germany*	+ 1,06%	+ 1,01%	1,04%
LNIV, Lisbon, Portugal	93	90	92

* recovery compared with normal extraction procedure

5.7.3 Results of the samples

Table 17: Results of the samples that were extracted overnight

Partner	LUFA, Augustenberg, Germany	LNIV, Lisbon, Portugal	
Sample content (mg/kg)	Result compared with normal extraction procedure	Result 1 (mg/kg)	Result 2 (mg/kg)
0	Not reported	negative	Negative
0	Not reported	negative	Negative
20	- 1,4%	17,34	17,60
20	- 4,2%	17,46	16,81
45	Not reported	38,07	39,45
45	Not reported	40,45	38,77
70	Not reported	62,43	63,15
70	Not reported	58,47	58,00
120	- 2,1%	106,24	104,42
120	- 0,9%	102,85	101,93
Premixture	Not reported	11121,04	10395,38

The values seem to be slightly lower than those obtained with the normal extraction procedure. However, these data are far from sufficient to draw firm conclusions. In the evaluation meeting it has been discussed whether the possibility of overnight extraction is left open (see Chapter 6).

5.7.4 Remarks

Table 18: Remarks made by the partners

Partner	Remarks
LUFA, Augustenberg, Germany	Experiences from other trials: there are mineral feeds where the contents in polyether antibiotics is lowered by > 20% overnight Conclusion of German working group: if a figure results with overnight extraction that does not match the declared value, the analyses have to be repeated with extraction for 1 hour.
LNIV, Lisbon, Portugal	No remarks

5.8 Special requests 3: microbiological analysis

The following partners performed the microbiological analyses of the samples:

- Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany; A. Thalmann, K. Wagner
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Bral, R. van San

5.8.1 Recoveries

Both partners did not report recoveries.

5.8.2 Results of the samples

Table 19: Reported results of the samples analysed with the microbiological method

Partner	LUFA, Augustenberg, Germany	Rijksontledingslaboratorium (ROL), Tervuren, Belgium	
Sample content (mg/kg)	Reported microbiological activity (in mg/kg)		
0	Not reported	Not found	
0	Not reported	Not found	
20	Not detectable	20,0	
20	Not detectable	19,3	
45	33,0	43,5	
45	33,2	44,0	
70	62,5	67,4	
70	65,5	66,4	
120	102,9	120	
120	109,3	119	
Premixture	10195	10653	10805

The values for the feeds obtained by ROL are slightly higher than the mean values obtained with the CANFAS-method (and in very good agreement with the declared values) while the values for the premixture are similar to the mean values reported with the CANFAS-method.
The values reported by LUFA Augustenberg are slightly lower than the mean values obtained with the CANFAS-method, especially at lower contents. For the 20 mg/kg sample narasin is not detected at all.

5.8.3 Remarks

Table 20: Remarks made by the partners

Partner	Remarks
Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany	Method: Official method for monensin (agar diffusion)
Rijksontledingslaboratorium, Tervuren, Belgium	No remarks

6 EVALUATION AND CONCLUSIONS

The results of the collaborative study were evaluated in a meeting in Tervuren (Belgium) on June 19-20, 2001.
The relatively low values for all feed samples and for the recovery reported by lab 32 were discussed. Lab 32 indicated that the only possible reason could be the instability of the DMAB reagent. No problems were encountered with vanillin. While the recovery value of lab 32 is not a Grubbs outlier, the panel decided that the results of lab 32 should be taken into account in the statistical evaluation.
The results of the statistical evaluation, as described in par. 5.1, Table 6 have been accepted by the panel.

Consequently it can be concluded that for feedingstuffs the repeatability and reproducibility of the method is acceptable. The results obtained for the recovery and for the blind blank samples are also satisfactory. The overall conclusion is that for feedingstuffs the performance of the method is satisfactory.
The results (baseline noise) obtained by a number of laboratories and the remarks made by some laboratories indicate that the calibration curve should be shifted to a higher range. This will be changed in the method.
For the premixture the rsd_R (18,1 %) is far too high. Although not an outlier, lab 24 largely contributes to the unsatisfactory repeatability. Lab 24 will repeat the analysis and also send the sample to Thalmann. Lab 26 used a sample weight of 0,2 g for the premixture. This can possibly contribute to the low results of this lab. According to the panel, a value of approx. 7 % for the rsd_R of the premixture should be attainable. It was decided that for premixtures a new small-scale collaborative study will be organised (ca. 10 laboratories) with a modified method. The modifications in the method are as follows:

- weight is increased to 5 g
- the calibration curve will be shifted to higher concentrations (see above)
- more strict description of the calibration method, stating that the concentration of the premixture extract should be in the middle of the calibration curve
- the mixing of the premixture prior to the weighing of the 5 g will be described more strictly (see instructions for nicarbazin)
- the extraction time will be fixed to 1 hour (the extraction overnight will become optional and will be described in the remarks)

The panel agreed with the conclusion (see par. 5.5 of this report) that quantification should be based on the factor A peak only.

The following remarks, related to the method description have been accepted:

- lab 11: in par. 8.1 "at room temperature" will be added in the text
- lab 26, remark 1
- lab 26, remark 2, par. 5.2: it will be considered to define a minimum weight for feeds and to describe separate procedures for feeds and premixtures, like in other methods (e.g. nicarbazin).
- lab 26, remark 5; however, it is important to describe or to be sure that the sample extracts are always diluted by a factor of 3 or more.
- The use of stainless steel tubing in the post-column reactor and detector should be avoided
- A remark will be added about the suitability of vanillin for post-column derivatisation, stating that a full validation with vanillin has not been performed

ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Eli Lilly and Company, Mr. D. Towell, 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)

cc. Driessen, Van der Kamp/J. de Jong

to addressee

DATE
13 February 2001

SUBJECT
Collaborative study CANFAS
narasin

ENCLOSURE(S)
6

OUR REFERENCE
01/0004565/rik/rikjjo

HANDLED BY
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09098104 te Arnhem

THE INTERNET
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Dear colleague,

With separate post the samples for the collaborative study for narasin will be sent to you by Mr. Towell (Eli Lilly). We expect the samples will be sent to you this week. You will receive the following samples :

- 10 feed samples, with the text "additive: NARASIN" and with a sample code; these samples constitute 4 blind duplicates of feed samples containing narasin (contents in the range between 10 and 150 mg/kg) and 1 blind duplicate of a blank feed
- 1 premixture containing narasin, content in the range between 0,5 and 3 %.

For the feed samples you are asked to do a single determination per sample, the premixture must be analysed in duplicate.

For recovery purposes, a blank sample, with the text "blank feed for narasin recovery purposes" will be included.

The method which has to be used is included as Annex 1 (please note that this method is a *modified* version of the method which was distributed prior to the kick-off meeting).

Annex 2 contains the reporting form. This form has already been 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 (please send the results to the following E-mail address: j.j.m.driessen@rikilt.wag-ur.nl). Of course you can also fill in the form and send it by fax or normal mail.
The **deadline** for reporting the results is **13 April 2001**. This deadline is shorter than for the other analytes because we want to organise the evaluation meeting for all the analytes before the summer. Hopefully, it is not a problem for you to stick to this deadline.

Annex 3 contains instructions for handling (milling, storage) of the samples.

Annex 4 is a questionnaire. 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.

Annex 5 is a second questionnaire regarding the factors of narasin.

QUALIFIED BY STERLAB

Annex 6 contains information about special requests. We hope that, next to the regular determinations, you are prepared to volunteer to do some extra work.

The reference standard of narasin which has to be used will be send to you by mr. Towell (Eli Lilly), together with the samples. Please take note of the microbiological potency and the moisture content of this reference standard (see Annex 5).

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

Kind regards,

dr. Jacob de Jong
CANFAS co-ordinator

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

RIKILT
State Institute for Quality Contr of Agricultural Products

DATE
13 February 2001

OUR REFERENCE
01/0004565/rik/rikjjo

cc
Mrs. D. Bennink, European Commission, DG Research, CII/3, Brussels
Mr. D. Towell, Eli Lilly and Company Ltd., Speke Operations, Liverpool

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 1 - Description of the method

CANFAS/NAR/09102000/A.THALMANN

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.

f, see remark 8.4

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 95 °C, 7.0 m with 0.33 mm ID and water bath or reactor oven for operation at 90 °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.

5.2 Extraction
Depending on the concentration expected 0.200-20.0 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 at least 1 h. Let settle the coarse particles. If necessary an aliquot is

diluted 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	90 °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: 5 ml the stock solution (3.16) are diluted with the extraction solvent (3.10) to 50 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 Preparation of the calibration solution: 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 (100 ml see 5.2)
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 it is possible to let stand the extracts over night with most of the samples. 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 is 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

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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

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

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis:

Analyte: NARASIN

	Unit	Result (mg/kg)
Sample code		
313376		
313393		
313397		
313403		
313418		
313437		
313439		
313461		
313473		
313482		

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

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 3 - Instructions for handling of the samples

1. Storage

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

2. Milling (see par. 5.1)

- Feed samples: grind the feed samples with a mill equipped with a 1 mm screen
- Premix: premix samples should not be milled

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

Mix the entire sample thoroughly

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - 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:

- Blind positive feed samples
- Blind blank feed sample
- 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 OCTOBER 2000 - NARASIN

Annex 5 - 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

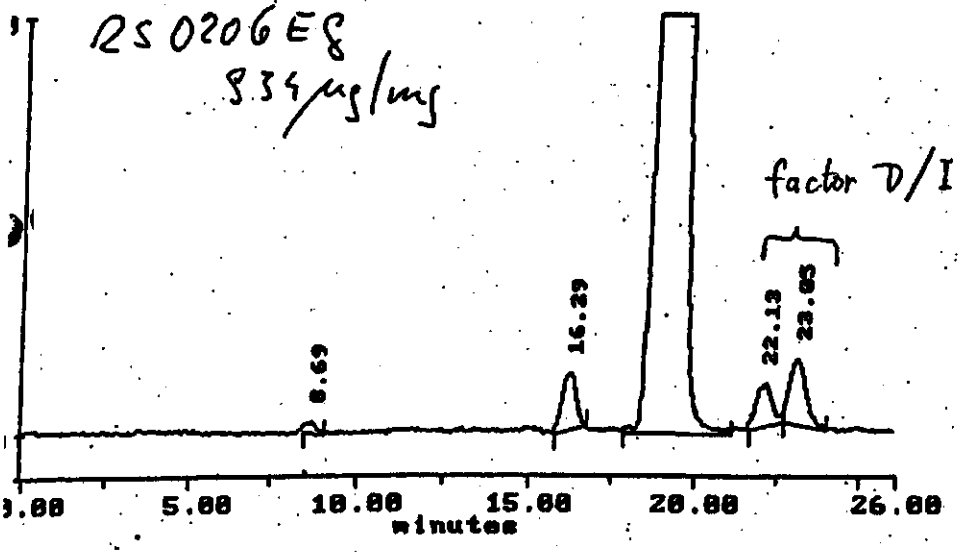
Thank you for your cooperation !

D-6000 HPLC Manager Report

ted: Nov 5, 1998 15:52 Reported: Nov 5, 1998 16:44

Title : Ionophor-Antibiotika Bestimmung
: NAREG Data File : NAREG002.RW Channel : 1
: EG NAR 5-50 STD1- Inj 2 Vial No. = 1 Vol = 100 ul

Channel 1



Channel 1

uantitation : HEIGHT
ation Method : EXT-STD
ount : 1.000
Factor1 : 1.000

RT	NAME	HEIGHT	mg/kg	R-FACTOR	RRT	BC
16.29	NAR 1	135	100.000	7.394E-01	16.22	BB
18.92	NAR 2	13173	100.000	7.591E-03	18.89	BB
22.13	NAR 3	99	100.000	1.003E+00	22.08	BB
23.05	NAR 4	156	100.000	6.380E-01	23.11	BB
		13563	400.000			

jection Level : 5

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 6 - Special requests

Volunteers are asked to do the following *additional* work:

- post-column reaction with vanillin (see page 2 of this annex)
- extraction: overnight (see par. 5.2)
- use of a microbiological method (please indicate which method and add a description)

Please report the results in a copy of annex 4 and clearly describe your modification, conditions, etc. Please also include representative chromatograms.

Thanks in advance for doing the additional work

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 6, page 2 - Special requests

Conditions for post-column derivatisation with vanillin

- Vanillin ≥ 98% (HPLC)
- Methanol, HPLC-grade
- Sulfuric acid, 95-97%, p.a.
- Vanillin reagent:
  Dissolve 10 g of vanillin in a mixture of 250 ml of methanol and 5.0 ml of sulfuric acid. Mix well and sonicate for some min under vacuum at room temperature. This solution has to be prepared daily prior to use and has to be cooled with ice water during use.

- Flow rate reagent pump: 0.8 ml/min
- Reactor temperature: 95°C
- Detection wavelength: 520 nm

Other conditions are not changed

APPENDIX 2

Homogeneity of samples

Sample No	F-Nr.	mg/kg narasin	Average	s	CV%
133011	140	17,54			
133012	141	19,86			
133013	142	19,66			
133014	143	17,69			
133015	144	19,12			
133016	145	18,81			
133017	146	18,45			
133018	147	18,54			
133019	148	19,68			
133020	149	19,77	**18,91**	**0,85**	**4,51**
133021	150	41,66			
133022	151	39,56			
133023	152	39,82			
133024	153	42,28			
133025	154	42,88			
133026	155	40,45			
133027	156	42,39			
133028	157	42,50			
133029	158	41,83			
133030	159	42,19	**41,56**	**1,18**	**2,84**
133031	160	68,42			
133032	161	63,46			
133033	162	65,68			
133034	163	63,35			
133035	164	67,46			
133036	165	63,00			
133037	166	63,20			
133038	167	63,27			
133039	168	65,32			
133040	169	64,33	**64,75**	**1,93**	**2,98**
133041	170	114,57			
133042	171	113,86			
133043	172	113,94			
133044	173	117,27			
133045	174	114,12			
133046	175	113,85			
133047	176	114,71			
133048	177	113,86			
133049	178	116,47			
133050	179	114,99	**114,76**	**1,20**	**1,04**

CANFAS

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

Homogeneity test collaborative study

Additive : **Narasin**
Product : **Premixture: 1,2%**

Date of determination : October 23th, 2000

Sample	Content %	Duplicate average %
1605	1,13	1,16
1605	1,16	
1605	1,20	
1606	1,29	1,27
1606	1,23	
1606	1,29	
1607	1,18	1,16
1607	1,12	
1607	1,18	
1608	1,27	1,26
1608	1,20	
1608	1,30	
1609	1,17	1,17
1610	1,14	1,14
1611	1,21	1,21
1612	1,13	1,13
1613	1,24	1,24
1614	1,28	1,28

Homogeneity OK Crtiterion : $CV_{between}$ = < 7%		
Average	**1,2**	
SD (between samples)	**0,056**	
CV (between samples)	**4,7**	Result Grubb's test
Grubb's test, single lower	**1,279**	**no outlier**
Grubb's test, single upper	**1,386**	**no outlier**
Grubb's test, double lower	**0,6047**	**no outliers**
Grubb's test, double upper	**0,5310**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,046**
CV (within samples)	(CV (%))	**3,9**

APPENDIX 3

Sample codes

Sample codes supplied to the participants in the narasin collaborative study

NARASIN number of participants 13	NAR broiler I 20ppm NAR 1a	NAR broiler I 20ppm NAR 1b	NAR broiler II 45ppm NAR 2a	NAR broiler II 45ppm NAR 2b	NAR broiler I 70ppm NAR 3a	NAR broiler I 70ppm NAR 3b	NAR broiler II 120ppm NAR 4a	NAR broiler II 120ppm NAR 4b	VIRG broiler 2ppm NAR blank 1a	VIRG broiler 2ppm NAR blank 1b
Participant code										
11	113373	113441	113477	113479	113383	113400	113425	113464	113447	113386
13	133407	133450	133399	133487	133375	133432	133500	133377	133486	133462
23	233463	233410	233492	233427	233453	233434	233467	233469	233414	233416
24	243409	243457	243421	243484	243474	243435	243420	243483	243501	243390
26	263465	263502	263452	263412	263419	263424	263455	263493	263470	263449
29	293426	293431	293445	293471	293490	293460	293454	293499	293489	293384
30	303385	303496	303448	303379	303436	303494	303456	303428	303402	303430
31	313376	313439	313403	313473	313437	313418	313397	313393	313461	313482
32	323440	323398	323378	323395	323488	323406	323382	323446	323417	323481
33	333381	333451	333497	333392	333478	333396	333485	333491	333476	333459
35	353468	353438	353374	353422	353408	353380	353405	353466	353444	353387
37	373391	373472	373458	373389	373433	373429	373475	373404	373423	373480
41	413388	413401	413498	413394	413415	413443	413413	413442	413495	413411

APPENDIX 4

Narasin reference standard profile

Eli Lilly and Company Limited
Speke Operations
Fleming Road
Speke
Liverpool L24 9LN
UK

January 17th 2001

The following has been extracted from the current Lilly reference standard profile document for Narasin :

* Effective Date: November 22, 2000 Compound: 079891
* Supersedes: March 28, 2000 Revision: 21
Expiry Date: March 28, 2001

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.

Handling: Please refer to current MSDS for caution and handling information.

Storage: 125 mg quantities in heat sealed amber glass ampoules with argon overlay at freezer temperature, -10° to -25°C.

D. P. Towell
Compliance Team

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 11

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**
Lab-name:
Contact person: e-mail:
fax:
telephone:

Date of analysis: 9-26.April, 2001

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
113373		20.6
113383		61.9
113386		ND
113400		67.5
113425		111.6
113441		18.5
113447		ND
113464		102.6
113477		43.1
113479		42.9

Sample / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	11402	10708

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: .....9th – 26 April 2001.....

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:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

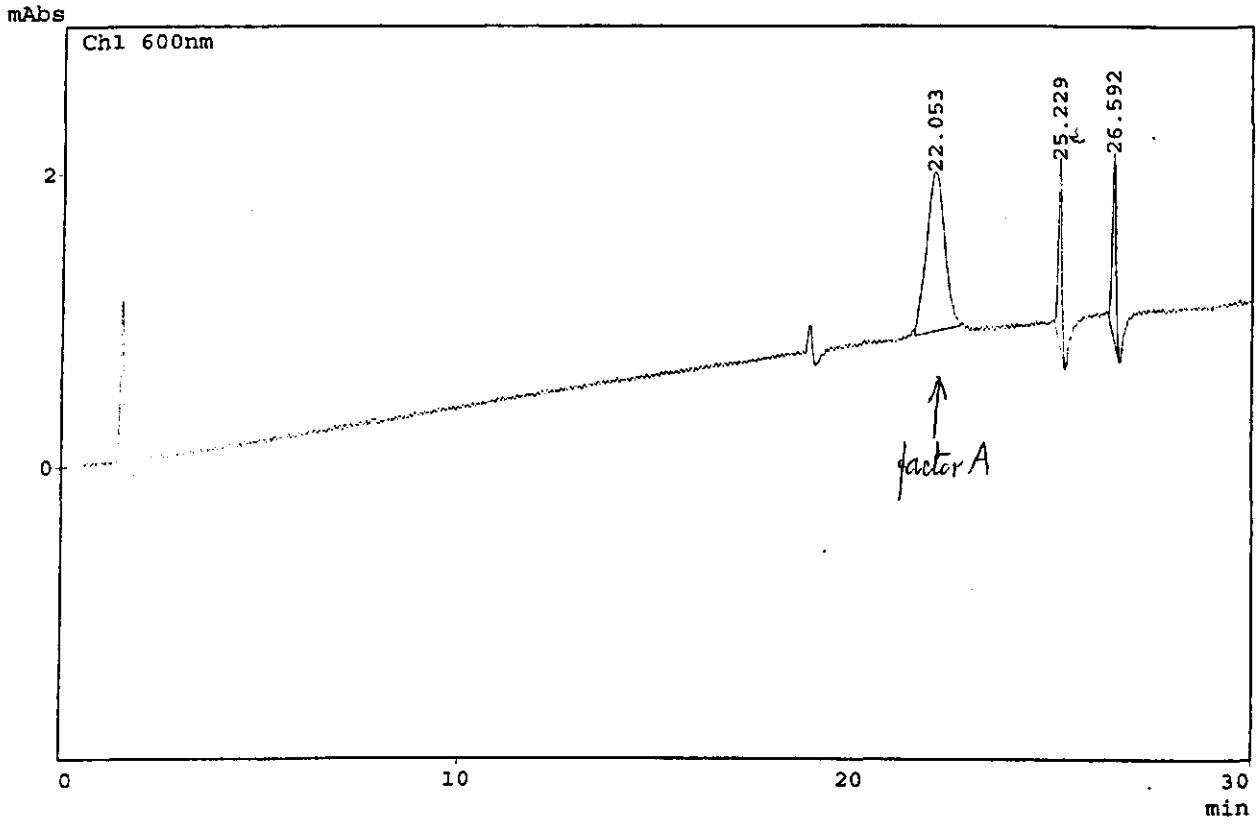
- Percentage recovery: 102.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 106. % and 97.. %
- Spiking level: ...50..... mg/kg

CLASS-LC10 Ver.=1.63 SYS=1 REPORT.NO=81 DATA=NA250406.K01 01/04/25 14:30:37

11

Sample : premix b
ID :
Sample Amount : 0.2107
Type : Unknown
Detector : SPD-M10Avp
Operator : lju
Method Name : NARASIN.MET

*** Chromatogram ***



*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN	IDNO	CONC
1	1	22.053	34617	V	0.9987(0.9488)	0.9994(0.9688)	1	10708.1250
			34617					10708.1250

01/04/25 09:52:03

CLASS-LC10 Ver.=1.63 SYS=1 REPORT.NO=89 DATA=NA250412.K01 01/04/25 20:49:38

Sample : 113479 b

ID :

Sample Amount : 5.0273

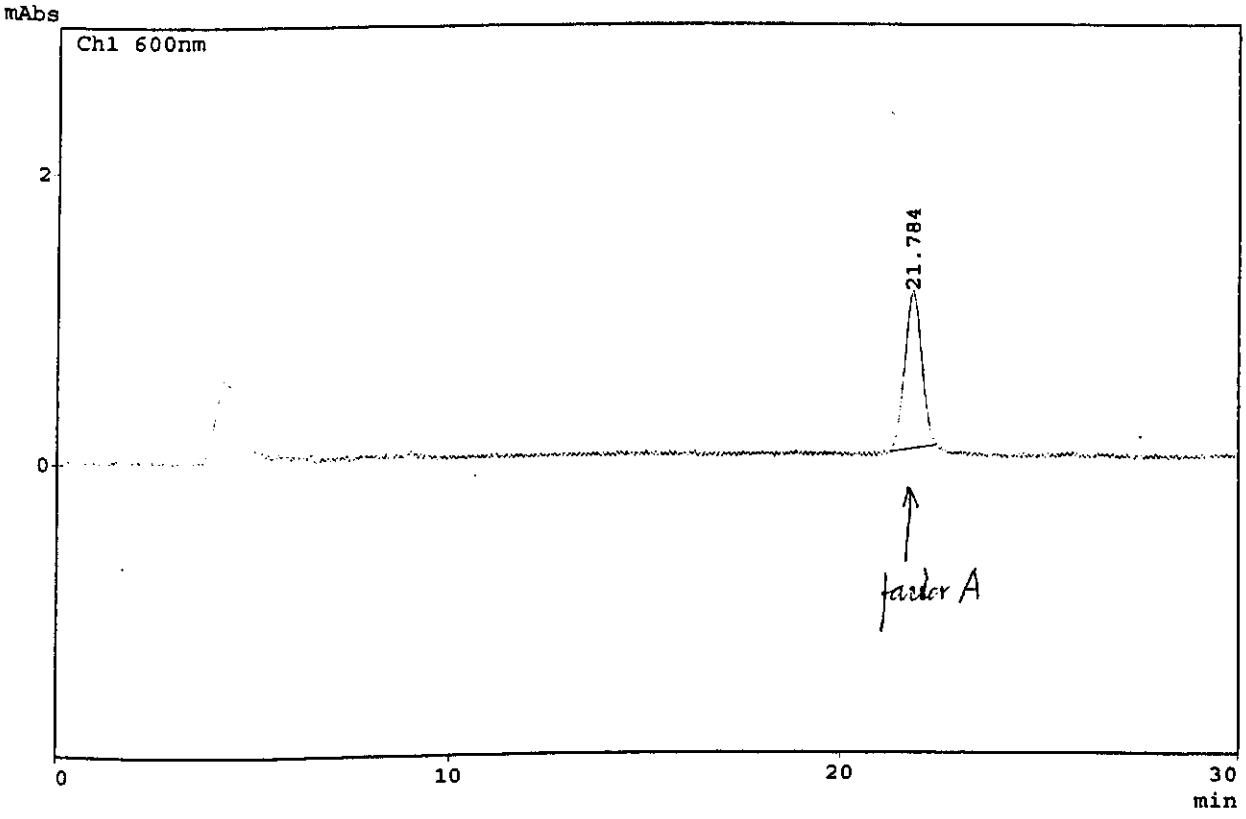
Type : Unknown

Detector : SPD-M10Avp

Operator : lju

Method Name : NARASIN.MET

*** Chromatogram ***



** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN	IDNO	CONC
1	1	21.784	32910		0.9975(0.9233)	0.9991(0.9685)	1	42.8736
			32910					42.8736

CLASS-LC10 Ver.=1.63 SYS=1 REPORT.NO=17 DATA=NA0904A9.K01 01/04/09 18:43:37

Sample : 113386 b

ID : nara09

Sample Amount : 1

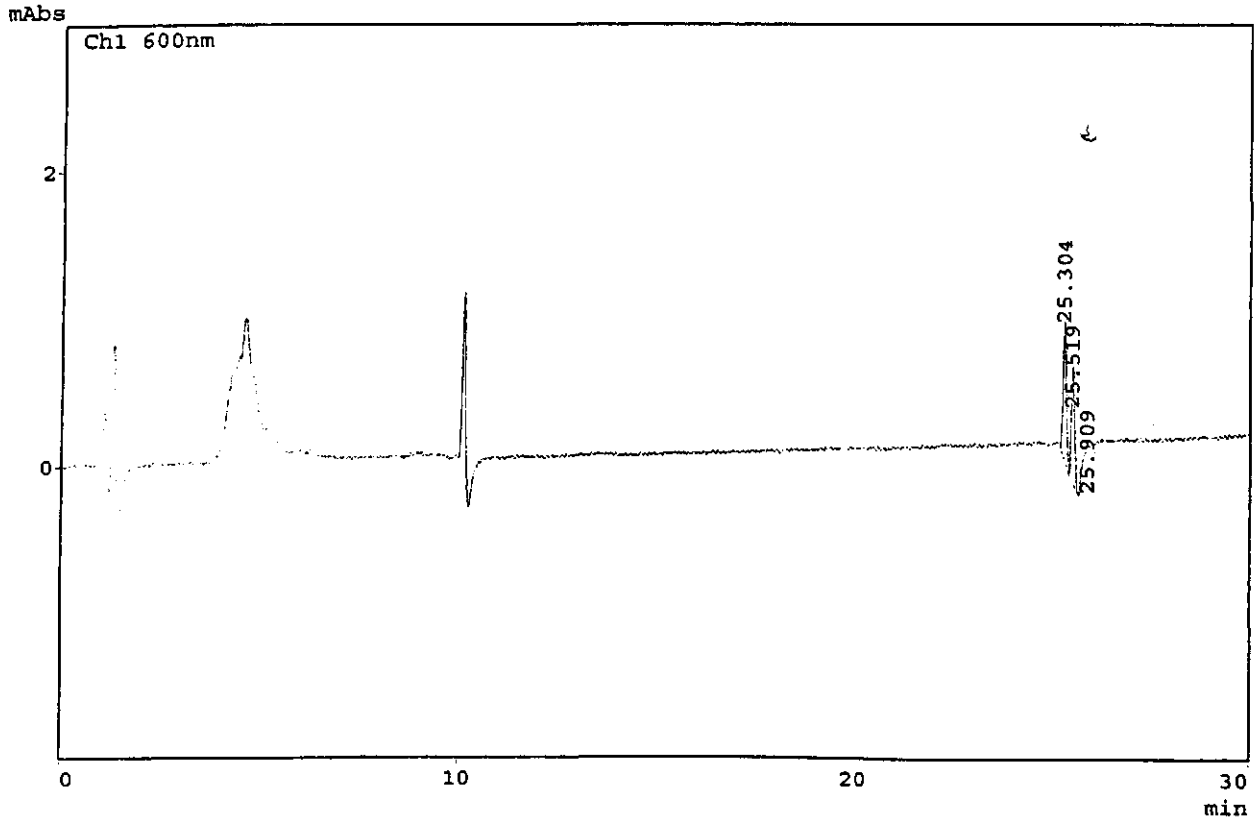
Type : Unknown

Detector : SPD-M10Avp

Operator : lju

Method Name : NARASIN.MET

*** Chromatogram ***



*** Peak Report ***

!! No Identified Peak !!

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 13

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
133375		64,9
133377		114,1
133399		42,2
133407		18,5
133432		66,4
133450		17,9
133462		< 0,5
133486		< 0,5
133487		43,6
133500		112,6

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		11402	11520

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

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

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:

- Blind positive feed samples X
- Blind blank feed sample X
- Premixture Y

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

Recovery results:

- Percentage recovery: 97,0 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 96,6 % and 97,4 %
- Spiking level: ..5,0.... mg/kg

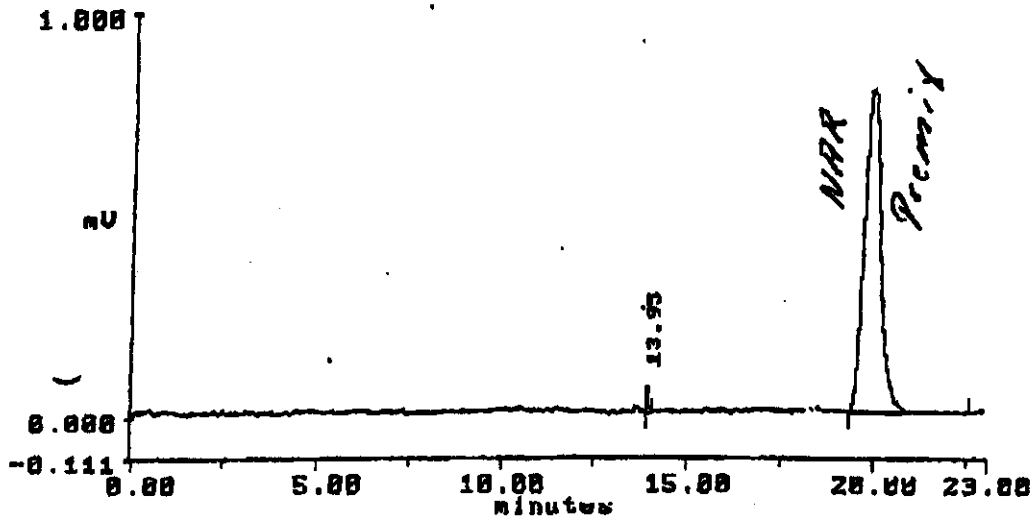
Analyzed: Mar 1, 2001 14:43 Reported: Mar 1, 2001 15:06

Method Title :
Method : POLY1 Data File : POLY1853.RW Channel : 1
Sample : F 750a>10000 UNK - Inj 1 Vial No. = 1 Vol = 100 ul

Premix

Channel 1

Premix + Nar



(13)

Channel 1

Peak Quantitation : HEIGHT
Calculation Method : EXT-STD
Sample Amount : 1.000
Scale Factor1 : 1.000

NO	RT	NAME	HEIGHT	mg/kg	R-FACTOR	RRT	BC
2	19.96	Narasin	799	114.305	1.431E-01	19.94	BB
TOTAL			799	114.305			

X

11430,5

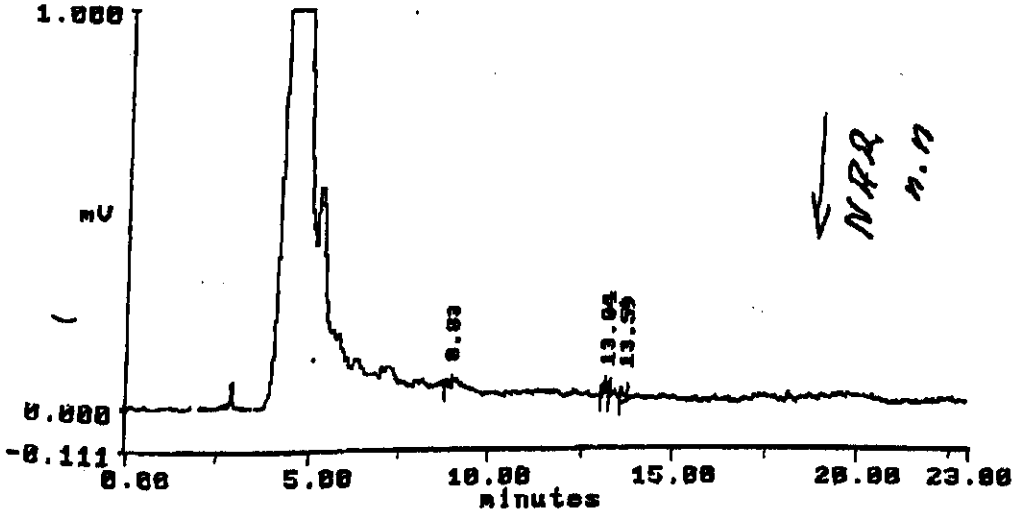
Peak Rejection Level : 5

Analyzed: Feb 23, 2001 9:16 Reported: Feb 23, 2001 11:13

Method Title :
Method : POLY1 Data File : POLY1844.RW Channel : 1
Sample : F 752 UNK - Inj 1 Vial No. = 4 Vol = 100 ul

133462 Channel 1 Blind blank

(13)



Channel 1

Peak Quantitation : HEIGHT
Calculation Method : EXT-STD
Sample Amount : 1.000
Scale Factor1 : 1.000

NU	RT	NAME	HEIGHT	mg/kg	R-FACTOR	RRT	BC
3	13.21	Monensin	82	1.855	4.170E-02	12.99	BB
TOTAL			82	1.855			

Peak Rejection Level : 5

133462/F752

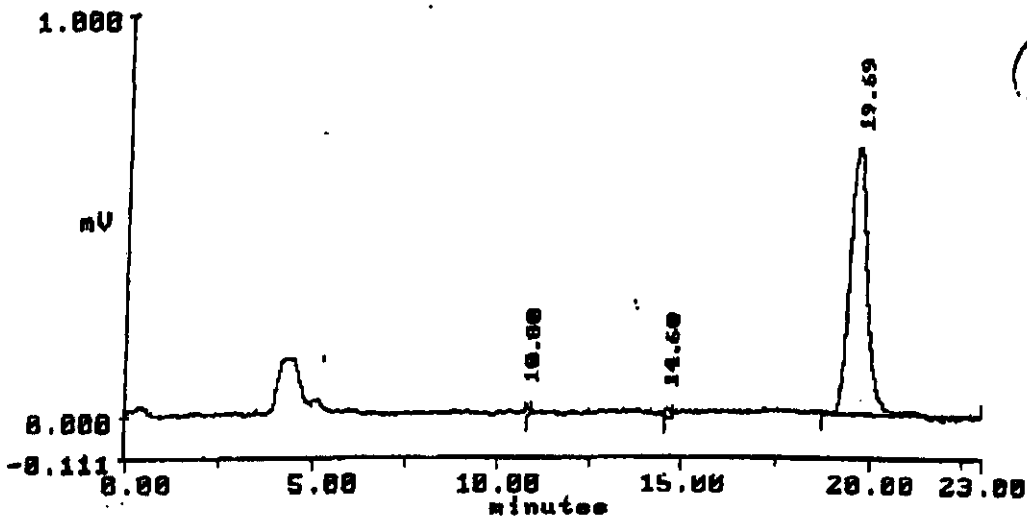
Analyzed: Mar 12, 2001 13:56 Reported: Mar 12, 2001 14:43

Method Title :
Method : POLY1 Data File : POLY1859.RW Channel : 1
Sample : Blinda>50Rec. UNK - Inj 1 Vial No. = 2 Vol = 100 ul

Channel 1

Blind, Control (recovery)

(13)



Channel 1

Peak Quantitation : HEIGHT
Calculation Method : EXT-STD
Sample Amount : 1.000
Scale Factor1 : 1.000

NO	RT	NAME	HEIGHT	mg/kg	R-FACTOR	RRT	BC
8	19.69	Narasin	659	97.404	1.476E-01	19.65	BB
TOTAL			659	97.404			

Peak Rejection Level : 5

Recovery 97.4%

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 23

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

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis: 04.18.01

Analyte: NARASIN

Sample code	Unit	Result (mg/kg)
233410		19,3
233414		< 2
233416		< 2
233427		44,8
233434		69,1
233453		64,1
233463		18,3
233467		117
233469		119
233492		45,4

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		10420	10500

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 24

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis: 6-7/03/2001

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
243390		0
243409		20,1
243420		113,6
243421		41,1
243435		63,3
243457		16,4
243474		67,9
243483		112,9
243484		41,6
243501		0

Sample / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	5244	6634

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: 6th - 7th March 2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: SpheriSorb C18 5µ (250 x 4.6) mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,5 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results: *

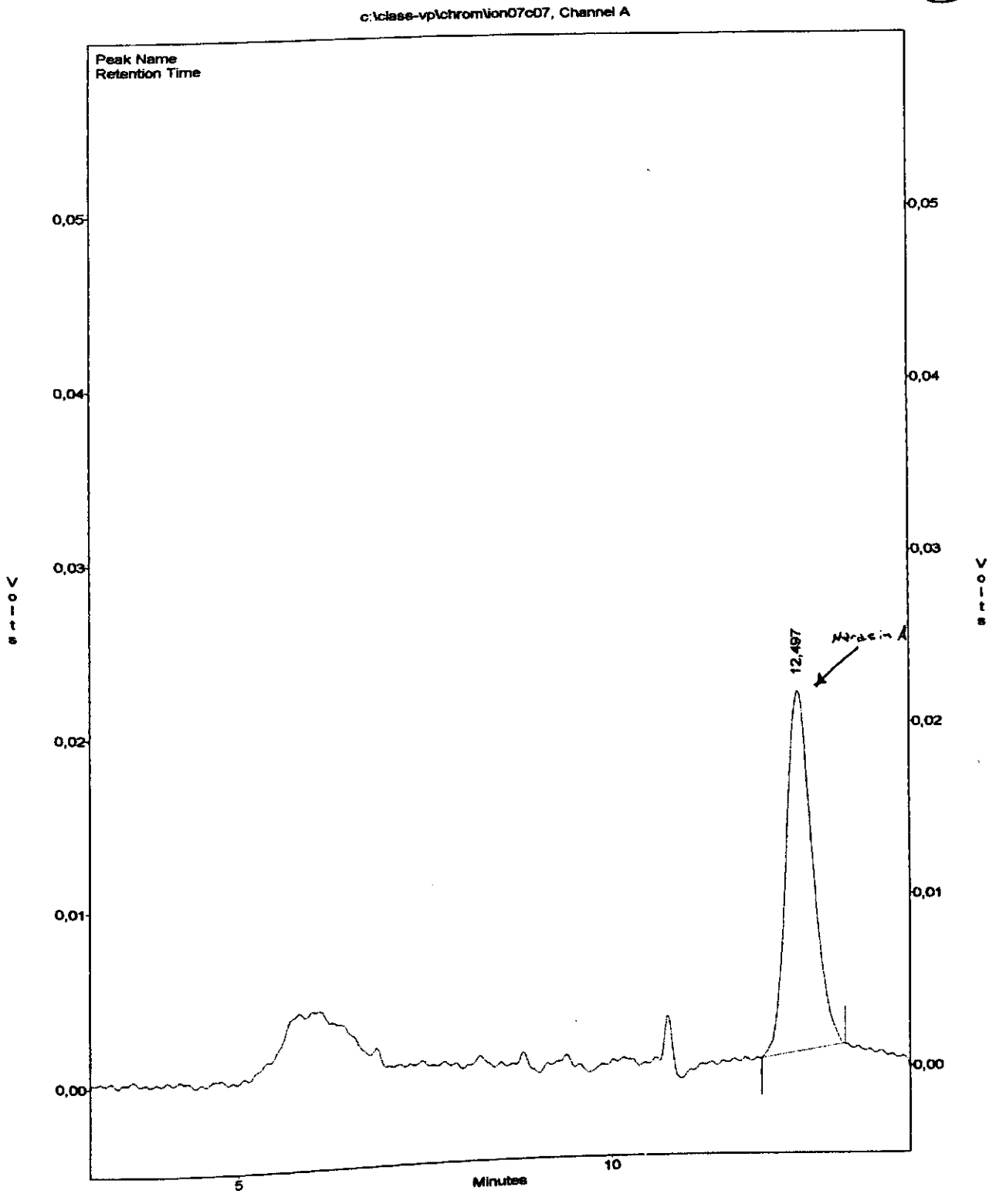
- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg

* We performed single determinations of feed samples spiked at two different levels:
level 1 : 5 mg/kg ⇒ Recovery = 76,6 %
level 2 : 12,5 mg/kg ⇒ Recovery = 93,5 %
} Average ≈ 85 %

Representative chromatogram of a blind positive feed sample

SAMPLE CODE: 243484

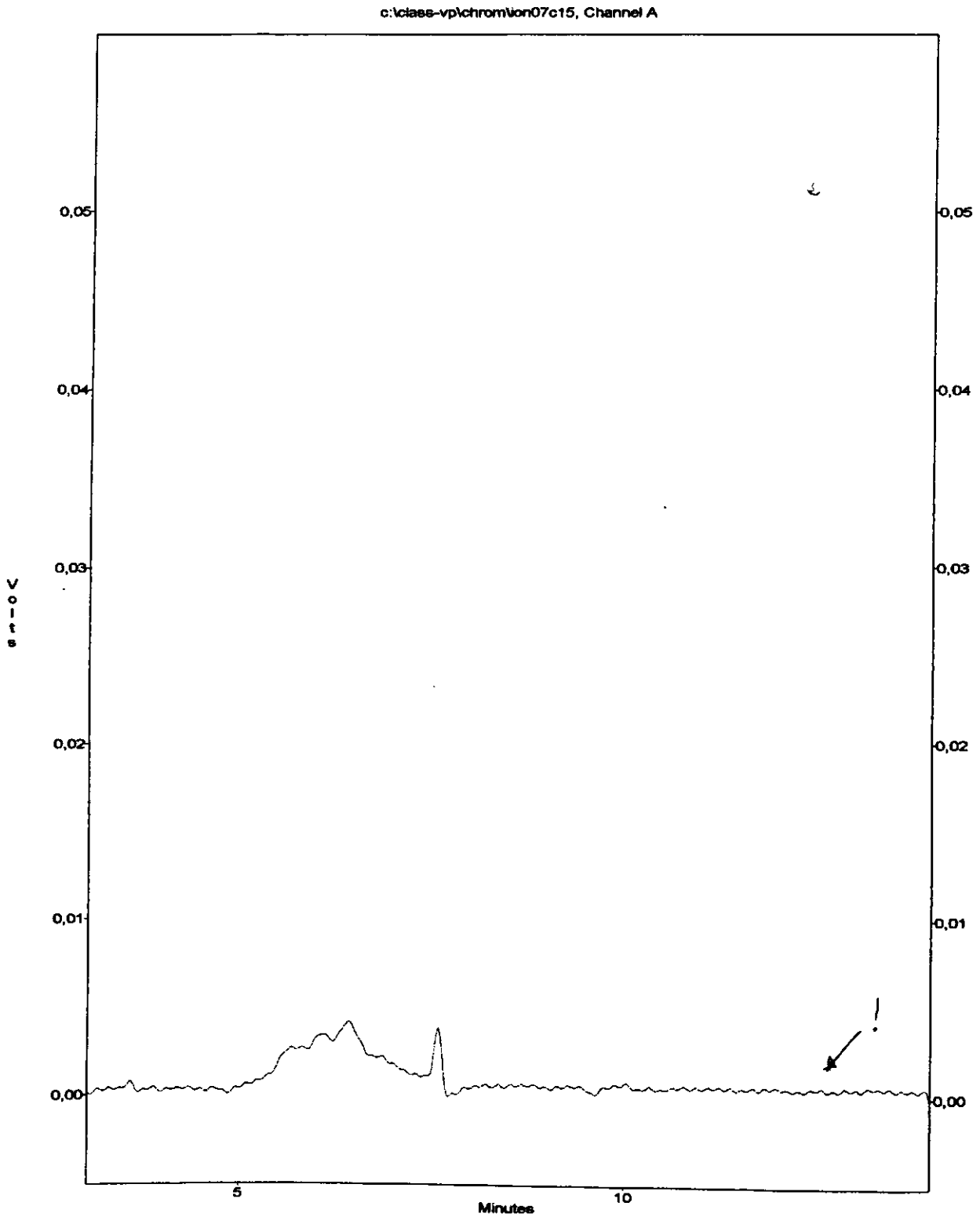
(24)



Representative chromatogram of a blank feed sample

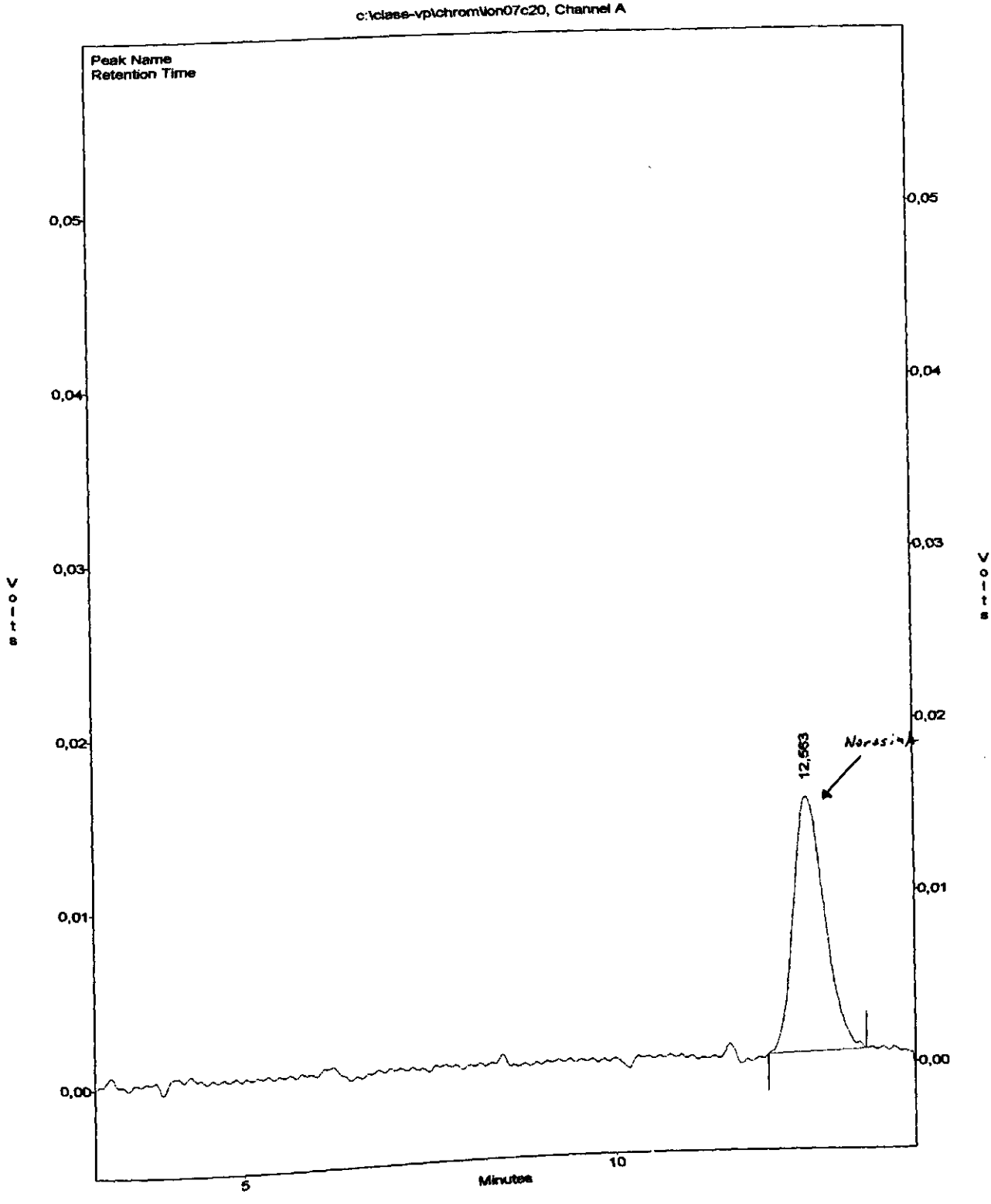
SAMPLE CODE : 243501

(24)



Representative chromatogram of a blind positive ~~premixture~~

SAMPLE CODE: PREMIXTURE



APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 26

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
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis: 04/04/2001

Analyte: NARASIN

	Unit	Result (mg/kg)
Sample code		
263412		33,6
263419		64,7
263424		63,1
263449		2,8
263452		42,5
263455		103,8
263465		17,9
263470		0,6
263493		103,7
263502		20,4

Unit / Sample	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	8212	10063

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: 04/04/2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ODS 3 10µm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 0.5 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

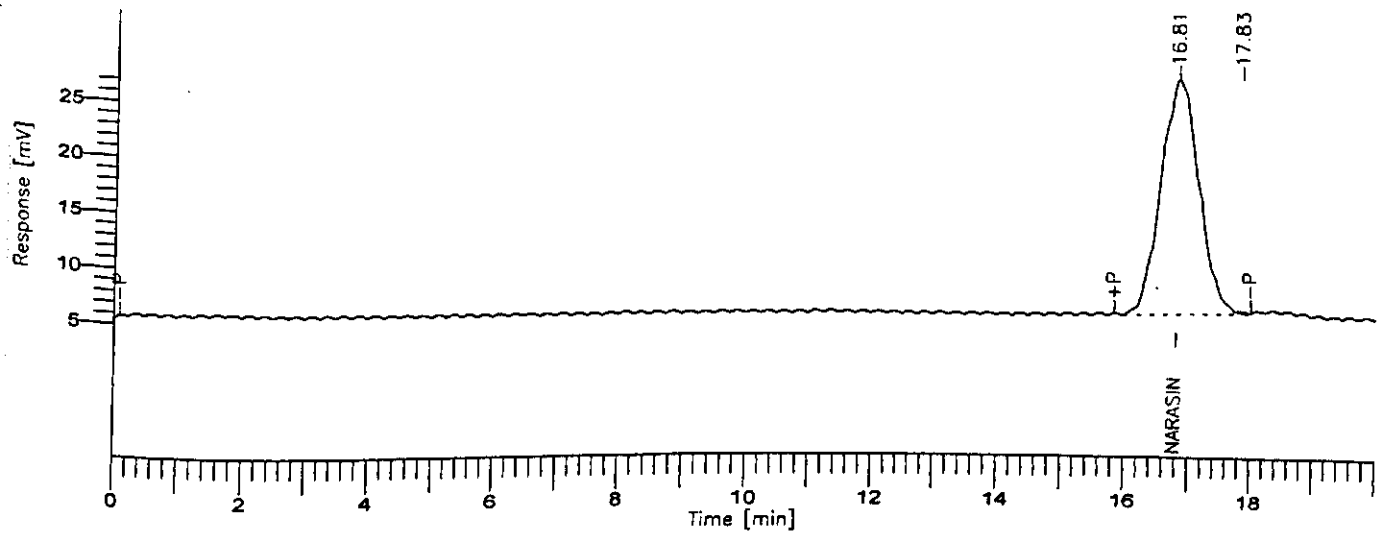
Recovery results:

- Percentage recovery: 92 %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 91 % and 92 %
- Spiking level: 51 mg/kg

Software Version: 4.1<2F12>
Date: 05/04/01 16:21

(26)

Sample Name : Premix 1
Data File : C:\TC4\CANFAS\NARASIN\LIN_AN~1\DATA019.RAW Date: 04/04/01 19:07
Sequence File: C:\TC4\CANFAS\NARASIN\LIN_AN~1\NARASIN1.SEQ Cycle: 19 Channel
Instrument : BOX_2 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



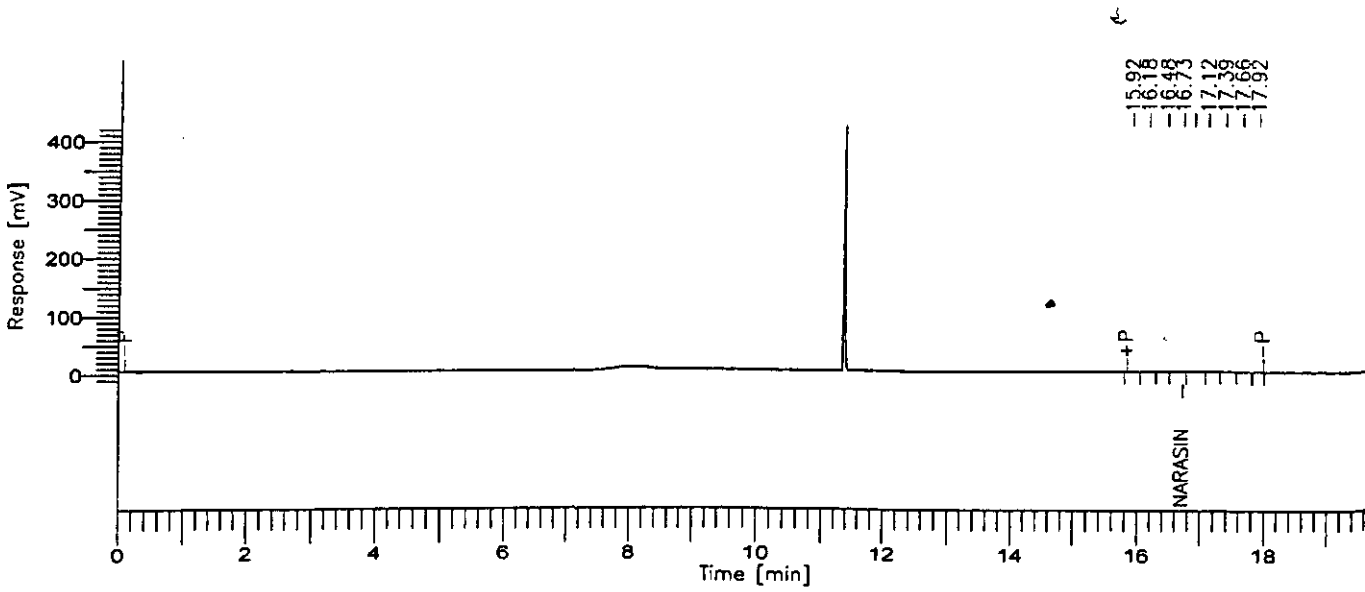
DEFAULT REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	16.808	883397.00	21221.76	99.86	99.86	BE	41.63
2	17.825	1265.00	136.14	0.14	0.14	EB	9.29
		884662.00	21357.91	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)
All components were found	

Software Version: 4.1<2F12>
Date: 05/04/01 16:22
Sample Name : 263449
Data File : C:\TC4\CANFAS\NARASIN\LIN_AN~1\DATA036.RAW Date: 05/04/01 00:
Sequence File: C:\TC4\CANFAS\NARASIN\LIN_AN~1\NARASIN1.SEQ Cycle: 36 Channe
Instrument : BOX_2 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



DEFAULT REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	15.922	1969.00	221.61	3.02	3.02	*BB	8.88
2	16.181	2245.50	229.52	3.44	3.44	BV	9.78
3	16.478	5056.10	519.31	7.74	7.74	VV	9.74
4	16.725	15459.80	1199.28	23.68	23.68	VV	12.89
5	16.900	22251.20	1424.80	34.08	34.08	VV	15.62
6	17.117	10434.80	963.20	15.98	15.98	VV	10.83
7	17.390	4584.50	426.08	7.02	7.02	VV	10.76
8	17.658	2287.60	247.48	3.50	3.50	VB	9.24
9	17.918	1006.00	138.96	1.54	1.54	*BB	7.24
		65294.50	5370.24	100.00	100.00		

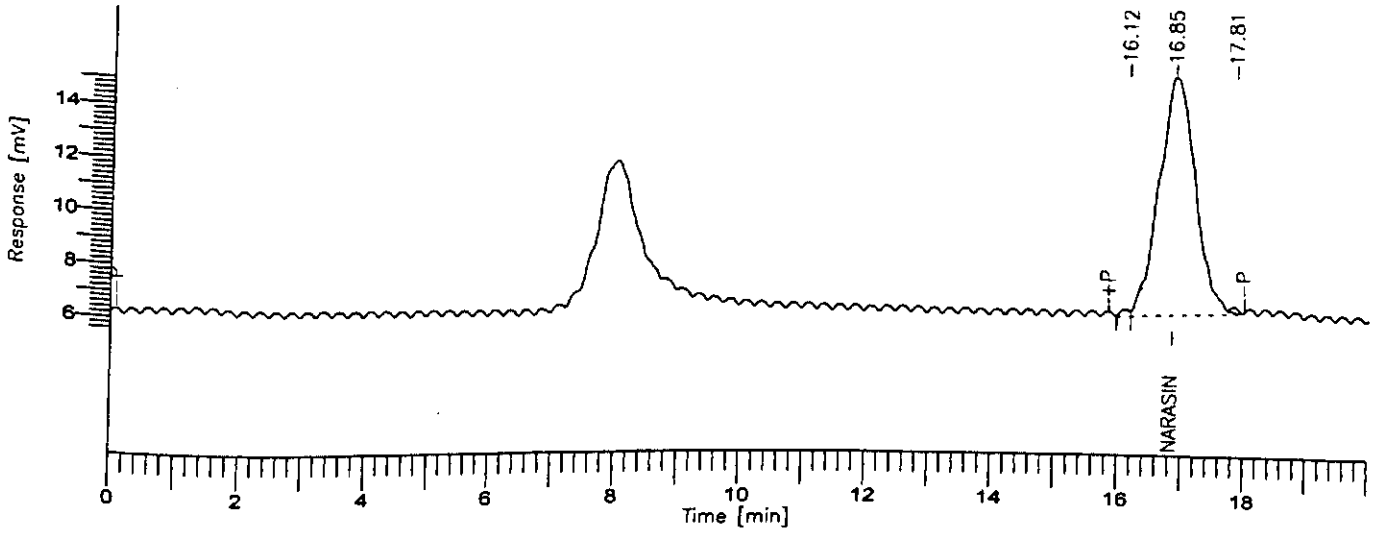
Missing Component Report
Component Expected Retention (Calibration File)

All components were found

Software Version: 4.1<2F12>
Date: 05/04/01 16:22

(26)

Sample Name : 263465
Data File : C:\TC4\CANFAS\NARASIN\LIN_AN~1\DATA047.RAW Date: 05/04/01 04:45
Sequence File: C:\TC4\CANFAS\NARASIN\LIN_AN~1\NARASIN1.SEQ Cycle: 47 Channel
Instrument : BOX_2 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



DEFAULT REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	16.120	2889.13	275.19	0.79	0.79	BV	10.50
2	16.852	359897.87	8988.04	98.71	98.71	VE	40.04
3	17.814	1832.00	200.23	0.50	0.50	EB	9.15
		364619.00	9463.45	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)
All components were found	

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 29

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
Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis: 23-27.04.01

Analyte: NARASIN

Sample code	Unit	Result (mg/kg)
293384		0
293426		16,96
293431		15,87
293445		44,26
293454		119,5
293460		68,1
293471		45,88
293489		0
293490		65,5
293499		114,4

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		12800	13060

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: 23-27 April 2001

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: 0.9 ml/min
- Injection volume: 200 µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

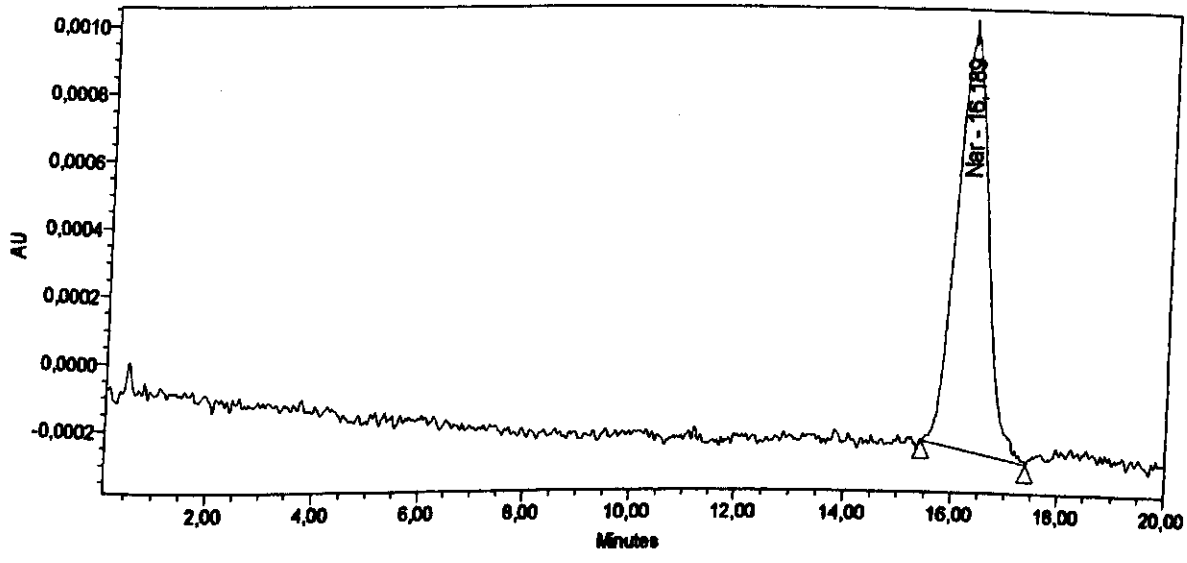
- Percentage recovery: 100.8%
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 50 mg/kg

Project Name: Narasin_CANFAS

SAMPLE INFORMATION

Sample Name:	PM I Dil 1/50	Processing Method:	Nar 24 Ap
Vial:	31	Run Time:	20,0 Minutes
Injection #:	1	Proc. Chnl. Descr.:	PDA 600,0 nm
Injection Volume:	200,00 ul		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Nar	16,189	51704	1248	1,280	ug/ml

Result : mg/kg narasin

$$C = \frac{100 \times A \times D}{w}$$

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

W=0,5g
D=50

C 12800 mg/kg narasin

Reemisheee

(29)

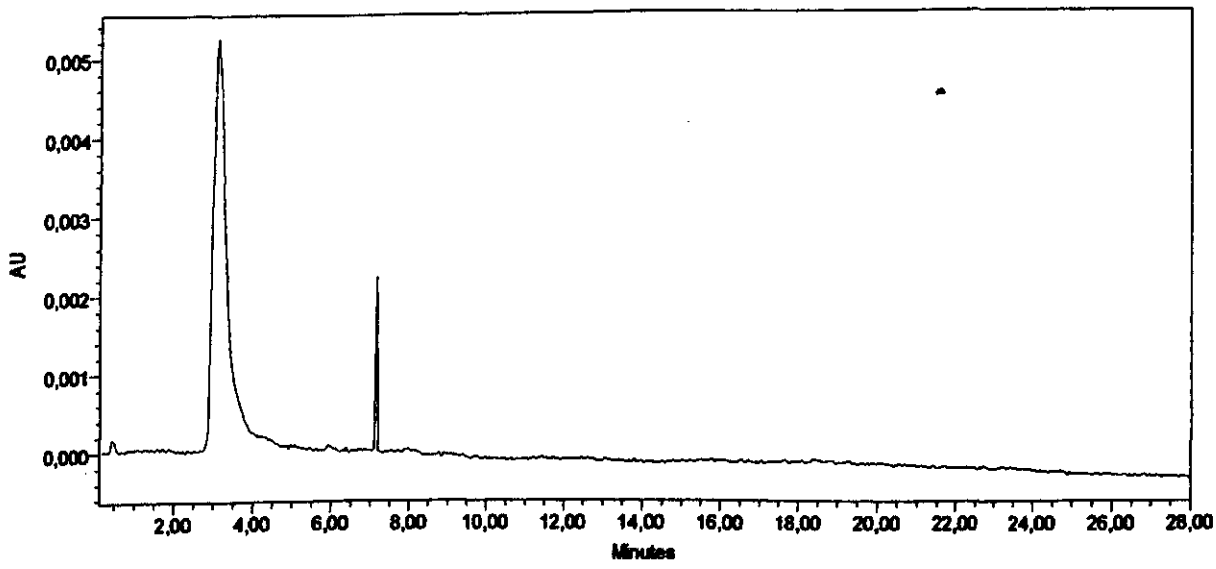
Project Name: Narasin_CANI-AS

SAMPLE INFORMATION

Sample Name: 384
Vial: 108
Injection #: 1
Injection Volume: 200,00 ul

Processing Method: Nar area 11_04
Run Time: 28,0 Minutes
Proc. Chnl. Descr.: PDA 600,0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Nar	22,859				

Result : mg/kg narasin

$$C = \frac{100 \times A \times D}{w}$$

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

w = 10g
D = 1

C _____ mg/kg narasin

Blind blank feed sample

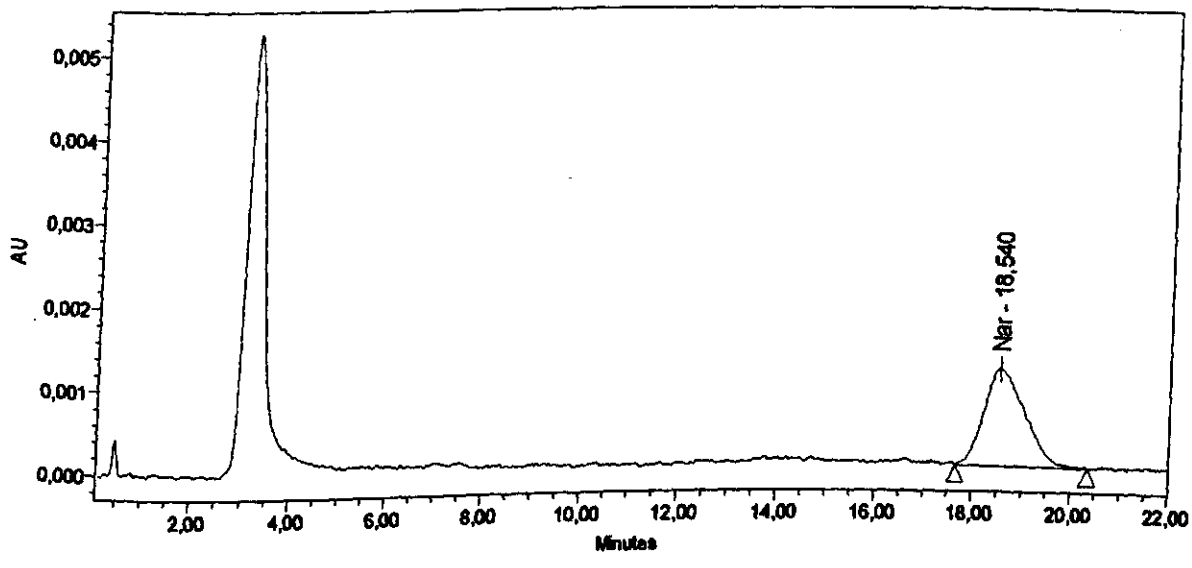
Project Name: Narasin_CANFAS

SAMPLE INFORMATION

Sample Name: 426
Vial: 35
Injection #: 1
Injection Volume: 200,00 ul

Processing Method: Nar 24 Ap
Run Time: 22,0 Minutes
Proc. Chnl. Descr.: PDA 600,0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Nar	18,540	68211	1183	1,696	ug/ml

Result : mg/kg narasin

$$C = \frac{100 \times A \times D}{w}$$

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

W = 10g
D = 1

c 16,96 mg/kg narasin

Blind positive feed sample

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: ....03.12.01.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: .....Kromasil C18 150 x 4.6 mm.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ...0.7..... ml/min
- Injection volume: 100...µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

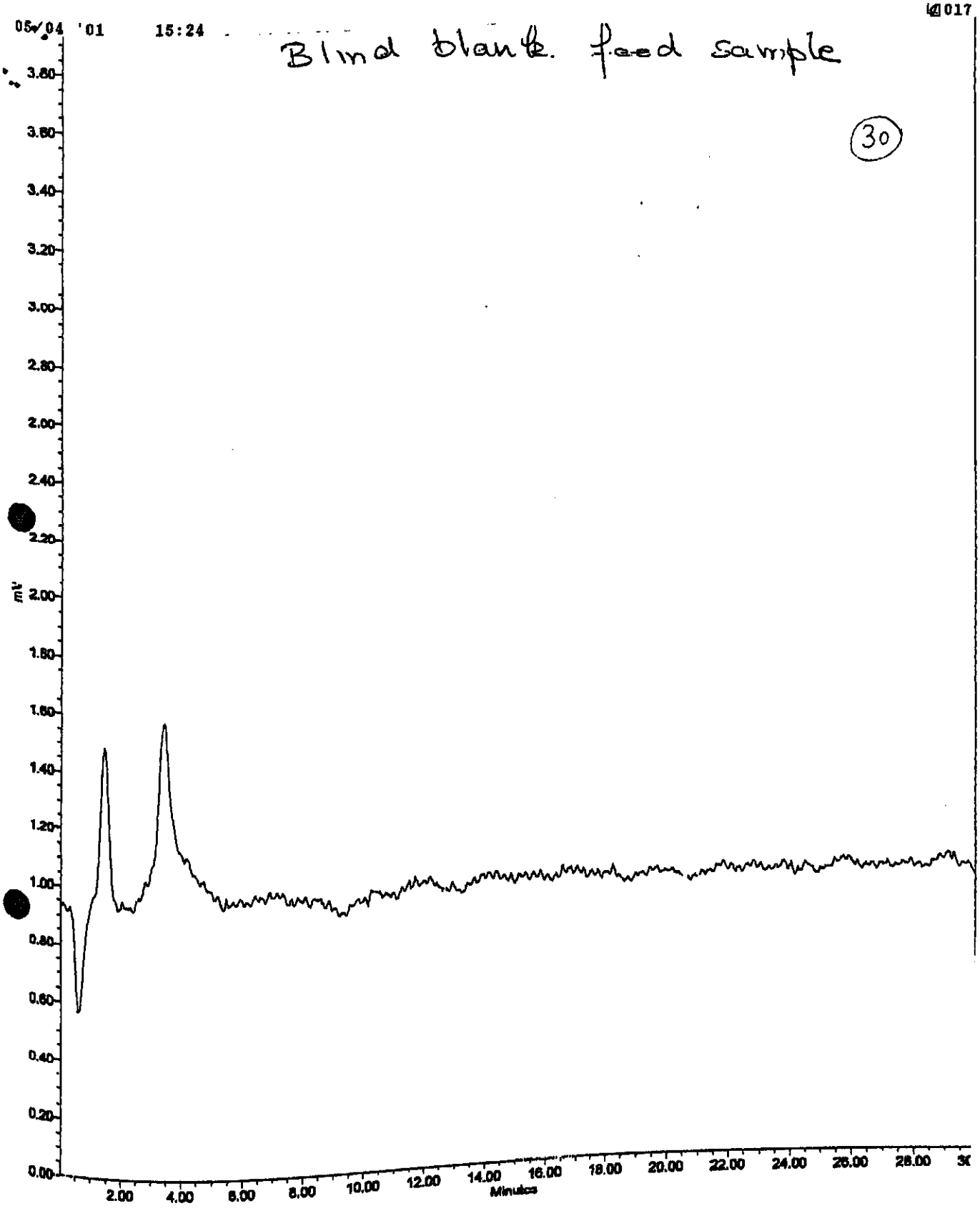
Recovery results:

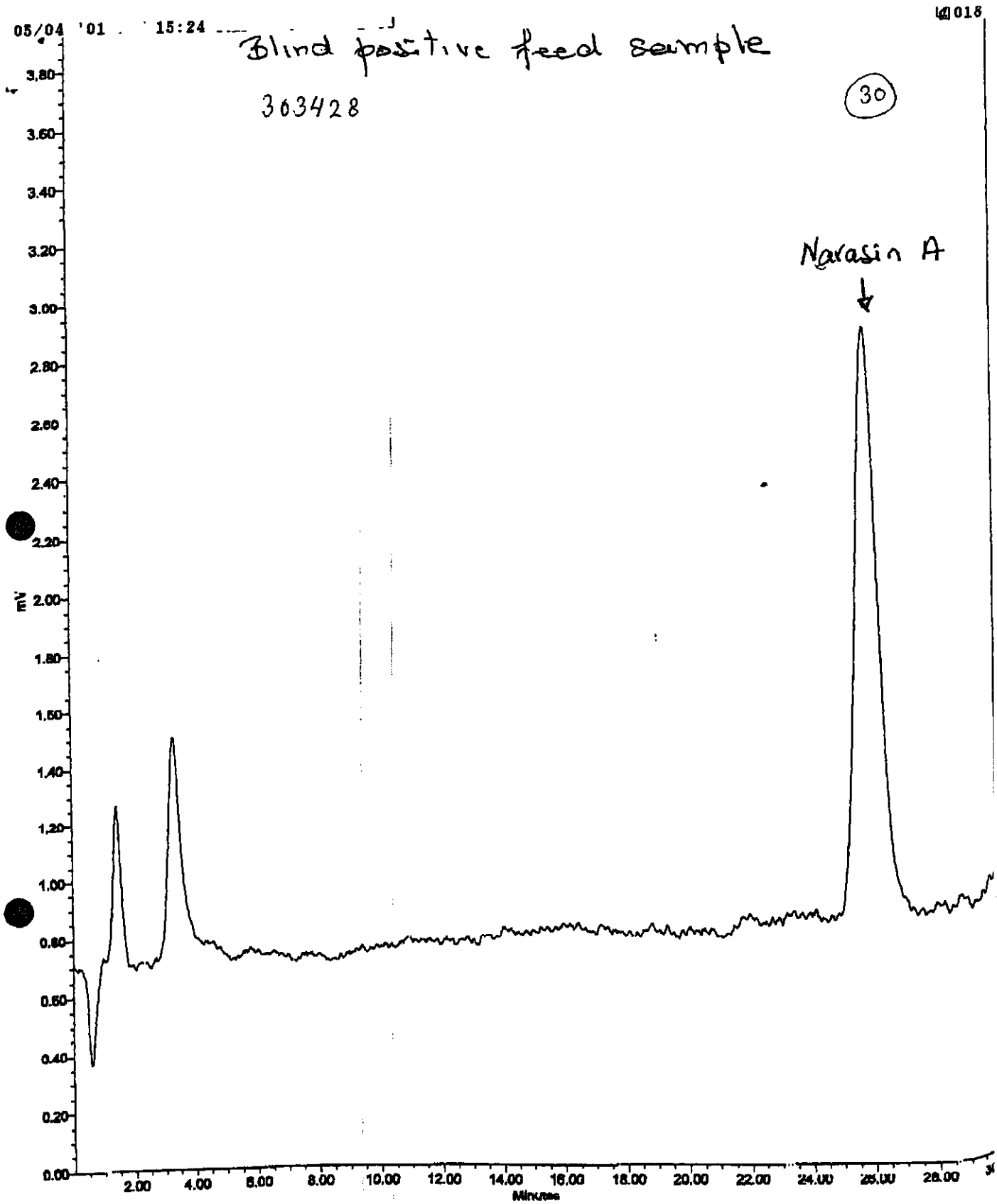
- Percentage recovery: 98 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..51.. mg/kg

05/04 '01 15:24

Blind blank. feed sample

(30)

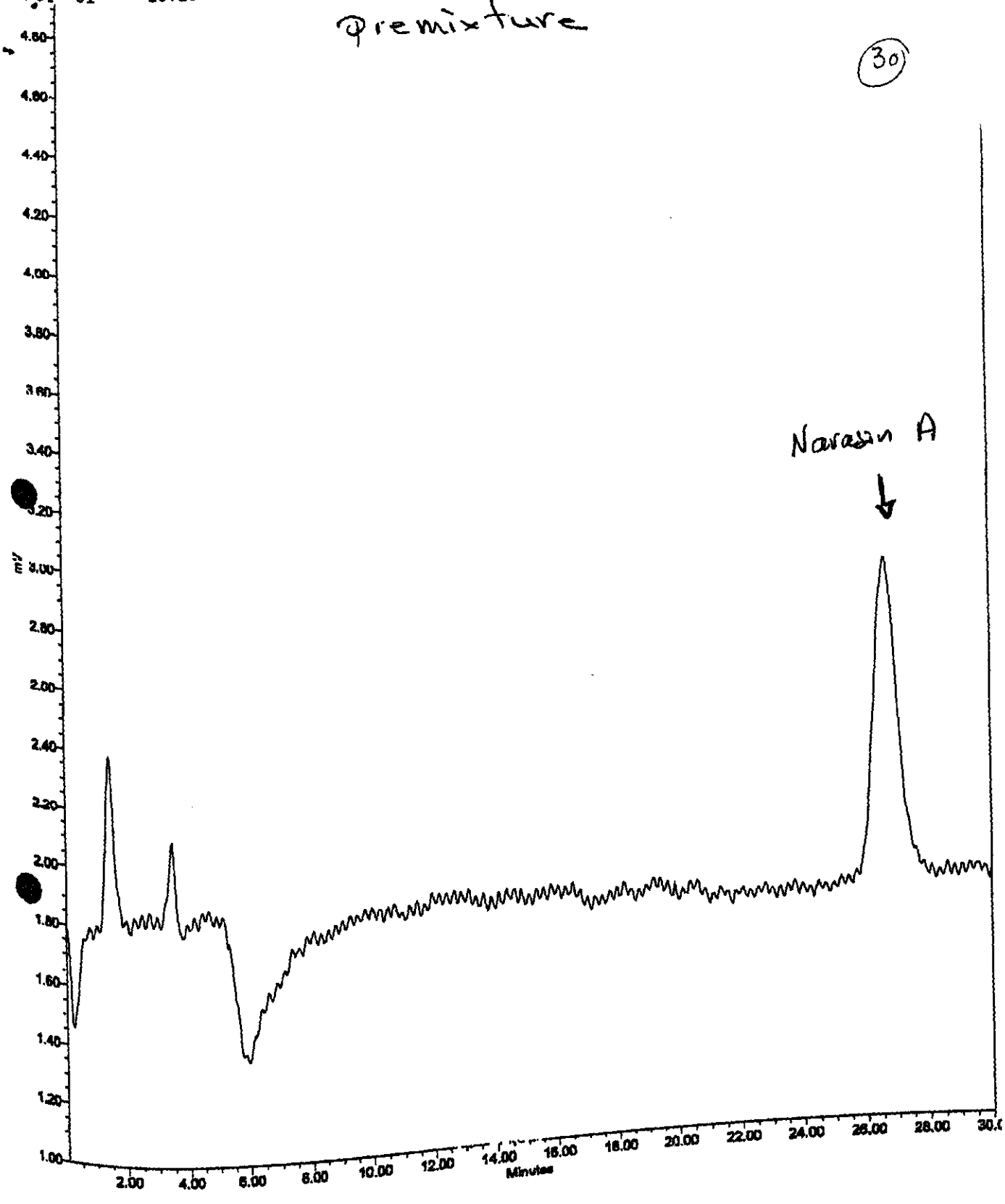



05/04 '01 15:24
Blind positive feed sample
303428
30
Narasin A
mV
Minutes

05/04 '01 15:25

premixture

(30)



APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 31

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis: 20.02.01

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
313376		17,7
313393		111,9
313397		109,3
313403		41,2
313418		64,1
313437		66,4
313439		18,0
313461		0
313473		40,4
313482		0

Sample / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	12102	11646

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: .....20..-..02..-.2001.....

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:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

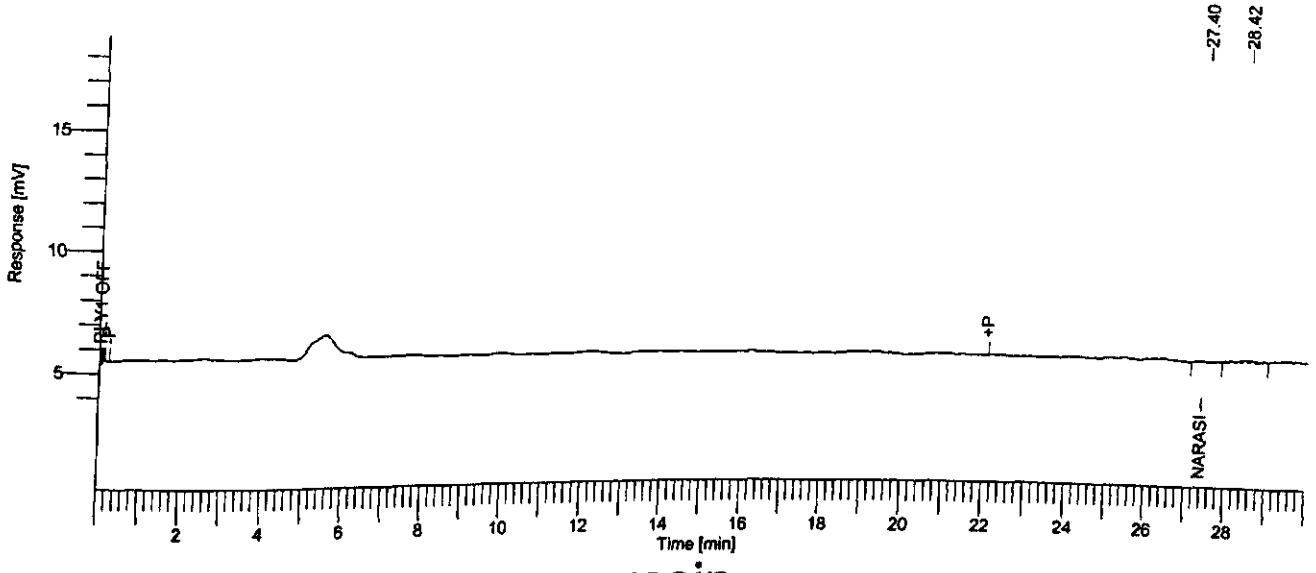
- Percentage recovery: .100,3%
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99,2 % and 102,5 %
- Spiking level: ...50... mg/kg

Software Version	: 6.1.1.0.0:K20	Date	: 2/21/01 8:12:03 AM
Sample Name	: blan -1 (3*	Data Acquisition Time	: 2/20/01 5:01:18 PM
Instrument Name	: HPLC-1	Channel	: A
Rack/Vial	: 0/0	Operator	: I
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 6		

blank

(31)

Result File : \\ 004s\TCDATA\ . Residue\HPLC-1\narasin\200201-006-20010221-081155.rst
Sequence File : \\ 004s\TCdata\ . Residue\HPLC-1\narasin\narasin200201.seq



narasin

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	27.40	narasin	794.01	39.82	19.17	19.17	BV	19.9375
2	28.42		3347.31	78.45	80.83	80.83	VB	42.6675
			4141.32	118.28	100.00	100.00		

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

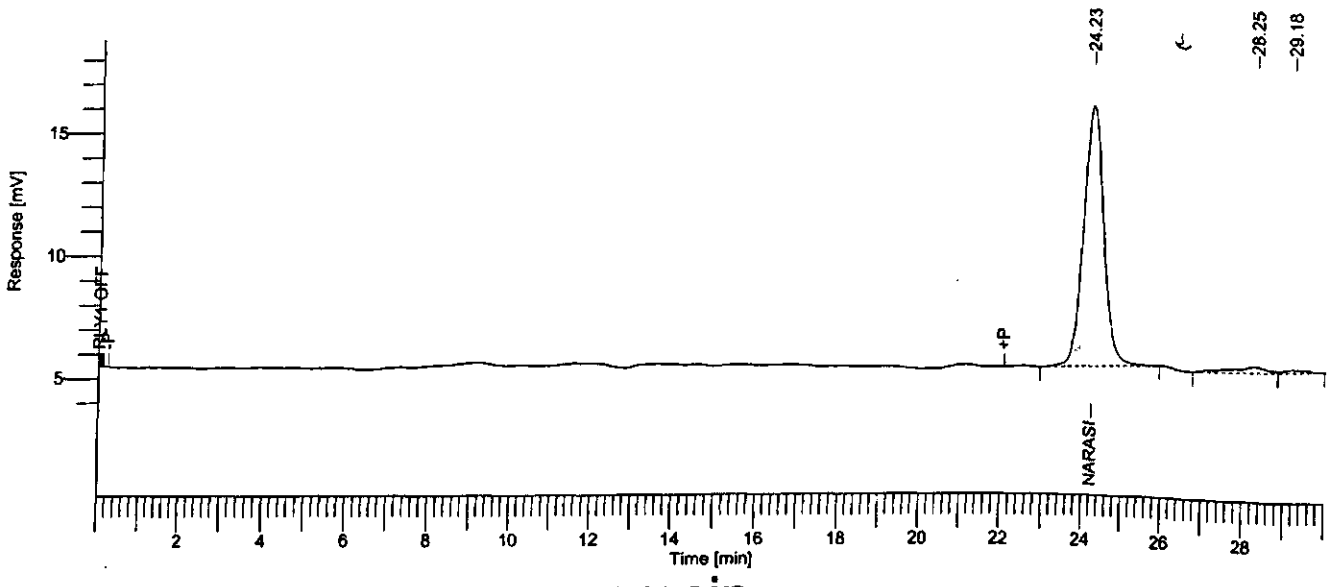
Blanco Voer

Software Version	: 6.1.1.0.0:K20	Date	: 2/21/01 8:12:06 AM
Sample Name	: 30258-a (3*	Data Acquisition Time	: 2/20/01 6:36:13 PM
Instrument Name	: HPLC-1	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 9		

premix

31

Result File : \ .004s\TCDATA\ Residue\HPLC-1\narasin\200201-009-20010221-081155.rst
Sequence File : \ .004s\TCdata\ Residue\HPLC-1\narasin\narasin200201.seq



narasin

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	24.23	narasin	381402.50	10748.73	94.43	94.43	BB	35.4835
2	28.25		16810.92	252.80	4.16	4.16	BV	66.4990
3	29.18		5679.08	136.21	1.41	1.41	VB	41.6928
			403892.50	11137.74	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)

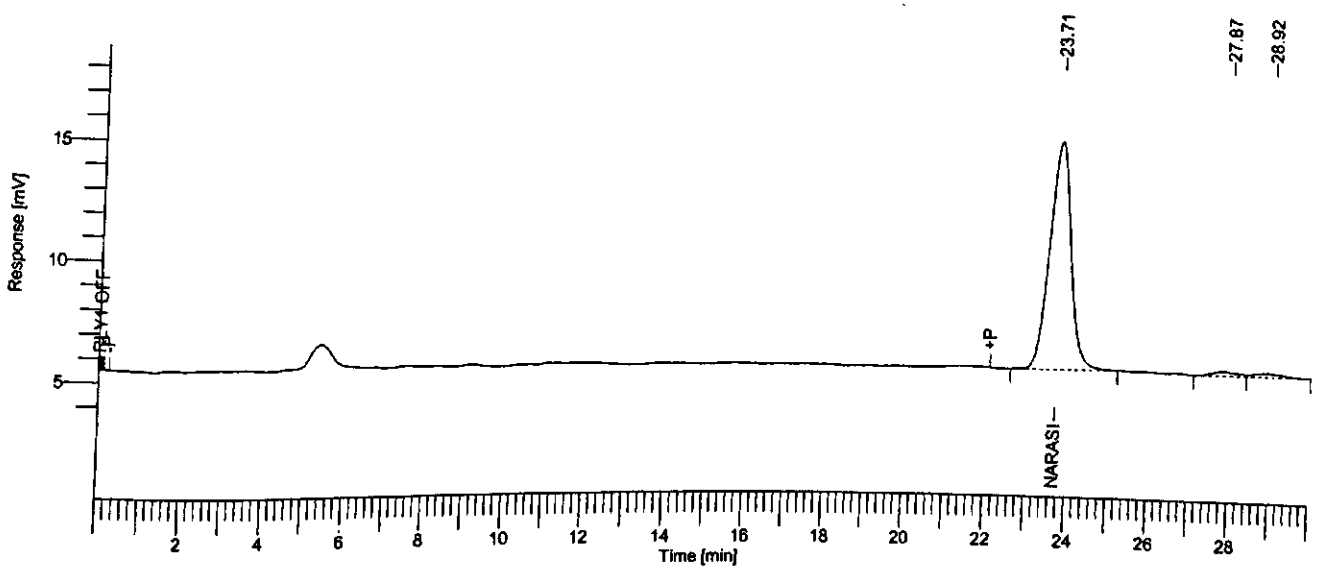
All components were found

Software Version	: 6.1.1.0.0:K20	Date	: 2/21/01 8:12:16 AM
Sample Name	: 30266 (3*	Data Acquisition Time	: 2/21/01 12:24:23 AM
Instrument Name	: HPLC-1	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 20		

313397

(31)

Result File : \\ .004s\TCDATA\ Residue\HPLC-1\narasin\200201-020-20010221-081156.rst
Sequence File : \ .004s\TCdata\ Residue\HPLC-1\narasin\narasin200201.seq



narasin

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	23.71	narasin	341857.00	9510.46	95.70	95.70	BB	35.9454
2	27.87		7624.24	172.14	2.13	2.13	BV	44.2900
3	28.92		7740.26	155.57	2.17	2.17	VB	49.7529
			357221.50	9838.17	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

Monsler 313397

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 32

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
323378	34,90	34,89
323382	96,43	97,69
323395	35,74	35,85
323398	15,14	15,58
323406	55,96	56,69
323417	negative	negative
323440	15,07	15,18
323446	93,04	93,49
323481	negative	negative
323488	51,69	52,43

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		10041,48	10450,68

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Waters Spherisorb, 5 µm, S5 ODS 2, 250 mm X 4.6 mm
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: MeOH + Phosphate buffer (97+3)
- Flow-rate: 1.0 ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

- Percentage recovery: 82.3%
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 82.28% and 82.32%
- Spiking level:.50 mg/kg

File C:\HPCHEM\1\DATA\NARCANF\DMABA005.D

Sample Name:

ode 323382/ - 5.0005g

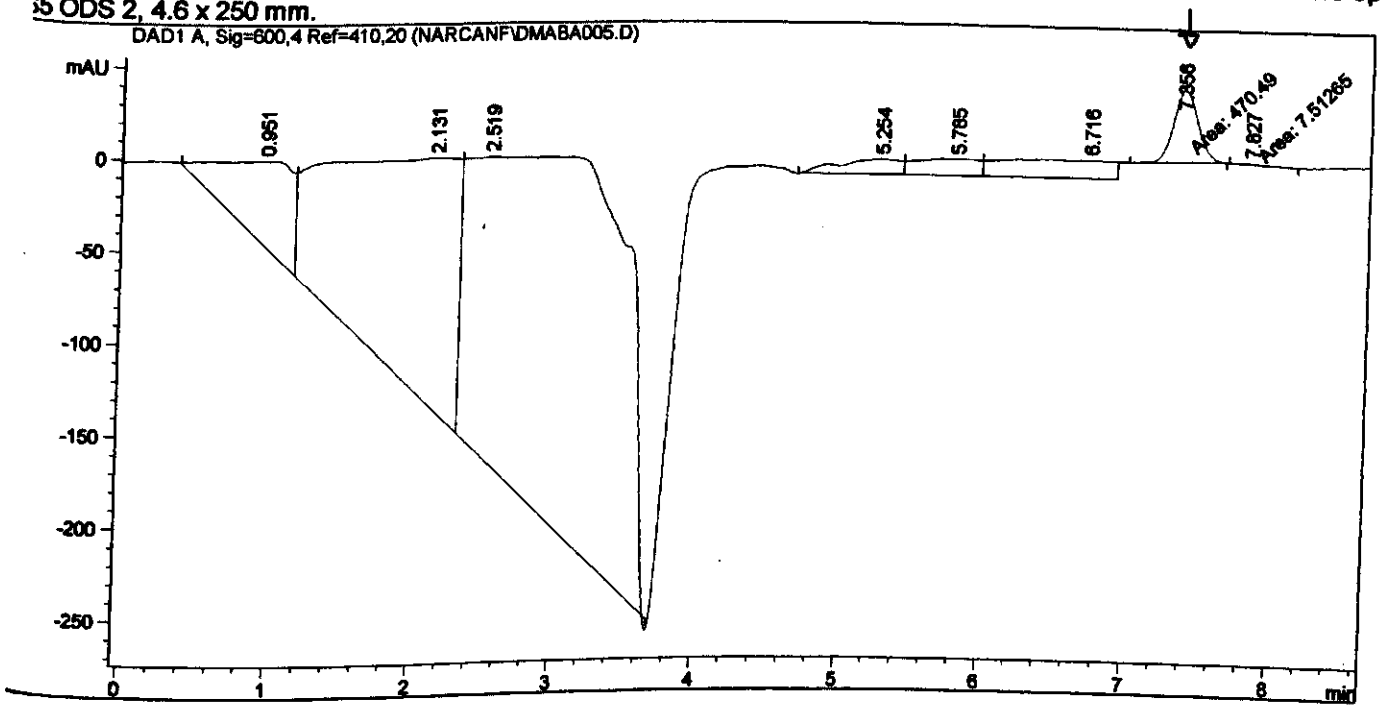
(32)

jection Date : 3/15/01 7:52:59 PM Seq. Line : 5
ample Name : 323382/ Vial : 10
cq. Operator Inj : 1
Inj Volume : 100 µl
cq. Method : C:\HPCHEM\1\METHODS\NARASIN .M
ast changed : 3/15/01 7:50:24 PM by
(modified after loading)
nalysis Method : C:\HPCHEM\1\METHODS\NARASIN .M
ast changed : 3/16/01 12:39:21 PM by
(modified after loading)

NARASIN (factor A)

Waters Sp

5 ODS 2, 4.6 x 250 mm.



External Standard Report

Sorted By : Signal
Calib. Data Modified : 3/16/01 12:34:33 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=600,4 Ref=410,20

RetTime [min]	Type	Height [mAU]	Amt/Height [ug/ml]	Amount	Grp	Name
7.356	MM	40.60791	6.01554e-2	2.44278		NAR A
7.827	MM	5.72264e-1	5.18771e-2	2.96874e-2		NAR D+I
Totals :				2.47247		

rument 1 3/16/01 12:42:57 PM

ata File C:\HPCHEM\1\DATA\NARCANF\DMABA008.D Sample Name: 323398

Code 323398 - 5.0002g

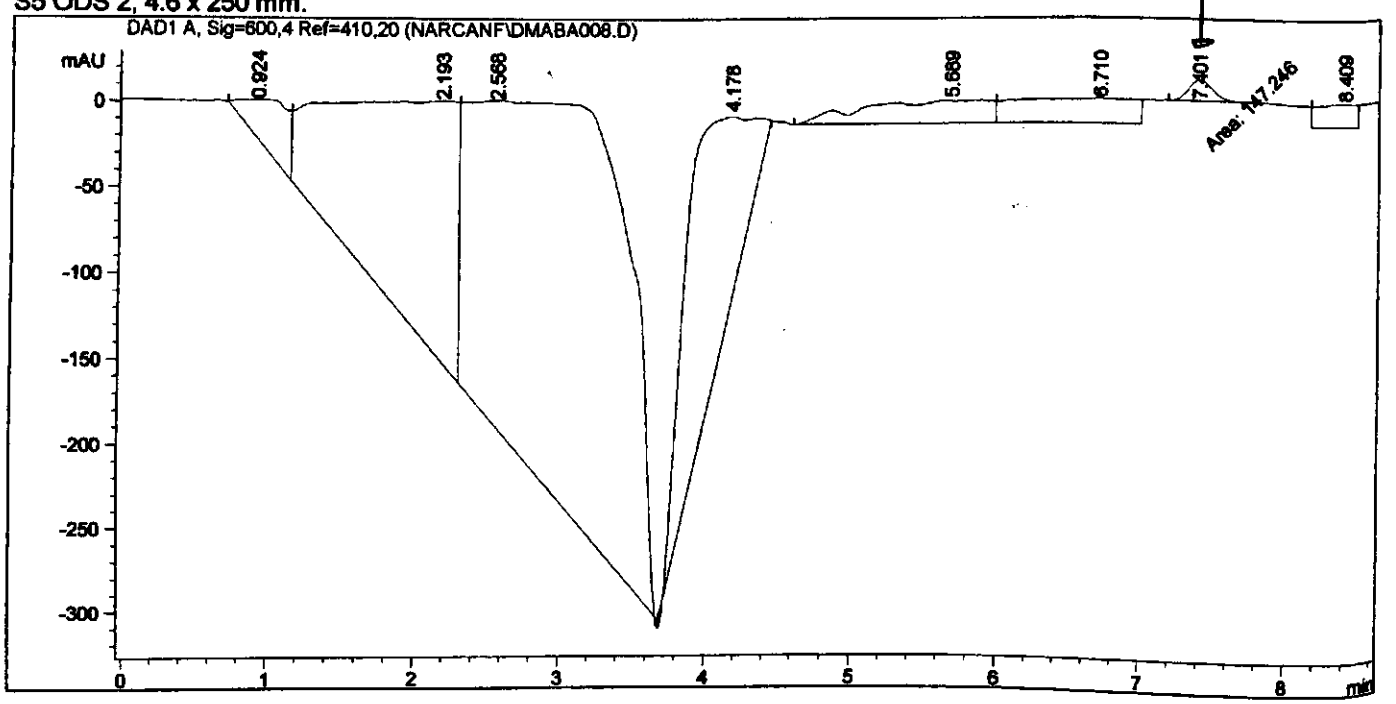
32

Injection Date : 3/15/01 8:27:14 PM Seq. Line : 8
Sample Name : 323398 Vial : 13
Acq. Operator Inj : 1
Inj Volume : 100 µl
Acq. Method : C:\HPCHEM\1\METHODS\NARASIN .M
Last changed : 3/15/01 8:24:38 PM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\NARASIN .M
Last changed : 3/16/01 12:50:55 PM by
(modified after loading)

nana hw
(factor A)

Waters Sp

S5 ODS 2, 4.6 x 250 mm.



External Standard Report

Sorted By : Signal
Calib. Data Modified : 3/16/01 12:34:33 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=600,4 Ref=410,20

RetTime [min]	Type	Height [mAU]	Amt/Height [ug/ml]	Amount	Grp	Name
7.401	MM	12.81608	5.90738e-2	7.57094e-1		NAR A
7.814	-	-	-			NAR D+I

Totals : 7.57094e-1

trument 1 3/16/01 12:52:40 PM

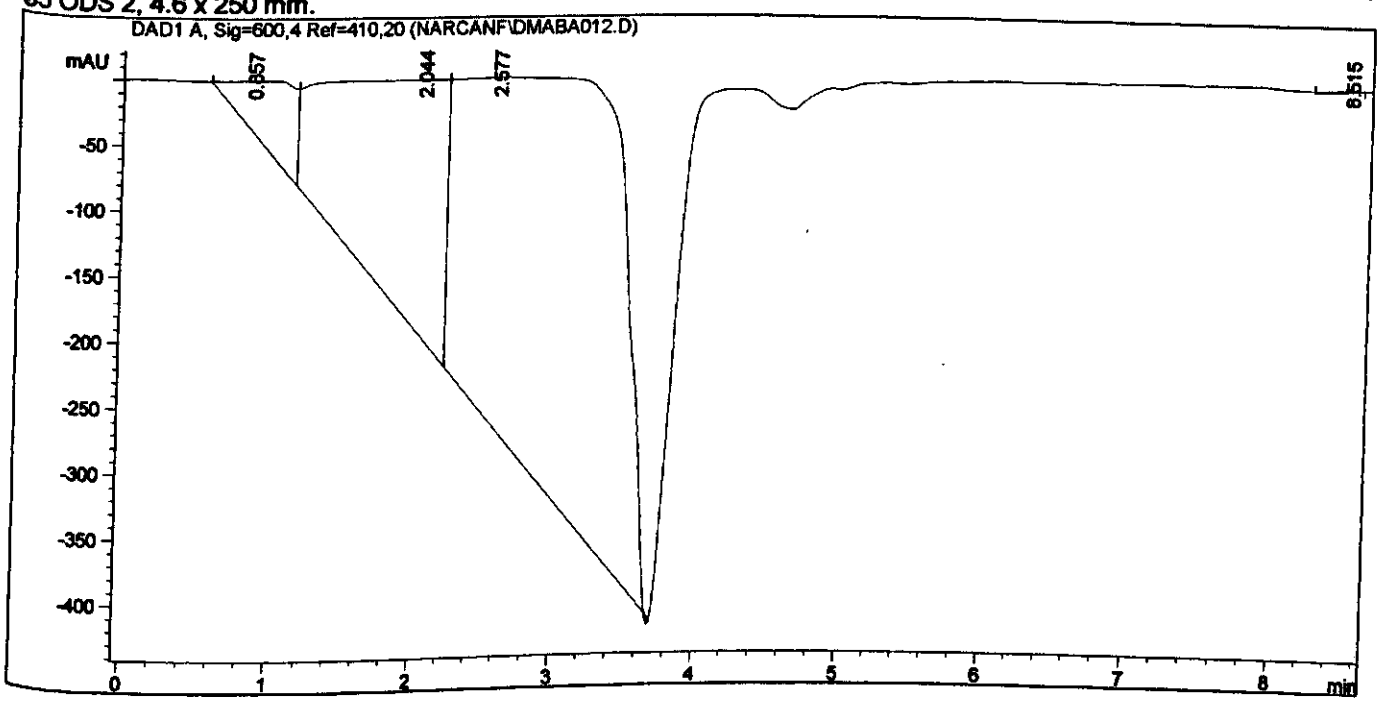
Data File C:\HPCHEM\1\DATA\NARCANF\DMABA012.D

Code 323417 - 5.0010g

32

Injection Date : 3/15/01 9:13:02 PM Seq. Line : 12
Sample Name : 323417 Vial : 17
Acq. Operator : Inj : 1
Inj Volume : 100 µl
Acq. Method : C:\HPCHEM\1\METHODS\NARASIN .M
Last changed : 3/15/01 9:10:26 PM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\NARASIN .M
Last changed : 3/16/01 12:50:55 PM by
(modified after loading)

S5 ODS 2, 4.6 x 250 mm. Waters Sp



External Standard Report

Sorted By : Signal
Calib. Data Modified : 3/16/01 12:34:33 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=600,4 Ref=410,20

RetTime [min]	Type	Height [mAU]	Amt/Height [ug/ml]	Amount	Grp	Name
7.419	-	-	-			NAR A
7.814	-	-	-			NAR D+I

Totals : 0.00000

Data File C:\HPCHEM\1\DATA\NARCANF\DMABA024.D

Code premix - 1.0009g

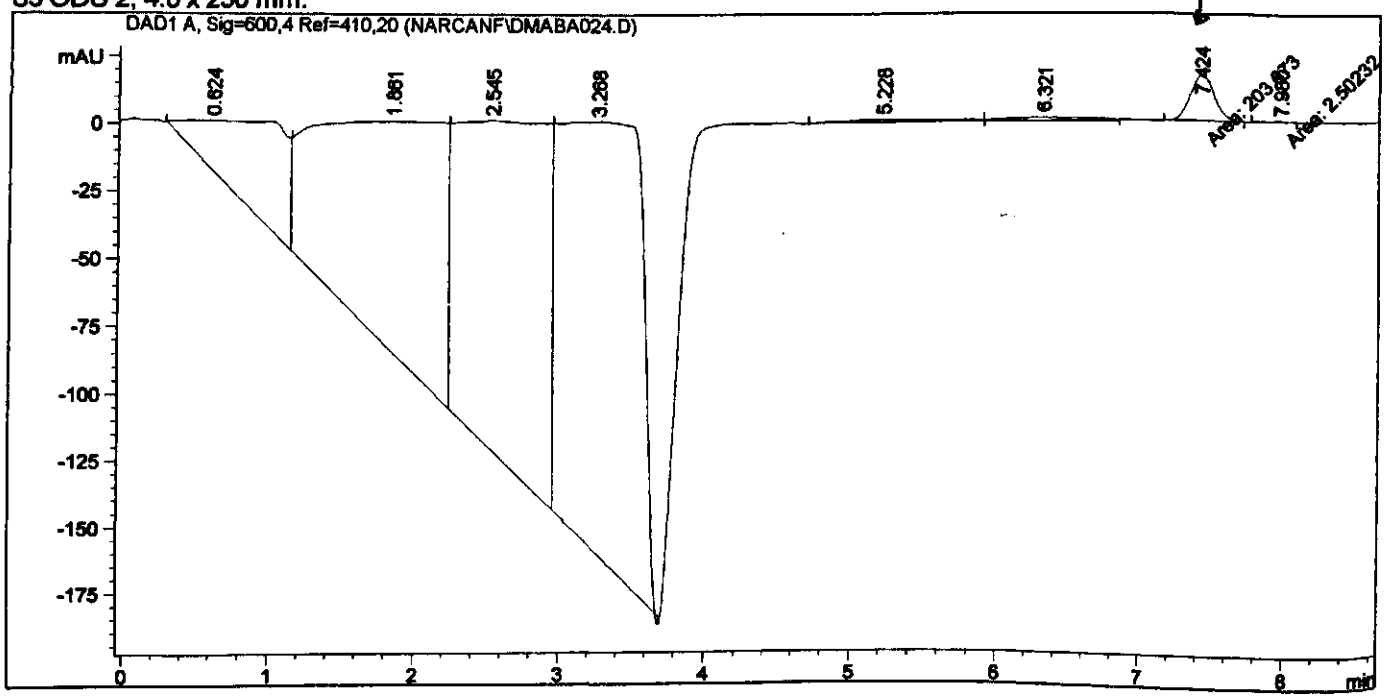
32

Injection Date : 3/15/01 11:30:40 PM Seq. Line : 24
Sample Name : Premix2 Vial : 29
Acq. Operator : Inj : 1
Inj Volume : 100 µl
Acq. Method : C:\HPCHEM\1\METHODS\NARASIN ..M
Last changed : 3/15/01 11:28:07 PM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\NARASIN .M
Last changed : 3/16/01 2:58:34 PM by
(modified after loading)

Narasin
(factor A)

Waters Sp

S5 ODS 2, 4.6 x 250 mm.



External Standard Report

Sorted By : Signal
Calib. Data Modified : 3/16/01 12:34:33 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=600,4 Ref=410,20

RetTime [min]	Type	Height [mAU]	Amt/Height [ug/ml]	Amount	Grp	Name
7.424	MM	17.62598	5.95050e-2	1.04883		NAR A
7.980	MM	2.09057e-1	0.00000	0.00000		NAR D+I

Totals : 1.04883

Instrument 1 3/16/01 2:59:49 PM

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 33

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**
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis: 16-02-2001

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
333381		17,0
333392		38,7
333396		59,7
333451		16,4
333459		afwezig
333476		afwezig
333478		59,3
333485		105,5
333491		105,9
333497		39,7

Sample / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	8474	8501

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: ....16/02/01....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ....Hypersil ODS 3mm 15 cm....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: ....
- Flow-rate: ....0,6. ml/min
- Injection volume: ..50....µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

- Percentage recovery: 98.. %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ...80.. mg/kg

Sample Set Name NAR16

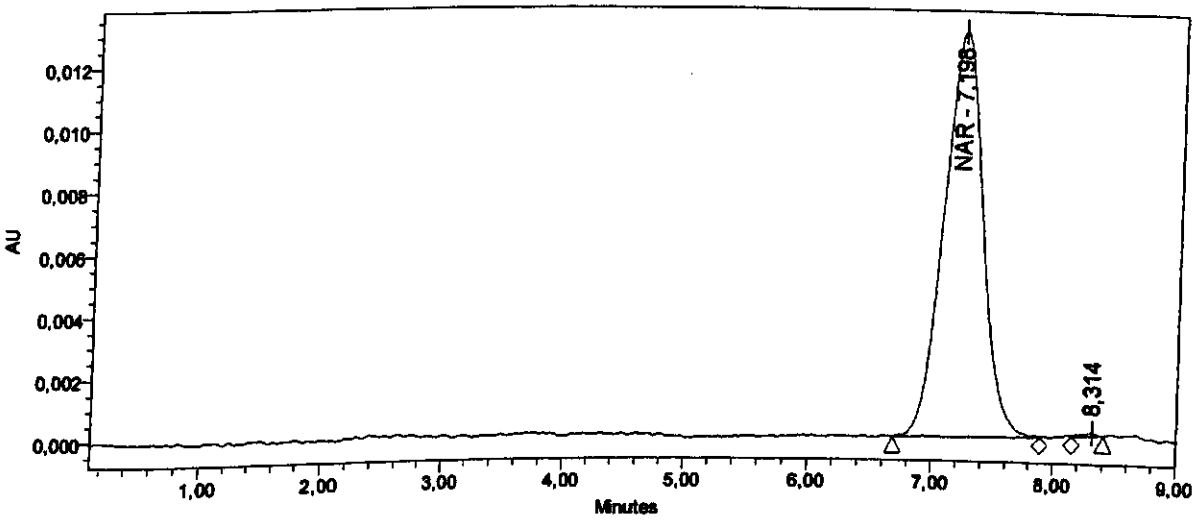
User Name RVSA

Current Date 16/02/01

Current Time 12:37:52

Remi Lère

Auto-Scaled Chromatogram



SampleName 0848/01 Vial 12 Injection 2 Channel 996 Date Acquired 16/02/01 11:46:00

Sample Set Name NAR16

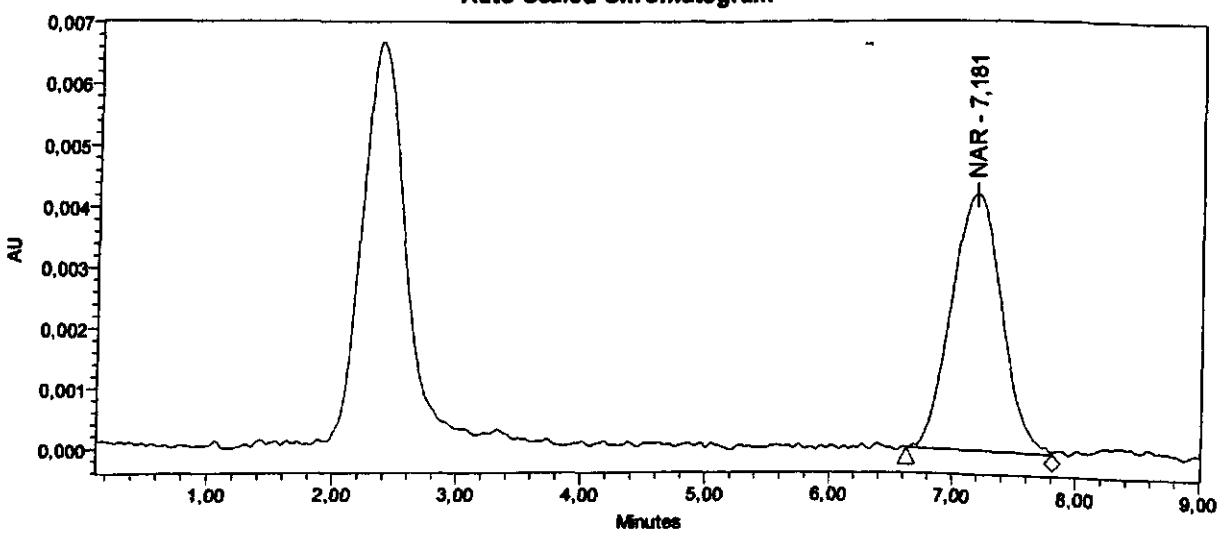
User Name RVSA

Current Date 16/02/01

Current Time 12:37:15

333381

Auto-Scaled Chromatogram



SampleName 0850/01 Vial 10 Injection 1 Channel 996 Date Acquired 16/02/01 11:14:24

Sample Set Name NAR16

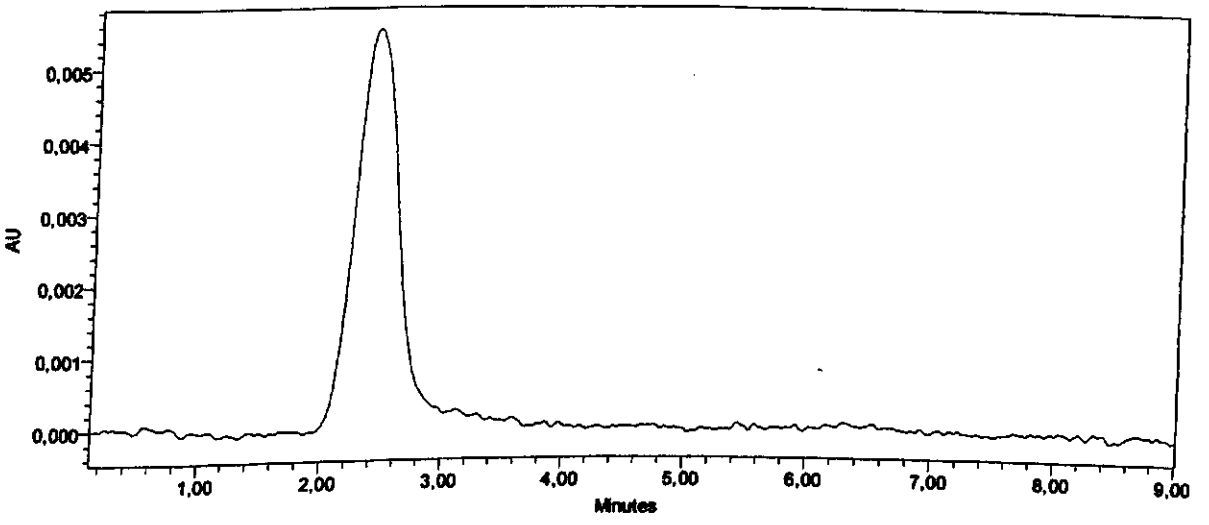
User Name RVSA

Current Date 16/02/01

Current Time 12:36:22

333476

Auto-Scaled Chromatogram



SampleName 0841/01 Vial 3 Injection 1 Channel 996 Date Acquired 16/02/01 10:00:32

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 35

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
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis: 09-04-2001

Analyte: NARASIN

	Unit	Result (mg/kg)
Sample code		
353374		44
353380		67
353387		< 1
353405		116
353408		70
353422		42
353438		19
353444		< 1
353466		108
353468		18

Unit / Sample	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture	12036	12583

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: 9 april 2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Chromsepher C8 100*3.0 mm (2x)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: see remarks ml/min
- Injection volume: 100 µl

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

Recovery results:

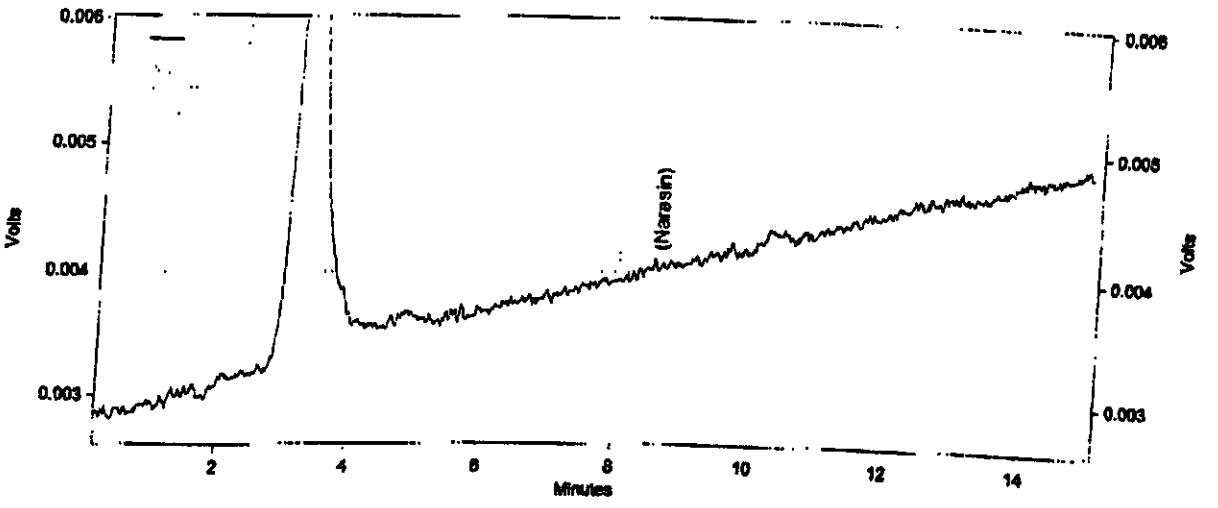
- Percentage recovery: 94.5 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: 93.6 % and 95.3 %
- Spiking level: 100 mg/kg

Fax :

Thank you for your cooperation !

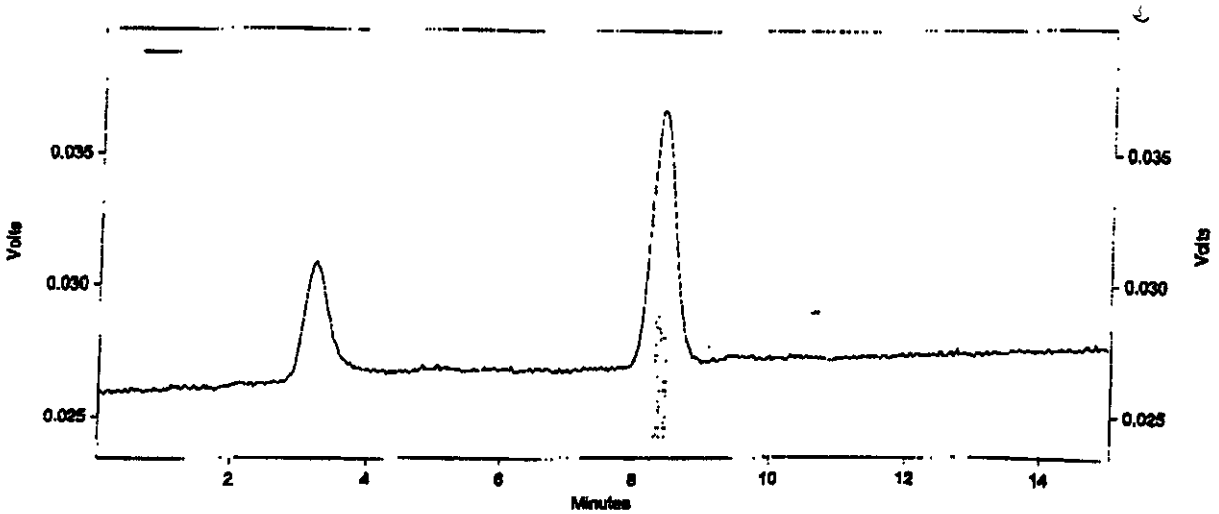
35

2



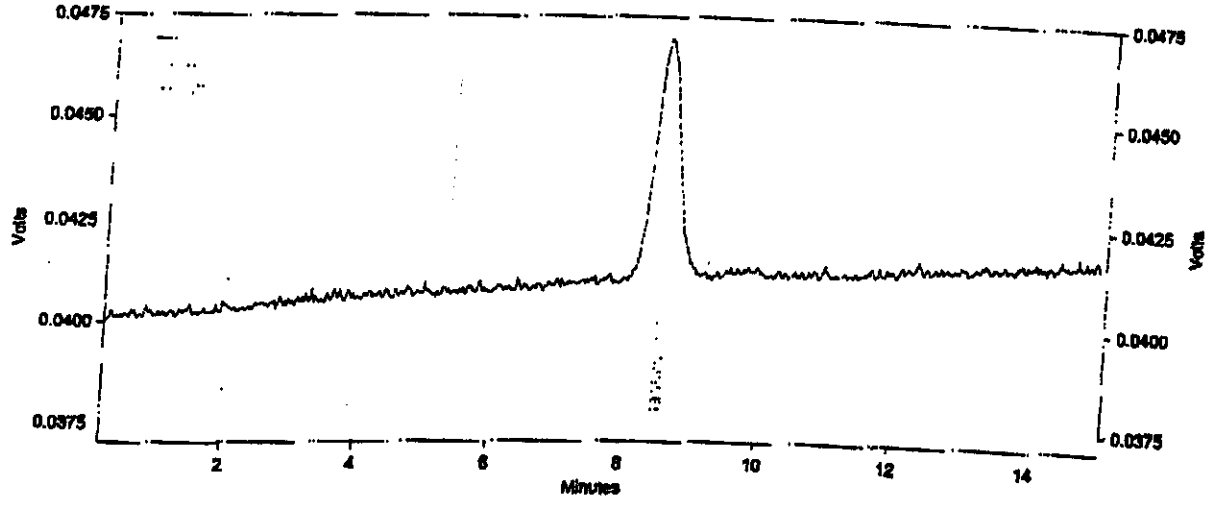
\\Fs_ \VOL1\DATA\Elite_Admin\Projects\Ionophor :\Data\Narasin_010409bw_006, UV-Detector

Blank



\\Fs_ \VOL1\DATA\Elite_Admin\Projects\Ionophor \Data\Narasin_010409bw_015, UV-Detector

353438



\\Fs_ \VOL1\DATA\Elite_Admin\Projects\Ionophor \Data\Narasin_010409bw_023, UV-Detector

premix

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 37

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
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis: 11-12 april 2001

Analyte: NARASIN

Sample code	Unit	Result (mg/kg)
373389		38,6
373391		17,3
373404		114,6
373423		-
373429		64,8
373433		60,1
373458		40,7
373472		19,4
373475		102,0
373480		-

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		10038,5	11213,8

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

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

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: HYPERSIL 5µm BDS C18 (25cm × 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:

- Blind positive feed samples
- Blind blank feed sample
- 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: ..50.... mg/kg (diluted extract so it would fall within calibration range as outlined in method).

(A) 99.2% (C) 89.7%

(A) 102% + 96.3% (C) 89.2% + 90.2%

9th April: Samples extracted + aliquoted into vials (along with calibration standards). Stored in fridge prior to LC analysis.

10th April (A) Sample extracts run undiluted against calibration curve as outlined in method

11th/12th April (B) Calibration curve increased 10 fold (ie 2.5, 5.0, 10, 20, 25 µg/g) and sample extracts run undiluted.

(C) Sample extracts RUN diluted in order to fall within calibration curve as outlined in method.

(37)

Annex 2

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

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis: SEE ANNEX 4.

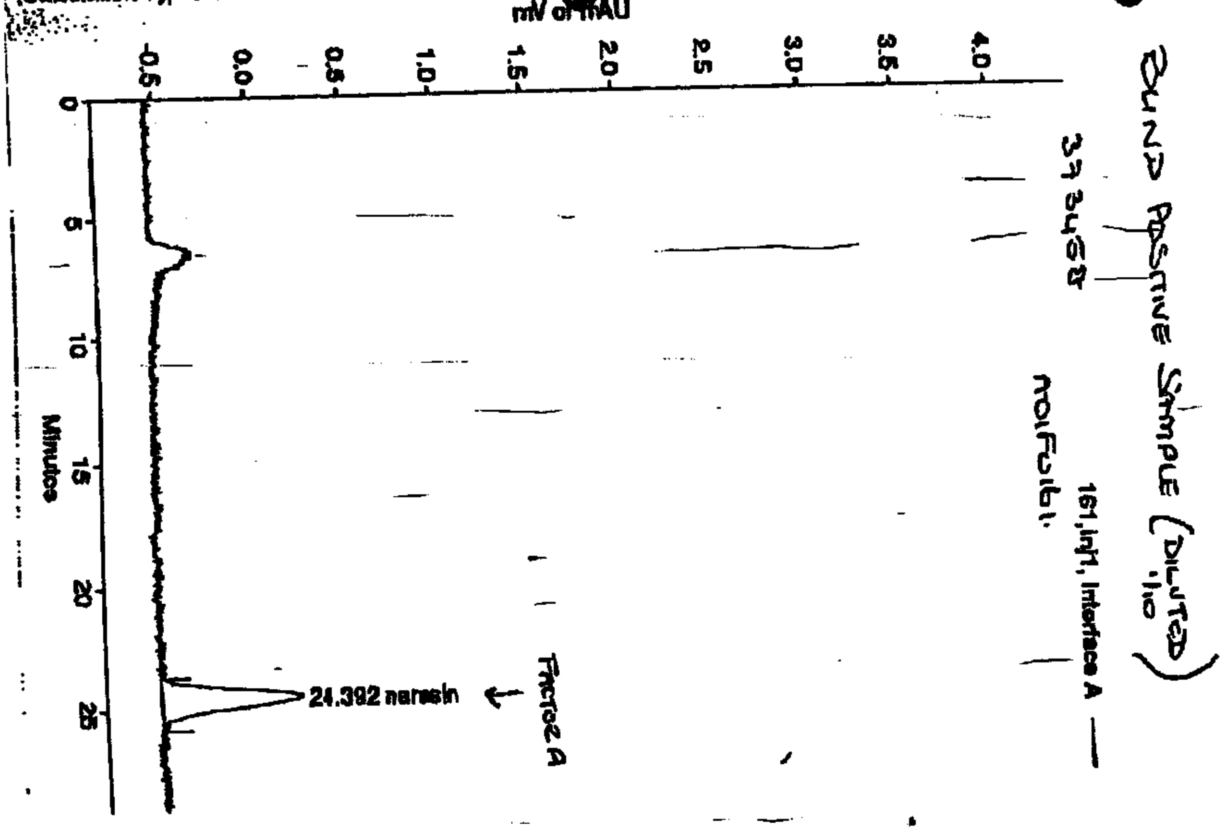
Analyte: NARASIN

Sample code	Unit	(A) Result (mg/kg)	(B)	(C) (see Annex 4)
373389		40.0	40.1	38.6
373391		17.4	17.6	17.3
373404		106.7	106.1	114.6
373423		ND	[redacted]	[redacted]
373429		63.4	63.1	64.8
373433		62.1	61.6	60.1
373458		40.0	40.0	40.7
373472		17.9	18.4	17.4
373475		106.7	105.1	102.0
373480		ND	[redacted]	[redacted]

↑ results selected

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		100,385	112,158

Signal 1: Interface A
Calculation Type: External Standard (Area)



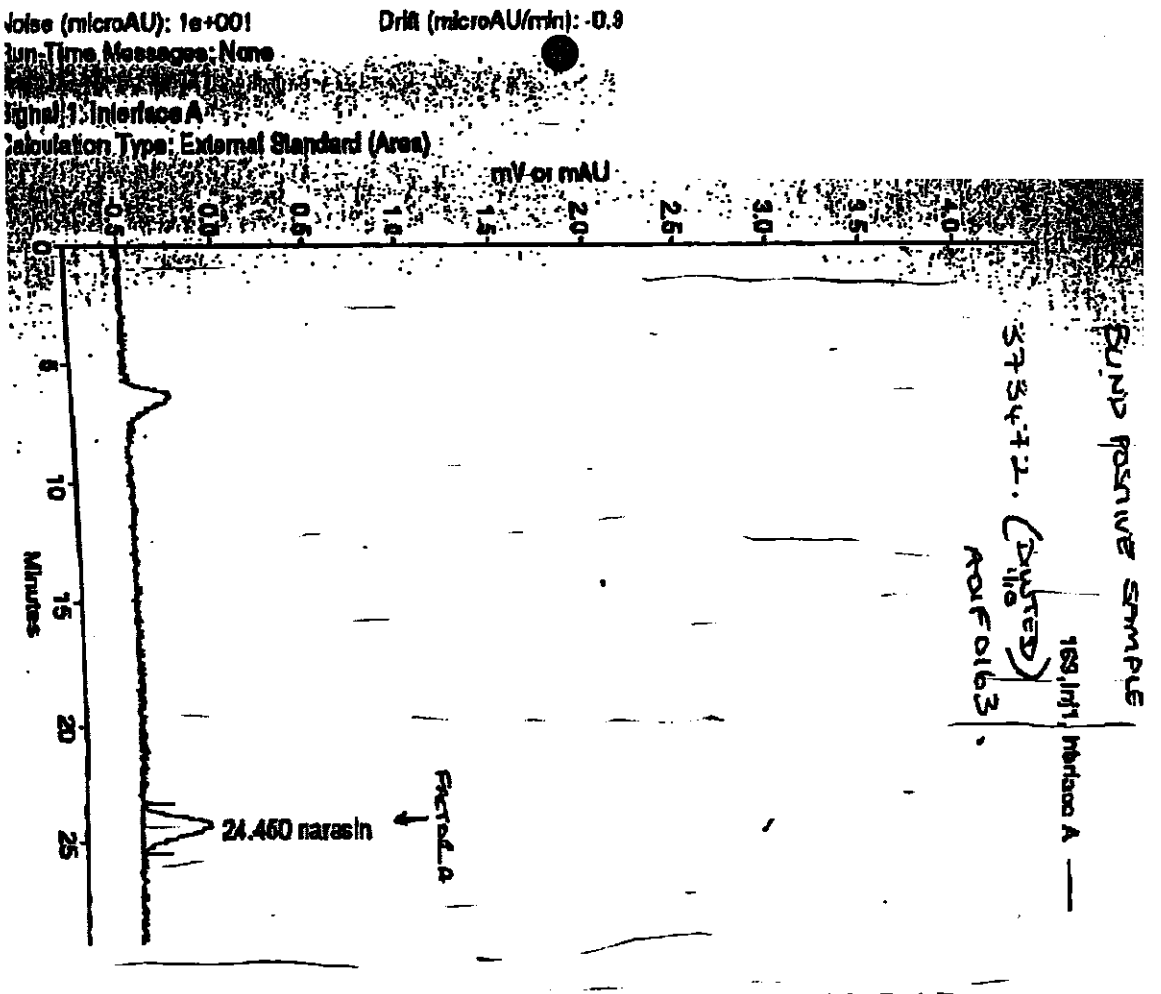
Component	RT(min)	Area	Height	ug/ml	Peak Type
narasin	24.392	39942	755	0.82111	Modified
Totals		39942	755	0.82111	

System: SYSTEM1 on Comm Port 1
Acquisition Method: D:\TSP\SYSTEM1\Methods\narasin.ACM
Calculation Method: D:\TSP\SYSTEM1\Methods\narasin.CAM
Report Method: D:\TSP\SYSTEM1\Methods\narasin.RPM

Analyst: ps

PC1000 Ver 3.0.1
10-04-101 12:29:22
23-04-101 15:17:28
29-05-98 11:30:44

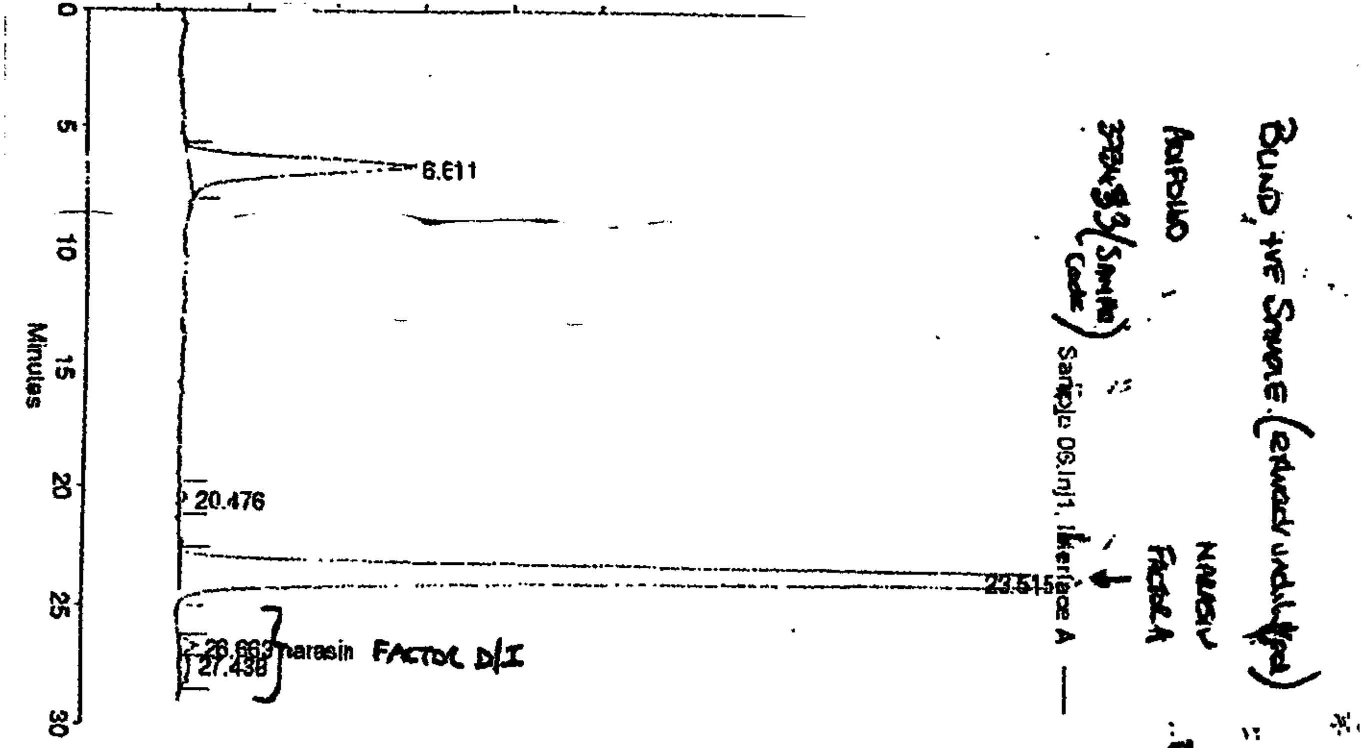
(37)



Component	RT(min)	Area	Height	ug/ml	Peak Type
narasin	24.450	19165	388	0.39399	Modified
Totals		19165	388	0.39399	

System: SYSTEM1 on Comm Port 1 Analyst: ps
Acquisition Method: D:\TSP\SYSTEM1\Methods\narasin.AQM
Calculation Method: D:\TSP\SYSTEM1\Methods\narasin.CAM
Report Method: D:\TSP\SYSTEM1\Methods\narasin.RPM

PC1000 Ver 3.0.1
10-04-101 12:29:22
23-04-101 15:17:28
29-05-98 11:30:44



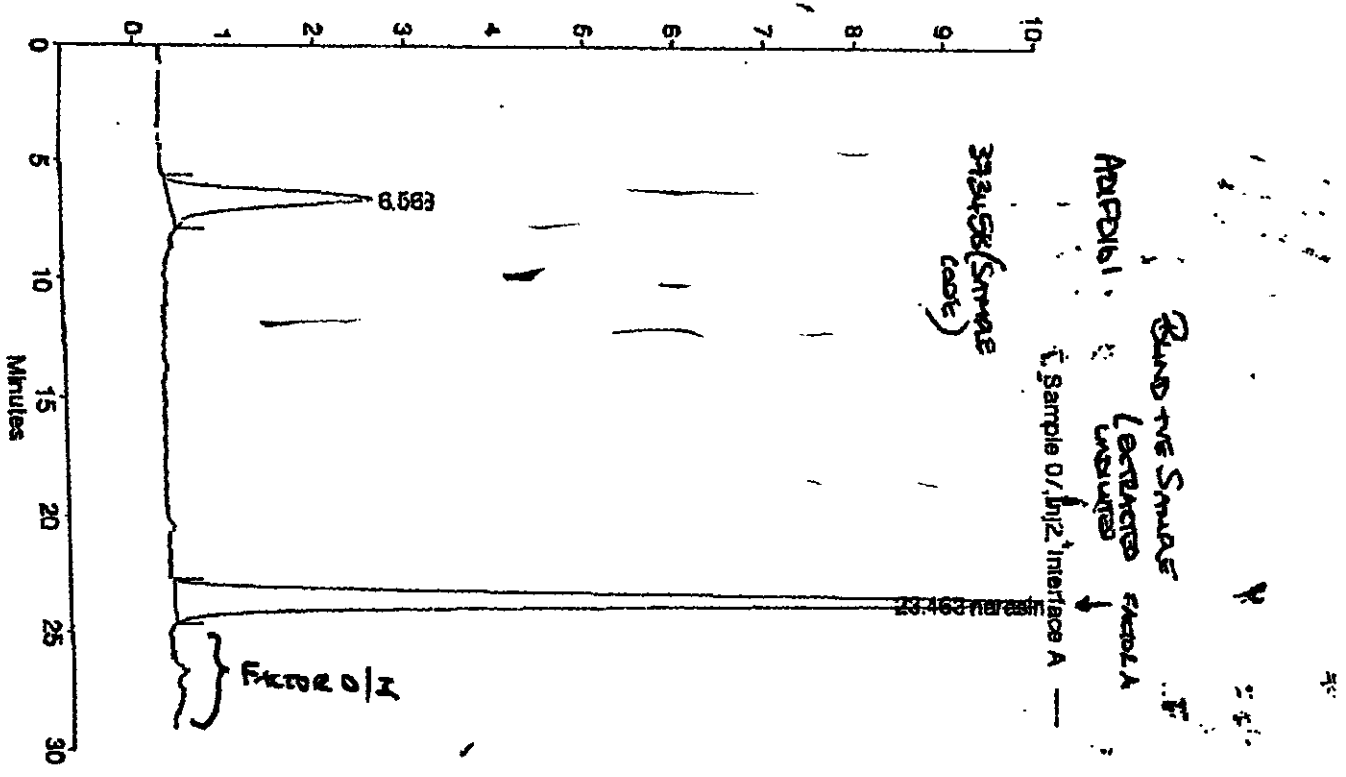
Component	RT(min)	Area	Height	ug/ml	Peak Type
Unident0001	6.61`	120340	2563	0.0	Resolved
Unident0002	20.476	2383	128	0.0	Modified
Unident0003	23.515	647148	18224	0.0	Resolved
narasin	26.663	5983	222	0.11775	Modified
Unident0005	27.438	7764	151	0.0	Modified
Totals		783598	21288	0.11775	

System: Reprocess
Acquisition Method: D:\TSP\SYSTEM1\Methods\narasin.AQM
Analyst: ps
PC1000 Ver 3.0.1
10-04-101 12:29:22

(37)

1st run PC1000 Monensin/PS
Mode: Reprocessed Data
Original Results: D:\TSP\SYSTEM1\Data\narcollab100401.RES

Reported On: 13-04-101 11:11:13

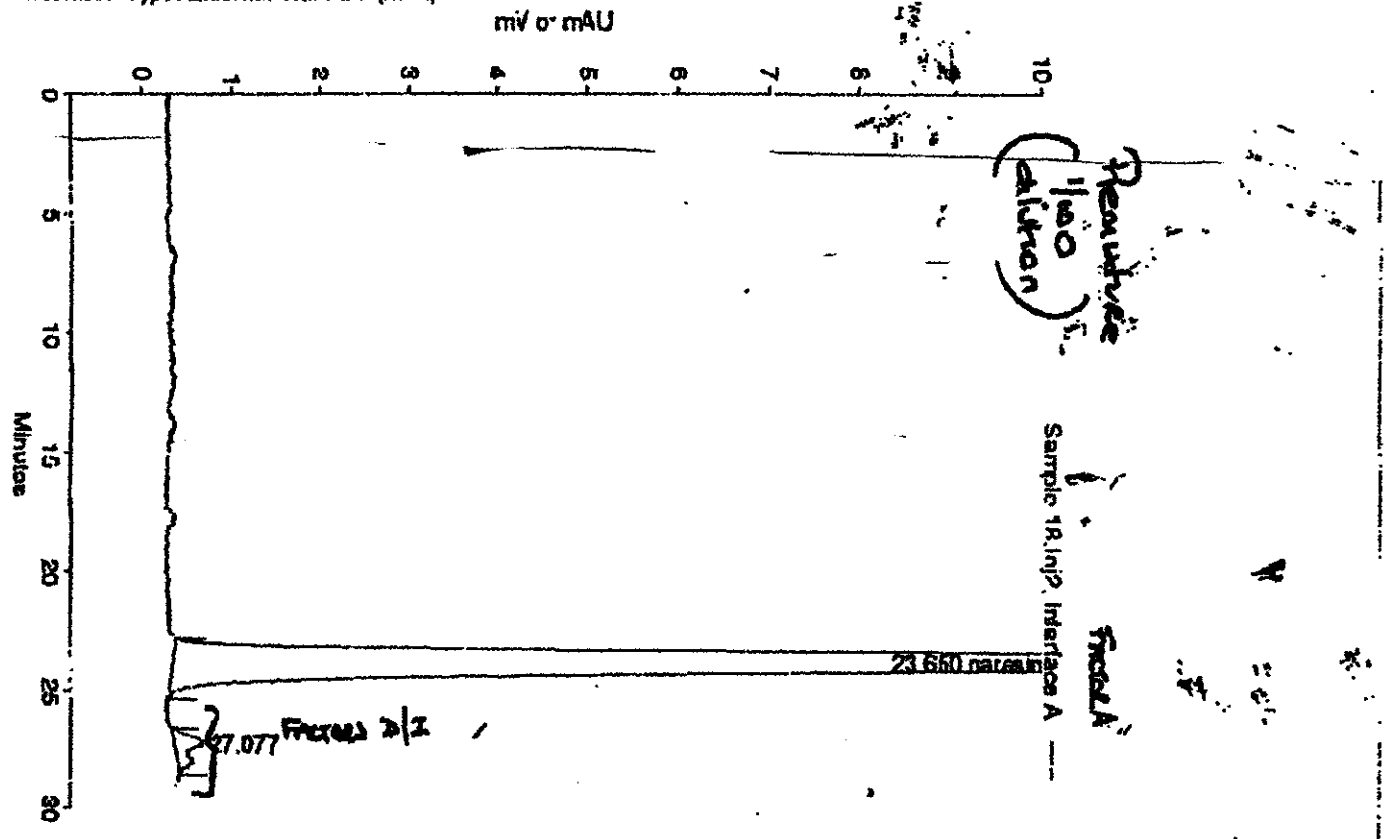


Component	RT(min)	Area	Height	ug/ml	Peak Type
UnidentC001	6.568	107357	2287	0.0	Resolved
narasin	23.463	406958	11325	8.0032	Modified
Totals		514315	13592	8.0032	

System: Reprocess Analyst: ps
Acquisition Method: D:\TSP\SYSTEM1\Methods\narasin.ACQ
Calculation Method: D:\TSP\SYSTEM1\Methods\narasin.CAM
Report Method: D:\TSP\Methods\narps.RPM

PC1000 Ver 3.0.1
10-04-101 12:29:22
13-04-101 10:38:10
13-04-101 10:37:16

Signal 1: Interface A
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	ug/ml	Peak Type
narasin	23.650	1038168	24756	20.432	Modified
Unident0002	27.077	18861	333	0.0	Resolved
Totals		1057049	25089	20.432	

System: Reprocess Analyst ps PC1000 Ver 3.0.1
Acquisition Method: D:\TSP\SYSTEM1\Methods\narasin.AQM 10-04-101 12:29:22
Calculation Method: D:\TSP\SYSTEM1\Methods\narasin.CAM 13-04-101 10:38:10
Report Method: D:\TSP\Methods\narps.RPM 13-04-101 10:37:16

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 41

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis: 17.04.2001

Analyte: **NARASIN**

Sample code	Unit	Result (mg/kg)
413388		20,5
413394		47,1
413401		20,4
413411		0
413413		113,6
413415		69,5
413442		114,7
413443		69,7
413495		0
413498		45,2

Sample	Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Premixture		11007	11420

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - NARASIN

Annex 4 - Questionnaire

Date(s) of analysis: 12.4.2001 and 17.4.2001

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:

- Blind positive feed samples
- Blind blank feed sample
- Premixture

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

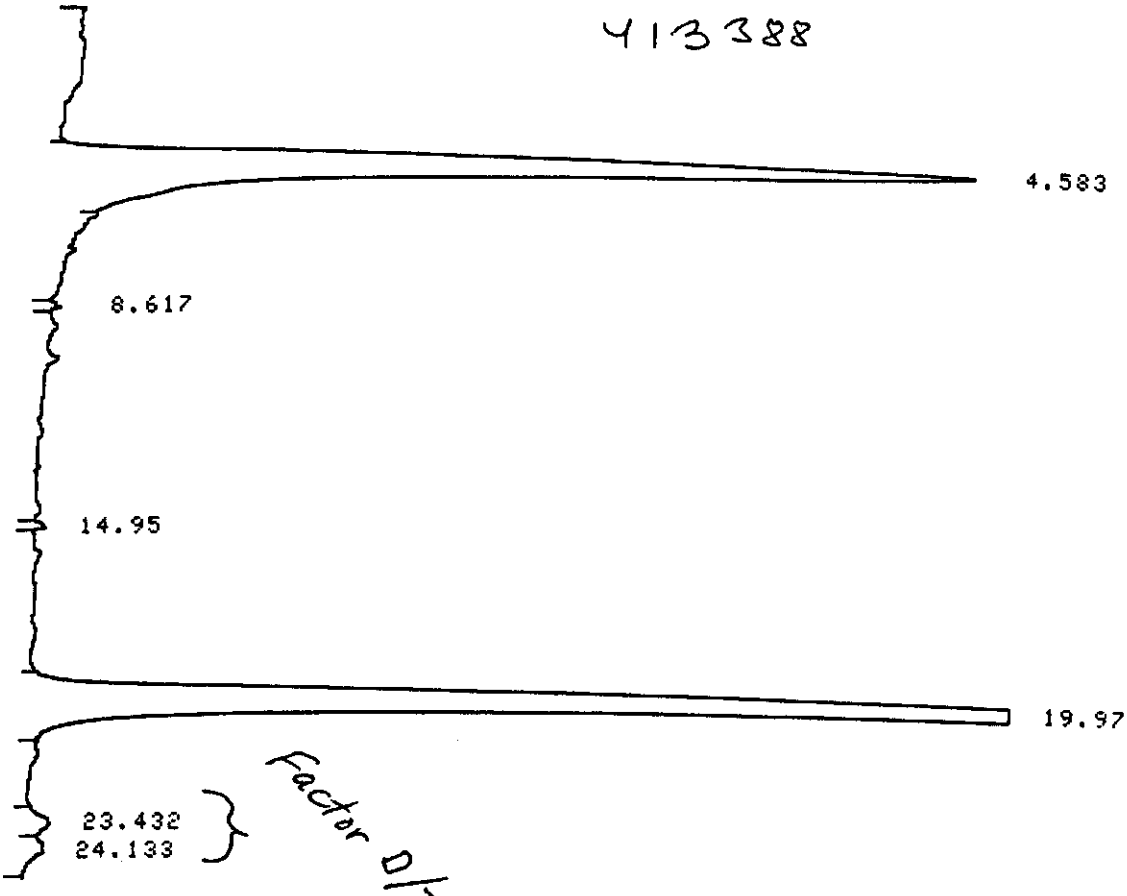
Recovery results:

- Percentage recovery: 104 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 106 % and 103 %
- Spiking level: 50 mg/kg

(41)

START 1
START 1

413388



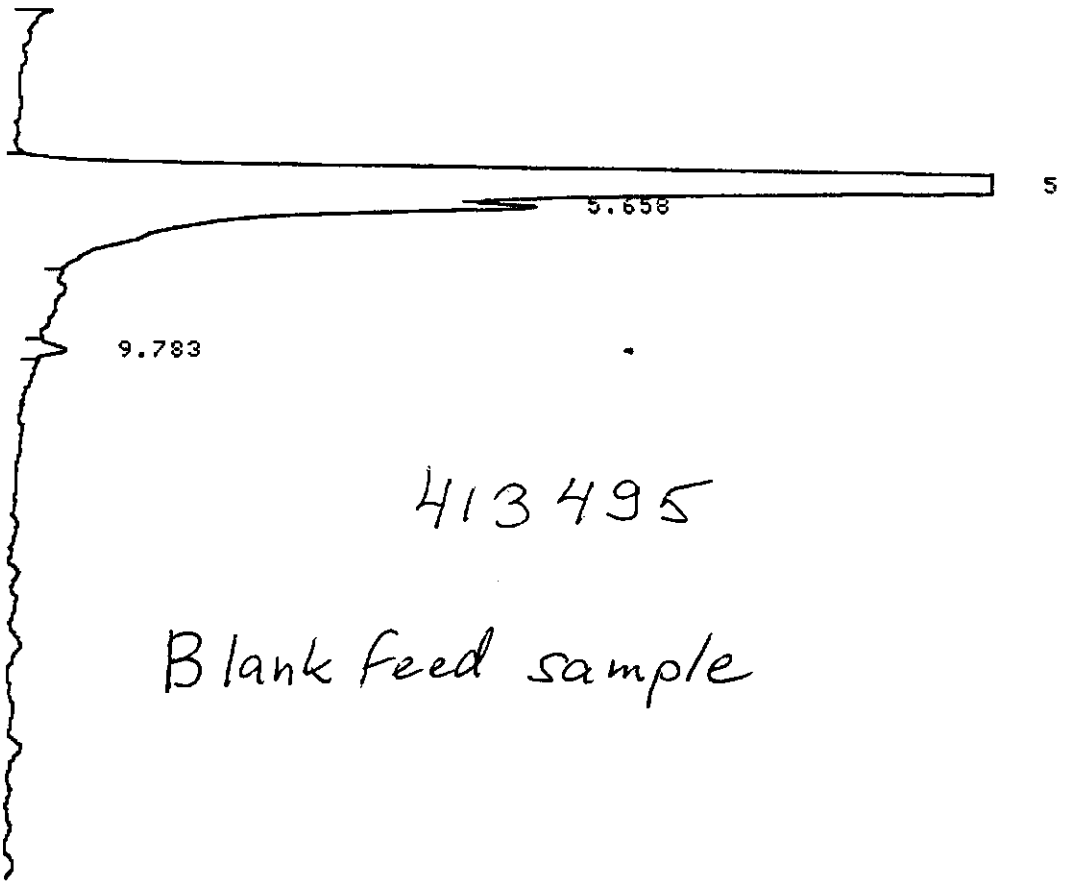
CHROMATOGRAM 3 MEMORIZED

CHROMATOPAC C-R3A FILE 0
SAMPLE NO 0 METHOD 3021
REPORT NO 5386

PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
1	4.583	1899			33.8956	
		72769ar				
2	19.973	3703			66.1044	
		105472ar				
	TOTAL	5601			100	

(41)

START 1
START 1



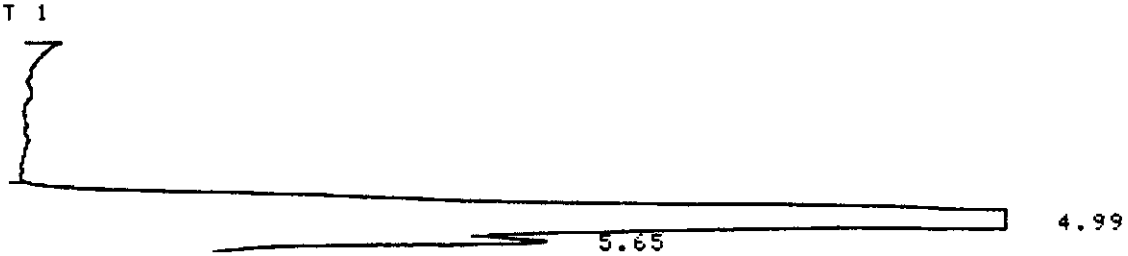
413495

Blank feed sample

CHROMATOPAC C-R3A FILE 0
SAMPLE NO 0 METHOD 3021
REPORT NO 5407

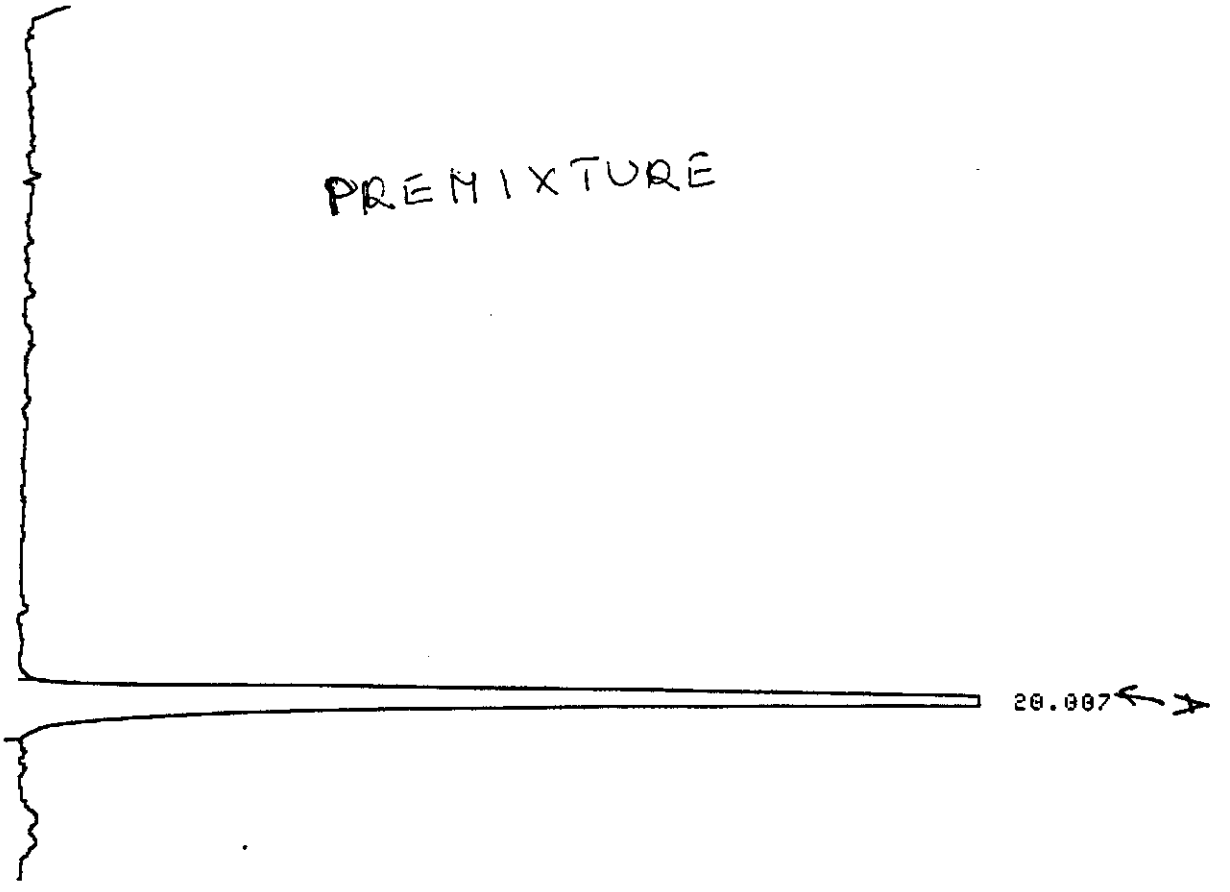
PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
1	5	3583			77.5588	
		142846ar				
2	5.658	1037	V		22.4412	
		35792ar				
	TOTAL	4620			100	

START 1
START 1



START 1

START 1



CHROMATOGRAM 7 MEMORIZED

CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 5452

FILE 0
METHOD 3021

PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
1	20.007	2530			100	
		80258ar				
	TOTAL	2530			100	