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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 virginiamycin in feedingstuffs by HPLC

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CONTENTS page

SUMMARY

This report describes the results of a collaborative study of an HPLC method for the antibiotic virginiamycin in four feeds. 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: Virginiamycin M1 is extracted from wetted feedingstuffs with ethyl acetate in an ultrasonic bath. A part of the extract is purified with tandem Solid Phase Extraction (SPE) using Silica and OASIS HLB cartridges. The eluate of the solid phase extraction is evaporated under nitrogen and the residue is dissolved in HPLC eluent. From this fraction a part is injected on a liquid chromatography system with a 'reversed phase' column. Isocratic elution is used and UV detection at 235 nm is applied.

The samples which were prepared for the collaborative study were a broiler feed and a bull feed with declared virginiamycin contents of 2 and 5 mg/kg resp., 1 blank broiler feed and 1 blank piglet feed. The samples were sent to the participants as blind duplicates. The participants were asked to do duplicate determinations per sample.

Results were reported by 12 laboratories. Statistical evaluation was performed according to ISO 5725. From the results it can be concluded that the repeatability and the reproducibility of the method are unsatisfactory. The measurement uncertainty of quantitative results is much larger than generally considered as acceptable. Thus, the applicability of the method is restricted to semi-quantitative use, which means that any quantitative result should only be reported together with a clear statement of the measurement uncertainty.

From the results it can be concluded that the method is suitable as a screening method to discriminate between positive samples containing 2 mg/kg virginiamycin (or more) and negative samples. From the information obtained in this collaborative study it is not possible to deduce if the method is also suited to discriminate between samples containing 1 mg/kg (the target value from the Project Plan) and negative samples.

The results of the collaborative study were evaluated in a meeting attended by the participants. The panel agreed with the conclusions stated above about repeatability, reproducibility and applicability of the method.

The method will not be recommended for adoption as an official method. It was agreed that no further work will be done in the CANFAS-project. New work will be started in SIMBAG-FEED, taking into account the results of the CANFAS-project.

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 virginiamycin. Virginiamycin is an antibiotic which was registered for use in feeds for poultry, piglets, pigs, calves, laying hens, cattle for fattening and sows with contents ranging from 5 - 50 mg/kg. Since July 1999 the use of virginiamycin as a feed additive is banned in the EU. In order to allow adequate control of possible illegal use, the objective was to develop an HPLC method with UV-detection that allows the determination of virginiamycin at sub-additive levels, viz. depending on the type of feed down to 1 - 4 mg/kg.
The method was developed and validated by RIKILT, Wageningen, The Netherlands (see report C.A.J. Hajee, Final report on method development and validation for the determination of virginiamycin in animal feeds, 08-11-1999). The method is based on the detection of virginiamycin M1 as the marker compound and UV detection at 230 nm. It proved to be necessary to apply a tandem clean-up with Sep-Pak silica gel and OASIS HLB cartridges. The overall LOQ-level was estimated at 2.44 mg/kg. Recoveries at the LOQ level ranged from 38 to 67 %, repeatabilities from 7 to 26 % and within-lab reproducibilities from 13 - 27 %. The applicability of the method is restricted to semi-quantitative, screening purposes only.
Subsequently, the method was subjected to between-lab validation by the National Veterinary Institute (NVI), Uppsala, Sweden (see report A. Stepinska, February 2000) and Danish Plant Directorate (DPD), Lyngby, Denmark (see report A. Plöger, 07-02-2000). The results of DPD were similar to the results of RIKILT with repeatabilities from 5 - 33 % and recoveries from 22 - 90 %. The results of NVI were better: higher and less fluctuating recoveries (64 - 89 %), better repeatabilities (1 - 5 %). Based on the results of NVI, new chromatographic conditions were adopted which make it possible to lower the LOQ to 1 mg/kg (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). In this meeting it was decided to organise a small-scale collaborative study with 2 positive feed samples (broiler and bull feed) and 2 blank samples. An important issue for the collaborative study would be to show the possibility to discriminate between positive and negative samples. Participating laboratories were given the opportunity to familiarise themselves with the method, using feed samples with stated contents of virginiamycin. Also prior to the production of the materials for the collaborative study, separate batches of the materials were produced for homogeneity and stability testing. The between-sample homogeneity was satisfactory but the within-sample homogeneity of the bull feed was too high. Interpretation of the results of stability testing was difficult due to the variability already observed during homogeneity testing. Best results were obtained with storage at room temperature. For bull feed and broiler feed less than 50 % breakdown was observed in 4 months.
The samples that were prepared for the collaborative study were a broiler feed and a bull feed with declared virginiamycin contents of 2 and 5 mg/kg respectively and 2 blank feeds. The feed samples were sent to the participants as blind duplicates. Before these samples were shipped, the between- and within-sample homogeneity of the feed samples containing virginiamycin was checked with satisfactory results (see par. 3.1.2).
Together with 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
- Departement voor Kwaliteit van Dierlijke Producten, Melle, Belgium; H. de Ruyck, H. de Ridder, L. Batjoens, P. de Neve
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, N. Simões
- Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy; G. Brambilla, C. Cartoni, M. Fiori.
- Istituto Zooprofilattico Sperimentale della Lombardia e dellémilia Ronagna, Reparto Chimico, Brescia, Italy; E. Faggionato, A. Baiguera.
- Istituto Zooprofillatico Sperimentale delle regioni Lazio e Toscana, Roma, Italy; A. Ubaldi, A. di Lullo.
- Laboratorio Nacional de Sanidad y Produccion Animal - M.A.P.A., Santa Fe, Spain; R. Checa-Moreno, A. Ariza-Avidad.
- Laboratory of the Government Chemist, Teddington, United Kingdom; G. Merson, J. Cowles, P. Ponnampalavanar.
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.
- National Veterinary Institute, Uppsala, Sweden; E. Nordkvist, A. Stepinska
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Bral, R. van San.
- RIKILT, Wageningen, The Netherlands; C.A.J. Hajee, R. Regnat
- Staatliche Landwirtschaftliche Unersuchungs- und Forschungsanstalt (LUFA), Augustenberg, Germany; A. Thalmann, K. Wagner
- State Laboratory Dublin, Ireland; P. Shearan, A. Murphy

3 MATERIALS

3.1 Samples for collaborative study

3.1.1 Sample composition

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

Table 1: Specifications of the samples

Type of feed	Declared content	Units	Subcontractor	Date of production
Broiler feed	2	mg/kg	IPC – Dier, Barneveld (NL)	06/09/2000
Bull feed	5	mg/kg	IPC – Dier, Barneveld (NL)	06/09/2000

The complete composition of the feeds is given in Appendix 2 (in Dutch).
The main composition of the two feeds is given in Table 2.

Table 2: Main composition of the two feeds

Product / Ingredient	Broiler	Bull
Crude protein (%)	17,0	18,1
Crude fat (%)	7,9	5,2
Starch (%)	40,8	1,3
Crude fibre (%)	3,5	9,4
Crude ash (%)	5,5	not known
Moisture (%)	11,4	11,3
Virginiamycin (mg/kg)	2	5

The composition of the feeds was equal to the composition of the products that were produced by IPC-Dier in October 1999 for homogeneity and stability testing (see Report on homogeneity and stability studies of samples for the collaborative studies for virginiamycin, J.J.M. Driessen and J. de Jong, RIKILT, Wageningen, NL, June 2000).

The feed products have been prepared in a quantity of 500 kg each. To achieve a maximum degree of homogeneity halfway through the production 54 kg of broiler feed and 72 kg of bull feed are withdrawn from the stream for subsampling activities and put into sacks of 18 kg. After

discarding the top layer (ca. 2 kg) about 40 - 50 subsamples of approx. 250 grams have been taken (manual distribution with a shovel) from each of these sacks. The subsamples were stored in double paper sacks and were numbered in the order in which they were filled.
All subsamples have been stored at room temperature (ca. 20 °C).
Next to the above mentioned samples which contained virginiamycin, two blind blank feeds were sent to the participants as well as a blank feed labelled "blank feed for virginiamycin recovery purposes" (see Appendix 1). The blind blank feeds concerned a broiler feed with 20 mg nicarbazin per kg and a piglet feed with 2,5 mg carbadox per kg/7,5 mg olaquindox per kg. The 20 ppm nicarbazin containing broiler feed has also been applied as recovery blank. These feeds were analysed at RIKILT prior to the collaborative studies. The blank broiler feed contained no detectable amounts of virginiamycin or interfering substances. In the blank piglet feed a signal of approx. 0,02 mg/kg was measured at the retention time of virginiamycin (while this signal is much smaller than the limit of detection (estimated at 1 mg/kg) exact quantification is not possible).

3.1.2 Sample homogeneity

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

Table 3: Results of homogeneity tests for virginiamycin in broiler feeds

Results / Product	Declared content (mg/kg)	Measured content (mg/kg)	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Broiler feed	2	0,94	35,8	37,4
Bull feed	5	2,17	26,5	21,6

The mean values for the measured contents are only ca. 45 % of the declared contents. This observation is similar to previous findings (see Report on homogeneity and stability studies of samples for the collaborative studies for virginiamycin, J.J.M. Driessen and J. de Jong, RIKILT, Wageningen, NL, June 2000) and is mainly caused by the relatively low recovery of the method. 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, 12-10-2000) the maximum limit for CV_{hom} was set to 60 %. All between- and within-sample CV's fulfil this requirement. Thus, it is concluded that the samples are sufficiently homogeneous.

3.1.3 Sample logistics

The feed samples were sent as blind duplicates. The codes are given in Appendix 4. The samples were sent to the participants by courier service on 2 October 2000 together with a letter with instructions (Appendix 1). During transport no special precautions were taken with regards to the temperature of the samples.

3.2 Reference standard

The reference standard was supplied by Dr. A. Plöger, Danish Plant Directorate, Lyngby (DK). According to the specifications (see Appendix 5), the reference standard (Lot Nr. 98162-QCS) has a microbiological potency of 225 % (2250 μg/mg).
The expiration date of the reference standard was January 2000. For this reason the content and identity was checked with LC-UV and LC-MS respectively. The content was compared by RIKILT with two similar reference standards also originating from Pfizer with an expiration date of January 2003. The results showed that there is no significant difference in the content of virginiamycin M1 between the three standards The identity of virginiamycin M1 was confirmed by means of LC-MS^3 (see report of C.A.J. Hajee, RIKILT, included in Appendix 5).

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 was recommended in the method is a Lichrospher C18 or Hypersil BDS C18, 250 mm x 4,6 mm with a particle size of 5 µm). The mobile phase described in the method is water/acetonitrile/formic acid 100% (600:400:3 v/v/v/). Two laboratories used a different mobile phase.
The HPLC conditions (Column and mobile phase) used by the participants are shown in Table 4.

Table 4: HPLC-conditions

Partner	Column	Mobile phase
11	Lichrospher C18, 250 mm x 4,6 mm	As described in the method
15	As described in the method.	As described in the method.
21	LC 18 supelcosil 25 cm x 4,6 mm + supelguard LCB	As described in the method.
22	Hyperlil ODS BDS C18, 250 x 4,6; 5 µm	As described in the method.
25	As described in the method.	Acetonitrile:water:formic acid = 450:550:3 (v/v/v)
26	Spherisorb ODS-2	As described in the method.
28	As described in the method.	As described in the method.
29	Waters Sperisorb ODS-2 5 µm; 4,6 x 250 mm	As described in the method.
31	As described in the method.	As described in the method.
32	Waters Symmetry, C18, 5 µm, 4,6mm x 250 mm	As described in the method.
37	Hypersil BDS C18; 25 cm x 4,6 mm, 5 µm	As described in the method.
38	Hypersil ODS C-18, 250 x 4,6 mm; 5 µm	Water:acetonitrile:Acetic acid = 650:350:3 (v/v/v)

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 Horwitz equation gives a prediction for the relative standard deviation (RSD) under reproducibility condition, based on the empirical formula: $RSD\_R,\% = 2^{[1 - 0.5 \log(c)]}$, where c refers to the decimal level. 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).

5 RESULTS

For each participant the reported results for the samples, the completed questionnaire and representative chromatograms are annexed in Appendix 6.

5.1 Statistical evaluation

The results reported by the participants are given in Table 9. Figure 1 demonstrates the Mandel h and k plots of these results. The values for the statistical parameters (mean, relative standard deviations for repeatability and reproducibility), *taking into account all the results of all participants,* are also given in Table 9.
Lab 31 is a Cochran outlier for one of the 2 mg/kg samples.
Lab 37 is a Grubbs' upper outlier at both levels and a Cochran outlier for one of the 5 mg/kg samples. Lab 37 was contacted to ascertain that the reported values were correct. The lab responded that no irregularities could be traced and that, in accordance with the instructions, the results were not corrected for recovery. For the 5 mg/kg sample it should be stressed that the reported values (6,05 - 8,04 mg/kg) are closer to the dosed content than the values reported by most of the other laboratories. The same applies to the duplicate values for one of the 2 mg/kg samples (2,28 and 2,42 mg/kg). For this reason it is not straightforward that the results of lab 37 should be removed from the statistical evaluation. Other arguments to retain the results of lab 37 are the results of tasks 1 - 3 of the project (low and fluctuating recoveries, high rsd,'s, inexplicable results during the stability studies; see Second Annual Report CANFAS, J. de Jong, 12-08-2000). This point has been discussed during the evaluation meeting (see chapter 6).
The values for the statistical parameters after removal of all the results of lab 37 and the results of one of the 2 mg/kg samples of lab 31 (Cochran outlier) are given in Table 10. Figure 2 demonstrates the Mandel h and k plots of these results.
The rsd_r values in Table 9 and 10 have been calculated from the duplicate results of individual samples, which means that at each level 24 pairs of duplicates have been used in the statistical analysis. The reason is that in a number of cases the differences between the mean values of the 2 duplicate samples of the same level are larger than the differences for the duplicate analyses in the same sample. See for example lab 15, 5 mg/kg sample: difference between the means of the 2 samples is 1,75 - 0,34 = 1,41 mg/kg; difference between the duplicate analysis in one sample is 0,03 mg/kg for the first and 0,14 mg/kg for the second sample. The relatively large differences obtained in some laboratories between the 2 samples of the same level may be attributed to inhomogeneity between samples. In order to estimate the effects of sample heterogeneity from the differences between the two samples analyzed in each laboratory also a more elaborate analysis was made in addition to the standard analysis of ISO 5725-2. In this analysis along the lines of ISO 5725-3 three variance components were estimated: laboratory differences, sample differences and repeatability differences. Variance components were estimated using residual maximum likelihood (REML) as implemented in the statistical package Genstat release 4.2, both on the complete data set, and on the set after discarding outliers.

The results from the REML variance component analyses, expressed relatively to the sample means, are shown in Tables 5 and 6.

Table 5: Variance components (relative to mean) calculated from all data

	2 mg/kg	5 mg/kg	Pooled
Laboratory	1,828	0,8206	1,324
Samples within laboratory	2,038	0,0478	1,043
Replicates	0,0295	0,0372	0,0334

Table 6: Variance components (relative to mean) calculated after discarding outliers

	2 mg/kg	5 mg/kg	Pooled
Laboratory	0,2163	0,3696	0,2930
Samples within laboratory	0,0822	0,1012	0,0921
Replicates	0,0215	0,0289	0,0253

From these variance components the performance characteristics can be calculated as shown in Tables 7 and 8.

Table 7: Relative standard deviations calculated from all data

	2 mg/kg	5 mg/kg	Pooled
rsd_r (%)	17,2	19,3	18,3
rsd_R (excluding sample heterogeneity) (%)	136,3	92,6	116,5
rsd_R (no correction for heterogeneity) (%)	197,4	95,2	154,9

Table 8: Relative standard deviations calculated after discarding outliers

	2 mg/kg	5 mg/kg	Pooled
rsd_r (%)	14,7	17,0	15,9
rsd_R (excluding sample heterogeneity) (%)	48,8	63,1	56,4
rsd_R (no correction for heterogeneity) (%)	56,6	70,7	64,1

These analyses show that:

- Pooling of the rsd's over the two concentration levels (2 and 5 mg/kg) is a reasonable option after discarding of the outliers;
- The contribution of sample heterogeneity to the total variance is sizeable (0,09), and cannot be ignored (in comparison to the other variance components 0,29 and 0,025);
- The largest contribution to the total variance (after discarding outliers) is due to between-laboratory differences (0,29).

Consequently the rsd_R values in this analysis, when corrected for sample heterogeneity, are smaller than in the simple ISO 5725-2 analysis (e.g. 56 % in comparison to 64 % for the pooled data after discarding outliers). However, they are still too large in consideration of the HORRAT criterion.

According to the Project Plan, the rsd_r-values should be $\leq$ 10 %. For both samples the values are higher. On the other hand the values are similar to the values obtained in tasks 1 and 2 of the project (see par. 1; these values were obtained with spiked samples) and even better than the values obtained for within-sample homogeneity (see par. 3.1.2, Table 3).
Thus it can be concluded that, with regards to the repeatability, the results of the collaborative study are in line with the results of within- and between-lab validation of the method.
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 Tables 11 (all results) and 12 (without the Grubbs' and Cochran outliers). The HORRAT ratios should be lower than 2. Although improved results are obtained after removal of the Grubbs' outliers, for both samples the HORRAT ratios (3,20 and 4,67) are still too high.

Table 9: Virginiamycin results for the positive feeds and statistical evaluation with all results

	Result (mg/kg)							
Sample	VIRG 2 mg/kg		VIRG 2 mg/kg		VIRG 5 mg/kg		VIRG 5 mg/kg	
Lab								
11	0,27	0,37	0,63	0,68	3,32	2,87	3,83	4,30
15	0,68	0,69	0,57	0,40	0,35	0,32	1,82	1,68
21	0,40	0,50	0,40	0,60	1,40	1,40	2,40	2,20
22	0,55	0,53	0,58	0,54	1,24	1,31	1,42	1,71
25	0,21	0,21	0,22	0,22	0,51	0,58	0,73	0,70
26	1,30	1,10	1,30	1,10	3,00	3,90	2,70	3,00
28	0,70	0,43	0,68	0,64	1,81	0,91	1,51	1,73
29	0,82	0,71	0,22	0,17	0,83	1,14	0,91	1,04
31	0,63	0,61	$1,33^{Co}$	$0,58^{Co}$	2,28	2,66	2,91	2,24
32	0,11	0,10	0,12	0,12	0,16	0,51	0,19	0,55
37	$7,88^{Guo}$	lost sample	$2,28^{Guo}$	$2,42^{Guo}$	$8,04^{Co/Guo}$	$6,05^{Co/Guo}$	$7,99^{Guo}$	$7,26^{Guo}$
38	0,52	0,54	0,79	0,78	1,45	1,61	0	0

number of labs	12	12
m (mg/kg)	0,79	2,09
rsd_r (%)	17,1	19,3
rsd_R (%)	148	93,3

Key to symbols:

$result^{Co}$ = Cochran outlier

$result^{Guo}$ = Grubb's upper outlier

Table 10: Virginiamycin results for the positive feeds and statistical evaluation after discarding outliers

	Result (mg/kg)							
Sample	VIRG 2 mg/kg		VIRG 2 mg/kg		VIRG 5 mg/kg		VIRG 5 mg/kg	
Lab								
11	0,27	0,37	0,63	0,68	3,32	2,87	3,83	4,30
15	0,68	0,69	0,57	0,40	0,35	0,32	1,82	1,68
21	0,40	0,50	0,40	0,60	1,40	1,40	2,40	2,20
22	0,55	0,53	0,58	0,54	1,24	1,31	1,42	1,71
25	0,21	0,21	0,22	0,22	0,51	0,58	0,73	0,70
26	1,30	1,10	1,30	1,10	3,00	3,90	2,70	3,00
28	0,70	0,43	0,68	0,64	1,81	0,91	1,51	1,73
29	0,82	0,71	0,22	0,17	0,83	1,14	0,91	1,04
31	0,63	0,61	*1,33*^{Co}	*0,58*^{Co}	2,28	2,66	2,91	2,24
32	0,11	0,10	0,12	0,12	0,16	0,51	0,19	0,55
37	*7,88*^{Guo}	*lost sample*	*2,28*^{Guo}	*2,42*^{Guo}	*8,04*$^{Co/Guo}$	*6,05*$^{Co/Guo}$	*7,99*^{Guo}	*7,26*^{Guo}
38	0,52	0,54	0,79	0,78	1,45	1,61	0	0

number of labs	11	11
m (mg/kg)	0,54	1,62
rsd_r (%)	14,7	17,0
rsd_R (%)	56,3	69,4

Remark 1: Italic printed results are not taken into account in the statistical evaluation!

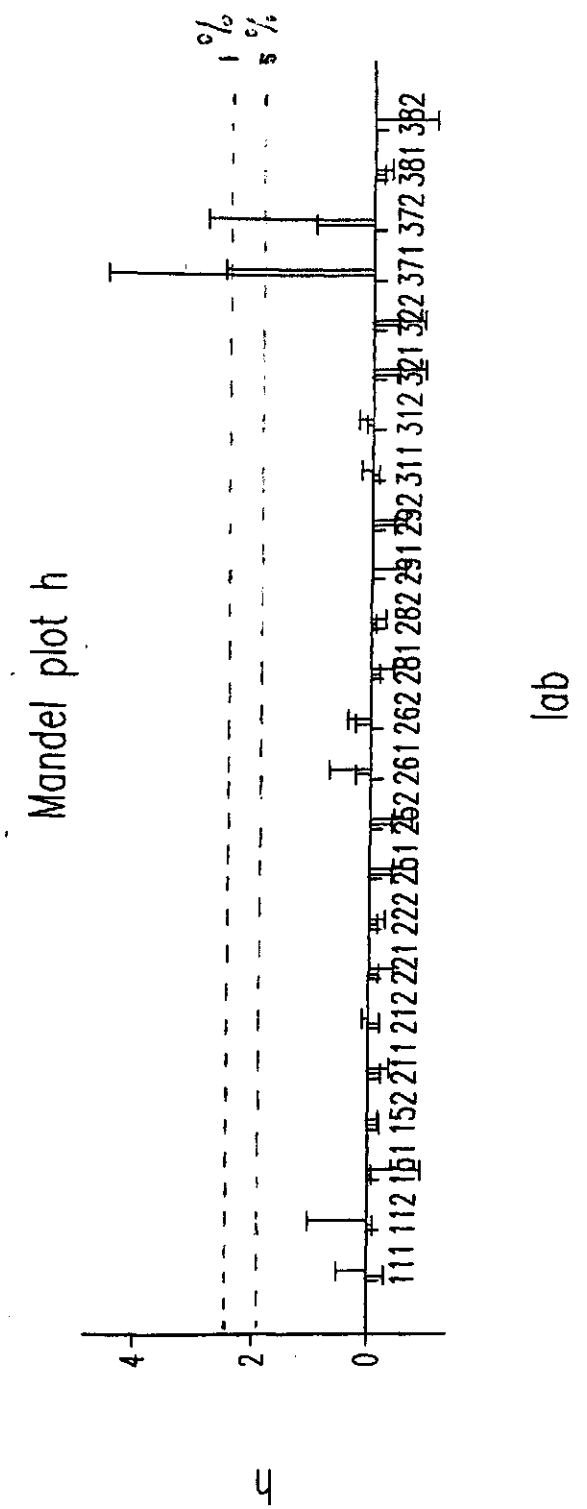
Key to symbols:

result^{Co} = Cochran outlier

result^{Guo} = Grubb's upper outlier

Figure 1: Mandel h and k plots of the results reported by the participants taking into account all the results of all participants

Lab code XX1 refers to the first set of a 2 mg/kg and a 5 mg/kg sample of lab XX, lab code XX2 refers to the second set



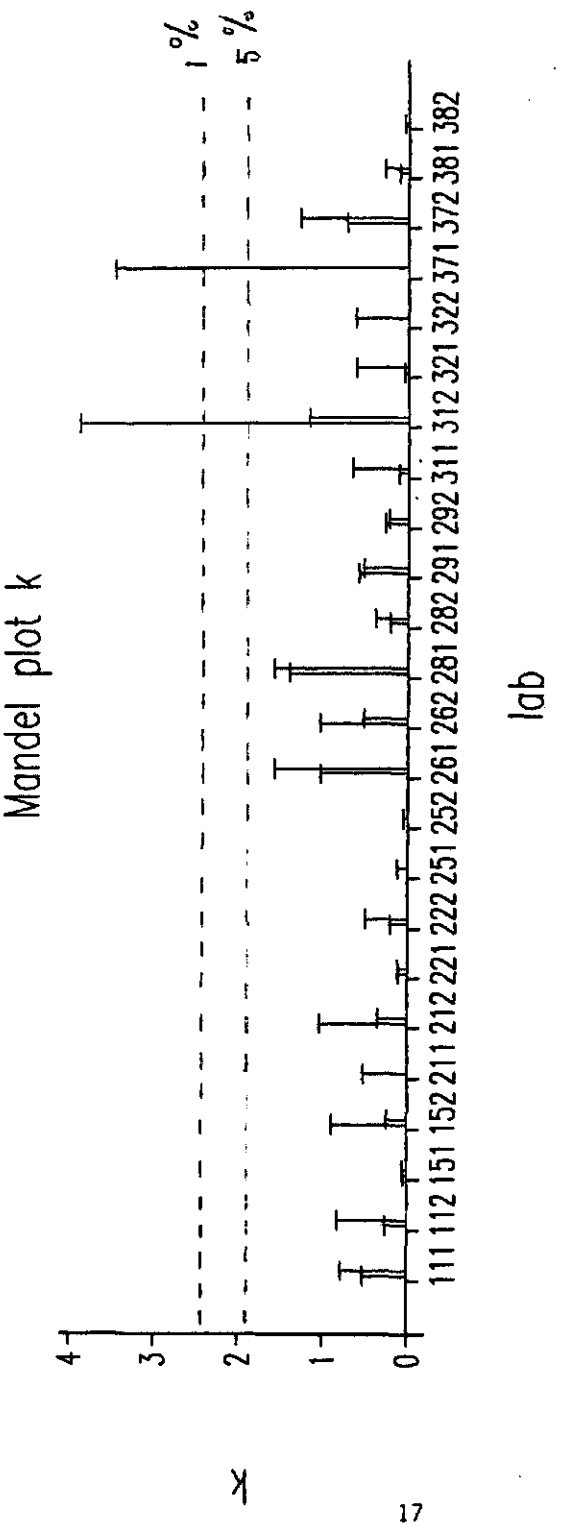
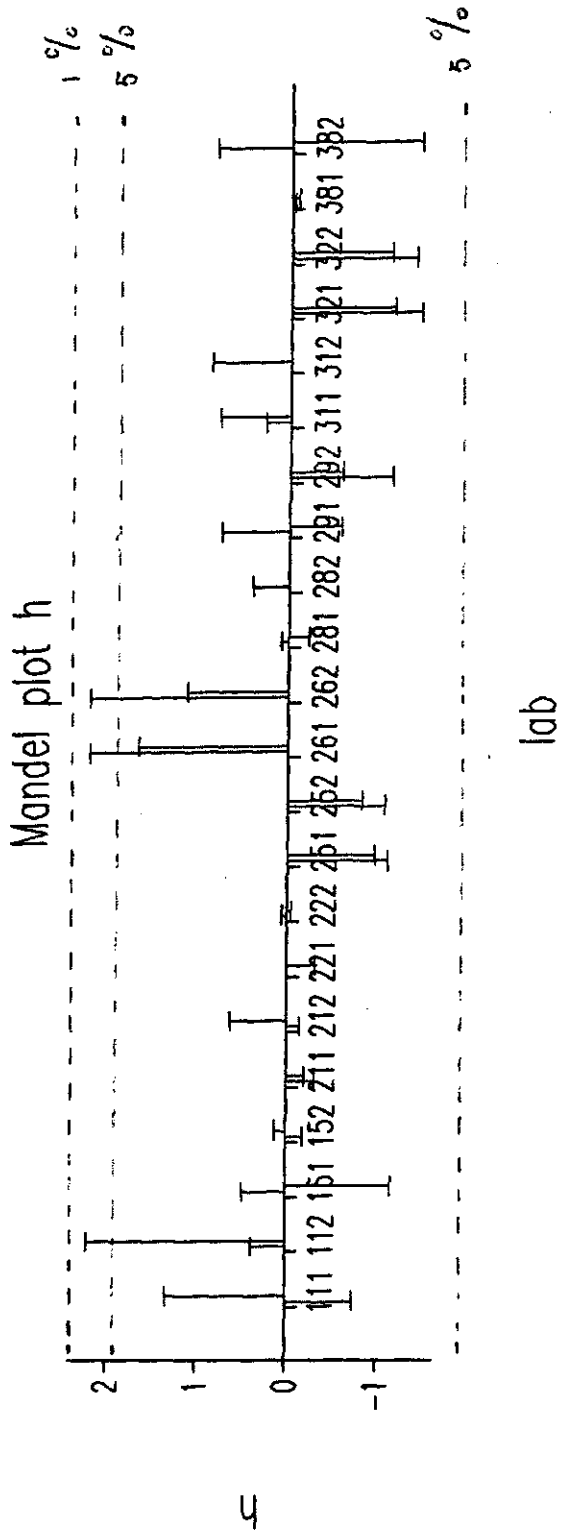


Figure 2: Mandel h and k plots of the results reported by the participants after discarding Outliers.

Lab code XX1 refers to the first set of a 2 mg/kg and a 5 mg/kg sample of lab XX, lab code XX2 refers to the second set



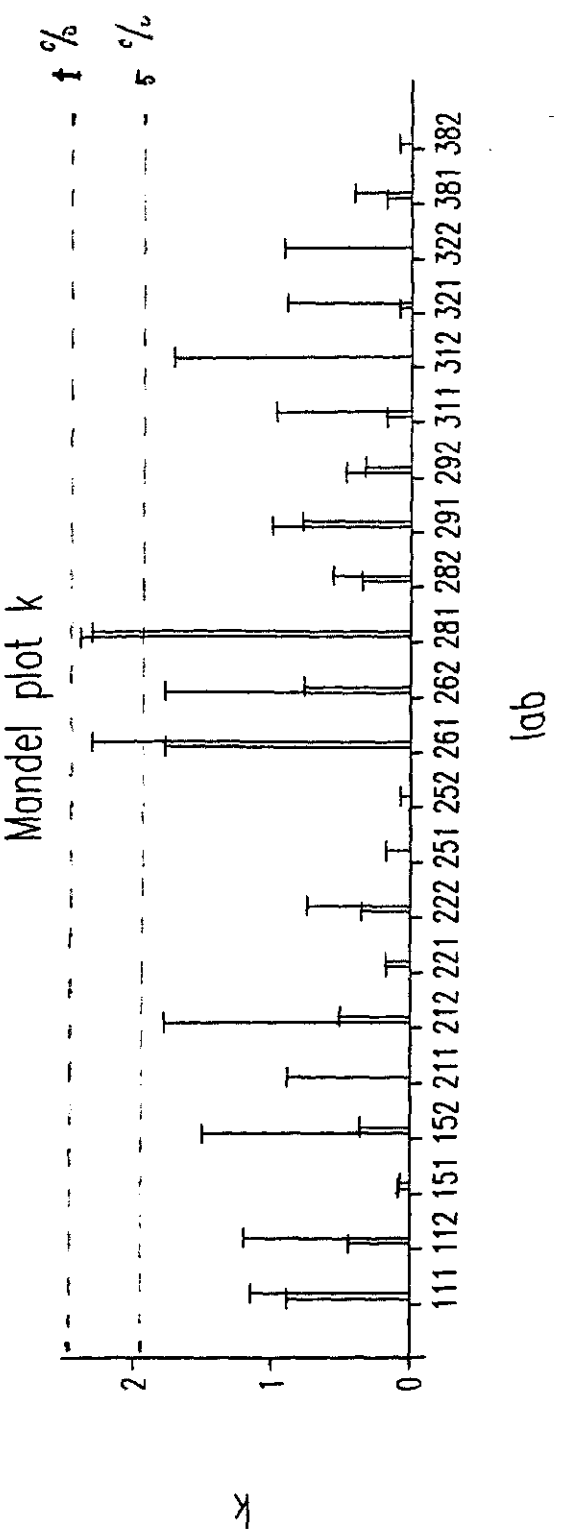


Table 11: Horrat ratios of the virginiamycin collaborative study (all results)

Mean (mg/kg)	Predicted rsd_R (%)	Established rsd_R (%)	Horrat[1]	Conclusion
0,79	16,58	148	8,92	Reproducibility NOT OK
2,09	14,32	93,3	6,51	Reproducibility NOT OK

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

Table 12: Horrat ratios of the virginiamycin collaborative study (without Grubbs' and Cochran outliers)

Mean (mg/kg), after discarding outliers[1]	Predicted rsd_R (%)	Established rsd_R (%)	Horrat[2]	Conclusion
0,54	17,555	56	3,20	Reproducibility NOT OK
1,62	14,879	69,4	4,67	Reproducibility NOT OK

[1] = outliers are lab 31 (one of the 2 ppm samples) and lab 37 (all samples)

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

From the values for rsd_r and rsd_R it can be concluded that both the repeatability and the reproducibility of the method are unsatisfactory high. This is in accordance with the results of task 1 (method development and within-lab validation) and task 2 (between-lab validation) of the project and the conclusions drawn at the kick-off meeting (see par. 1). Consequently, the application of the method is restricted to semi-quantitative use.

5.2 Blank samples

The results for the two blank feed samples (broiler and piglet feed) are reported separately in Tables 13 and 14.

Table 13: Reported results of the participants for the blank samples of broiler feed

Partner	Blank sample 1		Blank sample 2	
	Result 1	Result 2	Result 1	Result 2
11	0 mg/kg	0 mg/kg	0 mg/kg	0 mg/kg
15	Blank	Blank	Blank	Blank
21	0,0 N.D.	0,0 N.D.	0,0 N.D.	0,0 N.D.
22	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg
25	Nd	Nd	Nd	Nd
26**	0,4	0,3	0,3	0,2
28***	<LOD	<LOD	<LOD	<LOD
29	0	0	0	0
31	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg
32	Negative	Negative	Negative	Negative
37	ND	ND	ND	ND
38	0	0	0	0

** Lab 26 estimated their limit of quantification (LOQ) at 0,2 mg/kg.

*** Lab 28 estimated their limit of detection (LOD) at 0,12 mg/kg.

Note: lab 32 estimated concentrations for the 2 samples were 0,02 and 0,03

Table 14: Reported results of the participants for the blank samples of piglet feed

Partner	Blank sample 1		Blank sample 2	
	Result 1	Result 2	Result 1	Result 2
11	0 mg/kg	0 mg/kg	0 mg/kg	0 mg/kg
15	Blank	Blank	Blank	Blank
21	0,0 N.D.	0,0 N.D.	0,0 N.D.	0,0 N.D.
22	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg
25	Nd*	Nd*	Nd*	Nd*
26**	0,3	0,2	0,3	0,3
28***	<LOD	<LOD	Around LOD	<LOD
29	0	0	0	0
31	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg	< 0,5 mg/kg
32	Negative	Negative	Negative	Negative
37	Lost sample	ND	ND	ND
38	0	0	0	0

* revealed a high background that compromised analyte detection

** Lab 26 estimated their limit of quantification (LOQ) at 0,2 mg/kg

*** Lab 28 estimated their limit of detection (LOD) at 0,12 mg/kg

Note: lab 32 estimated concentrations for the 2 samples were 0,12 and 0,14

Out of 12 laboratories, one lab (nr. 26) reported positive results for both feeds. These values are lower by a factor of 3-4 than the values obtained by this lab for the 2 mg/kg sample. Lab 25 reported a background in the piglet feed that roughly corresponds to a signal of 0,05 mg/kg. These values are lower by a factor of 4 than the values obtained for the 2 mg/kg sample.
The laboratories that did not report a quantitative value for the blank samples used various descriptions (see Table 13 and 14), viz. "blank", "< 0,5 mg/kg", "nd", "<LOD", "negative", "0". In order to get more insight in the meaning of these descriptions, these laboratories were asked to give information on the signals measured in the blank samples. From the information obtained until now, it is not possible to draw clear conclusions about this point. The information tends to indicate that some laboratories only measure a very small signal (if any) at the retention time of virginiamycin while others measure a relatively large signal (see the chromatograms in Appendix 6), like laboratories 25 and 26. Most probably, these differences are caused by differences in the chromatographic conditions. Due to this diversity it was not possible to model the statistical error of the signals measured in blank samples. For this reason it is not possible to draw conclusions about the occurrence of false-negative results when the method is applied for screening at contents lower than 2 mg/kg.

5.3 Recoveries

Table 15: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Recovery 3 in %	Recovery average in %
11	76	80		78
15	63	65		64
21	68	67		68
22	82	79		80
25	43			43
26	87	85		86
28	78	51		65
29	54	37		46
31	56	42	45	48
32	42	43		43
37	43	37		40
38	60	93		77

Mean recoveries ranged from 40-86%. These results are in accordance with the results obtained previously in tasks 1 and 2 of the project (see chapter 1).

5.4 Remarks

Table 16: Remarks made by the partners

Partner	Remarks
11	No remarks
15	No remarks
21	- We would like to know why in the final calculation you don't consider the water volume used to wet the feed at the beginning of the extraction step. - We had some problems during the elution of the Sep-Pak Silica cartridges into the oasis HLB ones. In fact the Oasis HLB run dry before the elution solvent has passed through the Sep-Pak Silica cartridges. (critical)
22	- The virginiamycin content was calculated from the peak area by reference to the calibration graph. - The virginiamycin contents (mg/kg) are expressed as microbiological activities.
25	The extraction procedures on spiked samples at 10 ppm gave a recovery rate of around 43%. The absolute values found should be corrected by the recovery factor above mentioned. Of the following samples the peak area of the analyte found was lower than that of the standard solution 1 µg/ml: 256850 0,21** 0,21** 256931 0,22** 0,22**
26	- The extraction and clean-up method procedure was straight forward and easy to follow - The details concerning the preparation of the standards need some clarification - The final volume of 500 µl could be increased to 1 ml. This volume is very small. It is our practice to inject samples twice when there is no internal standard present. As a result the chromatography had to be re-run with the extract being placed into liners in the autosample vials.
28	Sample preparation: 5 ml of water is not sufficient for a complete moistering of some feed samples -> it seems to be better to increase this volume and to optimise the volume of extraction solvent. HPLC-determination: There is no complete base line separation of the virginiamycin peak even after 11 minutes of RT for the first analysis section! The chromatograms are very bad. It is very difficult to quantify and to calibrate accurately with standard solutions, which have very nice chromatograms. It seems to be better to quantify with spiked samples (matrix calibration). This is more convenient for residue analysis. Analysis with another column type didn't give nice HPLC-separation for virginiamycin. This determination method is not satisfactory optimised!

Partner	Remarks
29	- We analysed several times the samples you sent, and we couldn't agree more about the lack of homogeneity within the same sample. - As for recovery rates, we had great difficulties concerning your BLANK sample. We got better results when we spiked two different broiler feeds at the level of 2 mg/kg (49% and 64%) and two different bull feeds at the level of 5 mg/kg (89% and 100%), probably because of the type of feed matrix. - As for step 6.4.2, we never got a Na_2SO_4 pellet, but we always added the 5 ml of anhydrous ethyl acetate/n-hexane 1:1 v/v, as recommended in the protocol. - Finally, as for step 6.4.7, the residues were reconstituted in 1,0 ml of HPLC mobile phase, instead of 0,5 ml as recommended. This volume wasn't enough for the needle (in the HPLC) to get liquid injected. So, in step 7 - Expression of results - V_t is 1,0 ml instead of 0,5 ml. - The values sent weren't corrected for the recovery rate.
31	- The applied LOQ for the method is 0,5 mg/kg. - All quantitive results are based on peak height. A minor interference at R_t ca. 11,35 min. made this approach necessary.
32	No remarks
37	The biggest difficulty we had was at the elution from Sep-Pak Silica to Oasis HLB cartridge stage. In order to elute from the Silica Sep-Pak it was necessary to have a high vacuum and positive pressure (manually applied with syringe).
38	Please note that samples labelled as 386860 and 386947 has been quantified around our Limit of Determination (LD= 0,6 mg/kg).

5.5 Special requests

5.5.1 Microbiological analysis

The following partners performed microbiological analyses on the feed samples, applying the official EU-method (Directive 84/4/EEC):

- Rijksontledingslaboratorium (ROL), Tervuren, Belgium; K. Haustraete, A. Fontaine.
- Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Augustenberg, Germany; A. Thalmann, K. Wagner

Rijksontledingslaboratorium (ROL) measured a recovery of 94 % (level: 10 mg/kg).
Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Augustenberg didn't report the recovery.
Both laboratories didn't report remarks.
The results of the analysed samples are reported in Table 15.

Table 17: Results of the samples analysed with the microbiological method

Partner	Rijksontledingslaboratorium (ROL), Tervuren, Belgium		Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Augustenberg, Germany
Sample content (mg/kg)	Result 1 (mg/kg)	Result 2 (mg/kg)	Result (mg/kg)
Blank piglet (0)	<1	<1	0,0
Blank piglet (0)	<1	<1	0,0
Blank broiler (0)	<1	<1	0,0
Blank broiler (0)	<1	<1	0,0
2	1,20	1,46	1,7
2	1,25	1,06	0,0
5	2,79	3,09	2,5
5	3,38	2,91	2,1

The values obtained by ROL are higher than the mean values obtained with the CANFAS method. The negative results obtained by LUFA Augustenberg for one of the 2 mg/kg samples indicate that at this low content, problems may be encountered with the sensitivity of the method.

5.5.2 LC-MS/MS

The following partner performed the analysis by LC-MS/MS

- Departement voor Kwaliteit van Dierlijke Producten (DVK-CLO), Melle, Belgium; H. de Ruyck, H. de Ridder, L. Batjoens, P. de Neve

The mobile phase used was as described in the method. The HPLC column used was an Alltima C18, 150x2,1 mm, 5 µm (Alltech).

Reported recovery results: 82% and 64% (average 73%)

Remarks of DVK-CLO to this method:
For LC-MS/MS analysis it is recommended to use an internal standard for compensation of shifted ionisation.
The analyses are done with electrospray ionisation, the sensitivity is not so high, but high enough for this purpose of determining such high virginiamycin levels.
MS-conditions: see tune page report.
Diagnostic ions: -parent M+1= 526,3
-daughters M+1= (508,4), 354,8, 337,3
->see spectra of full scan, daughter scan

The results of the analysed samples are reported in Table 18.
Appendix 7 contains the completed questionnaire, applied LC-MS-conditions and representative mass spectra.
The mass spectra of the broiler feed containing 2 mg/kg virginiamycin and the blind blank feed clearly show the improvements in selectivity and sensitivity compared tot the LC-UV method.

Table 18: Results of the samples analysed with LC-MS/MS

Sample content (mg/kg)	Result 1 (mg/kg)	Result 2 (mg/kg)
Blank piglet (0)	<LOD	<LOD
Blank piglet (0)	<LOD	<LOD
Blank broiler (0)	<LOD	<LOD
Blank broiler (0)	<LOD	<LOD
2	1,49	1,07
2	1,36	1,32
5	3,09	1,51
5	2,40	2,92

The values obtained with LC-MS are higher than the mean values obtained with the CANFAS method (and also higher than the values obtained by DVK-CLO with the CANFAS method). While the same sample pre-treatment is applied for LC-UV and LC-MS this result cannot be explained.

6 EVALUATION AND CONCLUSIONS

From the results of the collaborative studies it can be concluded that the repeatability and the reproducibility of the method are unsatisfactory. The measurement uncertainty of quantitative results is much larger than generally considered as acceptable. Thus, the applicability of the method is restricted to semi-quantitative use, which means that any quantitative result should only be reported together with a clear statement of the measurement uncertainty. This is in accordance with the results of task 1 (method development and within-lab validation) and task 2 (between-lab validation) of the project and the conclusions drawn at the kick-off meeting.
From the results it can be concluded that the method is suitable as a screening method to discriminate between positive samples containing 2 mg/kg virginiamycin (or more) and negative samples. From the information obtained in this collaborative study it is not possible to deduce if the method is also suited to discriminate between samples containing 1 mg/kg (the target value from the Project Plan) and negative samples.

The results of the collaborative study were evaluated in a meeting in Tervuren (Belgium) on June 19-20, 2001.
After thorough discussion the panel decided to discard the results of lab 37 from the statistical evaluation. This decision is primarily based on the Grubbs outliers at all levels and supported by the chromatographic results.
Based on the unsatisfactory results for repeatability, reproducibility and recovery (see par. 5.1, Tables 6 and 8) it is concluded that the method is only suitable as a semi-quantitative screening method. The method can discriminate between positive samples containing 2 mg/kg and blank samples but it is uncertain if all laboratories could discriminate between samples containing 1 mg/kg and blank samples.
The method will not be recommended for adoption as an official method. It was agreed, also by mrs. Dyanne Bennink (scientific officer), that no further work will be done in the CANFAS-project. New work will be started in SIMBAG-FEED, taking into account the results of the CANFAS-project.

The following remarks, related to the method description have been accepted:
- Lab 26, second remark
- Lab 26, third remark, par. 6.4.7 of the method: the option will be described to reconstitute the residue in 1 ml HPLC mobile phase in stead of 0.5 ml.

The method description will be modified and the final method, together with the results of the collaborative study will be sent to the European Commission (CEMA).

ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Dr. A. Plöger, Danish Plant Directorate is thanked for supplying the virginiamycin reference standard.

APPENDIX 1

Letter with instructions, sent with the samples (with five annexes)

cc. Driessen, J. de Jong

DATE
2 October 2000

SUBJECT
collaborative study CAN
virginiamycin 71.316.2

ENCLOSURE(S)
5

OUR REFERENCE
00/0022095

HANDLED BY
Dr. J. de Jong

DIRECT (TELEPHONE) LINE
+31 317 47 55 81

E-MAIL
j.dejong@RIKILT.WAG-U

Dear colleague,

Please find enclosed the samples for the collaborative study for virginiamycin:

- 8 feed samples, with the text "additive: VIRGINIAMYCIN" and with a sample code; these samples constitute 2 blind duplicates of feed samples containing virginiamycin (contents in the range between 1 and 5 mg/kg) and 2 blind duplicates of a blank feed.

The samples must be analysed in *duplicate*.

For recovery purposes we have included a blank sample, with the text "blank feed for virginiamycin recovery purposes".

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 will also be send to you by E-mail as an Excel 5.0 file. We strongly prefer to get the results back in electronic form by E-mail (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 **8 December 2000**.

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 contains information about special requests. We hope that, next to the regular determinations, you are prepared to volunteer to do extra work with LC-MS.

The reference standard of virginiamycin which has to be used (98162-QCS) was already sent to you with my letter of 31 May 2000. This reference standard has a microbiological potency of 225 % or 2250 µg/mg.

RIKILT
State Institute for Quali
Control of Agricultural
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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

cc mrs. I. de Froidmont-Görtz, European Commission, DG Research, CII/3, Brussels

RIKILT
State Institute for Quality Contr
of Agricultural Products

DATE
2 October 2000

OUR REFERENCE
00/0022095

RIKILT

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 1 - Description of the method

CANFAS/VIRG/26092000/C. HAJEE

Detection of Virginiamycin in animal feeding stuffs by a High Performance Liquid Chromatographic method with UV detection

1 INTRODUCTION

Virginiamycin has been banned recently as a feed additive. Consequently, in addition to microbiological screening methods, there is a need for instrumental analytical methods that are capable of detecting Virginiamycin in feeding stuffs at levels well below additive level.

2 SCOPE AND FIELD OF APPLICATION

This operating procedure describes a method for the detection of the Virginiamycin M1 as a marker analyte for the total Virginiamycin content in animal feeding stuffs using high performance liquid chromatography and UV detection at 235 nm. The scope of the method is **screening** for the presence of virginiamycin in animal feeding stuffs at sub-additive levels.
Applicability of this method has been demonstrated for pigs, piglets, calves and poultry feed containing at least 1 mg/kg Virginiamycin.
Cattle feed containing at least 3 mg/kg Virginiamycin.
Sows and laying hens feed containing 4 mg/kg Virginiamycin.
Throughout this SOP the Virginiamycin content is expressed as mg/kg based on the microbiological activity, determined with Virginiamycin M1 as target compound. To obtain the content expressed on a weight to weight basis (w/w), the microbiological activity based content has to be divided by the microbiological potency of the reference standard material (usually around 220 %).

3 PRINCIPLE

Virginiamycin M1 is extracted from wetted feeding stuffs with ethyl acetate in an ultrasonic bath. A part of the extract is purified with tandem Solid Phase Extraction (SPE) using Silica and OASIS HLB cartridges. The eluate of the solid phase extraction is evaporated under nitrogen and the residue is dissolved in HPLC eluent. From this fraction a part is injected on a liquid chromatography system with a 'reversed phase' column. Isocratic elution is used and UV detection at 235 nm is applied.

4 REAGENTS AND MATERIALS

Only use reagents of recognised analytical grade.

4.1 Products used in their commercially available form

4.1.1 Water, HPLC quality. Conductivity < 10 MΩ.cm²

4.1.2 Acetonitrile, HPLC gradient grade

4.1.3 Methanol, HPLC gradient grade

4.1.4 Ethyl acetate, HPLC gradient grade

4.1.5 n-Hexane, GR

4.1.6 Sodium sulphate, anhydrous GR for analysis

4.1.7 Ammonium acetate, GR

4.1.8 Formic Acid, 100%, GR

4.1.9 Acetic acid, 100%, GR

4.1.10 Sep-Pak Silica Classic SPE cartridge, 690 mg (Waters, WAT 051900)) equipped with a solvent reservoir of 20 ml

4.1.11 OASIS HLB SPE cartridge, 60 mg, 3 mL, (Waters, WAT 094226)

4.1.12 Disposable polypropylene centrifuge tubes of 50 ml with screw cap (Greiner, Germany)

4.1.13 Disposable polypropylene 14 ml tubes with screw cap (Greiner, Germany)

4.1.14 Sample vials used for HPLC (1.2 ml)

4.1.15 Sep-Pak vac adapter (Waters, WAT 054260)

4.1.16 Sep-Pak classic male/male adapter (Waters, WAT 024310)

4.1.17 Disposable polypropylene syringes, 5 ml

4.1.18 Disposable polypropylene syringes, 20 ml

4.1.19 Disposable membrane filters, 0.22 µm, Ø 49 mm, Durapore (Millipore, GVWP04700)

4.2 Solutions

4.2.1 Conditioning solvent Silica Sep-Pak
Ethyl acetate / n-hexane 1:1 v/v. Mix 250 ml ethyl acetate (4.1.4) with 250 ml n-hexane (4.1.5). Dry and store the solution on anhydrous sodium sulphate (4.1.6).

4.2.2 Ammonium acetate, 1 M
Weigh 38.5 gram ammonium acetate (4.1.7), transfer into a 500 ml volumetric flask, dissolve and make up to 500 ml with water (4.1.1).

4.2.3 Ammonium acetate buffer 0.1 M, pH 4.0
Mix 100 ml 1 M ammonium acetate (4.2.2) with 800-ml water (4.1.1) in a 1000-ml volumetric flask. Adjust to pH 4.0 with formic acid (4.1.8) or acetic acid (4.1.9) and make up to 1000 ml with water (4.1.1).

4.2.4 Silica Sep-Pak elution solvent
Methanol / 0.1 M ammonium acetate buffer pH 4.0 1:3 v/v. Mix 250 ml methanol (4.1.3) with 750 ml ammonium acetate buffer 0.1 M pH 4.0 (4.2.3).

4.2.5 Washing solvent OASIS HLB
Methanol/water 1:1 v/v. Mix 100 ml methanol (4.1.3) with 100 ml water (4.1.1).

RIKILT

4.2.6 Mobile phase for HPLC

Water / acetonitrile / acid 600:400:3 v/v/v. Mix 600 ml water (4.1.1) with 400 ml acetonitrile (4.1.2) and 3 ml formic acid (4.1.8). Filter through a 0.22 µm filter (4.1.19) and degas with helium prior to usage.

4.3 Analytical standards

4.3.1 Virginiamycin reference standard

The reference standard should preferably be supplied with a certificate of analysis containing at least the chemical purity and the guaranteed microbiological potency / activity (Pfizer, Pharmaceutical Group, Antibiotics Plant, Rixenart, Belgium).

The reference mix usually contains about 57% Virginiamycin M1 and 19% Virginiamycin S1 and has a microbiological potency of around 220%.

4.4 Preparation of standard solutions

4.4.1 Stock solution virginiamycin 500 µg/ml m.a.

Weigh approximately 10 mg (± 0.1 mg) of Virginiamycin reference standard (4.3.1) (take account of the w/w-purity and of the microbiological potency of the reference standard). Dissolve the virginiamycin in an appropriate volume methanol (4.1.3) to produce a concentration of 500 µg/ml based on microbiological activity (take account of the w/w-purity and of the microbiological potency of the reference standard). The appropriate volume methanol can be weighed taking account of the density (1L methanol =0.79 kg).

This solution can be stored for 2 months at a temperature of 4 °C (± 4 °C).

4.4.2 Diluted stock solution Virginiamycin 50 µg/ml

Pipet 1000 µl stock solution (4.4.1) in a 10.00-ml volumetric flask, adjust to volume with LC mobile phase (4.2.6) and mix. This solution can be stored for one week at a temperature of 4 °C (± 4 °C).

4.4.3 Spiking solution Virginiamycin 50 µg/ml

Pipet 1000 µl stock solution (4.4.1) in a 10.00-ml volumetric flask, adjust to volume with methanol (4.1.3) and mix. This solution can be stored for one week at a temperature of 4 °C (± 4 °C).

4.4.4 Calibration solutions used for the calibration curve

These solutions can be stored and are stable for one week in a refrigerator at a temperature of 4 °C (± 4 °C).

4.4.4.1 Calibration solution 1.0 µg/ml (≡ 0.2 mg/kg in feed assuming 100% recovery)

Pipet 200 µl of diluted stock solution (4.4.2) into a 10.0-ml volumetric flask, adjust to volume with HPLC mobile phase (4.2.6) and mix thoroughly.

4.4.4.2 Calibration solution 2.5 µg/ml (≡ 0.5 mg/kg in feed assuming 100% recovery)

Pipet 500 µl of diluted stock solution (4.4.2) into a 10.0-ml volumetric flask, adjust to volume with HPLC mobile phase (4.2.6) and mix thoroughly.

4.4.4.3 Calibration solution 5.0 µg/ml (≡ 1.0 mg/kg in feed assuming 100% recovery)

Pipet 1000 µl of diluted stock solution (4.4.2) into a 10.0-ml volumetric flask, adjust to volume with HPLC mobile phase (4.2.6) and mix thoroughly.

4.4.4.4 Calibration solution 10.0 µg/ml (≡ 2.0 mg/kg in feed assuming 100% recovery)

Pipet 2000 µl of diluted stock solution (4.4.2) into a 10.0-ml volumetric flask, adjust to volume with HPLC mobile phase (4.2.6) and mix thoroughly.

4.4.4.5 Calibration solution 25.0 µg/ml (≡ 5.0 mg/kg in feed assuming 100% recovery)

Pipet 5000 µl of diluted stock solution (4.4.2) into a 10.0-ml volumetric flask, adjust to volume with HPLC mobile phase (4.2.6) and mix thoroughly.

4.4.4.6 Calibration solution 50.0 µg/ml (≡ 10.0 mg/kg in feed assuming 100% recovery)

Identical to diluted stock solution (4.4.2).

NOTE: Sections 4.4.2- 4.4.4 have been updated since the kick-off meeting.

5 APPARATUS

Common laboratory apparatus and, in particular, the following:

5.1 Vortex shaker (for example IKA fibrofix, VF1)

5.2 Ultrasonic bath (for example Branson art. 2210)

5.3 Centrifuge with preferably at least 24 positions for 50-mL tubes (for example MSE Mistral 3000 E)

5.4 SPE unit suitable for 12 or more cartridges equipped with a vacuum pump

5.5 Evaporation station (for example Pierce)

5.5.1 Reacti Therm (art.no. 18790)

5.5.2 Reacti Vap (art.no 18780)

5.6 pH meter (for example Schott art. CG 840)

5.7 High performance liquid chromatography system consisting of the following:

5.7.1 An autosampler or manual injector set to inject 100 µl.

5.7.2 A pump set to deliver a constant mobile phase flow rate of 1.0 ml/min.

5.7.3 A guard column packed with pellicular C18 material

5.7.4 An analytical column, length 250 mm, internal diameter 4.6 mm, packed with 5-µm Lichrospher C18 or Hypersil BDS C18 stationary phase particle material (for instance Chrompack)

5.7.5 A detector allowing the measurement of absorbance of UV light at a wavelength of 235 nm, with integrator / recorder.

The resulting average retention time for Virginiamycin M1 is 10 min ± 2 min.

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

6 PROCEDURE

6.1 Preparation of test samples

Feed test samples must be milled and mixed prior to assay. Grind feed samples through a mill (5.8) equipped with a 1-mm screen. After milling, mix the entire sample thoroughly. Store the sample in such a way that deterioration and changes in its composition are prevented.

6.2 Weighing test portion feed samples

Feed test samples should be at room temperature before taking into the procedure. Feed test samples are homogenised manually prior to weighing.

6.2.1 Blank feed

For the performance of the recovery test (6.2.2) a blank feed should be analysed to check that neither virginiamycin nor interfering substances are present. The blank feed should be similar in type to that of the sample and virginiamycin or interfering substances should not be detected.

6.2.2 Recovery test

A recovery test should be carried out by analysing the blank feed that has been fortified by addition

of a quantity of virginiamycin, similar to that present in the sample. Weigh to the nearest 0.01g, 5 g feed test sample (6.1) into a 50-ml tube (4.1.12). To fortify at a level of 4mg/kg, transfer 400 µl spiking solution (4.4.3) to the blank feed, mix thoroughly, for instance with a vortex mixer (5.1) and leave for 10 minutes mixing again several times before proceeding with the extraction procedure (6.3).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 6.2.1), 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 virginiamycin 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.

6.2.3 Feed test samples

Weigh to the nearest 0.01g, 5 g feed test sample (6.1) into a 50-ml tube (4.1.12).

6.3 Extraction procedure

Add 5 ml water (4.1.1), mix vigorously, for instance with a vortex mixer (5.1), to evenly distribute the water in the feed, wait for 10-15 minutes and mix vigorously again. *The water should be taken up completely by and distributed evenly in the feed* ***(critical)***.

Add 20 ml ethyl acetate (4.1.4), cap the tube, mix ± 30 sec vigorously for instance with a vortex mixer (5.1), wait for 10 minutes and mix vigorously again. *The feed layer should be loosely distributed in the ethyl acetate layer* ***(critical)***.

Place the tube for 30 min in an ultrasonic bath (5.2). Take the tube(s) out of the ultrasonic bath and shake by hand two to three times during the ultrasonication time.

Centrifuge (5.3) for 10 min at ambient temperature at 3500 rpm (= 3000*g).

Weigh approximately 5 gram anhydrous sodium sulphate (4.1.6) in a clean 50-ml polypropylene tube (4.1.12).

Transfer 10 ml of the upper layer i.e. the ethyl acetate fraction to the clean polypropylene tube containing the anhydrous sodium sulphate.

Add 10 ml n-hexane (4.1.5) to the 10 ml ethyl acetate extract and mix, for instance with a vortex mixer (5.1). Centrifuge (5.3) for 5 min at ambient temperature at 3500 rpm (=3000*g).

6.4 SPE sample clean-up procedure

6.4.1 Conditioning of the Sep-Pak Silica cartridge

Attach the adapter (4.1.16) and a 20-mL syringe reservoir (4.1.18) to the Sep-Pak Silica cartridge (4.1.10) and place it on the SPE unit (5.4). Rinse the Sep-Pak Silica cartridge with 2.5 ml anhydrous ethyl acetate / n-hexane 1:1 v/v (4.2.1).

6.4.2 Application of the extract to the Sep-Pak Silica cartridge

Carefully decant the clear supernatant and pass it through the conditioned Sep-Pak Silica cartridge. **Do not allow the cartridge to run dry! Solvent flow not higher than 2 ml/min.**

Rinse the sodium sulphate pellet by adding 5 ml anhydrous ethyl acetate / n-hexane 1:1 v/v (4.2.1) to the tube and mix for instance on a vortex mixer (5.1).

Centrifuge (5.3) for 5 min at ambient temperature at 3500 rpm (=3000*g).

Isolate the supernatant and apply it to the Sep-Pak Silica cartridge. After the liquid has completely passed through the Sep-Pak Silica cartridge, allow the cartridge to run dry. Wash the cartridge with 2 ml acetonitrile (4.1.2). Allow the cartridge to run dry. Remove and discard the syringe reservoir from the cartridge. Dry the cartridge for 30 min by applying vacuum with the SPE unit.

6.4.3 Pre-treatment of the OASIS HLB Cartridge

Place an OASIS HLB cartridge (4.1.11) on the SPE unit (5.4). Successively activate the OASIS HLB cartridge with 1 ml methanol (4.1.3) and condition with 1 ml ammonium acetate buffer 0.1 M, pH 4.0 (4.2.3)

6.4.4 Elution of the Sep-Pak Silica cartridge

Attach a 5–mL syringe reservoir (4.1.17) to the dried Sep-Pak Silica cartridge (6.4.2) and connect it to the conditioned OASIS HLB cartridge (6.4.3) (Sep-Pak Silica on top) using an SPE adapter (4.1.15).
Elute the dried Sep-Pak Silica cartridge with 5 ml elution solvent (4.2.4) and allow the eluate to pass directly through the OASIS HLB cartridge. **Solvent flow not higher than 2 ml/min.** The analyte is retained on the OASIS HLB cartridge. Disconnect and discard the Sep-Pak Silica cartridge.

6.4.5 Elution of the OASIS HLB cartridge
Wash the OASIS HLB cartridge with 2.5 ml washing solvent (4.2.5). Allow the OASIS HLB cartridges to run dry. Elute the OASIS HLB cartridge with two 2.5-ml volumes ethyl acetate (4.1.4) and collect both portions in the same 14-ml polypropylene tube (4.1.13).

6.4.6 Evaporation of the solvents
Place the polypropylene tube containing the ethyl acetate fractions obtained at 6.4.5, in the evaporation station (5.5). Adjust the temperature to 50 °C and evaporate the ethyl acetate fractions under a mild nitrogen gas flow (argon or helium can be used also).

6.4.7 Reconstitution of the residues
Reconstitute the residues in 0.5 ml HPLC mobile phase (4.2.6) and mix vigorously, for instance on a vortex mixer (5.1). Ultrasonicate the reconstituted sample solution for 5 minutes and mix again. In case an autosampler is used tranfer the sample solution to an autosampler vial of suitable dimensions.

6.5 HPLC determination

6.5.1 Parameters
The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.
Liquid chromatographic column (5.7.4): 250 mm □ 4.6 mm, Lichrospher C18 or Hypersil BDS C18, 5 µm packing, or equivalent.
Mobile phase (4.2.6): Mixture of acetonitrile (4.1.2), water (4.1.1) and formic acid (4.1.8), 400+600+3 (v+v+v).
Flow rate: 1.0 ml/min.
Detection wavelength: 235 nm.
Injection volume: 100 µL.
Analysis time: 40 min
Check the stability of the chromatographic system, injecting several times the calibration solution (0) containing 10.0 µg/mL virginiamycin, until constant peak areas (heights) and retention times are achieved.

6.5.2 Chromatographic series
The sequence in a chromatographic series should be injection of calibration solutions (4.4.3), blank HPLC solvent (4.2.6), blank feed (6.2.1), recovery samples (6.2.2), blank HPLC solvent (4.2.6), feed samples (6.4.7) and calibration solutions (4.4.3).

6.5.2.1 Calibration graph
Inject each calibration solution (4.4.3) and determine the peak areas (heights) for each concentration. Plot a calibration graph using the peak areas (heights) of the calibration solutions as the ordinates and the corresponding concentrations in µg/mL as the abscissae.

6.5.2.2 Sample solution
Inject the sample extracts (6.4.7) using the same injection volume as taken for the calibration solutions and determine the mean peak height (area) of the virginiamycin M1 peak.

RIKILT

7 EXPRESSION OF RESULTS

Expression of the results is merely an estimation of the virginiamycin content. The method is intended for screening purposes only.

From the area (height) of the virginiamycin M1 peak of the sample solution determine the virginiamycin concentration of the sample solution in µg/mL by reference to the calibration graph (6.5.2.1).

Use the following formula to estimate the virginiamycin content W in mg/kg in the feed samples:

$$W = c_s * \frac{V_e}{V_{SPE} * m} * V_t$$

W	=	content of Virginiamycin in mg/kg m.a. (**not corrected for recovery**)
c_s	=	virginiamycin concentration in sample solution (µg/mL)
m	=	test portion of animal feed in gram (= 5 g)
V_e	=	volume of ethyl acetate in extraction in ml (= 20 ml)
V_{SPE}	=	volume of feed extract taken to SPE clean up in ml (= 10 ml)
V_t	=	volume of sample extract in ml (= 0.5 ml)

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: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
316838		
316843		
316872		
316874		
316897		
316932		
316940		
316942		

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 3 - Instructions for handling of the samples

1. Storage
Store the samples at room temperature until analysis.

2. Milling
Grind the feed samples with a mill equipped with a 1 mm screen

3. Mixing of the test samples before weighing
Mix the entire sample thoroughly

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

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

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ..... mg
- Volume of methanol: ..... ml
- Concentration of the stock solution: ..... µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - □ As described in the method
  - □ Other: .....
- Mobile phase:
  - □ As described in the method
  - □ Other: .....
- Flow-rate: ..... ml/min
- Injection volume: .....µl
- Retention time of virginiamycin M1: ..... min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

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

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

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

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

Thank you for your cooperation !

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 5 - Special requests

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

LC-MS or LC-MS-MS of the feed samples

The extracts resulting from the LC-UV method can be used. If it is not possible to perform the LC-MS analysis directly, the extracts must be stored frozen.
As an example, the conditions applied by RIKILT are described in the appendix attached. Representative mass spectra are included.

Please report the results in a copy of annex 4 and give additional information on LC-MS mode(s) applied, conditions, diagnostic ions, etc. Please include representative mass spectra. Please also state if it was possible to fulfil the criteria for confirmation as described in the document SANCO/1805/2000 (document on revision of criteria, Revision of Commission Decision 93/256/EC)

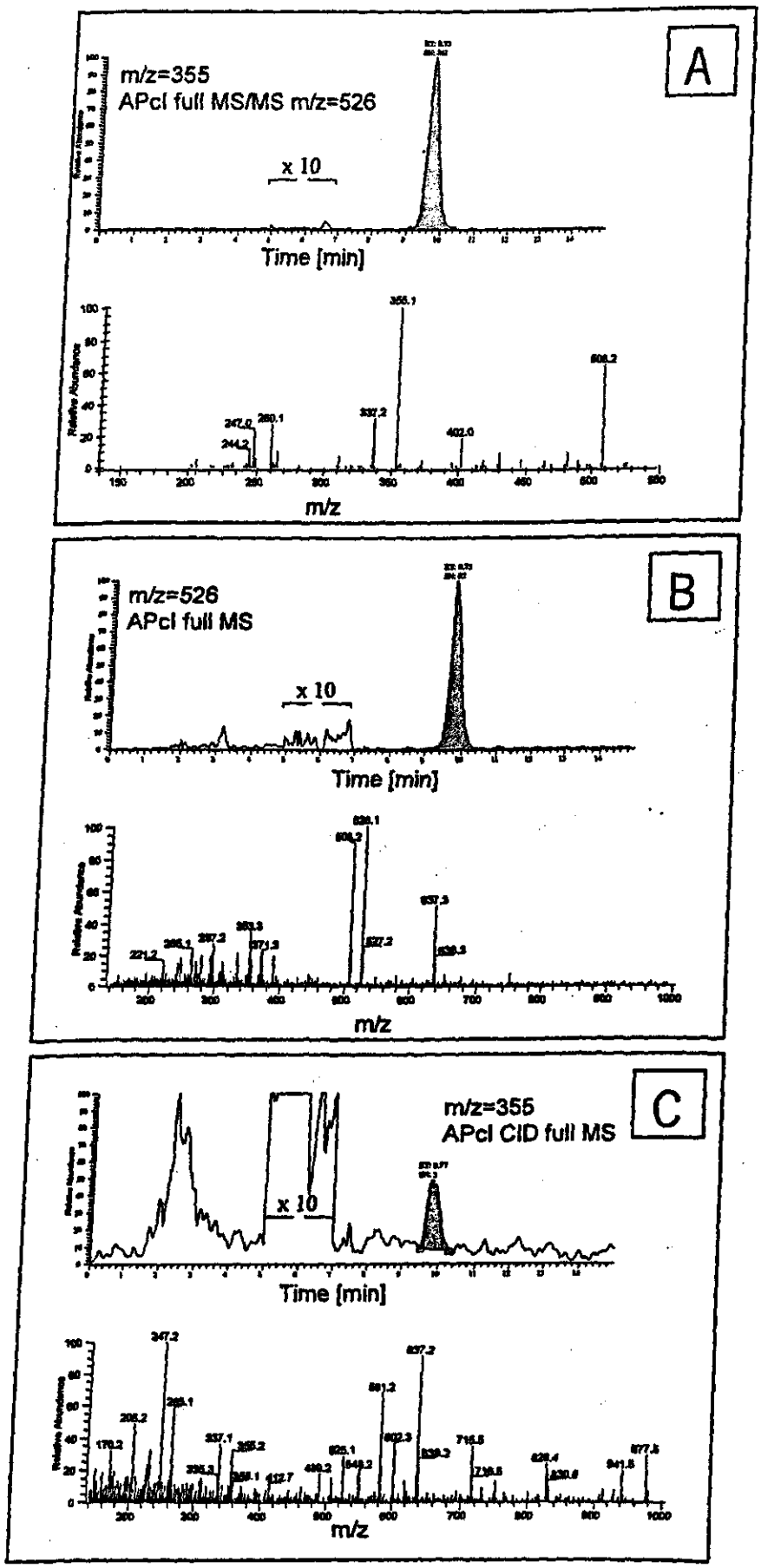


Figure 1. Example chromatograms of virginiamycin M1 in an extract of piglet feed spiked at LOQ 2.2 mg/kg m.a. with virginiamycin obtained after injection into an LC system coupled to MS detection in A. single reaction monitoring (SRM) LC-MS^2 mode; B. single ion monitoring (SIM) LC-MS mode; and C. SIM Collision Induced Dissociation (CID) LC-MS mode

std. virginiamycine 00-012
CQ

Divert Valve: not used during run

MS Detector Settings:

Segment 1 Information

Duration (min): 13.91
Number of Scan Events: 1
Tune Method: virg

Scan Event Details:
1: Pos ·(526.0)->·(508.0)->o(135.0-600.0)
MS2: Amp. 20.0% Q 0.250 Time 50.000 IsoW 2.1
MS3: Amp. 27.0% Q 0.250 Time 50.000 IsoW 2.6

egment 2 Information

uration (min): 6.09
umber of Scan Events: 1
une Method: virg

can Event Details:
1: Pos ·(824.0)->o(225.0-1000.0)
MS/MS: Amp. 25.0% Q 0.250 Time 50.000 IsoW 2.2

APPENDIX 2

Composition of the feed samples

BESTMIX - Afdruk mengopdracht - 06/09/00 - IPC DIER BARNEVELD

2 132.00 Vleeskuiken korrel
Vleeskuiken korrel
test Rikilt Virginiamycine 2 mg/kg

Broiler

Grondstof	Silo	%	Gewicht kg	Tol. +/-Afw.	Cumul Gew. kg	Charge 1	Charge 2
Weegschaal DW 1							
460 Tapioca65%zetmeel	(4)	30.00	150.00	4.50	150.00	✓	.....
77 Soja 45/46(arg/braz)	(9)	23.20	116.00	3.48	266.00	✓	.....
Weegschaal DW 2							
145 Tarwe (voer)	(9)	29.80	149.00	4.47	149.00	✓	.....
40 Mais	(12)	5.56	27.80	0.83	176.80	✓	.....
Bijstort SP4							
105 Vismeel 65.9% re	(0)	3.00	15.00	0.45	15.00	✓	.....
Bijstort SP7							
78 L-lysine HCl	(0)	0.06	0.30	0.00	0.30	✓	.....
79 DL-Methio-nine	(0)	0.14	0.70	0.01	1.00	✓	.....
117 Krijt/kalksteen	(0)	0.30	1.50	0.02	2.50	✓	.....
228 Monocal Belgie	(0)	0.60	3.00	0.03	5.50	✓	.....
485 Zout	(0)	0.04	0.20	0.00	5.70	✓	.....
510 Prem kuiken Rikilt Virginiamycine 0,2 g/kg.	(0)	1.00	5.00	0.05	10.70	✓	.....
Vloeistoffen							
96 Vet (soja-olie)	(0)	1.50	7.50	0.23	7.50	✓	.....
100 Vet destr.<0.5%polym	(0)	4.80	24.00	0.72	31.50	✓	.....
			Totaal :		500.00		

RETOURPRODUKT ....

INSTELLINGEN

T.R. : all. 50%
V.Z. : grof/~~fijn~~ ..80.. %
Z.F. : .2,5.... mm
H.M. : hoog/laag toeren
kringloop : ja/nee
L.M. : voormengen 60. sec
namengen 300. sec
M.D. : ..-.. l/h

Meel temp : .55. °C korrel temp 70 °C
Matrijs diam. : 2,5 x 35 mm
K.P. : .25. Amp
Laagdikte Ko : .35 cm
Zeef Ko : .fijn mm
Kruimelen : ja/neen
Holmen : 56,6 %
Vocht : %

BESTMIX - Afdruk mengopdracht - 06/09/00 - IPC DIER BARNEVELD

2 260.00 Vleesstierenbrok
Vleesstierenbrok 18%
5 mg/kg virginiamycine

Bull

Grondstof	Silo	%	Gewicht kg	Tol. +/-Afw.	Cumul Gew. kg	Charge 8	Charge
Weegschaal DW 1							
113 Zonbl.schr.290re	(2)	1.50	7.50	0.23	7.50	✓	.....
266 Raapschrt 340 g re	(3)	15.00	75.00	2.25	82.50	✓	.....
191 Tapioca66%zetmeel HP	(4)	15.00	75.00	2.25	157.50	✓	.....
Weegschaal DW 2							
64 Palmp.schil9rc	(28)	15.00	75.00	2.25	75.00	✓	.....
Bijstort SP4							
34 Lynzaad	(0)	0.60	3.00	0.09	3.00	✓	.....
84 Sojaschroot 49/3.5rc	(0)	10.00	50.00	1.50	53.00	✓	.....
Bijstort SP6							
10 Citruspulp	(0)	13.00	65.00	1.95	65.00	✓	.....
29 Kokossch. <100rv	(0)	10.00	50.00	1.50	115.00	✓	.....
107 Maisgl.USA Standaard	(0)	10.40	52.00	1.56	167.00	✓	.....
Bijstort SP7							
117 Krijt/kalksteen	(0)	0.90	4.50	0.05	4.50	✓	.....
485 Zout	(0)	0.10	0.50	0.01	5.00	✓	.....
507 Prem stieren Rikilt	(0)	1.00	5.00	0.05	10.00	✓	.....
0,5 g/kg Virginia							
Vloeistoffen							
52 Melasse riet;<450	(0)	6.00	30.00	0.90	30.00	✓	.....
100 Vet destr.<0.5%polym	(0)	1.50	7.50	0.23	37.50	✓	.....
			Totaal :		500.00		

RETOURPRODUKT ....

INSTELLINGEN

T.R. : aut. 50%
V.Z. : grof/(fijn) ...80.. %
Z.F. : .2,5.... mm
H.M. : (hoog)/laag toeren
kringloop : ja/(nee)
L.M. : voormengen 60. sec
namengen 300. sec
M.D. : 185.. l/h

Meel temp : 56.. °C koeltemp 76 °C
Matrijs diam. : 2,5 x 35. mm
K.P. : .28.. Amp
Laagdikte Ko : .35 cm
Zeef Ko : fijn mm
Kruimelen : ja/(neen)
Holmen : 97,1 %
Vocht : %

APPENDIX 3

Homogeneity of samples

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 : Virginiamycin
Product : Feed sample: 2 ppm

Date of determination : September 21th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
18431 A	1,01	0,98
18431 B	0,95	
18432 A	0,98	1,20
18432 B	1,41	
18433 A	0,57	0,76
18433 B	0,94	
18434 A	1,59	1,18
18434 B	0,78	
18435 A	0,75	0,94
18435 B	1,12	
18436 A	1,09	0,86
18436 B	0,63	
18437 A	0,73	0,53
18437 B	0,32	
18438 A	0,78	0,72
18438 B	0,65	
18439 A	0,78	0,91
18439 B	1,03	
18440 A	1,75	1,28
18440 B	0,81	

Homogeneity	OK		
Criterion : $CV_{between}$ = < 60%			
Average		0,94	
SD (between samples)		0,24	
CV (between samples)		25,3	Result Grubb's test
Grubb's test, single lower		1,716	no outlier
Grubb's test, single upper		1,469	no outlier
Grubb's test, double lower		0,4815	no outliers
Grubb's test, double upper		0,5307	no outliers

Repeatability		
SD (within samples)	(sd_r)	0,35
CV (within samples)	(CV (%))	37,4

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 : **Virginiamycin**
Product : **Feed sample: 5 ppm**

Date of determination : September 22th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
18841 A	1,16	1,60
18841 B	2,04	
18842 A	1,46	1,62
18842 B	1,77	
18843 A	2,71	2,23
18843 B	1,75	
18844 A	2,10	2,42
18844 B	2,73	
18845 A	1,42	2,06
18845 B	2,70	
18846 A	2,11	2,10
18846 B	2,09	
18847 A	1,81	2,16
18847 B	2,50	
18848 A	2,06	2,10
18848 B	2,14	
18849 A	3,01	3,01
18849 B	3,00	
18850 A	2,61	2,44
18850 B	2,28	

Homogeneity OK Criterion : $CV_{between}$ = < 60%		
Average	**2,17**	
SD (between samples)	**0,41**	
CV (between samples)	**18,8**	Result Grubb's test
Grubb's test, single lower	**1,408**	**no outlier**
Grubb's test, single upper	**2,047**	**no outlier**
Grubb's test, double lower	**0,4652**	**no outliers**
Grubb's test, double upper	**0,3840**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,47**
CV (within samples)	(CV (%))	**21,6**

APPENDIX 4

Sample codes

Sample codes supplied to the participants in the virginiamycin collaborative study

VIRGINIAMYCIN number of participants 16	VIRG broiler 2ppm VIRG 1a	VIRG broiler 2ppm VIRG 1b	VIRG bull 5ppm VIRG 2a	VIRG bull 5ppm VIRG 2b	CARB/OLA piglet 2,5/7,5 VIRG blank 1a	CARB/OLA piglet 2,5/7,5 VIRG blank 1b	NIC broiler 20ppm VIRG blank 2a	NIC broiler 20ppm VIRG blank 2b
Participant code								
11	116891	116884	116943	116899	116856	116922	116918	116946
13	136934	136849	136845	136933	136841	136920	136837	136956
15	156957	156921	156873	156958	156852	156904	156842	156938
20	206859	206903	206889	206945	206885	206877	206929	206927
21	216894	216879	216875	216925	216919	216892	216853	216863
22	226951	226913	226887	226858	226926	226890	226840	226882
23	236871	236883	236901	236870	236915	236944	236831	236896
25	256850	256931	256955	256941	256898	256861	256908	256914
26	266893	266950	266848	266869	266833	266855	266939	266912
28	286886	286909	286935	286832	286954	286834	286911	286888
29	296857	296878	296910	296900	296867	296937	296876	296949
31	316932	316874	316942	316838	316897	316872	316940	316843
32	326923	326839	326881	326880	326936	326952	326916	326928
33	336851	336902	336907	336846	336953	336847	336866	336930
37	376868	376895	376854	376844	376906	376836	376917	376835
38	386860	386947	386864	386948	386865	386862	386924	386905

APPENDIX 5

Virginiamycin reference standard profile, identity and purity

PPG/MD Quality Operations
Analytical Resource Group
Pfizer Inc
Eastern Point Road

CERTIFICATE OF ANALYSIS
REFERENCE STANDARD

Virginiamycin
LOT 98162-QCS

Purity: 225% or 2250 µg/mg Virginiamycin when used as is

Manufacture Date: January 1998

PARAMETER	RESULT
Appearance	Brown fine powder
HPLC - Identification	Passes
IR - Identification	Compares to historical
Microbial Assay	225% or 2250 µg/mg
LOD	1.6%
Residue on Ignition	< 0.1%
Other (Solubility)	Passes
Water (KF)	1.4%

Purity assignment based on microbiological assay data with support from transfer data.

Notes: Bottles are labeled 98162-QCS-XX, where XX represents the subdivision. Source lot for this material is V980122.

Prepared by: _____ 12/23/98
K. A. Sullivan — Date
Special Testing & Analytical Development

Approved by: _____ 12/23/98
Dr. K. J. Dennis, Group Leader — Date
Special Testing & Analytical Development

LC-MS CHARACTERISATION OF VIRGINIAMYCIN REFERENCE STANDARD AND COMPARISON OF VIRGINIAMYCIN M1 CONTENT OF AVAILABLE VIRGINIAMYCIN REFERENCE STANDARDS FOR CANFAS COLLABORATIVE STUDIES

Author: C.A.J. Hajee, RIKILT
Date: 26 September, 2000

INTRODUCTION

Dr. A. Plöger from Danish Plant Directorate (DPD) donated a large quantity of virginiamycin reference standard originating from Pfizer, lot 98162-QCS. This is designated to be used during the collaborative studies for virginiamycin in animal feed. The supplied analysis certificate from Pfizer, however, stated an expiration date of January 2000. To check the integrity of the reference standard, it was compared to two similar reference standards also originating from Pfizer, with a stated expiration date of January 2003. One of the prerequisites for the use of the reference standard from DPD is that the available standards show comparable responses for the marker component virginiamycin M1.

PROCEDURE

General

Three vial portions of virginiamycin reference standard, labelled V2 (RIKILT), 00-012 (DPD, lot 98162-QCS) and 00-015 (NVI), were available for comparison. Each originated from Pfizer lot nr. 980122 with a certified microbiological activity of 2250 µg/mg.
Stock solutions were prepared from each reference standard vial at ~500 µg/mL in methanol. Diluted stock solutions at ~ 50 µg/mL were prepared by dilution in HPLC mobile phase. A 10-µg/mL standard solution was prepared by fivefold dilution of the diluted stock solution in mobile phase.

LC-UV analysis

Aliquots (50 µL) of the 10-µg/mL standard solutions were injected in duplicate into an LC UV system for the detection of virginiamycin in compound animal feeds.
Characteristics are:

LC column:	250 mm × 4.6 mm, Lichrospher C18, 5 µm packing.
Mobile phase:	Acetonitrile / water / formic acid 400+600+3 (v+v+v).
Flow rate:	1.0 ml/min.
Detection wavelength:	235 nm.
Injection volume:	50 µL.
Analysis time:	30 min

Specific response, i.e. virginiamycin M1 response per µg injected virginiamycin was calculated and compared.

LC-MS analysis

Aliquots (20 µL) of the 10-µg/mL 00-012 standard solution were injected in duplicate into an LC MS system. Identification of virginiamycin M1 was performed with MS^3-detection on the basis of diagnostic ions m/z 355, 337, 260 and 247. These fragmentations had already been identified in an earlier stage of the CANFAS project.

Characteristics of the LC-MS system were:

LC column:	250 mm × 4.6 mm, Lichrospher C18, 5 µm packing.
Mobile phase:	Acetonitrile / water / formic acid 400+600+3 (v+v+v).
Flow rate:	1.0 ml/min.
Detection wavelength:	Finnigan LCQ ion-trap mass spectrometer operated standard settings
Injection volume:	20 µL.
Analysis time:	20 min

MS^3-detection method applied for the identification of virginiamycin M1 comprised the following settings:

$$m/z = 526 \xrightarrow{IW=2.1;AA=20\%} m/z = 508 \xrightarrow{IW=2.6;AA=27\%} m/z = [135;600]$$

RESULTS

LC-UV analysis

Results of the LC analyses of each injected standard solution are shown in Table 1.

Table 1. Individual responses and derived specific responses for the available virginiamycine reference standard vial portions.

Injection volume	Nominal concentration.	Sample name	Response	Specific response
[mL]	[µg/mL]		[µV*s]	[µV*s/µg]
0,05	9,94	00-012 10 µg/mL	416082	837187,1
0,05	9,94	00-012 10 µg/mL	415544	836104,6
0,05	10	00-015 10 µg/mL	426126	852252
0,05	10	00-015 10 µg/mL	424628	849256
0,05	10,01	V2 10 µg/mL	403249	805692,3
0,05	10,01	V2 10 µg/mL	400868	800935,1

One-way analysis of variance with these data supports a hypothesis that there is no significant difference in virginiamycine M1 presence between the three vial portions.

LC-MS analysis

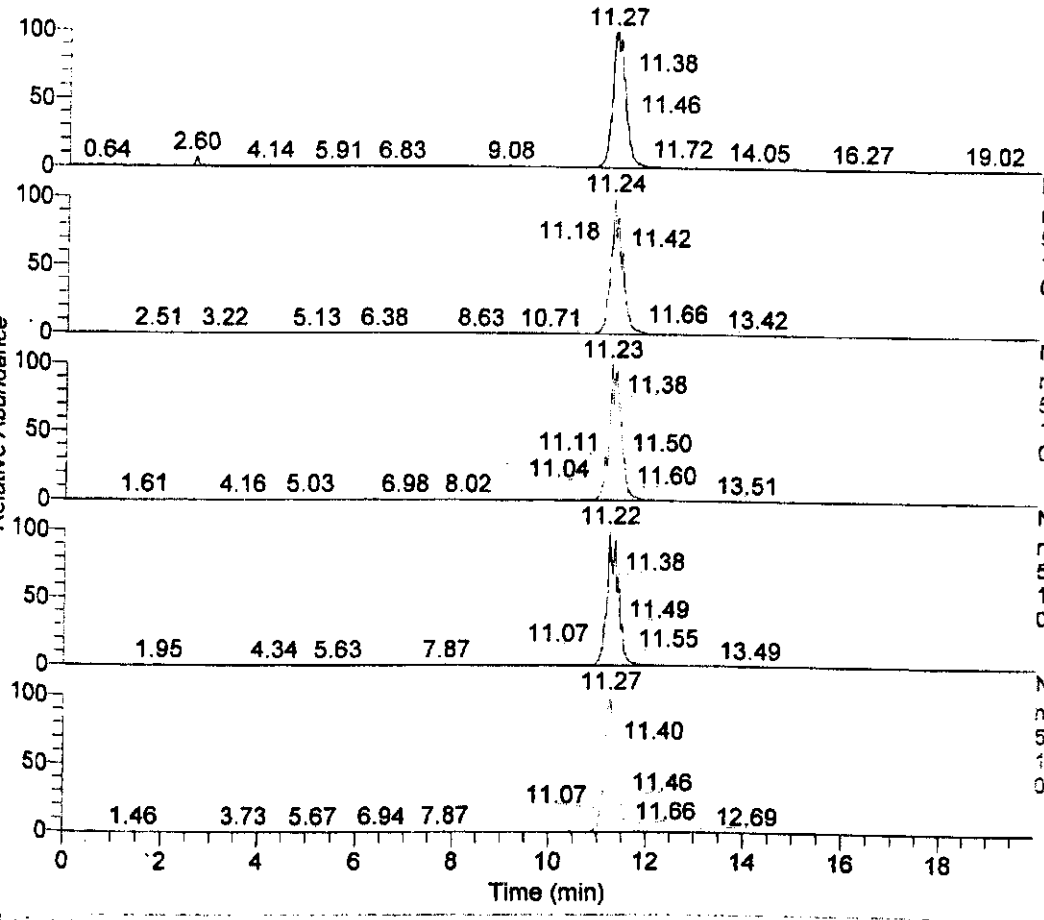
Results of the LC-MS^3 experiment are presented in Annex 1.

A total ion count (TIC) mass chromatogram showed a major peak at ~11.25 min corresponding to virginiamycin M1. A mass spectrum of this peak showed fragment ions at m/z 355, 337, 247 and 260, which is similar as found earlier. Mass chromatograms at m/z 355, 337, 247 and 260 of the 20-µL-aliquot of the 10-µg/mL solution containing virginimycin reference standard 00-012 (DPD) all showed the major peak at retention time ~11.25 min. Conclusion: virginiamycine M1 identity confirmed.

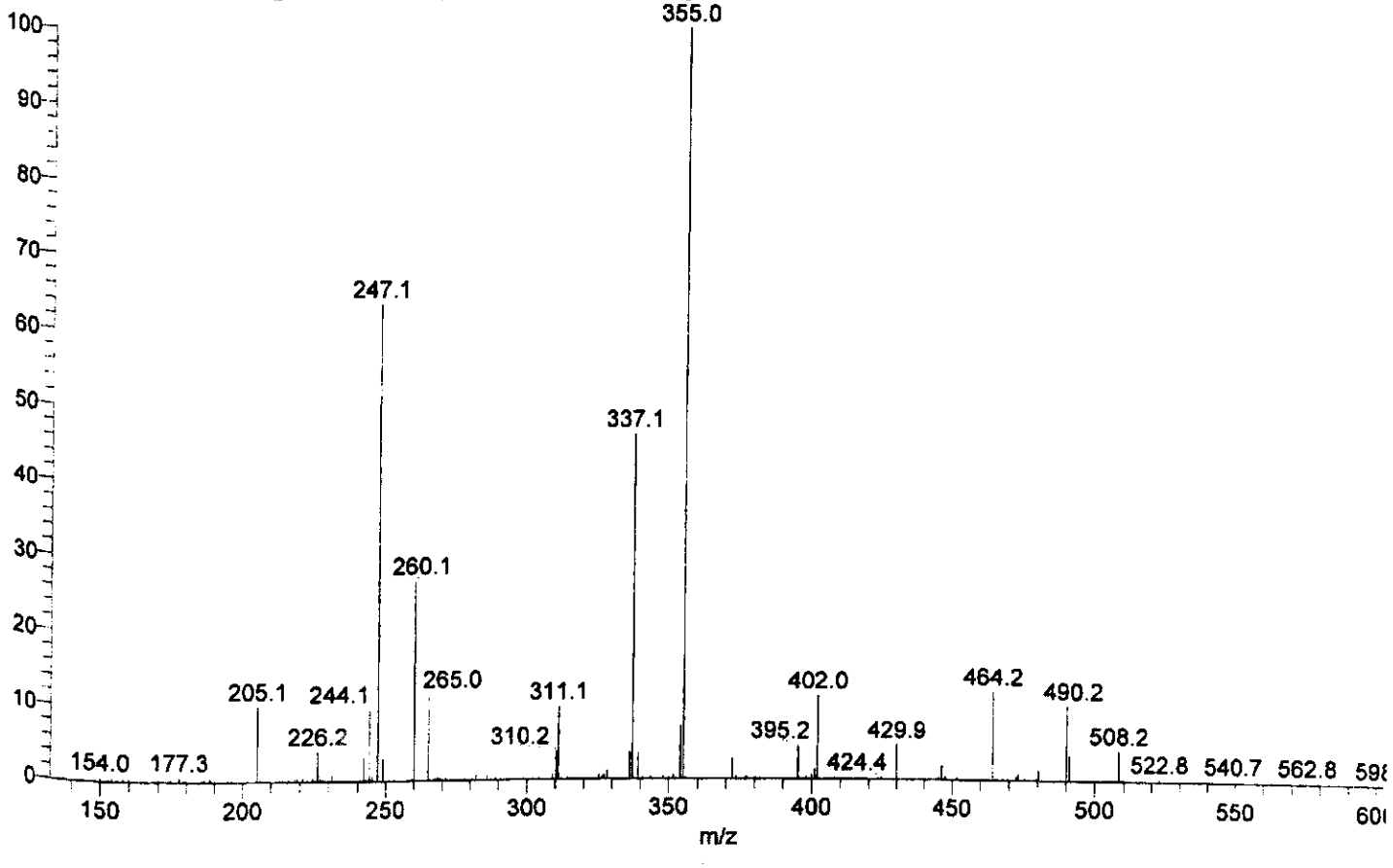
\000925\std. virginiamycine 00-012 09/25/00 02:13:14 PM

Annex 1.

T: 0.00 - 19.99 SM: 5G



virginiamycine 00-012#591-634 RT: 11.00-11.57 AV: 44 NL: 1.74E6
+ c APCI Full ms3 526.00@20.00 508.00@27.00 [135.00-600.00]



Antibiotics Plant
Rue de l'Institut, 87 A
B-1330 Rixensart, Belgium

Standaard NVI

meegehregen 17/2/00

Nr 55

Analysis nr January 1998

Certificate of Analysis

NAME OF PRODUCT : **VIRGINIAMYCIN**
Reference Standard

LOT Nr : V980122

PRODUCT CODE :

QUANTITY : 250 mg

PACKED IN :

MANUFACTURE DATE : January 1998
EXPIRY DATE : January 2003

STORAGE AND USE :

It is recommended that Virginiamycin Standard be stored at +4°C and be allowed to warm to room temperature prior to opening the container.
For a period longer than 1 month : Store the vials in a freezer at -21°C.

Do not dry before use.

1. **DESCRIPTION**	:	Brown fine powder having a characteristic odor and a very bitter taste.
2. **SOLUBILITY**	:	Passes
3. **IDENTITY** (HPLC)	:	Passes
4. **LOSS ON DRYING**	:	1.6 %
5. **RESIDUE ON IGNITION**	:	Less than 0.1 %
6. **VIRGINIAMYCIN** (Microbiological assay)	:	225 % or 2250 mcg/mg

Quality Assurance

PPG/MD Quality Operations
Analytical Resource Group
Pfizer Inc
Eastern Point Road

CERTIFICATE OF ANALYSIS
REFERENCE STANDARD

Virginiamycin
LOT 98162-QCS

Purity: 225% or 2250 µg/mg Virginiamycin when used as is

Manufacture Date: January 1998

PARAMETER	RESULT
Appearance	Brown fine powder
HPLC - Identification	Passes
IR - Identification	Compares to historical
Microbial Assay	225% or 2250 µg/mg
LOD	1.6%
Residue on Ignition	< 0.1%
Other (Solubility)	Passes
Water (KF)	1.4%

Purity assignment based on microbiological assay data with support from transfer data.

Notes: Bottles are labeled 98162-QCS-XX, where XX represents the subdivision. Source lot for this material is V980122.

Prepared by: 12/23/98
K. A. Sullivan — Date
Special Testing & Analytical Development

Approved by: 12/23/98
Dr. K. J. Dennis, Group Leader — Date
Special Testing & Analytical Development

APPENDIX 6

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:

Analyte: **VIRGINIAMYCIN**

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
116856	0	0
116884	0,27	0,37
116891	0,63	0,68
116899	3,32	2,87
116918	0	0
116922	0	0
116943	3,83	4,30
116946	0	0

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: October 24th - 27th, November 2nd. 2000

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: 44.4 mg
- Volume of methanol: 200 ml
- Concentration of the stock solution: 500 µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☒ As described in the method Lichrospher C18, 250mm x 4,6mm
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 100 µl
- Retention time of virginiamycin M1: 12 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

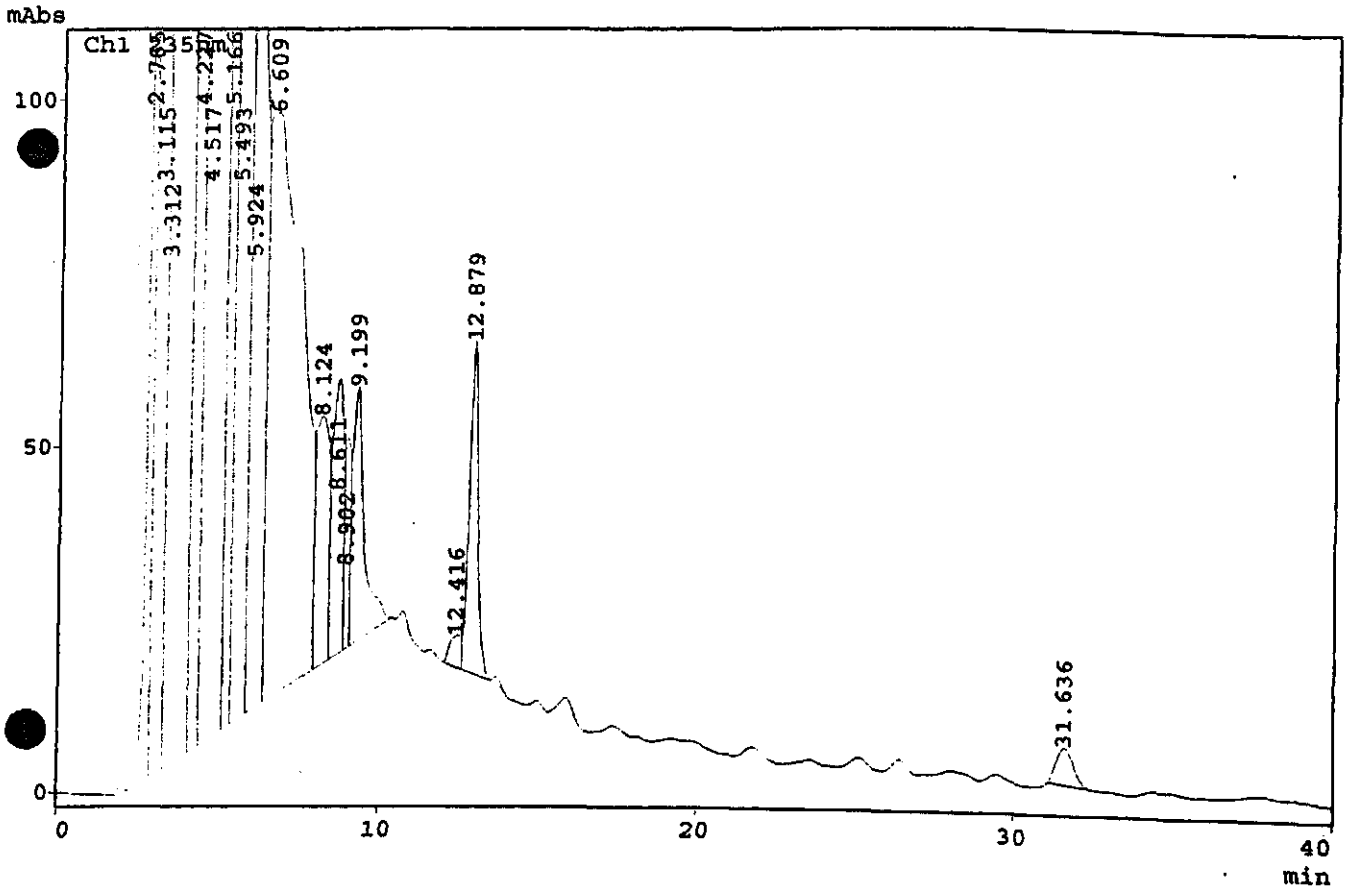
Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: 78 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 76 % and 80 %
- Spiking level: 4 mg/kg m.a.

LAS-LC10 Ver.=1.63 SYS=1 REPORT.NO=39 DATA=VIR02122.K01 00/11/02 21:46:43
Sample : 116922 om
) :
Sample Amount : 1
Type : Unknown
Detector : SPD-M10.
Operator :
Method Name : VIRGINIA.MET

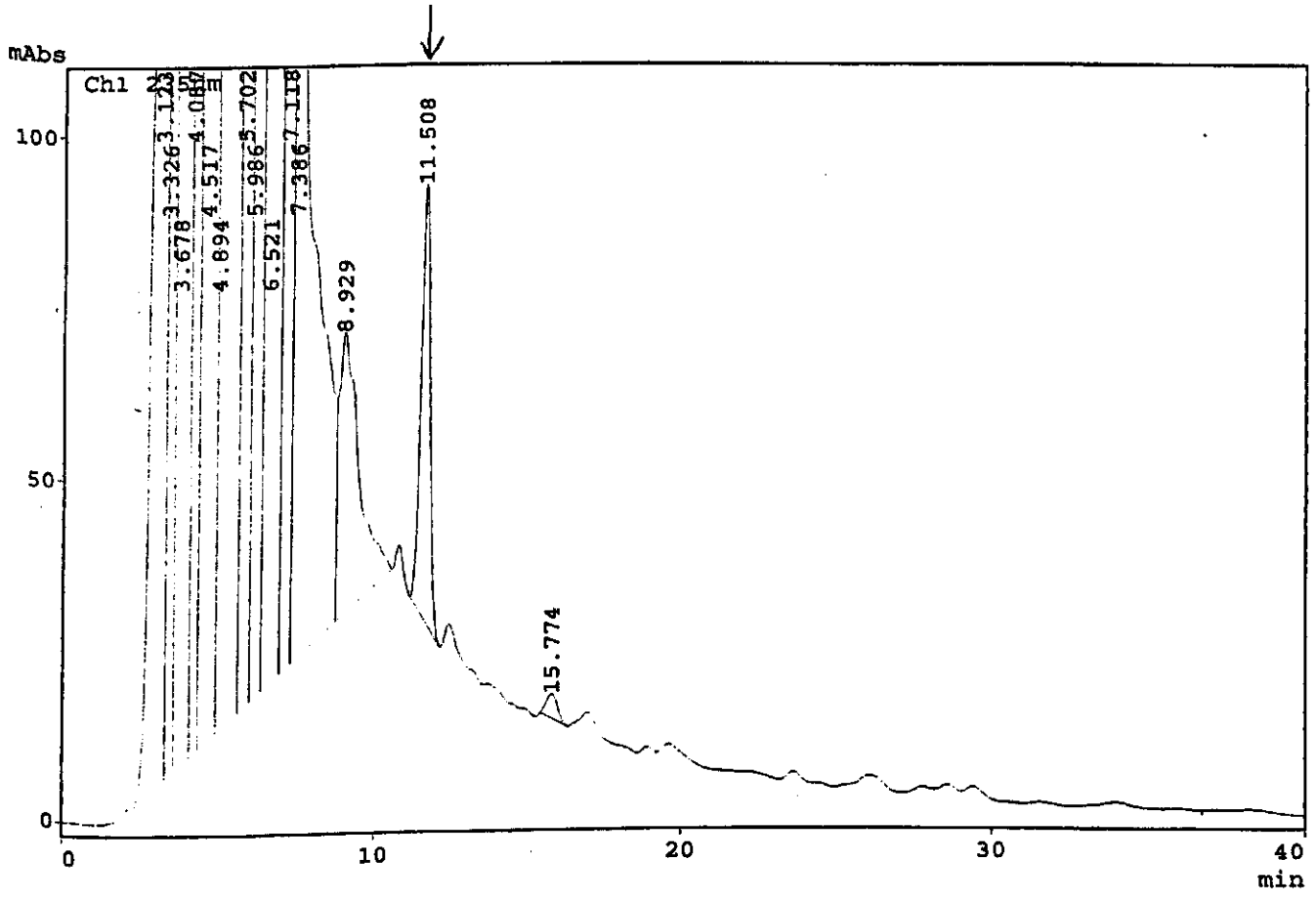
** Chromatogram ***



*** Peak Report ***
! No Identified Peak !!

CLASS-LC10 Ver.=1.63 SYS=1 REPORT.NO=41 DATA=VIR02120.K01 00/11/02 20:23:30
Sample : 116899 om
ID :
Sample Amount : 1
Type : Unknown
Detector : SPD-M10
Operator :
Method Name : VIRGINIA.MET

*** Chromatogram ***



** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN	IDNO	CONC
13	1	11.508	1330701		0.9346(1.0000)	0.9874(1.0000)	1	16.5984
			1330701					16.5984

/S = 3:32

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 15

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: **VIRGINIAMYCIN**

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
156842	blank	blank
156852	blank	blank
156873	0,35	0,32
156904	blank	blank
156921	0,68	0,69
156938	blank	blank
156957	0,57	0,4
156958	1,82	1,68

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: ....4-8 December 2000.....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ..11.12..mg
- Volume of methanol: .....50..... ml
- Concentration of the stock solution: .....500..... µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1.0..... ml/min
- Injection volume: ..100....µl
- Retention time of virginiamycin M1: ..11.2.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

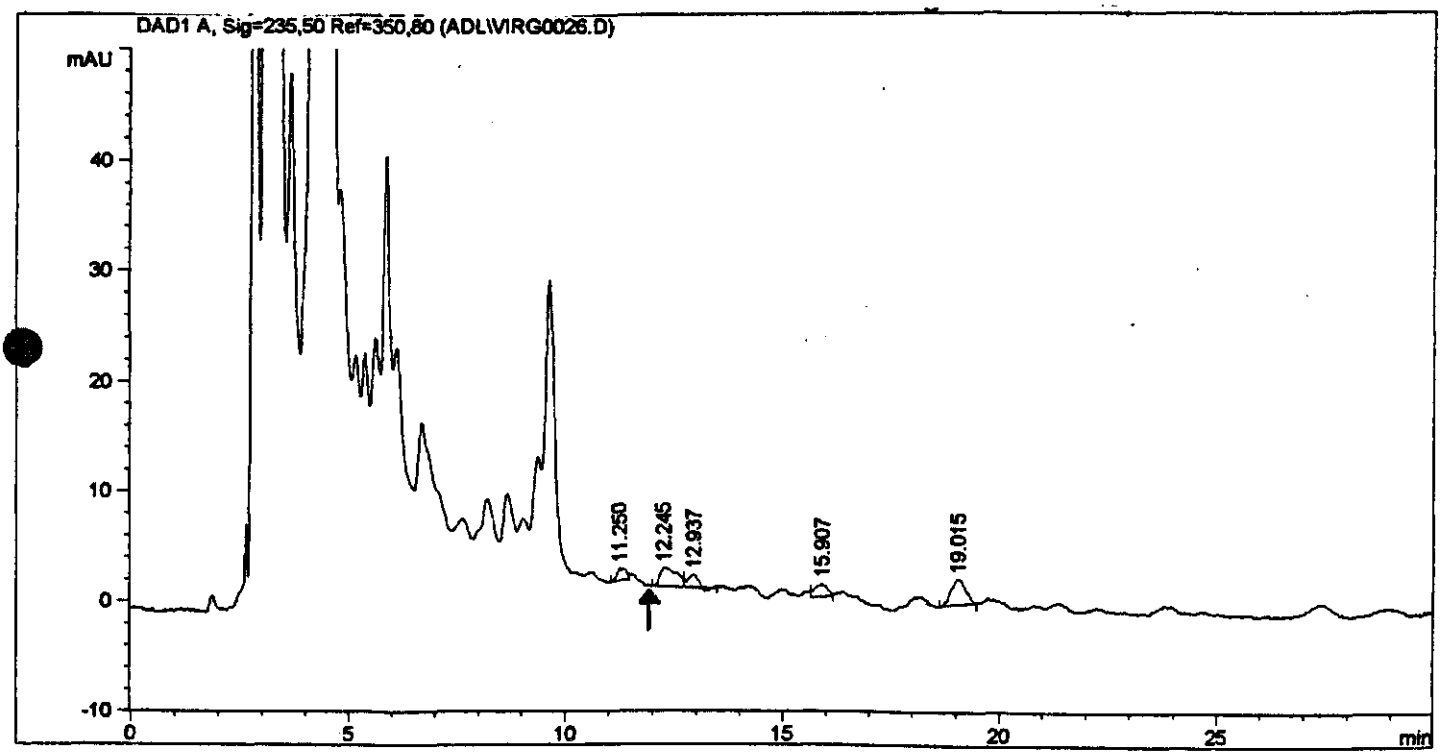
Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 95 % and 65 %
- Spiking level: ..178.. mg/kg ≡ 4.0 mg/kg m.a.

Data File C:\HPCHEM\1\DATA\ \VIRG0026.D Sample Name: 156938

15

Injection Date	: 12/8/00 1:07:16 AM	Seq. Line	: 26
Sample Name	: 156938	Vial	: 17
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/8/00 1:05:48 AM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/11/00 12:49:28 PM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Monday, December 11, 2000 12:49:15 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.956		-	-	-		Virginiamicin

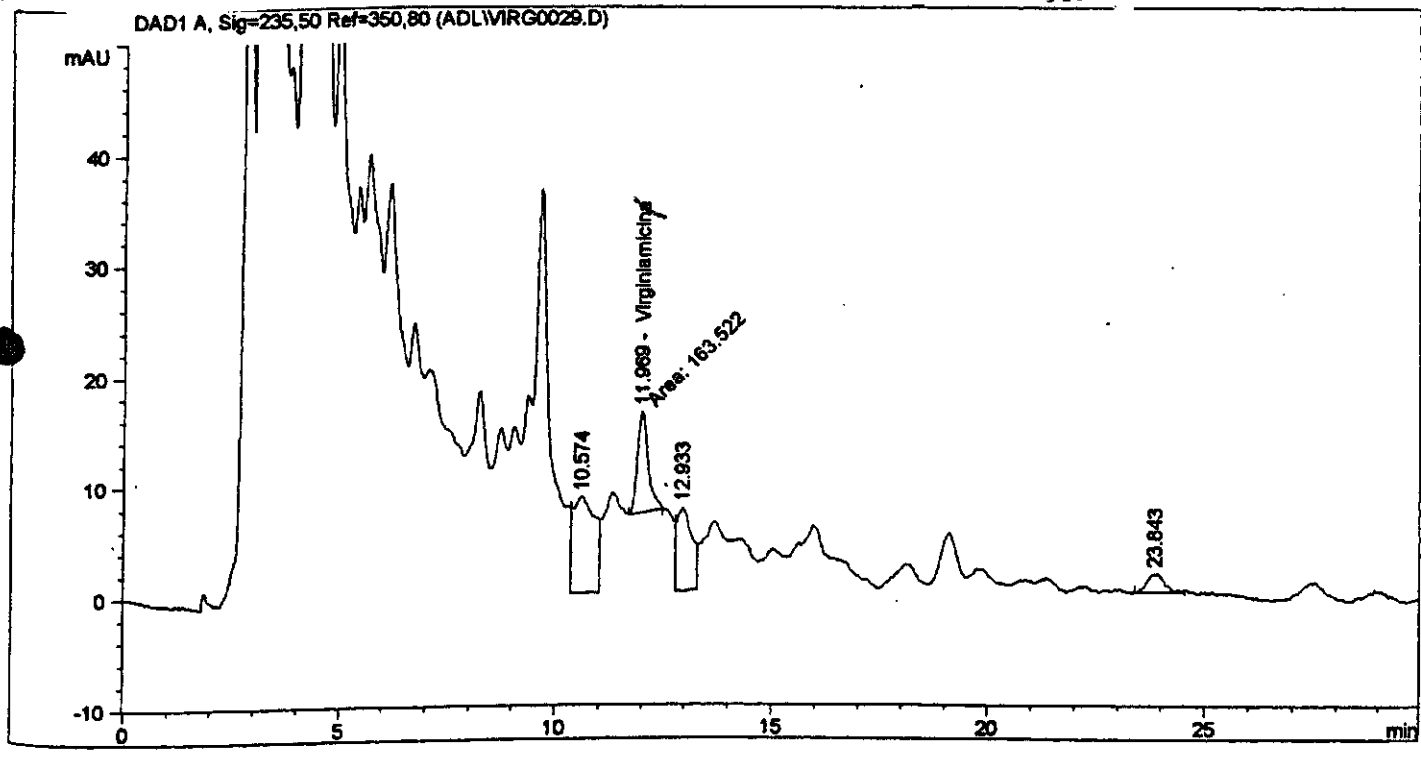
Totals : 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

(15)

Injection Date	: 12/8/00 2:43:19 AM	Seq. Line	: 29
Sample Name	: 156957	Vial	: 20
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/8/00 2:41:52 AM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/11/00 9:37:25 AM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Monday, December 11, 2000 9:37:25 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

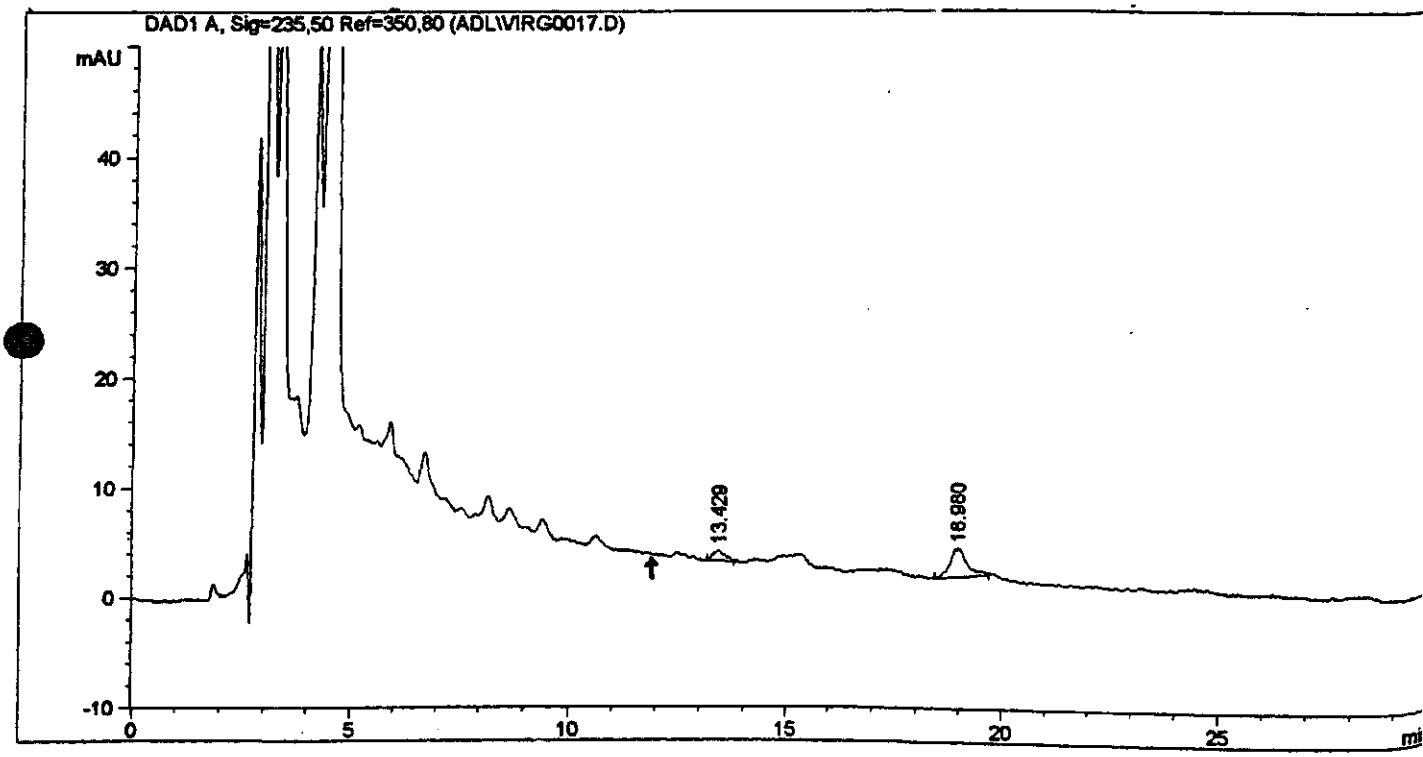
RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.969	MM	163.52209	1.73966e-2	2.84472		Virginiamicin

Totals : 2.84472 × 0.2 = 0.57 µg/kg m.a.

Results obtained with enhanced integrator!

*** End of Report ***

Injection Date	: 12/7/00 8:19:13 PM	Seq. Line	: 17
Sample Name	: 156904	Vial	: 14
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/7/00 8:17:47 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/11/00 9:37:25 AM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Monday, December 11, 2000 9:37:25 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.954		-	-	-		Virginiamicin

Totals : 0.00000

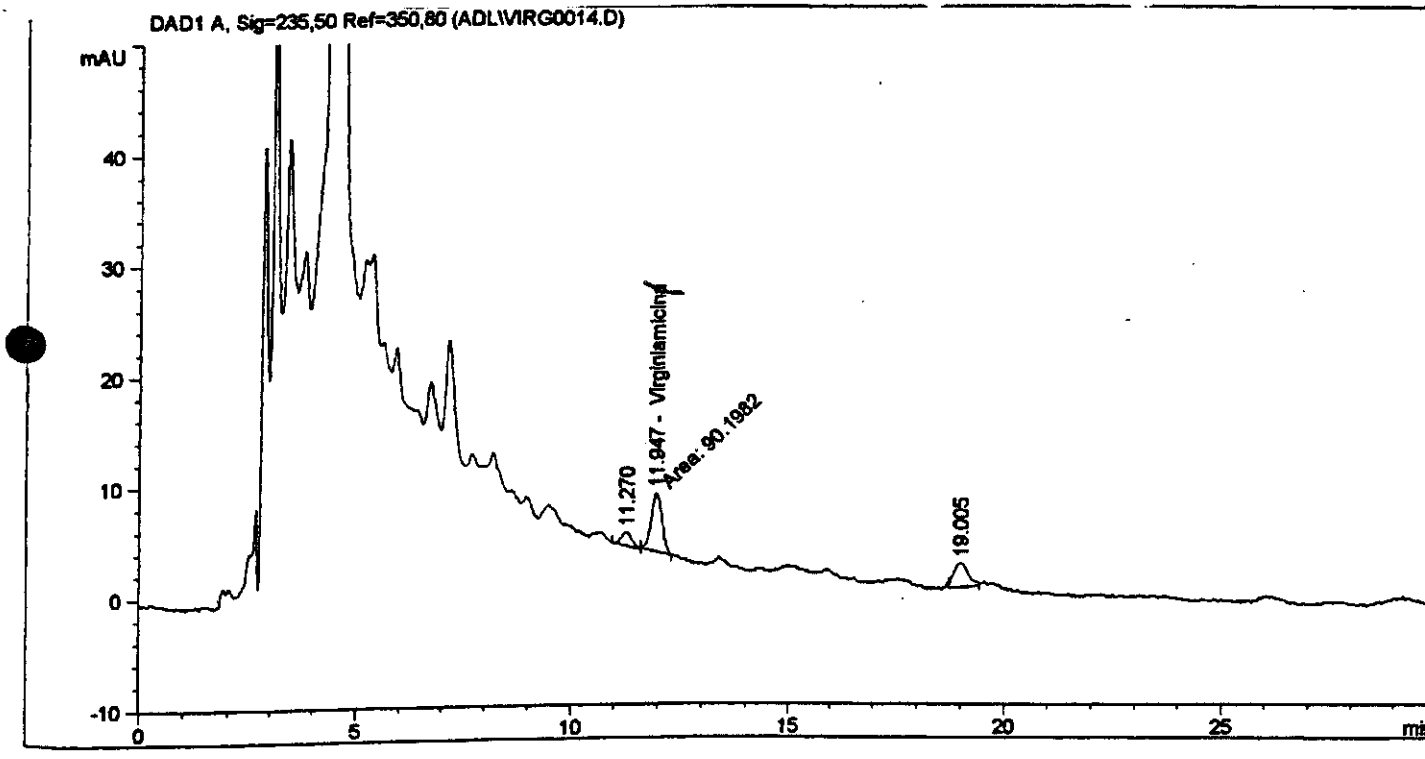
Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

(15)

Injection Date : 12/7/00 6:43:15 PM Seq. Line : 14
Sample Name : 156873 Vial : 11
Acq. Operator : Inj : 1
Inj Volume : 100 µl

Acq. Method : C:\HPCHEM\1\METHODS\MVALVIRG.M
Last changed : 12/7/00 6:41:48 PM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\MVALVIRG.M
Last changed : 12/11/00 9:37:25 AM by
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : Monday, December 11, 2000 9:37:25 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.947	MM	90.19824	1.75183e-2	1.58012		Virginiamicin

Totals :

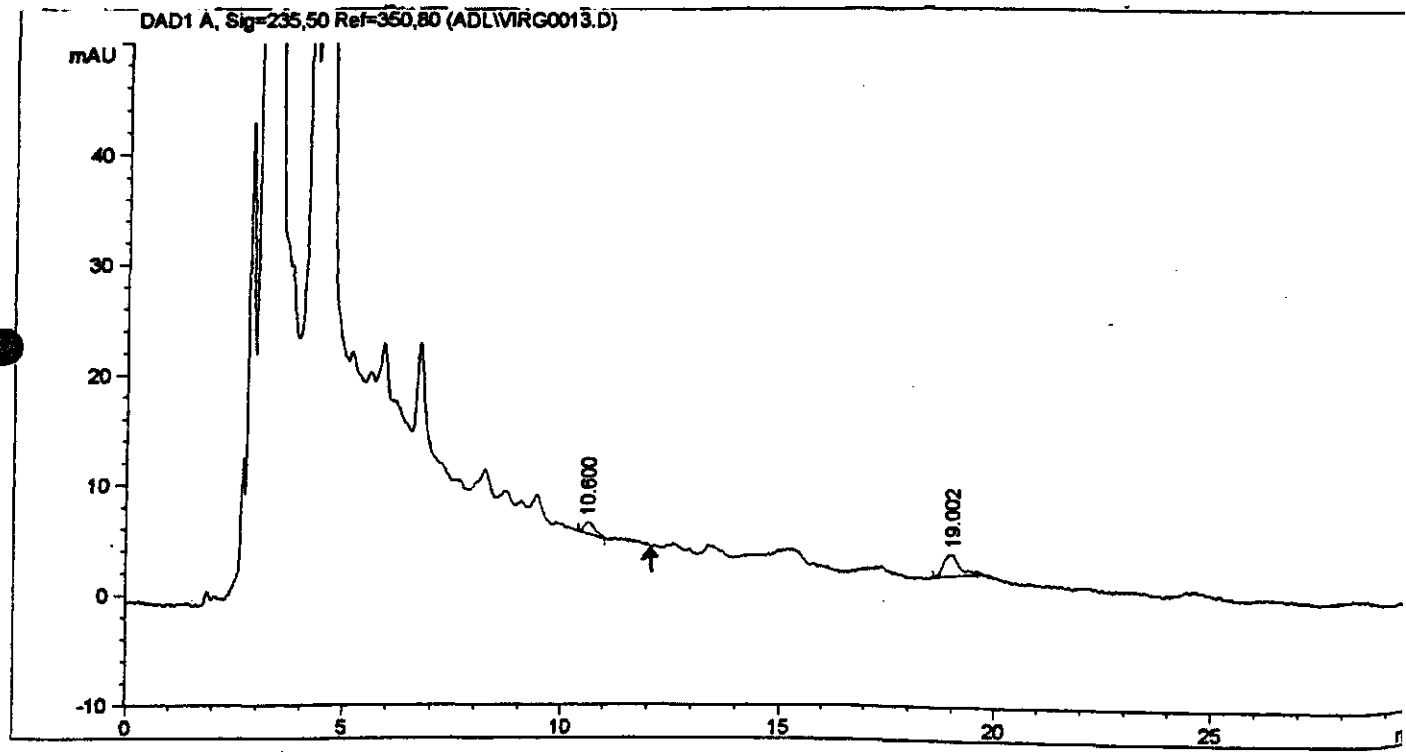
1.58012 × 0.2 = 0.32 ug/kg m.a.

Results obtained with enhanced integrator!

*** End of Report ***

15

Injection Date	: 12/7/00 6:11:14 PM	Seq. Line	: 13
Sample Name	: 156852	Vial	: 10
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/7/00 6:09:46 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/11/00 9:29:14 AM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Monday, December 11, 2000 9:29:14 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.954		-	-	-		Virginiamicin

Totals : 0.00000

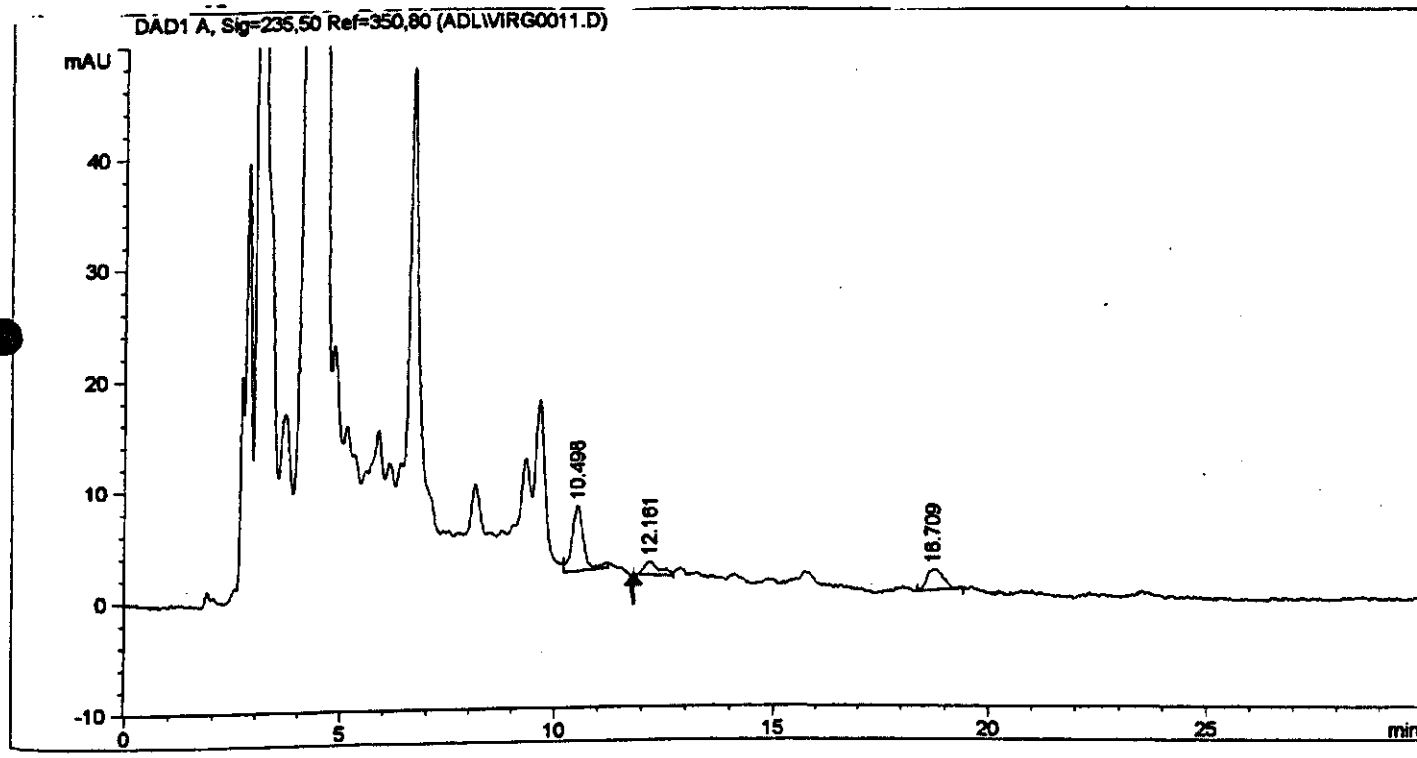
Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

Data File C:\HPCHEM\1\DATA\ \VIRG0011.D Sample Name: 156842

(15)

Injection Date	: 12/7/00 5:07:10 PM	Seq. Line	: 11
Sample Name	: 156842	Vial	: 8
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/7/00 5:05:44 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\MVALVIRG.M		
Last changed	: 12/11/00 9:29:14 AM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Monday, December 11, 2000 9:29:14 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.954		-	-	-		Virginiamicin

Totals : 0.00000

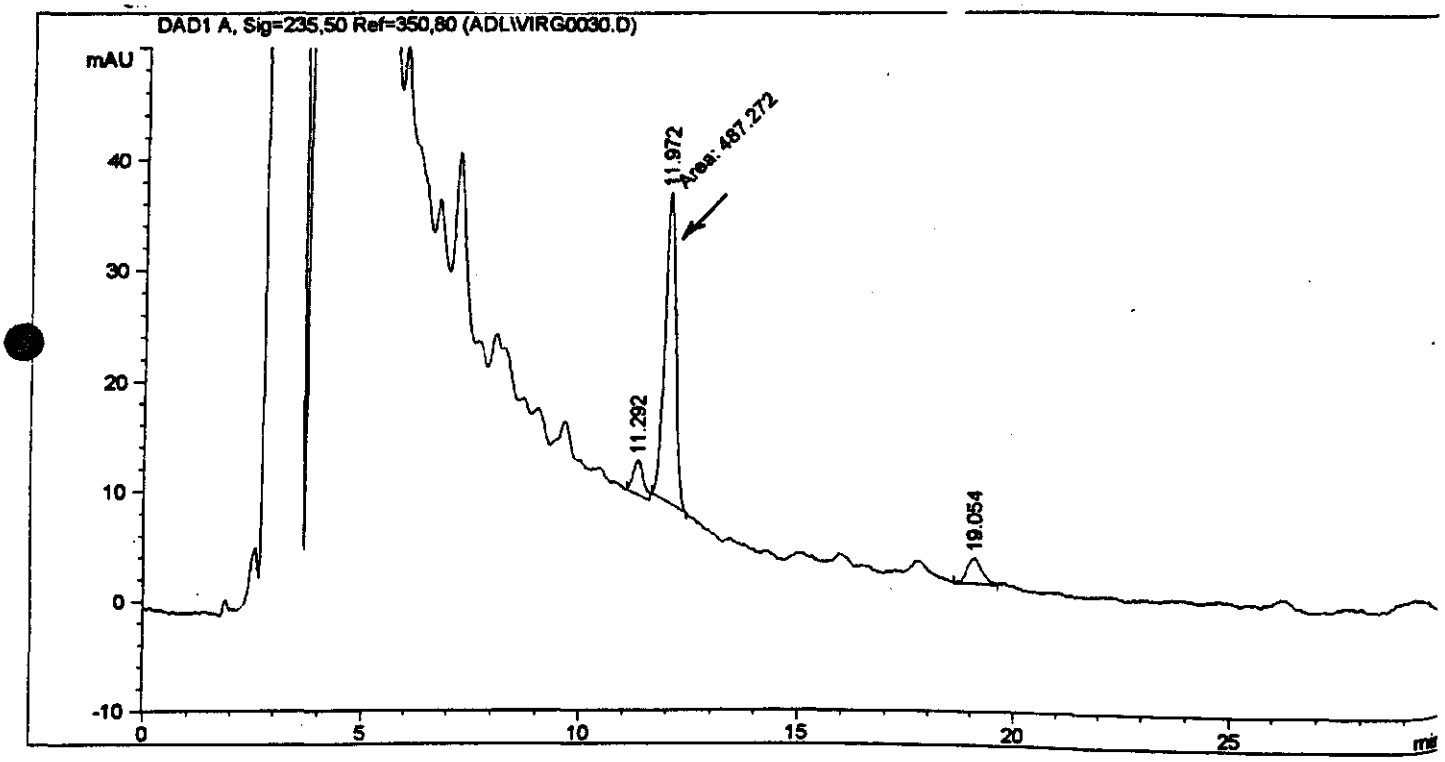
Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

(15)

Injection Date : 12/8/00 3:15:20 AM Seq. Line : 30
Sample Name : 156958 Vial : 21
Acq. Operator : Inj : 1
Inj Volume : 100 µl

Acq. Method : C:\HPCHEM\1\METHODS\MVALVIRG.M
Last changed : 12/8/00 3:13:52 AM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\MVALVIRG.M
Last changed : 12/11/00 10:22:48 AM by
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : Monday, December 11, 2000 8:59:41 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=235,50 Ref=350,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
11.972	MM	487.27155	1.72971e-2	8.42839		Virginiamicin

Totals : 8.42839 x 0.2 = 1.68 ug/kg m.a.

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 21

CANFAS

Development and Validation of HPLC-methods for the official cc
Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT

Subtitle: **Task 4 COLLABORATIVE STUDY**
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
216853	0,0 N.D.	0,0 N.D.
216863	0,0 N.D.	0,0 N.D.
216875	1,4	1,4
216879	0,4	0,5
216892	0,0 N.D.	0,0 N.D.
216894	0,4	0,6
216919	0,0 N.D.	0,0 N.D.
216925	2,4	2,2

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: 13/11/2000 and 23/11/2000

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: 111 mg
- Volume of methanol: 50 ml
- Concentration of the stock solution: 499,5 µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: LC 18 Supelcosil 25cm × 4,6 mm + SUPELGUARD LC
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1 ml/min
- Injection volume: 100 µl
- Retention time of virginiamycin M1: 10,0 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

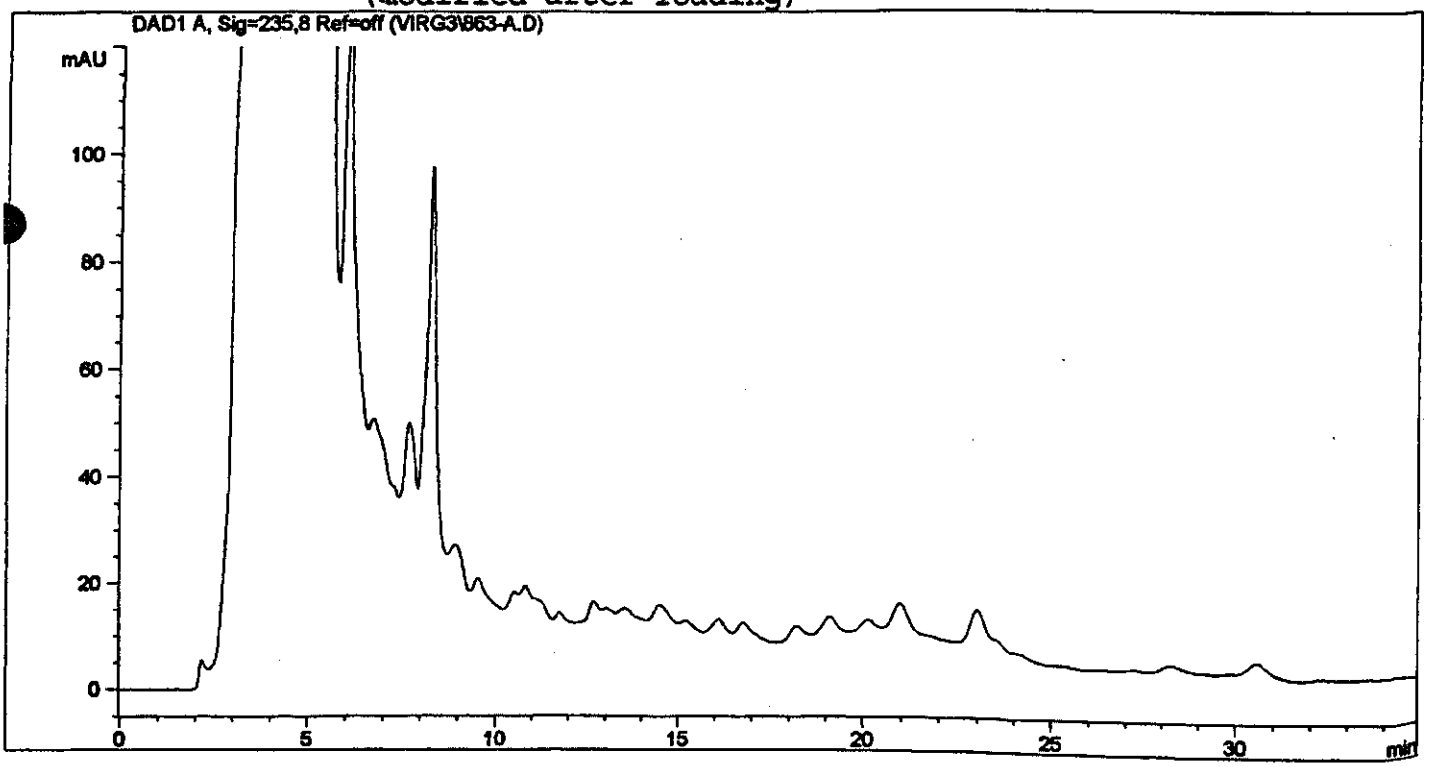
Recovery results:

- Percentage recovery: 67,5 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 68 % and 67 %
- Spiking level: 4,0 mg/kg

C:\HPCHEM\2\CROMAT\VIRG3\863-A.D Sample Name: 863-A

==========

tion Date	: 06/12/2000 3.07.39	Seq. Line	: 19
ple Name	: 863-A	Vial	: 14
cq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\2\ \VIRG .M		
Last changed	: 06/12/2000 3.05.50 (modified after loading)		
Analysis Method	: C:\HPCHEM\2\ \VIRG M		
Last changed	: 06/12/2000 10.44.05 (modified after loading)		



==========

External Standard Report

==========

Sorted By	:	Signal
Calib. Data Modified	:	06/12/2000 10.11.29
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,8 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/inj]	Grp	Name
10.096		-	-	-		VIRG-virginiamicin

Totals : 0.00000

Results obtained with enhanced integrator!

1 Warnings or Errors :

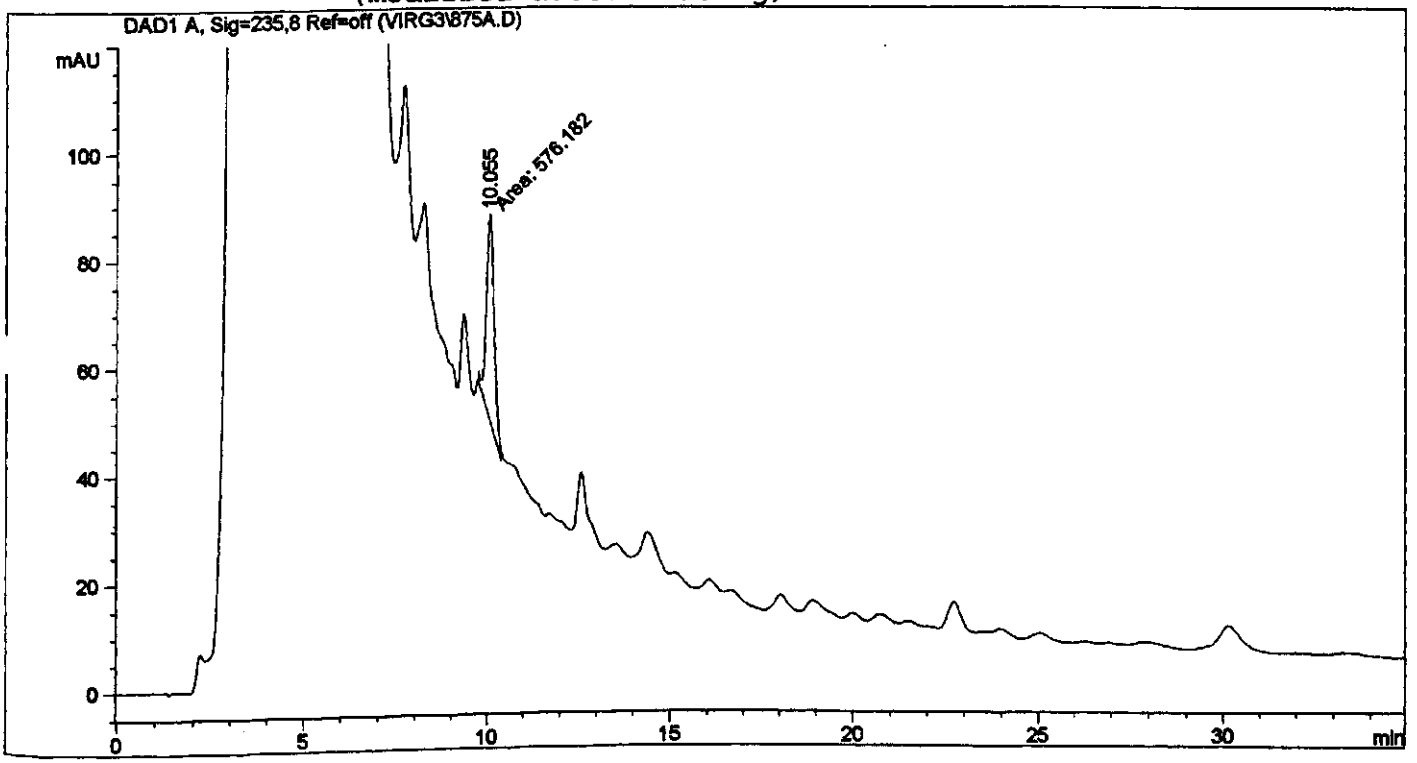
Warning : Calibrated compound(s) not found

==========

(21)

Injection Date : 05/12/2000 17.14.12 Seq. Line : 3
Sample Name : 875A Vial : 2
Acq. Operator : Inj : 1
Inj Volume : 100 µl

Acq. Method : C:\HPCHEM\2\ \VIRG .M
Last changed : 05/12/2000 17.12.22
(modified after loading)
Analysis Method : C:\HPCHEM\2\ \VIRG M
Last changed : 06/12/2000 10.44.05
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 06/12/2000 10.11.29
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=235,8 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/inj]	Grp	Name
10.055	MM	576.18176	1.19295	687.35580		VIRG-virginiamicin
Totals :				687.35580		

Results obtained with enhanced integrator!

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 22

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: **VIRGINIAMYCIN**

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
226840	<0.5	<0.5
226858	1,24	1,31
226882	<0.5	<0.5
226887	1,42	1,71
226890	<0.5	<0.5
226913	0,55	0,53
226926	<0.5	<0.5
226951	0,58	0,54

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: 00115 / 00122

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: 10,6 mg
- Volume of methanol: 25 ml
- Concentration of the stock solution: 552,37 µg microbiological activity/ml Virginiamycin M1

(Ref. std contains 57,9% virginiamycin M1)
(microbiological potency of 225%)

Chromatographic conditions:

- Column:
  - ☐ As described in the method Hypersil ODS BDS C18, 250 mm x 4,6, 5µ
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,8 ml/min
- Injection volume: 80 µl
- Retention time of virginiamycin M1: 11 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: 80 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 82 % and 79 %
- Spiking level: 1,1 mg/kg

NEW TIMED EVENTS FROM VIRGINIA

******* **EXTERNAL STANDARD TABLE** *******

********** 11-29-2000 13:32:57 Version 5.1 **********

* Sample Name: prov 1 Sample 226840 Data File: D:virg049

* Date: 11-14-2000 21:36:45 Method: VIRGINIA 11-29-2000 13:16:52 # 672 *

* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *

* Starting Peak Width: 50 Threshold: .05 Area Threshold: 50 *

****

Starting Delay: 0.00 Ending retention time: 45.00

Area reject: 50 One sample per 0.200 sec.

Amount injected: 80.00 Dilution factor: 1.00

Sample Weight: 1.00000

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	AREA/ HEIGHT	HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	11.511		0.0691	100.0000%	55288	1056	52.4 1			1.2500E-06

TOTAL AMOUNT = 0.0691

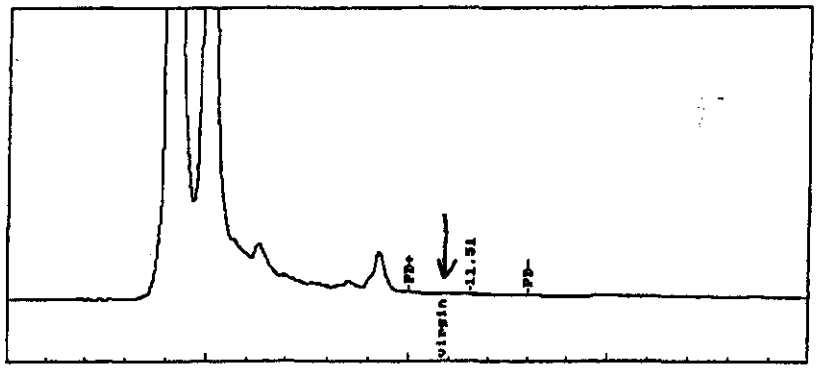
PEAKS NOT FOUND IN THIS RUN

NAME	ADJUSTED RET.TIME.	REFERENCE PEAK
virgin	10.83	virgin

Data File = D:virg049.PTS Printed on 11-29-2000 at 13:33:00

Start time: 0.00 min. Stop time: 20.00 min. Offset: 0 mv.

Low Value: 0 uv High Value: %2339739 uv Scale factor: 8.0



******* EXTERNAL STANDARD TABLE *******

********** 11-15-2000 10:32:47 Version 5.1 **********

* Sample Name: prov 2 (Sample 226858) Data File: D:VIRG050

* Date: 11-14-2000 22:23:46 Method: D:VIRGINIA 11-15-2000 10:32:35 # 610*

* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *

* Starting Peak Width: 10 Threshold: 1 Area Threshold: 100 *

****

Starting Delay: 0.00 Ending retention time: 30.00

Area reject: 100 One sample per 0.200 sec.

Amount injected: 80.00 Dilution factor: 1.00

Sample Weight: 1.00000

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	HEIGHT	AREA/ HEIGHT	BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	8.261		0.1085	1.6653%	86799	5837	14.9	2			1.2500E-0
2	8.948		0.0852	1.3084%	68195	2157	31.6	2			1.2500E-0
3	10.054		0.0263	0.4036%	21035	1183	17.8	1			1.2500E-0
4	10.837	virgin	6.2784	96.3658%	609134	34936	17.4	1	4	0	1.0307E-0
5	12.154		0.0167	0.2569%	13388	775	17.3	1			1.2500E-0

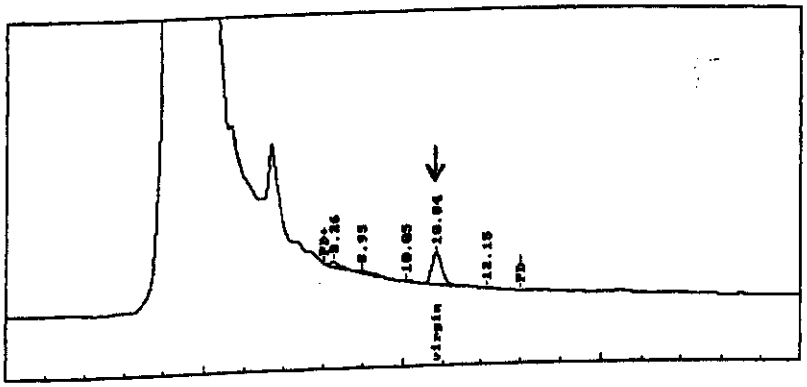
TOTAL AMOUNT = 6.5151

Areas, times, and heights stored in: D:VIRG050.ATB

Data File = D:VIRG050.PTS Printed on 11-15-2000 at 10:32:49

Start time: 0.00 min. Stop time: 20.00 min. Offset: 0 mv.

Low Value: 0 uv High Value: %2574000 uv Scale factor: 8.0



******* EXTERNAL STANDARD TABLE *******

********** 11-15-2000 10:33:33 Version 5.1 **********

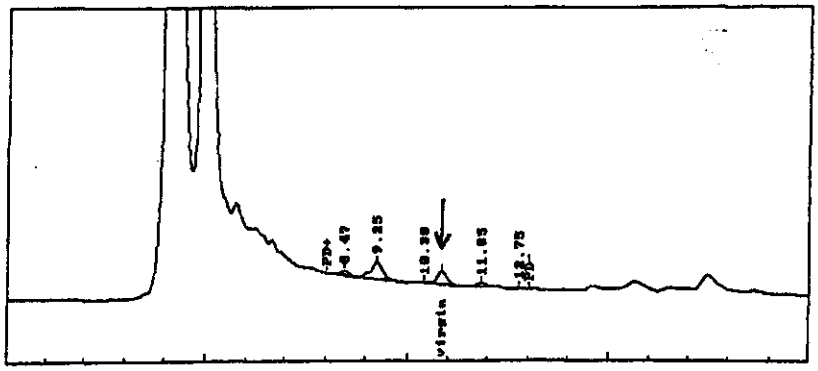
* Sample Name: prov 6 Sample 226913 Data File: D:VIRG054 *
* Date: 11-15-2000 01:31:51 Method: D:VIRGINIA 11-15-2000 10:32:35 # 610*
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 10 Threshold: 1 Area Threshold: 100 *

Starting Delay: 0.00 Ending retention time: 30.00
Area reject: 100 One sample per 0.200 sec.
Amount injected: 80.00 Dilution factor: 1.00
Sample Weight: 1.00000

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	AREA/ HEIGHT	HEIGHT	BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	8.471		0.0970	2.7220%	77605	5439	14.3	1			1.2500E-06
2	9.251		0.4962	13.9247%	396993	19763	20.1	1			1.2500E-06
3	10.378		0.0216	0.6055%	17264	969	17.8	2			1.2500E-06
4	10.844	virgin	2.8168	79.0398%	273287	14870	18.4	2	4	0	1.0307E-05
5	11.851		0.0939	2.6335%	75081	4437	16.9	1			1.2500E-06
6	12.748		0.0383	1.0745%	30634	2065	14.8	1			1.2500E-06

TOTAL AMOUNT = 3.5637

Areas, times, and heights stored in: D:VIRG054.ATB
Data File = D:VIRG054.PTS Printed on 11-15-2000 at 10:33:35
Start time: 0.00 min. Stop time: 20.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: %2591782 uv Scale factor: 8.0



APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 25

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: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
256850	0,21**	0,21**
256861	nd*	nd*
256898	nd*	nd*
256908	nd	nd
256914	nd	nd
256931	0,22**	0,22**
256941	0,51	0,58
256955	0,73	0,70

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: .....6-12-2000.....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ....5..... mg
- Volume of methanol: ..22,5..... ml
- Concentration of the stock solution: ..500.... µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: .....Mix of acetonitrile, water and formic acid, 450 + 550 + 3 (v+v+
- Flow-rate: ....1..... ml/min
- Injection volume: ...100...µl
- Retention time of virginiamycin M1: .6,63 min

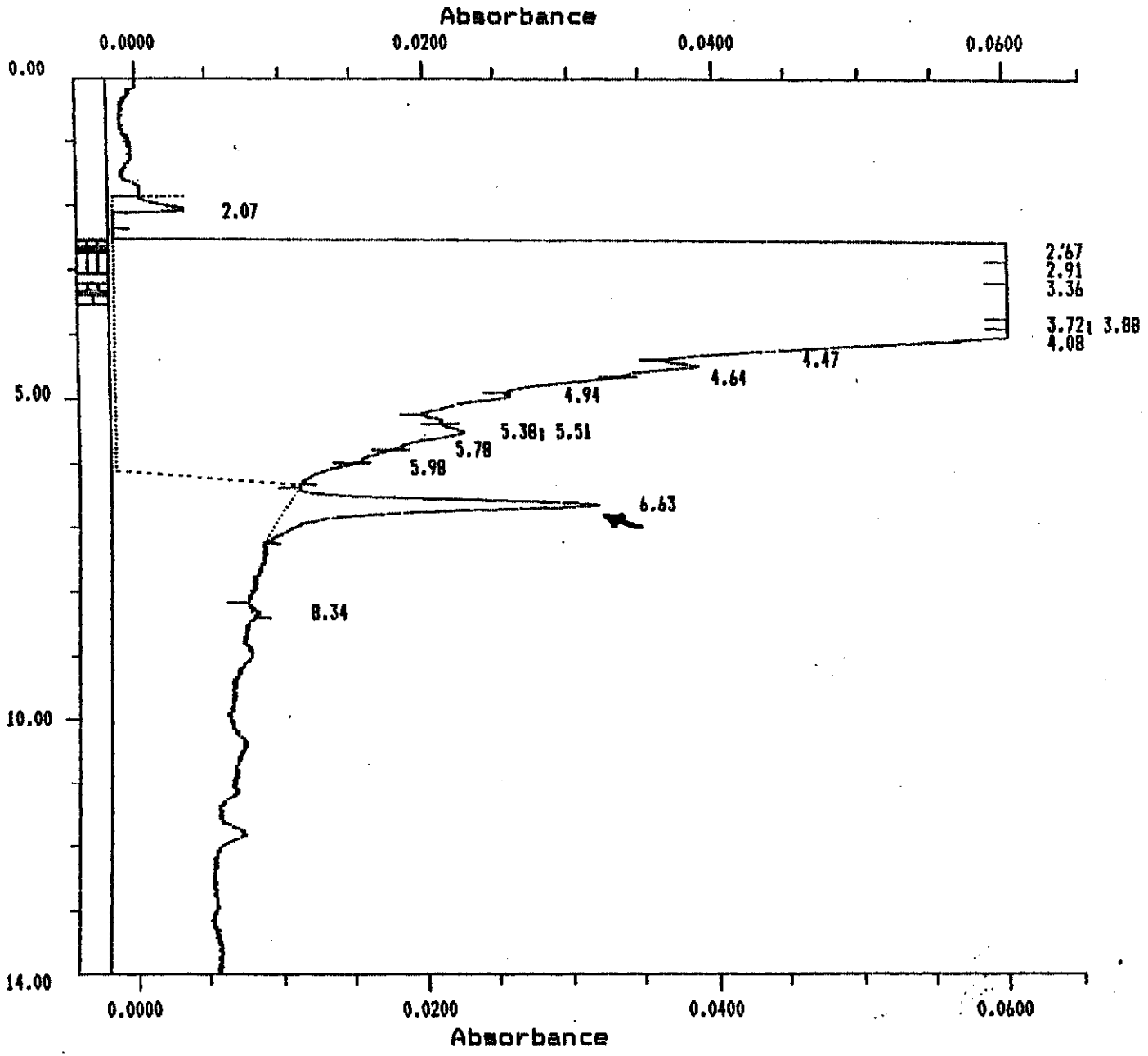
Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

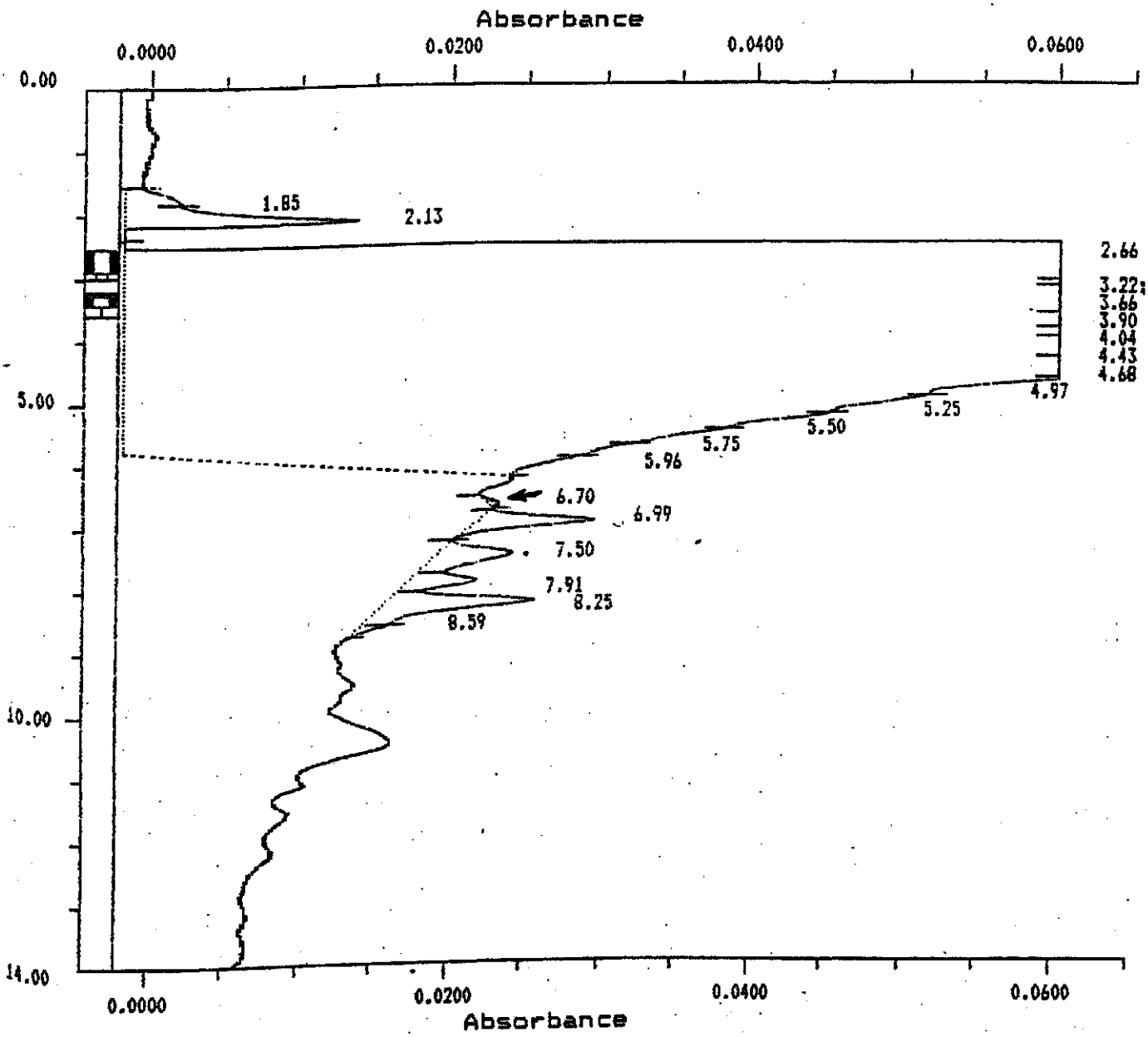
Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

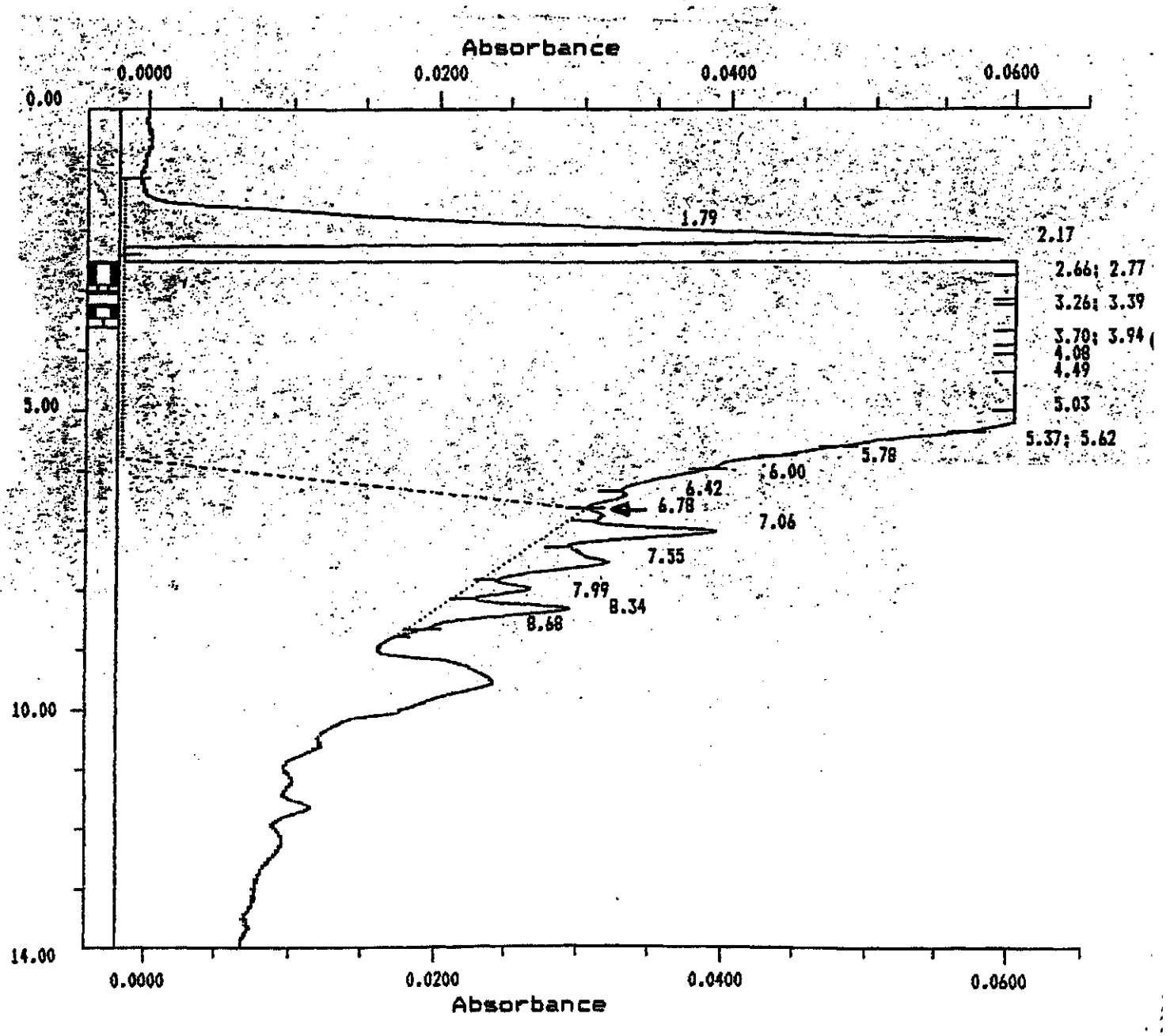
- Percentage recovery: 43.... %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ...10..... mg/kg


Absorbance
0.0000
0.0200
0.0400
0.0600
0.00
5.00
10.00
14.00
2.07
2.67
2.91
3.36
3.72
3.88
4.08
4.47
4.64
4.94
5.38
5.51
5.78
5.98
6.63
8.34
Absorbance

sample 256261



sample 256898



APPENDIX 6

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:

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
266833	0,3	0,2
266848	3	3,9
266855	0,3	0,3
266869	2,7	3
266893	1,3	1,1
266912	0,3	0,2
266939	0,4	0,3
266950	1,3	1,1

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: ....7/12/00....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ~~.....~~ 10.5 mg
- Volume of methanol: ..45.0.... ml
- Concentration of the stock solution: ..50.... µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: ....SPHERISORB ODS 2....
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: ....
- Flow-rate: ....1.0.... ml/min
- Injection volume: ..50....µl
- Retention time of virginiamycin M1: ..8.9.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

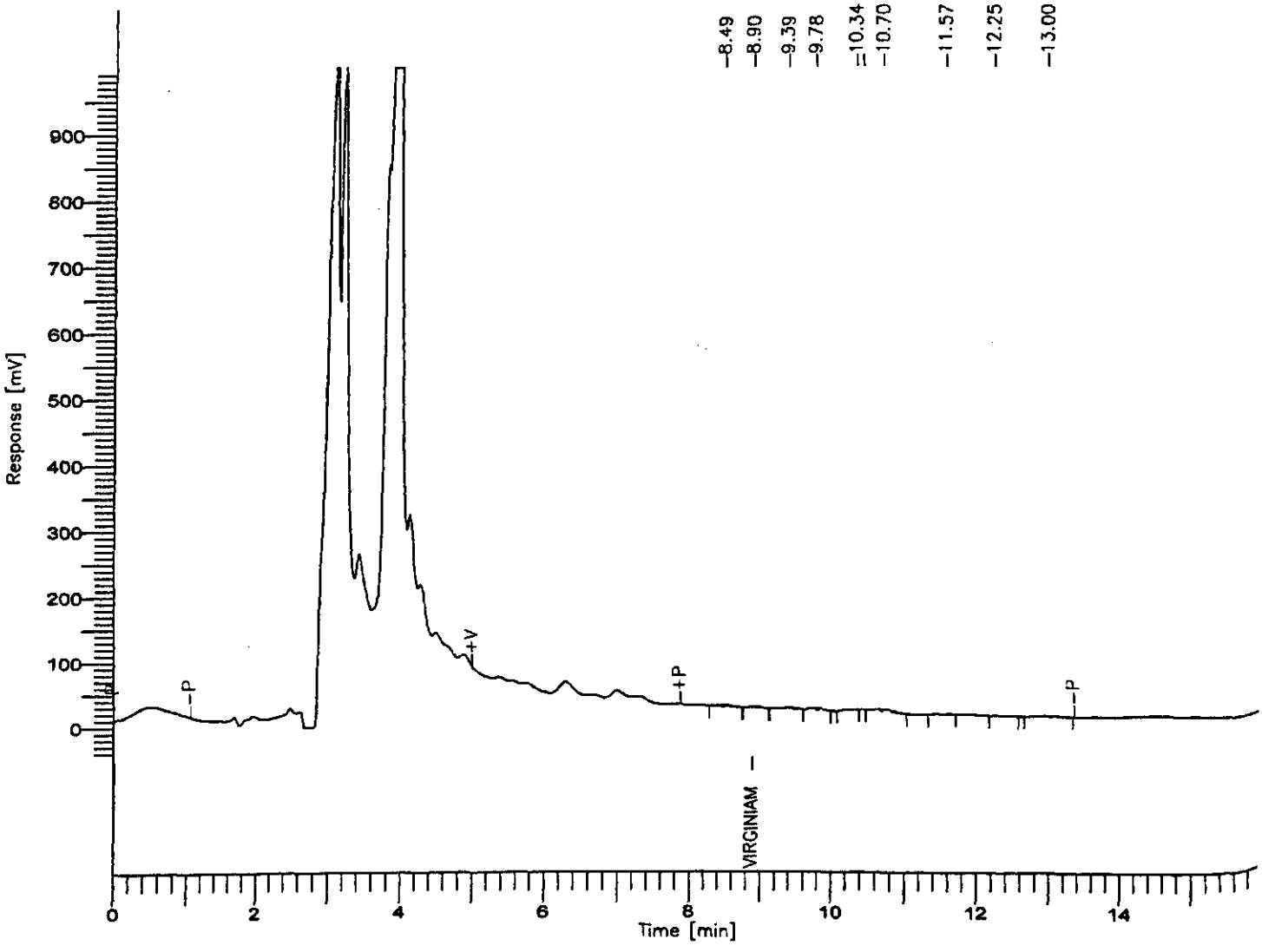
Recovery results:

- Percentage recovery: ..86.. %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: ..87.. % and ..85.. %
- Spiking level: ....4..... mg/kg

266833

(26)

Software Version: 4.1<2F12>
Date: 11/12/00 19:56
Sample Name : A3009155A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA017.RAW Date: 11/12/00 19:40
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 17 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00

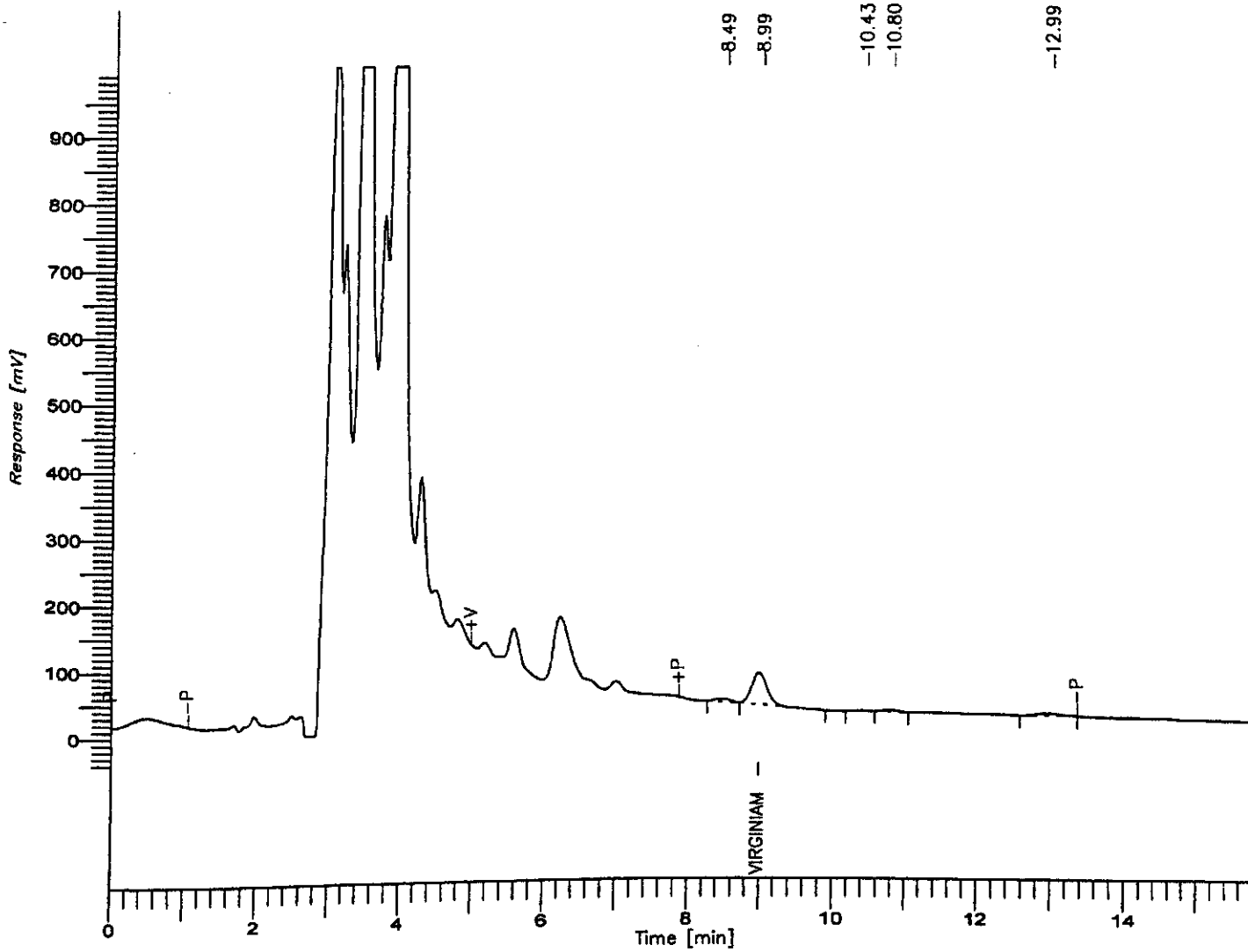


Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
2	8.898	Virginiamycin	11596.50	1241.90	*BB	9.34
			11596.50	1241.90		

266848

Software Version: 4.1<2F12>
Date: 11/12/00 21:08
Sample Name : A3009156A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA021.RAW Date: 11/12/00 20:52
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 21 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00

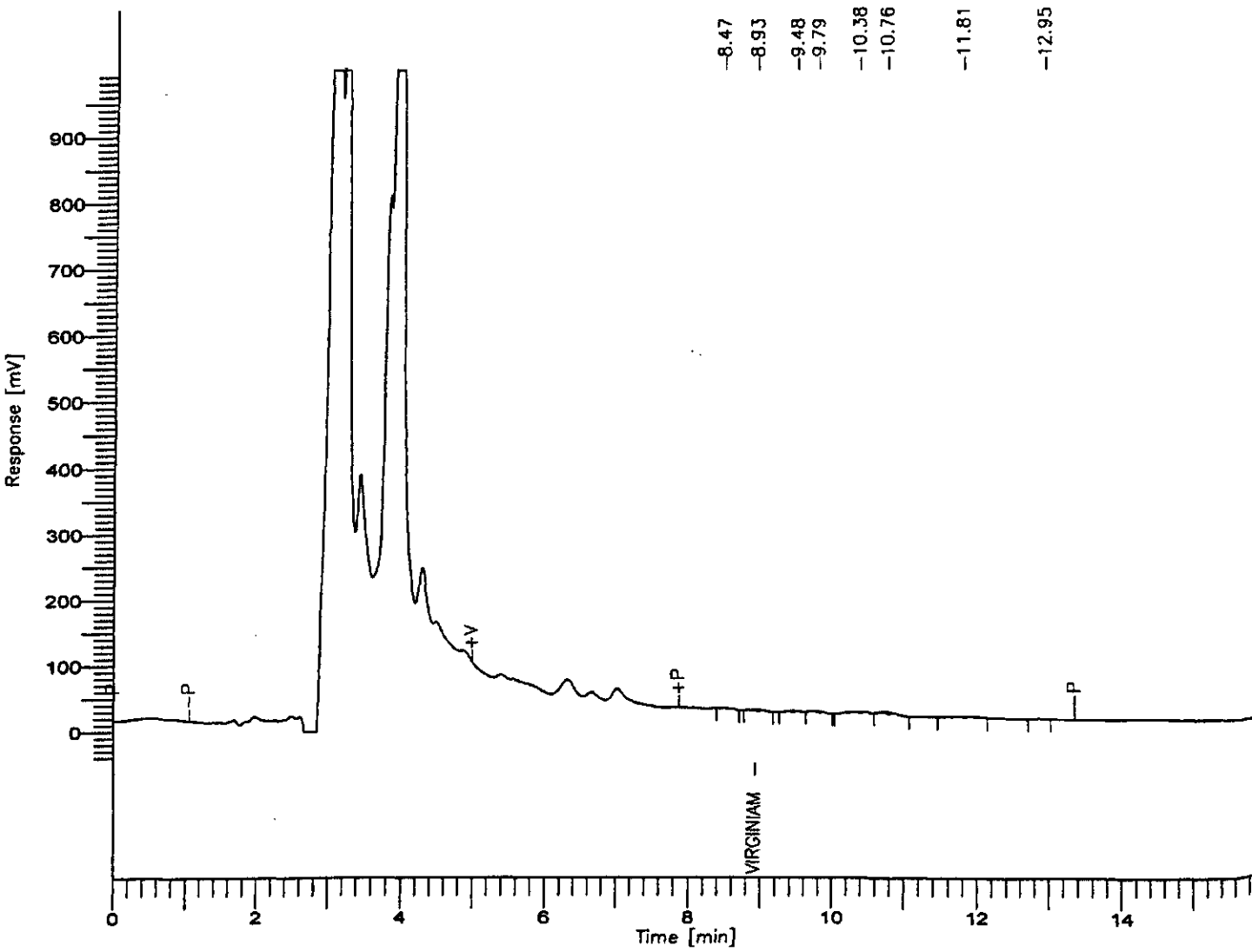


Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
2	8.994	Virginiamycin	701200.50	47649.47	*BB	14.72
			701200.50	47649.47		

266855

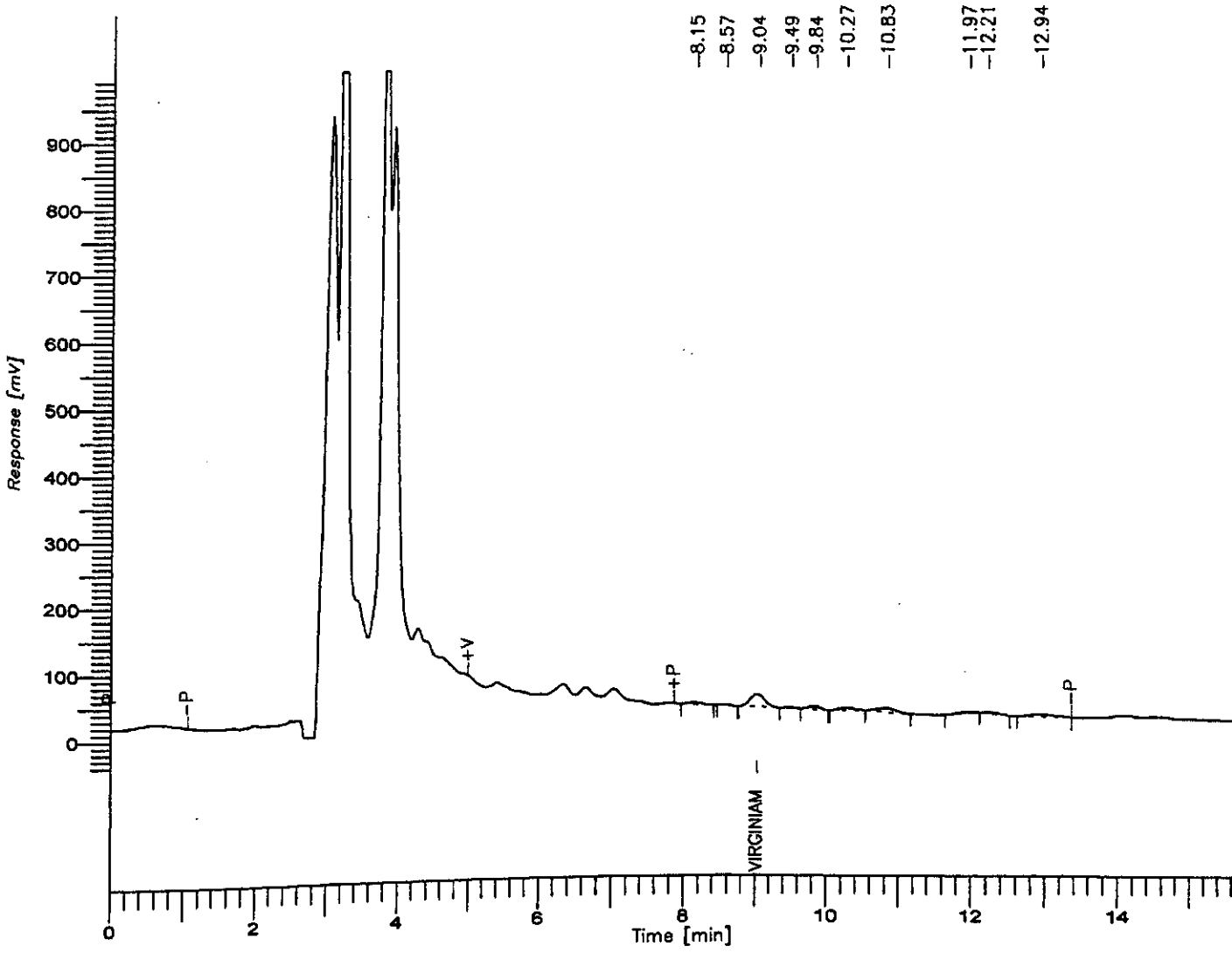
Software Version: 4.1<2F12>
Date: 11/12/00 22:20
Sample Name : A3009157A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA025.RAW Date: 11/12/00 22:03
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 25 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
2	8.933	Virginiamycin	22924.00	2077.60	*BB	11.03
			22924.00	2077.60		

Software Version: 4.1<2F12>
Date: 12/12/00 02:31
Sample Name : A3009159A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA039.RAW Date: 12/12/00 02:15
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 39 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



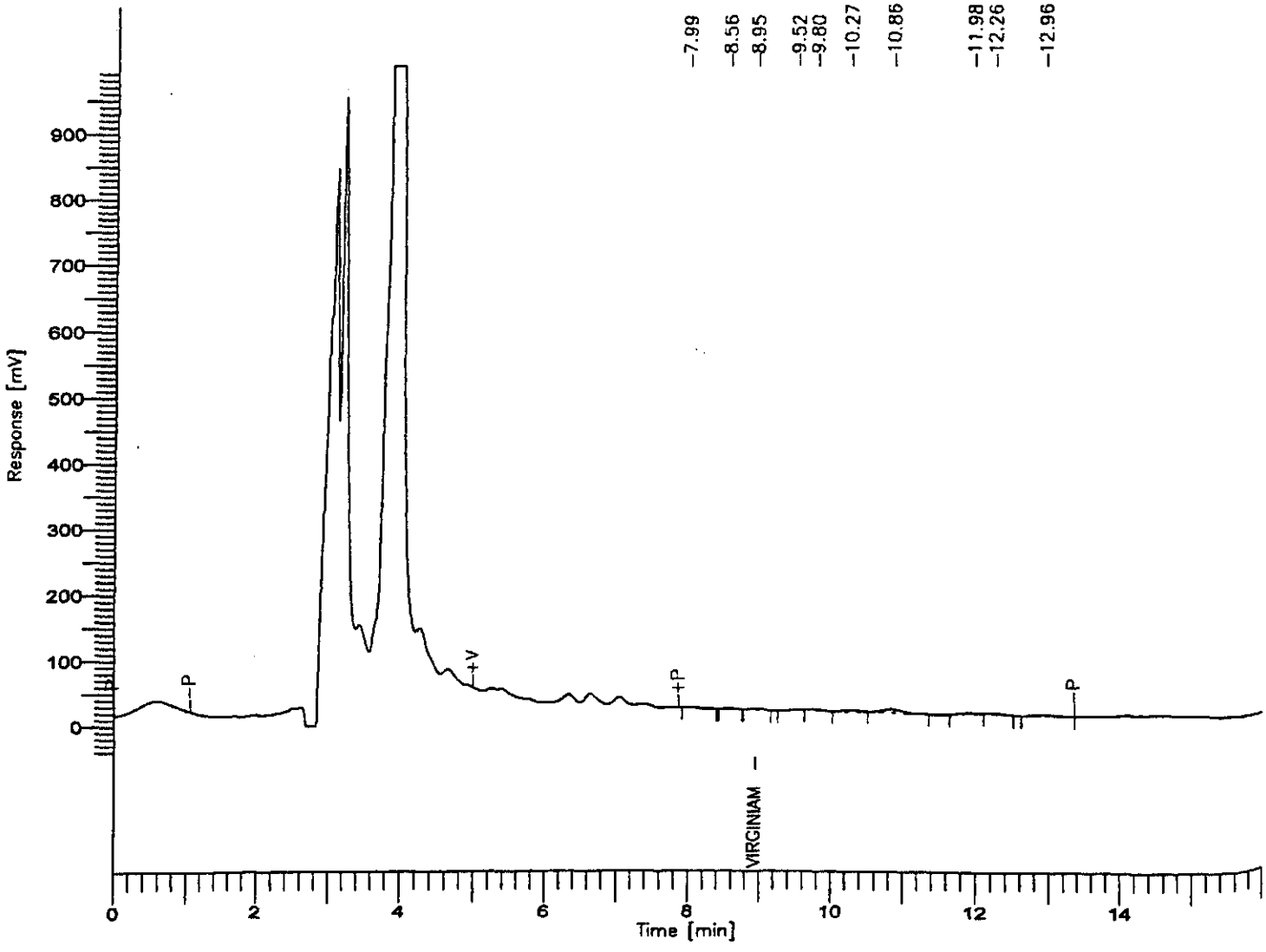
Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
3	9.036	Virginiamycin	277133.50	19228.78	*BB	14.41
			277133.50	19228.78		

266912

Software Version: 4.1<2F12>
Date: 12/12/00 03:43
Sample Name : A3009160A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA043.RAW Date: 12/12/00 03:27
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 43 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00

(26)

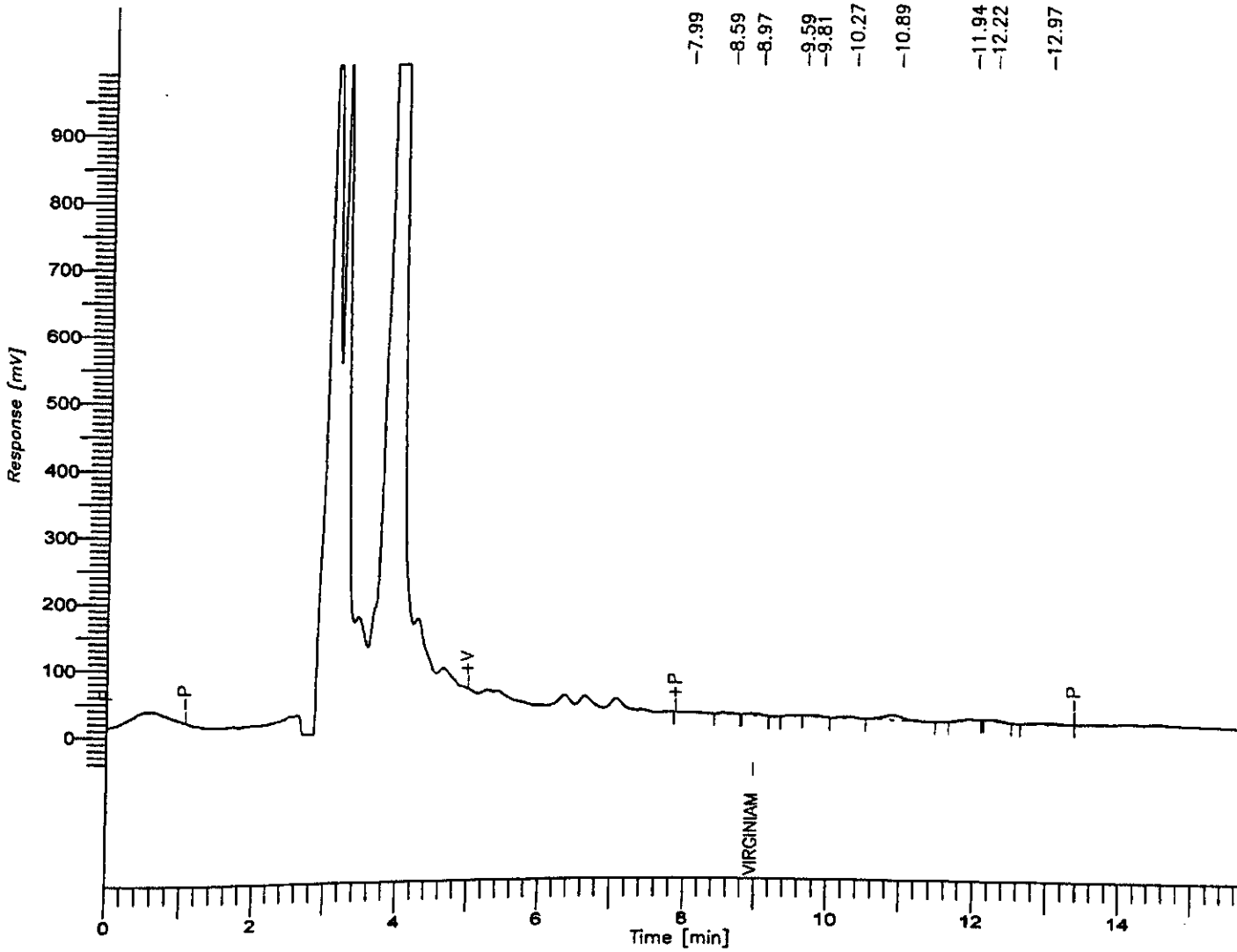


Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
3	8.954	Virginiamycin	27802.50	2313.17	*BB	12.02
			27802.50	2313.17		

266939

Software Version: 4.1<2F12>
Date: 12/12/00 04:55
Sample Name : A3009161A
Data File : C:\TC4\CANFAS\VIRGINIA\REINJ\DATA047.RAW Date: 12/12/00 04:3
Sequence File: C:\TC4\CANFAS\VIRGINIA\VIRGINI2.SEQ Cycle: 47 Channel : B
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



Virginiamycin in Feeds Report

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	BL	Area/Height [s]
3	8.969	Virginiamycin	34049.50	2927.05	*BB	11.63
			34049.50	2927.05		

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 28

HPLC-UV

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: ...20-24/11/00.....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ...10..... mg
- Volume of methanol: .....45..... ml
- Concentration of the stock solution: ...500.. µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1..... ml/min
- Injection volume: ..100...µl
- Retention time of virginiamycin M1: 11.6 min → 1st series; 10.4 min → 2nd series

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 78,3% and 51,1%
- Spiking level: .....4.... mg/kg

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: 20-24/11/00

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
286832	1,81	0,91
286834	< LOD	< LOD
286886	0,7	0,43
286888	< LOD	< LOD
286909	0,68	0,64
286911	< LOD	< LOD
286935	1,51	1,73
286954	around LOD*	< LOD

* the limit of detection (LOD) is estimated at 0,12 mg/kg

2nd series

rginiamycin

le : TEST__27.R01 std ~~22~~ 5 µg/ml
n : 25 Type : Sample

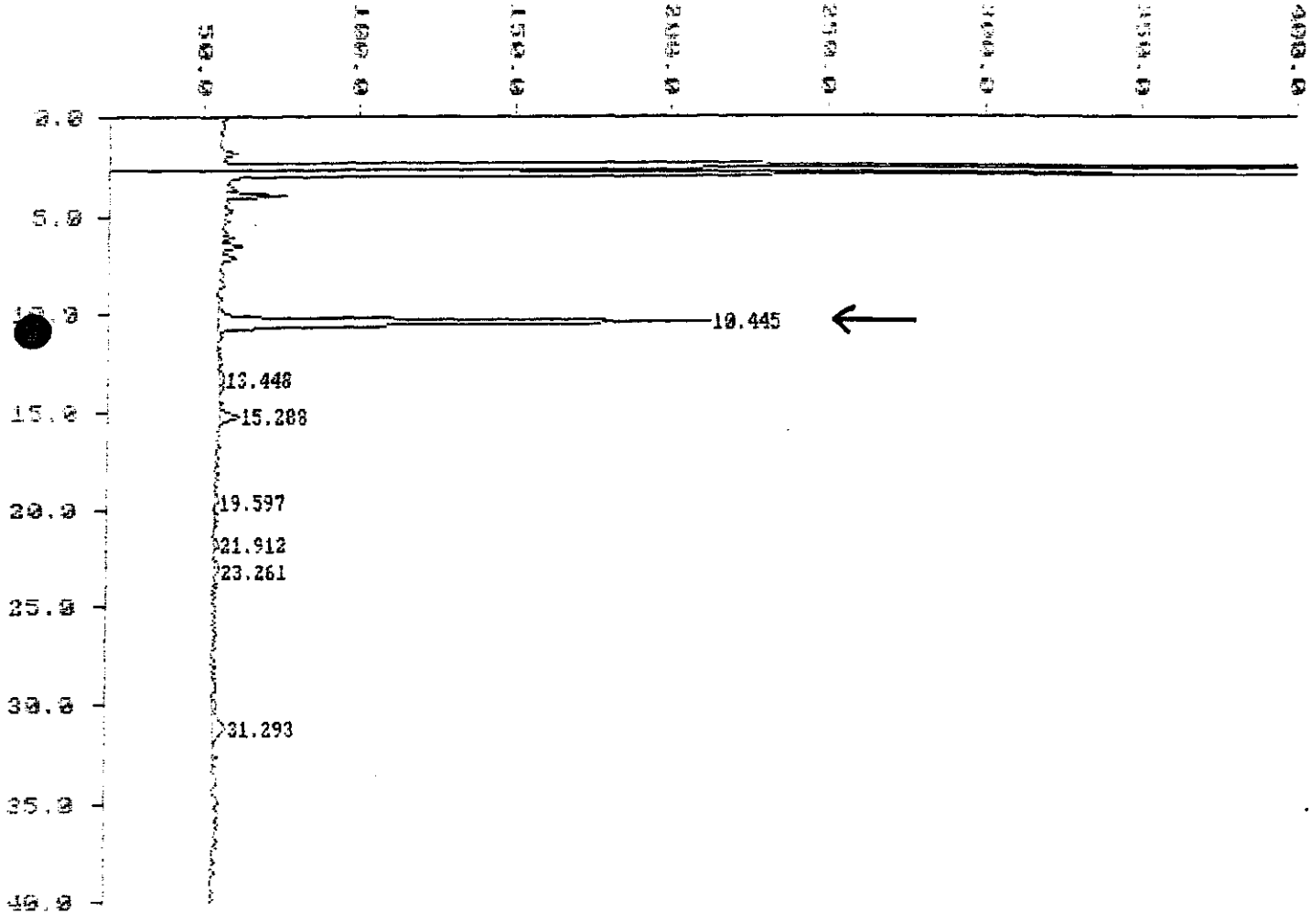
eport : 19:20:57 Nov 24 1985 Method : VIRGINIA [16:55:17 Nov 24 1985]

mple Amt : 1.000000e+000 Dilution: 1.000000e+000

EXTERNAL STANDARD , AREA)

RT	Area	Height	Conc	Name
[redacted]	2846342	157.685	4.9776	virginiamycin

(TEST__27.301) mV



Virginiamycine

~~Std 0.3~~ blank

File : TEST__11.R01 HDR
Run : 01 Type : Sample

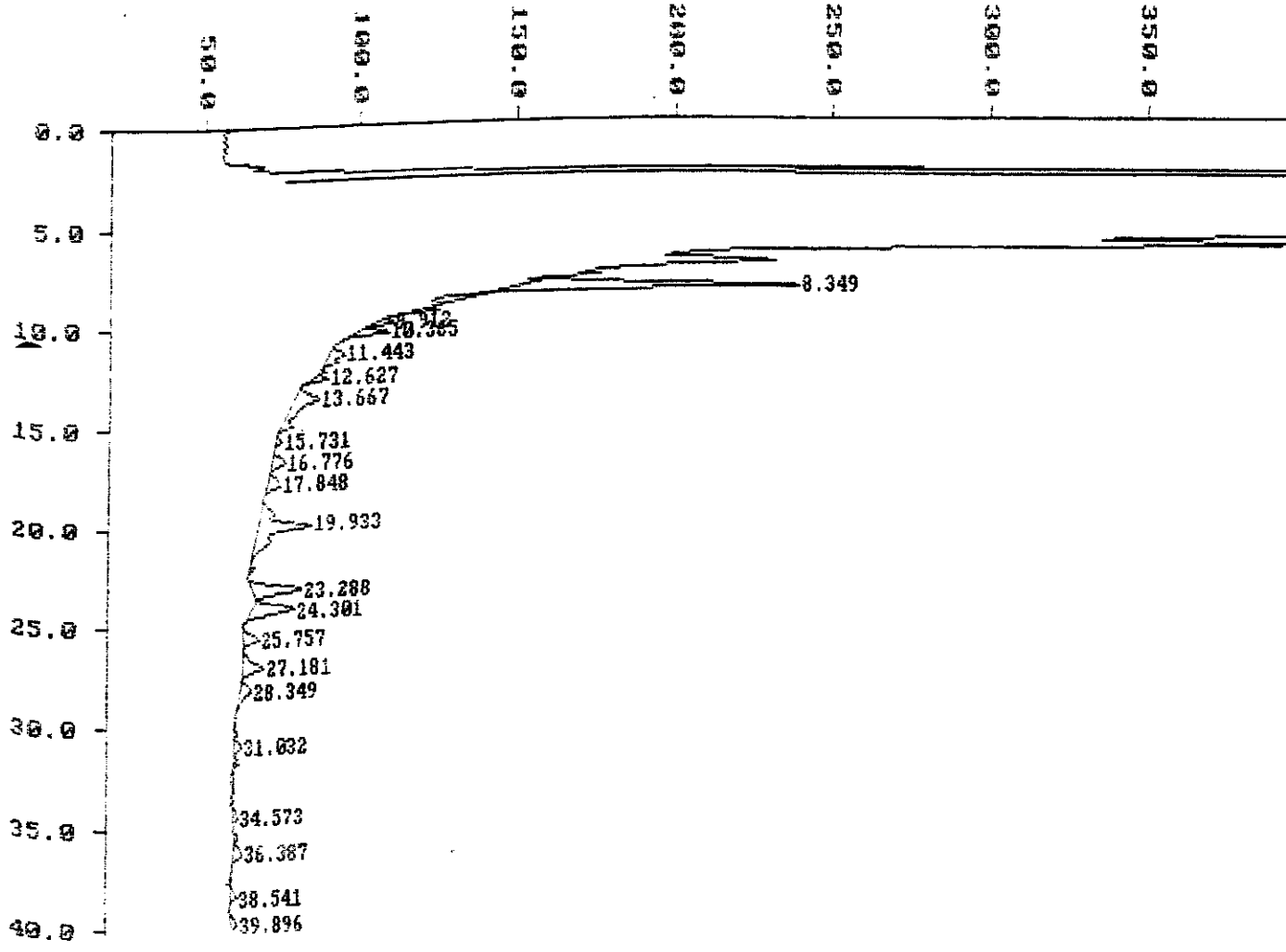
Report : 19:01:15 Nov 24 1985 Method : VIRGINIA [16:55:37 Nov 24 1985]

Sample Amt : 1.000000e+000 Dilution: 1.000000e+000

EXTERNAL STANDARD (AREA)

RT	Area	Height	Conc	Name
[illegible]55	94750	3.855	0.0276	virginiamycine

(TEST__11.R01) mV



rginiamycin

le : TEST__15.R01 d1= 286909-1
n : 13 Type : Sample

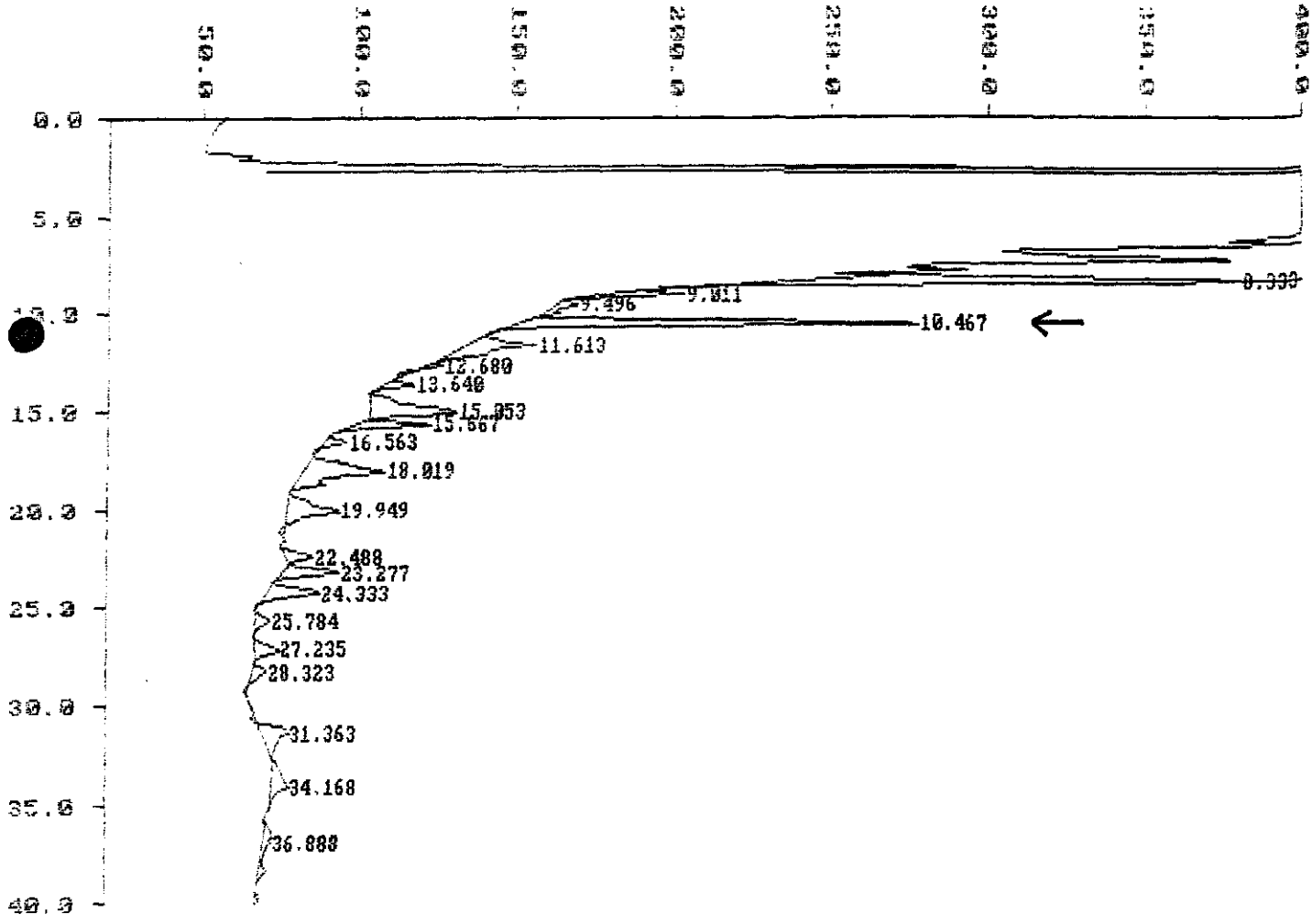
port : 19:06:03 Nov 24 1985 Method : VIRGINIA [16:55:37 Nov 24 1985]

mple Amt : 1.00000e+000 Dilution: 1.00000e+000

EXTERNAL STANDARD : AREA :

RT	Area	Height	Conc	Name
10.467	1963403	125.676	3.7863	virginiamycin

(TEST__15.R01) mV



Virginiamycin

f1=286954-1

le : TEST__19.R01
n : 17

Type : Sample

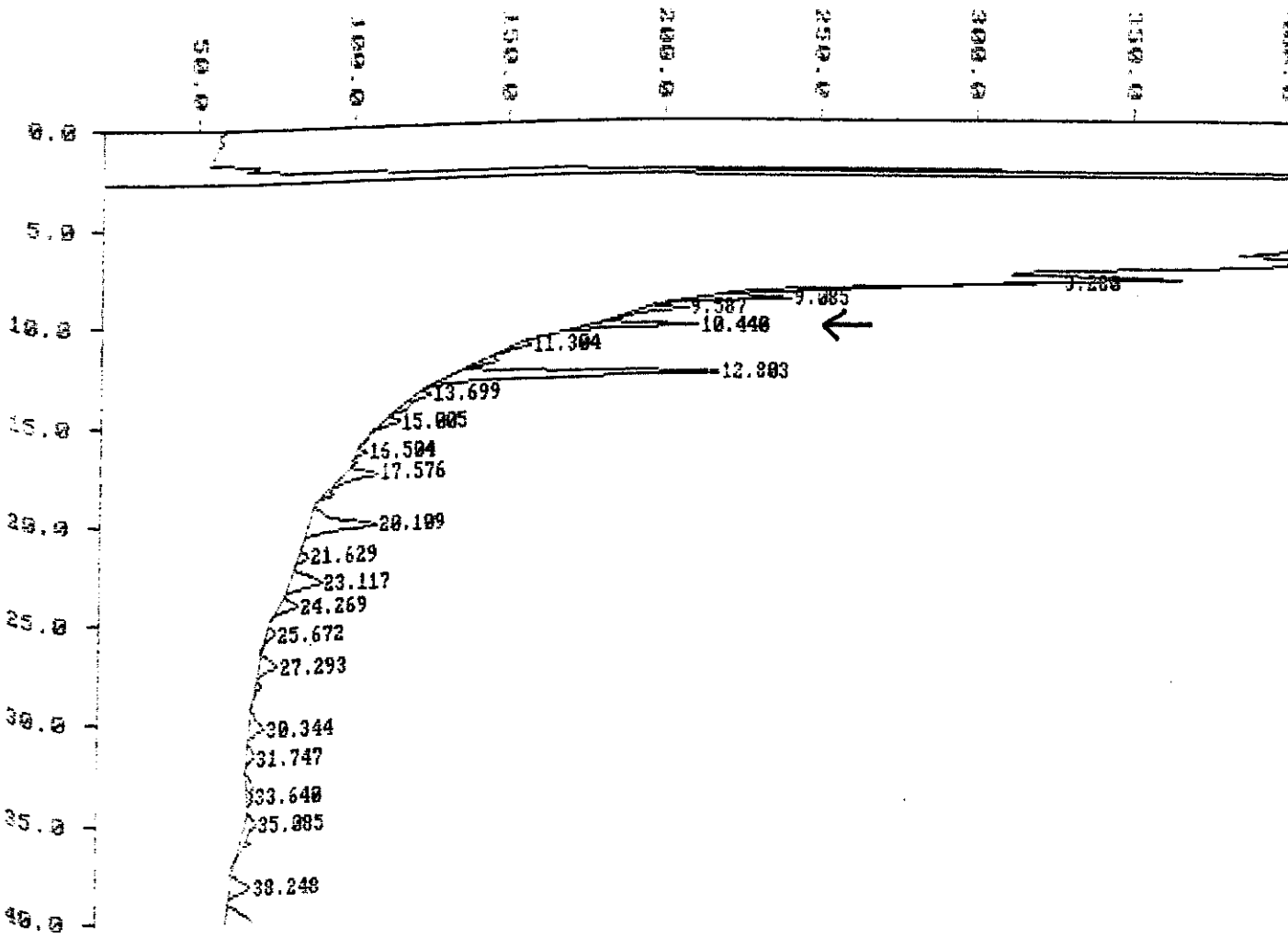
eport : 14:11:16 Nov 24 1988 Method : VIRGINIA [16:55:37 Nov 24 1988]

ample Amt : 1.000000e+000 Dilution: 1.000000e+000

EXTERNAL STANDARD (AREA)

RT	Area	Height	Conen	Name
9.440	404197	55.235	0.5639	virginiamycin

(TEST__19.R01) mU



APPENDIX 6

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:

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
296857	0,822	0,709
296867	0	0
296876	0	0
296878	0,224	0,171
296900	0,834	1,141
296910	0,906	1,035
296937	0	0
296949	0	0

Annex 4 - Questionnaire

Date(s) of analysis: ....24 November 2000.....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ..10,0.. mg
- Volume of methanol: ..25,65.. ml
- Concentration of the stock solution: ..500.. µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ....Waters Spherisorb ODS2 5µm, 4.6x250mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ...1,0..... ml/min
- Injection volume: .100..µl
- Retention time of virginiamycin M1: ...9,0. min/ 9,1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 54% and 37 %
- Spiking level: ....4.... mg/kg

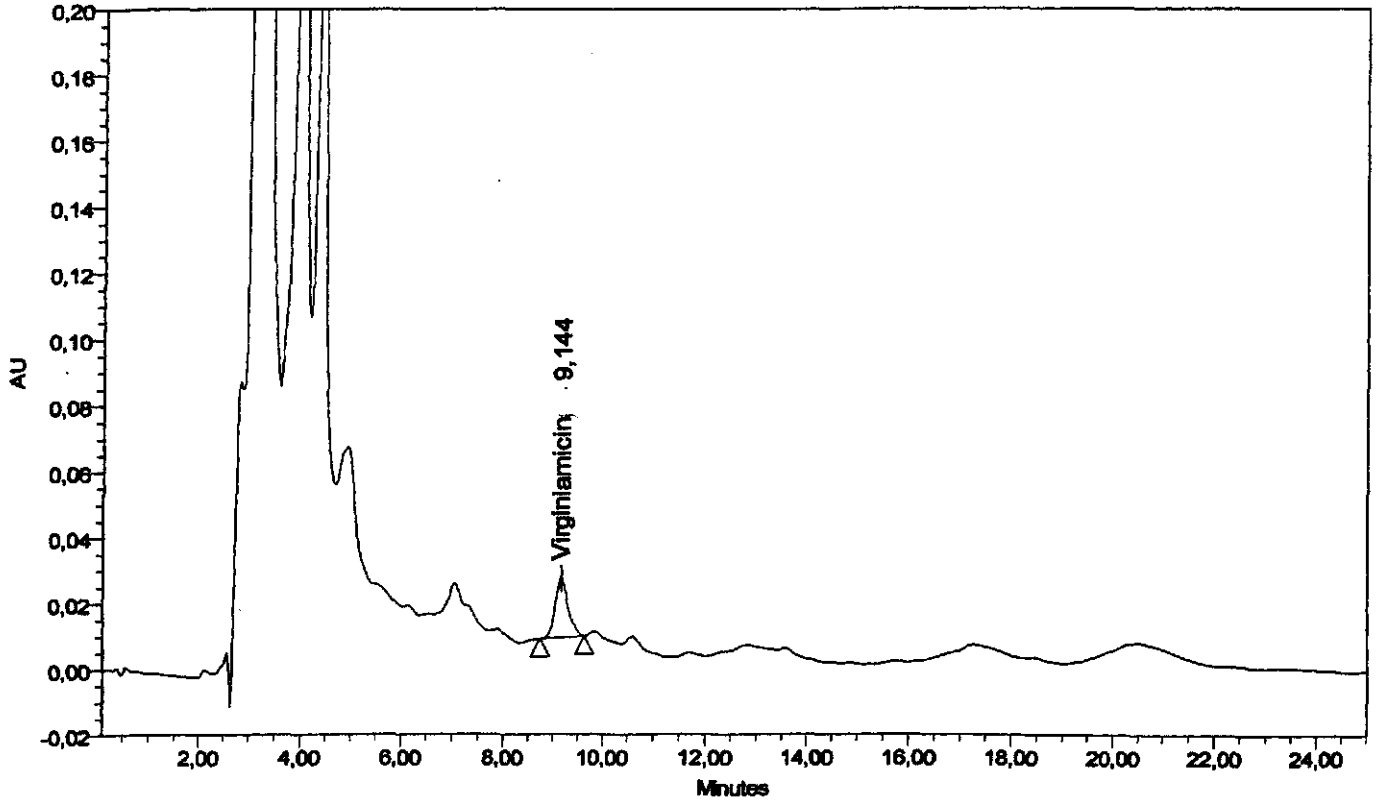
Project Virginiamic

Report

296857 ←

Vial:	10	"Sample Set":	Virginiamicin
Injection #	1	"Method Set":	Virginiamicin
Injection volume	100,00 ul		
Sample running time	25,0 Minutes	Canal:	PDA 235,0 nm

Chromatogram



	Compound	RT	Area	Height	Concentration	Units
1	Virginiamicin	9,144	329025	18153	2,042	ug/ml

Amount of Virginiamycin in sample (mg/kg): 0,82 mg/kg

Project: Virginiamicin

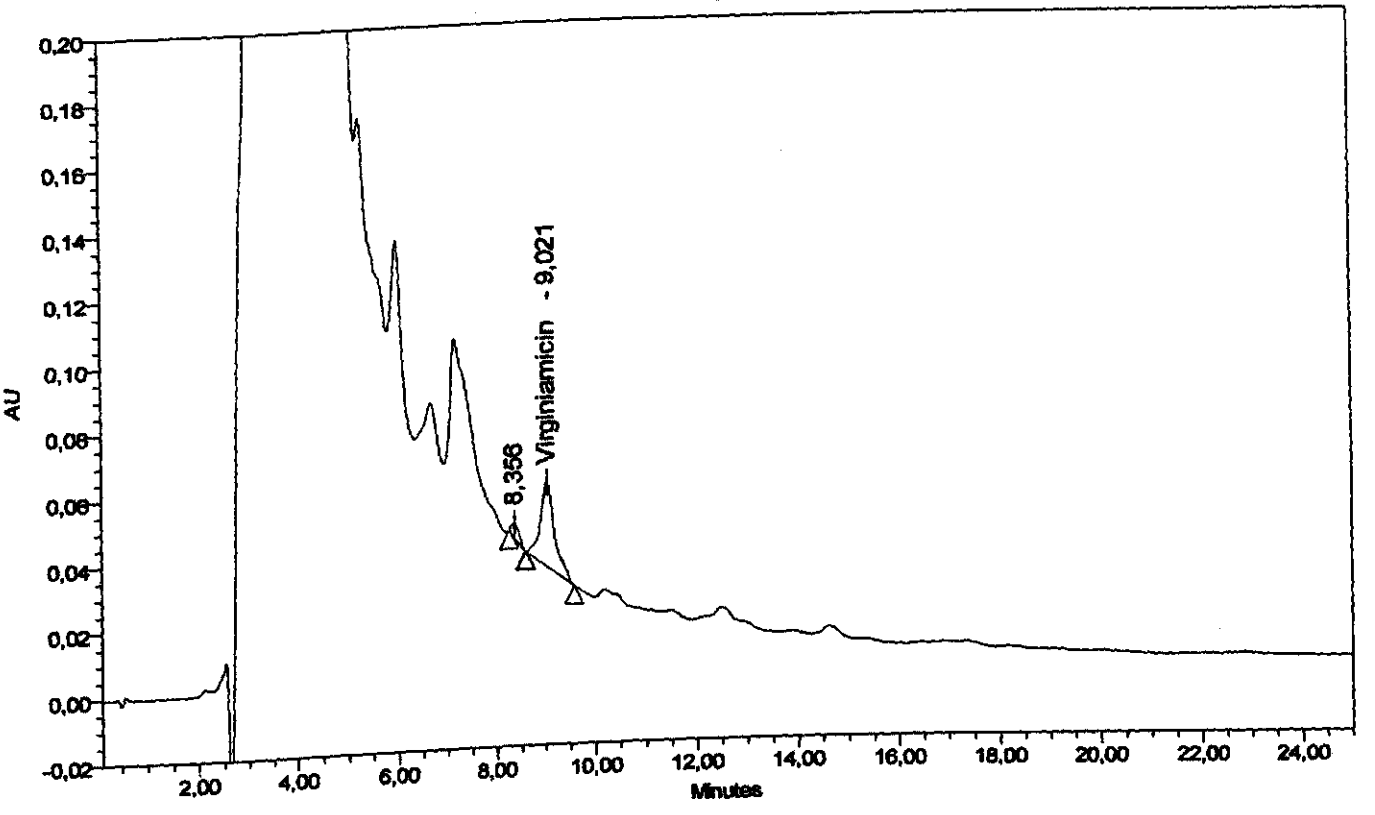
Report

06900 ←

al:	14
ection #	1
ection volume	100,00 ul
ample running time	25,0 Minutes

"Sample Set":	Virginiamicin
"Method Set":	Virginiamicin
Canal:	PDA 235,0 nm

Chromatogram



	Compound	RT	Area	Height	Concentration	Units
1		8,356	46463	4297		
2	Virginiamicin	9,021	510242	25726	2,851	ug/ml

Amount of Virginiamycin in sample (mg/kg): 1,14 mg/kg

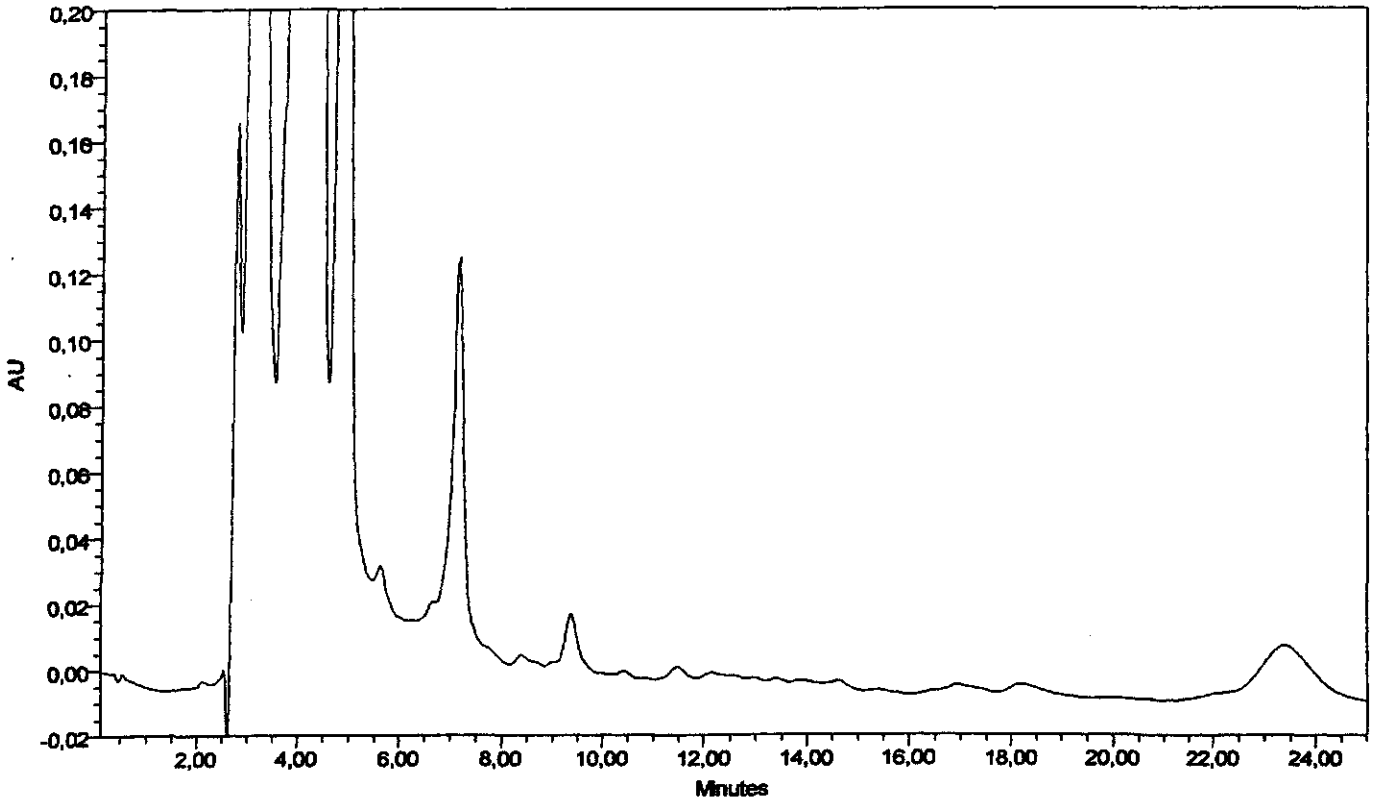
Project: Virginiamicin

Report

296949 ←

Vial:	17	"Sample Set":	Virginiamicin
Injection #	1	"Method Set":	Virginiamicin
Injection volume	100,00 ul		
Sample running time	25,0 Minutes	Canal:	PDA 235,0 nm

Chromatogram



	Compound	RT	Area	Height	Concentration	Units
1	Virginiamicin	9,013				

Amount of Virginiamycin in sample (mg/kg): —

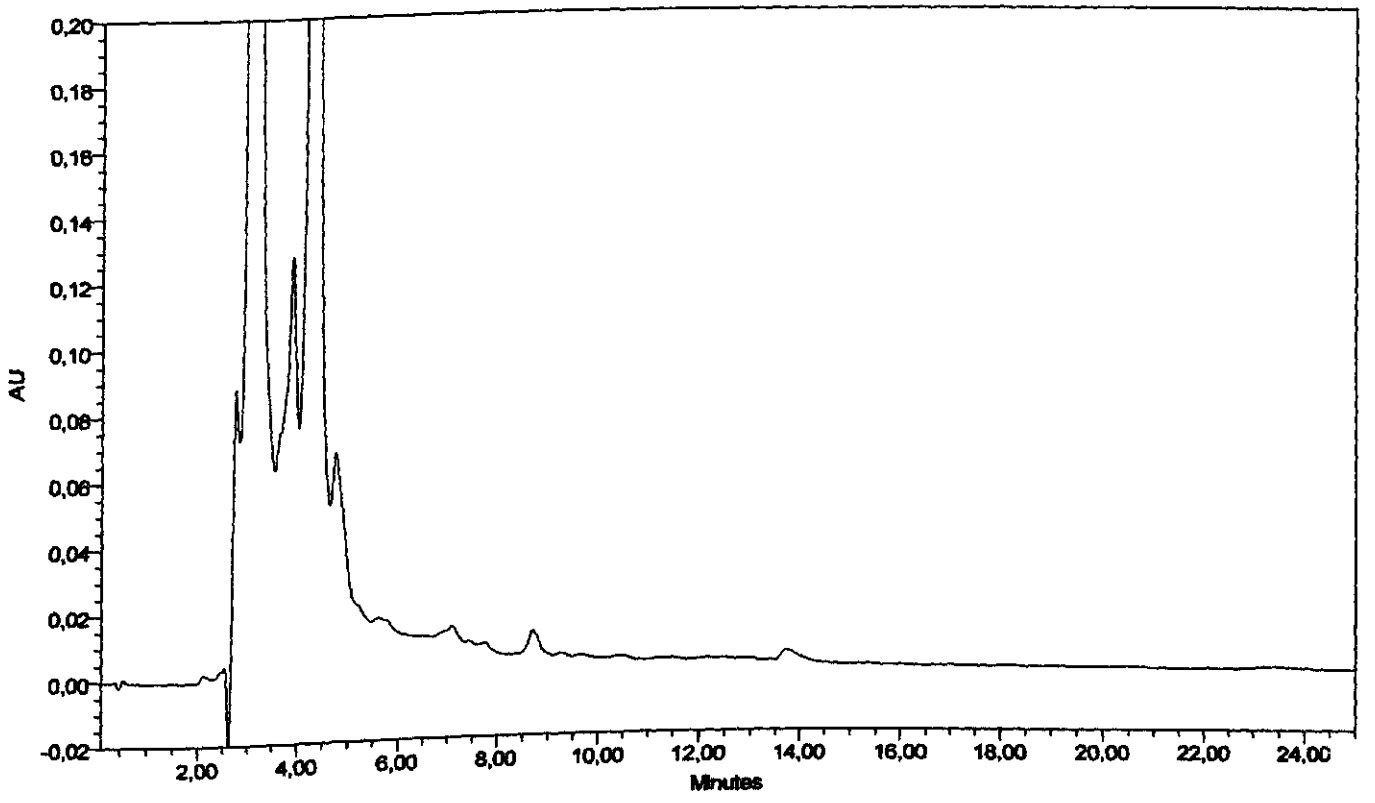
Project: Virginiamici

Report

296876 ←

Vial:	12	"Sample Set":	Virginiamicin
Injection #	1	"Method Set":	Virginiamicin
Injection volume	100,00 ul	Canal:	PDA 235,0 nm
Sample running time	25,0 Minutes		

Chromatogram



	Compound	RT	Area	Height	Concentration	Units
1	Virginiamicin	9,013				

Amount of Virginiamycin in sample (mg/kg): ———

APPENDIX 6

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:

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
316838	2,28	2,66
316843	< 0,5	< 0,5
316872	< 0,5	< 0,5
316874	0,63	0,61
316897	< 0,5	< 0,5
316932	1,33	0,58
316940	< 0,5	< 0,5
316942	2,91	2,24

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: ..... 18 - 10 - 2000 .....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ...9,11..... mg
- Volume of methanol: .41,2..... ml
- Concentration of the stock solution: ..497.... µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1,2..... ml/min
- Injection volume: ...50....µl
- Retention time of virginiamycin M1: .11,65 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: .48.. %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 1:2-1.0 mg/kg

→ triplicate : 56 %, 42 %, 45 %.

Software Version	: 6.1.1.0.0:K20	Date	: 10/19/00 10:48:07 AM
Operator	:	Sample Name	: 21039
Sample Number	: 007	Study	: 71.311.40
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: LC-5	Channel	: A
Interface Serial #	: NONE	A/D mV Range	: 2000
Delay Time	: 0.00 min	End Time	: 30.00 min
Sampling Rate	: 1.0000 pts/s		
Volume Injected	: 1.000000 µL	Area Reject	: 500.000000
Sample Amount	: 1.0000	Dilution Factor	: 1.00
Data Acquisition Time	: 10/18/00 9:39:39 PM	Cycle	: 14

Raw Data File : \\. .004S\TCDATA\NRC SSM\CANFAS Virg\001018\001018_014.raw
Result File : \\ .004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_014.rst
Inst Method : \. 004s\TCdata\nrc ssm\canfas virg\001018\001018 from \\. .004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_014.rst
Proc Method : \. 004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
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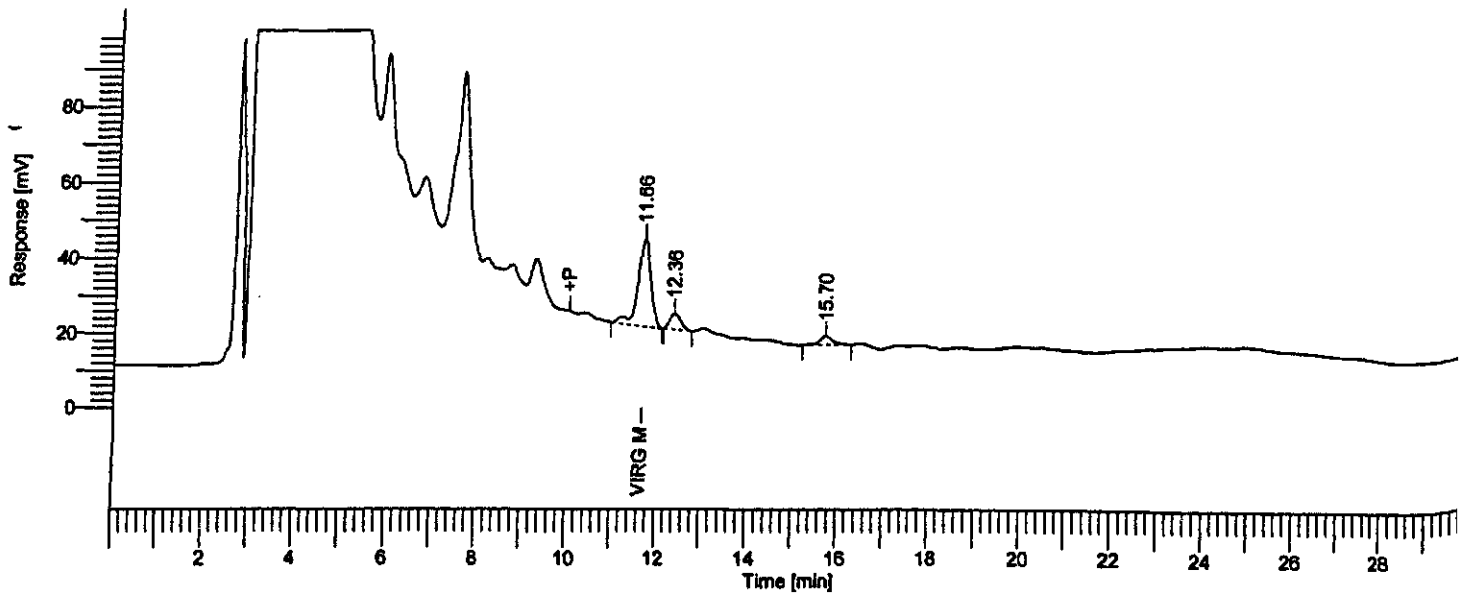
Sequence File : \. 004s\TCdata\NRC SSM\CANFAS Virg\001018\001018.seq



Quantitation results

Peak #	Ret Time [min]	Delta RT [%]	Component name	Area [µV*sec]	Height [uV]	Amount [mg/kg]	BL
1	11.655	0.0449	Virg M1	465974	23206	12	BB
2	12.362	------		72669	4244	0	BB
3	15.704	------		49194	2305	0	BB

Software Version	: 6.1.1.0.0:K20	Date	: 10/19/00 10:48:08 AM
Operator	: .	Sample Name	: 21040
Sample Number	: 008	Study	: 71.311.40
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: LC-5	Channel	: A
Interface Serial #	: NONE	A/D mV Range	: 2000
Delay Time	: 0.00 min	End Time	: 30.00 min
Sampling Rate	: 1.0000 pts/s		
Volume Injected	: 1.000000 µL	Area Reject	: 500.000000
Sample Amount	: 1.0000	Dilution Factor	: 1.00
Data Acquisition Time	: 10/18/00 10:10:25 PM	Cycle	: 15

Raw Data File : \\ ...004S\TCDATA\NRC SSM\CANFAS Virg\001018\001018_015.raw
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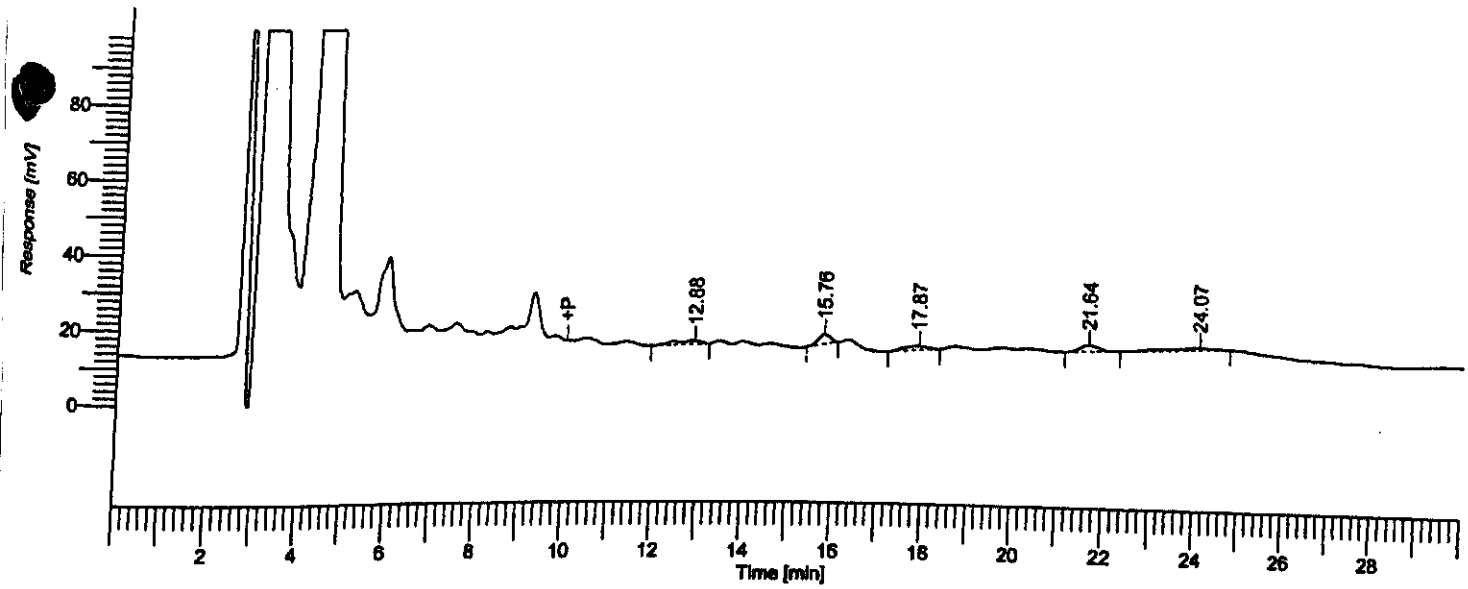
Proc Method : \ ...004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Calib Method : \ ...004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Sequence File : \ ...004s\TCdata\NRC SSM\CANFAS Virg\001018\001018.seq



Quantitation results

Peak	Ret Time [min]	Delta RT [%]	Component name	Area [µV*sec]	Height [uV]	Amount [mg/kg]	BL
	11.650	——	Virg M1	0	0	0	
1	12.881	——		44114	918	0	BB
2	15.759	——		46899	2510	0	BB
3	17.867	——		38445	1034	0	BB
4	21.640	——		52372	1782	0	BB
5	24.069	——		56321	690	0	BB

Software Version	: 6.1.1.0.0:K20	Date	: 10/19/00 10:48:09 AM
Operator	:	Sample Name	: 21041
Sample Number	: 009	Study	: 71.311.40
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: LC-5	Channel	: A
Interface Serial #	: NONE	A/D mV Range	: 2000
Delay Time	: 0.00 min	End Time	: 30.00 min
Sampling Rate	: 1.0000 pts/s		
Volume Injected	: 1.000000 µL	Area Reject	: 500.000000
Sample Amount	: 1.0000	Dilution Factor	: 1.00
Data Acquisition Time	: 10/18/00 10:41:11 PM	Cycle	: 16

Raw Data File : \...004S\TCDATA\NRC SSM\CANFAS Virg\001018\001018_016.raw
Result File : ...004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_016.rst
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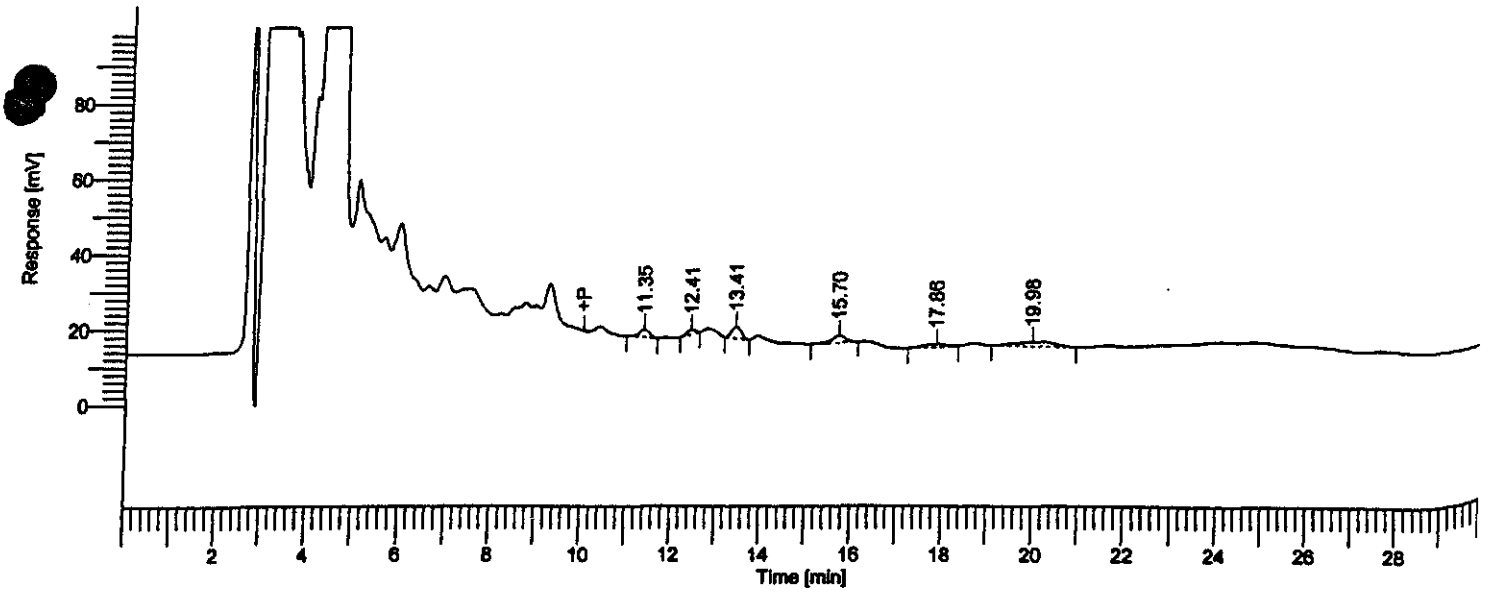
Proc Method : \...004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Calib Method : \...004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Sequence File : \...004s\TCdata\NRC SSM\CANFAS Virg\001018\001018.seq

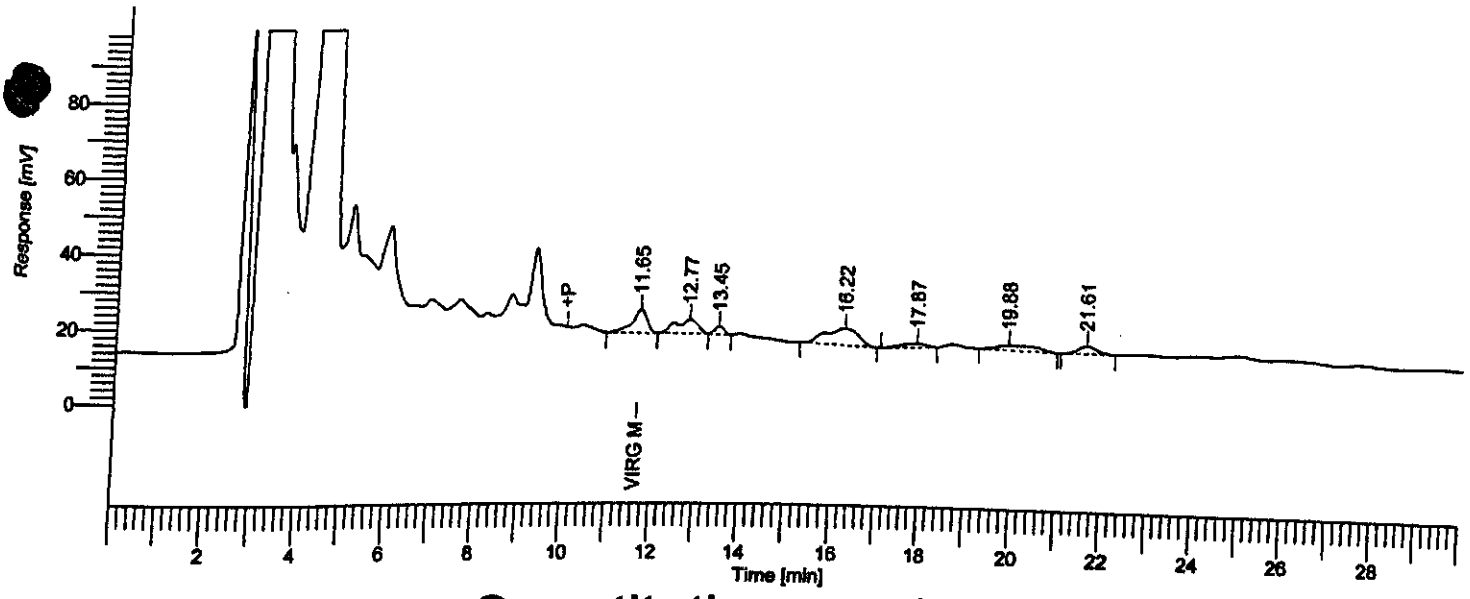


Quantitation results

Peak #	Ret Time [min]	Delta RT [%]	Component name	Area [µV*sec]	Height [uV]	Amount [mg/kg]	BL
1	11.350			32522	2042	0	BB
-	11.650		Virg M1	0	0	0	
2	12.411			16584	1296	0	BB
3	13.415			47130	3142	0	BB
4	15.704			40239	1952	0	BB
5	17.859			27331	804	0	BB
6	19.975			72259	1118	0	BB

Software Version	: 6.1.1.0.0:K20	Date	: 10/19/00 10:48:10 AM
Operator	:	Sample Name	: 21042
Sample Number	: 010	Study	: 71.311.40
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: LC-5	Channel	: A
Interface Serial #	: NONE	A/D mV Range	: 2000
Delay Time	: 0.00 min	End Time	: 30.00 min
Sampling Rate	: 1.0000 pts/s		
Volume Injected	: 1.000000 µL	Area Reject	: 500.000000
Sample Amount	: 1.0000	Dilution Factor	: 1.00
Data Acquisition Time	: 10/18/00 11:11:57 PM	Cycle	: 17

Raw Data File : \\ ,004S\TCDATA\NRC SSM\CANFAS Virg\001018\001018_017.raw
Result File : \\ ,004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_017.rst
Inst Method : ,004s\TCdata\nrc ssm\canfas virg\001018\001018 from \ :004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_017.rst
Proc Method : \ ,004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Calib Method : \ ,004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Sequence File : \ ,004s\TCdata\NRC SSM\CANFAS Virg\001018\001018.seq



Quantitation results

Peak #	Ret Time [min]	Delta RT [%]	Component name	Area [µV*sec]	Height [uV]	Amount [mg/kg]	BL
1	11.646	-0.0327	Virg M1	145486	6327	4	BB
2	12.772	———		133148	3753	0	BB
3	13.445	———		32422	2132	0	BB
4	16.222	———		228388	4398	0	BB
5	17.872	———		39616	1008	0	BB
6	19.880	———		83382	1358	0	BB
7	21.614	———		59304	2059	0	BB

Software Version	: 6.1.1.0.0:K20	Date	: 10/19/00 10:48:11 AM
Operator	:	Sample Name	: 21043
Sample Number	: 011	Study	: 71.311.40
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: LC-5	Channel	: A
Interface Serial #	: NONE	A/D mV Range	: 2000
Delay Time	: 0.00 min	End Time	: 30.00 min
Sampling Rate	: 1.0000 pts/s		
Volume Injected	: 1.000000 µL	Area Reject	: 500.000000
Sample Amount	: 1.0000	Dilution Factor	: 1.00
Data Acquisition Time	: 10/18/00 11:42:42 PM	Cycle	: 18

Raw Data File : \ .004S\TCDATA\NRC SSM\CANFAS Virg\001018\001018_018.raw
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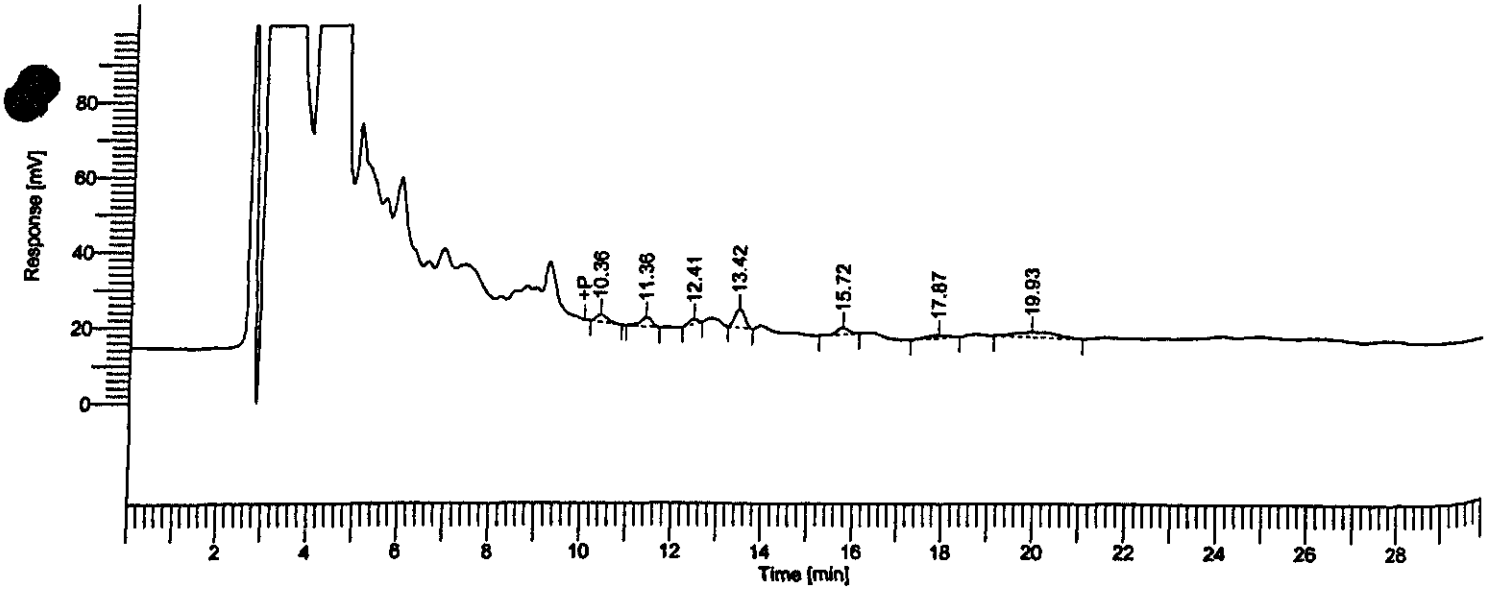
Inst Method : \ .004s\TCdata\nrc ssm\canfas virg\001018\001018 from \ 004s\TCdata\NRC SSM\CANFAS Virg\001018\001018_018.rst
Proc Method : \ .004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Calib Method : \ .004s\TCdata\nrc ssm\canfas virg\001018\001018.mth
Sequence File : \ .004s\TCdata\NRC SSM\CANFAS Virg\001018\001018.seq



Quantitation results

Peak	Ret Time [min]	Delta RT [%]	Component name	Area [µV*sec]	Height [uV]	Amount [mg/kg]	BL
1	10.356	----		33050	1995	0	BB
2	11.359	----		42356	2561	0	BB
-	11.650	----	Virg M1	0	0	0	
3	12.414	----		17120	1335	0	BB
4	13.419	----		75800	4925	0	BB
5	15.719	----		31060	1770	0	BB
6	17.874	----		26658	772	0	BB
7	19.927	----		85882	1305	0	BB

APPENDIX 6

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: VIRGINIAMYCIN

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
326839	0,11	0,1
326880	0,16	0,51
326881	0,19	0,55
326916	Negative	Negative
326923	0,12	0,12
326928	Negative	Negative
326936	Negative	Negative
326952	Negative	Negative

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Calculation for the stock solution (4.4.1.):

- Amount of virginiamycin reference standard weighed: 10 mg
- Volume of methanol: 39.5 ml
- Concentration of the stock solution: 550 µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Waters Symmetry, C18, 5 um, 4.6mmX250mm (Part Nº WAT 054215)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 100 (µL)
- Retention time of virginiamycin M1: 9.50 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: 42.5 %
- Single/duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: 41.74% and 43.35%
- Spiking level: 4 mg/kg

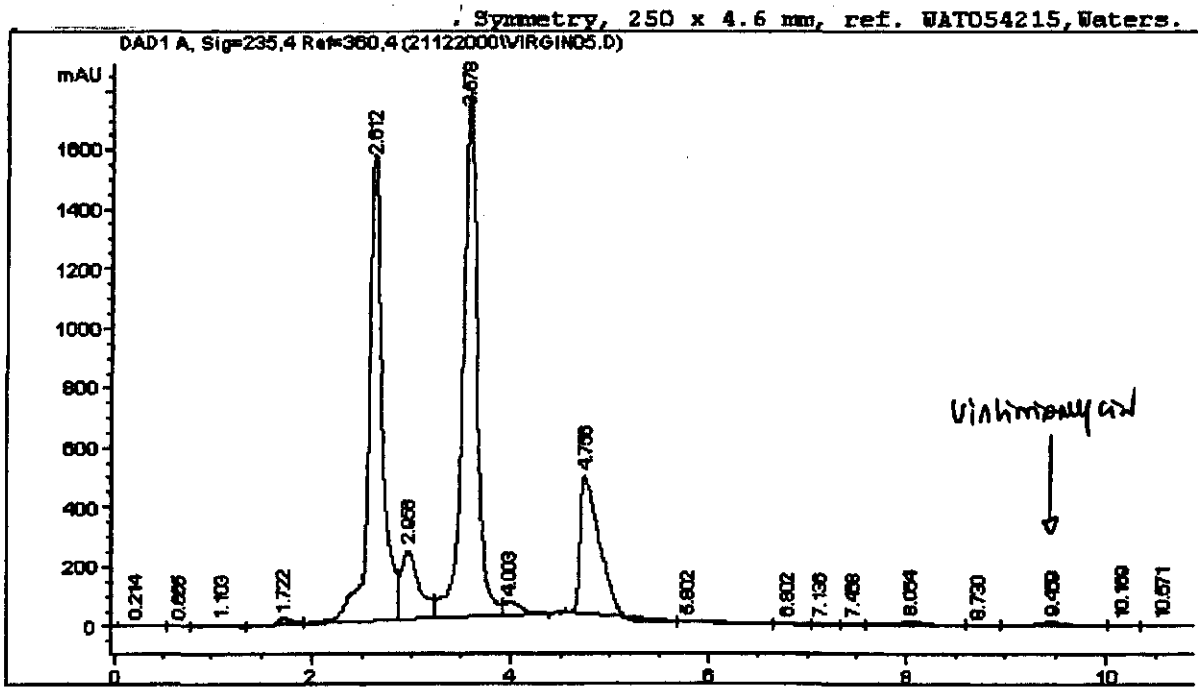
(32)

Data File C:\HPCHEM\1\DATA\21122000\VIRGIN05.D Sample Name: C2

4ppm, massa-5.00g. Virginia sample (4 ppm)

Injection Date	: 12/21/00 5:29:42 PM	Seq. Line	: 5
Sample Name	: C2	Vial	: 8
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/21/00 5:27:08 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 9:42:47 AM by , (modified after loading)		

, Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.459	VV	10.15324	1.70770e-1	1.73387		Virginiamycin

Totals : 1.73387

Results obtained with enhanced integrator!

ata File C:\HPCHEM\1\DATA\21122000\VIRGIN10.D

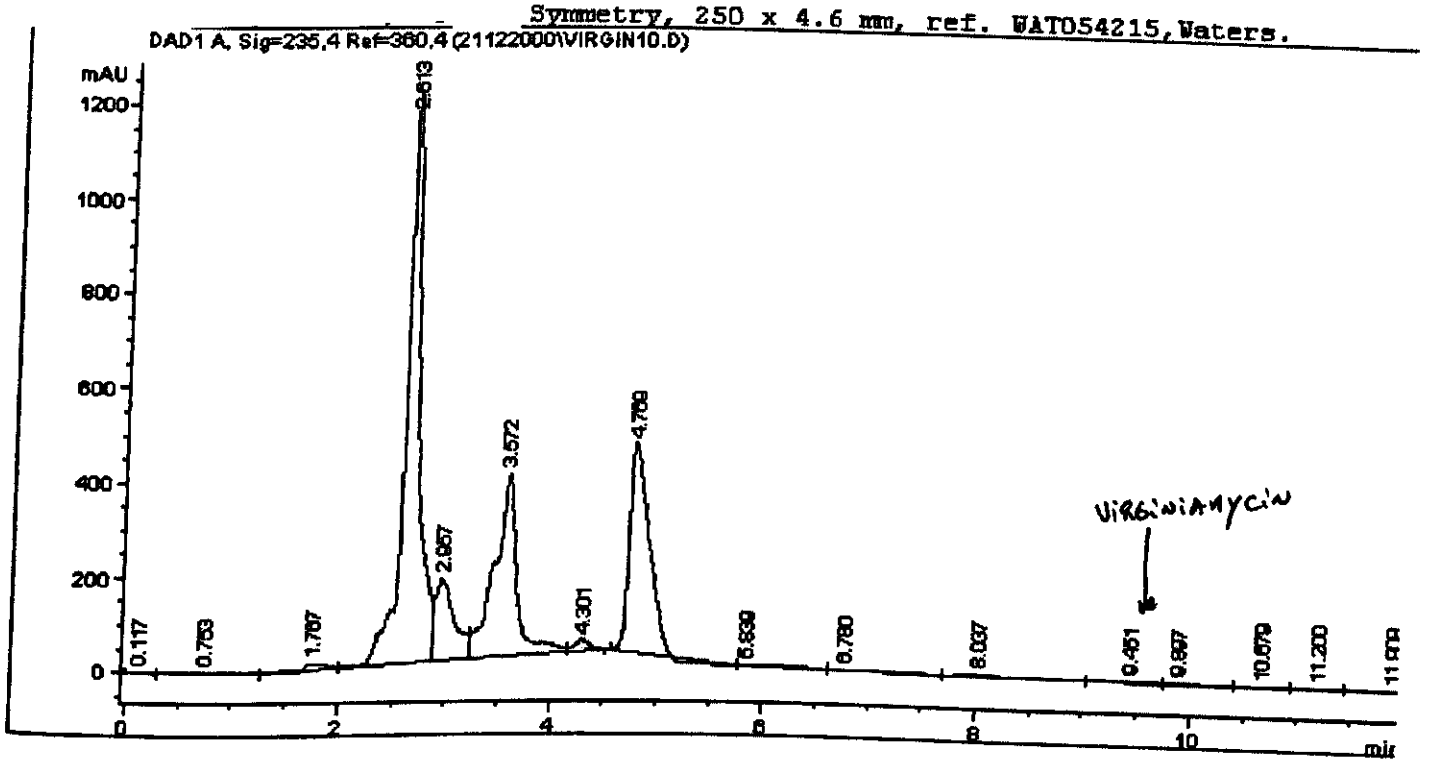
Sample Name: 326839

Code 326839, massa - 5.0028g

(32)

Injection Date	: 12/21/00 6:47:58 PM	Seq. Line	: 10
Sample Name	: 326839	Vial	: 10
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/21/00 6:45:25 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 9:44:42 AM by (modified after loading)		

Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.451	VV	2.81636	1.58496e-1	4.46382e-1		Virginiamycin

Totals : 4.46382e-1

Results obtained with enhanced integrator!

trument 1 12/22/00 9:44:43 AM

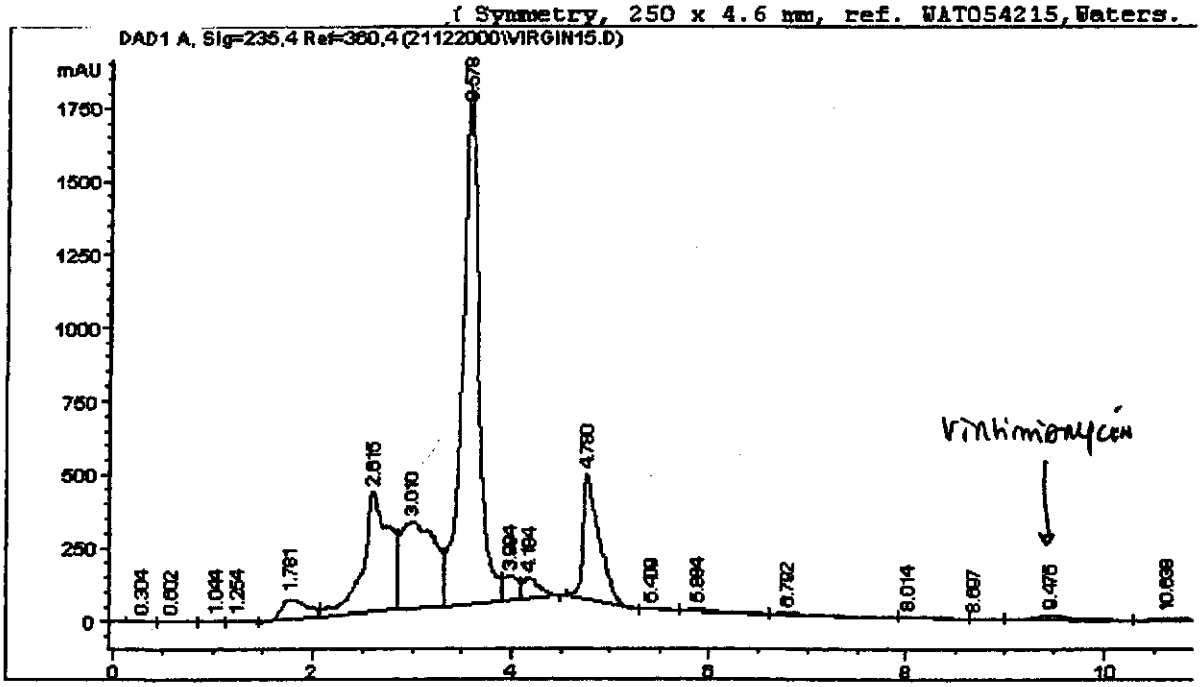
(32)

Data File C:\HPCHEM\1\DATA\21122000\VIRGIN15.D Sample Name: 326880/

Code 326880, massa - 5.0032g

Injection Date	: 12/21/00 8:06:12 PM	Seq. Line	: 15
Sample Name	: 326880/	Vial	: 14
Acq. Operator	: ;	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/21/00 8:03:36 PM by ; (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 9:46:28 AM by (modified after loading)		

{ Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.475	VP	14.82176	1.72254e-1	2.55311		Virginiamycin
Totals :				2.55311		

Results obtained with enhanced integrator!

Instrument 1 12/22/00 9:46:30 AM

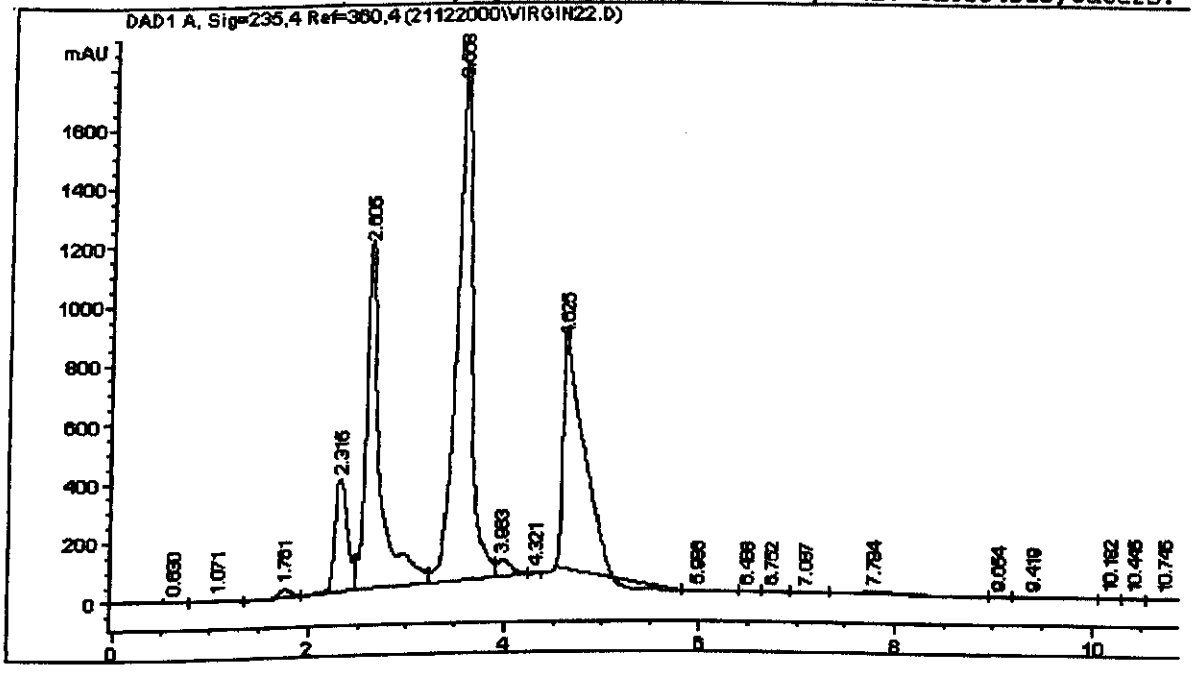
(32)

Data File C:\HPCHEM\1\DATA\21122000\VIRGIN22.D Sample Name: 326916/

Code 326916, massa - 5.00038g

Injection Date	: 12/21/00 9:56:07 PM	Seq. Line	: 22
Sample Name	: 326916/	Vial	: 20
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/21/00 9:53:33 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 10:11:41 AM by (modified after loading)		

Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.419	VP	1.34690	1.39965e-1	1.88518e-1		Virginiamycin
Totals :				1.88518e-1		

Results obtained with enhanced integrator!

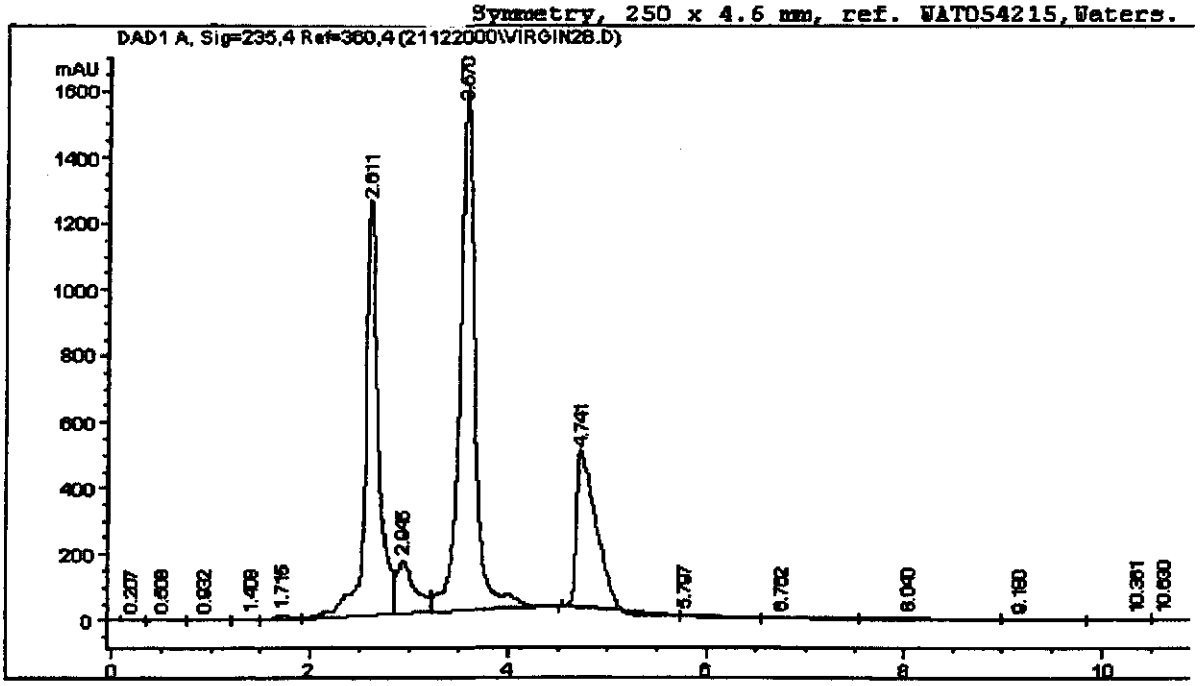
(32)

Data File C:\HPCHEM\1\DATA\21122000\VIRGIN28.D Sample Name: 326928

Code 326928, massa - 5.0020g

Injection Date : 12/21/00 11:30:08 PM Seq. Line : 28
Sample Name : 326928 Vial : 25
Acq. Operator : / Inj : 1
Inj Volume : 100 µl
Acq. Method : C:\HPCHEM\1\METHODS\VIRGINIA.M
Last changed : 12/21/00 11:27:41 PM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\VIRGINIA.M
Last changed : 12/22/00 10:15:05 AM by
(modified after loading)

Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By : Signal
Calib. Data Modified : Friday, December 22, 2000 9:38:37 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.180	VP	7.55841e-1	1.12191e-1	8.47986e-2		Virginiamycin

Totals : 8.47986e-2

Results obtained with enhanced integrator!

Instrument 1 12/22/00 10:15:06 AM

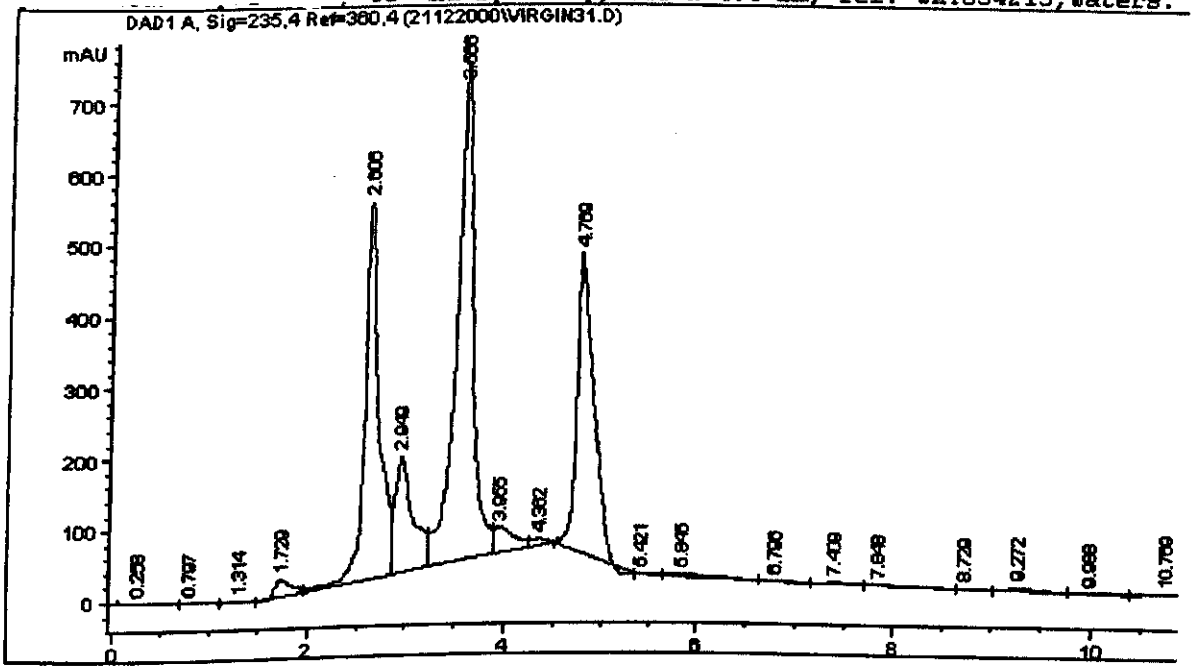
(32)

Data File C:\HPCHEM\1\DATA\21122000\VIRGIN31.D Sample Name: 326936

Code 326936, massa - 5.0026g

Injection Date	: 12/22/00 12:17:00 AM	Seq. Line	: 31
Sample Name	: 326936	Vial	: 28
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 12:14:32 AM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 10:16:44 AM by (modified after loading)		

Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.272	VV	4.34815	1.64480e-1	7.15184e-1		Virginiamycin
Totals :				7.15184e-1		

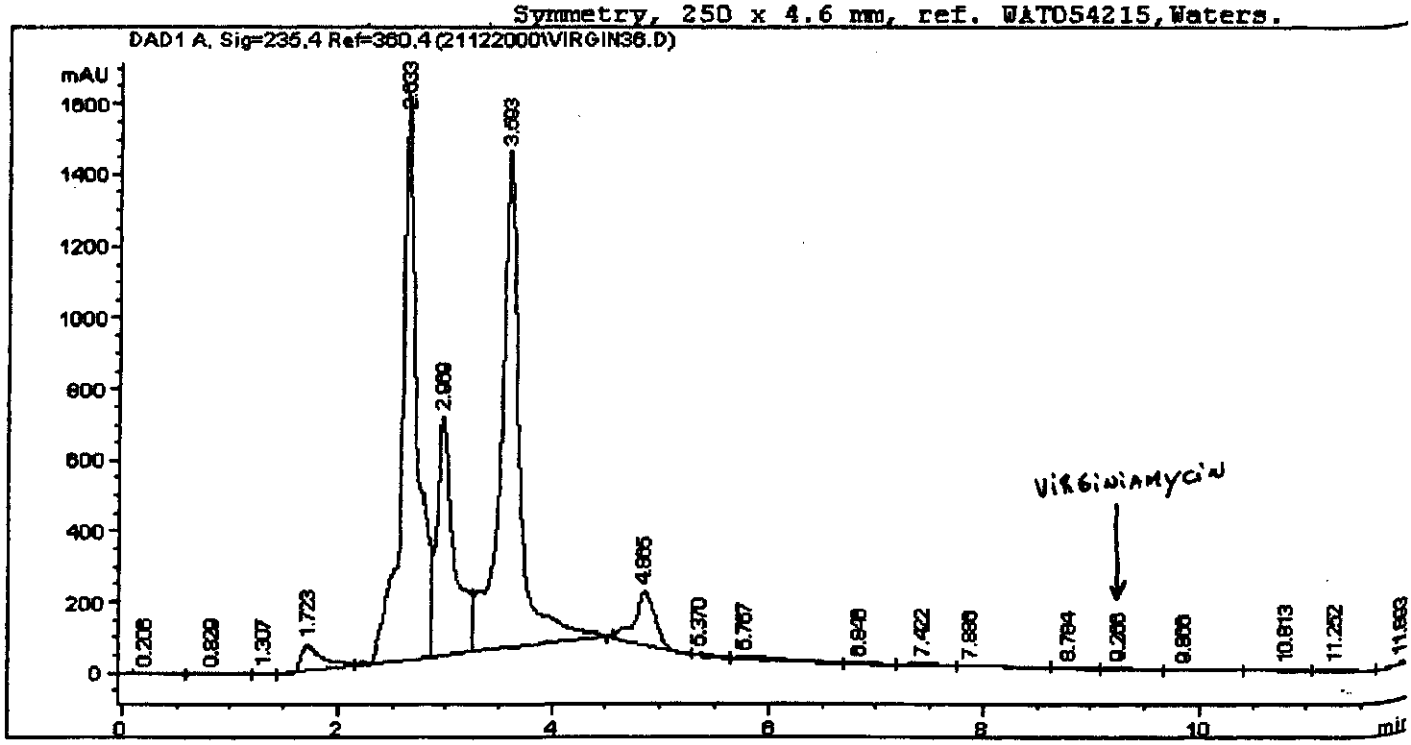
Results obtained with enhanced integrator!

Code 326952, massa - 5.0008g

32

Injection Date	: 12/22/00 1:35:19 AM	Seq. Line	: 36
Sample Name	: 326952/	Vial	: 32
Acq. Operator	:	Inj	: 1
		Inj Volume	: 100 µl
Acq. Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 1:32:48 AM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\VIRGINIA.M		
Last changed	: 12/22/00 10:18:51 AM by (modified after loading)		

Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, December 22, 2000 9:38:37 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=235,4 Ref=360,4

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
9.266	VP	4.34639	1.64475e-1	7.14873e-1		Virginiamycin

Totals : 7.14873e-1

Results obtained with enhanced integrator!

APPENDIX 6

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:

Analyte: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
376835	ND	ND
376836	Lost Sample	ND
376844	8,04	6,05
376854	7,99	7,26
376868	7,88	Lost Sample
376895	2,28	2,42
376906	ND	ND
376917	ND	ND

37

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Date(s) of analysis: 30th NOV 2000

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: 11 mg
- Volume of methanol: 35.69 ml
- Concentration of the stock solution: 500 µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☑ As described in the method Hypersil BDS C18 25cm x 4.6 mm, 5µm.
  - ☐ Other: .....
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 1 ml/min
- Injection volume: 100 µl
- Retention time of virginiamycin M1: ~~ min

Chromatograms: Please include representative chromatograms of:

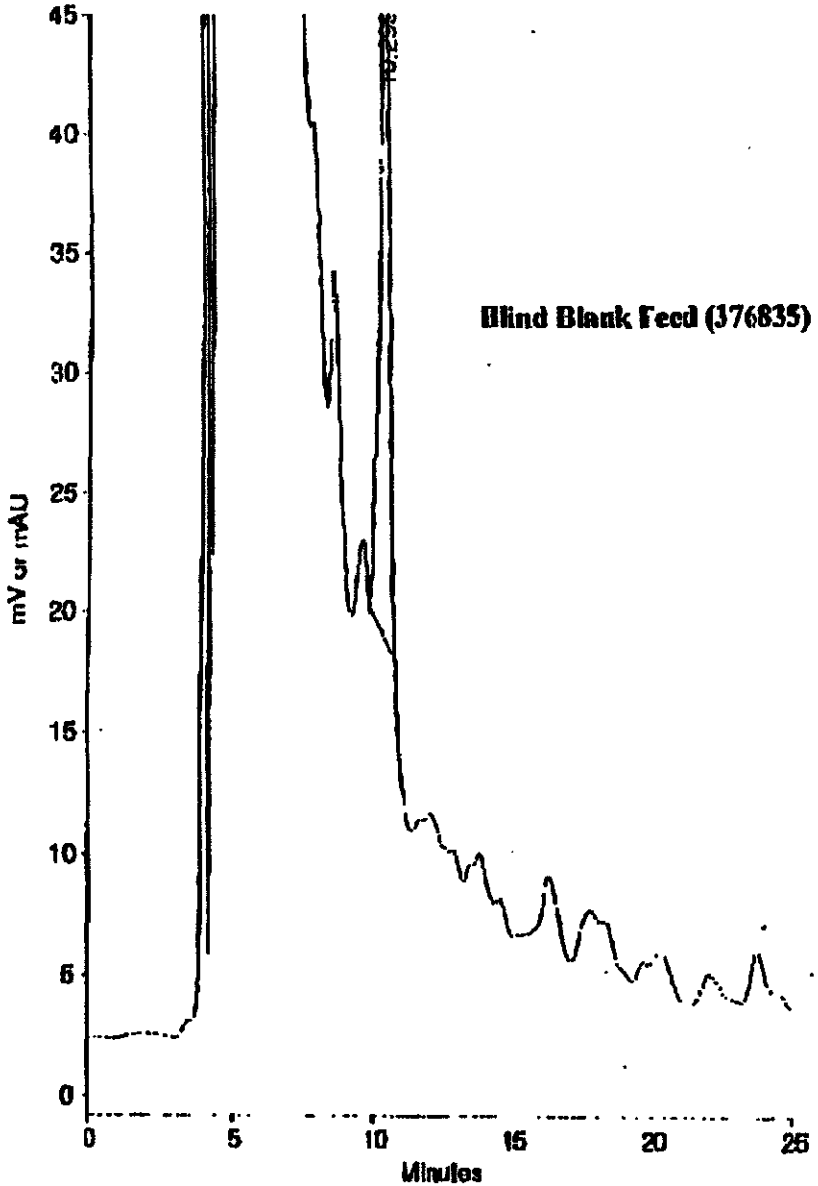
- Blind positive feed samples
- Blind blank feed samples

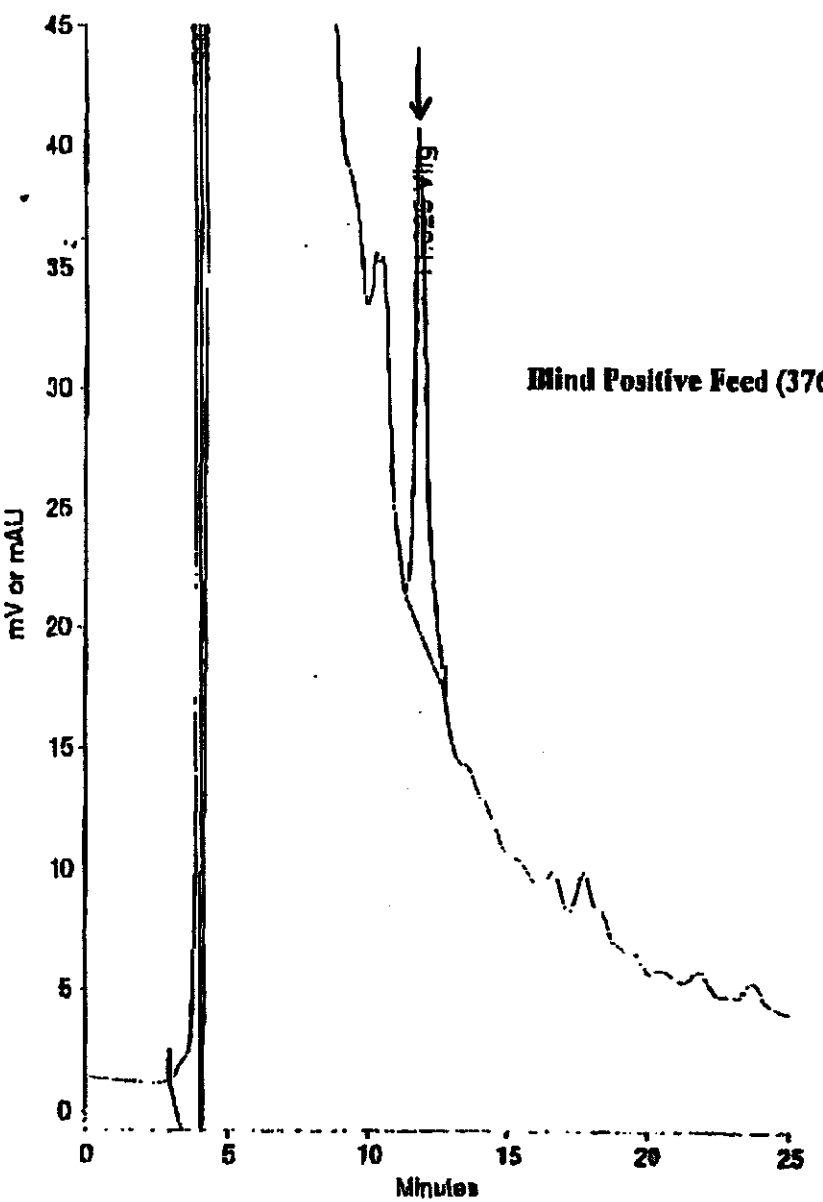
Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

- Percentage recovery: 40 %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 43 % and 37 %
- Spiking level: 4 mg/kg

Typical chromatograms for Virginiamycin





APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 38

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: VIRGINIAMYCIN

Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
Sample code		
386860	0,52	0,54
386862	0	0
386864	1,45	1,61
386865	0	0
386905	0	0
386924	0	0
386947	0,79	0,78
386948	0	0

CANFAS COLLABORATIVE STUDIES - VIRGINIAMYCIN

Annex 4 – Questionnaire

Date(s) of analysis: **12/05/00**

Calculation for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: **10.0** mg
- Volume of methanol: **100** ml
- Cocentration of the stock solution: **225** µg/ml microbiological activity

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - **X** Other: **Hypersil ODS C-18, 250 x 4,6 mm, 5 µm**
- Mobile phase:
  - ☐ As described in the method
  - **X** Other: **Water/Acetonitrile/Acetic Acid (650:350:3)**
- Flow-rate: **1** ml/min
- Injection volume: **20** µl
- Retention time of virginiamycin M1: **18** min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank samples

Please indicate the virginiamycin M1 peak with an arrow

Recovery results:

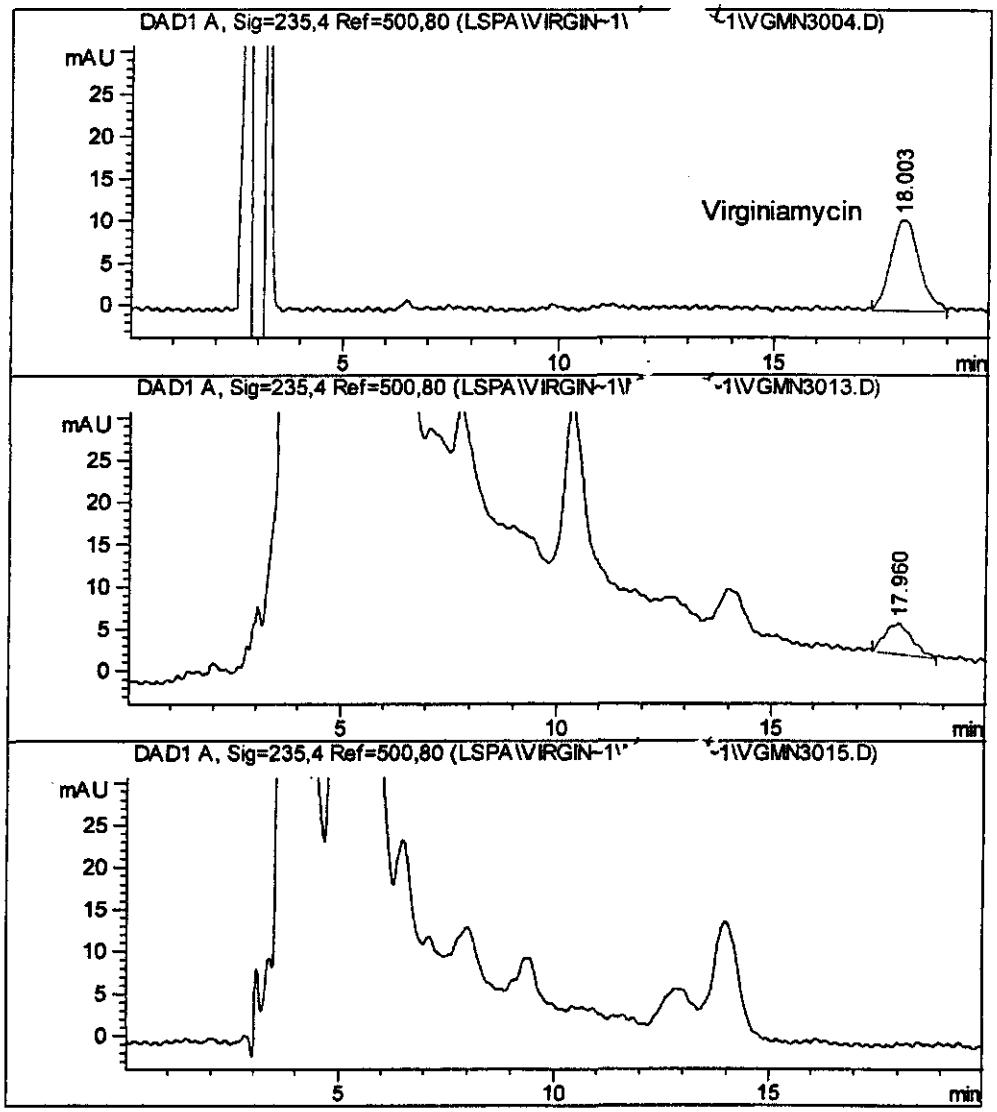
- Percentage recovery: ... %
- Single / duplicate determinations: ☐ single **X** duplicate
- If duplicate, please give both percentages: **60** % and **93** %
- Speaking level: **4,5** mg/kg **(m.a.)**

CANFAS COLLABORATIVE STUDIES - VIRGINIAMYCIN

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

Please note that samples labeled as 386860 and 386947 has been quantified around our Limit of Determination. (LD = 0,6 mg/Kg)

Chromatograms for standard (45 ppm m.a.), sample (386864) and blank



Appendix 7

Results of special requests:

LC-MS/MS results

Departement voor Kwaliteit van Dierlijke Producten (DVK-CLO), Melle, Belgium

LC-MS/MS

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - VIRGINIAMYCIN

Annex 4 - Questionnaire

Laboratory: .....DVK-CLO.....

Contact person: .....H.DE RUYCK.....

Date(s) of analysis: .....20-27/11/00.....

Calculations for the stock solution (4.4.1):

- Amount of virginiamycin reference standard weighed: ....10.... mg
- Volume of methanol: .....45.... ml
- Concentration of the stock solution: ...500. µg microbiological activity/ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ...Alltima C18 5µm 150x2.1 mm (Alltech)
- *Mobile phase:*
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....0.25. ml/min
- Injection volume: ....5....µl
- Retention time of virginiamycin M1: 6.0 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- *Blind blank feed samples*

Please indicate the virginiamycin M1 peak with an arrow

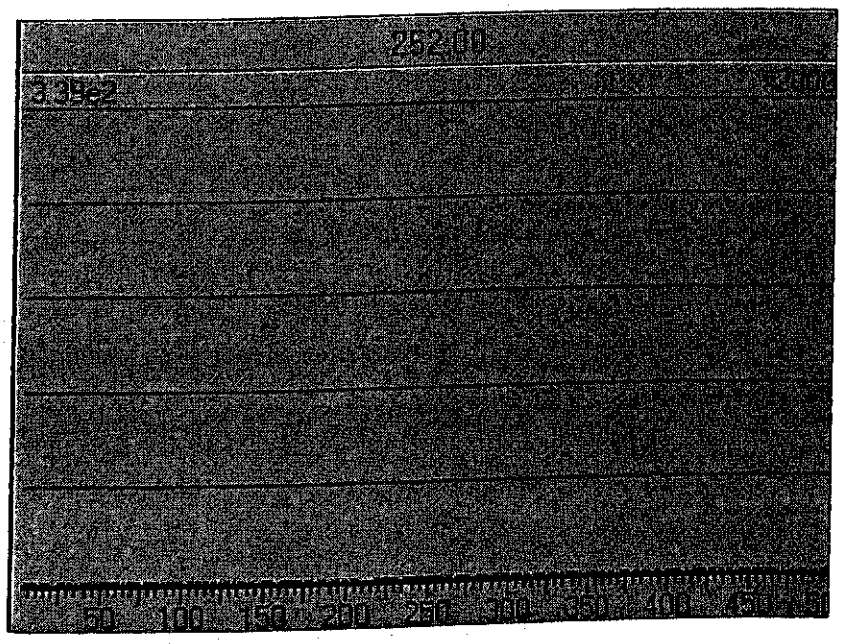
Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 81.5 % and 64.1 %
- Spiking level: .....4... mg/kg

Tuning Method Report **Page 1**

Method: C:\MASSLYNX\ACQUDB\MSMS

Printed: Thu Dec 07 16:41:37 2000



Dau 421.20

→ print out when MS was not in operation!
⇒ set values are correct!

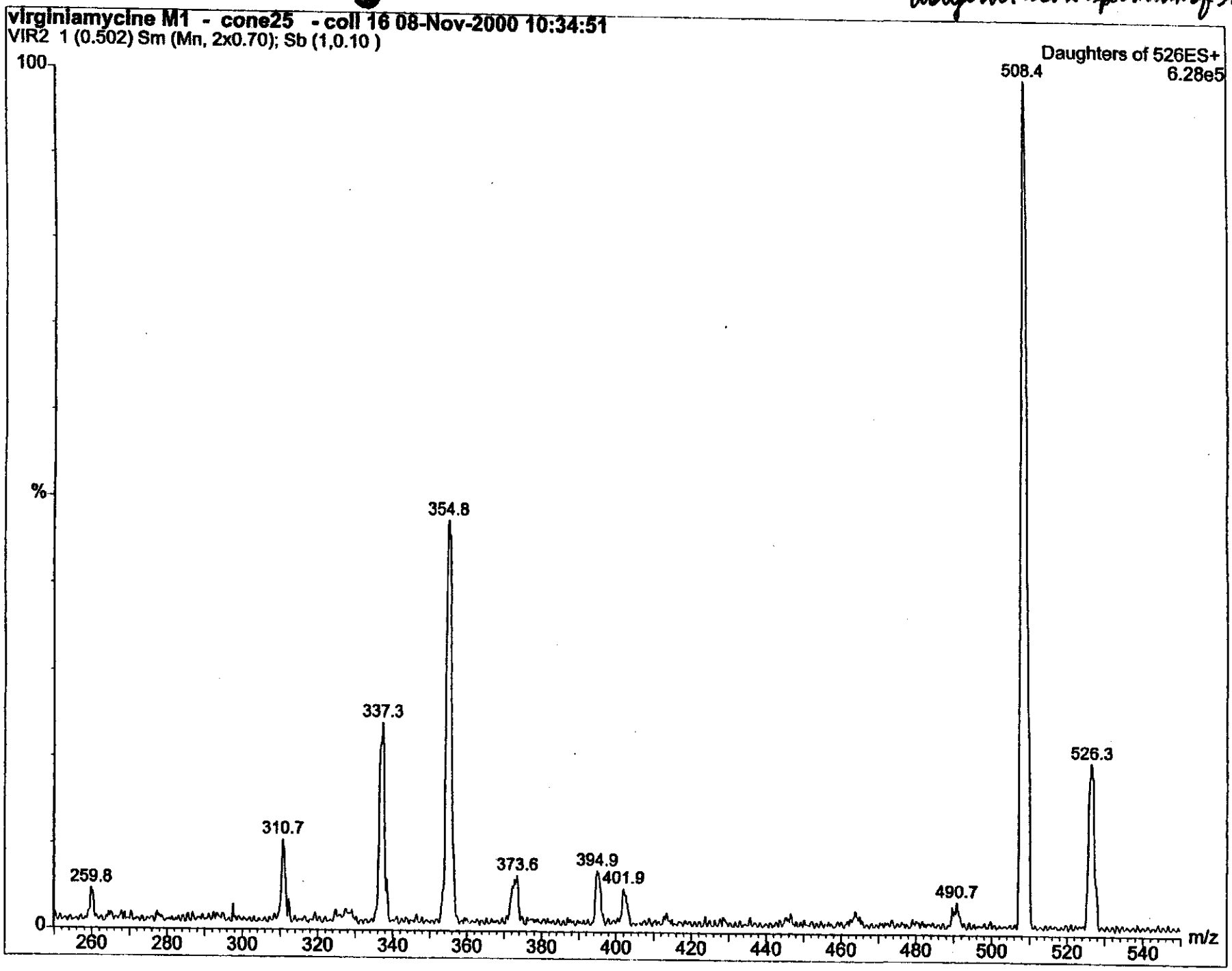
SOURCE (ESP+)	Set	Rdbk
Capillary	3.50	0.02
Cone	25 ~~45~~	-3
Extractor	2	-1
RF Lens	0.50	
Source Block Temp.	130	~~94~~
Desolvation Temp.	250	~~26~~

Analyser	Set	Rdbk
LM Res 1	11.0	
HM Res 1	11.0	
IEnergy 1	1.5	
Entrance	6	-0
Collision	~~23~~ 16	-1
Exit	6	-0
LM Res 2	11.0	
HM Res 2	11.0	
IEnergy 2	2.0	
Multiplier	643	4

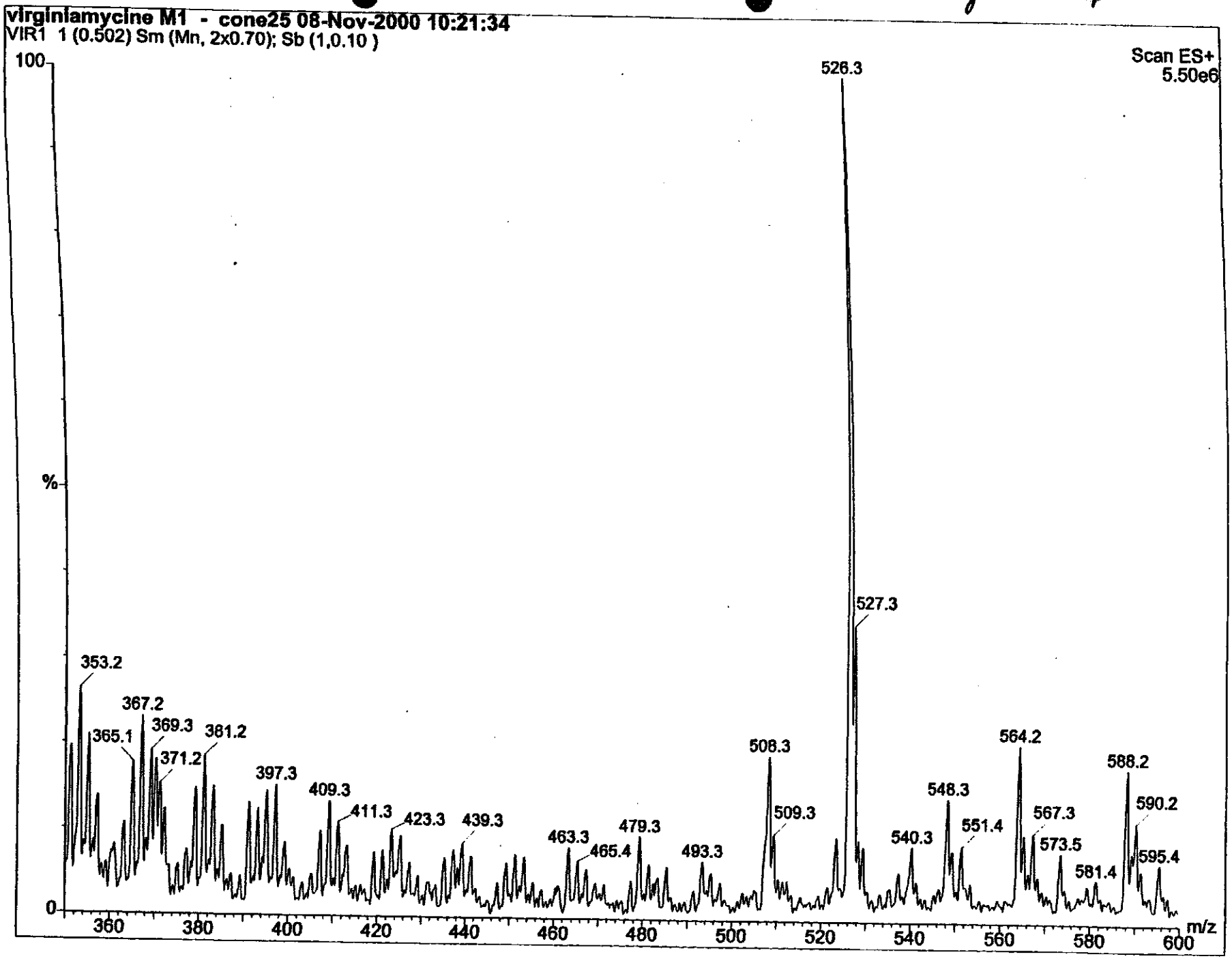
Pressures	Rdbk
Analyser Vacuum	5.0e-6
Gas Cell	2.0e-5

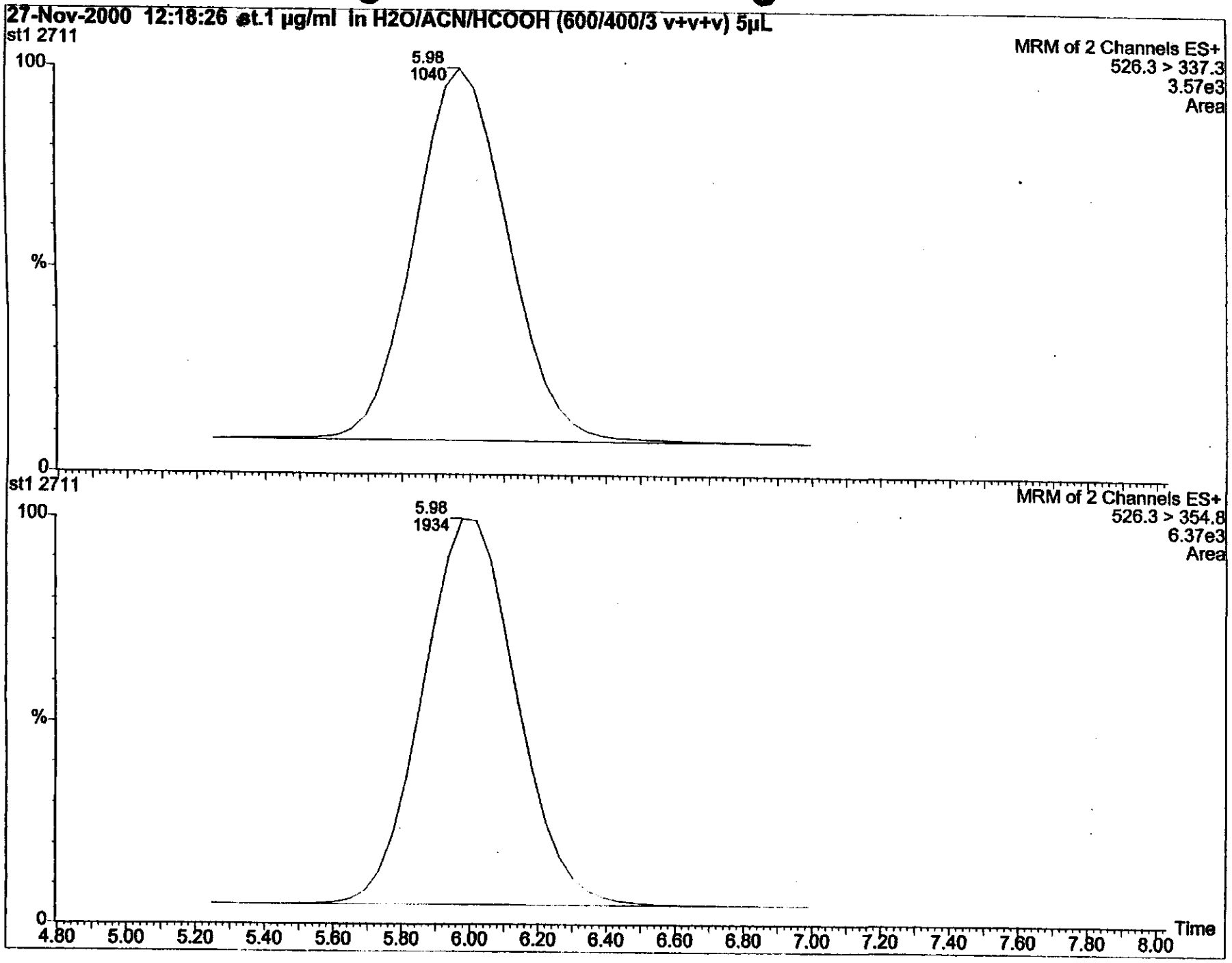
Gas Flows	Rdbk	
Nebuliser	~~<20.0~~	80L/h
Drying	~~<20.0~~	600L/h

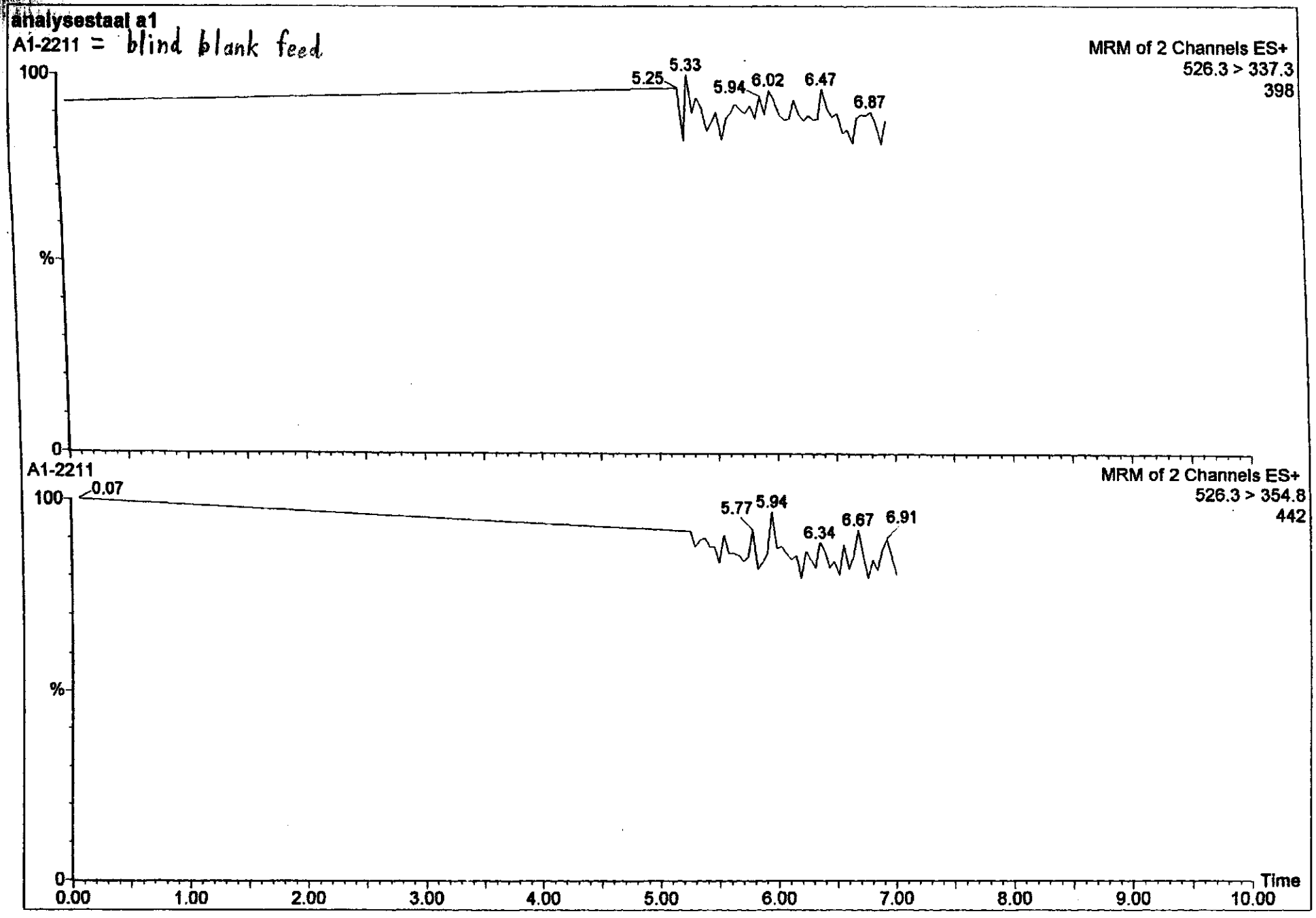
daughter scan spectrum of 526

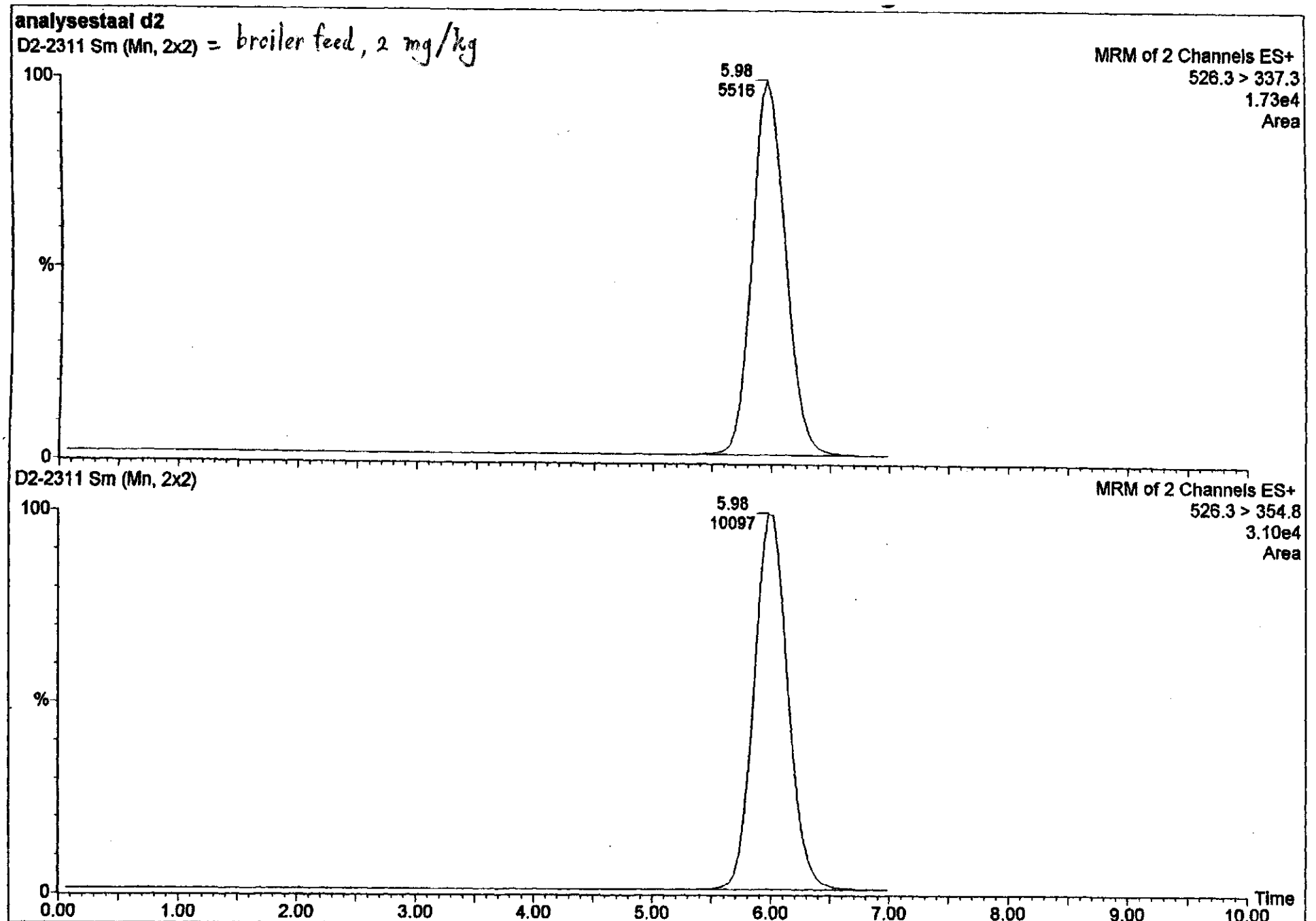


full scan spectrum




27-Nov-2000 12:18:26 st.1 µg/ml in H2O/ACN/HCOOH (600/400/3 v+v+v) 5µL
st1 2711
MRM of 2 Channels ES+
526.3 > 337.3
3.57e3
Area
5.98
1040
100
%
0
st1 2711
MRM of 2 Channels ES+
526.3 > 354.8
6.37e3
Area
5.98
1934
100
%
0
4.80
5.00
5.20
5.40
5.60
5.80
6.00
6.20
6.40
6.60
6.80
7.00
7.20
7.40
7.60
7.80
8.00
Time


analysestaal a1
A1-2211 = blind blank feed
MRM of 2 Channels ES+
526.3 > 337.3
398
100
%
0
5.25
5.33
5.94
6.02
6.47
6.87
A1-2211
MRM of 2 Channels ES+
526.3 > 354.8
442
100
%
0
0.07
5.77
5.94
6.34
6.67
6.91
Time
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00


analysestaal d2
D2-2311 Sm (Mn, 2x2) = broiler feed, 2 mg/kg
MRM of 2 Channels ES+
526.3 > 337.3
1.73e4
Area
5.98
5516
100
%
0
D2-2311 Sm (Mn, 2x2)
MRM of 2 Channels ES+
526.3 > 354.8
3.10e4
Area
5.98
10097
100
%
0
Time
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00