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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)

Co-ordinator: Dr. J. de Jong

**FINAL REPORT**

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

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## SUMMARY

This report describes the results of a collaborative study of an HPLC method for the coccidiostat maduramicin in four broiler feeds, one turkey feed and one premixture. The collaborative study forms part of the EU-project "Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS-SMT4-CT98-2216).
The principle of the method is as follows: The sample is extracted with methanol. The content of maduramicin is determined by reversed-phase high performance liquid chromatography (HPLC) with post-column derivatisation with vanillin and VIS-detection at 520 nm.
The samples which were tested in the collaborative study were 3 broiler feeds with declared maduramicin contents of 2,5, 4,5 and 9 mg/kg, 1 turkey feed with 5 mg/kg, 1 blank broiler feed and 1 premixture with declared content of 450 mg/kg maduramicin. The feed samples were sent to the participants as blind duplicates, the premixture as a single sample. The participants were asked to do a single determination per sample for the feed samples and to analyse the premixture in duplicate.
Results were reported by 10 laboratories. Statistical evaluation was performed according to ISO 5725.
The results of the collaborative study were evaluated in a meeting attended by the participants. The panel has accepted the results of the statistical evaluation, as described in Table 7 (including the results of lab 26). It can be concluded that the repeatability and reproducibility of the method is acceptable, both for feedingstuffs and premixtures. The results obtained for the blind blank feeds and for the recovery are also acceptable. The panel agreed that the method can be recommended for adoption as an official method.
Three laboratories used dimethylaminobenzaldehyde (DMAB) for post column derivatisation. The results do not differ significantly from the results with vanillin.

# 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 maduramicin. Maduramicin is a coccidiostat which is registered for broiler and turkey feeds at a content of 5 mg/kg.
The method for feeds and premixtures was developed and validated by the Bundesamt und Forschungszentrum für Landwirtschaft (BFL), Vienna, Austria (see Final report on method development and validation for maduramicin, B. Stoisser, 26-05-1999). Subsequently, the method for feeds and premixtures was subjected to between-lab validation by the Universität Hamburg, Institut für Angewandte Botanik, Germany (see report H.-A. Putzka, 24-01-2000) and the National Veterinary Institute (NVI), Uppsala, Sweden, (see report A. Stepinska, May 2000) with satisfactory results (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).
Prior to the collaborative study, a kick-off meeting was organised (Brussels, 13-14/6/2000) and participating laboratories were given the opportunity to familiarise themselves with the method, using feed samples with stated contents of maduramicin. Also prior to the production of the materials for the collaborative study, separate batches of the materials were produced for stability testing, indicating that maduramicin is unstable in feeds and premixtures at room temperature and at 37 °C. At 4 °C about 10 % breakdown is observed after 4 months. For this reason it was decided to store and ship the samples frozen and to ask the participants to analyse the samples in a short period of time after receipt.
The samples which were prepared for the collaborative study were 3 broiler feeds with declared maduramicin contents of 2,5, 4,5 and 9 mg/kg, 1 turkey feed with a content of 5 mg/kg, 1 blank feed and 1 premixture with declared content of 450 mg/kg maduramicin. The feeds with 2,5 and 9 mg maduramicin per kg have been included in order to assure that the method is applicable for contents 2 times lower and 2 times higher than the permitted content.
The feed samples were sent to the participants as blind duplicates, the premixture as a single sample. The participants were asked to do a single determination per sample for the feed samples and to analyse the premixture in duplicate. Before these samples were shipped, the between-sample homogeneity was checked with satisfactory results (see par. 3.1.2).
Apart from the samples, a letter with instructions, reporting forms, etc. was sent to the participants (see Appendix 1).
This report describes the results of the collaborative study.

## 2 PARTICIPANTS

The following laboratories/persons participated in the collaborative study.

- Bundesambt und Forschungszentrum für Landwirtschaft (BFL), Wien, Austria; B. Stoisser, M. Wieshaider
- Hoffmann-La Roche Ltd., Basel, Switzerland; P. Hofmann, A. Zuber
- IEEB, Bordeaux, France; J.P. Antalick, T. Gron.
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, C. Saldanha.
- Laboratory of the Government Chemist, Teddington, United Kingdom; G. Merson, J. Cowles, K. Needham.
- LUFA - Augustenberg, Karlsruhe, Germany; A. Thalmann, K. Wagner
- LUFA-ITL Kiel, Kiel, Germany; F.H. Johannsen, Kollwitz.
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- 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; H.J. van der Kamp, H.C.H. Kleijnen
- Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany; H. Putzka, D. Böhm.

# 3 MATERIALS

## 3.1 Samples for collaborative study

### *3.1.1 Sample composition*

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

Table 1: Specifications of the samples

| Type of feed / premixture | Declared content | Units | Subcontractor | Date of production |
|---|---|---|---|---|
| Broiler feed I, 4 % fat | 2,5 | mg/kg | Hoffmann-La Roche | 29/09/2000 |
| Broiler feed I, 4 % fat | 9 | mg/kg | Hoffmann-La Roche | 29/09/2000 |
| Broiler feed II, 8 % fat | 4,5 | mg/kg | Hoffmann-La Roche | 29/09/2000 |
| Turkey feed | 5 | mg/kg | Hoffmann-La Roche | 29/09/2000 |
| Premixture for broiler feed | 450 | mg/kg | Hoffmann-La Roche | 25/08/2000 |

The broiler feeds contained wheat, broken rice, soya extruded, corn gluten feed and pig fat in the usual industrial quantities. The turkey feed contained wheat, corn, soya expellers, rape, peas, potato protein and pig fat in the usual industrial quantities. The complete composition of the feeds is stored in the files of the co-ordinator (confidential). The premixture was based on wheat middlings as carrier material and contained regular contents of vitamins, minerals and trace elements.

The composition of the turkey feed and the premixture was the same as the composition of the products that were produced by Hoffmann-La Roche in August 1999 for stability testing (see Report on homogeneity and stability studies of samples for the collaborative studies for maduramicin, B. Stoisser, BFL, Vienna, Austria, 31.01.2000). For the broiler feeds, the composition had to be adapted because, due to the BSE-measures, some of the ingredients were no longer available.

The feed products have been prepared in a quantity of 30 kg each (10 kg went to waste, 20 kg were used). 50 subsamples of approx. 250 grams have been taken (manual distribution with a shovel). The subsamples were stored in minigrip PE sacks.
The premixture has been prepared in a quantity of 5 kg. From the premixture 35 subsamples of approx. 100 g have been taken (manual distribution with a shovel) and supplied in minigrip PE sacks.
All subsamples have been stored frozen (- 20 °C).
Next to the above mentioned samples which contained maduramicin, a blind blank feed was sent to the participants as well as a blank feed labelled "blank feed for maduramicin recovery check"

(see Appendix 1). The blind blank feed was the broiler I type feed (see above). This feed was analysed at F. Hoffmann-La Roche Ltd prior to the collaborative studies and was found to contain no detectable amounts of maduramicin or interfering substances. The blank feed for maduramicin recovery check was the same broiler I type feed.

*3.1.2 Sample homogeneity*

The homogeneity of the samples was studied by BFL 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 / premixture are attached in Appendix 2. Table 3 gives a summary of these results.

Table 3: Results of homogeneity tests for maduramicin in feeds and premixture

| Results / Product | Declared content (mg/kg) | Measured content (mg/kg) | Homogeneity results | |
|---|---|---|---|---|
| | | | Between sample CV (%) | Within sample CV (%) |
| Broiler feed I. 4 % fat | 2,5 | 2,69 | 4,4 | 6,2 |
| Broiler feed I. 4 % fat | 9 | 9,89 | 4,2 | 7,9 |
| Broiler feed II. 8 % fat | 4,5 | 5,03 | 2,4 | 4,3 |
| Turkey feed | 5 | 5,12 | 3,1 | 3,7 |
| Premixture | 450 | 487 | 4,4 | 4,6 |

According to the Project Plan the CV's for homogeneity should not exceed 2 times the CV's for repeatability ($CV_{hom} \leq 2\ CV_r$). Based on previous results of within-lab validation (see Second Annual Report CANFAS, J. de Jong, 12-08-2000) the maximum limit for $CV_{hom}$ was set to 15 % for the feed containing 2,5 mg/kg and 12 % for the other feeds and the premixture.
All between- and within-sample CV's fulfil these requirements. 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 3. The premixture was sent as a single sample and was labelled as such. The samples were sent to the participants from Hoffmann-La Roche by courier service on 14 November 2000. The samples were shipped frozen on dry ice.

## 3.2 Reference standard

The reference standard was supplied by dr. P. Hofmann, Hoffmann-La Roche, Basel, Switzerland. The certificate of analysis of the reference standard (Lot No. AC 9745-1C) is described in Appendix 4. The purity of this standard, expressed as the ammonium-salt, is 93,3 %. This information is described on the label of the reference standard vials and should be used in the calculations (see method). In addition to the information already given, the participants were instructed by E-mail to set the purity of the reference standard to 93,3 %. Moreover, the participants were also instructed by E-mail that, contrary to the information on the certificate of analysis, the reference standard should be used as such and should not be dried.

# 4 METHODS

## 4.1 Method of analysis

The method of analysis is included as annex 1 to Appendix 1. The participants were instructed that this method has to be used without any modifications.

### *4.1.1. HPLC-conditions*

Various types of HPLC-columns were used (the column which is recommended in the method is a Hypersil BDS C18, 250x4,6mm, 5 µm).
The mobile phase described in the method is phosphate buffer 10 mM, pH=4,0 : methanol = 100:900 (v/v) . One laboratory 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 |
|---|---|---|
| 17 | As described in the method | As described in the method |
| 22 | As described in the method | As described in the method |
| 23 | Not reported | Not reported |
| 26 | Sperisorb ODS-2 | As described in the method |
| 27 | As described in the method | As described in the method |
| 29 | Spherisorb ODS-3, C18, 4,6x250 mm, 5 µm | As described in the method |
| 30 | Kromasil C18 150 x 4,6 mm 5µm | As described in the method |
| 31 | As described in the method | As described in the method |
| 35 | Lichrospher 100 RP18 (5 µm) | As described in the method |
| 36 | Hypersil ODS, 5 µm, 250x4 mm | 100 ml phosphate buffer pH 4, 80 ml tetrahydrofurane, to 1000 ml with methanol |

## 4.2 Method for statistical evaluation

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

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

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

## 5 RESULTS

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

### 5.1 Statistical evaluation

The results reported by the participants are given in Table 7.
The results of lab 29 for the 450 mg/kg sample were discarded for the following reason: due to the fact that the extract of the premixture was not diluted, the value for the peak area was much higher than the area value for the highest standard (see Remarks, Table 11).
Statistical analysis of the results (excl. lab 29, 450 mg/kg sample) showed that lab 30 is a Cochran outlier for the 5 mg/kg sample. The resulting values for the statistical parameters (mean, relative standard deviations for repeatability and reproducibility) are given in Table 7. According to the Project Plan, the $rsd_r$-values should be ≤ 10 %. For all samples this criterion is met and consequently it can be concluded that the repeatability is satisfactory.
The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744). The HORRAT ratios are given in Table 5. The HORRAT ratios should be lower than 2. For all five samples this criterion is met and established $rsd_R$-values are in line with values predicted by the Horwitz equation. Consequently it can be concluded that the reproducibility of the method is satisfactory.

Table 5: Horrat ratios of the Maduramicin collaborative study

| Mean after elimination of outliers[1] (mg/kg) | Predicted $rsd_R$ | Established $rsd_R$ | Horrat[2] | Conclusion |
|---|---|---|---|---|
| 2,616 | 13,844 | 15,270 | 1,10 | Reproducibility OK |
| 4,438 | 12,785 | 25,350 | 1,98 | Reproducibility OK |
| 5,052 | 12,538 | 16,120 | 1,29 | Reproducibility OK |
| 9,374 | 11,424 | 16,820 | 1,47 | Reproducibility OK |
| 464,3 | 6,349 | 11,450 | 1,80 | Reproducibility OK |

[1] = lab 29/sample 450ppm; lab 30/sample 5 ppm
[2] = Horrat is the ratio between the established $rsd_R$ and the predicted $rsd_R$

Lab 26 reported a recovery (80 %) much lower than the mean recoveries of the other laboratories, which are all higher than 90 % (see par. 5.3). The latter is in line with the results obtained in the method development and between-lab validation phases of the CANFAS project where recoveries were consistently higher than 90 % (see Second Annual Report CANFAS, J. de Jong, 12-08-2000). The Mandel h plot (see Figure 1) shows low results for lab 26 across all levels. This could be caused by a systematic low recovery for the method in this lab. Together with the results, lab 26 had already indicated that they had a number of problems with the

chromatography, mainly with regards to the sensitivity of the method (see par. 5.4). Lab 26 was contacted to try to ascertain the cause of the discrepant behaviour. The lab suggested that the low results could be caused by degradation of the samples because "the final analysis was undertaken at the extreme limit of the time permitted. We may have experienced a degree of sample degradation even though the samples were kept frozen throughout the time period between receipt and final analysis". The suggested sample degradation is not very likely because a number of other labs performed the analyses later than lab 26.

During the evaluation meeting it has been discussed with relation to the above-mentioned reasons whether the results of lab 26 should be discarded or not. The results of the discussions are described in Chapter 6 of this report.
When the results of lab 26 are discarded, statistical analysis of the results shows that lab 30 is still a Cochran outlier for the 5 mg/kg sample. The resulting values for the relative standard deviations for repeatability are not very different from the values including lab 26 (see Table 8). The resulting values for the relative standard deviations for reproducibility and the HORRAT ratios are slightly improved (see Table 6).

Table 6: Horrat ratios of the Maduramicin collaborative study after discarding lab 26

| Mean after elimination of outliers[1] (mg/kg) | Predicted $rsd_R$ | Established $rsd_R$ | Horrat[2] | Conclusion |
|---|---|---|---|---|
| 2,679 | 13,794 | 13,570 | 0,98 | Reproducibility OK |
| 4,543 | 12,740 | 25,070 | 1,97 | Reproducibility OK |
| 5,184 | 12,490 | 14,690 | 1,18 | Reproducibility OK |
| 9,654 | 11,374 | 14,140 | 1,24 | Reproducibility OK |
| 472,0 | 6,334 | 9,195 | 1,45 | Reproducibility OK |

[1] = lab 29/sample 450ppm; lab 30/sample 5 ppm
[2] = Horrat is the ratio between the established $rsd_R$ and the predicted $rsd_R$

The corresponding Mandel h and k plots are shown in Figure 2.

**Table 7. Maduramicin in one turkey feed, four broiler feeds and one premixture for broiler feed**

| | Result (mg/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | MAD 2,5 mg/kg | | MAD 4,5 mg/kg | | MAD 5 mg/kg | | MAD 9 mg/kg | | MAD 450 mg/kg | |
| Lab | | | | | | | | | | |
| 17 | 2,51 | 2,67 | 4,75 | 4,71 | 4,94 | 4,97 | 9,38 | 9,41 | 503 | 514 |
| 22 | 2,64 | 2,57 | 4,87 | 5,08 | 5,52 | 5,04 | 9,44 | 9,52 | 485 | 510 |
| 23 | 3,18 | 3,28 | 5,91 | 6,49 | 6,76 | 6,44 | 12,07 | 12,90 | 501 | 507 |
| 26 | 2,20 | 1,90 | 3,30 | 3,70 | 4,00 | 4,00 | 6,20 | 7,50 | $340,8^{Gdls}$ | |
| 27 | 2,65 | 2,67 | 4,77 | 4,73 | 5,21 | 4,90 | 9,56 | 9,56 | 456 | 474 |
| 29 | 2,41 | 2,84 | 5,01 | 5,25 | 5,79 | 5,57 | 10,90 | 10,63 | *644* | *711* |
| 30 | 3,00 | 3,10 | 2,40 | 1,70 | *6,60*$^{Co}$ | *5,30*$^{Co}$ | 8,90 | 10,40 | 491 | 511 |
| 31 | 2,30 | 3,00 | 4,40 | 4,90 | 4,90 | 4,90 | 9,00 | 9,10 | 444 | 469 |
| 35 | 2,20 | 1,90 | 4,50 | 3,80 | 4,00 | 3,90 | 8,70 | 7,30 | $378^{Gdls}$ | $385^{Gdls}$ |
| 36 | 2,80 | 2,50 | 4,70 | 3,80 | 5,00 | 5,10 | 8,60 | 8,40 | 444 | 479 |

| | | | | | |
|---|---|---|---|---|---|
| number of labs | 10 | 10 | 9 | 10 | 9 |
| m (mg/kg) | 2,62 | 4,44 | 5,05 | 9,37 | 464 |
| $rsd_r$ (%) | 8,53 | 8,19 | 3,29 | 6,18 | 3,15 |
| $rsd_R$ (%) | 15,3 | 25,4 | 16,1 | 16,8 | 11,4 |

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

Key to symbols:

result$^{Co}$ = Cochran outlier

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

Table 7: Maduramicin in one turkey feed, four broiler feeds and one premixture for broiler feed

**Table 8. Maduramicin in one turkey feed, four broiler feeds and one premixture for broiler feed**

| | Result (mg/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | MAD 2,5 mg/kg | | MAD 4,5 mg/kg | | MAD 5 mg/kg | | MAD 9 mg/kg | | MAD 450 mg/kg | |
| Lab | | | | | | | | | | |
| 17 | 2,51 | 2,67 | *4,75* | *4,71* | 4,94 | 4,97 | 9,38 | 9,41 | 503 | 514 |
| 22 | 2,64 | 2,57 | 4,87 | 5,08 | 5,52 | 5,04 | 9,44 | 9,52 | 485 | 510 |
| 23 | 3,18 | 3,28 | 5,91 | 6,49 | 6,76 | 6,44 | 12,07 | 12,90 | 501 | 507 |
| 26 | *2,20* | *1,90* | *3,30* | *3,70* | *4,00* | *4,00* | *6,20* | *7,50* | *341* | |
| 27 | 2,65 | 2,67 | 4,77 | 4,73 | 5,21 | 4,90 | 9,56 | 9,56 | 456 | 474 |
| 29 | 2,41 | 2,64 | 5,01 | 5,25 | 5,79 | 5,57 | 10,90 | 10,63 | *644* | *711* |
| 30 | 3,00 | 3,10 | 2,40$^{Gls}$ | 1,70$^{Gls}$ | *6,60*$^{Co}$ | *5,30*$^{Co}$ | 8,90 | 10,40 | 491 | 511 |
| 31 | 2,30 | 3,00 | 4,40 | 4,90 | 4,90 | 4,90 | 9,00 | 9,10 | 444 | 469 |
| 35 | 2,20 | 1,90 | 4,50 | 3,80 | 4,00 | 3,90 | 8,70 | 7,30 | 378$^{Gls}$ | 385$^{Gls}$ |
| 36 | 2,80 | 2,50 | 4,70 | 3,80 | 5,00 | 5,10 | 8,60 | 8,40 | 444 | 479 |

| | | | | | |
|---|---|---|---|---|---|
| number of labs | 9 | 9 | 8 | 9 | 8 |
| m (mg/kg) | 2,68 | 4,54 | 5,18 | 9,65 | 472 |
| $rsd_r$ (%) | 8,37 | 8,17 | 3,40 | 5,48 | 3,10 |
| $rsd_R$ (%) | 13,6 | 25,1 | 14,7 | 14,1 | 9,2 |

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

Key to symbols:

result$^{Co}$ = Cochran outlier

result$^{Gls}$ = Grubb's lower straggler

Table 8: Maduramicin in one turkey feed, four broiler feeds and one premixture for broiler feed after elimination of lab 26

Figure 1: Mandel h and k plots after elimination of lab 29 (450 mg/kg) and lab 30 (5 mg/kg)

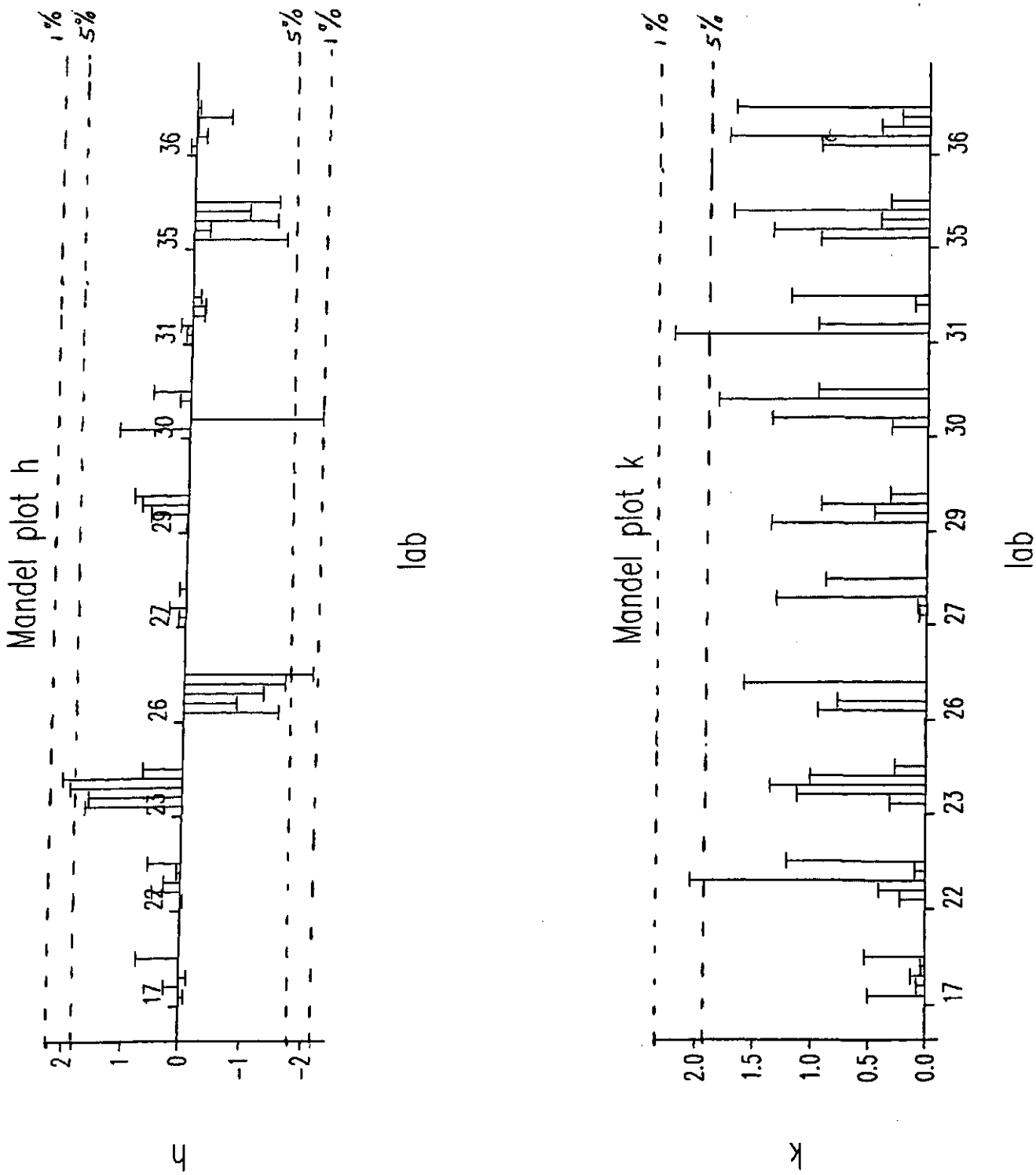
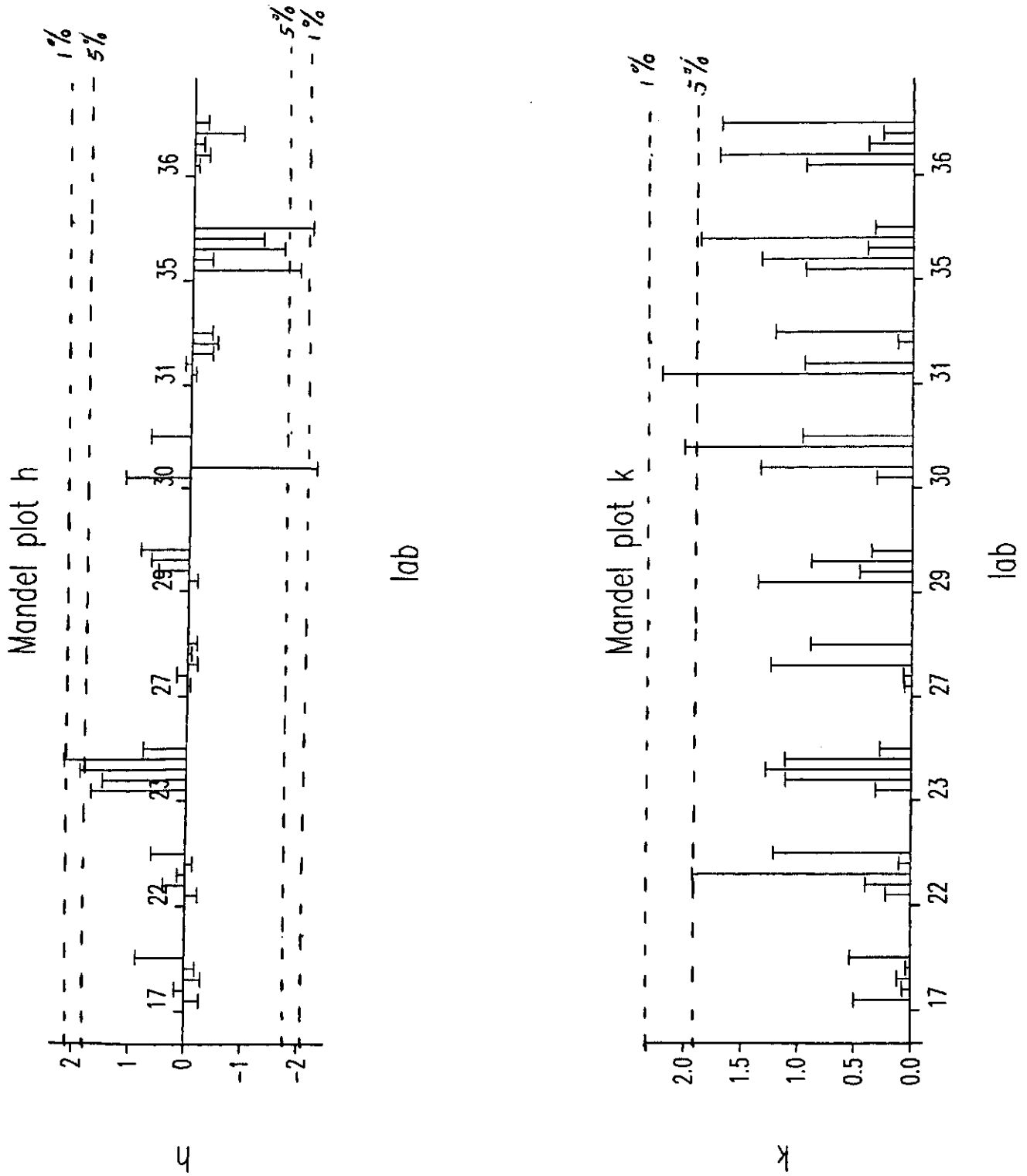


Figure 2: Mandel h and k plots after elimination of lab 29 (sample 450 mg/kg), lab 30 (sample 5 mg/kg) and all results of lab 26



## 5.2 Blank samples

Table 9: Reported results of the participants for the blank samples

| Partner | Blank sample 1 | Blank sample 2 |
|---|---|---|
| 17 | - | - |
| 22 | <1 | <1 |
| 23 | <0,39 | <0,39 |
| 26 | 0,8 | 0,7 |
| 27 | Not detectable | Not detectable |
| 29 | 0 | 0 |
| 30 | <1 | <1 |
| 31 | 0 | 0 |
| 35 | <2 | <2 |
| 36 | 0 | 0 |

One laboratory (nr 26) detected a signal in the blank samples, corresponding to 0,8 and 0,7 mg/kg. This signal was below their limit of quantification, which was estimated at 1 mg/kg.

## 5.3 Recoveries

Table 10: Recoveries

| Partner | Recovery 1 in % | Recovery 2 in % | Recovery average in % |
|---|---|---|---|
| 17 | 99 | 100 | 100 |
| 22 | 99 | 103 | 101 |
| 23 | Not reported | | |
| 26 | 80 | | 80 |
| 27 | 103 | | 103 |
| 29 | 100 | 110 | 105 |
| 30 | 103 | | 103 |
| 31 | 99<br>111 | 101<br>106 | 100 (day 1)<br>108 (day 2) mean: 104 |
| 35 | 97 | 95 | 96 |
| 36 | | | 99 (feeds)<br>99 (premixes) |

Except for lab 26, all recoveries were higher than 90% (see also paragraph 5.1). The result of lab 26 (80% recovery) is a straggler according to the Grubbs' test.

## 5.4 Remarks

Table 11: Remarks made by the partners

| Partner | Remarks |
|---|---|
| 17 | No remarks |
| 22 | The lower limit 0,2 µg/ml was the lowest standard concentration achievable due to the ripple in the base-line. |
| 23 | Not reported |
| 26 | - The extraction and clean up was straightforward and easy to follow.<br>- We had a great number of problems with the chromatography.<br>- The documentation does not mention the need to eliminate all stainless steel fittings between the mixing chamber and detection<br>- Even with the recommended length of tubing between the reaction coil and the detector we found it impossible to eliminate the baseline noise.<br>- As a result of the baseline noise we found it very difficult to integrate the lowest standard consistently even with a 100 µl injection loop<br>- After several runs we experienced an increase in back pressure generated by the mixing chamber<br>- Due to the above and pressure of time we are able to submit only 1 result for the premix. |
| 27 | There were no problems. On the four days, when analyses have been done, the linearity of the calibration graphs area vs. Concentration (from 0,2 µg/ml to 2,0 µg/ml) can be described as follows:<br>r= 0,999987; 0,999852; 0,999947; 0,999724<br>Two remarks to the method description:<br>- In the chapter "Apparatus"(4.3.3) and in the chapter "Parameters" (5.3.1) you should write 5 µm for the particle size of reversed phase material in the analytical column instead of 5 µ.<br>In the chapter "Parameters, mobile phase" (5.3.1) the mixing proportion of the mixture of phosphate buffer solution with methanol (100 + 900 [V+V]) does not correspond exactly to that one described in the chapter "Reagents" (3.7) because of the phenomenon of volume contraction. I would like to write "mixture (Vmi) of phosphate-buffer solution (Vp) with methanol 100/1000 (Vp/Vmi)". |
| 29 | We made some little modifications on the method such as:<br>- The decanted supernant was placed in the refrigerator overnight, in feedingstuffs and in premixtures (5.2.1, 5.2.2) and not in the freezer for 2-3 hours.<br>- There was no dilution for premix, but maybe it would be more correct to do one because the value for the peak area was much higher than the area value for the highest standard (187 µg/ml).<br>- The reaction temperature was settled at 98-100 °C.<br>- The injection was of 200 µl and not 50 µl because of the detector sensitivity. |

| Partner | Remarks |
|---|---|
| 30 | The method is not sensitive enough. The risk of interference of other ionophores is important. |
| 31 | Temperature of reaction was 98 °C instead of 95 °C. This method has to be considered as semi quantitative because of the low signal to noise ratio. The results were strongly influenced by the integration with the Turbochrom program. It was not possible to fix a clear baseline. |
| 35 | No remarks |
| 36 | We used our in-house method. (Note form the co-ordinator: this method only differs with regards to the chromatographic conditions and consequently it can be regarded as equivalent. No recovery experiments have been performed; recovery experiments are part of our in-house validation. |

## 5.5 Special requests

The following partner performed another method (Cyanamid method, pre-column derivatisation with dansylhydrazine)

- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine.

The following partners performed the post-column derivatisation reaction with DMAB (dimethylaminobenzaldehyde) instead of with vanillin:

- LUFA - Augustenberg, Karlsruhe, Germany; A. Thalmann, K. Wagner,
- Hoffmann-La Roche Ltd., Basel, Switzerland; P. Hofmann, A. Zuber
- Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany; H. Putzka, D. Böhm.

### *5.5.1 HPLC conditions*

Table 12: HPLC conditions

| Partner | Column | Mobile phase |
|---|---|---|
| Rijksontledingslaboratorium Tervuren, Belgium | Hypersil ODS, 25 cm x 4,9 mm | TBAS solution : acetonitril = 20:80 (v/v) |
| LUFA - Augustenberg, Karlsruhe, Germany | Hypersil ODS, 5 µm 250x4,6 mm | As described in the method |
| Hoffmann-La Roche Ltd., Basel, Switzerland | Hypersil ODS, 5 µm, 250 x 4 mm | 100 ml Phosphate buffer pH4, 80 ml tetrahydrofurane, to 1000 ml with methanol |
| Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany | As described in the method | As described in the method |

### *5.5.2 Recoveries*

Table 13: Recoveries

| Partner | Recovery 1 in % | Recovery 2 in % | Recovery average in % |
|---|---|---|---|
| Rijksontledingslaboratorium Tervuren, Belgium | 95 | | 95 |
| LUFA - Augustenberg, Karlsruhe, Germany | 104 | 100 | 102 |
| Hoffmann-La Roche Ltd., Basel, Switzerland | | | 99,4 (feed)<br>99,3 (premixes) |

### *5.5.3 Remarks*

Table 14: Remarks made by the partners

| Partner | Remarks |
|---|---|
| Rijksontledingslaboratorium Tervuren, Belgium | The Cyanamid method was followed because with the proposed CANFAS method problems with sensitivity and problems with the post-column derivatisation (air bubbles and non stabile system) occurred. |
| LUFA - Augustenberg, Karlsruhe, Germany | The reason for post-column derivatisation with DMAB instead of with vanillin was that the reaction with vanillin was too weak |
| Hoffmann-La Roche Ltd., Basel, Switzerland | No remarks |
| Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany | As to post-column derivatization with "DMAB-reagent" one test was made with 3 different standard solutions and 2 sample extracts. The conditions were nearly the same as with the prescribed vanillin method, i.e. we used the same post column reactor coil and the temperature of the water bath was 93°C. Reagents 3.13. and 3.14 were mixed (1+1/v+v) and thus added by 1 pump, but the flow rate of the post-column reagent was only 0,4 ml/min (the same as with the vanillin reagent), the flow rate of the mobile phase was 0,4 ml/min as well, injection volume was 50 µl, wavelength for detection 600 nm. Result: The corresponding signals for the area were about 5% higher than those caused by the vanillin reagent (on an average). |

*5.5.4 Results of the samples*

Table 15: Results reported by the partners

| Partner | Rijksontledingslaboratorium Tervuren, Belgium | | LUFA - Augustenberg, Karlsruhe, Germany | | Hoffmann-La Roche Ltd., Basel, Switzerland |
|---|---|---|---|---|---|
| Method | Cyanamid | | DMAB | | DMAB |
| Sample content (mg/kg) | Reported result (mg/kg) | | | | |
| 0 | <1 | | 0 | | 0 |
| 0 | <1 | | 0 | | Not analysed |
| 2,5 | 2,6 | | 2,4 | | 2,9 |
| 2,5 | 2,9 | | 2,3 | | Not analysed |
| 4,5 | 4,7 | | 4,9 | | 3,9 |
| 4,5 | 4,6 | | 4,6 | | Not analysed |
| 5 | 4,9 | | 4,6 | | Not analysed |
| 5 | 5,3 | | 4,1 | | Not analysed |
| 9 | 9,3 | | 9,1 | | 8,7 |
| 9 | 8,6 | | 8,9 | | Not analysed |
| Premixture | 276 | 276 | 443 | 528 | Not analysed |

Representative chromatograms of LUFA-Augustenberg and Hoffmann-La Roche are included in Appendix 6.

The results obtained with DMAB do not differ significantly from the mean values obtained with vanillin (see paragraph 5.1). Applying pre-column derivatisation with dansylhydrazine, for feeds the same conclusion can be drawn but for the premixture significantly lower values are obtained. The reason for this is not clear.

# 6 EVALUATION AND CONCLUSIONS

The results of the collaborative study were evaluated in a meeting in Tervuren (Belgium) on June 19-20, 2001.
After thorough discussions the panel decided that the results of lab 26 should be taken into account in the statistical evaluation. The relatively low results at all levels (see Mandel h plot, Fig. 1) do not justify removal of the results because only 1 value is below the 5 % indicator line. The problems with the sensitivity of the method that were reported by lab 26 are no reason to remove the results because some other laboratories experienced the same problem. During the meeting the question was raised if the recovery value reported by lab 26 (80 %) is an outlier. This was checked later on by Jaap Driessen (RIKILT): lab 26 is a Grubbs' straggler, but not a Grubbs' outlier.

The panel has accepted the results of the statistical evaluation, as described in Table 7 (including the results of lab 26). Consequently it can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blind blank feeds and for the recovery are also acceptable. The panel agreed that the method can be recommended for adoption as an official method.

The co-ordinator will send an enquiry to the participants about the type of detector that has been used, any special arrangements to increase the sensitivity in the VIS region and about the data acquisition system applied.
The following points will be changed in the method:

- The use of stainless steel tubing in the post-column reactor and detector should be avoided
- A remark will be added about the suitability of DMAB for post-column derivatisation, stating that a full validation with DMAB has not been performed

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

- Lab 27, remarks about particle size and mobile phase composition

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), CEN and ISO.

## ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Dr. P. Hofmann, Hoffmann-La Roche, Basel, Switzerland, is thanked for supplying the maduramycin reference standard.
Dr. H. van de Voet, Biometris, Wageningen University and Research Centre is thanked for statistical advice.

# APPENDIX 1

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

cc Driessen, De Jong

to addressee

DATE
25 October 2000

SUBJECT
collaborative study CANFAS
maduramicin 71.316.24

ENCLOSURE(S)
5

OUR REFERENCE
00/0024096

HANDLED BY
Dr. J. de Jong

DIRECT (TELEPHONE) LINE
+31 317 47 55 81

E-MAIL
j.dejong@RIKILT.WAG-UR.nl

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CHAMBER OF COMMERCE REGISTRATION NO.
09098104 te Arnhem

THE INTERNET
www.rikilt.wageningen-ur.nl

Dear colleague,

With separate post the samples for the collaborative study for maduramicin will be sent to you by dr. Hofmann (Hoffmann-La Roche). We expect the samples will be sent within one or two weeks from now. You will receive the following samples :

- 10 feed samples, with the text "additive: MADURAMICIN" and with a sample code; these samples constitute 4 blind duplicates of feed samples containing maduramicin (contents in the range between 1 and 15 mg/kg) and 1 blind duplicate of a blank feed
- 1 premixture containing maduramicin, content in the range between 200 and 1000 mg/kg.

**As discussed in the kick-off meeting, please store the samples frozen and perform the analysis within 2 weeks after receipt.**

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

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

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

Annex 2 contains the reporting form. This form has already been send to you by E-mail as an Excel 5.0 file. We strongly prefer to get the results back in electronic form by E-mail (please send the results to the following E-mail address: j.j.m.driessen@rikilt.wag-ur.nl). Of course you can also fill in the form and send it by fax or normal mail.
The **deadline** for reporting the results is **22 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 post-column derivatisation with DMAB.

The reference standard of maduramicin which has to be used will be send to you by Mr. Hofmann (Hoffmann-La Roche) together with the samples. In the calculations please take into account the purity of the reference standard.

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
collaborative studies

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

cc
Mrs. D. Bennink, European Commission, DG Research, CII/3, Brussels

**RIKILT**
**State Institute for Quality Contr of Agricultural Products**

DATE
**25 October 2000**

OUR REFERENCE
**00/0024096**

**Annex 1 - Description of the method**

CANFAS/MAD/09102000/B.STOISSER

# DETERMINATION OF MADURAMICIN-AMMONIUM

## 1 PURPOSE AND SCOPE

The method is for the determination of maduramicin in feedingstuffs and premixtures. The usual concentration of maduramicin in feedstuffs is 5 mg/kg, in premixtures 500 mg/kg. The limit of determination is 2 mg/kg. The limit of detection is 0,5 mg/kg.

## 2 PRINCIPLE

The sample is extracted with methanol. The content of maduramicin is determined by reversed-phase-high-performance-liquid chromatography (HPLC) with post column derivatisation with vanillin using a Vis-detector.

## 3 REAGENTS

### 3.1 Methanol, HPLC-grade

### 3.2 1,5-Dimethylhexylamine

### 3.3 Sulfuric acid, 95-97%, p.a.

### 3.4 Ortho-phosphoric acid, appr. 85%, p.a.

### 3.4.1 Diluted o-phosphoric acid:

Dissolve 10 ml of o-phosphoric acid (3.4) to 100 ml with demineralised water

### 3.5 Potassium dihydrogen phosphate, p.a.

### 3.6 Phosphate buffer solution 10 mmol/l, pH=4,0:

Dissolve 1.36 g of potassium dihydrogen phosphate (3.5) in 500 ml of demineralised water. Add 3.0 ml of o-phosphoric acid (3.4) and 10 ml of 1,5-dimethyl hexylamine (3.2) . Adjust the pH to 4.0 with diluted o-phosphoric acid (3.4.1) and fill with demineralised water to 1000 ml.
The solution can be stored some weeks, but if fungus grow, prepare a new one.

### 3.7 Mobile phase:

Dilute 100 ml of phosphate-buffer solution 10 mmol/l, ph=4 (3.6) with methanol (3.1) to 1000 ml.

### 3.8 Vanillin ≥ 98% (HPLC)

### 3.8.1 Vanillin reagent:

Dissolve 10 g of vanillin (3.8) in a mixture of 250 ml of methanol (3.1) and 5.0 ml of sulfuric acid (3.3). Mix well and sonicate for some min under vacuum at room temperature. This solution has to be prepared daily prior to use and has to be cooled with ice water during use.

### 3.9 Maduramicin $K^+$- salt (8.4)

The purity-grade accounted as $NH_4^+$-salt has to be taken into consideration at the following operations

#### 3.9.1 Stock-standard-solution 100 µg/ml:

Dissolve in a 100 ml volumetric flask to the nearest 0.1 mg10 mg of reference standard (3.9) with methanol (3.1) to 100 ml. This solution should be stored at 4°C not longer than 1 month.

#### 3.9.2 Standard solution 10 µg/ml:

Dilute 10.0 ml of the stock-standard-solution (3.9.1) to 100 ml with methanol (3.1) in a 100 ml volumetric flask

Standard solutions should be stored at 4°C not longer than 1 week.

#### 3.9.3 Standard solution 1 µg/ml

Dilute 2.0 ml of the stock-standard-solution (3.9.1) to 200.0 ml with methanol (3.1) in a 200 ml volumetric flask.

Standard solution should be prepared freshly

#### 3.9.4 Calibration solutions

Into a series of 50 ml graduated flasks transfer 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 ml of the intermediate standard solution (3.9.2). Make up to the mark with methanol and mix. These solutions correspond to 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 µg of maduramicin per ml respectively.

Calibration solutions should be prepared freshly

## 4 APPARATUS

### 4.1 Centrifuge

### 4.2 Ultrasonic bath

### 4.3 HPLC-equipment

with pump and column oven

#### 4.3.1 Autosampler with injection system,

suitable for injection of 50 µl

#### 4.3.2 (UV)-Vis-detector (8.1)

#### 4.3.3 Liquid chromatographic column

250x4.6 mm,5µ,Hypersil BDS C 18 or equivalent

#### 4.3.4 Reagent pump

#### 4.3.5 PEEK mixing chamber

#### 4.3.6 PTFE reaction coil (knitted) (8.3)

0.5 mm x 10 m (volume ≈2.0 ml)

#### 4.3.7 Reactor oven for the PTFE-reaction coil,

suitable to 100°C (or suitable water bath)

### 4.4 Freezer

### 4.5 Membrane-filter, PTFE , 0.22µm

## 5 PROCEDURE

### 5.1 General

#### 5.1.1 Blank feed

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

#### 5.1.2 Recovery test

A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of maduramicin, similar to that present in the sample. To fortify at a level of 5 mg/kg transfer 500 µl stock-standard solution (100 µg/ml (3.9.1)) to the flask. Add 10 g of the blank feed, mix thoroughly and leave for 10 min, mixing again several times before proceeding with the extraction step (5.2).

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

### 5.2 Extraction

#### 5.2.1 Feedingstuffs

Weigh to the nearest 0.01 g 10 g of the ≤ 1mm ground sample into a 250 ml volumetric flask and add 50 ml methanol (3.1) . Close the flask with a suitable method, and place in a ultrasonic bath at 50° C for 20 minutes. Shake vigorously, store and cool down to room temperature appr. 15 min, decant the clear supernant and place in freezer for 2 - 3 hours to settle down fat. Then centrifuge an aliquote for 1-2 min. After membrane (4.5) filtration 50 µl of this solution are injected into the HPLC-apparatus.

#### 5.2.2 Premixes

Weigh to the nearest 0.01 g 1 g of the ≤ 0.5mm ground sample into a 250 ml volumetric flask and add 50 ml methanol (3.1) . Close the flask with a suitable method, and place in an ultrasonic bath at 50° C for 20 minutes. Cool down to room temperature, shake vigorously , store some minutes and dilute an aliquote 1 : 10 of the clear supernatant with methanol and place in freezer for 2-3 hours to settle down fat. Then centrifuge an aliquote for 1-2 min. After membrane (4.5) filtration 50 µl of this solution are injected into the HPLC-apparatus.

### 5.3 HPLC determination

#### 5.3.1 Parameters:

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

| | |
|---|---|
| Analytical column (4.3.3) | 250 x 4.6 mm, 5µ , Hypersil BDS C 18 |
| Mobile phase (3.7) | Mixture of phosphate-buffer solution (3.6) with methanol (3.1), 100 + 900 (V + V) |
| Column oven: | 40°C |
| Flow rate: | 0.4 ml/min |

| | |
|---|---|
| Flow rate reagent pump: | 0.4 ml/min |
| Reactor temperature: | 95°C (8.2) |
| Detection wavelength: | 520 nm |
| Injection volume: | 50 µl |
| Retention time: | approx. 25 min |
| Run time: | 30 –35 min |

Check the stability of the chromatographic system, injecting several times the calibration solution (3.9.3) containing 1.0 µg/ml, until constant peak areas and retention times are achieved.
Working with the described conditions there is baseline separation from other ionophores like salinomicin, narasin, monensin, semduramicin.

### 5.3.2 Calibration graph

Inject each calibration solution (3.9.4) several times and determine the mean peak areas for each concentration. Plot a calibration graph using the mean peak areas of the calibration solutions as the ordinate and the corresponding concentrations in µg/ml as the abscissae.

### 5.3.3 Sample solution

Inject the sample extract (5.2) at least 2 times using the same volume as taken for the calibration solutions and determine the mean peak area of the maduramicin peaks.

## 6 CALCULATION OF THE RESULTS

From the mean area (8.5) of the maduramicin peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2)
The maduramicin content in mg/kg of the sample is given by the following formula:

$$\text{mg maduramicin/kg} = \frac{C \cdot 50 \cdot F}{M}$$

C = maduramicin concentration of the sample extract (5.2) in µg/ml

M = mass of the test portion in g

F = dilution factor according to (5.2)

## 7 VALIDATION OF THE RESULTS

### 7.1 Identity

The identity of maduramicin can be confirmed by co-chromatogrphy.
A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution. The amount of added maduramicin should be similar to the amount of maduramicin found in the sample extract.
Only the height of the maduramicin-peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of the

height, must be within ± 10% of the original width of the maduramicin peak of the unfortified sample extract.

### 7.2 Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed xx % relative to the higher result for maduramicin contents.

## 8 OBSERVATIONS

8.1 The detector is used in visual scope at 520 nm and should give sufficient light energy. Noise preferabably should be < $1.10^{-5}$ AU (250nm, 600nm)

8.2 A temperature of 92°C to 98°C is possible, high stability (± 1°C ) should be guaranteed

8.3 The length of the teflon tube (e.g. 1m ID 0.25 mm) between reagent-pump and mixing chamber and the length of the teflon tube (e.g. 3m ID 0.17 mm) between reactor and detector should be optimized if there are problems with bubbles

8.4 Maduramicin is very toxic. LD50 = 33mg/kg (rat).

8.5 Only area is allowed for calculation

# 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:** MADURAMICIN

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 312233 | | |
| 312247 | | |
| 312264 | | |
| 312307 | | |
| 312331 | | |
| 312334 | | |
| 312357 | | |
| 312359 | | |
| 312362 | | |
| 312367 | | |

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

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

### Annex 3 - Instructions for handling and analysis of the samples

1. Storage

Store the samples **frozen** until analysis

2. Analysis

Analyse the samples within 2 weeks after receipt

3. Milling

- Feed samples: grind the feed samples with a mill equipped with a 1 mm screen
- Premix: grind the premix sample with a mill equipped with a 0.5 mm screen. Take care of contamination of the milling equipment.

After milling, store the samples at 4 °C (or frozen if the time between milling and analysis is longer than one week).

4. Mixing of the test samples before weighing

Mix the entire sample thoroughly

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

***Annex 4 - Questionnaire***

Laboratory: .....

Contact person: .....

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

Dilution factor of the samples:

- Feed samples (specify for which feed samples): .....
  .....
- Premixture: .....

Chromatographic conditions:

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

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

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

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

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 - MADURAMICIN

### Annex 5 - Special requests

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

- post-column reaction with DMAB

Conditions for post-column derivatisation :

3.1 Methanol - HPLC grade

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

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

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

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

4.1.3 Post-column reactor (double pump or two single pumps) with mixing chamber, reaction coil of inert material (f.e. Teflon or Peek) for operation at 95 °C, 7.0 m with 0.33 mm ID and water bath or reactor oven for operation at 95 °C

| | |
|---|---|
| Flow rate of methanol-sulphuric acid-mixture (3.13) | 0.4 ml/min |
| Flow rate of DMAB-solution (3.14) | 0.4 ml/min |
| Temperature of the post-column reaction | 95 °C |
| VIS-Detector after post-column reaction | 600 nm |
| Volume of injections | 100 µl |

8.4 Post-column reaction

If only one pump for the post-column reaction is available, the reagents 3.13 and 3.14 may be mixed (1 + 1 v/v). Since DMAB undergoes quick auto-oxidation resulting in darkening of the solution this has to be kept protected from light in an ice bath and has to be used within 24 h.

*Other conditions are not changed*

Please report the results in a copy of annex 4 and clearly describe your conditions, etc. if different from the above mentioned conditions. Please also include representative chromatograms.

**Thanks in advance for doing the additional work**

# APPENDIX 2

## Homogeneity of samples

# Homogeneity study

## Broiler feed I (5% fat) supplemented with a nominal of 2,5mg/kg Maduramicin

| Sachet No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st Assay | 2,78 | 2,78 | 2,59 | 2,35 | 2,72 | 2,53 | 2,82 | 2,78 | 2,59 | 2,36 |
| 2nd Assay | 2,98 | 2,73 | 2,53 | 2,44 | 2,74 | 2,93 | 2,65 | 2,85 | 3,11 | 2,54 |
| | | | | | | | | | | |
| Mean | | Source of Variation | Sum of Squares | dof | Mean Square | F ratio | | | | |
| 2,69 | 145,2186 | Between Sachets | 0,4966 | 9 | 0,0552 | 2,0021 | F_crit_95%= | 3,02 | | |
| | 145,4942 | Within Sachets | 0,2756 | 10 | 0,0276 | | F_crit_99%= | 3,78 | | |
| | 144,7220 | Total | 0,7722 | 19 | | | | | | |
| | | | | | Analytical Deviation | 0,166012 | Sampling Deviation | 0,11751 | | |
| | | | | | CV% within | 6,171452 | CV% between | 4,36845 | | |
| | | | | | | | | | | |
| | | | | | CV% total | 7,561094 | | | | |

BFL

# Homogeneity study

## Broiler feed II (8% fat) supplemented with a nominal of 4,5 mg/kg Maduramicin

| Sachet No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st Assay | 4,77 | 5,28 | 5,33 | 5,15 | 4,71 | 5,21 | 5,18 | 5,12 | 5,10 | 4,67 |
| 2nd Assay | 4,81 | 5,35 | 4,73 | 4,73 | 5,15 | 5,31 | 4,77 | 5,19 | 5,20 | 4,76 |

| Mean | | Source of Variation | Sum of Squares | dof | Mean Square | F ratio | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5,03 | 505,8974 | Between Sachets | 0,6839 | 9 | 0,0760 | 1,6209 | F_crit_95%= | 3,02 | | |
| | 506,3662 | Within Sachets | 0,4688 | 10 | 0,0469 | | F_crit_99%= | 3,78 | | |
| | 505,2135 | Total | 1,1527 | 19 | | | | | | |
| | | | | | Analytical Deviation | 0,216518 | Sampling Deviation | 0,12064 | | |
| | | | | | CV% within | 4,307957 | CV% between | 2,40026 | | |
| | | | | | CV% total | 4,931506 | | | | |

BFL

# Homogeneity study

## Turkey feed supplemented with a nominal of 5 mg/kg Maduramicin

| Sachet No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st Assay | 5,32 | 4,85 | 5,22 | 4,91 | 5,04 | 5,06 | 4,83 | 4,67 | 5,07 | 5,24 |
| 2nd Assay | 5,52 | 4,69 | 5,36 | 5,04 | 5,11 | 5,43 | 5,24 | 5,15 | 5,18 | 5,50 |
| | | | | | | | | | | |
| Mean | | Source of Variation | Sum of Squares | dof | Mean Square | F-ratio | | | | |
| 5,12 | 525,3831 | Between Sachets | 0,7878 | 9 | 0,0875 | 2,4244 | F_crit_95%= | 3,02 | | |
| | 525,7441 | Within Sachets | 0,3610 | 10 | 0,0361 | | F_crit_99%= | 3,78 | | |
| | 524,5952 | Total | 1,1489 | 19 | | | | | | |
| | | | | | Analytical Deviation | 0,190013 | Sampling Deviation | 0,1604 | | |
| | | | | | CV% within | 3,710108 | CV% between | 3,1311 | | |
| | | | | | | | | | | |
| | | | | | CV% total | 4,854733 | | | | |

GBFL

# Homogeneity study

## Broiler feed (5% fat) supplemented with a nominal of 9 mg/kg Maduramicin

| Sachet No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st Assay | 9,20 | 10,04 | 9,11 | 10,55 | 9,24 | 9,61 | 9,23 | 9,85 | 10,32 | 8,93 |
| 2nd Assay | 10,47 | 9,03 | 9,48 | 10,40 | 10,36 | 10,33 | 10,27 | 11,57 | 9,25 | 10,52 |
| | | | | | | | | | | |
| Mean | | Source of Variation | Sum of Squares | dof | Mean Square | F ratio | | | | |
| 9,89 | 1958,5909 | Between Sachets | 3,1400 | 9 | 0,3489 | 0,5683 | F_crit_95%= | 3,02 | | |
| | 1964,7300 | Within Sachets | 6,1391 | 10 | 0,6139 | | F_crit_99%= | 3,78 | | |
| | 1955,4509 | Total | 9,2791 | 19 | | | | | | |
| | | | | | Analytical Deviation | 0,783524 | Sampling Deviation | 0,418 | | |
| | | | | | CV% within | 7,92399 | CV% between | 4,22735 | | |
| | | | | | | | | | | |
| | | | | | CV% total | 8,981095 | | | | |

BFL

# Homogeneity study

## Premix supplemented with a nominal of 450 mg/kg Maduramicin

| Sachet No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st Assay | 517 | 443 | 506 | 478 | 481 | 525 | 473 | 396 | 515 | 496 |
| 2nd Assay | 522 | 496 | 480 | 500 | 470 | 504 | 481 | 467 | 519 | 479 |
| | | | | | | | | | | |
| Mean | | Source of Variation | Sum of Squares | dof | Mean Square | F ratio | | | | |
| 487,40 | 4763915 | Between Sachets | 12740 | 9 | 1415,5333 | 2,8407 | F_crit_95%= | 3,02 | | |
| | 4768898 | Within Sachets | 4983 | 10 | 498,3000 | | F_crit_99%= | 3,78 | | |
| | 4751175 | Total | 17723 | 19 | | | | | | |
| | | | | | Analytical Deviation | 22,32263 | Sampling Deviation | 21,415 | | |
| | | | | | CV% within | 4,579941 | CV% between | 4,3938 | | |
| | | | | | | | | | | |
| | | | | | CV% total | 6,346752 | | | | |

BFL

# APPENDIX 3

## Sample codes

**Sample codes supplied to the participants in the *maduramicin* collaborative study**

| **MADURAMICIN**<br>number of participants 14 | MAD turkey 5ppm<br>MAD 1a | MAD turkey 5ppm<br>MAD 1b | MAD broiler 2,5ppm<br>MAD 2a | MAD broiler 2,5ppm<br>MAD 2b | MAD broiler 4,5ppm<br>MAD 3a | MAD broiler 4,5ppm<br>MAD 3b | MAD broiler 9ppm<br>MAD 4a | MAD broiler 9ppm<br>MAD 4b | MAD blank 1a | MAD blank 1b |
|---|---|---|---|---|---|---|---|---|---|---|
| Participant code | | | | | | | | | | |
| 13 | 132282 | 132327 | 132306 | 132272 | 132235 | 132236 | 132352 | 132293 | 132301 | 132251 |
| 17 | 172271 | 172242 | 172238 | 172273 | 172294 | 172303 | 172371 | 172298 | 172344 | 172269 |
| 22 | 222350 | 222337 | 222263 | 222252 | 222341 | 222267 | 222365 | 222287 | 222275 | 222280 |
| 23 | 232260 | 232254 | 232240 | 232281 | 232259 | 232253 | 232237 | 232336 | 232299 | 232353 |
| 26 | 262250 | 262266 | 262309 | 262368 | 262355 | 262261 | 262279 | 262277 | 262354 | 262268 |
| 27 | 272292 | 272278 | 272300 | 272323 | 272338 | 272257 | 272370 | 272241 | 272276 | 272234 |
| 29 | 292330 | 292361 | 292262 | 292246 | 292343 | 292305 | 292342 | 292322 | 292325 | 292295 |
| 30 | 302296 | 302328 | 302324 | 302274 | 302329 | 302316 | 302318 | 302290 | 302315 | 302356 |
| 31 | 312357 | 312233 | 312359 | 312331 | 312362 | 312334 | 312307 | 312367 | 312264 | 312247 |
| 32 | 322283 | 322349 | 322347 | 322312 | 322245 | 322372 | 322321 | 322335 | 322360 | 322326 |
| 33 | 332248 | 332311 | 332286 | 332255 | 332289 | 332302 | 332304 | 332243 | 332244 | 332285 |
| 35 | 352320 | 352284 | 352314 | 352310 | 352348 | 352256 | 352332 | 352358 | 352366 | 352265 |
| 36 | 362291 | 362313 | 362351 | 362258 | 362288 | 362317 | 362270 | 362363 | 362297 | 362340 |
| 38 | 382308 | 382333 | 382364 | 382369 | 382239 | 382319 | 382339 | 382346 | 382249 | 382345 |

# APPENDIX 4

## Maduramcyin reference standard profille

ALPHARMA
Animal Health Division

TECHNICAL CENTER
1 DUGGAR DRIVE
WILLOW ISLAND, WV 26134-9711
Phone: (304) 665-4191
Fax: (304) 665-4187

# Certificate of Analysis

NAME: Maduramicin DATE: August 3, 2000

Specifications

| | |
|---|---|
| Lot No: | AC 9745-1C |
| Purity: | 95.9% (by HPLC) |
| Standard Type: | Secondary Standard |
| Reference No: | TC 4122 |
| Expiration Date: | August 2002 |
| Drying Conditions: | Dry at 60°C, 28 mm Hg, for three hours prior to use. |
| Storage Conditions: | Store in dark container, at < 8°C |

Analyzed By: John E Fryman
John E. Fryman

Released By: Joseph B Henry
Joseph B. Henry
Manager,
Analytical Development

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 17

# 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:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 172238 | | 2,51 |
| 172242 | | 4,94 |
| 172269 | | 0 |
| 172271 | | 4,97 |
| 172273 | | 2,67 |
| 172294 | | 4,75 |
| 172298 | | 9,38 |
| 172303 | | 4,71 |
| 172344 | | 0 |
| 172371 | | 9,41 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 514 | 503 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 28.11.2000

Dilution factor of the samples:

- Feed samples (specify for which feed samples): 1 (10g/50ml)
- Premixture: 100 (1g/50ml/1ml/10ml)

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0.4 ml/min
- Injection volume: 50 µl
- Retention time of maduramicin: 24.5 min 24,9

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

- Percentage recovery: 99.7 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99.4 % and 100.0 %
- Spiking level: 5 mg/kg

(Spiking level 2,5 mg/kg Perc. recovery 96%, 100%)

**D-7000 HSM: MAD./ SAL./ MON.** Series: 0559

**Sample Name: Premix**

Sample Description:

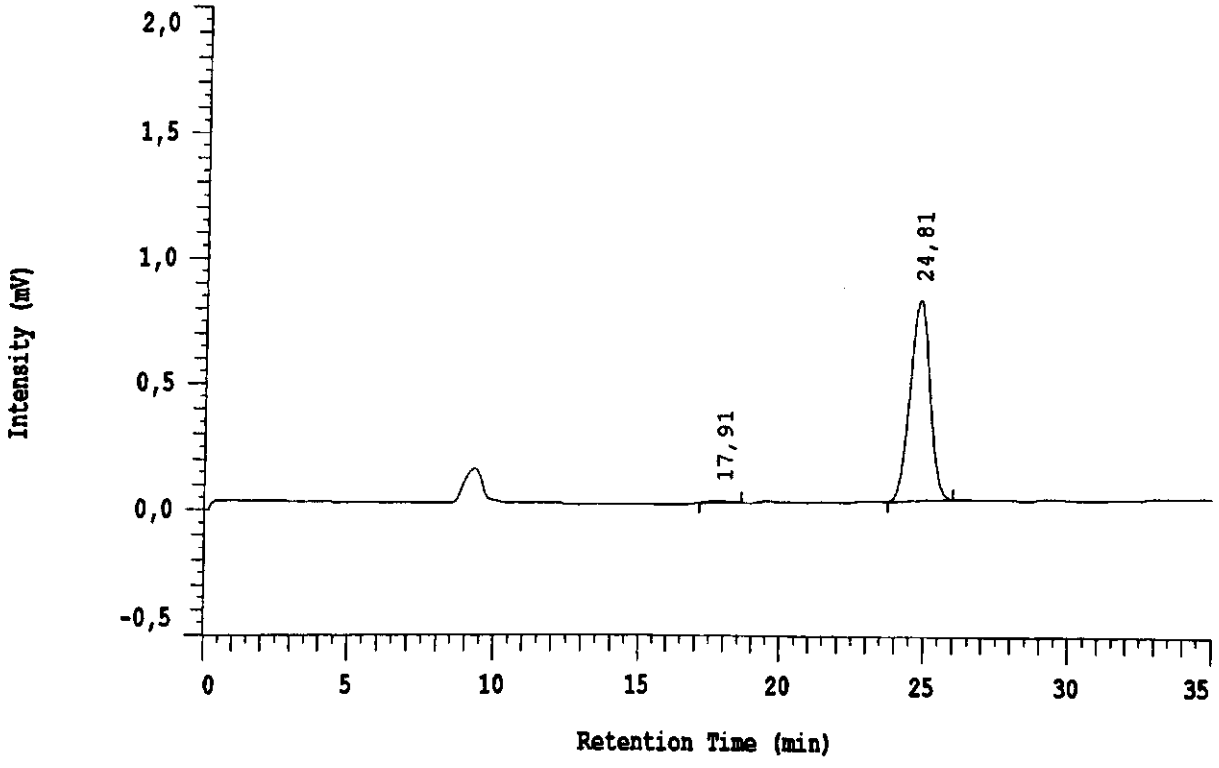
Analyzed: 28.11.00 15:35
Reported: 30.11.00 10:38
Processed: 30.11.00 10:38

Data Path: C:\Win32App\HSM\MADURAMYCIN\DATA\0559\

Application: MAD./ SAL./ MON.
Vial Number: 4
Injection from this vial: 1 of 1
Vial Type: UNK
Volume: 50,0 ul

Sample Description:

Chrom Type: HPLC Channel : 1




Acquisition Method: Maduramycin
Column Type: Hypersil BDS
Pump A Type: L-7100
Solvent A: MeOH/Buffer
Peak Quantitation: AREA
Sample Amount: 1,000
Calculation Method: EXT-STD
Scale Factor 1: 1,000

| Name | RT | Area | Height | Conc 1 | BC |
|---|---|---|---|---|---|
| | 17,91 | 475 | 9 | 0,000 | BB |
| Maduramycin | 24,81 | 37452 | 794 | 1,179 | BB |
| | | 37927 | 803 | 1,179 | |

Peak rejection level: 0

HPLC-pump: 0,4ml/min
Reagent-pump: 0,4ml/min
Collumn-temperature: 40°C
Reactor-temperature: 95°C

**D-7000 HSM: MAD./ SAL./ MON.** Series: 0560

**Sample Name: 172238**

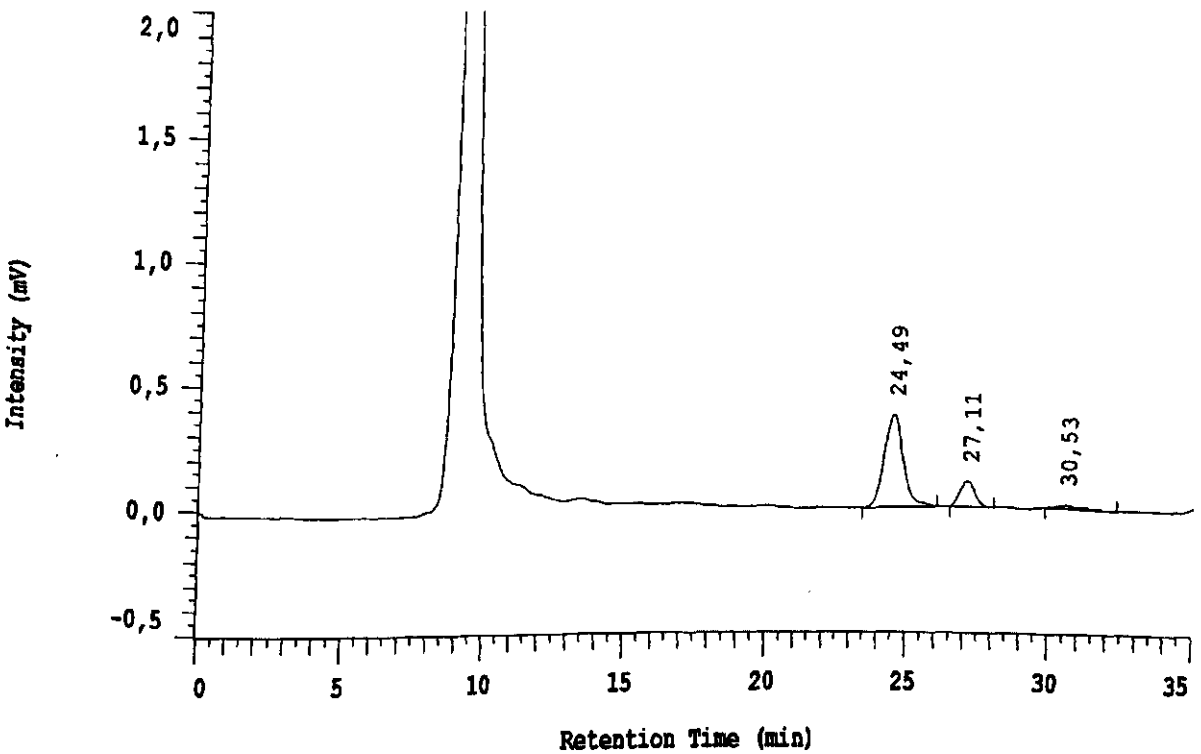
Sample Description:

Analyzed: 28.11.00 22:11
Reported: 30.11.00 10:46
Processed: 30.11.00 10:45

Data Path: C:\Win32App\HSM\MADURAMYCIN\DATA\0560\
Application: MAD./ SAL./ MON.
Vial Number: 13
Injection from this vial: 1 of 1
Vial Type: UNK
Volume: 50,0 ul

Sample Description:

Chrom Type: HPLC Channel : 1




Acquisition Method: Maduramycin
Column Type: Hypersil BDS
Pump A Type: L-7100
Peak Quantitation: AREA
Calculation Method: EXT-STD
Solvent A: MeOH/Buffer
Sample Amount: 1,000
Scale Factor 1: 1,000

| Name | RT | Area | Height | Conc 1 | BC |
|---|---|---|---|---|---|
| laduramycin | 24,49 | 16924 | 370 | 0,549 | BB |
| | 27,11 | 3760 | 103 | 0,000 | BB |
| | 30,53 | 835 | 13 | 0,000 | BB |
| | | 21519 | 486 | 0,549 | |

eak rejection level: 0

HPLC-pump: 0,4ml/min
Reagent-pump: 0,4ml/min
Collumn-temperature: 40°C
Reactor-temperature: 95°C

**D-7000 HSM: MAD./ SAL./ MON.** Series: 0560

## Sample Name: 172269

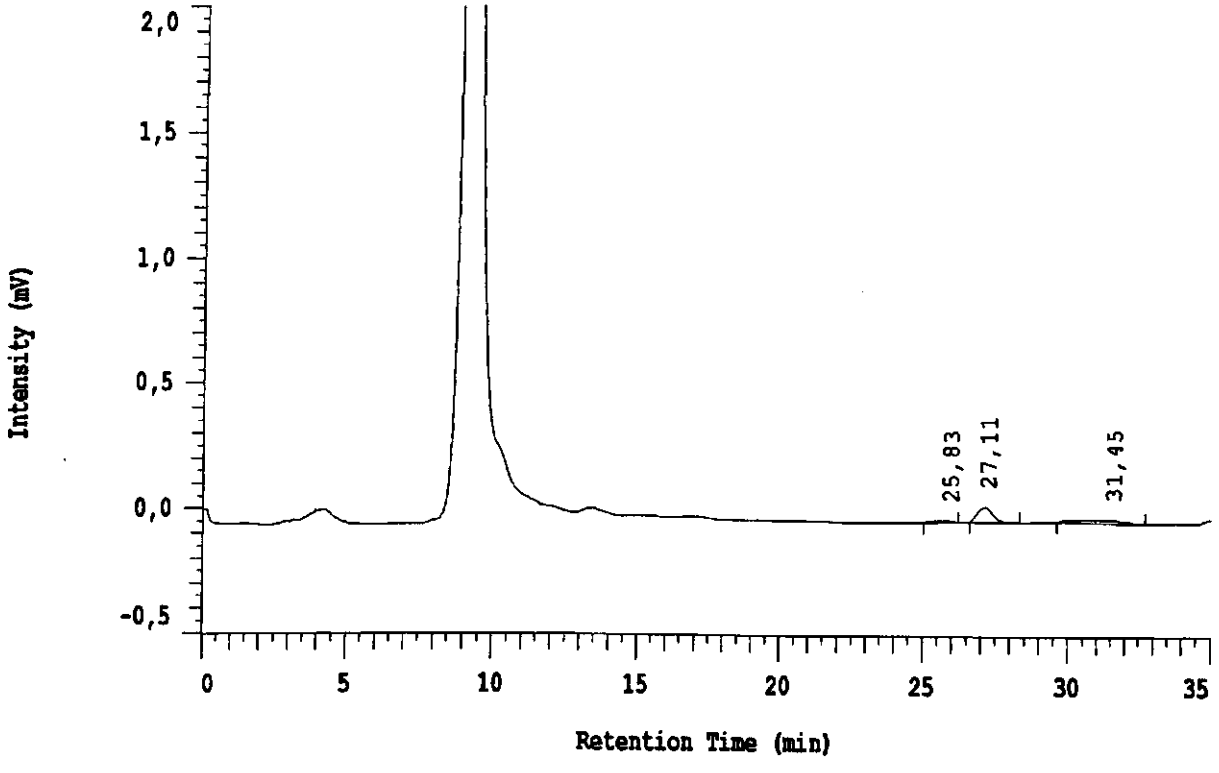
Sample Description:

Analyzed: 28.11.00 23:23
Reported: 30.11.00 11:03
Processed: 30.11.00 11:03

Data Path: C:\Win32App\HSM\MADURAMYCIN\DATA\0560\
Application: MAD./ SAL./ MON.
Vial Number: 15
Injection from this vial: 1 of 1
Vial Type: UNK
Volume: 50,0 ul

Sample Description:

Chrom Type: HPLC Channel : 1




Acquisition Method: Maduramycin
Column Type: Hypersil BDS
Pump A Type: L-7100
Peak Quantitation: AREA
Calculation Method: EXT-STD
Solvent A: MeOH/Buffer
Sample Amount: 1,000
Scale Factor 1: 1,000

| Name | RT | Area | Height | Conc 1 | BC |
|---|---|---|---|---|---|
| | 25,83 | 348 | 6 | 0,000 | BB |
| | 27,11 | 2273 | 60 | 0,000 | BB |
| | 31,45 | 1599 | 14 | 0,000 | BB |
| | | 4220 | 80 | 0,000 | |

Peak rejection level: 0

HPLC-pump: 0,4ml/min
Reagent-pump: 0,4ml/min
Collumn-temperature: 40°C
Reactor-temperature: 95°C

APPENDIX 5

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:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 222252 | | 2,64 |
| 222263 | | 2,57 |
| 222267 | | 4,87 |
| 222275 | | < 1 |
| 222280 | | < 1 |
| 222287 | | 9,44 |
| 222337 | | 5,52 |
| 222341 | | 5,08 |
| 222350 | | 5,04 |
| 222365 | | 9,52 |

| Sample / Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|
| Premixture | 485 | 510 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 00 11 23/25

Dilution factor of the samples:

- Feed samples (specify for which feed samples): F=1
- Premixture: F=10

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....

  Flow-rate: 0.4 ml/min
- Injection volume: 80 µl
- Retention time of maduramicin: 26 min

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

- Percentage recovery: 101 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99 % and 103 %
- Spiking level: 5.1 mg/kg

NEW TIMED EVENTS FROM MADURAMI

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

********** 12-08-2000 11:55:16 Version 5.1 **********

* Sample Name: prov nr 4 Data File: D:madu012

* Date: 11-23-2000 21:42:21Method: MADURAMI 12-06-2000 09:00:18 # 847 *

* 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: 32.00

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 |
|---|---|---|---|---|---|---|---|---|---|---|

TOTAL AMOUNT = 0.0000

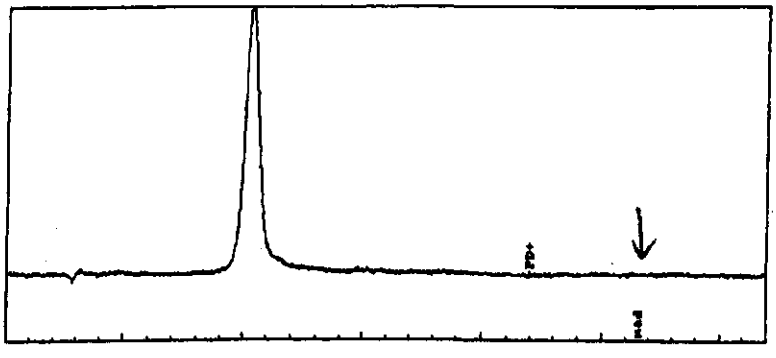
PEAKS NOT FOUND IN THIS RUN

| NAME | ADJUSTED RET.TIME. | REFERENCE PEAK |
|---|---|---|
| m | 26.54 | mad |

Data File = D:madu012.PTS Printed on 12-08-2000 at 11:55:20

Start time: 0.00 min. Stop time: 32.00 min. Offset: 0 mv.

Low Value: 0 uv High Value: 6102 uv Scale factor: 1.0



Sample 222275

NEW TIMED EVENTS FROM MADURAMI

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

********** 11-30-2000 08:29:22 Version 5.1 **********

* Sample Name: prov nr 1 Data File: D:madu041 *
* Date: 11-25-2000 20:54:40Method: MADURAMI 11-30-2000 08:27:00 # 806 *
* 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: 32.00
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 | HEIGHT | AREA/ HEIGHT | BL | REF PEAK | % DELTA RET TIME | CONC/AREA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 26.218 | mad | 0.6149 | 100.0000% | 35895 | 530 | 67.7 | 1 | 1 | 0 | 1.7132E-05 |

TOTAL AMOUNT = 0.6149

START TIME= 25.106 START HEIGHT= 69
STOP TIME= 27.442 STOP HEIGHT= 104
AREA = 32658
OLD AREA FOR PEAK# 1 = 35894.98 NEW AREA= 32658
OLD HEIGHT FOR PEAK # 1 = 529.881 NEW HEIGHT= 483.9104
REPRINT AREA REPORTS FOR NEW TABLES.

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

********** 11-30-2000 08:30:08 Version 5.1 **********

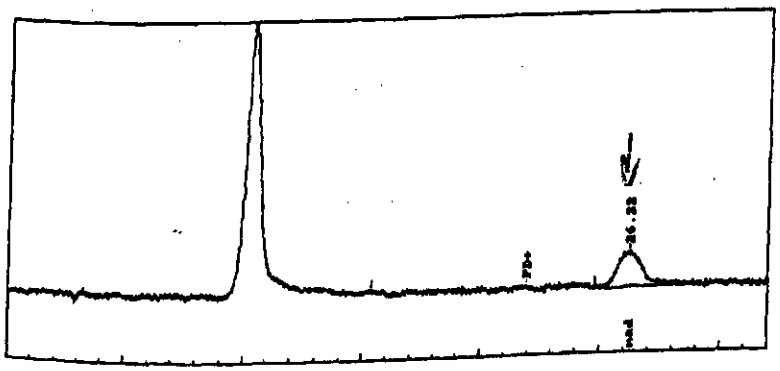
* Sample Name: prov nr 1 Data File: D:madu041 *
* Date: 11-25-2000 20:54:40Method: MADURAMI 11-30-2000 08:27:00 # 806 *
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 50 Threshold: .05 Area Threshold: 50 *

Starting Delay: 0.00 Ending retention time: 32.00
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 | HEIGHT | AREA/ HEIGHT | BL | REF PEAK | % DELTA RET TIME | CONC/AREA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 26.218 | mad | 0.5595 | 100.0000% | 32658 | 484 | 67.5 | 1 | 1 | 0 | 1.7132E-05 |

TOTAL AMOUNT = 0.5595

Data File = D:madu041.PTS Printed on 11-30-2000 at 08:30:10
Start time: 0.00 min. Stop time: 32.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 4119 uv Scale factor: 1.0



Sample 222252

NEW TIMED EVENTS FROM MADURAMI

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

********** 11-30-2000 08:33:57 Version 5.1 **********

* Sample Name: prov nr 3 Data File: D:madu043

* Date: 11-25-2000 22:02:03Method: MADURAMI 11-30-2000 08:27:00 # 806 *

* 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: 32.00
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 | HEIGHT | AREA/ HEIGHT | BL | REF PEAK | % DELTA RET TIME | CONC/AREA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 26.208 | mad | 0.9352 | 100.0000% | 54586 | 842 | 64.8 | 1 | 1 | 0 | 1.7132E-0 |

TOTAL AMOUNT = 0.9352

START TIME= 25.078 START HEIGHT= 130
STOP TIME= 27.829 STOP HEIGHT= 98
AREA = 57044
OLD AREA FOR PEAK# 1 = 54586.44 NEW AREA= 57044
OLD HEIGHT FOR PEAK # 1 = 841.9047 NEW HEIGHT= 821.1857
REPRINT AREA REPORTS FOR NEW TABLES.

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

********** 11-30-2000 08:34:38 Version 5.1 **********

* Sample Name: prov nr 3 Data File: D:madu043

* Date: 11-25-2000 22:02:03Method: MADURAMI 11-30-2000 08:27:00 # 806 *

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

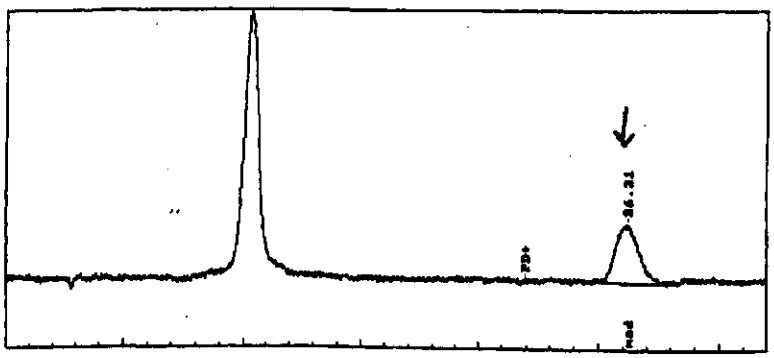
* Starting Peak Width: 50 Threshold: .05 Area Threshold: 50 *

Starting Delay: 0.00 Ending retention time: 32.00
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 | HEIGHT | AREA/ HEIGHT | BL | REF PEAK | % DELTA RET TIME | CONC/AREA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 26.208 | mad | 0.9773 | 100.0000% | 57044 | 821 | 69.5 | 1 | 1 | 0 | 1.7132E-0 |

TOTAL AMOUNT = 0.9773

Data File = D:madu043.PTS Printed on 11-30-2000 at 08:34:40
Start time: 0.00 min. Stop time: 32.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 4036 uv Scale factor: 1.0




Sample 222267

NEW TIMED EVENTS FROM MADURAMI
******* EXTERNAL STANDARD TABLE *******
******* 11-29-2000 16:03:30 Version 5.1 **********
Data File: D:madu024
* Sample Name: premix 1
* Date: 11-24-2000 04:26:35Method: MADURAMI 11-29-2000 14:41:00 # 801 *
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 50 Threshold: .05 Area Threshold: 50 *
**********
Starting Delay: 0.00
Area reject: 50
Amount injected: 80.00
Sample Weight: 1.00000
Ending retention time: 32.00
One sample per 0.200 sec.
Dilution factor: 1.00

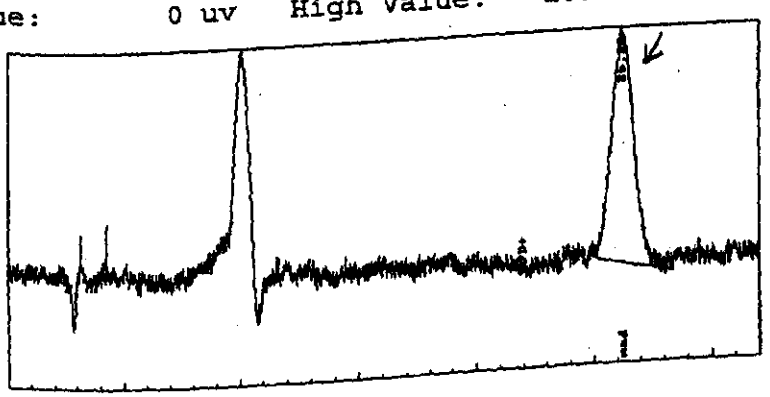
| PEAK NUM | RET TIME | PEAK NAME | CONCENTRATION in ug/ml | NORMALIZED CONC | AREA | HEIGHT | AREA/ HEIGHT | BL | REF PEAK | % DELTA RET TIME | CONC/AREA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 26.224 | mad | 0.9566 | 100.0000% | 60803 | 930 | 65.4 | 1 | 1 | 0 | 1.5732E-05 |
| | | TOTAL AMOUNT = | 0.9566 | | | | | | | | |

START TIME= 24.714 START HEIGHT= 153
STOP TIME= 28.055 STOP HEIGHT= 123
AREA = 65598
OLD AREA FOR PEAK# 1 = 60803.27 NEW AREA= 65598
OLD HEIGHT FOR PEAK # 1 = 929.8368 NEW HEIGHT= 938.3638
REPRINT AREA REPORTS FOR NEW TABLES.
******* EXTERNAL STANDARD TABLE *******
******* 11-29-2000 16:04:05 Version 5.1 **********
Data File: D:madu024
* Sample Name: premix 1
* Date: 11-24-2000 04:26:35Method: MADURAMI 11-29-2000 14:41:00 # 801 *
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 50 Threshold: .05 Area Threshold: 50 *
**********
Starting Delay: 0.00
Area reject: 50
Amount injected: 80.00
Sample Weight: 1.00000
Ending retention time: 32.00
One sample per 0.200 sec.
Dilution factor: 1.00

| 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 | 26.224 | mad | 1.0320 | 100.0000% | 65598 | 938 | 69.9 | 1 | 1 | 0 | 1.5732E-05 |
| | | TOTAL AMOUNT = | 1.0320 | | | | | | | | |

1 g → 50 ml
dil. ×10

Data File = D:madu024.PTS Printed on 11-29-2000 at 16:04:07
Start time: 0.00 min. Stop time: 32.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 1096 uv Scale factor: 1.0



Premix

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 23

# CANFAS

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

**Subtitle:** **Task 4 COLLABORATIVE STUDY**

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

**Date of analysis:**

**Analyte:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 232237 | | 12,07 |
| 232240 | | 3,18 |
| 232253 | | 5,91 |
| 232254 | | 6,76 |
| 232259 | | 6,49 |
| 232260 | | 6,44 |
| 232281 | | 3,28 |
| 232299 | | < 0,39 |
| 232336 | | 12,9 |
| 232353 | | < 0,39 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 506,91 | 501,15 |

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 26

# CANFAS

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

**Subtitle:** **Task 4 COLLABORATIVE STUDY**
**Lab-name:**
**Contact person:** e-mail:
fax:
telephone:

**Date of analysis:**

**Analyte:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 262250 | | 4,0 |
| 262261 | | 3,3 |
| 262266 | | 4,0 |
| 262268 | | 0,8 |
| 262277 | | 6,2 |
| 262279 | | 7,5 |
| 262309 | | 2,2 |
| 262354 | | 0,7 |
| 262355 | | 3,7 |
| 262368 | | 1,9 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | | 340,8 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 4/12/00

Dilution factor of the samples:

- Feed samples (specify for which feed samples): none
- Premixture: none

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: SPHERISORB ODS 2
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other:
- Flow-rate: 0.7 ml/min for post column reagent as well.
- Injection volume: ~~60~~ 100 µl
- Retention time of maduramicin: 23.5 min

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

- Percentage recovery: 80 %
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 5 mg/kg

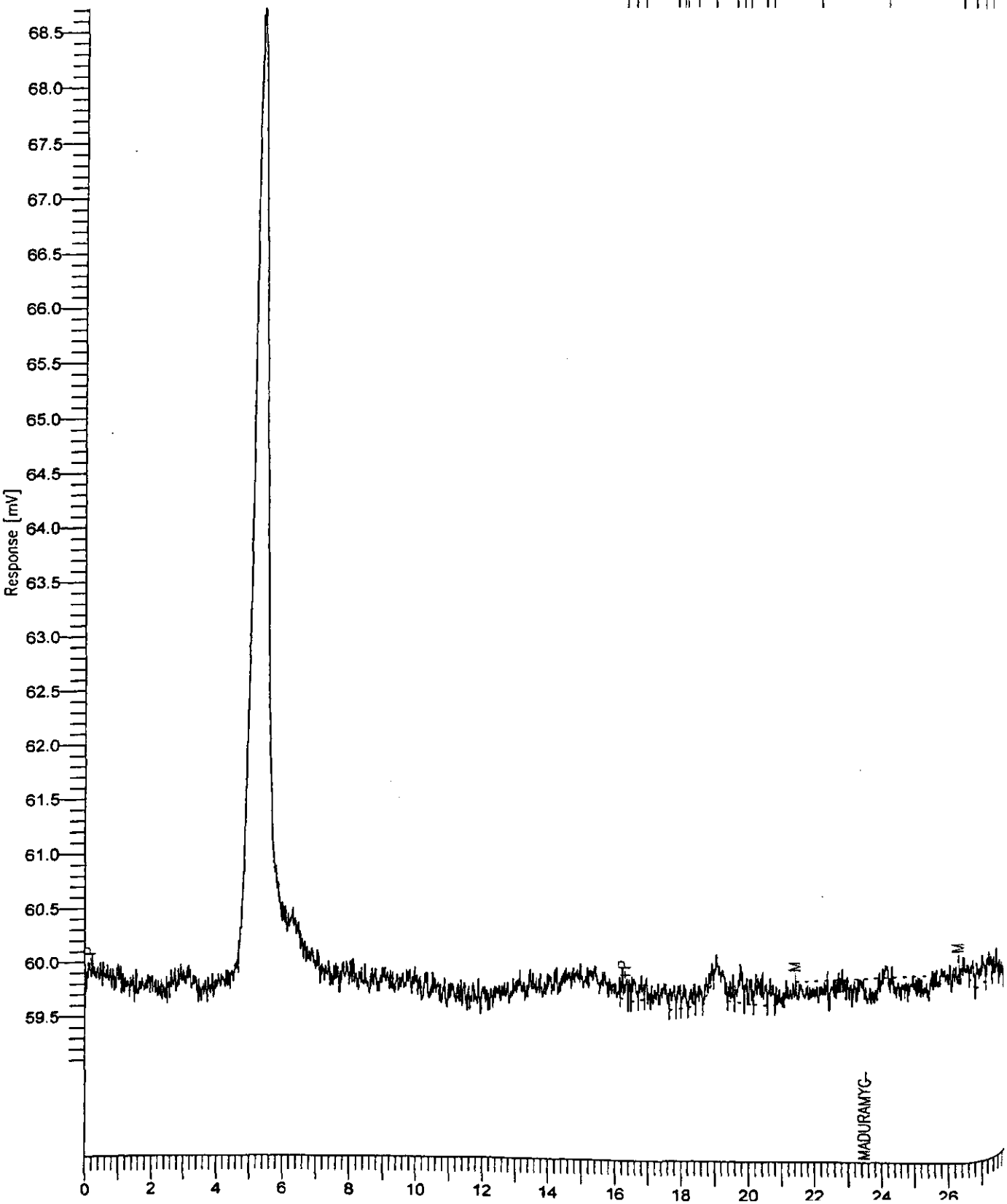
262268 Chromatogram

(26)

Sample Name : A3011167
FileName : C:\TC4\CANFAS\MADURA~1\DATA037.RAW
Method :
Start Time : 0.00 min End Time : 28.00 min
Scale Factor: 0.0 Plot Offset: 59 mV

Sample #: 19 Page 1 of 1
Date : 06/03/01 09:37
Time of Injection: 06/12/00 07:38
Low Point : 59.04 mV High Point : 68.71 mV
Plot Scale: 9.7 mV

16.21
16.78
17.78
18.38
18.90
19.53
19.94
20.41
22.09
24.10
26.34
26.71
27.20



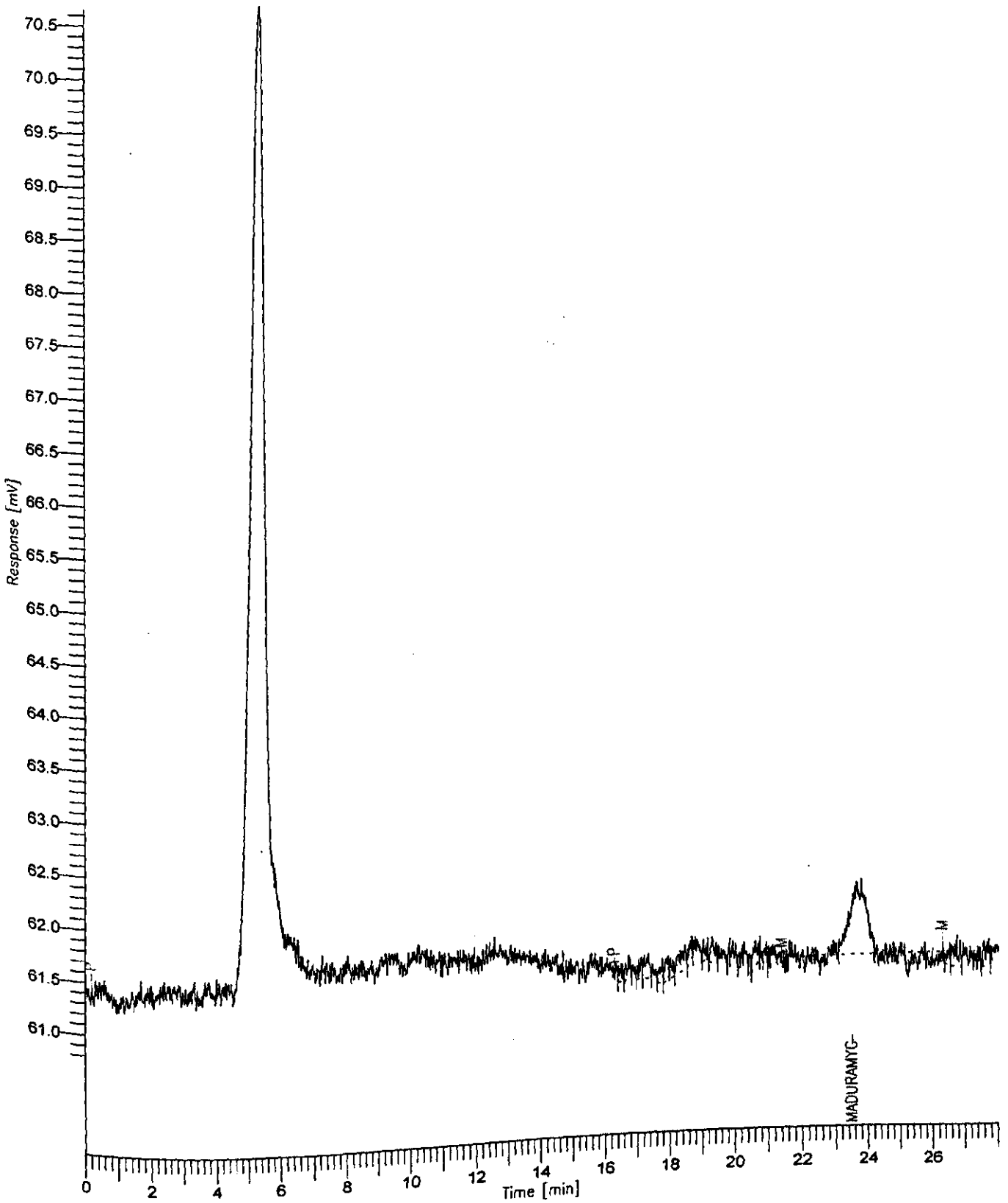
262309 Chromatogram

(26)

Sample Name : A3011170
FileName : C:\TC4\CANFAS\MADURA~1\DATA051.RAW
Method :
Start Time : 0.00 min End Time : 28.00 min
Scale Factor: 0.0 Plot Offset: 61 mV

Sample #: 26 Page 1 of 1
Date : 06/03/01 09:38
Time of Injection: 06/12/00 14:34
Low Point : 60.72 mV High Point : 70.65 mV
Plot Scale: 9.9 mV

16.51
17.13
17.78
18.48
19.16
19.83
20.28
20.94
21.43
23.78
24.27
25.73
26.52
27.72



262355

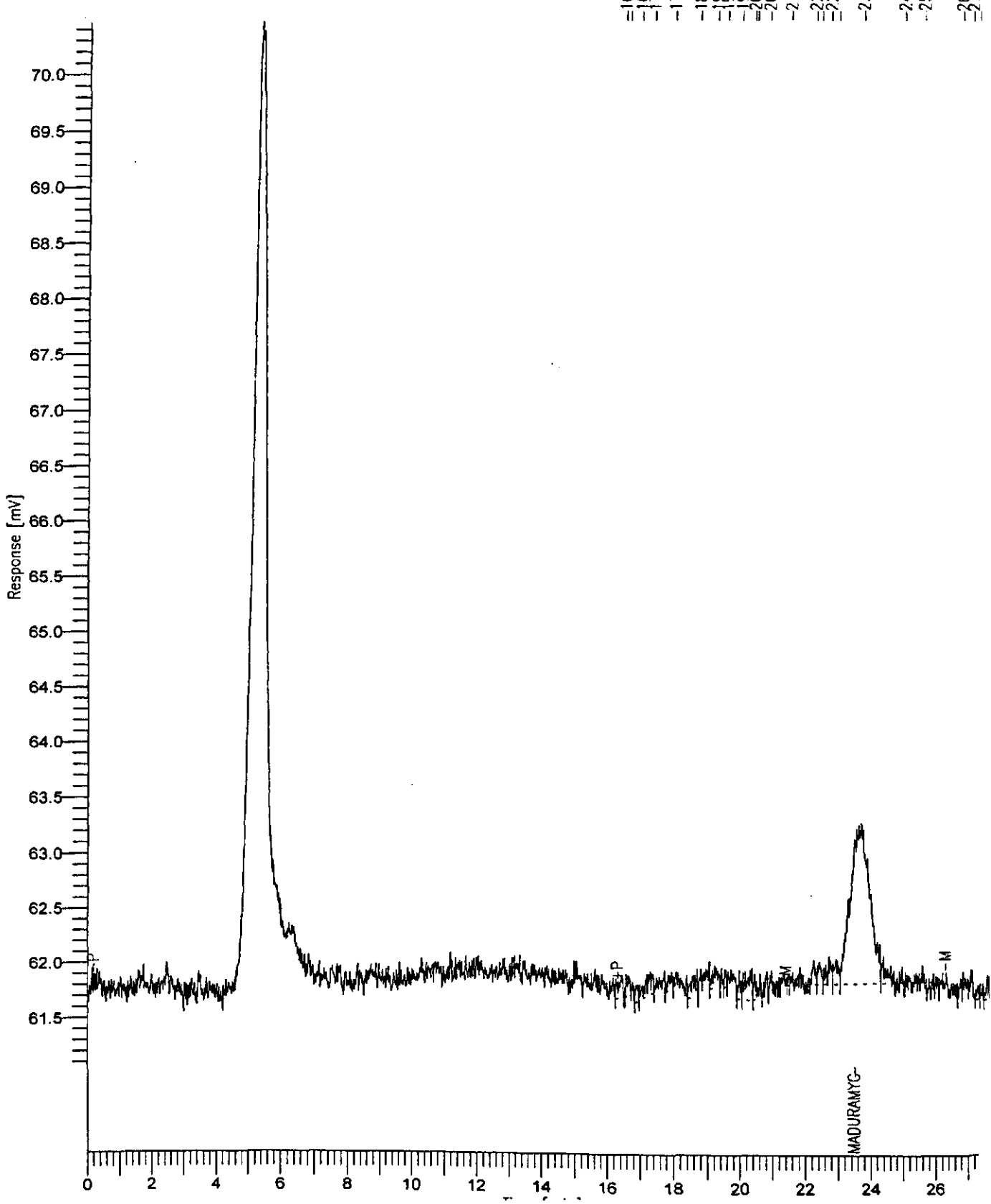
# Chromatogram

(2

Sample Name : A3011172
FileName : C:\TC4\CANFAS\MADURA~1\DATA055.RAW
Method :
Start Time : 0.00 min End Time : 28.00 min
Scale Factor: 0.0 Plot Offset: 61 mV

Sample #: 28 Page 1 of 1
Date : 06/03/01 09:38
Time of Injection: 06/12/00 16:33
Low Point : 61.08 mV High Point : 70.46 mV
Plot Scale: 9.4 mV

16.40 16.89 17.29 17.92 18.68 19.21 19.52 19.97 20.36 20.82 21.48 22.23 22.70 23.68 24.94 25.55 26.76 27.08



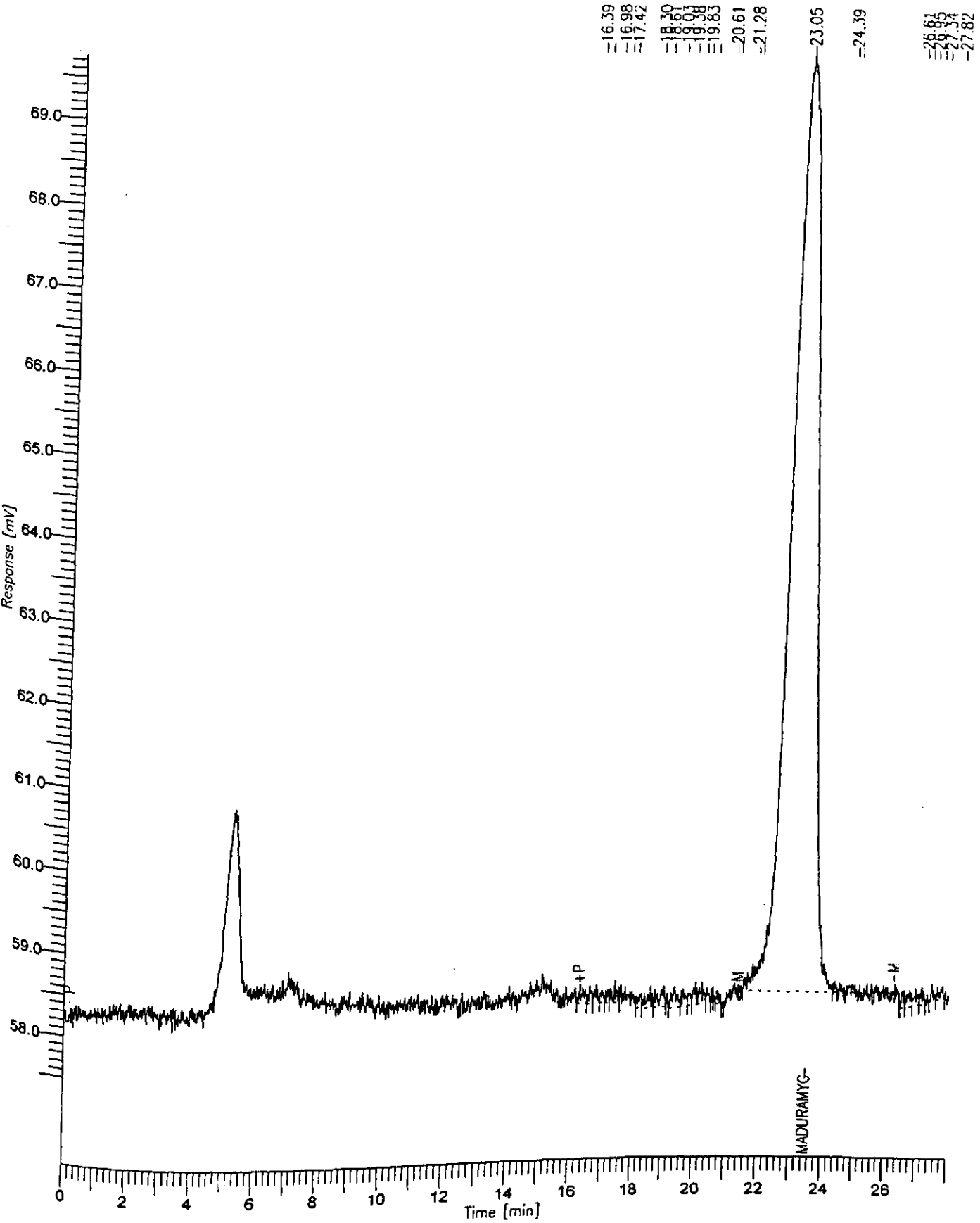
PREMIX

# Chromatogram

(26)

Sample Name : A3011175
FileName : C:\TC4\CANFAS\MADURA-1\DATA021.RAW
Method : MADUR.MTH
Start Time : 0.00 min End Time : 28.00 min
Scale Factor: 0.0 Plot Offset: 58 mV

Sample #: 11 Page 1 of 1
Date : 06/03/01 11:23
Time of Injection: 05/12/00 23:42
Low Point : 57.50 mV High Point : 69.75 mV
Plot Scale: 12.2 mV



# APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 27

# 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:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 272234 | | not detectable |
| 272241 | | 9,56 |
| 272257 | | 4,77 |
| 272276 | | not detectable |
| 272278 | | 5,21 |
| 272292 | | 4,90 |
| 272300 | | 2,65 |
| 272323 | | 2,67 |
| 272338 | | 4,73 |
| 272370 | | 9,56 |

| Sample / Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|
| Premixture | 456,1 | 474,4 |

**CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN**

**Annex 4 - Questionnaire**

Date(s) of analysis: 16.11. to 24.11. 2000

Dilution factor of the samples:

- Feed samples (specify for which feed samples): all samples F=1
- Premixture: F=10

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,4 ml/min mobile phase and 0,4 ml/min Vanillin reagent
- Injection volume: 50 µl
- Retention time of maduramicin: 24-25 min reactor oven temperature: 93°C (= water bath)

**Chromatograms: Please include representative chromatograms of:**

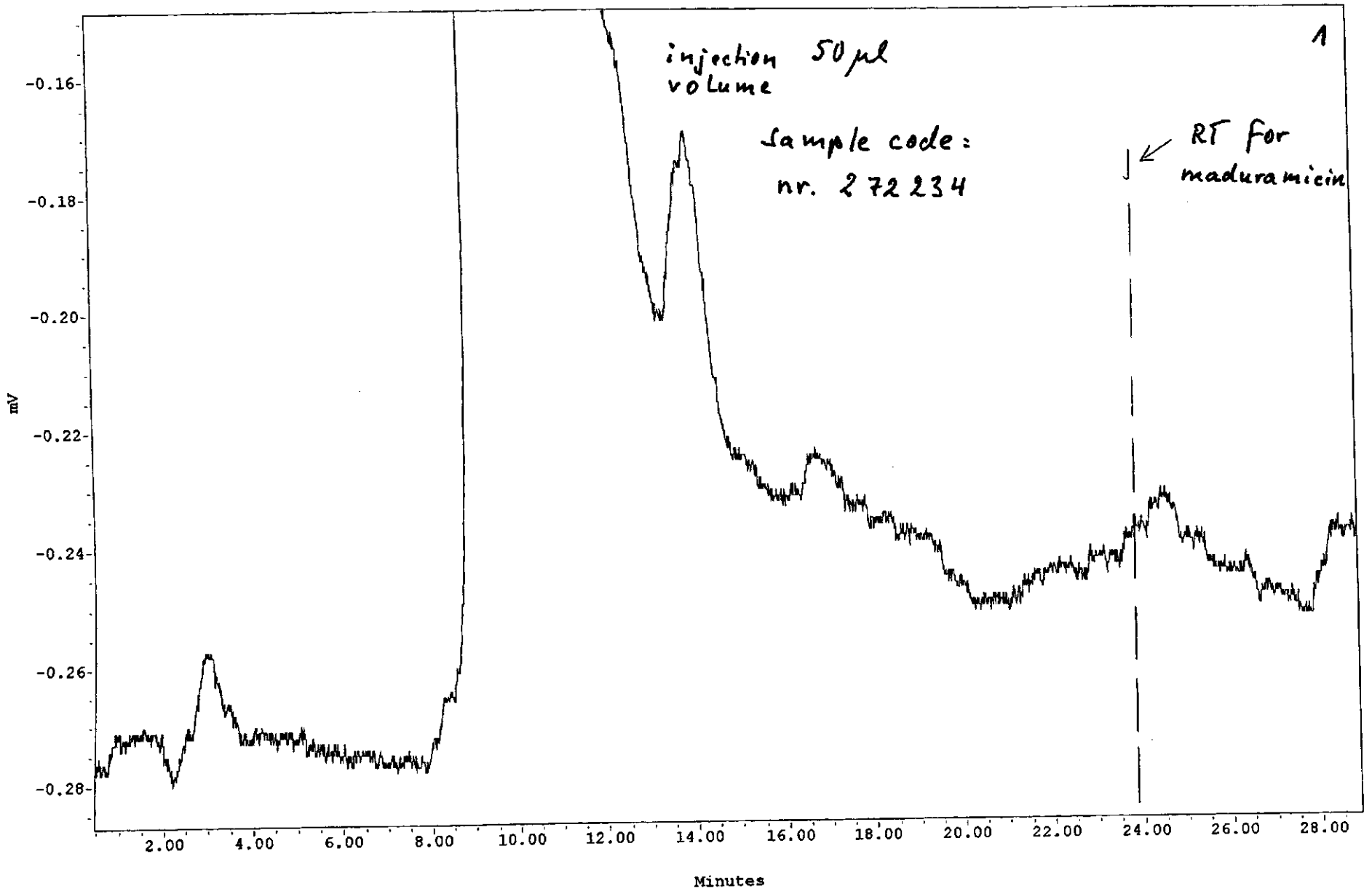
- Blind positive feed samples
- Blind blank feed sample
- Premixture

*Please indicate the maduramicin peak with an arrow*

Recovery results:

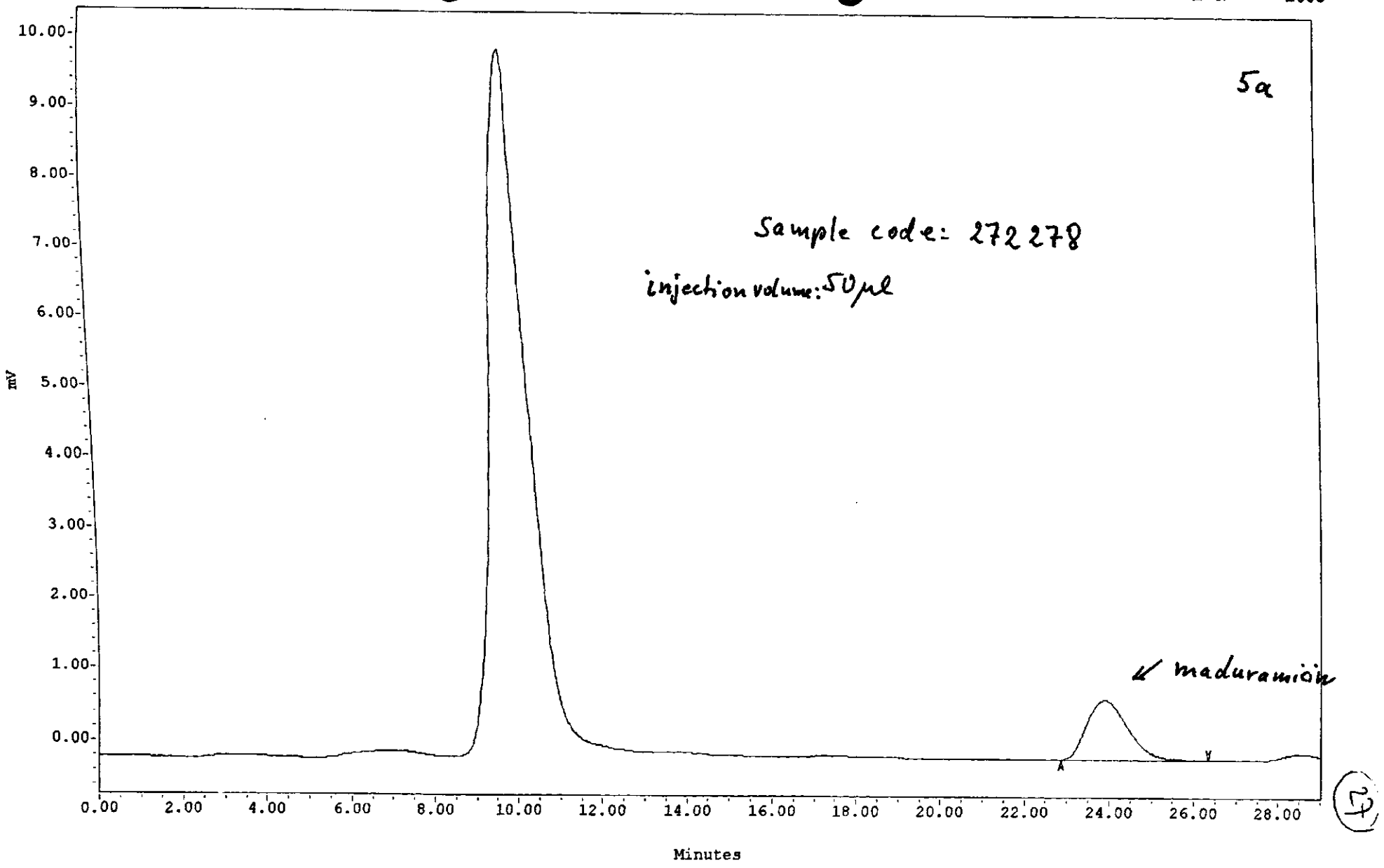
- Percentage recovery: 102,9 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ~ 5 mg/kg

16 NOV. 2000




(22)

SampleName: MAD 1 Vial: 9 Inj: 1 Ch: SATIN Type: Unknown




SampleName: MAD 5 Vial: 10 Inj: 1 Ch: SATIN Type: Unknown

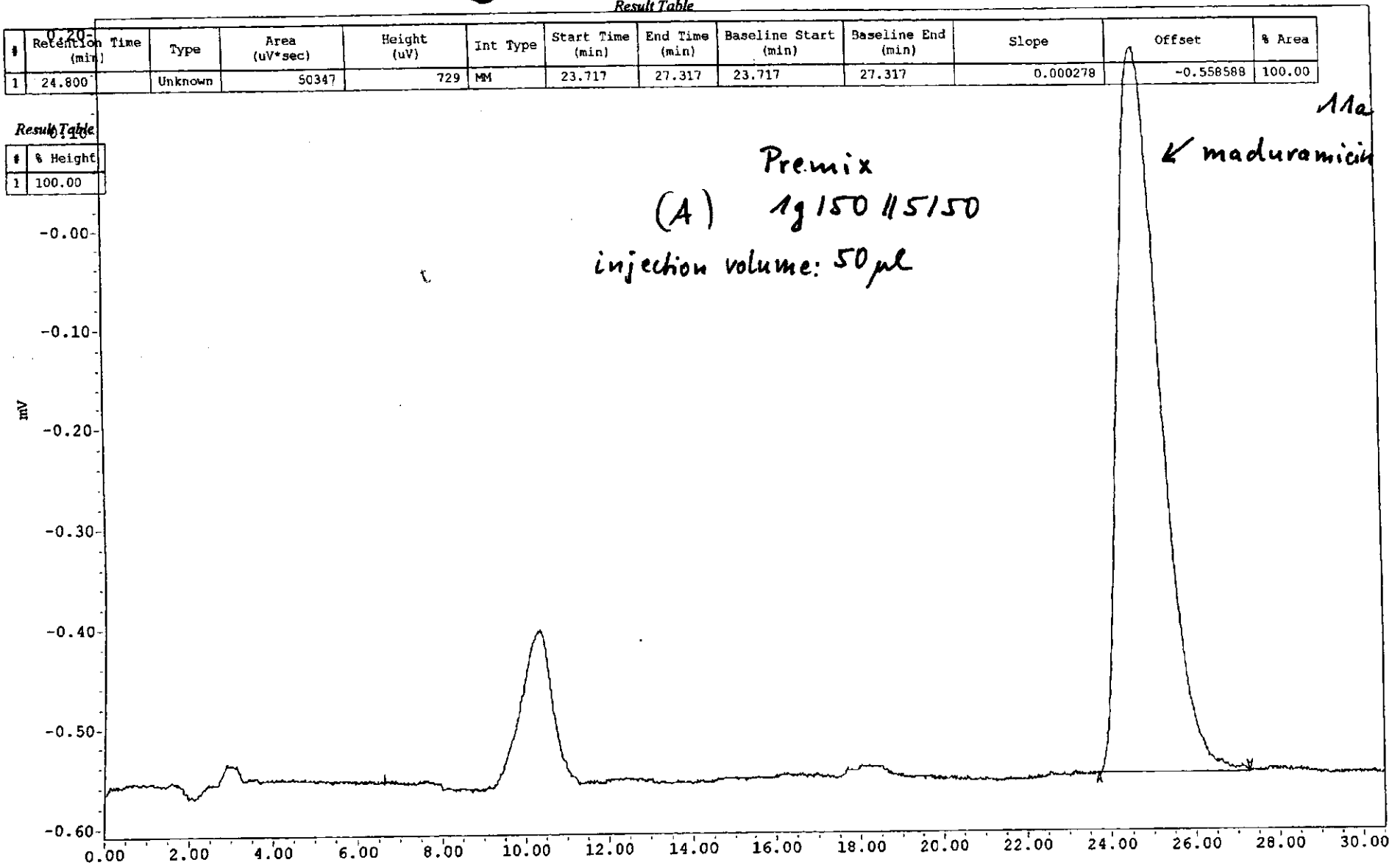
12 4. NOV. 2000

*Result Table*

| # | Retention Time (min) | Type | Area (uV*sec) | Height (uV) | Int Type | Start Time (min) | End Time (min) | Baseline Start (min) | Baseline End (min) | Slope | Offset | % Area |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 24.800 | Unknown | 50347 | 729 | MM | 23.717 | 27.317 | 23.717 | 27.317 | 0.000278 | -0.558588 | 100.00 |

*Result Table*

| # | % Height |
|---|---|
| 1 | 100.00 |




SampleName: MAD Pre A Vial: 7 Inj: 1 Ch: SATIN Type: UnknownPa

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 29

# CANFAS

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

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

e-mail:
fax:
telephone:

**Date of analysis:**

**Analyte:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 292246 | | 2,41 |
| 292262 | | 2,84 |
| 292295 | | 0 |
| 292305 | | 5,01 |
| 292322 | | 10,90 |
| 292325 | | 0 |
| 292330 | | 5,79 |
| 292342 | | 10,63 |
| 292343 | | 5,25 |
| 292361 | | 5,57 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 643,60 | 710,50 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 15/16 January 2001

Dilution factor of the samples:

- Feed samples (specify for which feed samples): ——
- Premixture: ——

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Spherisorb ODSB C18 5µm 4,6x250mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,4 ml/min
- Injection volume: 200 µl
- Retention time of maduramicin: 20,0-20,4 min

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

- Percentage recovery: 104,8 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99,5 % and 110,0 %
- Spiking level: 5 mg/kg

(29)

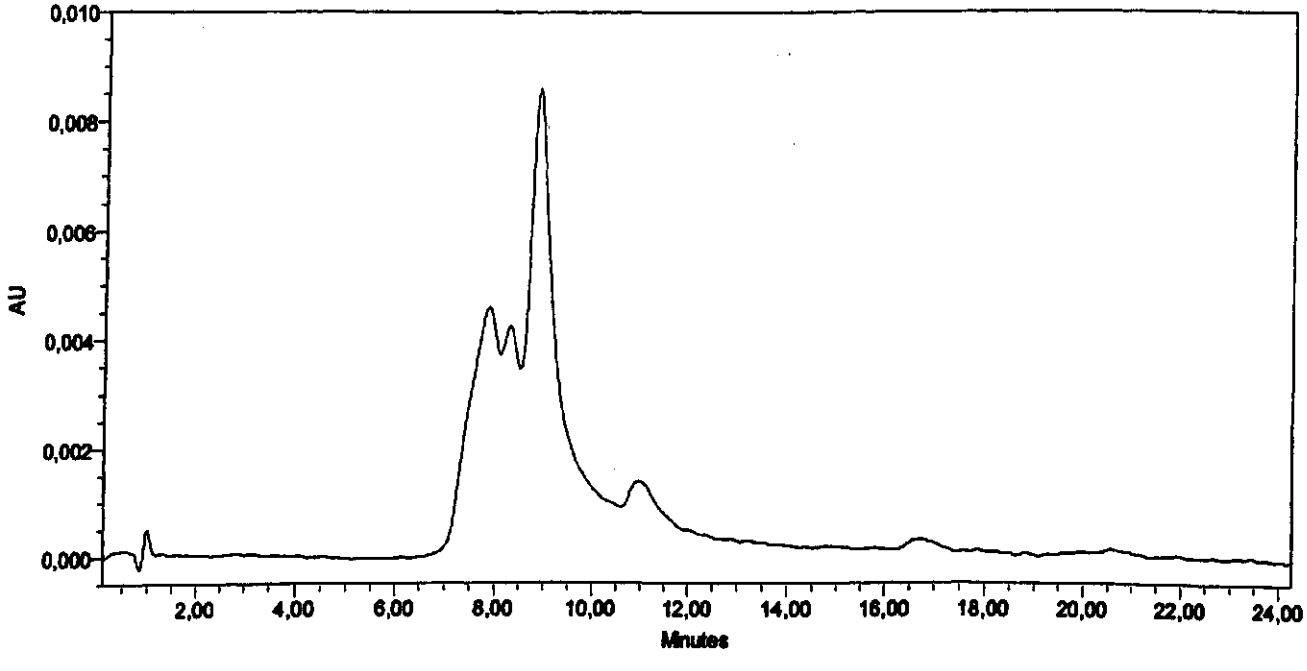
# Maduramicin Report

Project Name: Maduramicin

## SAMPLE INFORMATION

| | | | |
|---|---|---|---|
| Sample Name: | Sample 292325 | Date Acquired: | 16-01-2001 19:47:26 |
| Sample Type: | Unknown | Acq. Method Set: | Maduramicin CANFAS |
| Vial: | 38 | Date Processed: | 16-01-2001 20:22:46 |
| Injection #: | 1 | Processing Method: | MAD 16 01 2001 |
| Injection Volume: | 200,00 ul | Proc. Chnl. Descr.: | PDA 520,0 nm |
| Run Time: | 30,0 Minutes | | |

**Auto-Scaled Chromatogram**




**Peak Results**

| | Name | RT | Area | Height | Amount | Units |
|---|---|---|---|---|---|---|
| 1 | MAD | 20,432 | | | | |

Blind blank feed sample

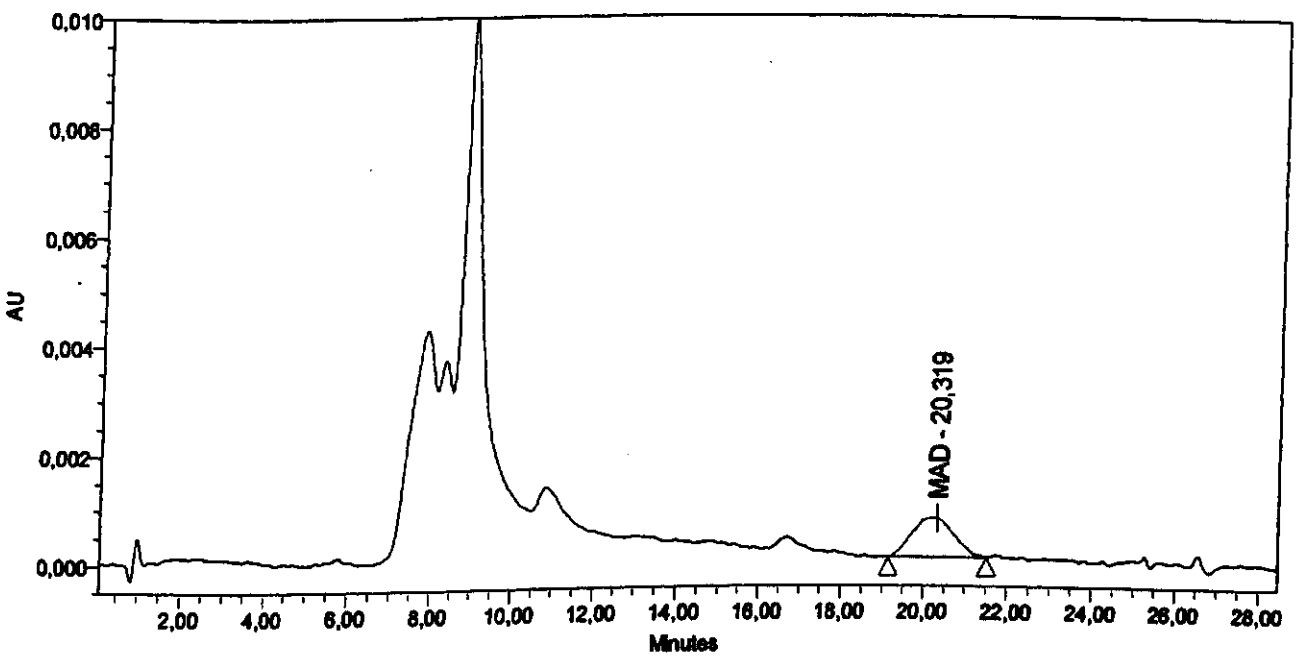
# Maduramicin Report

Project Name: Maduramicin

## SAMPLE INFORMATION

| | | | |
|---|---|---|---|
| Sample Name: | Sample 292262 | Date Acquired: | 16-01-2001 19:13:37 |
| Sample Type: | Unknown | Acq. Method Set: | Maduramicin CANFAS |
| Vial: | 40 | Date Processed: | 16-01-2001 20:22:47 |
| Injection #: | 1 | Processing Method: | MAD 16 01 2001 |
| Injection Volume: | 200,00 ul | Proc. Chnl. Descr.: | PDA 520,0 nm |
| Run Time: | 30,0 Minutes | | |

**Auto-Scaled Chromatogram**




**Peak Results**

| | Name | RT | Area | Height | Amount | Units |
|---|---|---|---|---|---|---|
| 1 | MAD | 20,319 | 51066 | 729 | 0,568 | ug/ml |

↓

2,84 mg/kg

Blind positive feed sample

29

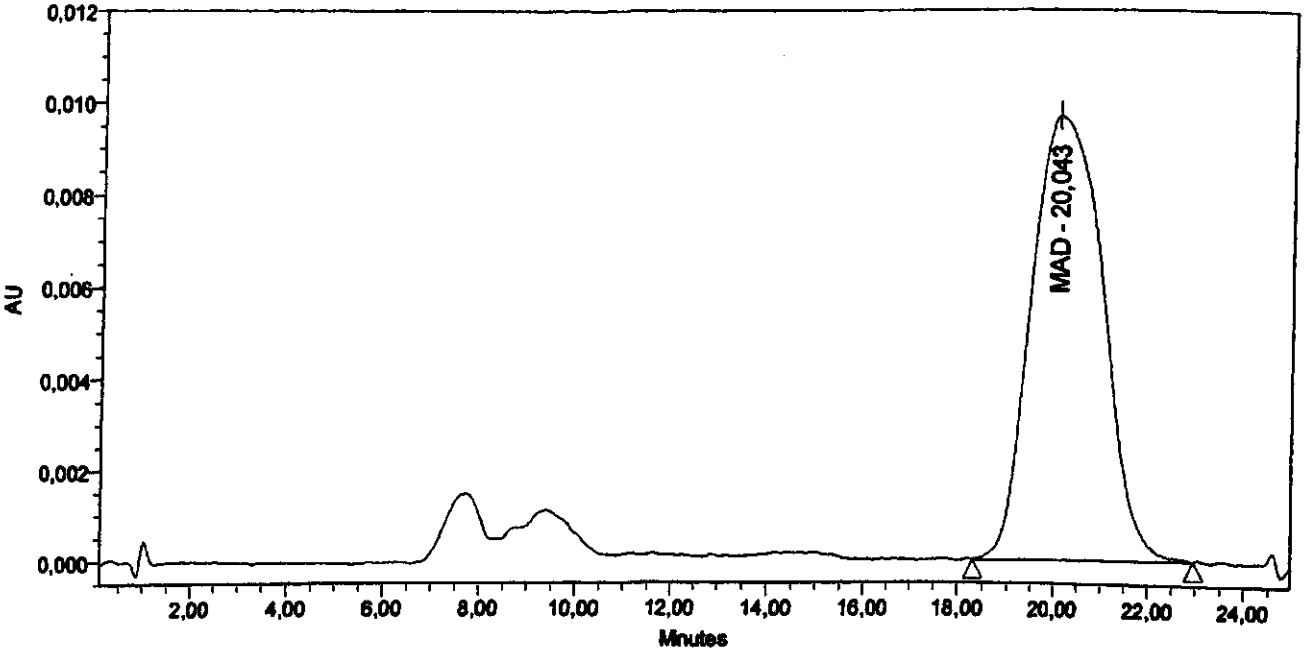
# Maduramicin Report

Project Name: Maduramicin

## SAMPLE INFORMATION

| | | | |
|---|---|---|---|
| Sample Name: | PM 12 II | Date Acquired: | 16-01-2001 15:29:50 |
| Sample Type: | Unknown | Acq. Method Set: | Maduramicin CANFAS |
| Vial: | 36 | Date Processed: | 16-01-2001 20:22:46 |
| Injection #: | 1 | Processing Method: | MAD 16 01 2001 |
| Injection Volume: | 200,00 ul | Proc. Chnl. Descr.: | PDA 520,0 nm |
| Run Time: | 30,0 Minutes | | |

**Auto-Scaled Chromatogram**




**Peak Results**

| | Name | RT | Area | Height | Amount | Units |
|---|---|---|---|---|---|---|
| 1 | MAD | 20,043 | 1021690 | 9658 | 12,872 | ug/ml |

↓

643,60 mg/kg

Premixture

# APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 30

# 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:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 302274 | | 3,0 |
| 302290 | | 8,9 |
| 302296 | | 6,6 |
| 302315 | | < 1 |
| 302316 | | 2,4 |
| 302318 | | 10,4 |
| 302324 | | 3,1 |
| 302328 | | 5,3 |
| 302329 | | 1,7 |
| 302356 | | < 1 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 491 | 511 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: ....12/19/00.....

Dilution factor of the samples:

- Feed samples (specify for which feed samples): ...10g...to...50ml.....
  .....
- Premixture: .....1g...to...50ml.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: ...Kromasil.....C18.....150...x..4,6.mm.....5µm.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....0,4.... ml/min
- Injection volume: .50....µl
- Retention time of maduramicin: 23,4 min

**Chromatograms: Please include representative chromatograms of:**

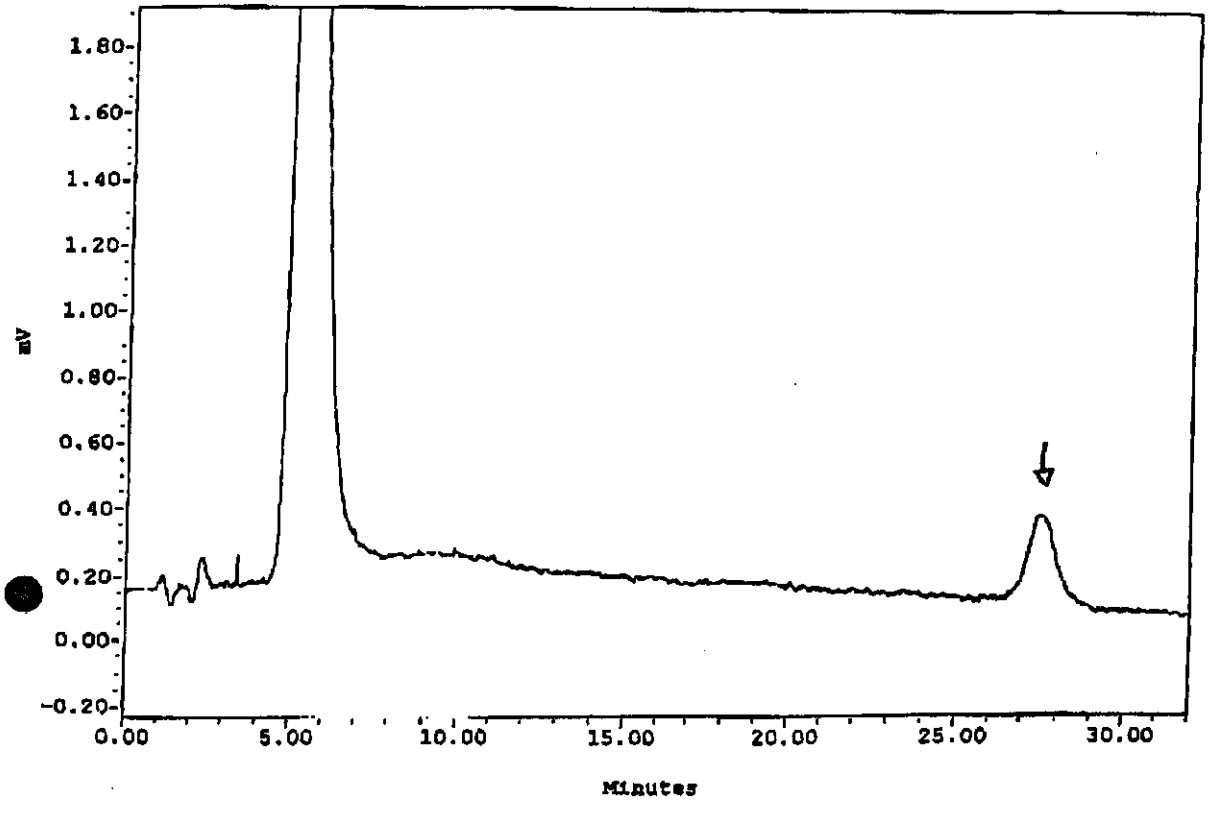
- Blind positive feed samples
- Blind blank feed sample
- Premixture

*Please indicate the maduramicin peak with an arrow*

Recovery results:

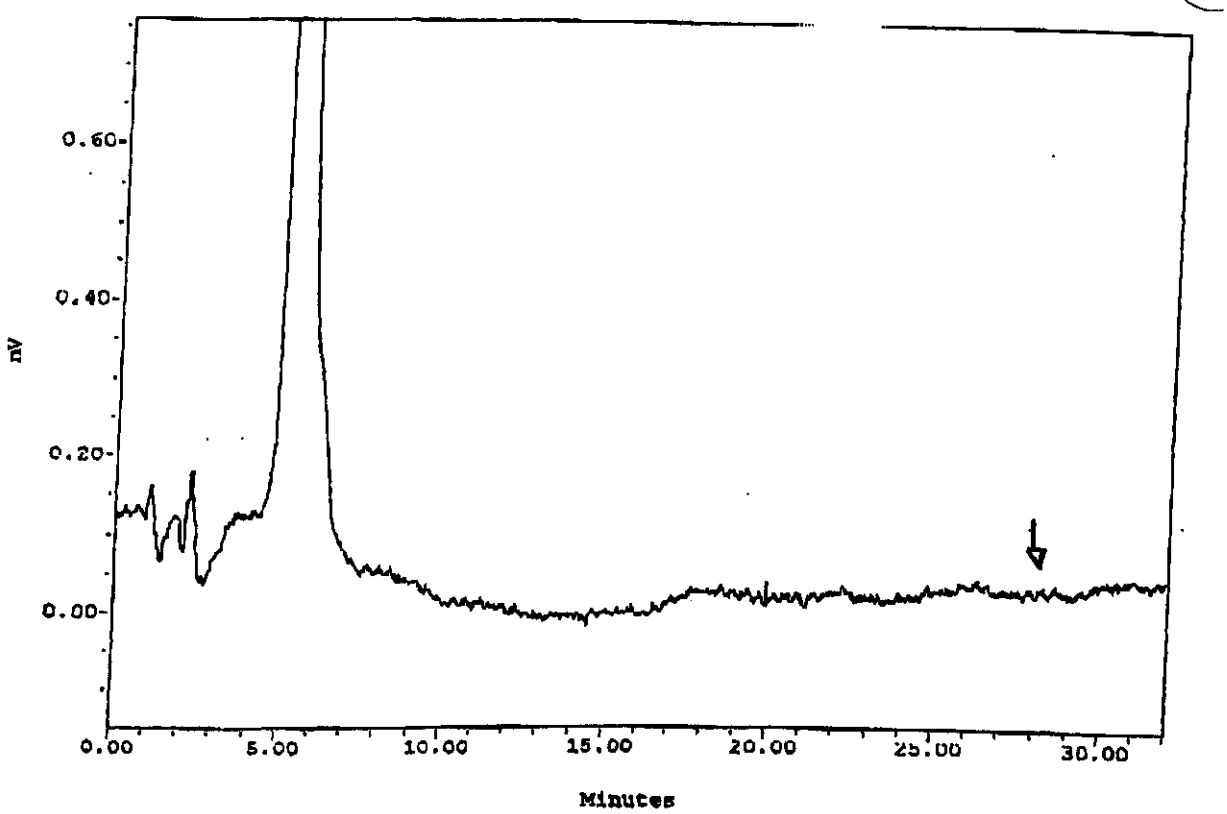
- Percentage recovery: 103 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: .....5... mg/kg

(30)



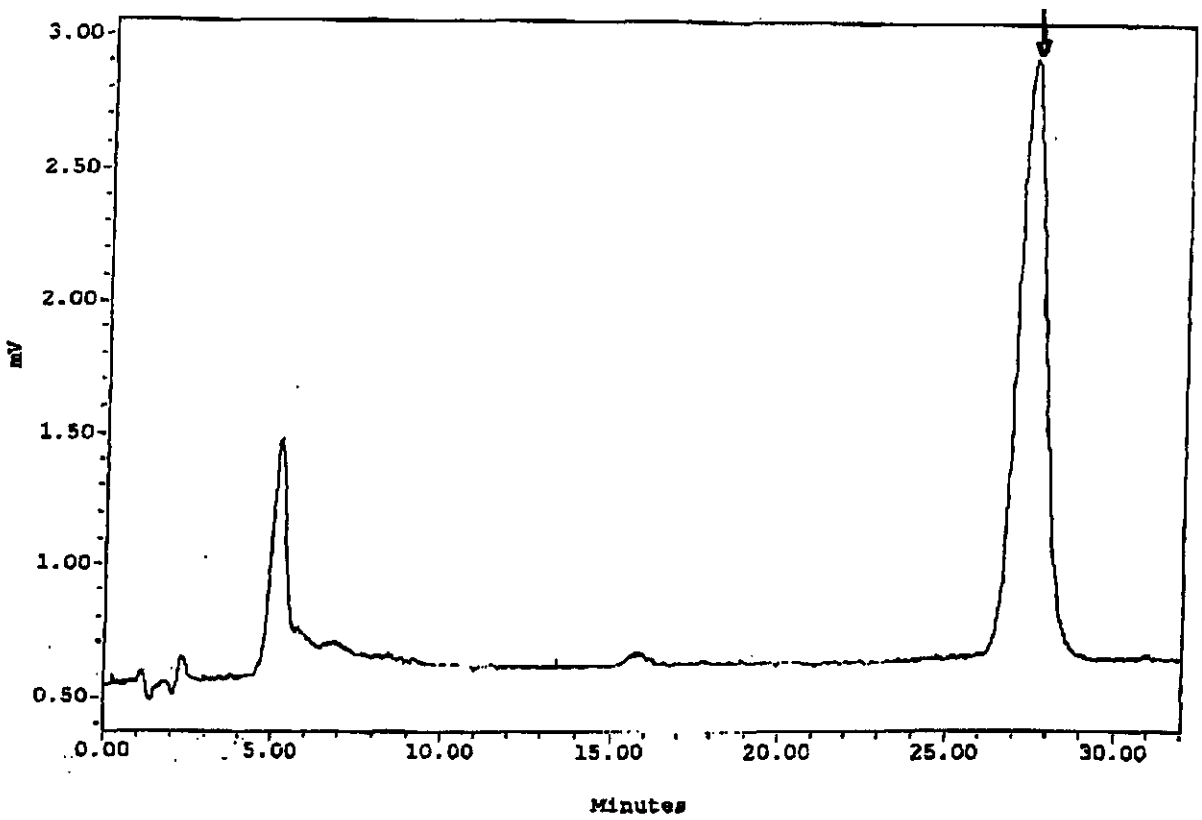

SampleName: 302328 Vial: 10 Inj: 1 Ch: SATIN Type: Unknown

Positive feed sample (5,3 ppm)




SampleName: 302356 Vial: 12 Inj: 1 Ch: SATIN Type: Unknown

blank feed sample




SampleName: PREMIX 1 Vial: 16 Inj: 1 Ch: SATIN Type: Unknown

Premixture (491 ppm)

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 31

# CANFAS

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

**Subtitle:** **Task 4 COLLABORATIVE STUDY**
**Lab-name:**
**Contact person:** e-mail:
fax:
telephone:
**Date of analysis:**

**Analyte:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 312233 | | 4,9 |
| 312247 | | 0 |
| 312264 | | 0 |
| 312307 | | 9,0 |
| 312331 | | 2,3 |
| 312334 | | 4,4 |
| 312357 | | 4,9 |
| 312359 | | 3,0 |
| 312362 | | 4,9 |
| 312367 | | 9,1 |

| Sample / Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|
| Premixture | 469 | 444 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 6-12-2000 + 7-12-2000

Dilution factor of the samples:

- Feed samples (specify for which feed samples): .... Undiluted
- Premixture: F = 10

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,4 ml/min
- Injection volume: 100 µl
- Retention time of maduramicin: 23,5 min

**Chromatograms: Please include representative chromatograms of:**

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

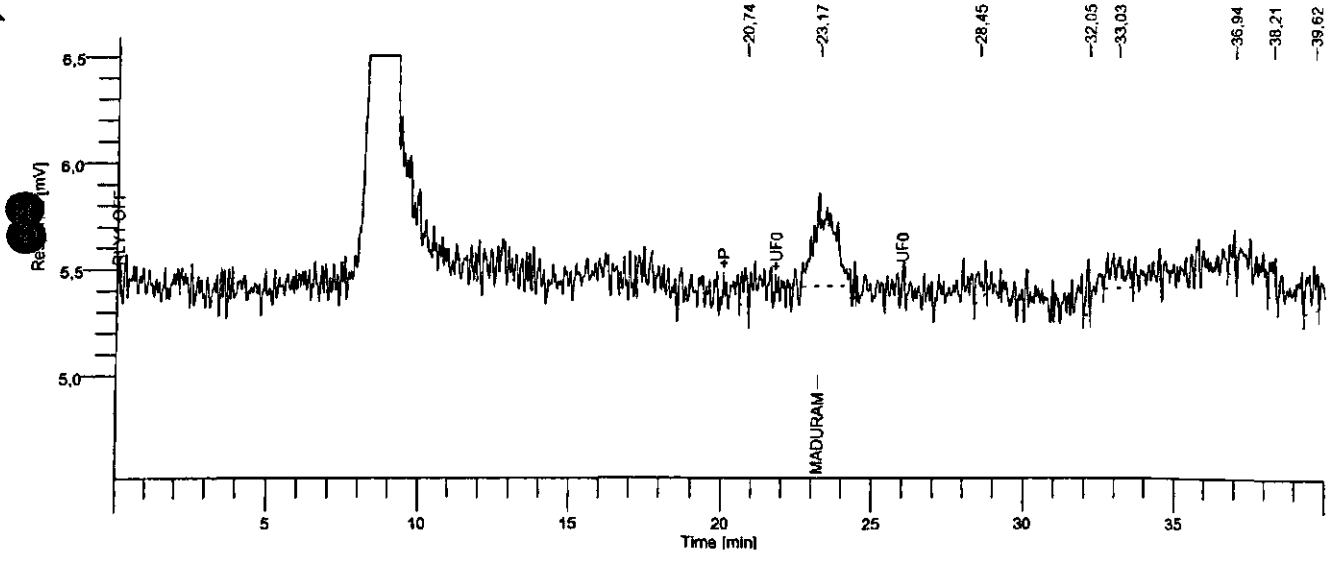
*Please indicate the maduramicin peak with an arrow*

Recovery results:

| | 6-12-2000 Day 1 | 7-12-2000 Day 2 |
|---|---|---|
| • Percentage recovery: | 99,6% | 108,4 % |
| • Single / duplicate determinations: | ☐ single ☒ duplicate | |
| • If duplicate, please give both percentages: | 98,6 % and 100,6 % | 110,5 - 106,2 % |
| • Spiking level: | 5 mg/kg | 5 mg/kg |

| | | | |
|---|---|---|---|
| Software Version | : 6.1.1.0.0:K20 | Date | : 8-12-2000 9:20:49 |
| Sample Name | : 24860 | Data Acquisition Time | : 7-12-2000 17:04:44 |
| Instrument Name | : HPLC-1 | Channel | : A |
| Rack/Vial | : 0/0 | Operator | : |
| Sample Amount | : 1,000000 | Dilution Factor | : 1,000000 |
| Cycle | : 13 | | |

Result File : \\I
Sequence File : \\




maduramycin (NH4+)

| Peak # | Time [min] | Component Name | Area [µV·s] | Height [µV] | Area [%] | Norm. Area [%] | BL | Area/Height [s] |
|---|---|---|---|---|---|---|---|---|
| 1 | 20,74 | | 2163,00 | 188,18 | 5,43 | 5,43 | BB | 11,4944 |
| 2 | 23,17 | maduramycin | 20915,41 | 436,55 | 52,49 | 52,49 | MM | 47,9102 |
| 3 | 28,45 | | 2067,00 | 139,01 | 5,19 | 5,19 | BB | 14,8694 |
| 4 | 32,05 | | 1478,00 | 234,63 | 3,71 | 3,71 | BB | 6,2992 |
| 5 | 33,03 | | 4980,00 | 97,19 | 12,50 | 12,50 | BB | 51,2401 |
| 6 | 36,94 | | 1907,50 | 221,36 | 4,79 | 4,79 | BB | 8,6171 |
| 7 | 38,21 | | 2413,00 | 176,27 | 6,06 | 6,06 | BB | 13,6896 |
| 8 | 39,62 | | 3919,00 | 208,73 | 9,84 | 9,84 | BB | 18,7751 |
| | | | 39842,91 | 1701,93 | 100,00 | 100,00 | | |

Missing Component Report

| Component | Expected Retention (Calibration File) |
|---|---|

All components were found

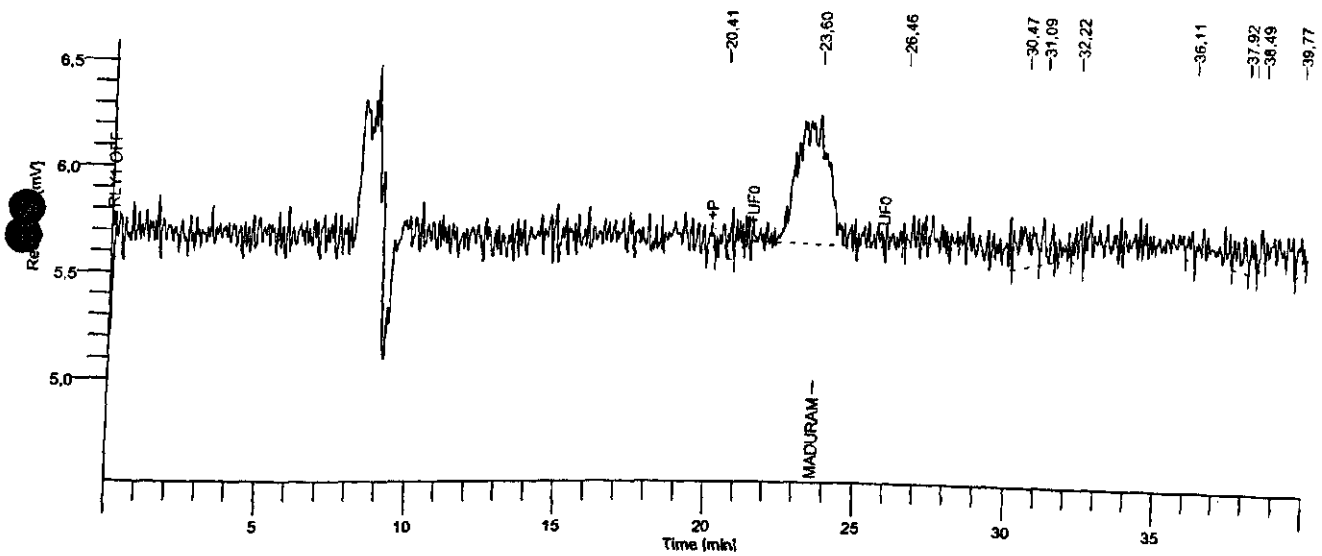
312331 ≡ 2,3 mg/l

premix



| | | | |
|---|---|---|---|
| Software Version | : 6.1.1.0.0:K20 | Date | : 8-12-2000 9:27:17 |
| Sample Name | : 24863-b : | Data Acquisition Time | : 7-12-2000 19:51:21 |
| Instrument Name | : HPLC-1 | Channel | : A |
| Rack/Vial | : 0/0 | Operator | : |
| Sample Amount | : 1,000000 | Dilution Factor | : 1,000000 |
| Cycle | : 17 | | |

Result File : \\
Sequence File : \\




maduramycin (NH4+)

| Peak # | Time [min] | Component Name | Area [µV·s] | Height [µV] | Area [%] | Norm. Area [%] | BL | Area/Height [s] |
|---|---|---|---|---|---|---|---|---|
| 1 | 20,41 | | 3649,00 | 160,87 | 5,09 | 5,09 | BB | 22,6829 |
| 2 | 23,60 | maduramycin | 45399,16 | 602,66 | 63,33 | 63,33 | MM | 75,3313 |
| 3 | 26,46 | | 383,00 | 108,15 | 0,53 | 0,53 | BB | 3,5412 |
| 4 | 30,47 | | 7126,63 | 201,58 | 9,94 | 9,94 | BV | 35,3539 |
| 5 | 31,09 | | 3012,87 | 209,37 | 4,20 | 4,20 | VB | 14,3901 |
| 6 | 32,22 | | 2429,00 | 178,60 | 3,39 | 3,39 | BB | 13,6004 |
| 7 | 36,11 | | 1702,50 | 156,83 | 2,37 | 2,37 | BB | 10,8559 |
| 8 | 37,92 | | 2979,30 | 172,37 | 4,16 | 4,16 | BV | 17,2845 |
| 9 | 38,13 | | 1660,20 | 167,09 | 2,32 | 2,32 | VB | 9,9358 |
| 10 | 38,49 | | 1240,00 | 176,03 | 1,73 | 1,73 | BB | 7,0443 |
| 11 | 39,77 | | 2110,00 | 145,53 | 2,94 | 2,94 | BB | 14,4991 |
| | | | 71691,66 | 2279,08 | 100,00 | 100,00 | | |

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

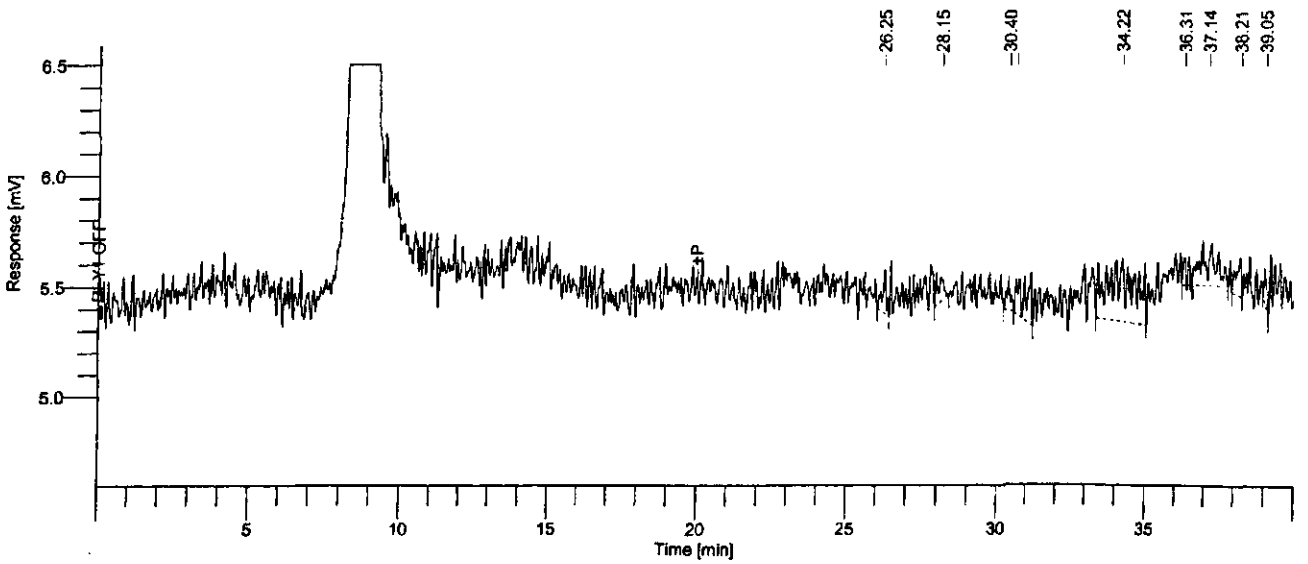
Premisc

nr. 312264 (31)



| | | | |
|---|---|---|---|
| Software Version | : 6.1.1.0.0:K20 | Date | : 12/7/00 4:21:33 PM |
| Sample Name | : 24858 | Data Acquisition Time | : 12/7/00 3:41:25 PM |
| Instrument Name | : HPLC-1 | Channel | : A |
| Rack/Vial | : 0/0 | Operator | : |
| Sample Amount | : 1.000000 | Dilution Factor | : 1.000000 |
| Cycle | : 11 | | |

Result File : \\ (
Sequence File : \




maduramycin (NH4+)

| Peak # | Time [min] | Component Name | Area [µV·s] | Height [µV] | Area [%] | Norm. Area [%] | BL | Area/Height [s] |
|---|---|---|---|---|---|---|---|---|
| - | 23.00 | maduramycir. | 0.00 | 0.00 | 0.00 | 0.00 | | |
| 1 | 26.25 | | 1751.00 | 104.94 | 4.86 | 4.86 | BB | 16.6863 |
| 2 | 28.15 | | 1725.50 | 111.39 | 4.79 | 4.79 | BB | 15.4901 |
| 3 | 30.40 | | 1338.50 | 145.41 | 3.72 | 3.72 | BB | 9.2052 |
| 4 | 30.65 | | 4127.00 | 152.17 | 11.47 | 11.47 | BB | 27.1208 |
| 5 | 34.22 | | 16368.00 | 270.34 | 45.47 | 45.47 | BB | 60.5451 |
| 6 | 36.31 | | 1295.63 | 122.70 | 3.60 | 3.60 | BV | 10.5595 |
| 7 | 37.14 | | 6454.80 | 98.28 | 17.93 | 17.93 | VB | 65.6773 |
| 8 | 38.21 | | 1971.00 | 167.86 | 5.48 | 5.48 | BB | 11.7419 |
| 9 | 39.05 | | 965.00 | 164.66 | 2.68 | 2.68 | BB | 5.8605 |
| | | | 35996.43 | 1337.75 | 100.00 | 100.00 | | |

Missing Component Report

| Component | Expected Retention (Calibration File) |
|---|---|
| maduramycir | 23.000 |

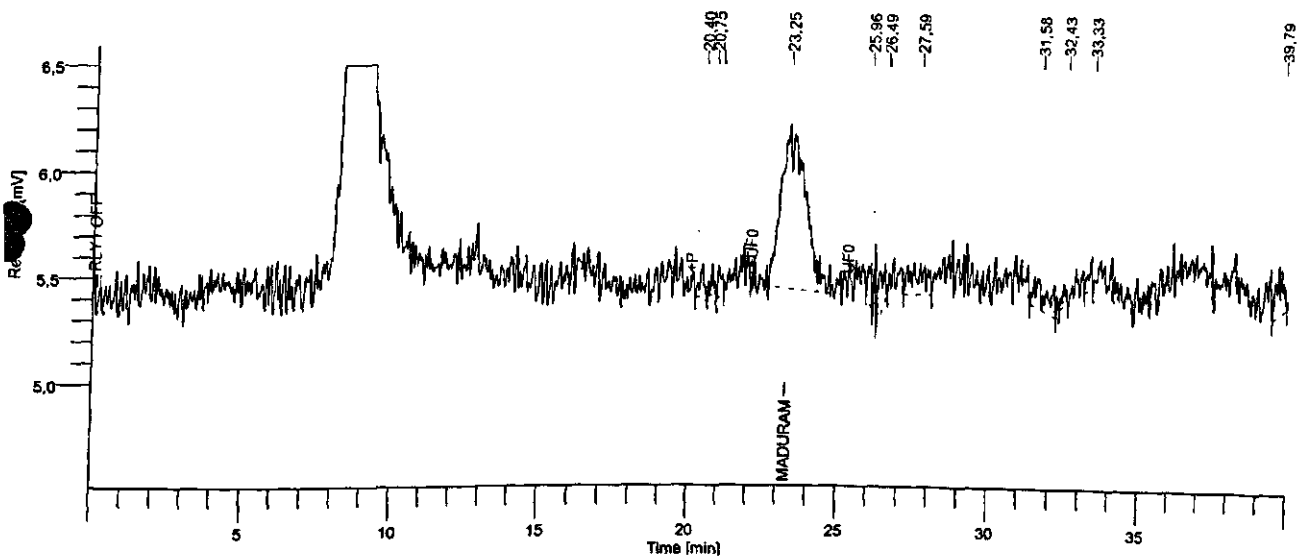
blanco voor

M. 312362 (21)



| | | | |
|---|---|---|---|
| Software Version | : 6.1.1.0.0:K20 | Date | : 8-12-2000 11:04:04 |
| Sample Name | : 24861 | Data Acquisition Time | : 7-12-2000 17:46:23 |
| Instrument Name | : HPLC-1 | Channel | : A |
| Rack/Vial | : 0/0 | Operator | : |
| Sample Amount | : 1,000000 | Dilution Factor | : 1,000000 |
| Cycle | : 14 | | |

Result File : \\ ... ul
Sequence File : \r ...seq




maduramycin (NH4+)

| Peak # | Time [min] | Component Name | Area [µV·s] | Height [µV] | Area [%] | Norm. Area [%] | BL | Area/Height [s] |
|---|---|---|---|---|---|---|---|---|
| 1 | 20,40 | | 1446,57 | 148,58 | 2,12 | 2,12 | BV | 9,7363 |
| 2 | 20,75 | | 1028,11 | 158,51 | 1,51 | 1,51 | VB | 6,4859 |
| 3 | 20,96 | | 1204,00 | 147,81 | 1,76 | 1,76 | BB | 8,1454 |
| 4 | 23,25 | maduramycin | 45697,05 | 774,05 | 66,95 | 66,95 | MM | 59,0359 |
| 5 | 25,96 | | 1511,00 | 155,96 | 2,21 | 2,21 | BB | 9,6881 |
| 6 | 26,49 | | 2652,00 | 181,97 | 3,89 | 3,89 | BB | 14,5741 |
| 7 | 27,59 | | 4840,50 | 50,46 | 7,09 | 7,09 | BB | 95,9245 |
| 8 | 31,58 | | 4084,00 | 146,25 | 5,98 | 5,98 | BB | 27,9257 |
| 9 | 32,43 | | 868,50 | 106,17 | 1,27 | 1,27 | BB | 8,1805 |
| 10 | 33,33 | | 1246,00 | 150,36 | 1,83 | 1,83 | BB | 8,2869 |
| 11 | 39,79 | | 3680,00 | 198,86 | 5,39 | 5,39 | BB | 18,5059 |
| | | | 68257,73 | 2218,98 | 100,00 | 100,00 | | |

Missing Component Report

| Component | Expected Retention (Calibration File) |
|---|---|

All components were found

312362 ≡ 4,5 mg /kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 35

# CANFAS

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

**Subtitle:** Task 4 COLLABORATIVE STUDY

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

**Date of analysis:**

**Analyte:** MADURAMICIN

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 352256 | | 4,5 |
| 352265 | | < 2 |
| 352284 | | 4,0 |
| 352310 | | 2,2 |
| 352314 | | 1,9 |
| 352320 | | 3,9 |
| 352332 | | 8,7 |
| 352348 | | 3,8 |
| 352358 | | 7,3 |
| 352366 | | < 2 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 385 | 378 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 10/01/01

Dilution factor of the samples:

- Feed samples (specify for which feed samples): 50
- Premixture: 500

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Lichrosphere 100 RP 18 (5 µm)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other:
- Flow-rate: 0,4 ml/min
- Injection volume: 50 µl
- Retention time of *maduramicin*: 22,5 min

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

Recovery results:

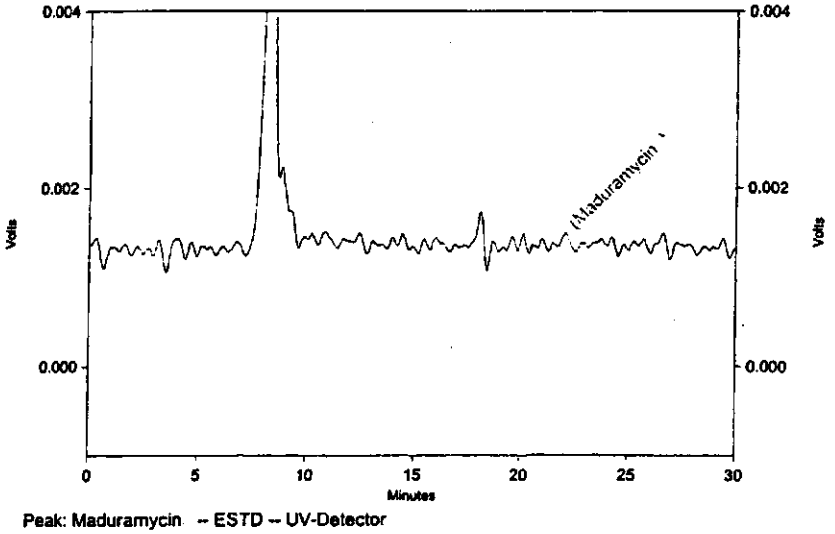
- Percentage recovery: 96,3 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 97,2 % and 95,3 %
- Spiking level: 10 mg/kg

# Maduramycin

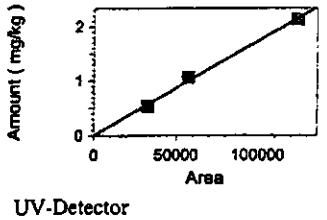
**Monster: 7 49-0514** ≈ 352265

Gebruiker: asc
Runtijdstip: 01-09-2001 13:43:47
Inweeg: 9.9859
Verdunning : 50

Methode: \\Fs_1 \VOL1\DATA\Elite_Admin\Projects\Testlab\Method\I
File: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Data
Sequence: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Sequence.




Peak: Maduramycin -- ESTD -- UV-Detector




UV-Detector
Results

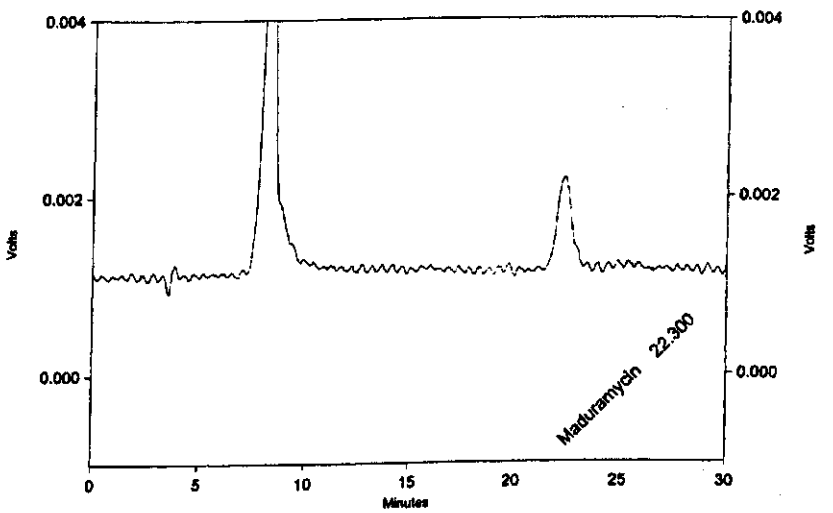
| Name | Retention Time | Area | Height | ESTD concentration | Units |
|---|---|---|---|---|---|
| Maduramycin | | | | 0.000 BDL | mg/kg |

# Maduramycin

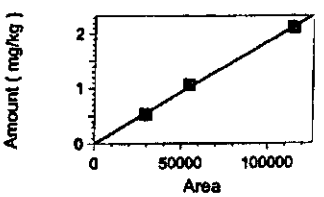
**Monster: 3 49-0512** = 352256

Gebruiker:
Runtijdstip: 01-09-2001 13:43:38
Inweeg: 10.1907
Verdunning : 50

Methode: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Method\Maduramycin
File: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Data\Maduramycin
Sequence: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Sequence\Maduramycin




Peak: Maduramycin – ESTD – UV-Detector




UV-Detector
Results

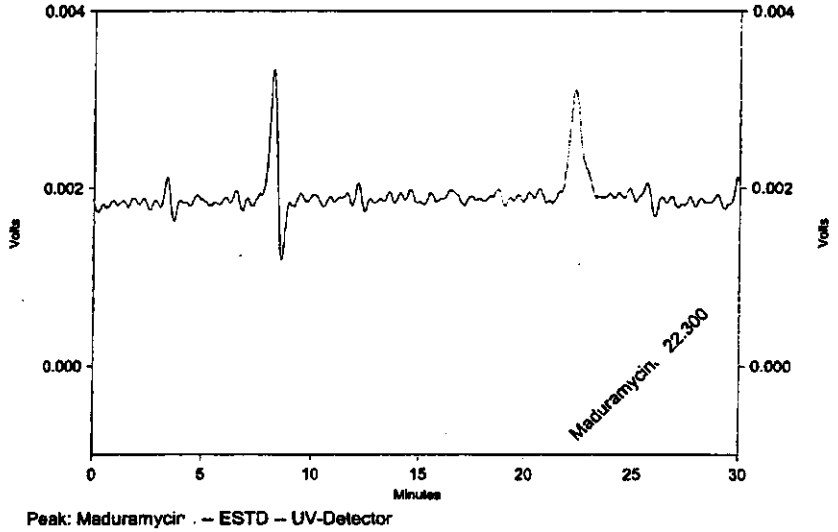
| Name | Retention Time | Area | Height | ESTD concentration | Units |
|---|---|---|---|---|---|
| Maduramycin | 22.300 | 49658 | 1036 | 4.549 | mg/kg |

# Maduramycin

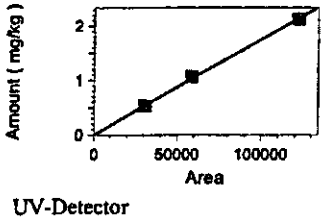
**Monster: 23 49-0522 pr.** = premix

Gebruiker: asc
Runtijdstip: 01-09-2001 13:44:06
Inweeg: 1.0425
Verdunning : 500

Methode: \\Fs_ .VOL1\DATA\Elite_Admin\Projects\Testlab\Method\
File: \\Fs . \VOL1\DATA\Elite_Admin\Projects\Testlab\Data\
Sequence: \\Fs_ \VOL1\DATA\Elite_Admin\Projects\Testlab\Sequence\




Peak: Maduramycin . – ESTD – UV-Detector




UV-Detector
Results

| Name | Retention Time | Area | Height | ESTD concentration | Units |
|---|---|---|---|---|---|
| Maduramycin | 22.300 | 45712 | 1160 | 384.558 | mg/kg |

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 36

# 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:** **MADURAMICIN**

| Sample code | Unit | Result (mg/kg) |
|---|---|---|
| 362258 | | 2,8 |
| 362270 | | 8,6 |
| 362288 | | 4,7 |
| 362291 | | 5,0 |
| 362297 | | 0 |
| 362313 | | 5,1 |
| 362317 | | 3,8 |
| 362340 | | 0 |
| 362351 | | 2,5 |
| 362363 | | 8,4 |

| Sample | Unit | Result 1 (mg/kg) | Result 2 (mg/kg) |
|---|---|---|---|
| Premixture | | 444 | 479 |

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Date(s) of analysis: 20.12.2000

Dilution factor of the samples:

- Feed samples (specify for which feed samples): 10 g in 50 ml
- Premixture: 1.0 g in 100 ml / 5 ml diluted to 20 ml

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Hypersil ODS, 5 microm, 250 x 4 mm
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: 100 ml phosphate buffer pH 4, 80 ml tetrahydrofuran, to 1000 ml with methanol
- Flow-rate: 0.4 ml/min
- Injection volume: 50 µl
- Retention time of maduramicin: 16 min

**Chromatograms: Please include representative chromatograms of:**

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

*Please indicate the maduramicin peak with an arrow*

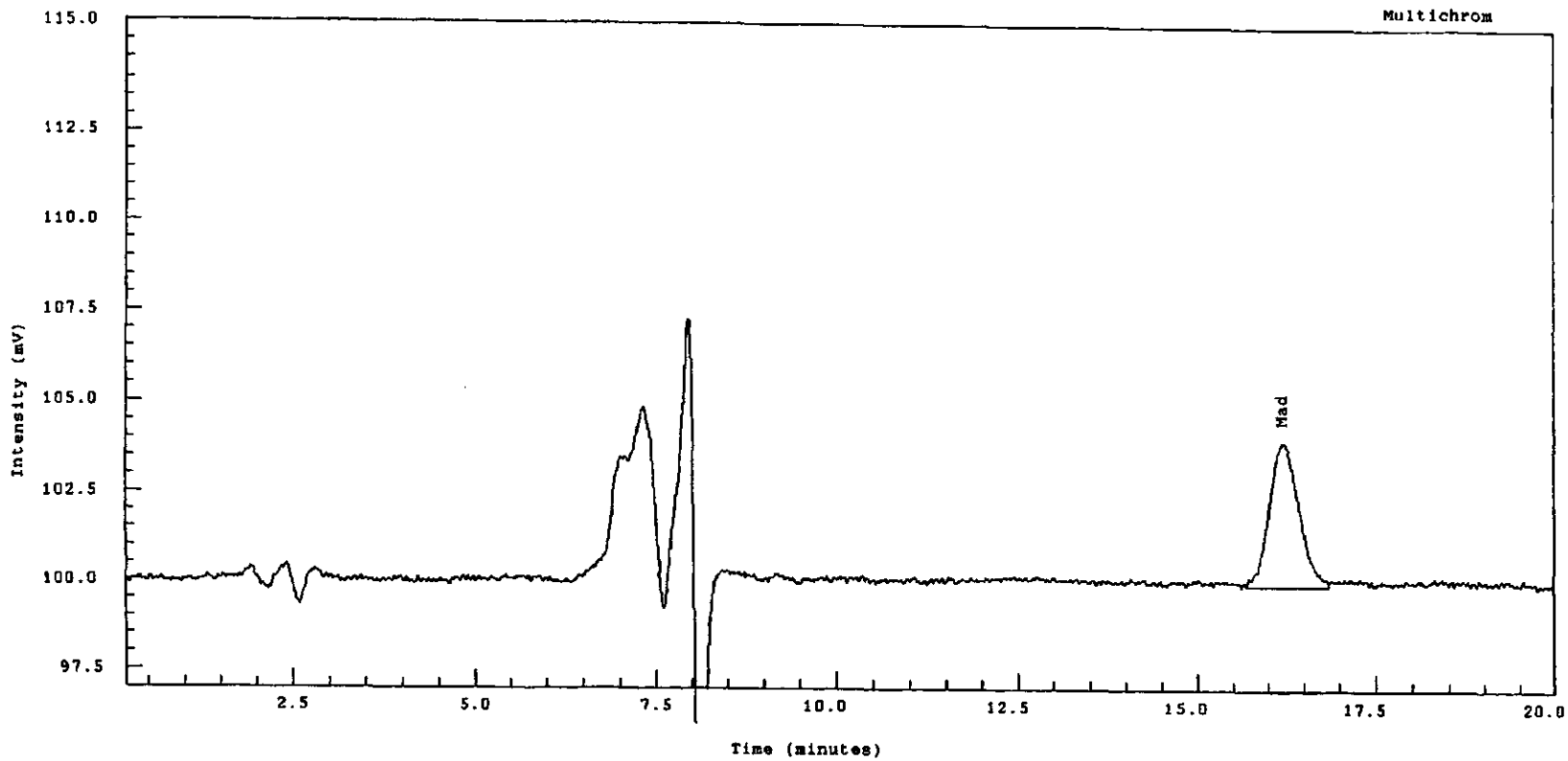
Recovery results:

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

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_20DEC001027,7,1.

Premix




*Instrument* :

Channel Title : Channel #3

Lims ID : 1

Acquired on 20-DEC-2000 at 12:41

Reported on 21-DEC-2000 at 16:08

*Method* : MAD-103

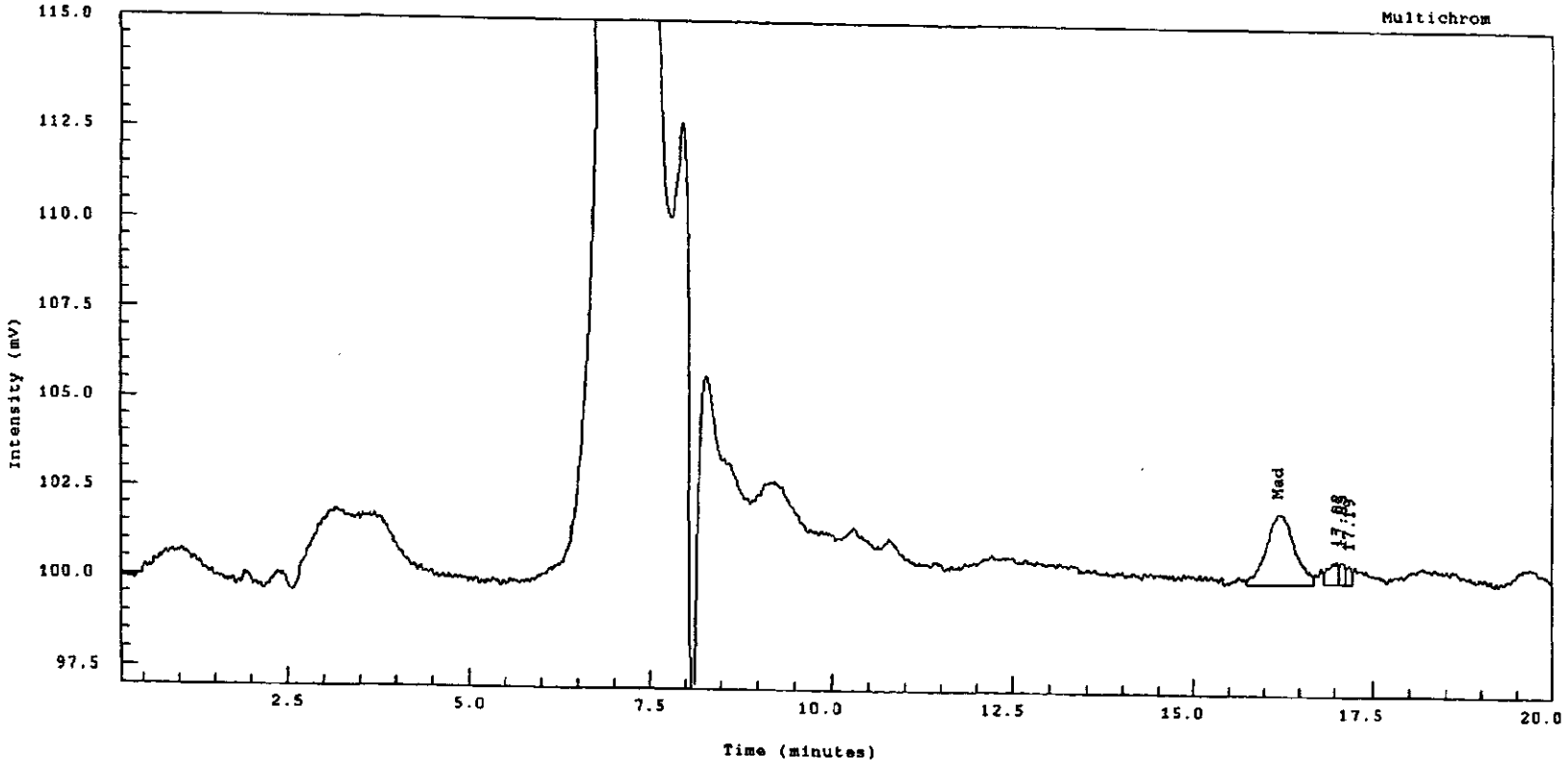
Calibration : MA201200

Run Sequence : MA201200

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_20DEC001027,9,1.

258 - 5




Instrument :

Channel Title : Channel #3

Lims ID : 1

Acquired on 20-DEC-2000 at 13:24

Reported on 21-DEC-2000 at 16:08

Method : MAD-103

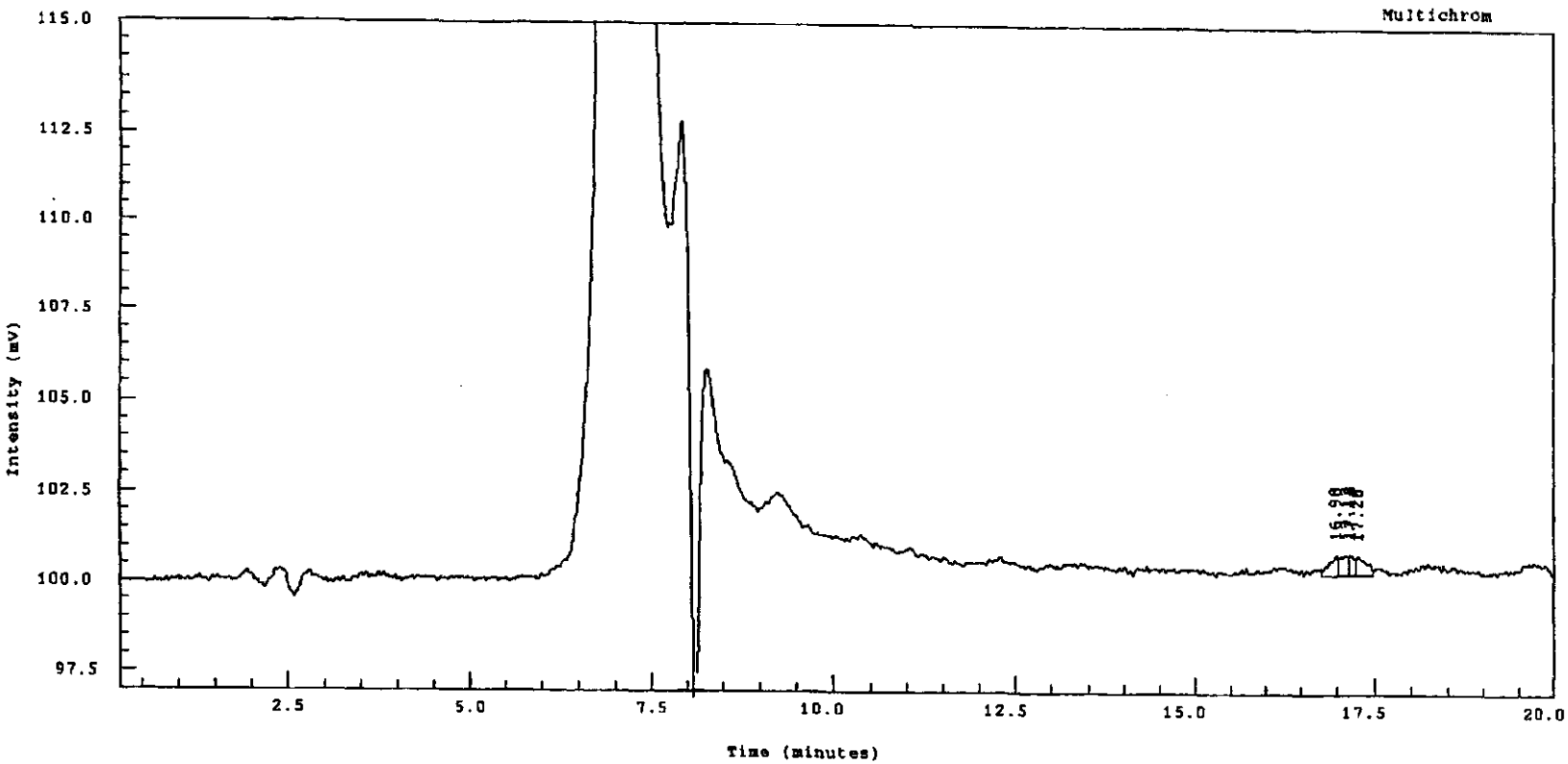
Calibration : MA201200

Run Sequence : MA201200

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_20DEC001027,8,1.

Blank




Instrument :

Channel Title : Channel #3

Lims ID : 1

Acquired on 20-DEC-2000 at 13:02

Reported on 21-DEC-2000 at 16:08

Method : MAD-103

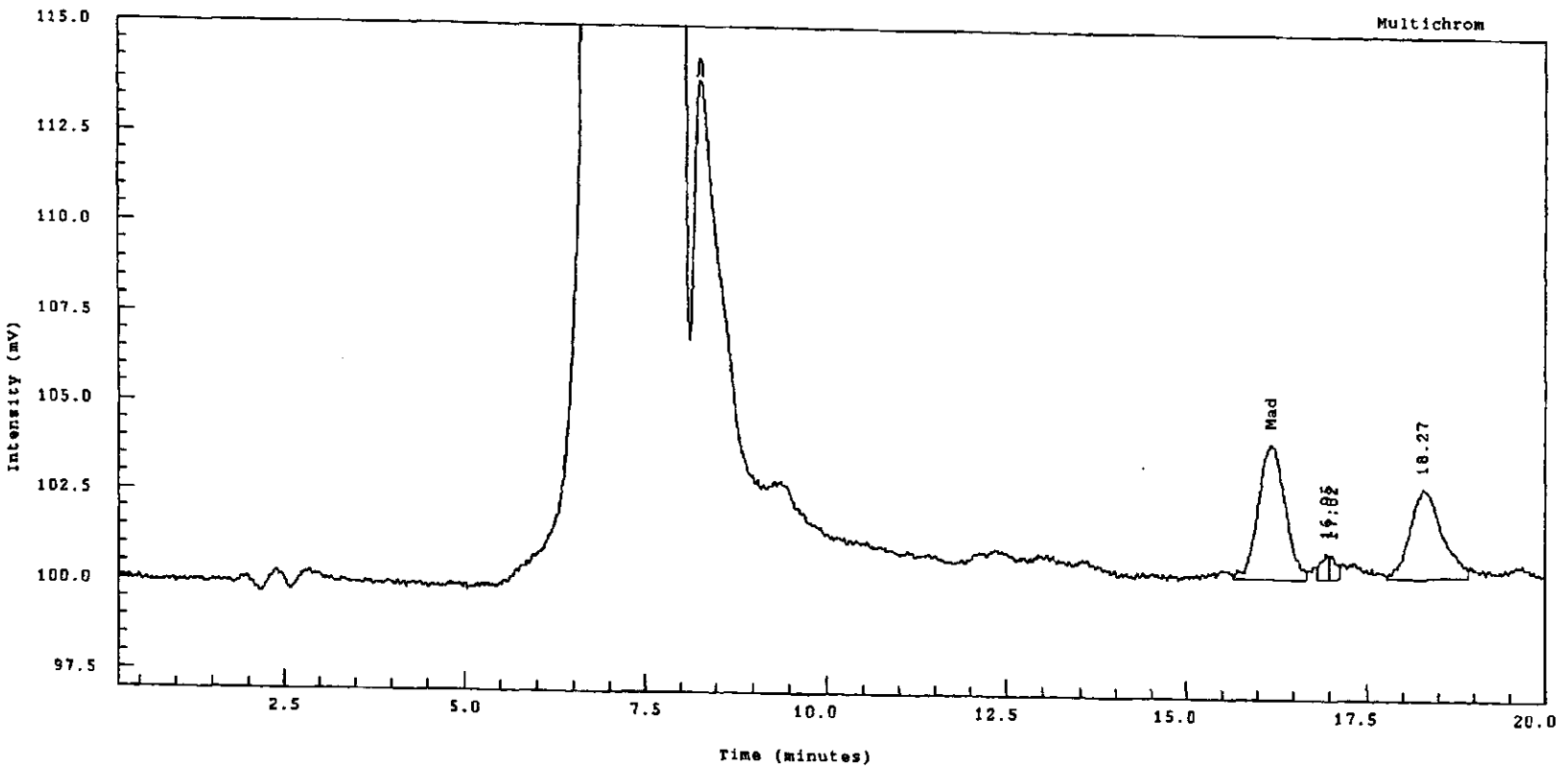
Calibration : MA201200

Run Sequence : MA201200

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_20DEC001027,12,1.

291 - 8




Instrument :

Channel Title : Channel #3

Lims ID : 1

Acquired on 20-DEC-2000 at 14:39

Reported on 21-DEC-2000 at 16:10

Method : MAD-103

Calibration : MA201200

Run Sequence : MA201200

Appendix 6

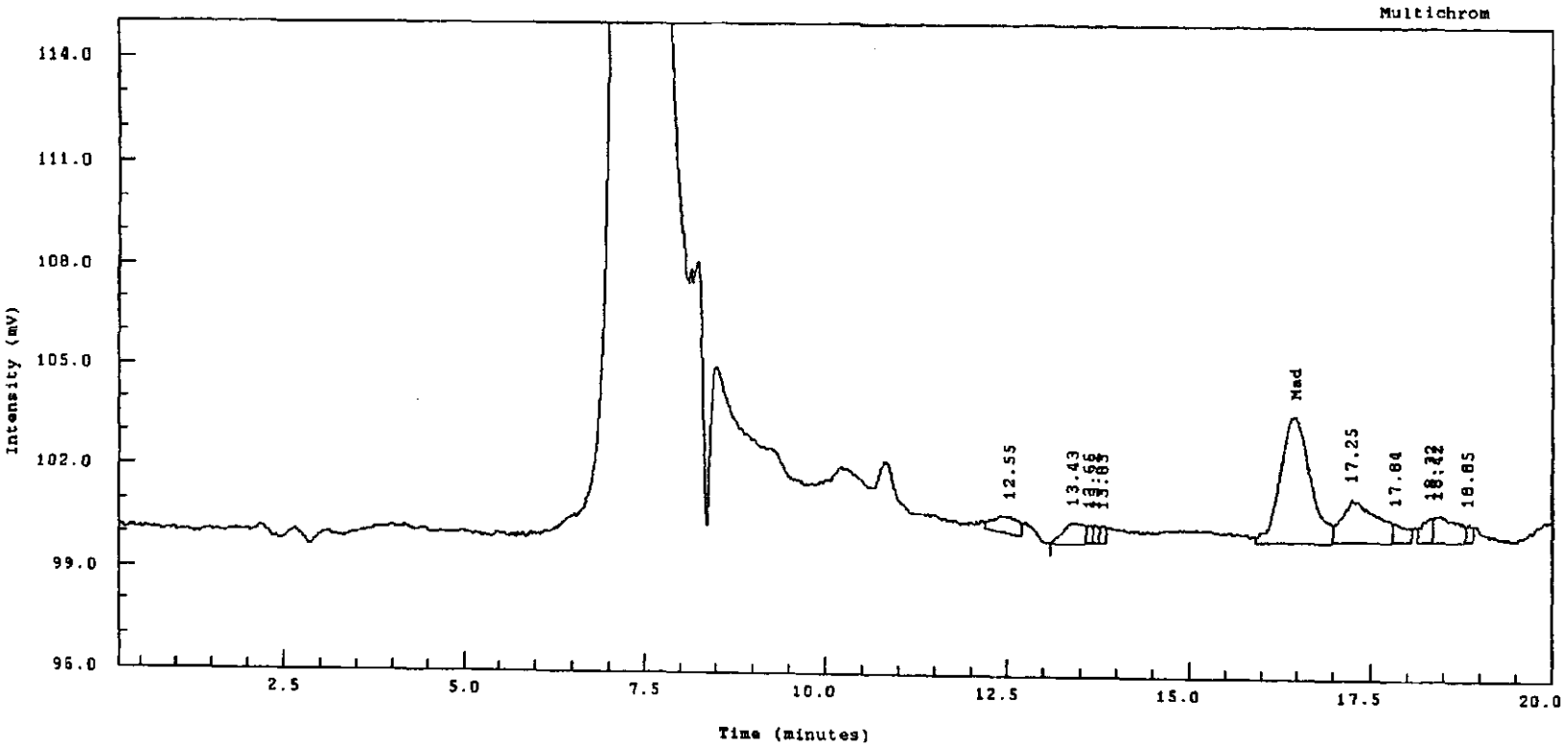
Results of special requests:

Results of Hoffmann-La Roche applying post-column derivatisation with DMAB

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_21DEC001100,9,1.

DMBA , 2,5 mg/kg




Instrument :

Channel Title : Channel #3

Lims ID : 1

Acquired on 21-DEC-2000 at 14:37

Reported on 21-DEC-2000 at 16:05

Method : MAD-103

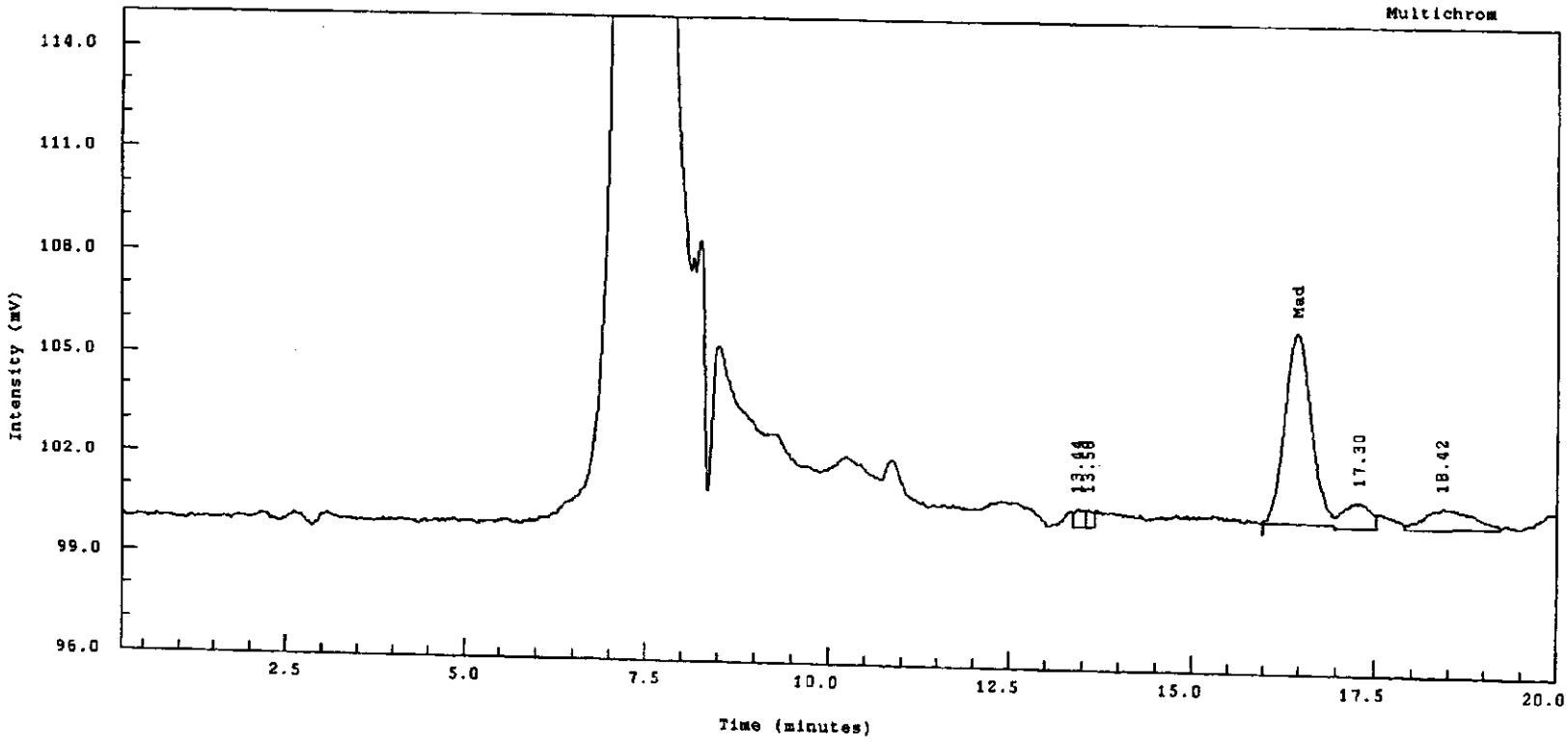
Calibration : MA2112DM

Run Sequence : MA2D1200

MULTICHROM 2.20h

Analysis Name : [K03_MFA] 3 MA_21DEC001100,7,1.

DMBA , 4,5 mg/kg




Instrument :

Channel Title : Channel #3

Lims ID : 1

Acquired on 21-DEC-2000 at 13:48

Reported on 21-DEC-2000 at 16:04

Method : MAD-103

Calibration : MA2112DM

Run Sequence : MA201200

Appendix 6

Results of special requests:

Results of LUFA-Augustenberg applying post-column derivatisation with DMAB

## CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - MADURAMICIN

**Annex 4 - Questionnaire**

Laboratory: LUFA Karlsruhe

Contact person: THALHAMER

Date(s) of analysis: 17.01. - 21.01.2001

Dilution factor of the samples:

- Feed samples (specify for which feed samples): Nic Annex 1 5.2.1
- Premixture: Nic Annex 1 5.2.2 1:10

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Hypersil ODS, 5 µm 250 x 4,6
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,7 ml/min
- Injection volume: 100 µl
- Retention time of maduramicin: 5,45 min

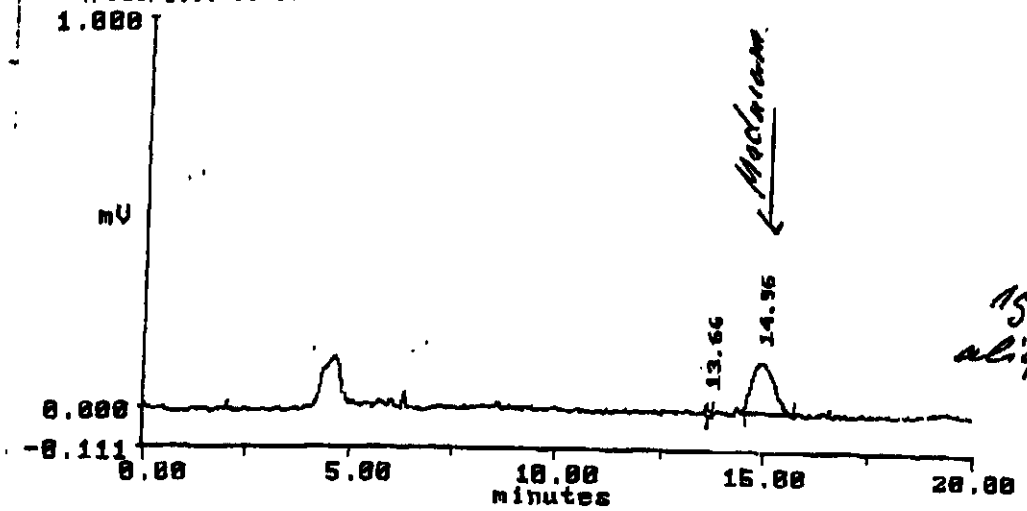
**Chromatograms: Please include representative chromatograms of:**

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

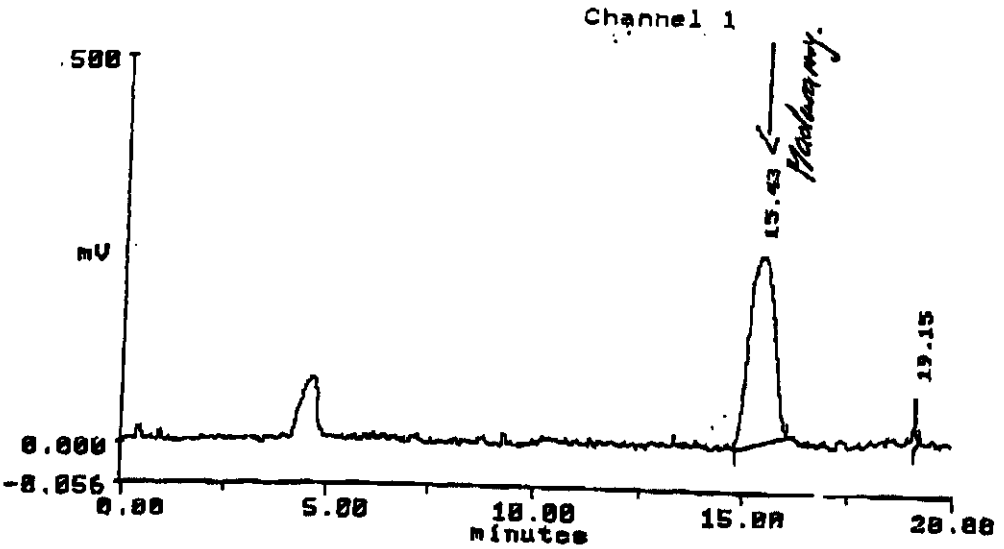
*Please indicate the maduramicin peak with an arrow*

Recovery results: 101,85

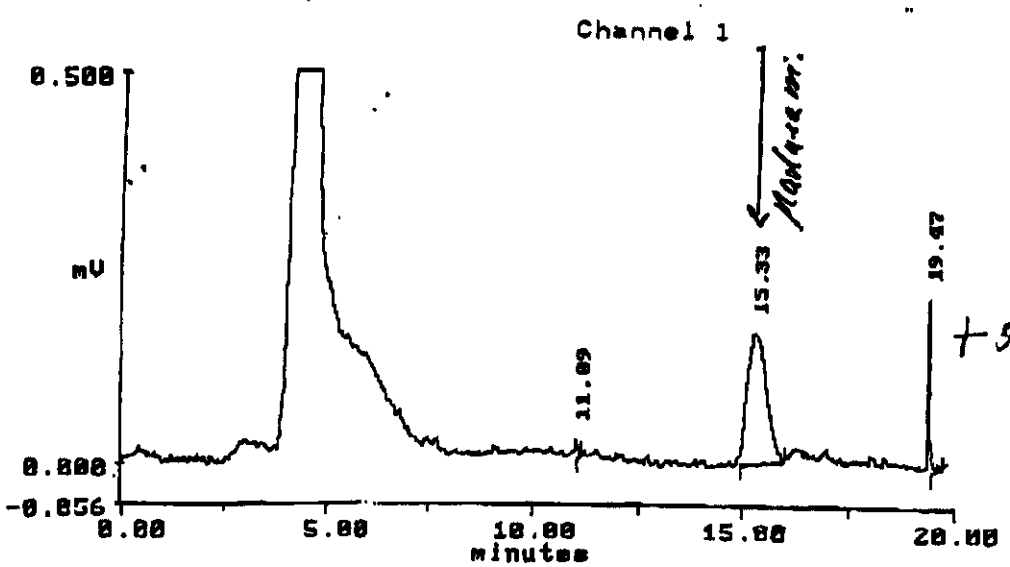
- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 5 mg/kg 103,98 / 99,16




Premix
15 + 50
aliquote 1:10




Std.
≙ 210 µg/ml




Blank Feed
+ 500 µl stock-stand.
(100 µg/ml)