Project 70311.40

Results of the second collaborative study on zinc bacitracin in animal feed (supplements) with a modified microbiological method

Projectleader(s): J. de Jong, H.J. van Egmond

Rapport 97.07 16 April 1997

RESULTS OF THE SECOND COLLABORATIVE STUDY ON ZINC BACITRACIN IN ANIMAL FEED (SUPPLEMENTS) WITH A MODIFIED MICROBIOLOGICAL METHOD

H.J.van Egmond

Section: Microbiology & Biotechnology

Co-workers: W.Driessen-van Lankveld, E. van Neer-Streutjens

State Institute for Quality Control of Agricultural Products (RIKILT-DLO)
Bornsesteeg 45, 6708 PD Wageningen
P.O. Box 230, 6700 AE Wageningen
The Netherlands
Telephone +31 317 475400
Telefax +31 317 417717

MAILING LIST

INTERNAL:
Director
Author(s)
Heads of research departments (programleaders) (4x)
Projectleader(s) (2x)
In- and external communications department (2x)
Library (3x)
"Werkgroep analysemethoden diervoeders en toevoegingen", H.Keukens

EXTERNAL:
Alpharma, Norway, mr. R.Sandvik
Laboratoire Interrégional, France, dr. J.Michard
LUFA Augustenberg, Germany, dr. A.Thalmann
Plantedirektoratet, Denmark, dr. T.Hoeg
Rijksontledingslaboratorium, Belgium, dr. K.Haustraete
Staffordshire County Council, County Laboratory, England, mr. B.Dredge
State Laboratory Abbotstown, Ireland, dr. D.Hayes
Swiss Federal Research Station for Animal Production, Switzerland, dr. J.L.Gafner
TNO-Voeding, Zeist, ir. A.Bovee
LUFA Kiel, Germany, ms. I.Blank
Bayerische Landesanstalt für Ernahrung, Germany, dr. E.Bucher
DLG Centrallaboratoreum, Denmark, ms. I.Grunnet
Bioteknologisk Institut, Denmark, ms. M.Virsoe
CCL/Cehavé, Veghel, dr. R.Margry

ABSTRACT

Results of the second collaborative study on zinc bacitracin in animal feed (supplements) with a modified microbiological method.

Report 97.07 16 April 1997

H.J. van Egmond

State Institute for Quality Control of Agricultural Products (RIKILT-DLO)
P.O. Box 230, 6700 AE Wageningen, the Netherlands

0 figures, 1 table, 3 annexes, 7 pages, 2 references

To confirm the promising results of a study in May 1996, a second collaborative study on zinc bacitracin in animal feed (supplements) with a modified microbiological method was organized by RIKILT-DLO.
The study included five samples at different levels of zinc bacitracin: one concentrate (15%), one premix (8000 ppm), one milk replacer (80 ppm), one mash pig feed (50 ppm) and one mash turkey feed (10 ppm) and was performed by fourteen official and private European laboratories.

Thirteen of the fourteen laboratories reported their analytical results. The results showed good recoveries (96-109%) at the five levels of zinc bacitracin. The variation between the laboratories (VC_R) was acceptable and varied from 5.4-17.5%. The results are similar to the results of the study in May 1996.

Keywords: zinc bacitracin, animal feeds, modified microbiological method, collaborative study, premix, concentrates, final feeds.

FOREWORD

The author wishes to thank the participants of this collaborative study for their contribution and Reidar Sandvik (fa. Alpharma AS) for supplying the samples and for technical support.

CONTENT page

1 INTRODUCTION

In May 1996 a collaborative study on zinc bacitracin in animal feed (supplements) with a modified microbiological method was organized by RIKILT-DLO. This modified method differs from the official EEC method (Official Journal of the European Communities (18.1.84) no. L15/35-38) in the following points:

- increase of the sensitivity by adding neomycin to the testmedium
- decrease of the zinc bacitracin concentration in S_8 and U_8 to 0.05 IU/ml
- no evaporation step
- dilutions in methanol/buffer mixture.

The results of this study with eight participants were promising (RIKILT-DLO report 96.23 (1)) and it was decided to confirm the results of this first study by a second study with more participants.

The following laboratories were invited to participate in this second collaborative study:

- State Laboratory Abbotstown, Dublin (Ireland)
- Rijksontledingslaboratorium, Antwerpen (Belgium)
- State Institute for Quality Control of Agricultural Products RIKILT-DLO, Wageningen (The Netherlands),
- Plantedirektoratet, Lyngby (Denmark),
- Laboratoire Interregional, Rennes (France),
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt Augustenberg, Karlsruhe (Germany),
- Swiss Federal Research Station for Animal Production, Posieux (Switzerland),
- Alpharma AS, Oslo (Norway) (largest European producer of zinc bacitracin),
- TNO-Voeding, Zeist (The Netherlands),
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt Kiel, Kiel (Germany),
- Bayerische Landesanstalt für Ernahrung, Münich (Germany),
- DLG Centrallaboratoreum, Odense (Denmark),
- Bioteknologisk Institut, Kolding (Denmark),
- Staffordshire County Council, County Laboratory, Stafford (England)

The results of this second collaborative study are summarised in this report.

2 MATERIAL AND METHODS

2.1 Samples

The test samples for this collaborative study were arranged and distributed by Alpharma AS, Oslo, Norway.

* The Albac 15% Granulated concentrate, lot 114264 was manufactured by Alpharma As, Oslo, Norway.
* The premix with 8000 ppm and milk replacer with 80 ppm were produced by Celtic Nutrition Animale, Rennes, France.
* The mash pig feed and mash turkey feed (50 and 10 ppm zinc bacitracin) were produced by Alpharma AS, Oslo, Norway. Pelleted feed free of antibiotic was obtained from a local supplier. This feed was milled and mixed with zinc bacitracin in the laboratories of Alpharma AS.

The samples were distributed to the participants immediately after preparation (November 1996). Each participant also received zinc bacitracin reference material (standard) and a neomycin standard. Participants were asked to report the sample weights, extraction volumes, dilutions, recorded inhibition zones and final result on forms.

2.2 Method

The modified microbiological method was sent to the participants a few weeks before the samples were sent. A copy of the method is included in this report (appendix I).
The participants were asked to analyze the samples in threefold at three different days and to use the forms and standards supplied by Alpharma AS.

3 RESULTS

Within two months thirteen of the participants reported the results of the collaborative study to RIKILT-DLO. At RIKILT DLO the results were statistically analyzed according to ISO 5725 (2). The results from the laboratories were tested on stragglers and outliers with the Dixon test (test on mean values) and Cochran test (test on mean standard deviations).
The analytical results for the different samples are tabulated in Appendix II (tables 1 to 5).

The outliers and stragglers selected with the Cochran and Dixon test are excluded from the data-set. Three mean values and one single result have been removed from the data-set. These are indicated with footmarks in the tables.

In table 1 the statistical results for all samples are summarized.

Table 1: Final results of the study on zinc bacitracin with a modified microbiological method

Sample	Level (mg/kg)	n	X_{mean} (mg/kg)	Recovery (%) (*)	R (mg/kg)	CV_R (%)
concentrate Albac 15% Granulated lot 114264	150000	12	151000	101	22600	5.4
premix	8000	12	7651	96	1816	8.5
milk replacer	80	12	81.2	101	26.1	11.5
mash pig feed	50	12	53.7	107	15.4	10.3
mash turkey feed	10	13	10.9	109	5.3	17.4

(*) with the assumption that the theoretical value (dosage) is correct

Table 1 indicates that the results are satisfying and similar to the results of the study of May 1996. The recovery varied from 96-109% and is almost identical to the 96-109% range from May 1996. The variation between the laboratories (CV_R) varied from 5.4-17.4% and in May 1996 from 4.5-14.5%.

Some problems with growth of the teststrain were reported. The incubation temperature, amount of inoculum, quality of the assay-agar (supplier) and amount of neomycin-sulphate in the assay-agar will influence the growth of the bacteria. By varying the above mentioned parameters, all laboratories were able to obtain good growth of the bacteria.

A few laboratories reported problems to obtain inhibition-zones with the S_1 and U_1 (0.00625 IU zinc bacitracin/ml). The same parameters which support the growth of the bacteria will influence the sensitivity for zinc bacitracin. It is our experience that separate ingredients (Difco) with the prescribed amount of neomycin-sulphate gives good sharp zones of inhibition. The same results may be obtained by using Antibiotic medium no. 1 (Oxoid) with half the amount of neomycin-sulphate.

In the next version of the modified method, the amount of neomycin-sulphate will be mentioned as a guideline with a comment that the quantity may be changed if this produces better zones. Another modification of the method will be the incubation temperature (30-37°C instead of 30 ± 2°C).

The suggestions/comments which were added to the results are summarised in appendix III. From these suggestions it can be seen that no major problems occurred.

4 CONCLUSION

In a collaborative study with thirteen laboratories, the modified microbiological method for zinc bacitracin in animal feed (supplements) gave good results.
The recovery and reproducibility (CV_R) are satisfying at five different levels of zinc bacitracin. The recovery varied from 96-109% and the reproducibility (CV_R) from 5.4-17.4%. The results are similar to the results of a smaller study in May 1996.

LITERATURE

(1) Egmond, H.J. van - Results of a collaborative study on zinc bacitracin in animal feeds with a modified microbiological method. RIKILT-DLO report 96.23, August 1996.

(2) ISO 5725 - Precision of test methods - Determination of repeatability and reproducibility by inter laboratory testing. - Second edition (1986)

rikilt-dlo

The determination of zinc bacitracin in complete feeding stuffs, feed premixes, concentrates and milk replacers by diffusion in an agar medium.

1 SCOPE AND FIELD OF APPLICATION

This method describes the quantitative determination of zinc bacitracin in complete feeding stuffs, feed premixes, concentrates and milk replacers. The limit of determination for complete feeding stuffs, concentrates and feed supplements is 2,5 mg/kg, for milk replacers 5 mg/kg. [1]

2 PRINCIPLE

The sample is extracted at pH <2, with a mixture of methanol, hydrochloric acid, water and sodium sulphide. The sodium sulphide is to precipitate any soluble copper salts that may interfere with the assay. After additon of phosphate buffer pH 6.5, the extract is brought to pH 6.5-7.2 with a sodium hydroxide solution, and diluted with methanol/phosphate buffer mixture to an expected amount of 0,05 IE zinc bacitracin/ml.
Its antibiotic activity is determined by measuring the diffusion of zinc bacitracin in an agar medium (with neomycin sulphate) inoculated with Micrococcus luteus ATCC 10240.
Neomycin sulphate is added to the agar, to increase the sensibility of the micro-organism for zinc bacitracin. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed.

3 MICRO-ORGANISM

3.1 Maintenance of stock culture

Inoculate tubes containing slopes of culture medium (4.1) with Micrococcus luteus ATCC 10240 and incubate for 24 hours at 30°C. Store the culture in a refrigerator (0-5°C). Reinoculate every two weeks.

3.2 Preparation of the bacterial suspension [2]

Harvest the growth from a recently prepared agar slope (3.1) with 2-3 ml of sodium chloride solution (4.6). Use this suspension to inoculate 250 ml of culture medium (4.1) contained in a Roux flask and incubate for 18-20 hours at 30°C. Harvest the growth in 25 ml of sodium chloride solution (4.6) and mix. Dilute the suspension to 1/10 with sodium chloride solution (4.6). The light transmission of the suspension must be about 75%, measured at 650 nm in a 1 cm cell against sodium chloride solution (5.6). This suspension may be kept for one week at 0-5°C.

4 CULTURE MEDIA AND REAGENTS

4.1 Culture medium [3]

meat peptone	6	g
tryptone	4	g
yeast extract	3	g
meat extract	1,5	g
glucose	1	g
agar	10-20	g
demineralised water	1000	ml

pH 6,5 ± 0,1 (after sterilization)

[1] 1 mg feedingstuff grade zinc bacitracin is equivalent to 42 international units (IU).
[2] Other methods may be used provided that it has been established that they give similar bacterial suspensions.
[3] Any commercial culture medium of similar composition and giving the same results may be used.

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

rikilt-dlo

4.2 Assay medium [1]

tryptone	10	g
yeast extract	3	g
meat extract	1,5	g
glucose	1	g
agar	15	g
tween 80	1	ml
demineralised water	1000	ml

pH 6,5 ± 0,1 (after sterilization)

4.3 Standard zinc bacitracin of known activity (in IU/mg).

4.4 Methanol p.a.

4.5 Hydrochloric acid (d 1.18 to 1.19)

4.6 Sodium chloride solution (0,85%) (w/v)

Sodium chloride	8,5	g
demineralised water	1000	ml

Sterilize.

4.7 Sodium hydroxide solution 1 M.

4.8 Hydrochloric acid solution 0,1 M or 1,0 M

4.9 Phosphate buffer pH 6,5

potassium dihydrogen phosphate	(KH_2PO_4)	27,85	g
dipotassium hydrogen phosphate	(K_2HPO_4)	22,15	g
demineralised water until		1000	ml

final pH 6,5 ± 0,1

4.10 Methanol (4.4)/phosphate buffer pH 6,5 (4.9) mixture (3:7)

4.11 Methanol (4.4)/water/hydrochloric acid (4.5) mixture (80:17,5:2,5)

4.12 Sodium sulphide solution (0,5 M)

Sodium sulphide ($Na_2S\ xH_2O$ (x=7-9))	3	g
demineralised water	25	ml

4.13 Neomycin sulphate solution 0,071% (w/v) in water.

5 STANDARD SOLUTIONS

Weigh out a quantity of zinc bacitracin (4.3) corresponding to 2500 IU zinc bacitracin.
Dissolve the material with 5 ml 0,1 M HCl (4.8) by gentle shaking for 5 minutes, add 5 ml phosphate buffer pH 6,5 (4.9). Make to a volume of 50 ml with demineralised water and mix well.
This solution contains 50 IU zinc bacitracin/ml.

From this solution prepare by successive (1+1) dilution with methanol/phosphate buffer mixture (4.10) the following solutions:

S_8: 0,05 IU/ml
S_4: 0,025 IU/ml
S_2: 0,0125 IU/ml
S_1: 0,00625 IU/ml.

[1] Any commercial culture medium of similar composition and giving the same results may be used.

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

rikilt-dlo

6 PREPARATION OF THE EXTRACT AND ASSAY SOLUTIONS

6.1 Extraction

6.1.1 Complete feeding stuffs, premixes and concentrates.

Weigh out a quantity of sample of 1-30 g (See appendix A, for the exact amount), add 49,0 ml methanol/water/HCl mixture (4.11) and 1,0 ml of sodium sulphide solution (4.12) and shake firmly.
Check that the pH is below 2. If necessary, bring the pH below 2 with 1 M hydrochloric acid (4.8). Shake for 10 minutes. Add 50,0 ml phosphate buffer pH 6,5 (4.9), shake for 15 minutes and centrifuge. Take a suitable volume of the supernatant and adjust the pH to 6,5 - 7,2 by means of 1 M Sodium hydroxide solution (4.7). Dilute with methanol/phosphate buffer mixture (4.10) to obtain an expected zinc bacitracin content of 0,05 IU/ml (U8)

6.1.2 Milk replacers.

Weigh out a quantity of sample of 5-30 g (See appendix A, for the exact amount), add 98,0 ml methanol/water/HCl mixture (4.11) and 2,0 ml of sodium sulphide solution (4.12) and shake firmly.
Check that the pH is below 2. If necessary, bring the pH below 2 with 1 M hydrochloric acid (4.8). Shake for 10 minutes. Add 100,0 ml phosphate buffer pH 6,5 (4.9), shake for 15 minutes and centrifuge. Take a suitable volume of the supernatant and adjust the pH to 6,5 - 7,2 by means of 1 M Sodium hydroxide solution (4.7). Dilute with methanol/phosphate buffer mixture (4.10) to obtain an expected zinc bacitracin content of 0,05 IU/ml (U8)

6.2 Assay solutions

From solution U_8 prepare solutions U_4 (expected content: 0,025 IU/ml), U_2 (expected content: 0,0125 IU/ml), U_1 (expected content: 0,00625 IU/ml) by means of successive dilution (1+1) with methanol/phosphate buffer mixture (4.10).

7 ASSAY PROCEDURE

7.1 Inoculation of the assay medium

Inoculate the assay medium (4.2) with the bacterial suspension (3.2) and neomycin sulphate solution (4.13) 0,20 ml per 150 ml of assay medium at about 50°C.
By preliminary trials on plates with assay medium (4.2) determine the quantity of bacterial suspension required to give the largest and clearest zones of inhibition with the various concentrations of zinc bacitracin.

7.2 Preparation of the plates

Diffusion through agar is carried out in plates with the four concentrations of the standard solutions (S_8, S_4, S_2, S_1) and the four concentrations of the assay solutions (U_8, U_4, U_2, U_1).
These four concentrations of extract and standard must necessarily be placed in each plate. To this effect, select plates big enough to allow at least eight holes with a diameter of 9-13 mm and not less than 30 mm between centres to be made in the agar medium.

Pour into the plates a quantity of the medium (4.2) inoculated as in point 7.1 to give a layer of about 2 mm thick, Allow to set in a level position, bore the holes and place in them exactly measured volumes of assay and standard solutions (between 0,10 and 0,15 ml) per hole, according to the diameter). Apply each concentration at least four times so that each determination is subject to an evaluation of 32 zones of inhibition.

7.3 Incubation

Incubate the plates for 16-18 hours at 30 ± 2°C.

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

rikilt-dlo

8 EVALUATION

Measure the diameter of the zones of inhibition to the nearest 0,1 mm, by direct measurement or projection. Record the mean measurements for each concentration on semi-logarithmic graph paper showing the logarithm of the concentrations in relation to the diameters of the zones of inhibition. Plot the "best fit" lines of both the standard and the extract, for example as below.

Determine the "best fit" point for the standard lowest level (S_L) using the formula:

(a) $S_L = \frac{(7S_1 + 4S_2 + S_4 - 2S_8)}{10}$

Determine the "best fit" point for the standard highest level (S_H) using the formula:

(b) $S_H = \frac{(7S_8 + 4S_4 + S_2 - 2S_1)}{10}$

Similarly, calculate the "best fit" points for the extract lowest level (U_L) and the extract highest level (U_H) by substituting U_1, U_2, U_4 and U_8 for S_1, S_2, S_4 and S_8 in the above formulae.

Record the calculated S_L and S_H values on the same graph paper and join them to give the "best fit" line for the standard solution.

Similarly record U_L and U_H and join then to give the "best fit" line for the extract.

In the absence of any interference the lines should be parallel.
For practical purposes the lines can be considered parallel if the values (S_H - S_L) en (U_H - U_L) do not differ by more than 10% from their mean value.

If the lines are found to be non-parallel, either U_1 and S_1, and U_8 en S_8 may be discarded and S_L, S_H, U_L en U_H calculated, using the alternative formulae, to give alternative "best fit" lines:

$S_L = \frac{(5S_1 + 2S_2 - S_4)}{6}$ or $\frac{(5S_2 + 2S_4 - S_8)}{6}$

$S_H = \frac{(5S_4 + 2S_2 - S_1)}{6}$ or $\frac{(5S_8 + 2S_4 - S_2)}{6}$

and similarly for U_L en U_H. The alternative "best fit" lines should be checked for parallelism as before. The fact that the result has been calculated from three levels should be noted on the final report.

When the lines are considered as being parallel, calculate the logarithm of the relative activity (log A) by means of one of the following formulae:

<u>for four levels:</u> $\log A = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8)}{(U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2)} \times 0.602$

<u>for three levels:</u> $\log A = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4)}{(U_4 + S_4 - U_1 - S_1)} \times 0,401$

or

$\log A = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8)}{(U_8 + S_8 - U_2 - S_2)} \times 0,401$

If the relative activity is found to be outside the range of 0,5 to 2,0 then repeat the assay making appropriate adjustments to the extract concentrations or, if this is not possible, to the standard solutions.

When the relative activity cannot be brought into the required range, any result obtained must be considered as approximate and this should be noted on the final report.

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

Appendix A

Sample size and dilutions for complete feeding stuffs, premixes and concentrates.

rikilt-dlo

Expected amount of zinc bacitracin	Sample size and dilutions
5 mg/kg	30 g--50+50=100 | 20---25
10 mg/kg	20 g--50+50=100 | 15---25
20 mg/kg	20 g--50+50=100 | 15---50
80 mg/kg	20 g--50+50=100 | 15---200
200 mg/kg	10 g --50+50=100 | 15---250
1000 mg/kg	2 g--50+50=100 | 15---250
3000 mg/kg	2 g--50+50=100 | 20---100 | 10---100
1%	1 g--50+50=100 | 5---100 | 25---100

Sample size and dilutions for milk replacers.

Expected amount of zinc bacitracin	Sample size and dilutions
10 mg/kg	30 g--100+100=200 | 20---25
20 mg/kg	20 g--100+100=200 | 15---25
80 mg/kg	20 g--100+100=200 | 15---100

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

When the lines are considered as not being parallel, repeat the determination. If parallelism is still not achieved, the determination must be considered as unsatisfactory.

Real activity = supposed activity x relative activity

Express the result as: "equivalent with a microbiological activity of .... mg/kg zinc bacitracin".

9 REPEATABILITY

The difference between the results of two determinations carried out on the same sample by the same analyst should not exceed:
-2 mg/kg, in absolute values, for contents of zinc bacitracin up to 10 mg/kg
-20% related tot the highest value for contents from 10 to 25 mg/kg
-5 mg/kg, in absolute value for contents of 25 to 50 mg/kg
-10% related to the highest value for contents above 50 mg/kg.

Bornsesteeg 45, Postbus 230, 6700 AE Wageningen, The Netherlands
Telefoon: 0317 - 47 54 00, Telefax: 0317 - 41 77 17, Postbank: 425263

APPENDIX II

Table 1: results of the collaborative study on zinc bacitracin in concentrate Albac 15% granulated lot 114264 (150 g/kg)

Laboratory	Results (g/kg zinc bacitracin)			
	Day 1	Day 2	Day 3	Average
1	147	153	151	150
2	154	167	153	158
3	156	144	178	159
4	131	160	157	149
5	148	154	160	154
6	142	149	149	147
7	142	155	157	151
8	152	150	150	151
9	136	136	144	139
10	151	149	156	152
11	155	150	152	152
12	135	122	103	120 (*)
13	145	145	146	145

n	: 12	reproducibility	: 22.6 g/kg
X_{mean}	: 151 g/kg	$S_{(reproducibility)}$	: 8.1 g/kg
		$CV_{(reproducibility)}$	: 5.4%

(*) lab. no. 12 is outlier (Dixon)

Table 2: Results of the collaborative study on zinc bacitracin in premix (8000 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)			
	Day 1	Day 2	Day 3	Average
1	8114	8029	7993	8045
2	8223	8140	8347	8236
3	8873	8253	7936	8354
4	4120	3980	4420	4173 (*)
5	7395	6677	6804	6959
6	7426	7643	7302	7457
7	8160	8860	9015	8678
8	8061	8092	8000	8051
9	7014	7203	6701	6972
10	7843	7501	7904	7749
11	6810	6860	7040	6903
12	7287	7783	7035	7368
13	7077	7031	6989	7032

n	: 12	reproducibility	: 1816 mg/kg
X_{mean}	: 7651 mg/kg	$S_{(reproducibility)}$	: 649 mg/kg
		$CV_{(reproducibility)}$	: 8.5%

(*) lab. no.4 is straggler (Dixon)

Table 3: Results of the collaborative study on zinc bacitracin in milk replacer (80 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)			
	Day 1	Day 2	Day 3	Average
1	79.4	79.5	79.5	79.5
2	91.3	91.6	85.0	89.3
3	91.4	86.7	89.7	89.3
4	84.0	80.8	84.4	83.1
5	74.9	75.9	75.0	75.3
6	90	88	84	87.3
7	100	116	126	114.0 (*)
8	80	80	80	80
9	71.0	75.0	80.5	75.5
10	76	78	75	76.3
11	94.1	109.0	93.3	98.8
12	84.0	63.0	64.0	70.3
13	71.5	70.5	66.9	69.6

n	: 12	reproducibility	: 26.1 mg/kg
X_{mean}	: 81.2 mg/kg	$S_{(reproducibility)}$	: 9.3 mg/kg
		$CV_{(reproducibility)}$	: 11.5%

(*) lab. 7 is outlier (Dixon) and straggler (Cochran)

Table 4: Results of the collaborative study on zinc bacitracin in mash pig feed (50 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)			
	Day 1	Day 2	Day 3	Average
1	51.6	49.8	50.7	50.7
2	62.0	63.7	55.0	60.2
3	59.0	39.0 (**)	55.6	57.3
4	48.9	48.4	57.4	51.6
5	51.2	45.7	53.7	50.2
6	55	54	57	55.3
7	57	66.2	61.5	61.6
8	50	51	55	52.0
9	53.6	48.9	53.8	52.1
10	60	57	58	58.3
11	78.5	74.4	78.8	77.2 (*)
12	55	49	49	51.0
13	42.5	47.8	44.5	44.9

n	: 12	reproducibility	: 15.4 mg/kg
X_{mean}	: 53.3 mg/kg	$S_{(reproducibility)}$	: 5.5 mg/kg
		$CV_{(reproducibility)}$	: 10.3%

(*) lab. 11 is outlier (Dixon)

(**) unsatisfactory parallelism, this result of lab. 3 is an outlier (Cochran) and excluded from the results

Table 5: Results of the collaborative study on zinc bacitracin in mash turkey feed (10 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)			
	Day 1	Day 2	Day 3	Average
1	10.4	10.3	10.5	10.4
2	12.1	12.4	11.3	11.9
3	12.6	8.1 (*)	11.9 (*)	10.9
4	9.1	10.1	11.2	10.1
5	10.8	6.9	9.3	9.0
6	15	14	14	14.3
7	12.4	13.8	13.6	13.3
8	7.8	11.6	12.5	10.6
9	9.0	9.0	10.2	9.4
10	11	10	10	10.7
11	11.7	10.3	12.5	11.5
12	11	13	11	11.7
13	7.6	8.4 (*)	8.7	8.2

n	: 13	reproducibility	: 5.3 mg/kg
X_{mean}	: 10.9 mg/kg	$S_{(reproducibility)}$	: 1.9 mg/kg
		$CV_{(reproducibility)}$	: 17.4%

no stragglers or outliers

(*) unsatisfactory parallelism

APPENDIX III

Comments/suggestions from the different laboratories.

Lab.	Comment/suggestions
1	-
2	-
3	-
4	-
5	Assay-medium with separate ingredients (Difco), pH 6.5 gives good results. Other assay-media can give growth problems. Adjusting the pH to 6.8 will solve these problems. Stability of zinc bacitracin in premix extract may be enhanced by adding cystein or thioacetamid.
6	-
7	-
8	No inhibition zones with S_1 and U_1.
9	-
10	Plates were easier to read after more than 24 hours of incubation.
11	Instead of sodiumsulphide, cystein (2%) and thioacetamide (0.5%) is used.
12	No inhibition zones with S_1 and U_1.
13	Incubation at 36°C.