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

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ABSTRACT

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

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

In May 1996 a collaborative study on zinc bacitracin in animal feed (supplements) with a modified microbiological method was organized by RIKILT-DLO. Eight official and private European laboratories participated and the study included six samples at five levels of zinc bacitracin: two concentrates (15%), one premix (8000 ppm), one milk replacer (80 ppm), one mash broiler feed (50 ppm) and one mash turkey feed (20 ppm).

The analytical results showed a good recovery (96-114%) at all levels of zinc bacitracin. At the four lowest levels one laboratory produced significantly higher results. These results were not included in the statistical analysis. The variation within the laboratories (VC_r) varied from 4.2 to 9.5%. The interlaboratory variation (VC_R) was also satisfactory and varied from 4.5 to 14.5%.

Keywords: zinc bacitracin, animal feed, modified microbiological method, collaborative study, premix, concentrates, milk replacer, turkey feed, broiler feed.

FOREWORD

The author wishes to thank all 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 several European countries problems have been encounted with the official EEC method for zinc bacitracin (Official Journal of the European Communities (18.1.84) no. L15/35-38) in complete feeding stuffs, concentrates and premixes.
Over the last few years several studies have been organized, and they demonstrate unsatisfactory variation between the laboratories and problems with parallelism and recovery.

An English study (8 laboratories) in 1992 with 10 ppm samples showed a good mean recovery but the interlaboratory variation (CV_R) was very high (±60%). The results varied from 1 - 20 ppm. (1)
A European study (9 laboratories) in 1992 with 15%, 2000 and 20 ppm zinc bacitracin samples gave a satisfactory recovery, but the CV_R was 66.7% at the lowest level.(1)
In another European study (9 laboratories) in 1994 containing 2 samples (10 and 50 ppm), many laboratories reported problems with parallelism and recoveries and the CV_R was about 30%.(2)

The recovery problems arise when the sample extract has to be evaporated to obtain a concentration of 0.42 International Units (IU) zinc bacitracin/ml. When this step can be avoided (samples with higher concentrations of zinc bacitracin) the recovery is good. The problems with parallelism may be due to the effect of the diluent used (phosphate buffer pH 6.5) or Ph differences between test and standard solutions.

A modification of the official EC method (Appendix A) has been developed to overcome the above mentioned problems. The main modifications compared with the official EC method are:
-increase of the sensitivity by adding neomycin to the testmedium
-decrease of the zinc bacitracin concentration in the S_8 and U_8 to 0.05 IU/ml
-no evaporation step
-dilutions in methanol/buffer mixture.

To test the performance of this method a collaborative study with the participation of 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) and Alpharma AS, Oslo (Norway) (largest European producer of zinc bacitracin) was carried out. The results of the 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% Powder and Albac 15% Granulated were 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. These were supplemented with Albac 15% Lactodispersible manufactured by Alpharma AS, Oslo, Norway.
* The broiler and turkey feed (50 and 20 ppm zinc bacitracin) were produced by Bioteknologisk Institut, Kolding, Denmark. These feeds were supplemented with Albac 15% Granulated manufactured by Alpharma AS, Oslo, Norway.

The samples were distributed to the participants immediately after preparation (the second week of May 1996). Each participant also received zinc bacitracin reference material (standard), a neomycin standard and forms on which they were asked to report in sample weights, extraction volumes, dilutions and recorded inhibition zones.

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 all participants reported the results of the collaborative study to RIKILT-DLO.

At RIKILT DLO the results were statistically analyzed according to ISO 5725 (3). 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 6).

One laboratory (no. 3) produced too high results with the premix (8000 ppm), milk replacer (80 ppm) and final feeds (50 and 20 ppm). The Dixon test confirmed this on the 50 and 20 ppm level, but not on the 8000 and 80 ppm level. This is due to the higher variation of the other laboratories at these levels. However, the results from laboratory 3 at the four lowest levels suggest a systematic error, and are therefore excluded from the final results. With this laboratory further investigation is started to find the possible cause of the problem.

With sample A15G lot 114209 (15%) (table 2), the Dixon test selected laboratory no.5 as a straggler (significant higher results). With the other samples the results of laboratory no. 5 were not significantly higher, so all values were included in the final results for sample A15G lot 114209.

The Cochran test on outliers in the standard deviations selected laboratory no. 6 with the milk replacer (80 ppm) (table 4) as a straggler. This result is not excluded from the final results.

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 (%)	R (mg/kg)	CV_R (%)
concentrate A15P lot 114177	150000	8	147000	98	17251	4.2	18678	4.5
concentrate A15G lot 114209	150000	8	154000	103	27504	6.4	36687	8.5
premix	8000	7	8669	108	1095	4.5	2650	10.9
milk replacer	80	7	91.6	114	17.8	7.0	37.2	14.5
mash broiler feed	50	7	48.0	96	12.6	9.4	14.0	10.4
mash turkey feed	20	7	20.8	104	5.5	9.5	7.7	13.2

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

Table 1 indicates that the recovery, within laboratory variation (CV_r) and interlaboratory variation (CV_R) are satisfactory. The mean factor between R and r is 1.56.

No problems with parallelism of lines or invalid determinations were reported. Most laboratories added some suggestions/comments to the method, these are summarised in appendix III. From these suggestions it can be seen that no major problems occurred.
Some problems with sharpness of zones and sensitivity of the zones appeared, but they did not influence the results of the study.

4 CONCLUSION

The modified microbiological method for zinc bacitracin in animal feed (supplements) gave good results in this collaborative study. The recovery was good (96-114%) at all levels of zinc bacitracin. At the four lowest levels one laboratory produced significant higher results. These results were not included in the statistical analysis. The variation within the laboratories (CV_r) varied from 4.2 to 9.5%. The interlaboratory variation (CV_R) was also satisfactory and varied from 4.5 to 14.5%.

LITERATURE

(1) Bentley M. - Zinc Bacitracin Collaborative trial 1992 Comparing the official EEC Method (Official Journal of the European Communities 18.1.84 No: L15/35-38) with a Modified Method (AMC/MA/425 and AMC/MA/437). Staffordshire County Council, County Laboratory, England.

(2) Bentley M., Sandvik R. - Collaborative trial comparing the determination of zinc bacitracin in feeds according to the Official EU Method and a modified method, 1994. Alpharma AS. Animal Health Division P.O. Box 158 Skoyen, N-0212 Oslo, Norway, March 1995.

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

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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.

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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.

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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.

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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.

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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.

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Appendix A

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

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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

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APPENDIX II

Table 1: Results of the collaborative study on zinc bacitracin in concentrate A15P lot 114177 (150 g/kg)

Laboratory	Results (g/kg zinc bacitracin)				
	Day 1	Day 2	Day 3	Day 4	Average
1	149	154	148		150
2	148	144	149	147	147
3	142	158	158		153
4	147	138	144		143
5	143	149	159		150
6	136	130	151		139
7	148	143	146		146
8	151	145	146		147
n : 8 X_{mean} : 147 g/kg		repeatability $S_{(repeatability)}$: 17.3 g/kg $CV_{(repeatability)}$: 4.2%		reproducibility $S_{(reproducibility)}$: 18.7 g/kg $CV_{(reproducibility)}$: 4.5%	
no stragglers or outliers					

Table 2: Results of the collaborative study on zinc bacitracin in concentrate A15G lot 114209 (150 g/kg)

Laboratory	Results (g/kg zinc bacitracin)				
	Day 1	Day 2	Day 3	Day 4	Average
1	150	151	153		151
2	138	157	173	150	154
3	144	163	155		154
4	138	147	144		143
5	185	178	167		177 (*)
6	145	127	156		143
7	163	146	148		152
8	156	154	153		154
n : 8 X_{mean} : 154 g/kg		repeatability $S_{(repeatability)}$: 27.5 g/kg $CV_{(repeatability)}$: 9.82 g/kg : 6.4%		reproducibility $S_{(reproducibility)}$: 36.7 g/kg $CV_{(reproducibility)}$: 13.1 g/kg : 8.5%	
(*)lab 5 is straggler (Dixon test)					
n : 7 X_{mean} : 150 g/kg		repeatability $S_{(repeatability)}$: 27.8 g/kg $CV_{(repeatability)}$: 9.92 g/kg : 6.6%		reproducibility $S_{(reproducibility)}$: 27.8 g/kg $CV_{(reproducibility)}$: 9.92 g/kg : 6.6%	
without lab 5					

Table 3: 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	Day 4	Average
1	7715	7878	7748		7780
2	8780	8722	7738	8658	8474
3	11100	13030	12024		12051
4	7837	8000	7854		7897
5	8740	9060	9660		9153
6	9724	11169	10395		10429
7	8700	8937	8794/8562		8748
8	8164	8251	8310		8242
n : 8 X_{mean} : 9075 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 1385 mg/kg : 495 mg/kg : 5.4%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 4182 mg/kg : 1494 mg/kg : 16.5%
no stragglers					
n : 7 X_{mean} : 8669 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 1095 mg/kg : 391 mg/kg : 4.5%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 2650 mg/kg : 946 mg/kg : 10.9%
without lab.3					

Table 4: 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	Day 4	Average
1	81.7	78.4	84.0		81.4
2	88.4	79.0	88.9	90.3	86.6
3	126.9	139.9	147.8		138.2
4	80	87	85		84.0
5	110	104	105		106.3
6	115	126	96.6		112.5 (*)
7	85.4	78.6	83.6		82.5
8	89.8	89.6	89.4		89.6
n : 8 X_{mean} : 97.2 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 19.6 mg/kg : 7.0 mg/kg : 7.2%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 57.7 mg/kg : 20.6 mg/kg : 21.2%
(*) lab. 6 is straggler (Cochran test)					
n : 7 X_{mean} : 91.6 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 17.8 mg/kg : 6.4 mg/kg : 7.0%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 37.2 mg/kg : 13.3 mg/kg : 14.5%
without lab. 3					

Table 5: Results of the collaborative study on zinc bacitracin in mash broiler feed (50 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)				
	Day 1	Day 2	Day 3	Day 4	Average
1	50.8	50.4	49.9		50.4
2	37.8	39.5	47.9	47.9	43.3
3	77	72	57		68.7 (*)
4	43	52	45		46.7
5	44.5	51.0	46.7		47.4
6	60.5	52.2	48.3		53.7
7	43.3	53.2	46.5		47.7
8	52.3	47.4	46.0		48.6
n : 8 X_{mean} : 50.5 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 15.5 mg/kg : 5.6 mg/kg : 11.0%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 25.2 mg/kg : 9.0 mg/kg : 17.9%
(*) lab. 3 is straggler (Dixon test)					
n : 7 X_{mean} : 48.0 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 12.6 mg/kg : 4.5 mg/kg : 9.4%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 14.0 mg/kg : 5.0 mg/kg : 10.4%
without lab. 3					

Table 6: Results of the collaborative study on zinc bacitracin in mash turkey feed (20 mg/kg)

Laboratory	Results (mg/kg zinc bacitracin)				
	Day 1	Day 2	Day 3	Day 4	Average
1	22.1	21.9	22.6		22.2
2	19.3	16.4	20.0	18.2	18.5
3	24.7	35.6	33.6		31.3 (*)
4	19	18	18		18.3
5	21.4	19.5	21.2		20.7
6	24.5	25.5	20.0		23.3
7	18.8	16.6	23.7		19.7
8	21.8	24.6	24.2		23.5
n : 8 X_{mean} : 22.1 mg/kg		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 7.7 mg/kg : 2.7 mg/kg : 12.4%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 13.6 mg/kg : 4.8 mg/kg : 22.0%
(*)lab. 3 is straggler (Dixon test) and outlier (Cochran test)					
n : 7 X_{mean} : 20.8 mg/kg without lab.3		repeatability $S_{(repeatability)}$ $CV_{(repeatability)}$	: 5.5 mg/kg : 2.0 mg/kg : 9.5%	reproducibility $S_{(reproducibility)}$ $CV_{(reproducibility)}$	: 7.7 mg/kg : 2.7 mg/kg : 13.2%

APPENDIX III

Comments/suggestions from the different laboratories.

Lab.	Comment/suggestions
1	S_1 and U_1 a bit less sharp than the other zones. Tween 80 is not used.
2	no problems
3	high results. Tween 80 is not used. Antibiotic medium no.1 works also (is not used).
4	some problems with sensitivity.
5	Some zones of S_1 and U_1 were not sharp. In general no problems.
6	Increasing of incubation temperature (35°C) and inoculum gives better results (is not used).
7	No problems
8	Antibiotic med. no.1 and. 3 level assay is used. Method gives better results than the official one. Incubation of more than 24 hours gives better results. Important to have no delay between the different steps in the procedure.