

NINTH COMMISSION DIRECTIVE
of 31 July 1981
establishing Community methods of analysis for the official control of feedingstuffs

(81/715/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

HAS ADOPTED THIS DIRECTIVE:

Having regard to the Treaty establishing the European Economic Community,

Article 1

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs ⁽¹⁾, as last amended by the Act of Accession of Greece, and in particular Article 2 thereof,

Member States shall require that analyses for official controls of feedingstuffs, as regards their content of avoparcin and monensin sodium, be carried out in accordance with the methods described in the Annex.

Whereas that Directive requires that official control of feedingstuffs for the purpose of checking compliance with requirements under the provisions laid down by law, regulation or administrative action concerning the quality and composition of feedingstuffs be carried out using Community methods of sampling and analysis;

Article 2

Member States shall bring into force the laws, regulations or administrative provisions necessary to comply with this Directive on 1 December 1981 and shall forthwith inform the Community thereof.

Whereas Commission Directives 71/250/EEC ⁽²⁾, 71/393/EEC ⁽³⁾, 72/199/EEC ⁽⁴⁾, 73/46/EEC ⁽⁵⁾, 74/203/EEC ⁽⁶⁾, 75/84/EEC ⁽⁷⁾, 76/372/EEC ⁽⁸⁾ and 78/633/EEC ⁽⁹⁾, as last amended by the Directive of 30 July 1981, have already established a number of Community methods of analysis; whereas the progress of work since then makes it advisable to adopt a ninth set of methods;

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 31 July 1981.

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

For the Commission
The President
Gaston THORN

⁽¹⁾ OJ No L 170, 3. 8. 1970, p. 2.
⁽²⁾ OJ No L 155, 12. 7. 1971, p. 13.
⁽³⁾ OJ No L 279, 20. 12. 1971, p. 7.
⁽⁴⁾ OJ No L 123, 29. 5. 1972, p. 6.
⁽⁵⁾ OJ No L 83, 30. 3. 1973, p. 21.
⁽⁶⁾ OJ No L 108, 22. 4. 1974, p. 7.
⁽⁷⁾ OJ No L 32, 5. 2. 1975, p. 26.
⁽⁸⁾ OJ No L 102, 15. 4. 1976, p. 8.
⁽⁹⁾ OJ No L 206, 29. 7. 1978, p. 43.

ANNEX

1. DETERMINATION OF AVOPARCIN BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

The method is for the determination of avoparcin in feedingstuffs and premixes. The lower limit of determination is 2 mg/kg (2 ppm). The presence of polyether antibiotics may interfere in the determination.

2. PRINCIPLE

The sample is extracted with a mixture of acetone/water/hydrochloric acid. The antibiotic activity of the extract is determined by measuring the diffusion of avoparcin in an agar medium inoculated with *Bacillus subtilis*. 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: *BACILLUS SUBTILIS* ATCC 6633 (NCIB 8054)

3.1. Maintenance of stock culture

Inoculate tubes containing slopes of culture medium (4.1) with *Bacillus subtilis* and incubate overnight at 30 °C. Store the culture in a refrigerator at about 4 °C. Reinoculate every month.

3.2. Preparation of the spore suspension ⁽¹⁾

Harvest the growth from a recently prepared agar slope (3.1) by means of 2 to 3 ml of sterile water. Use this suspension to inoculate 300 ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30 °C. Harvest the growth in 15 ml of ethanol (4.2) after having checked sporulation under the microscope, and mix well. This suspension may be kept for at least five months at about 4 °C.

4. CULTURE MEDIA AND REAGENTS

4.1. Culture medium ⁽²⁾

Peptone	6.0 g
Tryptone	4.0 g
Yeast extract	3.0 g
Meat extract	1.5 g
Glucose	1.0 g
Agar	15.0 g
Water	1 000 ml
pH 6.5 (after sterilization).	

4.2. Ethanol 20 % (v/v): dilute 200 ml of ethanol with 800 ml of water.

4.3. Hydrochloric acid, d: 1.18 to 1.19.

(1) Other methods may be used provided that it has been established that they give similar spore suspensions.

(2) Any commercial culture medium of similar composition and giving the same results may be used.

- 4.4. Sodium hydroxide, 2 M solution.
- 4.5. Phosphate buffer, 0.1 M:
Potassium dihydrogen phosphate, KH_2PO_4 : 13.6 g.
Water to 1 000 ml.
Adjust pH to 4.5.
- 4.6. Mixture of acetone/water/hydrochloric acid (4.3): 65/32.5/2.5 (v/v/v).
- 4.7. Standard substance: avoparcin sulphate of known activity.

5. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of approximately 10 mg of the standard substance (4.7) in phosphate buffer (4.5) and dilute with this buffer to give a stock solution containing 100 μg avoparcin per millilitre. Stored in a stoppered flask at 4 °C, this solution is stable for up to seven days.

5.1. For premixes

From this stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8	4.0 $\mu\text{g}/\text{ml}$
S_4	2.0 $\mu\text{g}/\text{ml}$
S_2	1.0 $\mu\text{g}/\text{ml}$
S_1	0.5 $\mu\text{g}/\text{ml}$

5.2. For feedingstuffs

From the stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8	2.0 $\mu\text{g}/\text{ml}$
S_4	1.0 $\mu\text{g}/\text{ml}$
S_2	0.5 $\mu\text{g}/\text{ml}$
S_1	0.25 $\mu\text{g}/\text{ml}$

6. PREPARATION OF THE EXTRACT AND ASSAY SOLUTIONS

6.1. Premixes

Weigh, to the nearest 10 mg, sufficient sample to contain 10 to 100 mg avoparcin. Transfer to a 100 ml graduated flask with 60 ml of the mixture (4.6) and shake for 15 minutes on a mechanical shaker. Check the pH and adjust to pH 2, if necessary, with hydrochloric acid (4.3). Make up to volume with the mixture (4.6) and mix well. Filter a portion through suitable filter paper (e.g. Whatman No 1), discarding the first 5 ml of the filtrate. Take an aliquot and adjust the pH to 4.5 with sodium hydroxide solution (4.4). Dilute this solution with buffer (4.5) to obtain an expected avoparcin concentration of 4 $\mu\text{g}/\text{ml}$ (= U_8).

From this solution prepare solutions U_4 (expected content: 2 $\mu\text{g}/\text{ml}$), U_2 (expected content: 1 $\mu\text{g}/\text{ml}$) and U_1 (expected content: 0.5 $\mu\text{g}/\text{ml}$) by means of successive dilution (1 + 1) with buffer (4.5).

6.2. Feedingstuffs

Weigh out a quantity of sample of 50 g and shake 100 ml of mixture (4.6) for 30 minutes on a mechanical shaker. Clarify the extract by centrifugation (using stoppered centrifuge tubes), take an aliquot of the clarified extract (see table below) and adjust the pH to 4.5 with sodium hydroxide solution (4.4). Dilute this aliquot with buffer (4.5) to provide U_8 (see table below).

From this solution prepare solutions U_4 (expected content: 1 $\mu\text{g/ml}$), U_2 (expected content: 0.5 $\mu\text{g/ml}$) and U_1 (expected content: 0.25 $\mu\text{g/ml}$) by means of successive dilution (1 + 1) with buffer (4.5).

Presumed level of avoparcin (mg/kg)	5	7.5	10	15	20	40
Weight of sample (g (\pm 0.1 g))	50	50	50	50	50	50
Volume of mixture (4.6) (ml)	100	100	100	100	100	100
Volume of clarified extract (ml)	20	15	20	15	20	10
Final volume (ml): U_8	25	25	50	50	100	100
Expected U_8 concentration ($\mu\text{g/ml}$)	2	approx. 2	2	approx. 2	2	2

7. ASSAY PROCEDURE

7.1. Inoculation of the assay medium

Inoculate the assay medium (4.1) with the spore suspension (3.2) at 50 to 60 °C. By preliminary trials on plates with assay medium (4.1) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of avoparcin.

7.2. Preparation of the plates

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the assay solution (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 10 to 13 mm and not less than 30 mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200 mm in diameter and 20 mm high.

Pour into the plates a quantity of the medium (4.1), inoculated as in 7.1, to give a layer about 2 mm thick (60 ml for a plate of 200 mm diameter). 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 to 18 hours at 30 °C.

8. EVALUATION

Measure the diameter of the zones of inhibition to the nearest 0.1 mm. 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 solution and the extract, for example as below.

Determine the 'best fit' point for the standard highest level (SL) using the formula:

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

Determine the 'best fit' point for the standard highest level (SH) using the formula:

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

Similarly, calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) 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 SL and SH values on the same graph paper and join them to give the 'best fit' line for the standard solution. Similarly, record UL and UH and join them 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 (SH-SL) and (UH-UL) 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 or U_8 and S_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$(a') \text{ SL} = \frac{5 S_1 + 2 S_2 - S_4}{6} \text{ or } \frac{5 S_2 + 2 S_4 - S_8}{6}$$

$$(b') \text{ SH} = \frac{5 S_4 + 2 S_2 - S_1}{6} \text{ or } \frac{5 S_8 + 2 S_4 - S_2}{6}$$

and similarly for UL and UH. 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:

For four levels

$$(c) \text{ log. A} = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0.602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$(d) \text{ log. A} = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4) \times 0.401}{U_4 + S_4 - U_1 - S_1}$$

or

$$(d') \text{ log. A} = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0.401}{U_8 + S_8 - U_2 - S_2}$$

Real activity = supposed activity \times relative activity.

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.

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.

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 value, for contents of avoparcin from 2 and up to 10 mg/kg,
- 20 % related to the highest value for contents of 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.

2. DETERMINATION OF MONENSIN SODIUM BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

The method is for the determination of monensin sodium in feedingstuffs and premixes. The lower limit of determination is 10 mg/kg (10 ppm) ⁽¹⁾.

2. PRINCIPLE

The sample is extracted with 90 % methanol. The extract is submitted to appropriate procedures according to the monensin sodium content of the sample. The antibiotic activity is determined by measuring the diffusion of monensin sodium in an agar medium inoculated with *Bacillus subtilis*. 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. The sensitivity of this assay system is reduced in the presence of sodium ions.

3. MICRO-ORGANISM: BACILLUS SUBTILIS ATCC 6633 (NCIB 8054)

3.1. Maintenance of stock culture

Inoculate tubes containing slopes of culture medium (4.1) with *Bacillus subtilis* and incubate overnight at 30 °C. Store the culture in a refrigerator at about 4 °C. Reinoculate every month.

3.2. Preparation of the spore suspension ⁽²⁾

Harvest the growth from a recently prepared agar slope (3.1) by means of 2 to 3 ml of sterile water. Use this suspension to inoculate 300 ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30 °C. Harvest the growth in 15 ml of 20 % ethanol (4.3), after having checked sporulation under the microscope and mix well. This suspension may be kept for at least five months at about 4 °C.

4. CULTURE MEDIA AND REAGENTS

4.1. Culture medium ⁽³⁾

Tryptone	10.0 g
Yeast extract	3.0 g
Meat extract	1.5 g
Glucose	1.0 g
Agar (according to quality)	10.0 to 20.0 g
Water	1 000 ml
pH 6.5 (after sterilization).	

4.2. Assay medium

Glucose	10.0 g
Yeast extract	2.5 g
Dipotassium hydrogen phosphate, K ₂ HPO ₄	0.69 g
Potassium dihydrogen phosphate, KH ₂ PO ₄	0.45 g
Agar (according to quality)	10.0 to 20.0 g
Water	1 000 ml
pH 6 (after sterilization).	

⁽¹⁾ 1 mg monensin sodium is equivalent to 1 000 UK units.

⁽²⁾ Other methods may be used provided that it has been established that they give similar spore suspensions.

⁽³⁾ Any commercial culture medium of similar composition and giving the same results may be used.

- 4.3. Ethanol 20 % (v/v): dilute 200 ml of ethanol with 800 ml of water.
- 4.4. Methanol, anhydrous.
- 4.5. Methanol 90 % (v/v): dilute 900 ml of methanol (4.4) with 100 ml of water.
- 4.6. Methanol 50 % (v/v): dilute 500 ml of methanol (4.4) with 500 ml of water.
- 4.7. Aluminium oxide, granulated (alcoa F, 20 mesh; activated alumina UGI: F. Lancaster and Co., or equivalent).
- 4.8. Standard substances: monensin sodium of known activity (e.g. from the International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, UK-Surrey KT15 3NB).

5. APPARATUS

- 5.1. Rotary vacuum evaporator, with a 250 ml round-bottom flask.
- 5.2. Glass tube for chromatography, internal diameter: 25 mm, length: 400 mm, with an open end of 2 mm diameter.
- 5.3. Glass tube for chromatography, internal diameter: 11 mm, length: approximately 300 mm, with an open end of 2 mm diameter.

6. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of the standard substance (4.8) in methanol (4.4) and dilute to give a stock solution containing 800 µg monensin sodium per ml. Stored in stoppered flasks at 4 °C, this solution is stable for up to two weeks.

From this stock solution prepare by successive dilution with 50 % methanol (4.6) the following solutions:

S ₈	8.0 µg/ml
S ₄	4.0 µg/ml
S ₂	2.0 µg/ml
S ₁	1.0 µg/ml

7. PREPARATION OF THE EXTRACT

7.1. Extraction

7.1.1. Premixes

Weigh out a quantity of sample of 2 g, add 100 ml of 90 % methanol (4.5), homogenize and centrifuge for a few minutes. Dilute the supernatant solution with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 µg/ml (= U₈).

7.1.2. Feedingstuffs with a level of monensin sodium not lower than 50 ppm

Weigh out a quantity of sample of 10 to 20 g, add 100 ml of 90 % methanol (4.5), homogenize for 15 minutes and leave to settle.

Insert a cotton-wool plug at the narrow end of a glass tube (5.2) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80 mm high.

Decant the extract on to the aluminium oxide column and collect the filtrate. Dilute 30 ml of the filtrate to 50 ml with water. Make subsequent dilutions with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 µg/ml (= U₈).

7.1.3. *Feedingstuffs with a level of monensin sodium lower than 50 ppm (up to the limit of 10 ppm)*

Weigh out a quantity of sample of 10 to 20 g, add 100 ml of 90 % methanol (4.5) and homogenize for 15 minutes. Centrifuge till clear.

For a sample containing 20 ppm of monensin sodium, take 40 ml of the supernatant liquid. For a sample containing 10 ppm, take 80 ml and evaporate to dryness under vacuum on a rotary evaporator (5.1) at not more than 40 °C. Dissolve the residue in 10 ml of 90 % methanol (4.5).

Insert a cotton-wool plug at the narrow end of a glass tube (5.3) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80 mm high.

Decant the methanolic solution of the residue on to the aluminium oxide column and collect the filtrate. Wash the column with 10 ml of 90 % methanol (4.5) and combine the washings with the filtrate.

Evaporate the solution to dryness under vacuum on a rotary evaporator (5.1) at less than 40 °C. Dissolve the residue in 10 ml of anhydrous methanol (4.4) and make up to 20 ml with water. Centrifuge the solution at at least 4 000 r/min for at least five minutes. Make subsequent dilutions with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 µg/ml (= U₈).

7.2. **Assay solutions**

From solution U₈ prepare solutions U₄ (expected content: 4 µg/ml), U₂ (expected content: 2 µg/ml) and U₁ (expected content: 1 µg/ml) by means of successive dilution (1 + 1) with 50 % methanol (4.6).

8. **ASSAY PROCEDURE**

8.1. **Inoculation of the assay medium**

Inoculate the assay medium (4.2) with the spore suspension (3.2) at 50 to 60 °C. By preliminary trials on plates with assay medium (4.2) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of monensin sodium.

8.2. **Preparation of the plates**

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S₈, S₄, S₂, S₁) and the four concentrations of the assay solution (U₈, U₄, U₂, U₁). 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 10 to 13 mm and not less than 30 mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200 mm in diameter and 20 mm high.

Pour into the plates a quantity of the medium (4.2), inoculated as in 8.1, to give a layer about 2 mm thick (60 ml for a plate of 200 mm diameter). 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.

8.3. **Incubation**

Incubate the plates for approximately 18 hours at 35 to 37 °C.

9. **EVALUATION**

Measure the diameter of the zones of inhibition to the nearest 0.1 mm. 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 solution and the extract, for example as below.

Determine the 'best fit' point for the standard lowest level (SL) using the formula:

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

Determine the 'best fit' point for the standard highest level (SH) using the formula:

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

Similarly, calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) 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 SL and SH values on the same graph paper and join them to give the 'best fit' line for the standard solution. Similarly, record UL and UH and join them 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 (SH-SL) and (UH-UL) 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 or U_8 and S_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$(a') \text{ SL} = \frac{5 S_1 + 2 S_2 - S_4}{6} \text{ or } \frac{5 S_2 + 2 S_4 - S_8}{6}$$

$$(b') \text{ SH} = \frac{5 S_4 + 2 S_2 - S_1}{6} \text{ or } \frac{5 S_8 + 2 S_4 - S_2}{6}$$

and similarly for UL and UH. 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:

For four levels

$$(c) \log. A = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0.602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$(d) \log. A = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4) \times 0.401}{U_4 + S_4 - U_1 - S_1}$$

or

$$(d') \log. A = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0.401}{U_8 + S_8 - U_2 - S_2}$$

Real activity = supposed activity \times relative activity.

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.

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.

10. REPEATABILITY

The difference between the results of two determinations carried out on the same sample, by the same analyst, should not exceed:

- 20 % related to the highest value for contents of monensin sodium 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.