
**Animal feeding stuffs — Determination of
monensin, narasin and salinomycin
contents — Liquid chromatographic
method using post-column derivatization**

*Aliments des animaux — Détermination des teneurs en monensine,
narasine et salinomycine — Méthode par chromatographie liquide
utilisant la dérivatisation post-colonne*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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Animal feeding stuffs — Determination of monensin, narasin and salinomycin contents — Liquid chromatographic method using post-column derivatization

1 Scope

This International Standard specifies a high-performance liquid chromatographic (HPLC) method for the determination of the monensin, narasin and salinomycin contents of animal feeding stuffs, supplements (dry and liquid) and mineral premixtures. The method is not applicable to drug premixes (pharmaceutical products). Lasalocid and semduramicin cannot be determined by this method.

The limit of quantitation is approximately 1 mg/kg, 2 mg/kg and 2 mg/kg for monensin, salinomycin and narasin, respectively. A lower limit of quantitation can be achievable but this is to be validated by the user.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6498:1998, *Animal feeding stuffs — Preparation of test samples*

3 Principle

The ionophores monensin, narasin and salinomycin are extracted using methanol/water (90 + 10) with mechanical shaking for 1 h, then the extracts are filtered. The ionophores are determined by reverse-phase HPLC using post-column derivatization with vanillin and detection at 520 nm. Suspect positive trace-level samples and medicated feed samples containing unexpected ionophores are confirmed using a hexane extraction or post-column derivatization with dimethylaminobenzaldehyde (DMAB).

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

- 4.1 **Water**, HPLC grade, or equivalent (e.g. Milli-Q purified water).
- 4.2 **Methanol** (CH₃OH), HPLC grade.
- 4.3 **Sulfuric acid** (H₂SO₄), 97 % to 98 %.
- 4.4 **Acetic acid** (CH₃COOH), glacial, 97 % to 98 %.
- 4.5 **Sodium hydrogen carbonate** (NaHCO₃), minimum 99 % purity.
- 4.6 **Vanillin** (4-hydroxy-3-methoxybenzaldehyde), minimum 99 % purity.

4.7 Dimethylaminobenzaldehyde (DMAB), minimum 99 % purity.

4.8 Hexane [$\text{CH}_3(\text{CH}_2)_4\text{CH}_3$], distilled in glass.

4.9 Extraction solvent, methanol/water (90 + 10).

Combine 1 800 ml of methanol (4.2) and 200 ml of water (4.1) in a 2 l flask. Mix well.

4.10 Mobile phases

4.10.1 Post-column reaction system

While stirring gently, slowly add by pipette 20 ml of sulfuric acid (4.3) to 950 ml of methanol (4.2). Allow to cool, then add 30 g of vanillin (4.6) while stirring. Protect from light. Prepare fresh daily.

4.10.2 HPLC column

Use methanol (4.2)/water (4.1)/acetic acid (4.4) (940/60/1). Filter under vacuum using the equipment in 5.7.

4.11 Neutralized methanol

Add 1,0 g of sodium hydrogen carbonate (4.5) into 4 l of methanol (4.2). Mix well and filter if necessary through an 11 μm filter paper (e.g. Whatman No. 1)¹⁾. See Note to 4.13.

4.12 Reference standards

Composition or potency is required for each lot of reference standard.

4.12.1 Monensin sodium²⁾

4.12.2 Narasin²⁾

4.12.3 Sodium salinomycin³⁾

WARNING — Avoid inhalation of and exposure to the toxic standard materials and solutions thereof. Work in a fume-hood when handling the solvents and solutions. Wear safety glasses and protective clothing.

4.13 Ionophore stock standards, ca. 0,50 mg/ml.

Accurately weigh, to the nearest 0,1 mg, 25 mg of each standard (4.12.1 to 4.12.3) into separate 50 ml volumetric flasks. Dissolve in neutralized methanol (4.11) and dilute to volume. Prepare freshly every month. Store in a refrigerator.

Protect all standard solutions from light or prepare them in low actinic flasks.

NOTE The requirement for neutralized methanol has not been verified for salinomycin. It is not required if analysing monensin only, but is required for analysis of narasin.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Available from Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285, USA.

3) Available from Alpharma Inc., Animal Health Division, 1 Duggar Drive, Willow Island, WV, USA 26134-97111, and Hoechst Roussel Vet, D-65926 Frankfurt am Main, Gebaude H 790, Germany.

4.13.1 Monensin stock standard

Prepare as described in 4.13. Calculate the concentration of stock standard based on the principle component, monensin A. The minor component, monensin B, which elutes just before monensin A ^[4] is determined in test samples based on monensin A. Use the component composition identified on the reference standard profile sheet:

$$\rho_M = \frac{0,5 S_M}{100}$$

where

0,5 is the concentration of the stock standard (4.13), in milligrams per millilitre, recorded to three significant figures;

ρ_M is the concentration of the given component monensin A in the stock standard, in milligrams per millilitre;

S_M is the proportion of the given component monensin A in the reference standard according to the profile sheet, in percent.

EXAMPLE Reference standard lot RS0234 contains 93,71 % of monensin A on an "as-is" basis.

4.13.2 Salinomycin stock standard

Prepare as described in 4.13. Determine the concentration using the reference standard concentration value provided by the supplier ^[2]:

$$\rho_S = \frac{0,5 w}{1000}$$

where

ρ_S is the concentration of salinomycin in the stock standard, in milligrams per millilitre;

w is the concentration of the salinomycin standard given by the supplier, in micrograms per milligram.

EXAMPLE For lot WS-19B, the standard concentration is 986 µg/mg.

4.13.3 Narasin stock standard

Prepare as described in 4.13. Calculate the concentration of the stock standard based on the principle component, narasin A. The minor components (narasin D and I), which elute after narasin A ^[5], are determined in test samples based on narasin A. Use the component composition identified on the reference standard profile sheet:

$$\rho_N = \frac{0,5 S_N}{100}$$

where

ρ_N is the concentration of the component narasin A in the stock standard, in milligrams per millilitre;

S_N is the proportion of the given component narasin A in the reference standard according to the profile sheet, in percent.

EXAMPLE For reference standard lot RS0302, the percentage of each component on an "as-is" basis is:
 narasin A = 85,4 %,
 narasin D = 1,9 %,
 narasin I = 0,7 %.

4.14 Intermediate mixed standard solution, ca. 20 µg/ml, 40 µg/ml and 40 µg/ml monensin, salinomycin and narasin, respectively.

Transfer by pipette 10,0 ml, 20,0 ml and 20,0 ml of monensin, salinomycin and narasin stock standards (4.13), respectively, into a 250 ml volumetric flask. Dilute to volume with extraction solvent (4.9). Mix well. Prepare freshly every month.

4.15 Mixed HPLC standards

Prepare five mixed HPLC standard solutions by pipetting an aliquot of the mixed intermediate standard (4.14) into 100 ml low-actinic volumetric flasks and diluting to volume with extraction solvent (4.9), as specified in the Table 1. Mix well. Prepare freshly every month.

Table 1

Mixed HPLC standard identification	Amount of intermediate standard (4.14) ml	Approximate HPLC standard concentration µg/ml		
		Monensin	Salinomycin	Narasin
A	1	0,2	0,4	0,4
B	5	1	2	2
C	10	2	4	4
D	25	5	10	10
E	50	10	20	20

4.16 Single HPLC standards

4.16.1 Monensin, ca. 5 µg/ml.

Accurately pipette 1,0 ml of monensin stock standard (4.13.1) into a 100 ml low-actinic volumetric flask. Dilute to volume with extraction solvent (4.9). Mix well. Prepare freshly every month. Store in a refrigerator.

4.16.2 Salinomycin, ca. 10 µg/ml.

Accurately pipette 2,0 ml of salinomycin stock standard (4.13.2) into a 100 ml low-actinic volumetric flask. Dilute to volume with extraction solvent (4.9). Mix well. Prepare freshly every month. Store in a refrigerator.

4.16.3 Narasin, ca. 10 µg/ml.

Accurately pipette 2,0 ml of narasin stock standard (4.13.3) into a 100 ml low-actinic volumetric flask. Dilute to volume with extraction solvent (4.9). Mix well. Prepare freshly every month. Store in a refrigerator.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 HPLC system consisting of the following.

5.1.1 Pump, pulse free, flow capacity 0,1 ml/min to 2,0 ml/min.

5.1.2 Injection system, manual or autosampler, with loop suitable for 100 µl injections.

5.1.3 UV/VIS detector, with variable wavelength, suitable for measurements at 520 nm and 592 nm.

5.1.4 Integrator or computer data system.

5.1.5 Post-column reactor, with a 1,5 ml to 2,0 ml reaction coil, for operation at 98 °C.

The coil may be a commercially available knitted coil or it may be made using 7,5 m to 10 m of 316 SS tubing, 0,5 mm ID, coiled in a format to fit the reactor heating chamber. For example, wrap the coil in sufficient aluminium foil to make it fit snugly in the heater and to provide good heat transfer to the coil. A knitted coil is preferable. To ensure effective mixing of reagent and column effluent, use a vortex or static mixing tee (not a regular tee) before the reaction coil.

5.1.6 Post-column reagent pump, pulse free, with flow capacity 0,5 ml/min to 2,0 ml/min.

5.1.7 Analytical column.

NOTE A 5 µm C₁₈, 25 × 0,46 cm Nucleosil 120A, Partisil 5 ODS-3, or Waters Nova Pak (4 µm), or equivalent, has been found to be suitable.⁴⁾

5.1.8 Guard column, C₁₈.

5.2 Nitrogen evaporator, for evaporation of solvents under a stream of nitrogen.

5.3 Shaker, rotary or wrist-action.

5.4 Balances: analytical balance of 10 g capacity or greater with 0,1 mg readability, and another balance of 100 g capacity or greater with 0,01 g readability.

5.5 Erlenmeyer flasks, of capacities 125 ml, 250 ml and 500 ml, with glass stoppers.

5.6 Filter papers, Whatman No. 41 (15 cm), Whatman No. 42 (15 cm), and Whatman No. 1 (15 cm), or equivalent.⁴⁾

5.7 Solvent filtration system, all glass filter apparatus, suitable for 47 mm filter, and 47 mm diameter nylon filter of pore size 0,45 µm.

5.8 Sample filtration system, equipped with nylon or PTFE filter of pore size 0,45 µm.

5.9 Sieve, with 1 mm apertures.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497.

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

Grind the laboratory sample (> 200 g) so that it passes completely through a sieve with 1 mm apertures. For trace-level samples, grind the entire laboratory sample. Mix thoroughly.

4) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

8 Procedure

8.1 Preparation of quality control sample

The use of quality control samples and quality control charts is recommended.

With each set, include a sample spiked at approx. 100 mg/kg, 50 mg/kg, 50 mg/kg (for medication levels) or at 4 mg/kg, 6 mg/kg, and 6 mg/kg (for trace levels) for monensin, salinomycin and narasin, respectively.

EXAMPLE 1 4,0 ml of monensin stock standard added to 20 g of sample gives 100 mg/kg, and 2,0 ml each of salinomycin and narasin stock standard gives 50 mg/kg. All stock standards have approximately equal concentrations (0,50 mg/ml, see 4.13).

EXAMPLE 2 3,0 ml of mixed intermediate standard (4.14) added to 20 g of sample gives 3 mg/kg monensin and 6 mg/kg salinomycin and narasin.

Acceptable recovery for medication level samples (> 10 mg/kg) is between 95 % and 108 %. Acceptable recovery for trace-level samples (< 10 mg/kg) is between 90 % and 110 %.

8.2 Extraction

8.2.1 Dry feeds and premixes containing < 5 000 mg/kg

Accurately weigh a 20 g test portion into a 250 ml Erlenmeyer flask. For mineral premixes, add 5 g of sodium hydrogen carbonate. Add 100 ml of extraction solvent (4.9). Stopper the flask and shake vigorously for 1 h on the shaker (5.3).

8.2.2 Dry feeds and premixes containing > 5 000 mg/kg

Accurately weigh a 5 g test portion into a 500 ml Erlenmeyer flask. For mineral premixes, add 2 g of sodium hydrogen carbonate. Add 200 ml of extraction solvent (4.9). Stopper the flask and shake vigorously for 1 h on the shaker (5.3).

8.2.3 Liquid samples

Homogenize the sample by stirring the sample bottle contents on a magnetic stirrer or with a propeller mixer. Measure a 20 ml liquid sample into a tared 25 ml graduated cylinder. Weigh and transfer the sample to a 500 ml Erlenmeyer flask. Add 180 ml of methanol (4.2) (using some to rinse the graduated cylinder). Stopper the flask and shake vigorously for 1 h on the shaker (5.3).

8.2.4 Filter extract

Filter extracts through a No. 41 Whatman filter paper (5.6) into a 125 ml Erlenmeyer flask.

For extracts containing a high level of ionophores, dilute to the approximate concentration of HPLC standard D (4.15). The dilution required, D , can be calculated using the following formula:

$$D = \frac{w_s}{\rho_{\text{std}}} \times \frac{m_t}{V_e}$$

where

w_s is the sample target level, in milligrams per kilogram;

ρ_{std} is the concentration of the HPLC standard, in micrograms per millilitre;

m_t is the mass of the test portion, in grams;

V_e is the volume of extractant, in millilitres.

Pass the above extract or eluate through a 0,45 µm filter before proceeding to HPLC analysis.

8.3 HPLC analysis

8.3.1 HPLC conditions

a) HPLC separation parameters:

- column: as in 5.1.7
- mobile phase: as in 4.10.2
- flow rate: 0,7 ml/min
- wavelength: 520 nm
- chart speed: 0,5 cm/min
- injection volume: 100 µl
- guard column: as in 5.1.8 (change or repack the guard column frequently, especially when analysing trace level samples)
- attenuation: adjust to give 50 % to 60 % full-scale deflection for HPLC standard B (4.15) for low level sample, and HPLC standard D for samples containing medication levels.

b) Post-column reaction parameters:

- post-column reactor: as in 5.1.5
- mobile phase: as in 4.10.1
- flow rate: 0,9 ml/min
- reactor temperature: 98 °C

The system suitability criteria in 8.3.2 shall be met. The three ionophores and minor components should be baseline resolved, however, a minor component of salinomycin may appear as a shoulder peak on the front side of the narasin peak. Using the Nucleosil column (5.1.7), with the above conditions, retention times for monensin B, monensin A, salinomycin, narasin A and narasin (D + I) should be approximately 8,7 min, 9,8 min, 11,2 min, 12,8 min and 14,6 min, respectively.

The flow rates and mobile phase for the analytical column may be varied slightly, however, the total flow rate shall be between 1,5 ml/min and 1,6 ml/min to allow at least a 1 min reaction time. Sensitivity is determined by the reaction conditions and detector signal/noise.

Maduramicin is a potential interference when analysing trace levels of salinomycin; maduramicin elutes about 0,3 min before salinomycin. Semduramicin is a potential interference when analysing monensin; it elutes about 0,4 min before monensin B. Both maduramicin and semduramicin exhibit low sensitivity and can be effectively handled using the confirmation procedures in Clause 9.

8.3.2 System suitability

8.3.2.1 Resolution

Inject HPLC standard D (4.15).

Calculate the resolution factor, F_R , for each pair of adjacent peaks as follows:

$$F_R = 2 \left(\frac{d_2 - d_1}{w_1 + w_2} \right)$$

where

d_n is the distance on the baseline between the starting point of the chromatogram and the peak maximum, in centimetres;

w_n is the triangulated peak width at the baseline, in centimetres;

n is the number of the peak ($n = 1$ or 2).

EXAMPLE Data for calculation of resolution between monensin B (1) and monensin A (2):

$d_1 = 10,38$ cm, $w_1 = 0,46$ cm;

$d_2 = 11,98$ cm, $w_2 = 0,52$ cm.

$$F_R = 2 \frac{(11,98 - 10,38)}{0,46 + 0,52} = 3,3$$

The value of F_R shall be greater than 1,4 for all adjacent peaks. If this requirement is not met, adjust the HPLC conditions to improve the resolution, then repeat the injection and calculations.

8.3.2.2 Tailing factor

Calculate the tailing factor, F_T , as follows:

$$F_T = \frac{t_b - t_a}{2(t - t_a)}$$

where

t is the retention time of the given peak (time of the maximum of the fitted Gaussian curve), in minutes;

t_a is the retention time when the peak first reaches a height equal to 5 % of the peak maximum;

t_b is the retention time when the peak descends to a height equal to 5 % of the peak maximum.

The tailing factor, F_T , for monensin A, salinomycin and narasin A shall be less than 1,4.

8.4 Determination

8.4.1 Make replicate 100 μ l (full loop) injections of HPLC standard D (4.15) or the appropriate single standard (4.16) until the peak area is repeatable to within ± 1 % and a stable baseline is obtained. Inject 5 μ l of each stock standard, if necessary, to identify all the peaks. Check the linearity by injecting 100 μ l of each HPLC standard A, B, C, D and E (4.15). The HPLC response shall be linear (correlation factor not less than 0,999), and the intercept should not be significantly different from zero.

Quantification may be carried out using a single-point calibration or a calibration curve. The latter is preferable.

For single-point calibration, when analysing medication samples use the appropriate single standard (4.16) for quantitation (inject a mixed standard for qualitative purposes in case other ionophores are present in any of the samples). For trace-level samples, use mixed HPLC standard B (4.15) for quantification. Re-inject the standard solution after every 8 to 10 test solution injections.

For use of a calibration curve, inject the five mixed HPLC standards (4.15) at the start and end of the sample set. Construct a linear regression plot.

Inject 100 µl of the test solution. Measure the peak area. If the peak is larger than the single standard or outside the range of the calibration curve, dilute the test solution with extraction solvent (4.9). Do not reduce the injection volume.

NOTE Because test solutions receive no clean-up, retention times can be up to 0,5 min longer than for standard solutions. This occurs most often with monensin. However, the presence of factor B peak usually confirms the identity of monensin. If in doubt, peak identity can be confirmed by standard addition to the test solution, or confirmation with DMAB (9.2), or by use of the hexane extraction (9.3).

8.4.2 Shut off the system as follows. Turn off the post-column reagent flow before stopping the HPLC pump to prevent back flow of reagent into the analytical column. Wash the column and reactor for 30 min to 45 min with methanol (4.2).

9 HPLC confirmation

9.1 General

Positive trace-level samples and medication samples containing an unexpected ionophore should be confirmed to verify the correct identity of the peak(s). Either of the two following procedures may be used, however confirmation using DMAB is recommended. Hexane extraction cannot be applied to mineral premixtures or liquid samples, and results using hexane are less quantitative (approximately 90 %), but chromatograms for difficult samples may be significantly improved (less interfering peaks). See 10.5 for interpretation of results.

9.2 Post-column derivatization with DMAB

This may be applied to all types of feeds [6].

Follow steps 8.1 to 8.4 and HPLC system parameters (8.3.1), but use the following post-column reaction parameters.

a) Post-column reaction parameters:

- reagent flow: 0,8 ml/min
- reactor temperature: 95 °C
- wavelength: 592 nm.

b) Post-column reagent (mobile phase):

- while gently stirring, slowly add 20 ml of sulfuric acid (4.3) to 950 ml of methanol (4.2)
- allow to cool, then add 30 g of DMAB (4.7) while stirring
- protect from light; prepare freshly daily.

9.3 Hexane extraction

This cannot be applied to mineral or liquid samples.

Accurately weigh a 20 g sample into a 250 ml Erlenmeyer flask. Add 100 ml of hexane (4.8). Stopper the flask and shake overnight on the shaker (5.3). Filter sample solutions through No. 42 Whatman filter paper (5.6) into a 125 ml Erlenmeyer flask. Pipette 10 ml of extract into a tube and evaporate to dryness on the nitrogen evaporator (5.2). Dissolve the residue in 10 ml of extraction solvent (4.9). Filter a portion of the test solution before proceeding to the HPLC analysis (8.3).

10 Calculation of results

10.1 General

Calculate the ionophore content of the samples by comparing the peak area of each sample with the average peak area of the standards injected before and after the sample (for single-point calibration). For monensin and narasin, results are calculated in terms of microbiological activity by use of biopotency factors. Salinomycin results are calculated on a mass-by-mass basis.

10.2 Monensin

Monensin content (mg/kg) equals the biopotency of monensin A + B.

Use the following equation for calculation of the biopotency, B , of each component, in milligrams per kilogram:

$$B_M = \frac{A_M}{A_{As}} \times \rho_{As} \times \frac{V}{m} \times D \times F_M$$

where

A_M is the peak area of the given component (monensin A or B) in the test solution;

A_{As} is the peak area of monensin A in the standard solution (average);

ρ_{As} is the concentration of monensin A in the reference standard solution, in micrograms per millilitre;

m is the mass of the test portion, in grams;

V is the extractant volume, in millilitres, or total volume (for liquid samples);

D is the dilution factor (see 8.2.4 and 8.4.1);

F_M is the biopotency conversion factor of the given component (monensin A or B), which correlates the HPLC value to the microbiological activity:

$$F_A = 1,00$$

$$F_B = 0,28$$

The total monensin microbiological activity equals $B_A + B_B$, in milligrams per kilogram.

For calculation of the percent recovery of monensin in spiked samples, do not include F_M for either component in the calculation. For trace levels, monensin B may not be measurable.

The concentration of monensin B in test solutions is calculated on the basis of monensin A in the reference standard solution. See the example calculation.

EXAMPLE	Mass of test portion (m)	= 20,00 g
	Extractant volume (V)	= 100 ml
	Dilution factor (D)	= 4
	Concentration of monensin A in the HPLC standard solution (ρ_{As})	= 5,05 µg/ml
	Average peak area of monensin A in the HPLC standard solution (A_{As})	= 1 479 566
	Peak area of monensin A in the test solution (A_A)	= 1 450 878
	Peak area of monensin B in the test solution (A_B)	= 67 091
	$B_A = \frac{1\,450\,878}{1\,479\,566} \times 5,05 \times \frac{100}{20,00} \times 4 \times 1,00 = 99,04 \text{ mg/kg}$	
	$B_B = \frac{67\,091}{1\,479\,566} \times 5,05 \times \frac{100}{20,00} \times 4 \times 0,28 = 1,3 \text{ mg/kg}$	
	Total monensin content = 99,0 + 1,3 = 100,3 mg/kg	

10.3 Salinomycin

Use the following equation for the calculation of the salinomycin content, w_S , of the sample, in milligrams per kilogram:

$$w_S = \frac{A}{A_s} \times \rho_s \times \frac{V}{m_t} \times D$$

or

$$w_S = \rho_t \times \frac{V}{m_t} \times D$$

where

A is the peak area of salinomycin in the test solution;

A_s is the peak area of salinomycin in the HPLC standard solution (average);

ρ_s is the concentration of salinomycin in the HPLC standard solution, in micrograms per millilitre;

m_t is the mass of the test portion, in grams;

V is the extractant volume, in millilitres;

D is the dilution factor (see 8.2.4 and 8.4.1);

ρ_t is the salinomycin concentration in the test solution, in micrograms per millilitre, from the calibration curve.

EXAMPLE	Mass of test portion (m)	= 20,00 g
	Extractant volume (V)	= 100 ml
	Dilution factor (D)	= 1
	Concentration of salinomycin in the HPLC standard solution (ρ_s)	= 10,55 µg/ml
	Peak area of salinomycin in the standard solution (A_s)	= 1 575 475
	Peak area of salinomycin in the test solution (A)	= 1 568 882
	$w_S = \frac{1\,568\,882}{1\,575\,475} \times 10,55 \times \frac{100}{20,00} \times 10 = 52,5 \text{ mg/kg}$	

10.4 Narasin

The narasin content (mg/kg) equals the biopotency of narasin [A + (D + I)].

Use the following equation for calculation of the biopotency, B , of each component, in milligrams per kilogram:

$$B_N = \frac{A_N}{A_{As}} \times \rho_{As} \times \frac{V}{m_t} \times D \times F_N$$

or

$$B_N = \rho_N \times \frac{V}{m_t} \times D \times F_N$$

where

- A_N is the peak area of the given component [narasin A or (D + I)] in the test solution;
- A_{As} is the peak area of narasin A in the standard solution (average);
- ρ_{As} is the concentration of narasin A in the reference standard solution, in micrograms per millilitre;
- m_t is the mass of the test portion, in grams;
- V is the extractant volume, in millilitres;
- D is the dilution factor (see 8.2.4 and 8.4.1);
- F_N is the biopotency conversion factor of the given component [narasin A or (D + I)];
- ρ_N is the narasin concentration in the test solution, in micrograms per millilitre, from the calibration curve;
- F_A is 1,077;
- F_{D+I} is as calculated using the following equation

$$F_{D+I} = \frac{(c_{Ds} \times 1,510) + (c_{Is} \times 0,012)}{c_{Ds} + c_{Is}}$$

where

- c_{Ds} is the concentration of narasin D in the narasin standard, in percent;
- c_{Is} is the concentration of narasin I in the narasin standard, in percent;

and the biopotency conversion factors for the individual factors are

$$F_D = 1,510$$

$$F_I = 0,012$$

Refer to the Reference Standard Profile for the percentage of factors A, D and I in the current reference standard.

The concentration of factors (D + I) in sample extracts is calculated on the basis of factor A in the reference standard solution. See the example calculation.

EXAMPLE 1 For reference standard lot RS0302, $c_{Ds} = 1,9 \%$ and $c_{Is} = 0,7 \%$

$$F_{D+I} = \frac{(1,9 \times 1,510) + (0,7 \times 0,012)}{1,9 + 0,7} = 1,11$$

For calculation of the percent recovery of narasin in spiked samples, do not include F_n for either component in the calculation. For trace levels, narasin (D + I) may not be measurable.

EXAMPLE 2 Mass of test portion (m) = 20,00 g

Extractant volume (V) = 100 ml

Dilution factor (D) = 1,0

Concentration of narasin A in the HPLC standard solution (ρ_{As}) = 10,60 µg/ml

Average peak area of narasin A in the HPLC standard solution (A_{As}) = 1 559 626

Peak area of narasin A in the test solution (A_A) = 1 539 393

Peak area of narasin (D + I) in the test solution (A_{D+I}) = 77 376

$$B_A = \frac{1\,539\,393}{1\,559\,626} \times 10,60 \times \frac{100}{20,00} \times 1 \times 1,077 = 56,3 \text{ mg/kg}$$

$$B_{D+I} = \frac{77\,376}{1\,559\,626} \times 10,60 \times \frac{100}{20,00} \times 1 \times 1,11 = 2,92 \text{ mg/kg}$$

The total narasin content = 56,3 + 2,9 = 59,2 mg/kg

Report the final results to two significant figures if < 10 mg/kg, and three significant if > 10 mg/kg. Positive trace level samples and medicated samples suspected of containing unexpected ionophores should be confirmed. See Clause 9.

10.5 Interpretation of confirmation data

When confirming with DMAB as post-column reagent, results for the three ionophores should match those results obtained using vanillin. However, the peak size for each ionophore will be 1,3 to 1,5 larger with DMAB than with vanillin. The peak size ratio for DMAB/vanillin for samples should match that of the standards.

If maduramicin is suspected at a retention time similar to that of salinomycin, results using DMAB will be significantly lower since maduramicin is about six times less sensitive than salinomycin. A 5 mg/kg maduramicin sample would give a peak signal equivalent to about 1 mg/kg salinomycin; i.e. less than the limit of quantitation for salinomycin.

If semduramicin is present, it would appear as a resolved or merged peak before monensin B. When using vanillin, semduramicin is about 13 times less sensitive than either monensin A or B. When using DMAB, the semduramicin sensitivity is about 28 times less than monensin.

When confirming with hexane as extractant, results for the three ionophores should agree to within 10 % of those obtained with 90 % methanol as extractant. If either maduramicin or semduramicin is present, hexane extraction would give a result 40 % to 50 % of that obtained by the methanol extractant.

The above information shall be validated for each HPLC system.

Peak identity is confirmed if, by the procedure in Clause 8 and either of the confirmation procedures, the retention times differ by no more than 0,2 min and quantitative results are within ± 5 % (vanillin vs. DMAB) and ± 10 % (90 % methanol vs. hexane as extractant).

11 Precision

11.1 Interlaboratory test

Details of the interlaboratory test are summarized in Annex A. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The absolute difference between two independent single test results, expressed as a percentage of the mean determined value, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the repeatability limit (r) derived from the following equation:^[9]

$$r = 2 \times 2^{1/2} \times \text{RSD}(r) = 2,8 \times \text{RSD}(r)$$

where $\text{RSD}(r)$ is the repeatability relative standard deviation.

Repeatability values are similar for the three analytes, see Table 2.

11.3 Reproducibility

The absolute difference between two single test results, expressed as a percentage of the mean determined value, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit (R) derived from the following equation:^[9]

$$R = 2,8 \times \text{RSD}(R)$$

where $\text{RSD}(R)$ is the reproducibility relative standard deviation.

Repeatability and reproducibility criteria for medicated feeds (> 10 mg/kg), supplements and mineral premixes, derived from Tables A.1, A.2 and A.3, are as given in Table 2.

Table 2

Parameter	Monensin	Narasin	Salinomycin
RSD(r), maximum	5,23	4,46	4,31
RSD(r), average ^a	4,01	3,99	4,77
r , %, maximum	14,6	12,5	12,1
r , %, average ^a	11,2	11,2	9,46
RSD(R), maximum	6,75	6,54	5,67
RSD(R), average ^a	5,21	5,65	4,72
R , %, maximum	18,9	18,3	15,9
R , %, average ^a	14,6	15,8	13,2
^a Average values determined by analysis of variance. The study data indicate that analysis variability is independent of the analyte concentration and the type of feedstuff.			

Similar repeatability and reproducibility criteria for trace level feeds were obtained, except for the sample containing 3 mg/kg monensin (sample homogeneity appeared to be inadequate).

11.4 Limit of quantitation

Practical limits of quantitation are 1 mg/kg for monensin, and 2 mg/kg for narasin and salinomycin. Lower limits of quantitation may be possible, depending on the HPLC equipment being used, and would require validation by the user.

12 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this International Standard;
- all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- the test result obtained;
- if the repeatability has been checked, the final quoted result obtained.

Annex A (informative)

Results of interlaboratory test

A.1 Procedure

An interlaboratory test was organized and carried out in accordance with the Collaborative Guidelines of AOAC International ^[9,10] in 2002. Test samples were prepared by grinding a suitable amount of the dry test materials with a Retsch ZM1 (0,75 mm sieve) or Retsch SR3 (1,0 mm sieve) mill. The ground material was mixed manually, then subdivided into approximately 50 g portions using a Retsch rotary PTZ divider. Homogeneity of the subsamples was verified by analysing six randomly selected samples: test materials were judged to be homogeneous if the repeatability relative standard deviation was < 2%.

Candidate laboratories were selected on the basis of results from a method familiarization study involving the verification of system suitability and the analysis of 2 samples, 1 containing monensin and 1 containing salinomycin. The range of the method studied in the test was trace level (1 mg/kg) to drug premix level (200 g/kg). Twenty-five medicated samples, 9 trace level samples and reference standards of the 3 antibiotics were sent to the candidate laboratories. Ten laboratories returned acceptable data within a suitable timeframe. The data from one other laboratory was rejected since some requirements of the study protocol were not met.

The samples tested were as follows.

a) **Medicated samples** (all submitted as blind samples)

Monensin	Narasin	Salinomycin
2 finished feeds, in duplicate	2 finished feeds, in duplicate	3 finished feeds, in duplicate
1 supplement, in duplicate	1 drug premix, single sample	1 supplement, single sample
1 liquid supplement, in duplicate	1 mineral premix, in duplicate	
1 mineral premix, single sample	1 drug premix, single sample	
1 drug premix, single sample		

b) **Trace level samples** (all submitted as blind samples)

- 1 finished feed containing monensin and narasin, in duplicate
- 1 finished feed containing salinomycin, in duplicate
- 1 liquid supplement containing monensin, single sample
- 4 blank finished feed samples

A confirmation technique (alternative post-column reagent) was provided. Analysts were requested to apply the technique to all suspect positive trace-level samples.