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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

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 14183 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

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** (CH_3OH), HPLC grade.

4.3 **Sulfuric acid** (H_2SO_4), 97 % to 98 %.

4.4 **Acetic acid** ($CH_2CH_3CO_2H$), glacial, 97 % to 98 %.

4.5 **Sodium hydrogen carbonate** ($NaHCO_3$), minimum 99 % purity.

4.6 **Vanillin** (4-hydroxy-3-methoxybenzaldehyde), minimum 99 % purity.

4.7 Dimethylaminobenzaldehyde (DMAB), minimum 99 % purity.

4.8 Hexane [$CH_3(CH_2)_4CH_3$], 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 sodium[2]

4.12.2 Narasin[2]

4.12.3 Sodium salinomycin[3]

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.

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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]:

$$\rho_S = \frac{0{,}5\ w}{1\,000}$$

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 C_{18}, 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, C_{18}.

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.

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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:

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

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.