

5.2.05

AOAC Official Method 997.04 Monensin in Premix and Animal Feeds Liquid Chromatographic Method First Action 1997 Final Action 2004

(Applicable for determination of monensin activity in range of 5–200 000 mg/kg in all types of premixes and animal feeds.)

See Table 997.04 for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Monensin is extracted from premixes and feeds with methanol–water (90 + 10, v/v) and isolated by liquid chromatography with post-column derivatization. Acid-reaction product of monensin with vanillin is measured by variable wavelength detector at 520 nm. There is no interference from narasin, salinomycin, and lasalocid.

B. Apparatus

(a) *Liquid chromatograph*.—With recorder or integrator (manual or computer), pulse-dampened LC pump [for LC mobile phase, C(g)] and reagent pump [for vanillin reagent, C(h)] both operating at 0.7 mL/min; variable wavelength absorbance detector operating at 520 nm with rise time of 1.0 and range of 0.2 (or as needed); 90° Tee positioned in such way so inlet flows directly oppose one another; and stainless steel coil (6.1 m 0.51 mm id) enclosed in 98.0°C heater. (Comparable components may be substituted. Parameters may be modified to compensate for daily variations in instrument performance.)

Operating conditions: injection volume, 200 µL; column temperature, ambient; reaction coil temperature, 98°C; flow rate (both pumps), 0.7 mL/min. Approximate retention times: monensin A, 640 s; monensin B, 560 s; (see Figure 997.04A for chromatogram of typical system suitability test, Figure 997.04B for chromatogram of rumensin medicated article, and Figure 997.04C for chromatogram of monensin medicated feed.)

(Note: When not using system for 2–3 days, or at regular intervals during use periods, flush system with methanol to prolong life of components.)

(b) *LC column*.—250 4.6 mm id (Whatman Partisil 5 ODS-3, or equivalent). C18 guard column may be used to extend life of analytical column.

(c) *LC vials*.

(d) *Mechanical shaker*.—Wrist action shaker, or equivalent.

(e) *Filter*.—0.2–0.5 µm porosity; Teflon or chemical resistant.

(f) *Containers*.—500–1000 mL, glass or polypropylene.

(g) *Volumetric flasks*.—100 and 200 mL.

(h) *Magnetic stirrer*.

(i) *Teflon-coated stir bars*.

(j) *Balances*.—(1) Top loading and (2) analytical.

C. Reagents

(a) *Solvent*.—Methanol, LC grade.

(b) *Purified water*.—e.g., LC grade.

(c) *Sulfuric acid*.—Analytical grade.

(d) *Glacial acetic acid*.—ACS grade.

(e) *Vanillin*.—99% purity.

(f) *Neutralized methanol*.—Add 1 g NaHCO₃ to 4 L methanol. Mix well and, if needed, filter through 11 µm pore size filter paper.

(g) *LC mobile phase*.—Methanol–H₂O–glacial acetic acid solution (94 + 6 + 0.1, v/v/v). Degas with He prior to use.

(h) *Vanillin reagent*.—Methanol–sulfuric acid–vanillin solution (95 + 2 + 3, v/v/w). While stirring gently, add slowly and carefully 20 mL concentrated H₂SO₄ to 950 mL methanol, C(a). (*Caution*: Avoid splattering when adding H₂SO₄ to methanol, using pipet or other suitable equipment. Do not add H₂SO₄ to methanol by pouring from beaker or graduated cylinder.) Let methanol–H₂SO₄ solution cool to room temperature. Add 30.0 g vanillin while stirring gently. Vanillin reagent is stable up to 4 weeks at room temperature in dark amber or foil-covered container.

(i) *Extraction solution*.—Methanol–H₂O solution (90 + 10, v/v).

(j) *Monensin standard solutions*.—(1) *Monensin standard stock solution*.—1 mg/mL. Accurately weigh 100 mg monensin reference standard (available from Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 46285, USA) into 100 mL volumetric flask and dilute to volume with methanol, C(a). (2) *Monensin working standard solutions*.—(i) For rations with theoretical monensin concentrations >40 ppm. Quantitatively dilute monensin standard stock solution with extraction solution to obtain monensin concentrations of 5.0, 10.0, 20.0, and 40.0 µg/mL. (ii) For rations with theoretical monensin concentrations <40 ppm. Quantitatively dilute monensin standard stock solution with extraction solution to obtain monensin concentrations of 0.25, 1.25, 2.5, 3.75, and 5.0 µg/mL.

Monensin standard solutions are stable up to 4 weeks in refrigerator or at room temperature protected from direct sunlight.

(k) *Resolution solution*.—Containing 5–40 µg monensin/mL and 5–40 µg narasin/mL. Prepare resolution solution as follows: Accurately weigh ca 100 mg monensin sodium reference standard

Table 997.04. Interlaboratory study results for determination of monensin in premixes and animal feed by liquid chromatography with post-column derivatization

Sample	Mean activity	Monensin activity	s _r	s _R	RSD _r , %	RSD _R , %	r	R
Coban premix	130.2 mg/g	132 mg/g		3.6		2.8		10
Rumensin premix	190.3 mg/g	176 mg/g		6.5		3.4		18
Poultry feed	32.4 g/t (35.6 g/g)	30 g/t (33 g/g)	3.8	3.8	12	12	11	11
Poultry feed	114.8 g/t (126.3 g/g)	100 g/t (110 g/g)	7.0	11	6.1	9.8	20	31
Cattle feed 1	8.6 g/t (9.5 g/g)	7 g/t (7.1 g/g)	0.9	1.2	11	14	2.6	3.2
Cattle feed 2	9.0 g/t (9.9 g/g)	7 g/t (7.1 g/g)	1.4	1.4	15	15	3.8	3.8
Cattle feed 2	32.4 g/t (35.6 g/g)	30 g/t (33 g/g)	2.6	2.8	7.9	8.6	7.1	7.8
Liquid feed supplement	25.3 g/t (27.8 g/g)	25 g/t (27.5 g/g)	5.3	6.4	21	25	15	18

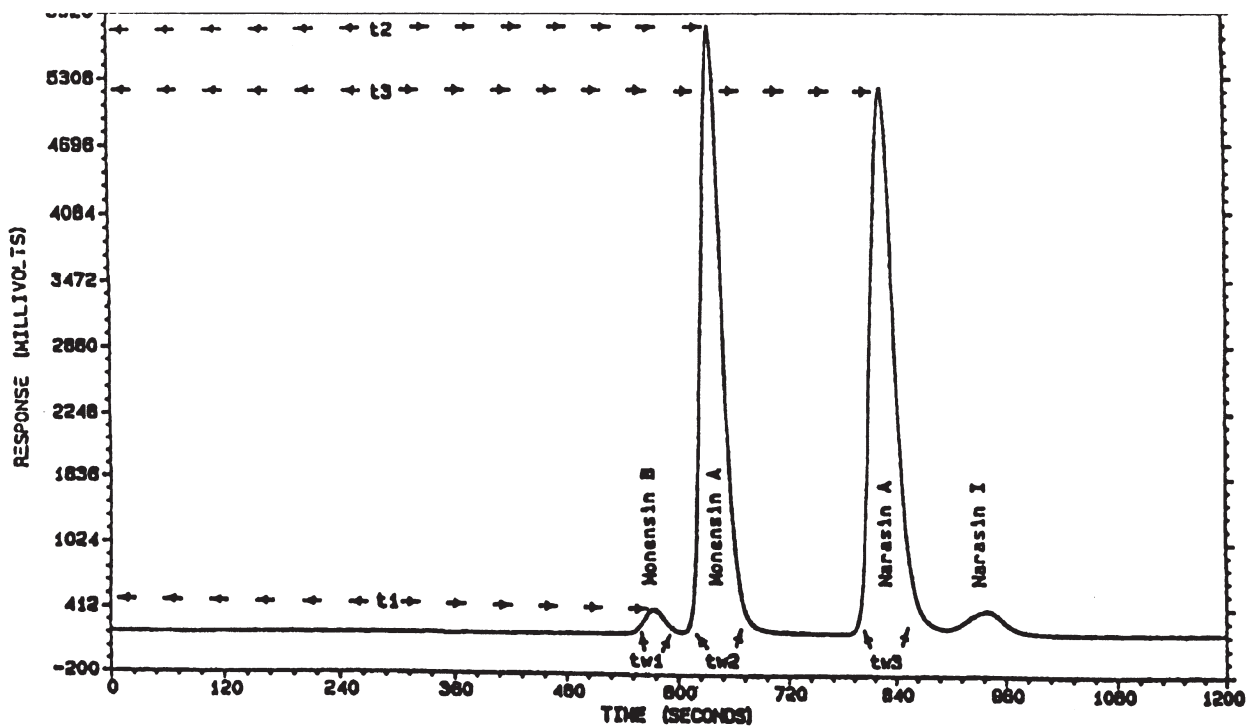


Figure 997.04A. Chromatogram of typical system suitability test (monensin and narasin).

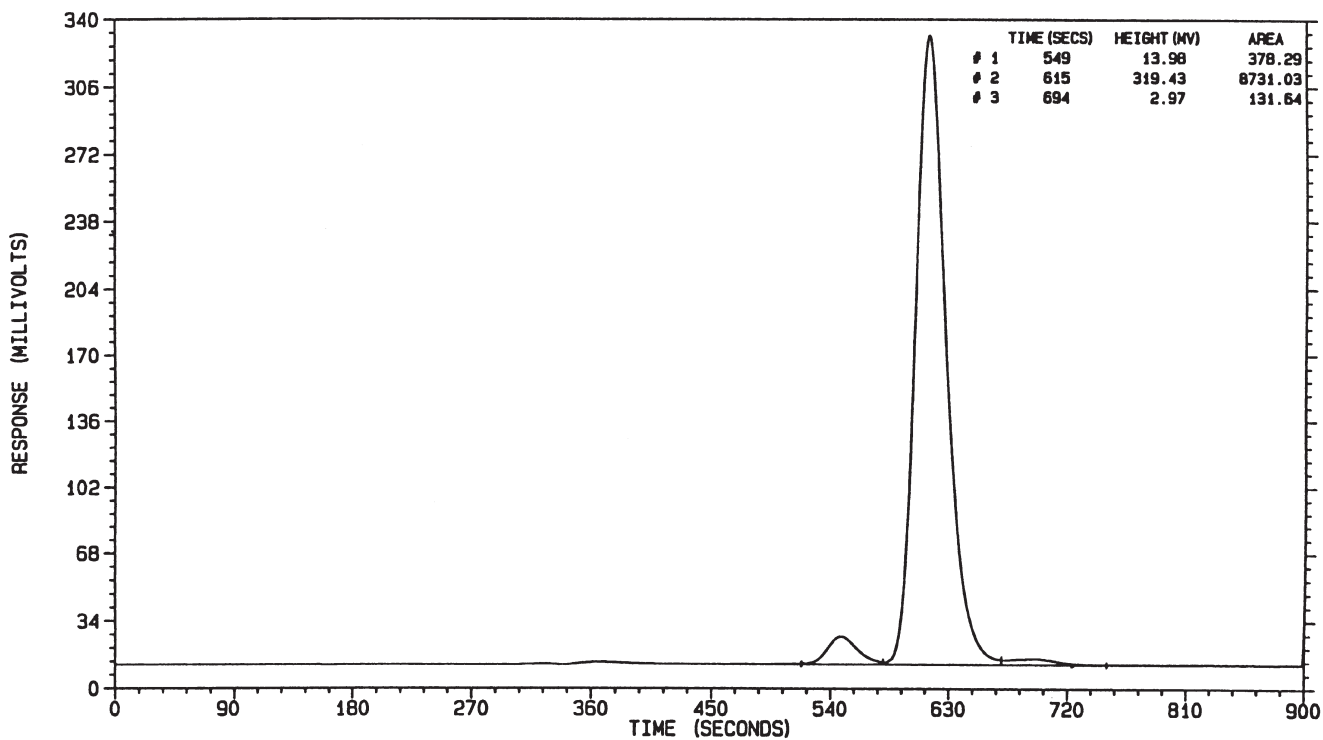


Figure 997.04B. Chromatogram of Rumensin 80 type A medicated article.

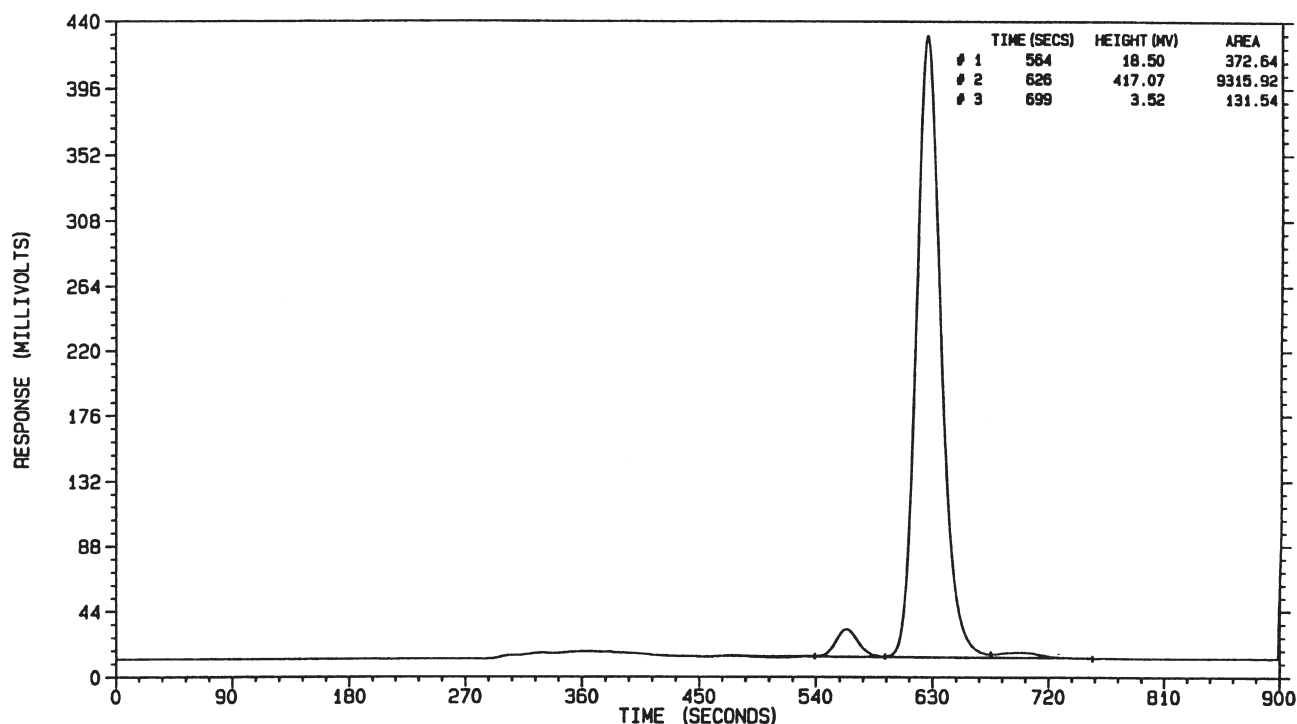


Figure 997.04C. Chromatogram of monensin type C medicated feed.

and 300 mg narasin reference standard (available from Eli Lilly and Co.) into 100 mL volumetric flask. Dilute to volume with neutralized methanol, C(f). Mix well. Pipet 2 mL into 200 mL volumetric flask and dilute to volume with extraction solution, C(i). Mix well. Resolution solution is stable up to 2 weeks in refrigerator or at room temperature protected from direct sunlight.

D. Preparation of Test Solution

(a) *Premix*.—Accurately weigh ca 5 g ground test portion into 500 mL jar or other suitable container. Add 200 ± 2 mL extraction solution, C(i), cap, and mix thoroughly 1 h on mechanical shaker. Let solids settle. Based on theoretical monensin concentration, dilute solution to obtain final monensin concentration within standard curve levels. Filter aliquot of extract and proceed to LC determination, F.

(b) *Feed including mineral mixes*.—Accurately weigh ca 50 g ground test portion into 500 mL jar or other suitable container. Proceed as in (a) starting with “Add 200 ± 2 mL extraction solution”.

(c) *Liquid feed supplements*.—(Note: Settling of insoluble matter in fortified liquid feed supplements presents sampling problem. Ensure adequate mixing of liquid feed supplements prior to sampling.)

Thoroughly mix laboratory samples by agitation with gyratory shaker, propeller mixer, magnetic stir bar, or equivalent apparatus depending on viscosity. While continuing to mix, rapidly (to prevent settling), transfer ca 40 g to weighed 200 mL volumetric flask and reweigh. Add 100–150 mL extraction solution, C(i), cap, and mix thoroughly. Dilute to volume with extraction solution, and let settle. Dilute aliquot of extract with extraction solution to obtain final

monensin concentration of ca 20 g/mL. Filter aliquot of diluted extract through 0.2–0.5 m filter, B(e), and proceed to LC Determination, F.

E. System Suitability

(a) *Resolution*.—Inject resolution solution, C(k), onto LC column. Calculate resolution factor, R_s , for each pair of adjacent peaks as follows (see Figure 997.04A):

$$R_s = \frac{t_2 - t_1}{(tw_1 + tw_2) 0.5}$$

where t_1, t_2, \dots, t_n = retention volume of peak maximum, cm; tw_1, tw_2, \dots, tw_n = triangulated peak width at baseline, cm.

Example: $t_1 = 10.65$ cm; $t_2 = 11.85$ cm; $t_3 = 15.55$ cm; $tw_1 = 0.85$ cm; $tw_2 = 0.95$ cm; $tw_3 = 1.00$ cm.

$$\begin{aligned} \text{Monensin B} - \text{monensin A}, R_s &= \frac{11.85 - 10.65}{(0.85 + 0.95) 0.5} \\ &= 1.33 \text{ (must be } \geq 1.25) \end{aligned}$$

$$\begin{aligned} \text{Monensin A} - \text{narasin A}, R_s &= \frac{15.55 - 11.85}{(0.95 + 1.00) 0.5} \\ &= 3.79 \text{ (must be } \geq 3.50) \end{aligned}$$

If resolution does not meet requirements specified, adjust LC conditions to improve resolution and re-inject resolution solution onto LC system.

(b) *Tailing factor*.—Tailing factor, T_f , for monensin factor A of reference standards must be < 1.4 . Calculate T_f as follows:

$$T_r = \frac{10\% \text{ width}}{(\text{retention time} - \text{time of front 10\% point}) \cdot 2}$$

where retention time = time of the maximum of the fitted Gaussian curve; 10% width = (time of back 10% point – time of front 10% point). A 10% point is where the response of a side of the peak reaches a height equal to 10% of the peak maximum.

If any of the above parameters do not meet system suitability, adjust mobile phase. After adjustment, parameters must meet system suitability prior to analysis.

(c) *Linearity of standard curve.*—The standard curve should be linear and the 95% confidence interval of the y -intercept must include zero.

(d) Limit of quantitation is ca 1 mg/kg. Factor B becomes unmeasurable as monensin levels decrease below 15 mg/kg.

F. LC Determination

(Note: The most critical parameters in LC system are water content, reactor temperature, H₂SO₄–vanillin concentrations, and flow rates.)

Before analyzing test solutions, ensure that LC system meets system suitability parameters by injecting resolution solution, C(k). Then inject 200 L standard solutions followed by test solutions.

Measure peak area response, PR , at retention volume of monensin factor A and monensin factor B for each test.

G. Calculations

Using measured responses, construct linear regression plot of standard curve of monensin factor A to determine concentration of monensin factors A and B in each test. Determine biopotency for each factor as follows:

$$\text{Biopotency} = \frac{V}{W_s} \cdot BCF \cdot f$$

g activity/mL (from standard curve)

where V = extraction volume, mL (e.g., 200 mL); W_s = weight of test portion, g; f = dilution factor; BCF = biopotency conversion factor [BCF (Factor A) = 1.000; BCF (Factor B) = 0.280].

Sum individual biopotency values for monensin A and monensin B to obtain total biopotency for monensin. (Note: Refer to the Reference Standard Profile [available from Eli Lilly and Co.] for percent Factor A content of the current Reference Standard.)

References: *J. AOAC Int.* **80**, 693(1997); **81**, 1096(1998).

Revised: March 2002