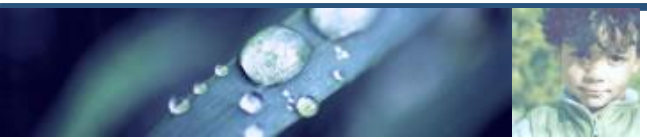

Development of an LC-MS/MS Method for the Determination of Tylosin in Feed at Medicated Levels

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AAFCO Laboratory Methods and Service Committee Meeting

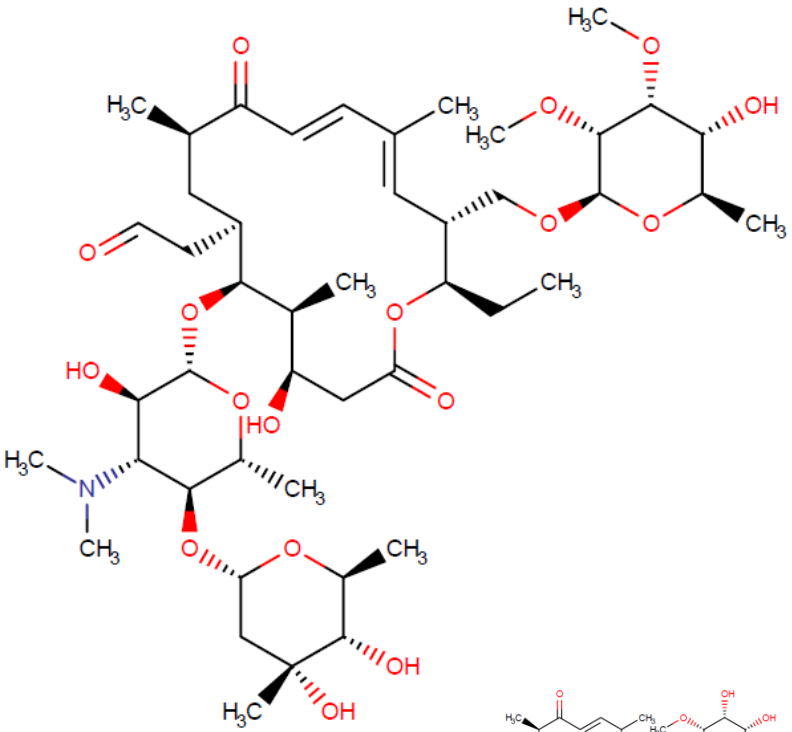
2017 Midyear Conference

Introduction

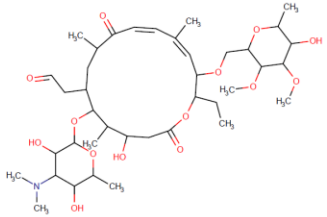
Tylosin in Type C Feeds

- **Chickens and Swine**
  - Increased weight gain and improved efficiency
- **Chickens**
  - The treatment and control of chronic respiratory disease
- **Swine**
  - Prevention of dysentery
  - Prevention and/or control of ileitis
- **Cattle**
  - Reduction in the incidence of liver abscesses in cattle

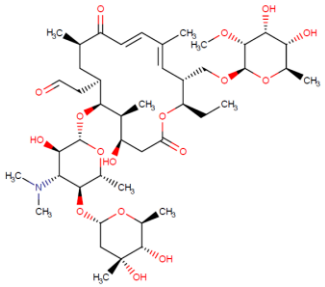
Tylosin Factors A, B, C and D



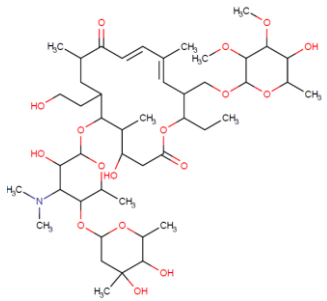
Tylosin (A); bcf* = 1.00



Desmycosin (B); bcf* = 1.26



Macrocin (C); bcf* = 1.01



Relomycin (D); bcf* = 0.33

*based on conversion factors using Staphylococcus aureus

Method Needs

- Should be able to determine tylosin A, B, C and D content
- Should apply to Type A, B and C medicated articles
  - Type A and Type B: 95 – 105 %
  - Medicated complete feeds > 10.0 mg/kg (Type C): 90 – 110 %
  - Residue levels in finished feeds < 10 mg/kg: > 80 %
- Should apply to feed matrices approved for use in the US
- Should have specificity for tylosin in the presence of interferences
- Desired MDL and MQL
  - Medicated products: 0.3 mg/kg / 1.0 mg/kg
  - Contamination analysis: 0.03 mg/kg / 0.1 mg/kg
- Linearity of standard curve:
  - r ≥ 0.999, and 95 % confidence limit of the y intercept includes zero.

Method Needs

- **Performance:**
  - **Performance of this method should be comparable to or exceed that of the FDA approved turbidimetric assay.**
  - **The method is to be rugged/robust and critical parameters are to be identified and controlled.**
  - **Method performance criteria are to be defined.**
  - **Familiarization plan is to be suggested which will demonstrate that the laboratory analyst can capably perform the method prior to analyzing samples. Quality control plan is to be suggested along with warning and out of control limits.**
- **Traceability:**
  - **Reference standards and acceptable sources are to be identified.**
  - **Standards are to be provided with assigned purity or potency and uncertainty value.**

Method Needs

- **Precision Repeatability:**
  - **Medicated products > 10 mg/kg: CVr = or < 5 %**
  - **Contamination analysis and medicated products < 10 mg/kg: CVr = or < 10 %.**
- **Precision Reproducibility:**
  - **Medicated products > 10 mg/kg: CVR = or < 10 %**
  - **Contamination analysis and medicated products < 10 mg/kg: CVR = or < 20 %.**
- **Range:**
  - **0.1 – 220,000 mg/kg (22%, 100 g/lb)**
  - **Recovery: Medicated products > 10 mg/kg: 90 – 110 %**
  - **Contamination analysis and medicated products < 10 mg/kg: > 80 %**

Sample preparation

1. Sampling
    1. Splitting
    2. Grinding
    3. Homogenizing
    4. Test portion size
2. Extraction
    1. Solvent: 90% Methanol
    2. Test portions weighed directly to a wide-mouth HDPE bottle
    3. QC fortification with liquid stock solutions
    4. Mechanical agitation for 1 hour
3. Cleanup
    1. Syringe filter extracts
    2. Dilute to fall within linear range of calibraiton standards

Instrument Method

HPLC

1. Reverse-phase separation
2. Gradient elution

Mass spectometry

1. Electrospray ionization
2. QQQ

RT	Analyte	MW (Da)	Transition (Da)	DP (volts)	CE (volts)	CXP (volts)
4.03	Tylosin	916.469	772.5	146	41	12
			174.3	146	55	10
4.02	Desmycosin	774.385	318.4	146	41	12
			174.2	146	55	10
4.43	Macrocin	902.535	758.421	26	39	12
			174.2	26	53	8
4.08	Relomycin	919.474	775.54	26	41	12
			174.1	26	75	8
4.41	Roxithromycin (I.S.)	837.4	679.4	31	10	29
			158.1	31	10	43

Verification

- **One quantifying transition**
- **At least one qualifying transition**

Sample ID	Tylosin T1	Tylosin T2	**Ion ratio**
2016-1340 A	40600	35400	**115%**
2016-1340 B	35600	37500	**95%**
2016-0821 A	78200	69200	**113%**
2016-0821 B	77000	63400	**121%**
2016-0823 A	82400	82000	**100%**
2016-0823 B	83300	85800	**97%**

Results – Trial 1, 10-g test portion

Table 1. Without internal standard correction; n = 3. Results given in ppm.

Sample ID	Replicate	Tylosin (A)	Desmycosin (B)	Macrocin (C)	Relomycin (D)
2016-1340	1	41.1	2.0	1.1	ND
	2	35.0	2.0	0.9	ND
	3	52.0	5.9	1.1	ND
	average	**42.7**	**3.3**	**1.0**	**NA**
	RSD	**20%**	**70%**	**9%**	**NA**
2016-0821	1	110.0	1.9	1.8	ND
	2	117.0	5.2	2.1	ND
	3	133.0	7.9	2.6	ND
	average	**120.0**	**5.0**	**2.2**	**NA**
	RSD	**10%**	**59%**	**18%**	**NA**
2016-0823	1	132.0	7.8	2.1	ND
	2	95.4	5.9	1.7	ND
	3	100.0	3.9	2.0	ND
	average	**109.1**	**5.9**	**1.9**	**NA**
	RSD	**18%**	**33%**	**10%**	**NA**

Results – Trial 1, 10-g test portion

Table 2. With internal standard correction; n = 3. Results given in ppm.

Sample ID	Replicate	Tylosin (A)	Desmycosin (B)	Macrocin (C)	Relomycin (D)
2016-1340	1	42.9	2.1	0.3	ND
	2	30.5	0.9	0.2	ND
	3	51.0	1.0	0.5	ND
	average	**41.5**	**1.3**	**0.3**	**NA**
	RSD	**25%**	**51%**	**46%**	**NA**
2016-0821	1	84.5	0.8	0.4	ND
	2	81.0	0.7	0.3	ND
	3	127.0	0.9	0.8	ND
	average	**97.5**	**0.8**	**0.5**	**NA**
	RSD	**26%**	**17%**	**47%**	**NA**
2016-0823	1	90.4	0.7	0.4	ND
	2	86.1	4.5	0.5	ND
	3	131.0	2.6	0.9	ND
	average	**102.5**	**2.6**	**0.6**	**NA**
	RSD	**24%**	**73%**	**44%**	**NA**

Results – Trial 1: Summary

Table 3. Without internal standard correction

Sample ID	Total Tylosin by LC-MS/MS n = 3 (mg/kg)	Guarantee (g/T)	Total Tylosin by LC-MS/MS (g/T)	Total Tylosin by Plate Assay (g/T)	Recovery by LC-MS/MS	Recovery by Plate Assay
2016-1340	47.9	40	**43**	37	**109%**	93%
2016-0821	128.5	100	**117**	92	**117%**	92%
2016-0823	118.5	100	**108**	93	**108%**	93%

Table 4. With internal standard correction

Sample ID	Total Tylosin by LC-MS/MS n = 3 (mg/kg)	Guarantee (g/T)	Total Tylosin by LC-MS/MS (g/T)	Total Tylosin by Plate Assay (g/T)	Recovery by LC-MS/MS	Recovery by Plate Assay
2016-1340	43.4	40	**39**	37	**99%**	93%
2016-0821	99.0	100	**90**	92	**90%**	92%
2016-0823	106.4	100	**96**	93	**96%**	93%

Results – Trial 2, 40-g test portion

Table 5. Without internal standard correction; n = 2. Results given in ppm.

Sample ID	Replicate	Tylosin (A)	Desmycosin (B)	Macrocin (C)	Relomycin (D)
2016-1340	1	40.6	1.9	0.4	ND
	2	35.6	1.6	0.4	ND
	average	**38.1**	**1.8**	**0.4**	**NA**
	RSD	**9%**	**11%**	**3%**	**NA**
2016-0821	1	78.2	3.1	0.4	ND
	2	77.0	2.8	0.4	ND
	average	**77.6**	**3.0**	**0.4**	**NA**
	RSD	**1%**	**5%**	**10%**	**NA**
2016-0823	1	82.4	3.2	0.7	ND
	2	83.3	3.6	0.5	ND
	average	**82.9**	**3.4**	**0.6**	**NA**
	RSD	**1%**	**9%**	**16%**	**NA**

Results – Trial 2, 40-g test portion

Table 6. With internal standard correction; n = 2. Results given in ppm.

Sample ID	Replicate	Tylosin (A)	Desmycosin (B)	Macrocin (C)	Relomycin (D)
1340	1	42.5	2.0	0.3	ND
	2	40.1	1.7	0.4	ND
	average	**41.3**	**1.8**	**0.3**	**NA**
	RSD	**4%**	**9%**	**12%**	**NA**
2016-0821	1	74.1	2.8	0.3	ND
	2	97.3	3.8	0.5	ND
	average	**85.7**	**3.3**	**0.4**	**NA**
	RSD	**19%**	**21%**	**39%**	**NA**
2016-0823	1	78.3	3.0	0.5	ND
	2	82.9	3.5	0.4	ND
	average	**80.6**	**3.3**	**0.5**	**NA**
	RSD	**4%**	**10%**	**15%**	**NA**

Results – Trial 1: Summary

Table 4. Without internal standard correction

Sample ID	Total Tylosin by LC-MS/MS, n = 2 (mg/kg)	Guarantee (g/T)	Total Tylosin by LC-MS/MS (g/T)	Total Tylosin by Plate Assay (g/T)	Recovery by LC-MS/MS	Recovery by Plate Assay
2016-1340	40.7	40	**37**	37	**92%**	93%
2016-0821	81.7	100	**74**	92	**74%**	92%
2016-0823	87.8	100	**80**	93	**80%**	93%

Table 4. With internal standard correction

Sample ID	Total Tylosin by LC-MS/MS, n = 2 (mg/kg)	Guarantee (g/T)	Total Tylosin by LC-MS/MS (g/T)	Total Tylosin by Plate Assay (g/T)	Recovery by LC-MS/MS	Recovery by Plate Assay
2016-1340	44.0	40	**40**	37	**100%**	93%
2016-0821	90.2	100	**82**	92	**82%**	92%
2016-0823	85.2	100	**77**	93	**77%**	93%

Quality Control

1. If an incurred LCS is not available, non-medicated negative control complete feed corresponding to the medicated feed should be used to prepare blanks and fortified samples.
2. A known amount of standard solution is added to the negative control feed to give appropriate fortification levels.
3. At least one fortified negative control should be prepared in duplicate for each batch.
4. At least one test portion of negative control feed should be analyzed to demonstrate specificity at the analyte retention time.

Parameter	Acceptance Criteria
Recovery	80 - 110%
Peak Symmetry	0.7-1.3
Repeatability (%RSD of Recovery)†	≤5% or ≤7.5%
Specificity	The signal measured in the negative control should be less than 10% of the chromatographic peak area of the lowest calibration standard.

†For drugs incorporated into medicated feeds at greater than 10 ppm, %RSD should be ≤5%. For drugs incorporated into medicated feeds at less than 10 ppm, the %RSD should be ≤7.5%.

Next Steps

1. Acquire reference material
2. Multi-day trial with multiple analysts
3. Add tylosin-urea adduct (TUA)
4. Perform trials with ruminant feeds with urea added
    1. Evaluate chemical hydrolysis
    2. Evaluate enzymatic hydrolysis
5. Suggestions?
6. Criticism/concerns?