MICROBIOLOGICAL RESIDUES METHOD FOR VIRGINIAMYCIN

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Virginiamycin

Background - Analytical

Fermentation Product

Multiple Factors – 2 Major + Minor

Synergistic Bio-potency

Microbiological Assay

Analytical Standard – 225 µg/mg

Fermentation Development

Microbiological Assay

All Products

Agar – Cut Well –Zone concentration

Micrococcus Luteus (ATCC-9341)

Range 0.2µg/ml TO 0.8 µg/ml

Final Solution: 20% MEOH.pH6 buffer

Products Assay

- Bulk
- Intermediate Use Concentration
- Fermentation Use Mixes
- Animal Feed Premixes
- Final Animal Feeds
- Ingredients (DDG)

Sample Preparation

- All except Feeds/Ingredients
- Extract 50% 0.1 M Citric Acid- Acetone
- Aliquot-dilute with 20% MEOH-pH 6 Buffer

Feeds Preparation

1. Shake extract with 50% Citric Acid-Acetone
2. Weight scaled for solvent
3. Paper filter and adjust to pH6
4. Wash aliquot- Pet ether and cyclohexane
5. Evaporate trace solvents
6. Dilute -20% MeOH – pH6 buffer
7. PAV $\pm$ 30% LOQ – 2 ppm

Plate Assay Critical Fscotrs

- Migration out of well into agar
- Solubility + other compounds
- Concentration decreases
- Matrix more complex
- Isolation need increases

Ingredients – Distillers Grain

- FDA letter November 1993
  - Specified 0.5 ppm Virginiamycin upper limit
- Method reference
  - Standard micro-assay with modified sample preparation

DDG Sample Prep 1993

- Shake extract MIBK 50 g/200 ml
- Centrifuge, filter, rotovap to 1 ml
- Dilute to 10 ml with 20% MeOH-buffer
- Wash with pet ether and cyclohexane
- Evaporate solvents
- Dilute to 5 ml with 20% MeOH.buffer

Implementation of 1993 Prep

- MIBK not a usable solvent – Replace with MeOH
- Locate lab that could do both chemical prep and bio-assay
- MeOH shake flasks – poor handling, filtration, extract volume
- Replace with Soxhlet 3 hrs 50 g/300 ml MeOH
- Easy transfer Soxhlet to Rotovap

Cont'd

- Wash Rotovap residue 3 x 25 Hexane
- Evaporate to dryness
- Residue up in 20 ml MeOH and 80 ml buffer
- Vacuum paper and 0.45 µm filter
  - Assay not reliable at this point
- Solid Phase SepPak C-18 SPE
- Clean-up 25 ml with 10 ml wash x 4
- Elute with 7.5 ml 70%MeOH buffer –dilute to 25 ml with buffer

Cont'd

- Wide array of samples okay
- Assay in place:
  - Commercial Laboratory
  - Phibro Ethanol Process Lab
- Assay performance data from Phibro Laboratory

Performance Data

Data Table 1.1 – Dry Distiller's Grains (DDG) with solubles

Replicate	Sample Volume	% Moisture	VM Spike Conc. (ppm)	VM Concentration recovered (ppm) [1]	% Recovery
1	50.03	10.9	0.50 ppm	0.51	102.9%
2	50.09	10.9	0.50 ppm	0.52	103.6%
3	50.12	10.9	0.50 ppm	0.50	99.6 %
4	50.06	10.9	0.50 ppm	0.53	105.9%
5	50.05	10.9	0.50 ppm	0.53	105.9%
1	50.14	10.9	1.0 ppm	1.03	102.7%
2	50.09	10.9	1.0 ppm	1.00	99.8%
3	50.00	10.9	1.0 ppm	1.03	103.0%
4	50.06	10.9	1.0 ppm	1.06	105.9%
5	50.01	10.9	1.0 ppm	1.04	103.8%
1	50.19	10.9	1.5 ppm	1.33	88.8%
2	50.19	10.9	1.5 ppm	1.34	89.6%
3	50.04	10.9	1.5 ppm	1.36	90.7%
4	50.03	10.9	1.5 ppm	1.34	89.1%
5	50.09	10.9	1.5 ppm	1.32	88.2%
Control	50.11	10.9	Unspiked	No Zone of Inhibition Present	NA

Data Table 1.2 – Wet Distiller's Grains (WDG) with solubles

Replicate	Sample Volume (g)	% Moisture	VM Spike Conc. (ppm)	VM Concentration recovered (ppm) [1]	% Recovery
1	50.11	66.1%	0.50 ppm	0.53	105.8%
2	50.22	66.1%	0.50 ppm	0.55	109.6%
3	50.16	66.1%	0.50 ppm	0.50	100.7%
4	50.10	66.1%	0.52 ppm	0.48	92.1%
5	50.02	66.1%	0.52 ppm	0.52	99.6%
1	50.13	66.1%	1.04 ppm	1.00	95.9%
2	50.04	66.1%	1.04 ppm	0.85	81.6%
3	50.05	66.1%	1.04 ppm	0.97	93.2%
4	50.02	66.1%	1.04 ppm	0.97	93.2%
5	50.10	66.1%	1.04 ppm	0.96	92.1%
1	50.02	66.1%	1.56 ppm	1.36	87.3%
2	50.15	66.1%	1.56 ppm	1.37	87.9%
3	50.10	66.1%	1.56 ppm	1.41	90.4 %
4	50.01	66.1%	1.56 ppm	1.40	89.6%
5	50.10	66.1%	1.56 ppm	1.31	84.0%
Control	50.02	66.1%	Unspiked	No Zone of Inhibition Present	NA

[1] Adjusted for concentration from ~ 50 gram solid to 25 ml final extraction volume

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Data Table 1.3 – Mean % Recovery and RSD

Replicates	Mean Concentration (ppm)	Mean % Recovery	RSD
DDG @ 0.5 ppm	0.52	103.6%	2.5
DDG @ 1.0 ppm	1.03	103.0%	2.1
DDG @ 1.5 ppm	1.34	89.3%	1.1
WDG @ 0.5/0.52 ppm	0.52	101.6%	6.5
WDG @ 1.04 ppm	0.95	91.2%	6.1
WDG @ 1.56 ppm	1.37	87.8%	2.8

Data Table 1.4 – Stability of Spiked DDG Extracts

	Date of DDG Spiking	First Bioassay	Result	Second Bioassay	Result	% Difference From First to Second Bioassay
DDG 0.5 ppm	Aug. 27	Sept. 6	0.53 ppm	Sept 24	0.51 ppm	3.8%
DDG 0.5 ppm	Aug. 25	Sept. 9	0.50 ppm	Sept 24	0.50 ppm	0%
DDG 1.0 ppm	Aug. 25	Sept. 9	1.03ppm	Sept 24	1.03 ppm	0%
DDG 1.0 ppm	Aug. 24	Sept. 9	0.96 ppm	Sept 24	0.93 ppm	3.2%
DDG 1.0 ppm	Aug. 24	Sept. 9	1.18 ppm	Sept 24	1.00 ppm	16.5 %

Data Table 1.5 – Stability of Spiked Solutions

	Preparation of Spike	Bioassay	Result	% Recovery
1.0 ppm Spike Solution	Aug. 24	Sept. 9	1.14 ppm	114%
1.0 ppm Spike Solution	Sept. 16	Sept 24	1.03 ppm	103%