Improved Procedures for the Determination of Oxytetracycline in Milk, Milk Products; Chicken Muscle, Liver; and Eggs

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The procedures used to determine residues of anitibotics in many types of commodities, raw or processes are based upon the classical cylinder-plate diffusion techniques (1). Perhaps the most comprehensive summary of procedures for antibiotic residues are those described by the FDA (2). In general, this publication is a compendium of sensitive and reproducible methods for assaying antibiotic residues.

The purpose of this manuscript is to report on the evaluation of improved procedures for determining oxytetracycline in milk and dairy products, chicken muscle tissue and livers, and eggs. The procedures used are based upon those developed for chlortetracycline in similar materials (3,4,5) and are compared to the procedures listed in the FDA compendium of methods and protocols.

Materials and Methods

Reagents:

1. Buffer pH 4.5 ± 0.1. Dissolve 13.6 g monobasic potonium phosphate in distilled water and dilute to 1 liter with distilled water. For egg assay stand curve, add 5 gram/liter Tween 20 to buffer.

2. Agar Culture Medium. This medium is available in dehydrated form as Difco Antibiotic Medium 2. The pH should be adjusted so that after sterilization the pH is 5.95 ± 0.05.

Bulletin of Environmental Contamination & Toxicology,
Vol. 7, No. 4, 1972, © by Springer-Verlag New York Inc.

3. Standard Solutions of Oxytetracycline. Dissolve oxytetracycline standard in 0.1 N hydrochloric acid to give a stock solution containing 1000 µg/ml activity. Dilute aliquots of the stock solution with pH 4.5 buffer to give a standard response curve of 0.025, 0.05, 0.10, 0.20 and 0.40 µg/ml. The 0.10 µg/ml standard is the reference concentration. The acid stock solution is stable 14 days under refrigeration.

4. Test Organism. Bacillus cereus var mycoides ATCC 11778 prepared according to the A.O.A.C. procedures (6). This stock culture solution is diluted 1:5 with sterile saline and can be maintained for several months if refrigerated and kept free from contamination. Zones for the 0.025 µg/ml should approximate 10.0 mm and standard reference concentration 0.10 µg/ml should approximate 18.0 mm. Extension of the curve to. 0.02 µg/ml is possible and these zones approximate 8.5 mm.

Apparatus:

1. High speed blender capable of handling pint Mason jars such as manufactured by the John Oster Company, Milwaukee, Wisconsin.

2. Microbiological Assay Equipment. Cylinder dispenser, petri dish bottoms or equivalent plates, porcelain covers glazed on the outside, stainless steel cylinders, incubators and ancillany equipment described in the A.O.A.C. procedures (6).

3. Centrifuge capable of handling 250 ml bottles.

4. pH meter.

Procedures

Preparation of Standard Curves: Two types of standard response curves can be developed. For recovery studies and/or actual residue determinations themselves, a standard curve of 0.025, 0.05, 0.01, 0.02, and 0.04 µg/ml in phosphate buffer of pH 4.5 is used. For actual residue studies, theuse of a standard response curve requires recovery data on blank product. Recoveries using 5, 10 and 15 µgs of oxytetracycline activity are sufficient. To prepare a compensatory curve in milk add 3.00, 6.00, 15.00, 30.00 and 60.00 µgs to 100 ml quantities of milk, adjust pH to 4.5 and bring total 150 ml with 4.5 buffer. This procedure should yield a response curve of 0.03, 0.05, 0.10, 0.20 and 0.40 µg/ml. Use 0.10 µg/ml as before. For other commodities prepare similar curve based upon the limit of detectability and range needed.

Preparation of Plates: Melt the agar and cool to 70°C before innoculating with the previously standardized organism suspension. Mix well. Add 6 ml of the seeded agar to the petri dishes, distributing the agar uniformly and allowing to harden on a level surface. Use plates within 1 hr of hardening. A complete description of the placing and filling of the cylinders, incubation times and temperatures are found in the A.O.A.C. procedures and the FDA methods and protocols (2,6).

Determination of Oxytetracycline in Various Materials:

1. Fresh and Evaporated Milk. Take 100 g of milk, adjust the pH of the milk to 4.5 with 3 N phosphoric acid and add sufficient pH 4.5 phsophate buffer to bring weight to 150 g. Mix well. Centrifuge at high speed for 10 min. The adjustments can also be made on a volume basis.

2. Cream. Take 50 g of cream and add 50 ml distilled water. Mix well. Adjust the pH to 4.5 with 3 N phosphoric acid and bring weight to 150 g with pH 4.5 phosphate buffer. Centrifuge at high speed for 10 min.

3. Non-Fat Dry Milk. Take 10 g dry powder and add 30 ml distilled water and mix well. Adjust pH to 4.5 with 3 N phosphoric acid and bring to a total weight of 50 g with pH 4.5 buffer. Mix well again. Centrifuge at high speed for 10 min.

4. Chicken Muscle and Liver. Place 25 g of chicken muscle tissue in a pint Mason jar and add 100 ml of pH 4.5 phosphate buffer. Blend at high speed for 2 min. Remove a portion and centrifuge for 10 min at high speed. For liver tissue, either take a 25 g sample or the whole liver and weigh in a tared pint Mason jar. Add pH 4.5 phosphate buffer equivalent to 4 times the weight of the liver. Blend for 2 min and proceed as previously described.

5. Eggs. Place a shelled egg in a tared Mason jar. After determining the weight, add 50 ml of distilled water and blend for ½ minute. Adjust pH to 4.5 with 3 N phosphoric acid and add pH 4.5 buffer and Tween 20 so that the final weight is 4 times the weight of the original shelled egg and the Tween 20 concentration is 0.5%. This mixture is reblended for 30 seconds at high speed followed by centrifuging for 10 min at high speed.

Results and Discussion

The rationale for the improvements, such as pH adjustment, centrifugation, single agar layer, high temperature seeding and spreading of agar, and the use of the surfactant have been discussed adequately in other manuscripts (3,4,5).

Recoveries of oxytetracycline from "spikes" milk samples using both the centrifuge and FDA procedures show interesting relationships. The average recoveries for the FDA procedure were 57.4% versus 84.3% for the centrifuge procedure, a ration of 1.47. The centrifuge procedure detects 0.02 μg/g with consistency at 0.15 μg/g oxytetracycline in milk.

TABLE 1

Recoveries of Oxytetracycline from "Spiked" Samples of Milk and Comparison Between the Centrifuge and FDA Procedures

Supplementation per 100 g milk µg	Procedure Recoveries FDA µg	Suggested %	Centrifuge µg	%
0.0	N.D.[1]	Blank	N.D.	Blank
2.0	N.D.	-	J.D.[2]	-
3.0	N.D.	-	3.5	116.6
3.0	N.D.	-	2.4	79.8
4.0	N.D.	-	2.5	62.5
5.0	N.D.	-	4.4	88.0
7.0	N.D.	-	6.1	87.2
9.0	N.D.	-	7.9	87.8
10.0	N.D.	-	8.5	85.0
12.0	N.D.	-	9.5	79.0
12.0	7.5	62.5	9.4	78.3
15.0	6.7	44.7	12.0	75.0
15.0	9.9	66.0	13.3	88.7
20.0	11.5	57.5	16.0	80.0
20.0	12.6	63.0	17.6	88.0
20.0	10.2	51.0	17.0	85.0
	Average	57.4		84.3

[1] Not Detected

[2] Just Detected (zone of inhibition approximates 8.5 mm)

Use of this methodology proved to be quite successful when applied to 2 other milk produces i.e. non-fat dry milk and evaporated milk. The procedure used for evaporated milk was identical to that used for fresh milk. Results were similar but there was a loss of sensitivity. In fresh milk, 0.03 µg/g was measurable by the centrifuge procedure; in evaporated milk 0.05 µg/g is detectable but quantitative measurement is only possible at the 0.06-07 µg/g. The average recovery for the centrifuge procedure was 87.0% versus 31.6% for the FDA procedure. The ratio of recoveries between the centrifuge procedure and the FDA method was 2.75. Consistent detection and quantitative analysis by the FDA procedure is possible at 0.10 µg/g.

TABLE 2

Recoveries and Comparison of Methods for Oxytetracycline in Evaporated Milk

Supplementation per 100 g milk	Procedure Recoveries FDA Suggested		Centrifuge	
µg	µg	%	µg	%
0	N.D.[1]	-	N.D.	-
2	N.D.		N.D.	-
3	N.D.		N.D.	-
5	N.D.		J.D.[2]	
7	N.D.		6.3	90.0
10	3.15	31.5	8.7	87.0
12	3.75	31.2	10.2	85.0
15	4.80	32.0	12.9	86.0
Average		31.6		87.0

[1] Not Detected

[2] Just Detected (zone of inhibition approximates 8.5 mm)

In fat free dry milk, the recoveries by the centrifuge procedure were slightly lower than found in the other milks 71.0% but the recoveries found using the FDA procedure average 17.6% with a ratio between the centrifuge and FDA methods 4.34.

TABLE 3

Recoveries and Comparison of Methods for Oxytetracycline in Non-Fat Dry Milk

Supplementation per 10 g milk	Procedure Recoveries FDA Suggested		Centrifuge	
µg	µg	%	µg	%
0	N.D.[1]	-	N.D.	-
2	N.D.	-	J.D.[2]	-
3	N.D.	-	2.0	66.6
5	0.8	16.0	3.6	72.0
10	1.7	17.0	7.6	76.0
12	2.0	16.7	9.0	75.0
15	3.1	20.7	9.8	65.3
Average		17.6		71.0

[1] Not Detected

[2] Just Detected (zone of inhibition approximates 8.5 mm)

Although recoveries of oxytetracycline using both methods appears to be low based upon a 10 g sample, the values when calculated to restitution are similar to those found in fresh milk. The 10 g sample is approximately equivalent to 100 g of liquid milk.

Recoveries from a cream product, half'n half, are essentially the same as were found for liquid milk, averaging 53.5% for the FDA procedure and 89.7% for the centrifuge procedure. The ratio of recoveries comparing the centrifuge procedure to the FDA method is 1.68.

TABLE 4

Recoveries and Comparison of Methods for Oxytetracycline in Half and Half

Supplementation per 50 g Cream	Procedure Recoveries			
	FDA	Suggested	Centrifuge	
µg	µg	%	µg	%
0	N.D.	-	N.D.	-
2	N.D.	-	2.2	110.0
3	N.D.	-	2.7	90.0
5	N.D.	-	4.1	82.0
7	3.3	47.1	5.7	81.4
10	5.3	53.0	8.3	83.0
12	6.8	56.6	11.3	94.1
15	8.6	57.3	13.1	87.3
Average		53.5		89.7

Recoveries using the centrifuge procedure on chicken tissues also were improved over those found using the FDA procedure. In edible chicken muscle the average recoveries using the centrifuge procedure were 82.8% versus 53.6% for the FDA procedure. The ratio of recoveries between the centrifuge and FDA procedure was 1.54.

TABLE 5

Recoveries and Comparison of Methods for Oxytetracycline in Chicken Liver Tissue

Supplementation per 25 g tissue	Procedure Recoveries			
	FDA	Suggested	Centrifuge	
µg	µg	%	µg	%
0.0	N.D.[1]	-	N.D.	-
2.0	N.D.	-	J.D.[2]	-
3.0	N.D.	-	2.2	75.0
5.0	3.1	62.0	4.0	80.0

µg	µg	%	µg	%
7.0	3.4	49.1	4.5	64.3
9.0	4.4	48.6	5.9	65.3
11.0	5.6	51.1	8.0	72.7
13.0	5.8	44.2	8.1	62.4
15.0	8.3	55.0	10.6	70.8
18.0	8.8	48.6	12.5	69.4
20.0	10.1	50.6	14.4	71.9
	Average	51.2		70.7

[1] Not Detected

[2] Just Detected (zones approximate 8.5 mm)

A comparison of a procedure consistent with the FDA concept, namely, 1 part egg + 2 parts pH 4.5 buffer versus the surfactant-centrifuge procedure was made. The average recoveries by the furfactant-centrifuge procedure was 64.7 versus 34.0 for the procedure consistent with FDA procedures; the ratio between methods was 1.90.

TABLE 7

Recoveries and Comparison of Methods for Oxytetracycline in Eggs

Supplementation per 50 g Eggs	Procedure Recoveries			
	FDA Suggested		Centrifuge-Surfactant	
µg	µg	%	µg	%
0.0	N.D.[1]	-	N.D.	-
2.0	N.D.	-	N.D.	-
3.0	N.D.	-	N.D.	-
4.0	N.D.	-	J.D.[2]	-
5.0	N.D.	-	3.0	60.0
6.0	N.D.	-	3.8	63.3
7.0	2.5	36.4	4.5	64.3
8.0	2.7	33.7	5.0	62.5
10.0	3.1	31.0	6.2	62.0
12.0	4.1	33.9	8.0	66.7
14.0	4.6	33.2	9.6	68.6
18.0	6.2	34.2	12.6	70.0
20.0	7.1	35.2	13.8	65.0
	Average	34.0		64.7

[1] Not Detected

[2] Just Detected (zones approximate 8.5 mm)

Summarizing the limits of detecabilities and analytical measurement for the individual procedures, it can be seen that the FDA suggested procedures range from 0.10 to 0.20 in detection and analytical measurement, while the centrifuge procedures range in detection from 0.02 to 0.10 μg/g and in analytical measurement from 0.03 to 0.12 μg/g. The limits of measurement for non-fat dry milk are listed both as a function of the dry powder and reconstituted.

TABLE 8

Product	FDA Suggested Procedures Compared Centrifuge			
	Detection μg	Measurement μg	Detection μg	Measurement μg
Milk	0.12	0.15	0.02	0.03
Evaporated Milk	0.10	0.10	0.05	0.07
Non-Fat Dry Milk (dry basis)	0.50	0.50	0.20	0.30
Non-Fat Dry Milk (Reconstituted)	0.05	0.05	0.02	0.03
Cream (Half'n Half)	0.14	0.14	0.04	0.04
Chicken Muscle	0.20	0.20	0.04	0.12
Chicken Liver	0.20	0.20	0.04	0.12
Eggs	0.14	0.14	0.08	0.10

The centrifuge modification, pH adjustment where applicable, and the use of surfactant when necessary have significanly improved the ability to measure residue of oxytetracycline in milk, eggs, and chicken tissue.

Acknowledgment

These studies were supported by U. S. Public Health Service Grant FD-00172-03.

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Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University - The State University of New Jersey, Department of Biochemistry and Microbiology, New Brunswick, New Jersey 08903.