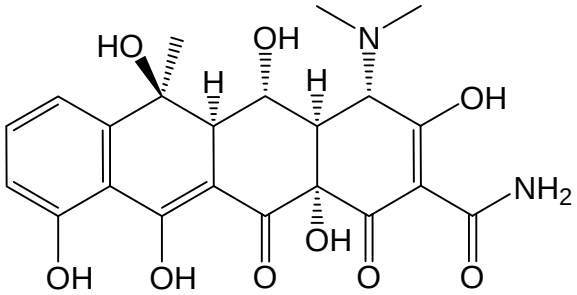
4 Alkyl trimethyl ammonium calcium oxytetracycline or oxytetracycline hydrochloride

Alkyl trimethyl ammonium calcium oxytetracycline, Oxytetracycline hydrochloride



Oxytetracycline

(4S,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,11,12a-hexahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,5a,6,12,12a-octahydrotetracene-2-carboxamide

$C_{22}H_{24}N_2O_9$ MW: 460.4 CAS No.: 79-57-2

[Summary of alkyl trimethyl ammonium calcium oxytetracycline or oxytetracycline hydrochloride]

Oxytetracycline (OTC) is a tetracycline antibiotic obtained by the incubation of *Streptomyces rimosus*. The one used as a feed additive is alkyl trimethyl ammonium calcium salt.

Alkyl trimethyl ammonium calcium salt is a coarse powder obtained by adding calcium carbonate and alkyl trimethyl ammonium chloride to the culture filtrate in the presence of alkali.

For physicochemical properties, OTC technical (i.e., alkyl trimethyl ammonium calcium salt) occurs as a yellow-brown to dark-brown powder, and has a characteristic odor. It is slightly soluble in hydrochloric acid (1 mol/L), and practically insoluble in water and in methanol.

OTC has a broad antibacterial spectrum covering Gram-positive bacteria, Gram-negative bacteria, rickettsia, chlamydia, etc., and has a growth promoting effect on chickens (including broilers), pigs, and cattle.

«Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds»

Alkyl trimethyl ammonium calcium oxytetracycline (OTC) and oxytetracycline hydrochloride were designated as feed additives as of July 24, 1976, but oxytetracycline hydrochloride was deleted from the list as of July 6, 1983.

OTC is a pure-grade antibiotic. The specifications for feeds containing this ingredient are specified in Appended Table 1, 1-(1)-C of the Ministerial Ordinance Concerning the Ingredient Specifications for Feeds and Feed Additives.

(in g(potency)/t)

Feed type	For chikens (except for broilers)	For broilers	For pigs	For cattle	
	Staring chicks Growing chicks	Starting broilers	Sucking piglets	Sucking calves	Calves
Added amount	5~55	5~55	5~70	20~50	20~50

The amount of OTC added to a commercial premix is roughly 4 to 50 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test methods - Plate method

1.1 Premix

[Feed Analysis Standards Chapter 9, Section 2, 4.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Oxytetracycline standard solution. Weigh accurately not less than 40 mg of oxytetracycline working standard[1], accurately add hydrochloric acid (0.01 mol/L) and dissolve to prepare a standard stock solution at concentration of 1 mg (potency)/mL[2].

At the time of use, accurately dilute a quantity of standard stock solution with Buffer No.1 to prepare high- and low-concentration standard solutions with concentrations of 5 and 1.25 µg (potency)/mL, respectively[3].

3) Culture medium: Medium F-4

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[4] as the test organism. Add 0.2 mL of a 100-fold diluted suspension of the test organism to 100 mL of the culture media.

5) Agar plate. Proceed by the agar well method.

6) Extracting solvent: A mixture of methanol and hydrochloric acid (4 mol/L) (49:1)[5]

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.1 to prepare high- and low-concentration sample solutions with concentrations of 5 and 1.25 µg (potency)/mL, respectively[6].

C. Quantification[7]

Proceed by the 2-2 dose method[8].

«Summary of analysis method»

This method is intended to determine the amount of OTC in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of methanol and hydrochloric acid (4 mol/L) (49:1) and diluting with Buffer No.1. None of the antibacterial substances approved to be used in combination with OTC interfere with the quantification of OTC.

The flow sheet of the analysis method is shown in Figure 9.2.4-1.

Sample (3.0-5.0 g)
↓
Extract with 100 mL of methanol-hydrochloride (4 mol/L) (49:1) .
(magnetic stirrer, 20 min)
↓
Filter (filter paper: No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.1to prepare high- and low-concentration sample solutions (5 and 1.25 μg(potency)/mL, respectively).
↓
Dispense to agar plates (allow to stand at 10-20 ℃ for 2 hours).
↓
Incubate (at 35-37 ℃ for 24 hours).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.4-1 Quantitative test method for alkyl trimethyl ammonium calcium oxytetracycline and oxytetracycline hydrochloride (premix)

References: Noriyuki Koyama: Research Report of Animal Feed, 6, 163 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Oxytetracycline hydrochloride	Vitamin premix	5~20	3	98.5~99.8	1.7
	Vitamin/mineral premix	5~20	3	98.6~99.6	1.6

«Notes and precautions»

[1] For the definition etc. of oxytetracycline working standard, refer to «Notes and precautions» [9] in Secton 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Secton 1, 1 of this Chapter.

Method of preparation: Example (when weighed in an amount of 50 mg)

When the labeled potency of the working standard is 908 μg (potency)/mg, 50 mg of the working standard contains 45,400 μg (potency) (i.e., 50 mg × 908 μg (potency)/mg). To prepare a standard stock solution with a concentration of 1,000 μg (potency)/mL, the required amount of solvent is thus calculated to be 45.4 mL (i.e., 45,400 μg (potency) / 1,000 μg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 45.4 mL of hydrochloric acid (0.01 mol/L) and dissolve to prepare a standard stock solution with a concentration of 1,000 μg (potency)/mL.

[3] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Secton 1, 1 of this Chapter.

An example method of preparation for oxytetracycline standard solution is shown in Table 9.2.4-1.

[4] For the number of bacteria, refer to «Notes and precautions» [33] in Secton 1, 1 of this Chapter.

[5] Corresponds to a mixture of methanol, water, and hydrochloric acid (980:13:7).

[6] For the method of preparation for the sample solution, refer to «Notes and precautions» [8] in Secton 1, 1 of this Chapter.

An example method of preparation is shown in Table 9.2.4-1.

Table 9.2.4-1 Method of preparation for oxytetracycline standard solution and sample solution

1) Method of preparation for oxytetracycline standard solution (premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	5	5
Amount (mL) of Buffer No.1	18	16	15	15
Concentration (μg(potency)/mL)	100	20	5	1.25

Note: "②mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

2) Method of preparation for the sample solution (premix, example)

When the analysis sample is collected in an amount equivalent to 50,000 μg (potency) of OTC, the concentration of OTC in the filtrate is calculated to be 500 μg (potency)/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	②	2	5
Amount (mL) of Buffer No.1	18	18	15
Concentration (μg(potency)/mL)	50	5	1.25

Note: "②mL" means "2 mL of the filtrate (500 μg(potency)/mL)".

[7] An example standard response line for OTC is shown in Figure 9.2.4-2.

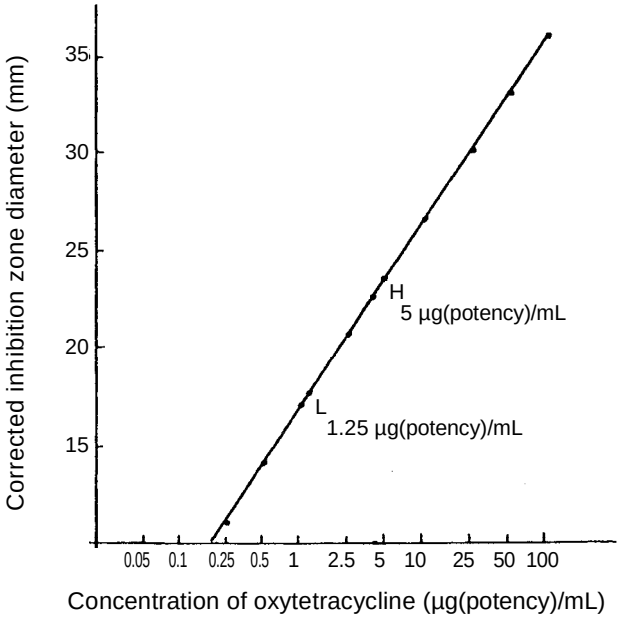


Figure 9.2.4-2 Standard response line for oxytetracycline (premix, example)

(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

[8] Refer to «Notes and precautions» [53] to [60] in Secton 1, 1 of this Chapter.

1.2 Feed

[Feed Analysis Standards Chapter 9, Section 2, 4.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Oxytetracycline standard solution. Weigh accurately not less than 40 mg of oxytetracycline working standard, accurately add hydrochloric acid (0.01 mol/L) and dissolve to prepare an oxytetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

i) When OTC content is not less than 20 g (potency)/t

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of Buffer No.1 and methanol (3:1) to prepare standard solutions with concentrations of 8, 4, 2, 1, and 0.5 µg (potency)/mL[1].

ii) When OTC contnt is less than 20 g (potency)/t

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of Buffer No.1 and methanol (3:1) to prepare standard solutions with concentrations of 1.6, 0.8, 0.4, 0.2, and 0.1 µg (potency)/mL[1].

3) Culture medium: Medium F-17

4) Spore suspension and amount of addition

i) When OTC content is not less than 20 g (potency)/t

Use *Bacillus cereus* ATCC 11778 as the test organism. Add about 0.1 mL of spore suspension with a concentration of 1×10^7 spores/mL to 100 mL of the culture medium.

ii) When OTC content is less than 20 g (potency)/t

Use *Bacillus cereus* ATCC 11778 as the test organism. Add about 0.2 mL of spore suspension with a concentration of 1×10^5 spores/mL to 100 mL of the culture medium.

5) Agar plate. Add the spore suspension to the culture medium that has been melted and maintained at 49 to 51°C, stir thoroughly, dispense 15 mL into Petri dishes to form a uniform layer, and allow to stand horizontally to solidify. To the layer again dispense[2] 5 mL of the culture medium to form a uniform layer, allow to stand horizontally to solidify, and then proceed by the agar well method.

6) Extracting solvent: A mixture of Buffer No.1, methanol, and hydrochloric acid (4 mol/L) (50:49:1)[3]

B. Preparation of sample solution

1) When OTC content is not less than 20 g (potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.4 mg (potency) as OTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 4.4 to 4.6 with

ammonia solution (6 mol/L)[4]. Transfer the whole amount of this liquid with Buffer No.1 to a 50-mL one-mark flask, add Buffer No.1 up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 2 µg (potency)/mL.

2) When OTC content is less than 20 g (potency)/t

i) When MN is not used in combination

Weigh accurately a quantity of the analysis sample (equivalent to 80 µg (potency) as OTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 4.4 to 4.6 with ammonia solution (6 mol/L)[4]. Transfer the whole amount of this liquid with Buffer No.1 to a 50-mL one-mark flask, add Buffer No.1 up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.4 µg (potency)/mL.

ii) When MN is used in combination[5]

Weigh accurately a quantity of the analysis sample (equivalent to 80 µg (potency) as OTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, adjust the pH to 1.0 or lower with hydrochloric acid, allow to stand for 1 hour, and again adjust the pH to 4.4 to 4.6 with ammonia solution (6 mol/L)[4]. Transfer the whole amount of the liquid with Buffer No.1 to a 50-mL one-mark flask, add Buffer No.1 up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.4 µg (potency)/mL.

C. Quantification[6]

Proceed by the standard response line method[7].

«Summary of analysis method»

This method is intended to determine the amount of OTC in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of Buffer No.1, methanol, and hydrochloric acid (4 mol/L) (50:49:1) and adjusting the pH to 4.4 to 4.6. In order to quantify OTC at low concentrations, this method employes a standard response line method using a test organism and culture medium that are more sensitive than those employed in a method to quantify OTC in a premix.

The flow sheet of the analysis method is shown in Figure 9.2.4-3.

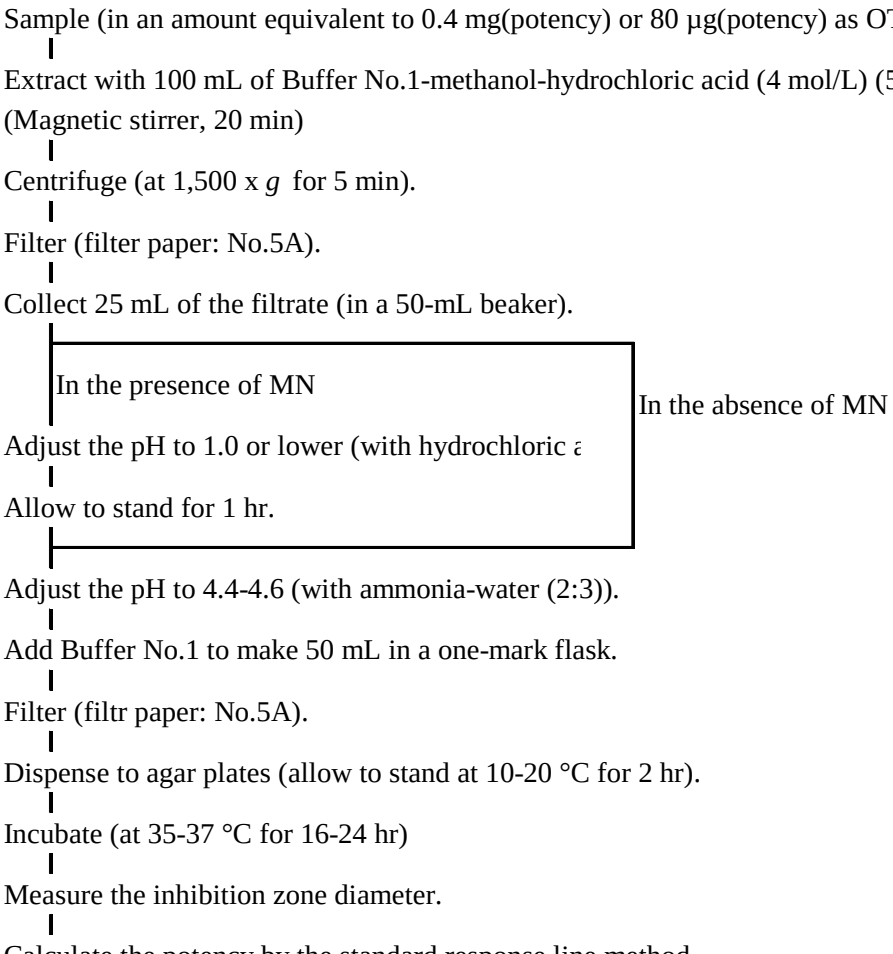


Figure 9.2.4-3 Quantitative test method for alkyl trimethyl ammonium calcium oxytetracycline and oxytetracycline hydrochloride (feed)

References: Kiyoshi Kanno: Research Report of Animal Feed, 6, 87 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Oxytetracycline hydrochloride	Starting chick formula feed	5-100	6	98.3-103.7	3.3
	Sucking piglet formula feed	5-100	6	104.4-109.2	5.8
	Sucking calves formula feed	5-100	6	100.9-106.9	7.4

・Collaborative study

Name of spiked component	Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-labo reproducibility RSDR (%)
Oxytetracycline hydrochloride	Calf formula feed	3	50	106.3	4.5	14.3

«Notes and precautions»

[1] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in

Section 1 of this Chapter.

An example method of preparation for oxytetracycline standard solution is shown in Table 9.2.4-2.

Table 9.2.4-2 Method of preparation for oxytetracycline standard solution

1) Method of preparation for oxytetracycline standard solution (a feed with OTC content (potency) of not less than 20 g (potency)/t, example)

Test tube No.	1	2	3	4	5	6
Amount (mL) of standard solution	②	2	10	10	10	5
Amount (mL) of Buffer No.1-methanol (3:1)	23	18	10	10	10	5
Concentration (μg(potency)/mL)	80	8	4	< 2 >	1	0.5

Note: "②mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

2) Method of preparation for oxytetracycline standard solution (a feed with OTC content (potency) of less than 20 g (potency)/t, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	2	4	10	10	10	5
Amount (mL) of Buff No.1-methanol (3:1)	23	18	16	10	10	10	5
Concentration (μg(potency)/mL)	80	8	1.6	0.8	<0.4>	0.2	0.1

Note: "②mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

[2] The edge of the inhibition zone produced by test organism *Bacillus cereus* ATCC 11778 is poorly defined because it grows strongly on the surface of the culture medium. This procedure reduces the superficial growth of the test organism, resulting in a clear-cut inhibition zone.

Alternatively, the superficial growth can also be reduced by incubating at 30°C as directed in the single-layered agar well method using 20 mL of Medium F-17. In this case, the amount of spore suspension to be added to the culture medium should be about 20 times the specified amount.

[3] Corresponds to a mixture of Buffer No.1, methanol, water, and hydrochloric acid (1,000:980:13:7).

[4] To facilitate the pH adjustment, it is advisable to add 5 to 10 mL of Buffer No.1 to 25 mL of the filtrate in advance.

[5] MN and SL have antibacterial effects on test organism *Bacillus cereus* ATCC 11778, the organism used for the quantification of OTC.

A study on feeds containing OTC and MN (80 g (potency)/t) or SL (50 g (potency)/t) has revealed that the presence of MN affects the quantified results of OTC in feeds containing OTC at a concentration of 20 g (potency)/t or lower. To remove this effect, treat the sample solution with hydrochloric acid when the analysis sample contains MN in combination with OTC. Refer to Monograph 1 Zinc bacitracin or manganese, 1.1, «Notes and precautions» [8] of this Section (p.****).

[6] Examples of the standard response line for OTC are shown in Figures 9.2.4-4 and Figure 9.2.4-5.

Liniarity is observed in the quantification range of OTC (between 0.5 and 8 μg (potency)/mL for Figure 9.2.4-4 and between 0.1 and 1.6 μg (potency)/mL for Figure 9.2.4-5).

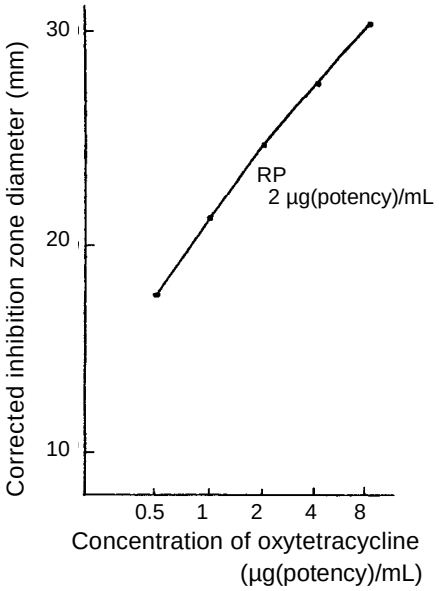


Figure 9.2.4-4 Standard response line for oxytetracycline (feed with OTC content of not less than 20 g (potency)/t, example)
(*Bacillus cereus* ATCC 11778,
Medium F-17, Agar well method)

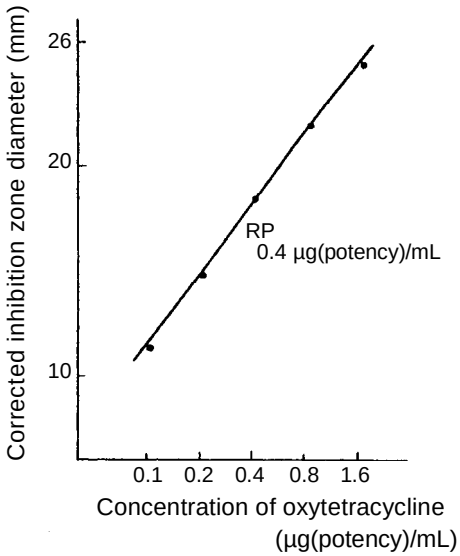


Figure 9.2.4-5 Standard response line for oxytetracycline (feed with OTC content of less than 20 g (potency)/t, example)
(*Bacillus cereus* ATCC 11778,
Medium F-17, Agar well method)

[7] Refer to «Notes and precautions» [53] to [57] and [61] in Secton 1, 1 of this Chapter.

2 Liquid chromatography [Feed Analysis Standards Chapter 9, Section 2, 4.2.2]

Scope of application: Feeds containing not less than 10 g (potency)/t of OTC

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Extracting solvent: A mixture of Buffer No.1, methanol, and hydrochloric acid (4 mol/L) (50:49:1)

3) Oxytetracycline standard solution. Weigh accurately not less than 40 mg of oxytetracycline working standard, accurately add hydrochloric acid (0.01 mol/L) and dissolve to prepare an oxytetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of standard stock solution with the extracting solvent so that each mL contains an amount equivalent to 0.1 to 5.0 μg (potency) as oxytetracycline to prepare several oxytetracycline standard solutions.

B. Quantification

Extraction. Weigh accurately 4 to 5 g of the analysis sample (equivalent to not more than 0.4 mg (potency) as OTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,000×*g* for 5 minutes, filter the supernatant liquid through a membrane filter (pore size of not exceeinng 0.5 μm), and use as a sample solution subject to liquid chromatography.

Liquid chromatography. Inject 20 μL each of the sample solution and oxytetracycline standard solutions into a liquid chromatograph to obtain chromatograms.

Operating conditions (example)

Detector: Fluorescent detector (excitation wave length: 380 nm; emission wavelength: 520 nm)

Column: Octadecylsilanized silica gel column (4.6 mm in internal diameter, 250 mm in length, 5 μm in particle size)[Note 1]

Eluent: A mixture of imidazole buffer[Note 2] and methanol (77:23)

Flow rate: 0.8 mL/min

Column oven temperature: 40°C

Calculation. Calculate the peak height or peak area from the obtained chromatogram[1] to prepare a calibration curve, and calculate the amount of oxytetracycline in the sample as the amount of alkyl trimethyl ammonium calcium oxytetracycline.

Note 1 Shim-pack VP-ODS (Shimadzu Corporation) or an equivalent column[2]

2 Weigh 68.08 g of imidazole, 10.72 g of magnesium acetate tetrahydrate, and 0.37 g of disodium dihydrogen ethylenediaminetetraacetic acid, dissolve in 750 mL of water, add about 25 mL of acetic acid to adjust the pH to 7.1 to 7.3, and add water to make 1,000 mL.

«Summary of analysis method»

This method is intended to determine the amount of OTC in a feed by extracting with a mixture of Buffer No.1, methanol, and hydrochloric acid (4 mol/L) (50:49:1), eluting the extract through a liquid chromatograph, separating the fluorescent derivative(s) produced by chelation of magnesium ion with OTC from the eluate using an octadecylsilanized silica gel (ODS) column, and detecting with a fluorescent detector.

The application of this method to a feed with OTC content of 5 g (potency)/t results in a decrease in recovery. Based on this, the scope of application of this method is restricted to feeds with OTC content of not less than 10 g (potency)/t.

The flow sheet of the analysis method is shown in Figure 9.2.4-6.

4-5 g of sample (not more than the amount equivalent ot 0.4 mg potency as OTC)
↓
Add 100 mL of extracting solvent and extract with stirring for 2
↓
Centrifuge (at 1,000×*g* for 5 min).
↓
Filter through membrane filter (pore size: not exceeding 0.5 μm
↓
LC-FL (Ex: 380 nm, Em: 520 nm)

Figure 9.2.4-6 Liquid chromatography method for alkyl trimethyl ammonium calcium oxytetracycline
(feed with OTC content of not less than 10 g (potency)/t)

References: Tetsuo Chihara, Tomotaro Yoshida, Tomoe Inoue: Research Report of Animal Feed, 29, 50 (2004)

History in the Feed Analysis Standards [27] New

«Validation of analysis method»

・Spike recovery and repeatability

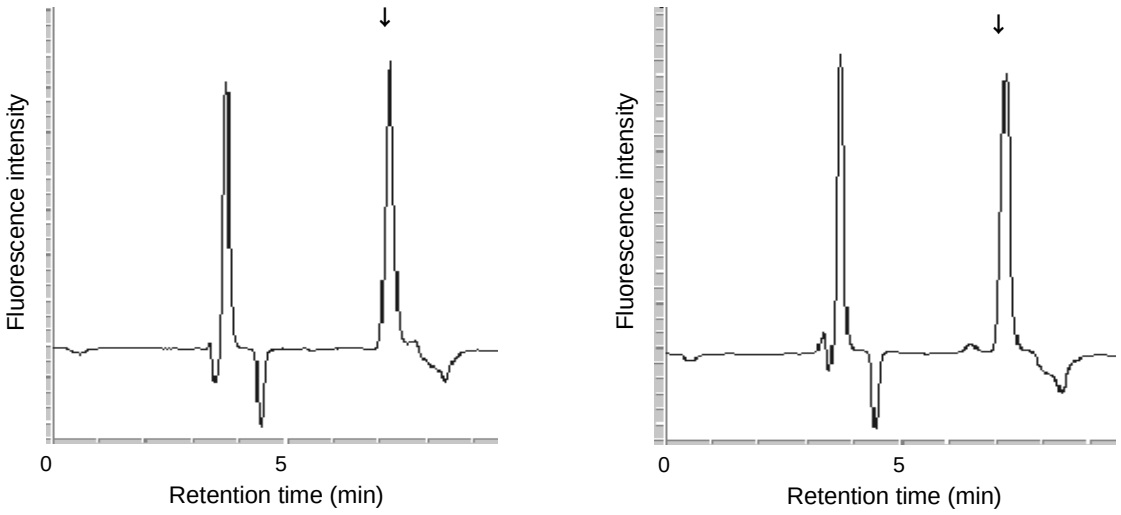
Name of spiked component	Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Alkyl trimethyl ammonium calcium oxytetracycline	Formula feed for mid-term chicks	10~100	3	95.9~100.7	2.1
	Formula feed for lactating piglets	10~100	3	96.6~103.7	3.5
	Formula feed for lactating calves	10~100	3	93.3~100.9	3.9

・Collaborative study

Name of spiked component	Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr（%）	Inter-lab reproducibility RSDR (%)
Alkyl trimethyl ammonium calcium oxytetracycline	Mid-term chick formula feed	7	50	95.6	1.3	3.9

«Notes and precautions»

[1] Example LC chromatograms obtained from OTC standard solution and spiked sample are shown in Figure 9.2.4-7.



OTC standard solution (equivalent to 50 ng (potency))

Formula feed for mid-term chicks (50 g (potency)/t)

Figure 9.2.4-7　Chromatograms for OTC

(The arrow indicates the peak of an OTC derivative.)

[2] As OTC has a strong tendency to bind to irons, tailing of peaks is likely to result from residual silanol groups remaining after end-capping of packing material and from traces of metals contained in the silica gel, although depending on the types of ODS columns. It is therefore necessary to confirm the absence of such effects by performing a trial run using the ODS column to be used. Additionally, the column used in the validation of this method was Shim-pack VP-ODS.