Antioxidant

[Summary of antioxidant]

Oxygen in air accelerates oxidation, transformation and decomposition of several feed ingredients such as animal fat and oil, fishmeal and vitamin A and carotene contained in feed, which may cause loss of protein and energy or deterioration of feed taste and quality. In addition, a problem arises that generated lipid peroxide may poison livestock.

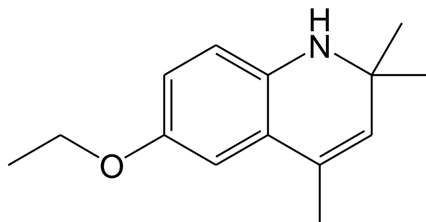
These problems on oxidation are accelerated at high temperature. Significant oxidation generates heat, which may cause spontaneous combustion for fish meal. A substance which prevents or reduces oxidation is called antioxidant. Three chemical substances, ethoxyquin, dibutylhydroxytoluene (BHT) and butylhydroxyanisol (BHA) are officially designated as the feed additives.

Actually, to protect fish meal against combustion during shipment (the products are likely to become high temperature because they are shipped from the production regions in the Southern Hemisphere through the equator), ethoxyquin is definitely added to the products in accordance with the international conventions.

These three types of antioxidant can be added to any feed. However, the amount contained in the feed (sum of three additives) is regulated to be less than 150 g/t to the final feed (feed, such as formula feed, that is directly supplied to livestock). Therefore, sum of the amount added to the final feed and the amount added to the ingredients such as fish meal must be less than 150 g/t.

Although the amount for the feed ingredients such as fish meal is not regulated, the amount of the sum of three antioxidants must be indicated in %. (This indication is not required for the final feed.)

1 Ethoxyquin



6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline

$C_{14}H_{19}NO$ MW: 217.31 CAS No.: 91-53-2

[Summary of ethoxyquin]

Ethoxyquin has an antioxidation action. This substance was designated as the feed additive in 1981 in order to prevent oxidation of animal fat and oil and fat in fish meal and has been used since then. Although ethoxyquin can be added to these ingredients without limitation, indication of the added amount is mandated.

For other details, see [Summary of antioxidant].

«Maximum Residue Limits in the Law Concerning Safety Assurance and Quality Improvement of Feeds» [Ordinance of the Standard of Feed and Feed Additives]

Contained amount (sum of Ethoxyquin, dibutylhydroxytoluene and dibutylhydroxytoluene) in feed (excluding ingredients and materials used to produce feed): 150 g/t or less

[Method listed in the Analytical Standards of Feeds]

1. Liquid chromatography (for formula feed and fish meal) [Analytical Standards of Feeds, Chapter 13, Article 1-(1)]

A. Reagent preparation

Ethoxyquin standard solution. Weigh accurately 50mg of ethoxyquin [$C_{14}H_{19}NO$], place in a 100mL brown volumetric flask, add acetonitrile and dissolve it. Add this solvent to the marked line to prepare ethoxyquin standard stock solution (1 mL of this solution contains 0.5 mg of ethoxyquin).

In use, dilute fixed quantities of this standard stock solution with methanol to prepare several ethoxyquin standard solutions containing amounts equivalent to 0.1 – 6 μg/mL of ethoxyquin.

B. Quantification

Extraction. Weigh 10.0 g of analysis sample and put it in a stoppered 200 mL brown Erlenmeyer flask. Add 100 mL of methanol to this flask, stir the content for 15 minutes, and leave the content for 3 minutes. Pour 5 mL of extract accurately into a 50 mL brown volumetric flask, add methanol up to the marked line, filter the solution through a membrane filter (pore size: 0.5 μm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography[1]. Inject 20 μL of sample solution and respective ethoxyquin standard solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions[2]

Detector: Fluorescence detector (extitation wavelength: 370nm, emission wavelength: 415nm)[3]

Column: Octadecylsilylated silica gel column[4] (internal diameter 3.9 mm, length 250 mm, particle size 5 μm)[*1]

Eluent: Dissolve 50 mg of dibutylhydroxytoluene to acetonitrile – water (4 : 1), adjust to 1 L[5].

Flow rate: 0.6 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram[6] and prepare a calibration curve and subsequently calculate the amount of ethoxyquin in the sample.

* 1. Puresil 5μC18 120 Å (Waters) or equivalent

«Summary of analysis method»

This method is to determine ethoxyquin in formula feed and fish meal by extracting with methanol and quantifying by a liquid chromatograph equipped with a fluorescence detector.

References: Toshiaki Hayakawa, Mayumi Isa: Reseach Report of Animal Feed, 20, 94 (1995)
Eiichi Ishikuro: Livestock Research, 37, 491 (1983)
Hisaaki Hiraoka, Rie Fukunaka: Research Report of Animal Feed, 31, 139 (2006)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for growing pig	50-300	3	96.2-97.6	2.8
Formula feed for carp	50-300	3	93.6-96.8	3.9
Formula feed for young yellowtail	50-300	3	91.8-95.5	3.2
65 % fish meal	50-300	3	99.5-109.0	7.3
Fish scrap meal	50-300	3	98.7-109.6	3.5

• Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Formula feed for trout	6	100	101.3	2.4	3.8	0.47

• Limit of determination: 1 mg/kg in sample

«Notes and precautions»

[1] As being measured with a fluorescence detector, rapid temperature fluctuation influences the fluorescence intensity. Measurement in a laboratory at constant temperature is preferable.

[2] Under this measurement condition, gradually reducing peak intensity of ethoxyquin may be found when analyzing many samples. To solve this problem, you may take either measure as below:

1) Changing eluent: In the section of eluent in measurement condition examples, change “water” to “10 mmol/L acetic acid solution” (dissolve 0.5 mL of acetic acid with 1 L of water) .

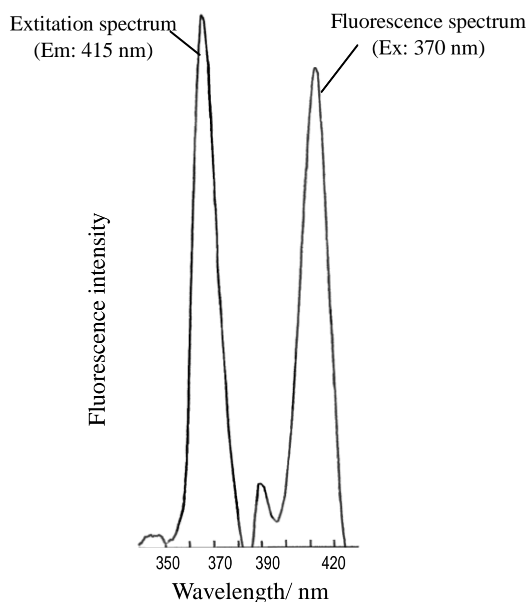
2) Preconditioning:

a) Wash the column and path with 10 mmol/L acetic acid solution from the previous day of analysis to 1 hour before starting analysis (at flow rate of 0.2 mL/min).

b) After washing the column and path for about 30 minutes one hour before starting measurement (at flow rate of 1.0 mL/min), flow the eluent for 30 minutes (at flow rate of 0.6 mL/min).

c) Immediately start measurement. Be sure to set measurement time within 3 hours.

[3] Figure 13.1.1-1 shows ethoxyquin excitation/fluorescence spectrum.



Measurement conditions;

Manufactured by Hitachi Ltd.
Fluorescence spectrophotometer Model 650-10

Ex. slit: 10 nm

Em. slit: 10 nm

Scan speed: 60 nm/min

Chart speed: 8 mm/min

Solvent: Acetonitrile – water (9 : 1)

Figure 13.1-1 Ethoxyquin excitation/fluorescence spectrum

[4] Any column is applicable as long as its end-capped packing material meets the requirements. The column used when developing and examining this analysis method is Puresil 5μC18 120Å.

[5] To quantify 10 ng or less of ethoxyquin, be sure to add dibutylhydroxytoluene to the eluent. Otherwise, effective reproducibility cannot be obtained due to the influence of oxygen dissolving in the eluent.

[6] Figure 13.1.1-2 shows an example of chromatogram

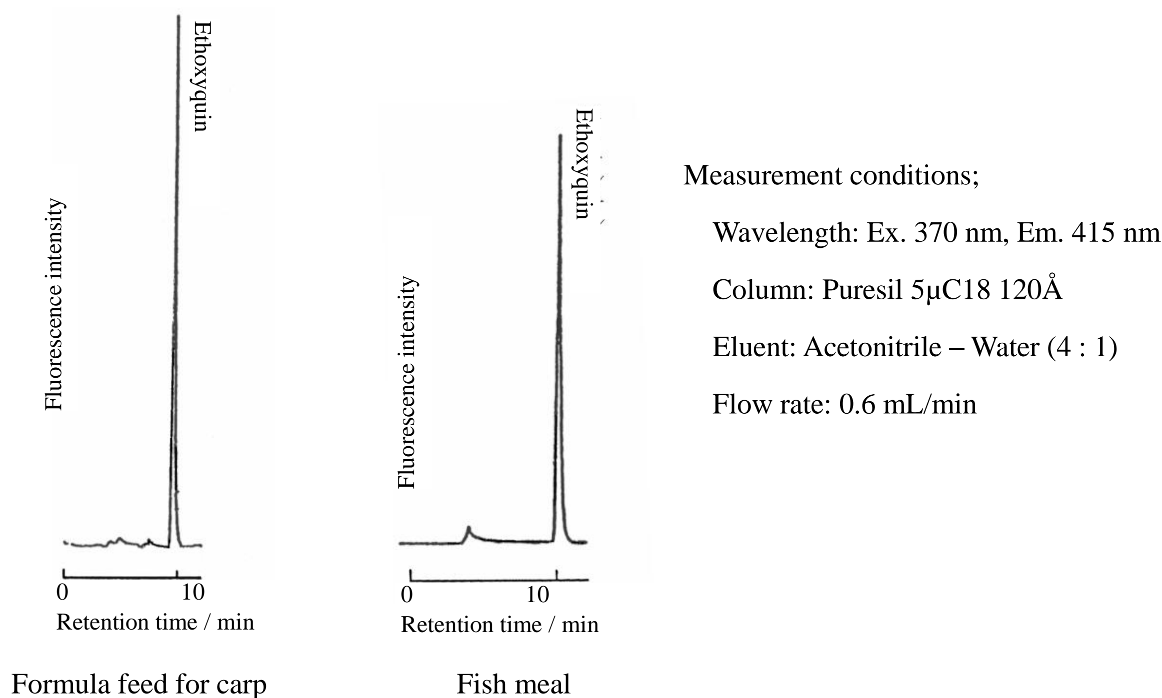


Figure 13.1-2. Chromatogram of Ethoxyguin in formula feed and fish meal

2. Liquid chromatography (for fat and oil 1) [Analytical Standards of Feeds, Chapter 13, Article 1-(2)]

A. Reagent preparation

Ethoxyquin standard solution. Weigh accurately 50 mg of ethoxyquin [$C_{14}H_{19}NO$], place in a 100 mL brown volumetric flask, add acetonitrile and dissolve it. Add this solvent to marked line to prepare ethoxyquin standard stock solution. (1 mL of this solution contains 0.5 mg of ethoxyquin).

In use, dilute fixed quantities of the standard stock solution with acetonitrile to prepare several ethoxyquin standard solutions containing amounts equivalent to 2 – 8 μg/mL of ethoxyquin.

B. Quantification

Extraction. Weigh 5 g of analysis sample accurately and put it in a 200 mL brown round-bottom flask. Add 0.5 g of pyrogallol[1] and 100 mL of potassium hydroxide solution in ethanol (5 w/v%) there. Connect a reflux condenser to this round-bottom flask and heat it for 30 minutes in a water bath at 80 °C. Saponify the fat and then leave the solution in the flask to cool it down.

Transfer the cooled solution into 500 mL brown separating funnel A. Wash the round-bottom flask three times each with 20 mL of water and add the washings to the separating funnel A. Then, add 100 mL of hexane to the separating funnel A, shake it for 10 minutes and leave to stand.

Transfer the water layer (lower layer) into 500 mL brown separating funnel B. Add 100 mL of hexane to the separating funnel B and repeat the same operation. Add hexane layer (upper layer) to the separating funnel A and wash it 3 times each with 50 mL of water. Transfer the hexane layer into a Erlenmeyer flask and dehydrate the content with an appropriate amount of sodium sulfate (anhydrous). Then, filter the solution through a filter paper (No. 5A) into a 300 mL brown recovery flask. Wash the

Erlenmeyer flask and the filter paper with a small amount of hexane sequentially. Filter the washings in the similar way.

Concentrate the filtrate under reduced pressure to 2 ~ 3 mL in a water bath at 40 °C, further dry up by the flow of nitrogen gas. Add acetonitrile to dissolve residues and transfer this solution into a 100 mL brown volumetric flask. Wash the recovery flask with a small amount of acetonitrile, add the washings to the volumetric flask and add acetonitrile up to the marked line. Filter this solution through a membrane filter (pore size: 0.5 μm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography[2]. Inject 20 μL of sample solution and respective ethoxyquin standard solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions

Detector: Fluorescence detector (Excitation wavelength: 370 nm, emission wavelength: 415nm)[3]

Column: Octadecylsilylated silica gel column (internal diameter 4.6 mm, length 150 mm, particle size 5μm)*1 [4]

Eluent: Acetonitrile – water (9 : 1)

Flow rate: 0.5 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram[5] to prepare a calibration curve and subsequently calculate the amount of ethoxyquin in the sample.

* 1. Shodex ODS pak F-411 (Showa Denko) or equivalent

«Summary of analysis method»

This method is to determine ethoxyquin in animal fat and oil, such as tallow and yellow grease by saponifying the sample with 5 % potassium hydroxide solution in ethanol, extracting ethoxyquin with hexane, purifying through liquid-liquid extraction and quantifying by a liquid chromatograph equipped with a fluorescence detector.

References: Eiichi Ishikuro, Kazuo Izumi: Research Report of Animal Feed, 8, 17 (1983)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Tallow	100-2,000	4	92.4-93.5	4.6
Yellow grease	100-2,000	4	90.9-96.1	5.6

«Notes and precautions»

[1] To protect samples against oxidation.

[2] Measurement in a room exposed to drastic temperature fluctuation will influence fluorescence intensity. To solve this problem, measurement in a constant temperature room is preferable. Some fluorescence detectors require 90 minutes until the lamp becomes stabilized. Thus, be sure to check the stabilization before starting measurement.

[3] See **«Notes and precautions»** [2] in Article 1.

[4] Any column is applicable as long as its end-capped packing material meets the requirements. The column used when developing and examining this analysis method is Shodex ODS pak F-411.

[5] Figure 13.1-3 shows an example of chromatogram.

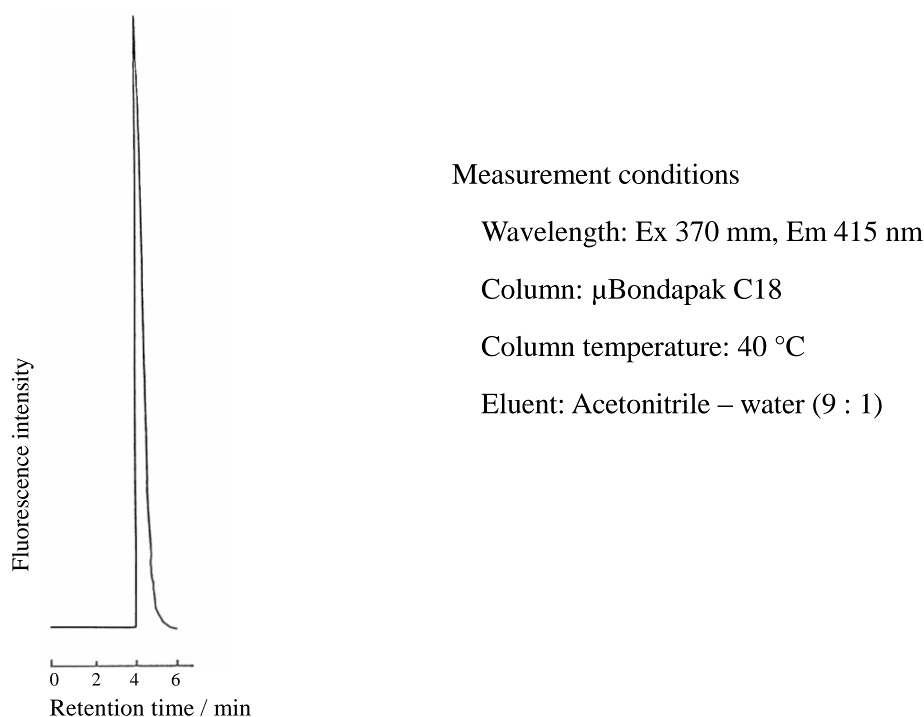


Figure 13.1-3. Chromatogram of ethoxyquin in yellow grease

3. Liquid chromatography (for fat and oil 2) [Analytical Standards of Feeds, Chapter 13, Article 1-(3)]

A. Reagent preparation

Ethoxyquin standard solution. Weigh accurately 50 mg of ethoxyquin [$C_{14}H_{19}NO$], place in a 100 mL brown volumetric flask, add acetonitrile and dissolve it. Then adjust it with water to the marked line to prepare ethoxyquin standard stock solution (1 mL of the solution contains 0.5 mg of ethoxyquin).

In use, dilute fixed quantities of the standard stock solution with acetonitrile – water (4 : 1) to prepare ethoxyquin standard solutions containing amounts equivalent to 0.1 – 8 μg/mL of ethoxyquin.

B. Quantification

Extraction. Weigh 10.0 g of analysis sample, pour it into a stoppered 200 mL brown Erlenmeyer flask and add 100 mL of acetonitrile to the flask. Shake the flask for 30 minutes. Transfer the extract into a 50 mL brown centrifuge tube and centrifuge it for 5 minutes with 1,000×*g*. Transfer 5 mL of supernatant solution accurately into a 100 mL brown volumetric flask and add acetonitrile – water (4 : 1) up to the marked line. Filter the solution through a membrane filter (pore size: 0.5 μm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography[1]. Inject 20 μL of sample solution and respective ethoxyquin standard solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions[2]

Detector: Fluorescence detector (Excitation wavelength: 370 nm, emission wavelength: 415 nm)[3]

Column: Octadecylsilylated silica gel column (internal diameter 4.6 mm, length 250 mm, particle size 5 μm)[*1] [4]

Eluent: Dissolve 50 mg of dibutylhydroxytoluene with acetonitrile – water (4 : 1) to make 1 L of solution in total. (Prepare the solution when using it.)

Flow rate: 0.6 mL/min

Column temperature :40 °C

Calculation. Calculate the peak height or peak area from obtained chromatogram[5] to prepare a calibration curve and subsequently calculate the amount of ethoxyquin in the sample.

* 1. Shodex C18M4E (Showa Denko) or equivalent

«Summary of analysis method»

Although the method to determine ethoxyquin in fat and oil is also designated in Article 2 of this document, the operations in the method are complicated. Thus, the method to determine ethoxyquin in formula feed and fish meal in Article 1 is also applied to fat and oil.

Figure 13.1-4 shows a flow sheet of analysis.

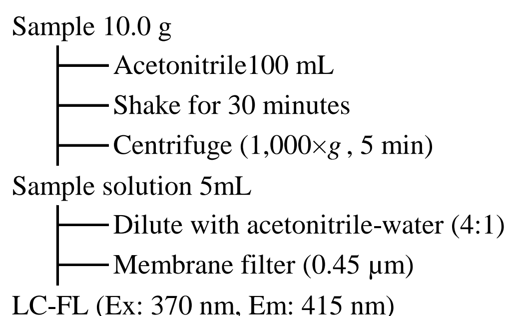


Figure 13.1-4. Flowsheet of analysis method for ethoxyquin

References: Mayumi Nishimura; Research Report of Animal Feed, 29, 68 (2004)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Chicken oil	250-1,000	3	90.4-91.2	0.7
Collected edible oil	250-1,000	3	89.5-91.5	0.4
Yellow grease	250-1,000	3	90.3-92.6	0.4

• Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Collected edible oil	6	500	95.4	1.6	4.0	0.63

• Limit of determination: 1 mg/kg in sample

«Notes and precautions»

[1] Measurement in a room exposed to drastic temperature fluctuation will influence fluorescence intensity. To solve this problem, measurement in a constant temperature room is preferable. Some

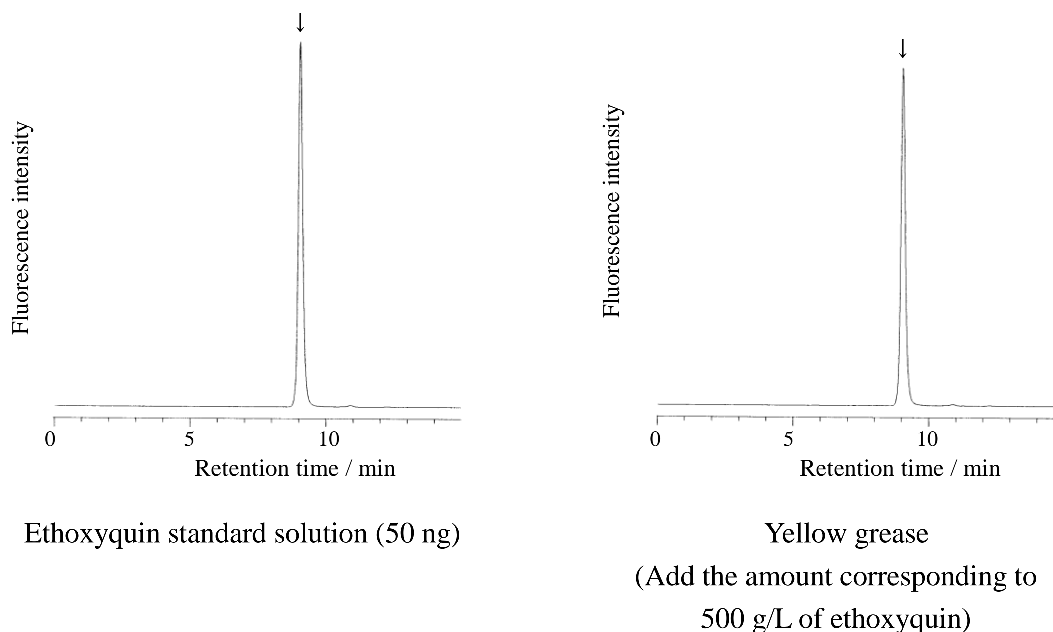
fluorescence detectors require 90 minutes until the lamp becomes stabilized. Thus, be sure to check the stabilization time required by your fluorescent detector before starting measurement.

[2] See **«Notes and precautions»** [2] in Article 1.

[3] See **«Notes and precautions»** [3] in Article 1.

[4] Any column is applicable as long as its end-capped packing material meets the requirements. The column used when developing and examining this analysis method is Shodex ODS pak F-411.

[5] Figure 13.1-5 shows an example of chromatogram.

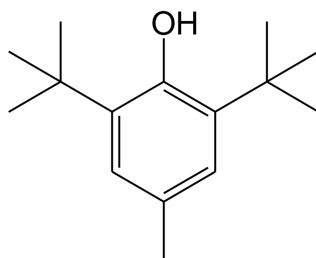


Ethoxyquin standard solution (50 ng)

Yellow grease
(Add the amount corresponding to
500 g/L of ethoxyquin)

Figure 13.1-5. Chromatogram of ethoxyquin
(Arrows indicate the peak of ethoxyquin.)

2 Dibutylhydroxytoluene (BHT)



2,6-bis(1,1-dimethylethyl)-4-methylphenol

$C_{15}H_{24}O$ MW: 220.35 CAS No.: 128-37-0

[Summary of dibutylhydroxytoluene]

Dibutylhydroxytoluene (BHT) was designated as the antioxidant xin 1981 and is added to fish meal and fat and oil.

«Maximum Residue Limits in the Law Concerning Safety Assurance and Quality Improvement of Feeds» [Ordinance of the Standard of Feed and Feed Additives]

Contained amount (sum of ethoxyquin, dibutylhydroxytoluene and butylhydroxyanisol) in feed (excluding ingredients and materials used to produce feed): 150 g/t or less

[Method listed in the Analytical Standards of Feeds]

1. Liquid chromatography (for formula feed and fish meal[1]) [Analytical Standards of Feeds, Chapter 13, Article 2-(1)]

A. Reagent preparation

1) Dibutylhydroxytoluene standard solution. Weigh accurately 25 mg of dibutylhydroxytoluene [$C_{15}H_{24}O$], place in a 250 mL brown volumetric flask, add methanol and dissolve it. Add this solvent to marked line to prepare dibutylhydroxytoluene standard stock solution. (1 mL of this solution contains 0.1 mg of dibutylhydroxytoluene).

   In use, dilute fixed quantities of the standard stock solution with methanol to prepare several dibutylhydroxytoluene standard solutions containing amounts equivalent to 2.5 – 15 mg/mL of dibutylhydroxytoluene.

2) Neutral alumina. Dry neutral alumina (with particle diameter of 63 ~ 200 µm (230 ~ 70 mesh))[*1] for column chromatography at 120 °C for 2 hours.

B. Quantification

Extraction. Measure 10.0 g of analysis sample, put it into a stoppered 200 mL brown Erlenmeyer flask and add 100 mL methanol to the flask. Stir the solution for 10 minutes and extract the content. Use the supernatant liquid of the extract as the sample solution to be provided for a column chromatography.

Column treatment. Fill 5 g of neutral alumina into a column tube[2] (with inner diameter of 7 mm) in a dry manner and prepare the column.

Pour the analysis sample into the column. Discharge the first 3 mL of effluent. Then, filter the

following 5 mL of effluent through a membrane filter[3] (pore size: 0.5 µm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography. Inject 20 µL of sample solution and respective dibutylhydroxytoluene standard solution into liquid chromatography to obtain chromatogram.

Example of measurement conditions

Detector: Ultraviolet spectrophotometer (Wavelength: 277 nm)[4]

Column: Octadecylsilylated silica gel column (internal diameter 4.6 mm, length 150 mm, particle size 5µm)*2 [5]

Eluent: Methanol – water (4 : 1)

Flow rate: 1.0 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram[6] to prepare a calibration curve and subsequently calculate the amount of dibutylhydroxytoluene in the sample.

* 1. Aluminiumoxid 90 aktiv neutral Art. 1077 (Merck) or equivalent

2. Shodex ODS pak F-411 (Showa Denko) or equivalent

«Summary of analysis method»

This method is to determine dibutylhydroxytoluene (BHT) in the sample by extracting with methanol, purifying with a neutral alumina column and quantifying by a liquid chromatograph equipped with an ultraviolet spectrophotometer.

References: Eiichi Ishikuro, Shuichi Shimada: Research Report of Animal Feed, 7, 20 (1981)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for finishing period broiler	50-100	4	100.4-100.9	1.7
Formula feed for piglet	50-100	4	99.4-99.5	2.1
Fish meal (3 types)	50	2 for each	98.3-9.6	1.8

• Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Fomula feed	5	50	104.1	8.1	2.0	0.92

• Limit of determination: 10 mg/kg in sample

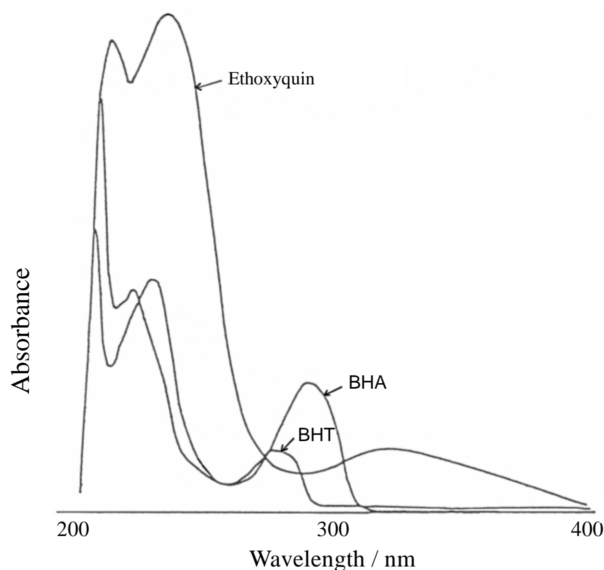
«Notes and precautions»

[1] This method can be applied to premix with a change of the sample amount or concentration of the standard solution, or dilution of the extracted solution, as required. In this case, BHT may be contained in the drug products such as vitamin A and vitamin D and it may be possibly extracted partially and added to the measured value as much as several mg/kg. However, the added amount is usually small enough to be ignored.

[2] A column with reservoir is useful.

[3] Made of polytetrafluoroethylene (PTFE) resin or equivalent.

[4] Figure 13.2-2 shows the absorption curve for BHT and other antioxidants



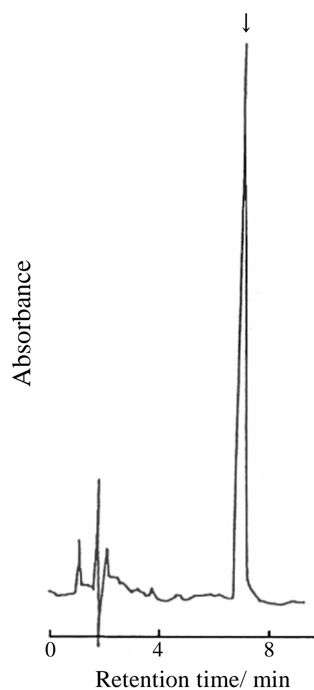
10 µg/mL methanol solution

Ref.: methanol

Figure 13.2-2. Absorption curve of antioxidants

[5] Any column is applicable as long as its end-capped packing material meets the requirements.

[6] Figure 13.2-1 show an example of chromatogram.



Measurement conditions;

Column: Shodex ODS pak F-411

Column temperature: 35 °C

Eluent:Methanol – water (4 : 1)

Flow rate: 1.0 mL/min

Detector : UV 277 nm

Sensitivity: 0.01 AUFS

Figure 13.2-1. Chromatogram of BHT in formula feed
(Arrow indicates the peak of BHT.)

2. Liquid chromatography (for fat and oil) [Analytical Standards of Feeds, Chapter 13, Article 2-(2)]

A. Reagent preparation

Dibutylhydroxytoluene standard solution[1]. Weigh accurately 25 mg of dibutylhydroxytoluene [$C_{15}H_{24}O$] in a 250 mg brown volumetric flask, add methanol and dissolve it. Add this solvent to marked line to prepare dibutylhydroxytoluene standard stock solution. (1 mL of this solution contains 0.1 mg of dibutylhydroxytoluene).

In use, dilute fixed quantities of the standard stock solution with methanol to prepare several dibutylhydroxytoluene standard solutions containing amounts equivalent to 2.5 – 15 mg/mL of dibutylhydroxytoluene.

B. Quantification

Extraction. Weigh 5 g of analysis sample accurately and dissolve with 10 mL of hexane in a 200 mL separating funnel. Add 50 mL of hexane saturated acetonitrile and 0.5 mL of acetic acid to the solution, shake the funnel and leave it. Transfer the acetonitrile layer (lower layer) into a 200 mL volumetric flask. Add 50 mL of hexane saturated acetonitrile and 0.5 mL of acetic acid to the residual solution and repeat the same operation twice. Add each acetonitrile layer to the volumetric flask above. Then, add acetronitrile up to the marked line[2] of the volumetric flask and filter the content through a membrane filter[3] (pore size: 0.5 µm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography. Inject 20 µL of sample solution and respective dibutylhydroxytoluene standard solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions

Detector: Ultraviolet spectrophotometer (wavelength: 277 nm)

Column: Octadecylsilylated silica gel column (internal diameter 4.6 mm, length 150 mm, particle size 5 µm)*1

Eluent: Methanol – water (4 : 1)

Flow rate: 1.0 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram to prepare a calibration curve and subsequently calculate the amount of dibutylhydroxytoluene in the sample.

* 1. Aluminiumoxid 90 aktiv neutral Art. 1077 (Merck) or equivalent

«Summary of analysis method»

This method is to determine BHT in fat and oil by dispersing sample homogeneously in a low polarity solvent (hexane), then extracting BHT to a medium polarity solvent (acetonitrile) and quantifying by a liquid chromatograph equipped with an ultraviolet spectrophotometer as in Article 1.

«Method validation»

• Limit of determination: 1 mg/kg in sample

«Notes and precautions»

[1] The range of concentration of the standard solution should be appropriately set with consideration of the concentration of dibutylhydroxytoluene in the sample.

[2] i) When concentration of dibutylhydroxytoluene in the sample is about 30 mg/kg or less

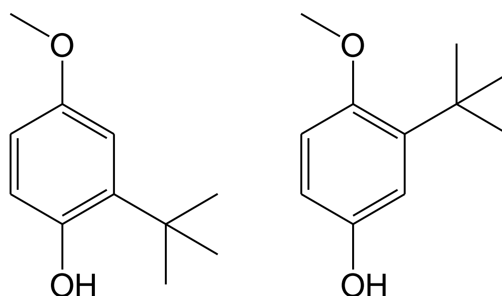
Transfer the lower layer (sample extract) into a 200 mL round-bottom flask and concentrate the extract under reduced pressure to 2 ~ 3 mL in a water bath at 50 °C or less. Move the content to a 25 mL volumetric flask, add methanol up to the marked line. Filter the solution through a membrane filter to obtain the sample solution subjected to liquid chromatography.

ii) When concentration of dibutylhydroxytoluene in the sample is about 500 mg/kg or more

Transfer the lower layer (sample extract) into a 200 mL volumetric flask, add acetonitrile up to the marked line. Dilute the solution appropriately and filter it through a membrane filter to obtain the sample solution subjected to liquid chromatography.

[3] Made of polytetrafluoroethylene (PTFE) resin or equivalent.

3 Butylhydroxyanisol (BHA)



2-tert-butyl-4-methoxyfenol; 3-tert-butyl-4-methoxyfenol

$C_{11}H_{16}O_2$ MW: 180.24 CAS No.: 25013-16-5 (mixture),

88-32-4 (2-tert-butyl-4-hydroxyanisole), 121-00-6 (3-tert-butyl-4-hydroxyanisole)

[Summary of butylhydroxyanisol]

Butylhydroxyanisol (BHA) was designated as the feed additive in 1981 and is added to fish meal and fat.

BHA is a mixture of 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole.

As both ingredients have the similar properties, they are hard to be separated. Even in the chromatogram for quantification explained in this document, the peaks of the two ingredients are not separated. Thus, BHA is quantified as a total amount.

«Maximum Residue Limits in the Law Concerning Safety Assurance and Quality Improvement of Feeds» [Ordinance of the Standard of Feed and Feed Additives]

Contained amount (sum of ethoxyquin, dibutylhydroxytoluene and butylhydroxyanisol) in feed (excluding ingredients and materials used to produce feed): 150 g/t or less

[Method listed in the Analytical Standards of Feeds]

1. Liquid chromatography (for formula feed and fish meal[1]) [Analytical Standards of Feeds, Chapter 13, Article 3-(1)]

A. Reagent preparation

1) Butylhydroxyanisol standard solution. Weigh accurately 25 mg of butylhydroxyanisol [$C_{11}H_{16}O_2$], place in a 250 mL brown volumetric flask correctly, add methanol and dissolve it. Then adjust it with this solvent to the marked line to prepare butylhydroxyanisol standard stock solution (1 mL of this solution contains 0.1 mg of butylhydroxyanisol).

   In use, dilute fixed quantities of this standard stock solution with methanol to prepare several butylhydroxyanisol standard solutions containing amounts equivalent to 2.5 – 15 µg/mL of butylhydroxyanisol.

2) Neutral alumina[2]. Dry neutral alumina (with particle diameter of 63 ~ 200 µm (230 ~ 70 mesh)) for column chromatography at 120 °C for 2 hours.

B. Quantification

Extraction. Weigh 10.0 g of analysis sample, put it into a stoppered 200 mL brown Erlenmeyer flask. Add 100 mL of methanol and stir for 10 minutes. Use the supernatant of the extract as the sample

solution to be provided for a column treatment.

Column treatment. Fill 5 g of neutral alumina into a column tube (with inner diameter of 7 mm) in a dry manner and prepare the column.

Place the sample solution into the column. Discharge the first 3 mL of effluent. Then, filter the following 5 mL of effluent through a membrane filter (pore size: 0.5 µm or less) to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography. Inject 20 µL of sample solution and respective butylhydroxyanisol standard solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions

Detector: Ultraviolet spectrophotometer (Wavelength: 290 nm)[3]

Column: Octadecylsilylated silica gel column (internal diameter4.6 mm, length 150 mm, particle size 5 µm)*1 [4]

Eluent: Methanol – water (3 : 2)

Flow rate: 0.8 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram[5] to prepare a calibration curve and subsequently calculate the amount of butylhydroxyanisol in the sample.

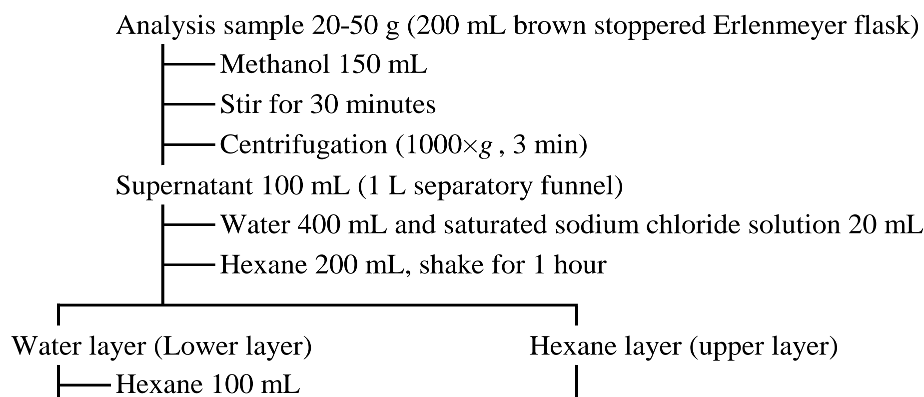
* 1. Shodex ODS pak F-411 (Showa Denko) or equivalent

«Summary of analysis method»

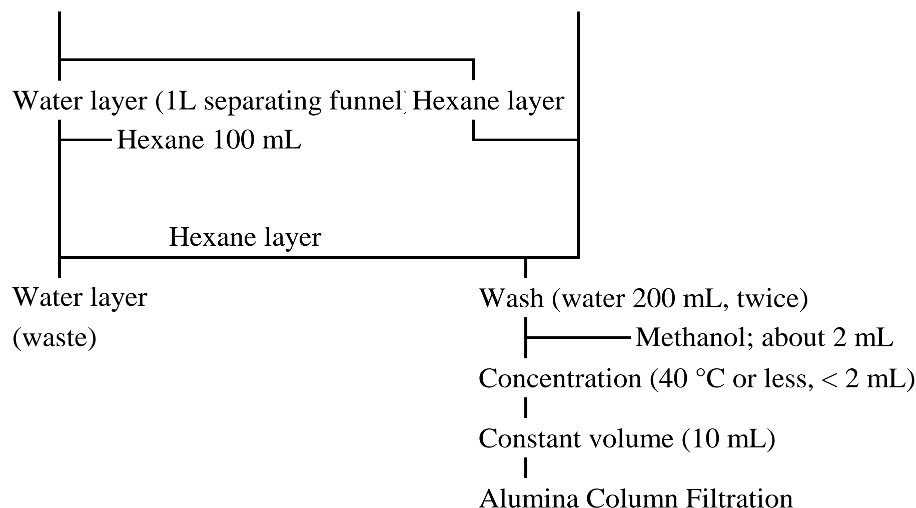
This method is to determine butylhydroxyanisol (BHA) in the sample by extracting with methanol, purifying with a neutral alumina column and quantifying by a liquid chromatograph equipped with an ultraviolet spectrophotometer.

To quantify BHA and BHT simultaneously, extraction and purification with the column are in accordance with the quantification method for BHA. Use methanol – water (39 : 11) as the eluent to be used in the liquid chromatography and set the measurement wavelength to 277 nm. However, this method shows slight deterioration of sensitivity for BHT.

Figure 13.3-1 shows the flowsheet in case the amount of BHT or BHA is low (BHT 10 mg/kg or less, BHA 5mg/kg or less).



To be continued to the next page



Following procedure is same as 2-1-B or 3-1-B in this chapter.

Calculation.

$$\text{BHT (or BHA) (mg/kg)} = \frac{3\times B}{4\times W}$$

B : Amount of BHT (or BHA) obtained from the calibration curve (ng)

W : Weight of sample used for analysis (g)

Figure 13.3-1 Analysis flow chart of small amount of BHT or BHA
(BHT 10mg/kg or less, BHA 5mg/kg or less)

References: Shuichi Shimada: Research Report of Animal Feed, 7, 28 (1981)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for finishing period broiler	50-100	4	102.0-102.4	0.8
Formula feed for piglet	50-100	4	101.0-102.5	0.4
Fish meal (3 types)	50	2 for each	99.2-02.3	2.6

• Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Formula feed	5	50	103.2	7.4	2.0	0.83

• Limit of determination: 5 mg/kg in sample

«Notes and precautions»

[1] This method can be applied to premix with a change of the sample amount or concentration of the standard solution, or dilution of the extracted solution, as required. In this case, BHA may be contained in the drug products such as vitamin A and vitamin D and it may be possibly extracted partially and added to the measured value as much as several mg/kg. However, the added amount is usually small enough to be ignored.

[2] This is the same preparation method as 2), A, Article 1 of “2 Dibutylhydroxytoluene”.

[3] See **«Notes and precautions»** [4], Article 1 of “2 Dibutylhydroxytoluene”.

[4] See **«Notes and precautions»** [5], Article 1 of “2 Dibutylhydroxytoluene”.

[5] Figure 13.3-2 shows an example of chromatogram.

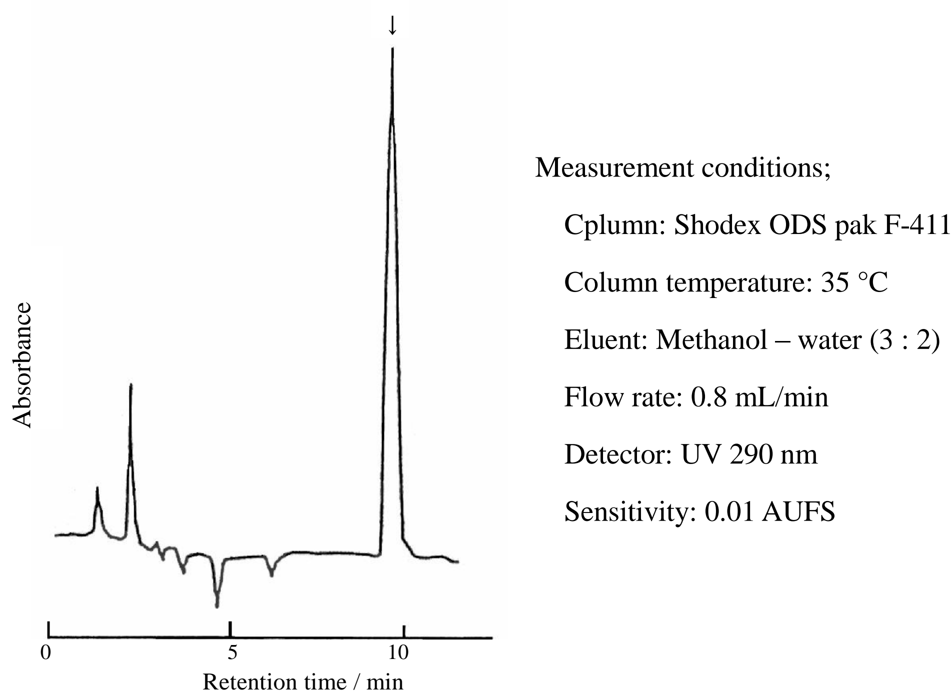


Figure 13.3-2. Chromatogram of BHA

2. Liquid chromatography (for fat and oil) [Analytical Standards of Feeds, Chapter 13, Article 3-(2)]

A. Reagent preparation

Butylhydroxyanisol standard solution. Weigh accurately 25 mg of butylhydroxyanisol [$C_{11}H_{16}O_2$], place in a 250 mL brown volumetric flask, add methanol and dissolve it. Add this solvent to the marked line to prepare butylhydroxyanisol standard stock solution (1 mL of this solution contains 0.1 mg of butylhydroxyanisol).

In use, dilute fixed quantities of this standard stock solution with methanol to prepare several butylhydroxyanisol standard solutions containing amounts equivalent to 2.5 – 15 μg/mL of butylhydroxyanisol.

B. Quantification

Extraction. Weigh 5 g of analysis sample accurately and dissolve with 10 mL of hexane in a 200 mL separating funnel. Add 50 mL of hexane saturated acetonitrile and 0.5 mL of acetic acid to the solution, shake the funnel and leave it. Pour the acetonitrile layer (lower layer) into a 200 mL volumetric flask. Add 50 mL of hexane saturated acetonitrile and 0.5 mL of acetic acid to the residual solution and repeat the same operation twice. Add each acetonitrile layer to the volumetric flask above. Then, add acetronitrile up to the marked line of the volumetric flask and filter the content through a membrane filter (pore size: 0.5 μm or less). to obtain the sample solution subjected to liquid chromatography.

Liquid chromatography. Inject 20 μL of sample solution and respective butylhydroxyanisol standard

solution into liquid chromatograph to obtain chromatogram.

Example of measurement conditions

Detector: Ultraviolet spectrophotometer (wavelength: 290 nm)

Column: Octadecylsilylated silica gel column (internal diameter 4.6 mm, length 150 mm, particle size 5 µm)[*1]

Eluent: Methanol – water (3 : 2)

Flow rate: 0.8 mL/min

Calculation. Calculate the peak height or peak area from obtained chromatogram to prepare a calibration curve and subsequently calculate the amount of butylhydroxyanisol in the sample.

* 1. Shodex ODS pak F-411 (Showa Denko) or equivalent

«Summary of analysis method»

This method is to determine butylhydroxyanisol (BHA) in fat and oil by dispersing the sample homogeneously in a low polarity solvent (hexane), then extracting BHA to a medium polarity solvent (acetonitrile) and quantifying by a liquid chromatograph equipped with an ultraviolet spectrophotometer as in Article 2 of “2 Dibutylhydroxytoluene”.

«Method validation»

• Limit of determination: 0.5 mg/kg in sample