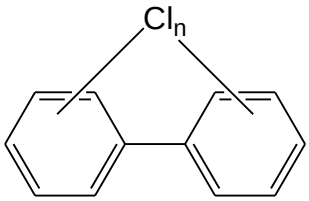
Harmful substances

[Summary of harmful substances]

It is difficult to define the range of harmful substances contained in feed. As specific chapters are already dedicated to harmful substances generally referenced in the context of feed including mycotoxins as represented by aflatoxin, heavy metals including arsenic, pesticide residue and residual antibiotics, hydrogen cyanide, histamine and other harmful substances are picked up within this chapter.

1 PCB

Polychlorinated biphenyl



[Summary of PCB]

PCB is an abbreviation standing for Polychlorinated biphenyl, a form of organic compound.

In March 1968, series of unexplained intoxication occurred in broiler and layer in Kyushu, Chugoku and Shikoku areas. The damage exceeded 2 million of chickens with symptoms of dyspnea, edema, lack of vitality and comatose. The symptoms were especially severe in chicks with many deaths. Although death rate among the layer were not as significant, decline in egg-laying rate was evident. Upon testing, the incident was ascertained to be poisoning caused by formula feed containing acidulated soap stock manufactured by an oil company in Kita-Kyushu. By mid-October, the onset of the ‘yusho’ expanded to humans, affecting the people – mostly in Fukuoka prefecture – subjected to rice bran oil manufactured by the same firm. Investigations indicated the harmful substance causing the symptoms were PCDFs and Co-PCBs deriving from the mixing of the PCB used in the deodorizing process during the manufacture of edible oil (refer to the document of dioxins) and the acidulated soap stock poison of the chicken was also concluded to be caused by PCB contamination.

PCB is a collective designation for various compounds sharing a similar structure, and the existence of at least 102 types of isomers is currently known. As a low chlorine compound, PCB is a smooth-running fluid, increasing viscosity as the chlorine quantity increases, transforming from tar-like to resinous conditions.

PCB is non-combustible, with resistance to heat, superior insulation properties, and chemical stability. The substance is difficult to decompose, and tends to accumulate within the fatty tissue of animals. PCB was conventionally used in heat transfer mediums, insulation oil and coating materials.

In the context of feed ingredients, residual PCB was detected in coastal fish meals and animal fat/oil.

«Maximum Limits in the Law Concerning Safety Assurance and Quality Improvement of Feeds» [Administrative Guidelines for Hazardous Substances in Feeds]

Provisional tolerable standards were designated for PCB within the "Measures for the prevention of PCB pollution" (Livestock Bureau Notice 47 livestock No.2452 dated Aug.24th, 1972). The standard specifies 5 ppm for fish meal (including fish scrap meal) and other animal by-product feed and 0.5 ppm in the case of formula feed.

[Method listed in the Analytical Standards of Feeds]

1 Gas chromatography*1

1.1 Feed [Analytical Standards of Feeds, Article 1.1-(1), Chapter 7]

Scope of application: Feed[1]

A. Reagent preparation

1) Decachlorinated biphenyl standard solution. Weigh accurately 20 mg of decachlorinated biphenyl [$C_{12}Cl_{10}$] into a 100 mL volumetric flask, add benzene to dissolve. Further add benzene to the marked line to prepare decachlorinated biphenyl standard stock solution. (1 mL of the solution contains 0.2 mg of decachlorinated biphenyl) .

At the time of use, accurately dilute fixed quantities of the standard stock solution with benzene, and prepare several decachlorinated biphenyl standard solutions containing 0.2 to 1 μg of decachlorinated biphenyl per 1 mL of the solution[2].

2) Magnesium silicate. Dry synthetic magnesium silicate (particle size 149 to 250 μm (100 to 60 mesh))*2 at 130 °C for 3 hours.

3) Silica gel. Desiccate silica gel used in column chromatography (particle size 44 to 149 μm (325 to 100 mesh))*3 at 130 °C for 3 hours add and mix with 3 v/w% quantity of water[3], and allow to settle overnight.

4) Diatom earth. Place 200 g of diatom earth*4 in a 5 liter-beaker, add 3 liters of hydrochloric acid (1+1) solution and stir. Allow solution to settle overnight and filter with sintered glass filter (G3), cleanse with warm water, then with 2 liters respectively of ethanol, ethyl acetate and hexane, and air-dry.

B. Quantification

Extraction. Measure and transfer 10.0 to 50.0 g of analysis sample into blender cup. Add 350 mL of acetonitrile – water (13 : 7) and stir for 5 minutes to extract. Then filter with sintered glass filter (G2).

Transfer a certain amount of filtrate (V_1)[4] into 1 liter-separating funnel, add 100 mL of hexane and shake for 2 minutes. Add 10 mL of sodium chloride saturated solution and 600 mL of water, shake for 30 seconds[5] and allow it to settle. Discard water layer (lower layer) and wash residual solution 3 times, each using 100 mL of water[6], transfer the hexane layer (upper layer) to a stoppered measuring cylinder, measure the quantity (V_2) and dehydrate with appropriate quantities of sodium sulfate (anhydrous)[7] for use as sample solution subject to column treatment I.

Column treatment I. Fill column tube (inner diameter: 22 mm) with 25 g of magnesium silicate and 8 g of sodium sulfate (anhydrous) sequentially in dry process[8], add hexane[9] to the column and flow down until liquid surface reaches 3 mm above the top of the column packing material.

Add sample solution (V_2) to the column, wash the measurement cylinder used with small amounts of hexane and add the washings to the column. Flow down at a rate of 5 mL/min until liquid surface reaches 3 mm above the top of the column packing material.

Place a 300 mL recovery flask under the column, add 200 mL of hexane – diethyl ether (47 : 3) to the column to elute PCB. Then condense the eluate[10] to approximately 5 mL in a water bath at 50 °C under reduced pressure to obtain sample solution subject to column treatment II.

Column treatment II. Suspend 5 g of diatom earth and 20 g of silica gel respectively in hexane and sequentially pour into column tube (inner diameter: 22 mm). Adjust column to enable solution to flow out until liquid surface lowers to level 3 mm above the top of the filler agent.

Place a 300 mL recovery flask below the column, add sample solution to the column. Wash recovery flask previously holding sample solution with small quantities of hexane and add the washings to the column. Adjust column to enable hexane to flow out until liquid surface lowers to level 3 mm above the top of the filler agent. Add 250 mL of hexane to the column to enable PCB to run off, then vacuum concentrate eluate to less than 5 mL by immersing in water maintained at 50 °C[11] and adjust quantity to exactly 5 mL by adding hexane. This solution shall be used as sample for synthesis of decachlorinated biphenyl.

Synthesis of decachlorinated biphenyl. Place 1 to 2 mL of sample solution[12] accurately into reaction tube[13], add several drops of chloroform, condense the content to approximately 0.1 mL in a water bath at over 80 °C[14], repeat procedure twice by adding 2 mL of chloroform respectively[15], then seal reaction tube after adding 0.2 mL of antimony chloride (V)[*5] [16]. Apply 165 to 175 °C of heat overnight, allow tube to cool down and open seal.

Add 1 to 3 drops of hydrochloric acid (1+1) solution[17] to reaction tube, subsequently add 1 mL of hydrochloric acid (1+1) solution to dissolve the residue. Then transfer the solution into a 30 mL separating funnel A. Wash the emptied reaction tube with 5 mL of hydrochloric acid (1+1) solution and then with 15 mL of hexane and transfer the washings into separating funnel A. Shake the separating funnel and allow it to settle. Transfer hydrochloric acid layer (lower layer) into alternate 30 mL separating funnel B, add 15 mL of hexane to separating funnel B, shake and allow it to settle. Transfer hydrochloric acid layer into third 30 mL separating funnel C, add 15 mL of hexane to separating funnel and shake it. Transfer hexane layer from separating funnels A, B and C into a 100 mL separating funnel, wash the hexane layer twice with 20 mL of water, once with 20 mL of sodium hydrogen carbonate (10 w/v%)[18] and twice with 20 mL of water.

Place a 300 mL recovery flask under a column (inner diameter: 22 mm, to be filled with 60 g of sodium sulfate (anhydrous) in dry process). Transfer hexane layer from the 100 mL separating funnel into column and elute decachlorinated biphenyl, wash separating funnel with small quantities of hexane and add washings to the column. Add 100 mL of hexane to column and elute in the similar way. Add several drops of methanol to effluent[19], condense the effluent to less than 5 mL in a water bath at 50 °C and adjust quantity to exactly 5 mL by adding hexane to prepare sample solution subject to gas chromatography.

Gas chromatography. Inject a certain amount of the sample and respective decachlorinated biphenyl

standard solutions into the gas chromatograph and obtain chromatogram.

Example of measurement conditions:

Detector: Electron capture detector (ECD)

Column tube: Glass, inner diameter 3 mm, length 2 m

Column packing material: Dimethylpolysiloxane (1 %)[*6]/ acid treated and silanized diatomaceous earth for gas chromatography (particle size 149 to 177 μm (100 to 80 mesh))[*7]

Carrier gas: N_2 (80 to 120 mL/min)

Column oven temperature: 210 °C

Injection port temperature: 230 °C

Detector temperature: 250 °C

Calculation. Calculate the peak area from obtained chromatogram to prepare calibration curve, and determine the total amount of PCB in the sample.

Total amount of PCB in the sample: (mg/kg) $= \dfrac{A \times 5 \times 10^3}{W \times B \times V} \times 0.6$

A : Weight of decachlorinated biphenyl obtained from calibration curve (μg)

B : Quantity of liquid used in synthesis of decachlorinated biphenyl (mL)/5

V : Quantity of liquid introduced to the gas chromatograph (μL)

W: $W_0 \times \dfrac{V_1}{350} \times \dfrac{V_2}{100}$

W_0 : Weight of sample used in analysis (g)

V_1 : Quantity of liquid used in liquid-liquid extraction (mL)

V_2 : Quantity of hexane extract liquid (mL)

* 1. Reagent for PCB test or equivalent are used as solvent.

2. Florisil (Floridin) or equivalent

3. Silicic Acid AR 2847 (Mallinckrodt) or equivalent

4. Celite 545 (Celite Corporation) or equivalent

5. Measuring PCB (Wako Pure Chemical Industries) or equivalent

6. Silicone OV-101 (Ohio Valley Specialty Chemical) or equivalent

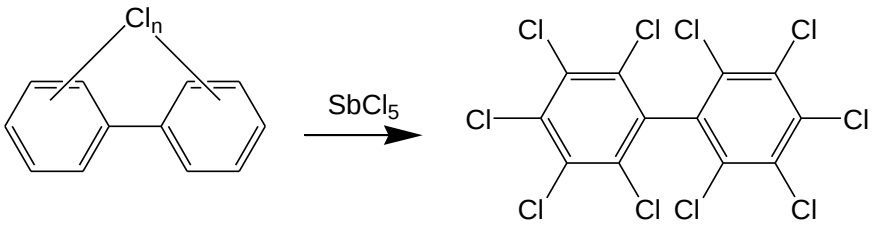
7. Chromosorb W-AW-DMCS (Celite Corporation) or equivalent

«Summary of analysis methods»

Numerous peaks are detected on chromatogram upon conducting gas chromatography using PCB extracted/refined from sample. It is therefore undesirable to quantify PCB on basis of specific peaks. Accordingly, this method chlorinates the biphenyl, quantifying the entire PCB content as decachlorinated biphenyl and calculates the PCB quantity by multiplying with coefficient.

To summarize the method, PCB in the sample is extracted by acetonitrile – water, removing the coloring agent, oil and fats with a magnesium silicate column, further separating DDTs and DDEs with a silica gel column. Then PCB is chlorinated and quantified using a gas chromatograph equipped with an electron capture detector as decachlorinated biphenyl. Result of the quantification is then multiplied by 0.6 to obtain the quantity of the PCB.

Coefficient 0.6 is a correction factor taking into consideration the increase in molecular weight (weighted mean) upon chlorification of PCB into decachlorinated biphenyl.



Reagents and water to be used in the analysis shall be of quality acceptable for use in pesticide residue/PCB testing, and must not contain substances inhibiting the analysis. Sufficiently clean equipments to be used in analysis with hexane or acetone.

References; Pesticide Analytical manual., 251 (1972 Jan.1st)
J. AOAC, 53, 761 (1970)
J. AOAC, 56, 987 (1973)

«Notes and precautions»

[1] May be applied to water and oil/fats content of formula feed and feed ingredients below 20 % respectively. In case of samples with higher water content, reduce quantity of water to be mixed with acetonitrile.

[2] It is desirable to maintain concentration of standard solution at levels close to the concentration of the sample solution. Use within range where the response of the gas chromatograph indicates linearity.

[3] Activity of silica gel differs depending on the lot. Precedential to the testing, use 3 μg of *p,p′*-DDE and 40 μg of PCB to conduct procedure specified for column treatment II processing to confirm separation of PCB from *p,p′*-DDE. If separation is insufficient, adjust quantity of water to be added to compensate for the activity of the silica gel.

[4] Normally, quantity shall be 250 mL.

[5] Insufficient shaking leads to the decline of recovery rate.

[6] Shake it gently, as hard shaking may cause solution to emulsion.

[7] If dehydration is insufficient, the activity of the magnesium silicate may decline in the subsequent column treatment process, causing coloring agents and oil/fats to elute.

[8] Vibrators should be used to facilitate process.

[9] Pour approximately 80 mL of hexane into column and cleanse by allowing hexane to flow out until surface of the liquid reaches a level 3 mm above the top of the column packing materials.

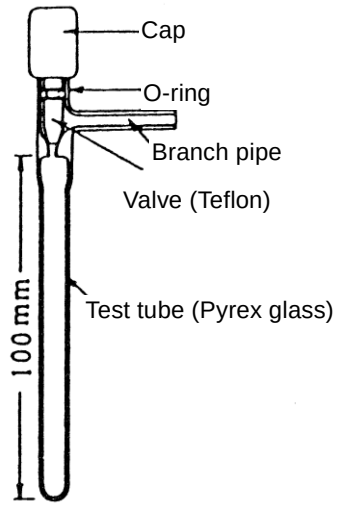


Figure7.1-1 Reaction tube

[10] This concentrate is applicable for the quantification of DDT and BHC (recovery rate may be low). When determining the PCB pattern on the gas chromatograph using Silicone DC-200 (Dow Chemicals), peaks are generated in the sequence of the chlorine quantity depending on the duration of

the retention time.

[11] PCB may volatilize upon exsiccation. The pattern for PCB can be determined from the silica gel column eluate.

[12] Appropriate quantity would contain 1~20 μg of PCB.

[13] Hydrol tube (896860-4010; Kimble / Kontes) or equivalent products may be utilized as the reaction tube (Figure. 7.1-1). The product is also used in the hydrolysis reactions for amino acid analysis.

[14] Chloroform may erupt as a result of bumping. Precautionary measures such as adding carborundum should be implemented in executing the procedure.

[15] Procedure substitutes hexane within solvent with chloroform, completely sublimating hexane.

[16] If the cleanup procedure or the sublimation of hexane is not sufficient, the solution might blacken upon the addition of antimony pentachloride.

[17] Solution reacts violently upon addition of hydrochloric acid (1+1) solution. The initial 1 to 3 drops of the acid should be added with caution.

[18] Procedure neutralizes the hydrochloric acid.

[19] Add methanol to facilitate the sublimation of chloroform.

1.2 Fat and oil [Analytical Standards of Feeds, Article 1.1-(2), Chapter 7]

A. Reagent preparation[1]

Follow the steps A of 1.1.

B. Quantification

Extraction. Weigh accurately 1 g of the analysis sample, place in a 50 mL separating funnel. Add 15 mL of hexane to dissolve, then add 30 mL of hexane saturated acetonitrile and allow solution to settle after shaking for 1 minute. Transfer acetonitrile layer (lower layer) to a 1 L separating funnel A containing 650 mL of water, 40 mL of sodium chloride saturated solution and 100 mL of hexane.

Repeat procedure twice while adding 30 mL of hexane saturated acetonitrile for each instance, transferring the acetonitrile layer to a 1 L separating funnel A. Shake and allow solution to settle, then transfer the water layer (lower layer) to an alternative 1 L separating funnel B. Add 100 mL of hexane to separating funnel B, shake funnel and allow solution to settle. Transfer the hexane layer from funnels A and B to a 300 mL separating funnel C, wash twice using 100 mL of water respectively. Transfer the hexane layer to an Erlenmeyer flask, dehydrate with appropriate quantities of sodium sulfate (anhydrous) to prepare the sample solution for the column treatment.

Column treatment I[2]. Follow the column treatment I in B of 1.1.

Column treatment II[2]. Follow the column treatment II in B of 1.1.

Synthesis of decachlorinated biphenyl[2]. Follow the decachlorinated biphenyl synthesis in B of 1.1.

Gas chromatography[2]. Follow the gas chromatography in B of 1.1.

Calculation[2]. Follow the calculation in B of 1.1. However, value *W* to be weight (g) of sample used in the analysis.

«Summary of analysis methods»

See the **«Summary of analysis method»** of 1.1 in this section.

«Notes and precautions»

[1] Same preparations as in A of 1.1.

[2] Same procedures as in B of 1.1.

1.3 Fish Soluble [Analytical Standards of Feeds, Article 1.1-(3), Chapter 7]

A. Reagent preparation[1]

Follow the steps A of 1.1.

B. Quantification

Extraction. Place 100 g of analysis sample (amount of moisture V_3 (mL)) into blender cup, and add 200 mL of acetonitrile solution and stir for 2 minutes to extract. Then filter with sintered glass filter (G2).

Pour fixed quantity of filtrate (V_1) into 1 liter- separating funnel, add 100 mL of hexane and shake for 2 minutes. Add 10 mL of sodium chloride saturated solution and 600 mL of water, shake for 30 seconds and allow it to settle. Discard water layer (lower layer) and cleanse residual solution 3 times, each using 100 mL of water. Then transfer the hexane layer to a stoppered measuring cylinder, measure the quantity (V_2) and dehydrate with appropriate quantities of sodium sulfate (anhydrous) for use as sample solution supplied to column treatment I.

Column treatment I[2]. Follow the column treatment in B of 1.1.

Column treatment II[2]. Follow the column treatment in B of 1.1.

Synthesis of decachlorinated biphenyl[2]. Follow the decachlorinated biphenyl synthesis in B of 1.1.

Gas chromatography[2]. Follow the gas chromatography in B of 1.1.

Calculation[2]. Follow the calculation in B of 1.1. However, value $V_1/350$ to be substituted with alternative value $V_4/(200+V_3)$.

«Summary of analysis methods»

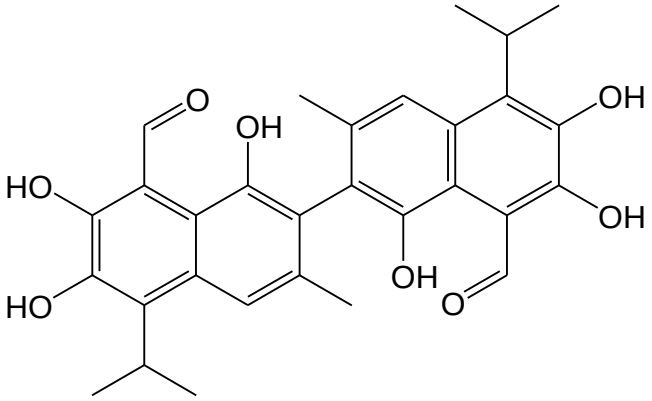
See the **«Summary of analysis method»** of 1.1 in this chapter.

«Notes and precautions»

[1] Same preparations as in A of 1.1.

[2] Same procedures as in B of 1.1.

2 Gossypol



2,2′-bis-(formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)
$C_{30}H_{30}O_8$ MW: 518.6 CAS No.: 303-45-7

[Summary of Gossypol]

Cottonseed is basically used as formula feed for cows, but contains toxic gossypol as its constituent. Livestock subjected to large quantities of the substance exhibit symptoms including anorexia, dyspnea, symptosis and decline in fertility. Content of gossypol in cottonseed is approximately 0.01 to 2 %, with deviation of concentration among the varieties.

[Method listed in the Analytical Standards of Feeds]

1 Liquid chromatography [Analytical Standards of Feeds, Article 2.1, Chapter 7][*1]

A. Reagent preparation

Gossypol standard solution. Weigh accurately 20 mg of gossypol[1] [$C_{30}H_{30}O_8$], place in a 100 mL brown volumetric flask, and add acetone to dissolve. Then add acetone to marked line to prepare gossypol standard stock solution[2] (1 mL of the solution contains 0.2 mg of gossypol).

At the time of use, accurately dilute fixed quantities of the standard stock solution with acetone – water – acetic acid (9 : 9 : 2), and prepare several gossypol standard solutions[3] containing 0.2 to 1 μg of gossypol per 1 mL of the solution.

B. Quantification

Extraction. Weigh accurately 5 g of the analysis sample[*2] (1 g for cottonseed), place in a 200 mL brown stoppered Erlenmeyer flask (300 mL for cottonseed), add 100 mL of acetic acid – water – phosphoric acid (85 : 15 : 1) (200 mL for cottonseed) and either seal or apply aluminum foil to flask. Extract by heating for 20 minutes immersed in boiling water[*3][4]. Shake and water-cool extract, transfer solution to centrifuge tube and centrifuge for 5 minutes at 1,000×*g*. Transfer 10 mL of supernatant solution accurately to 50 mL volumetric flask and add acetone – water (1 : 1) to marked line and allow solution to settle for 20 minutes at ambient temperature. Centrifuge solution for 5 minutes at 5,000×*g*, then filter supernatant solution using membrane filter (pore size: 0.45 μm)[5] to prepare sample solution subject to

liquid chromatography.

Liquid chromatography. Inject 20 µL of each sample solution and respective gossypol standard solutions into the liquid chromatograph and generate chromatogram.

Example of measurement conditions:

Detector: UV spectrophotometer (wavelength 254 nm)

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

Column temperature: 40 °C

Eluent: Methanol – water (9 : 1) solution adjusted pH to 2.6 using phosphoric acid

Flow rate: 1.0 mL/min

Calculation. Calculate the peak area from obtained chromatogram[6] to prepare calibration curve, and determine the amount of gossypol in the sample.

* 1. Quantification to be performed in shaded condition[7].

2. In case of formula feed mixed containing cottonseed, ground cottonseed tend to be distributed unevenly after the grinding process. Accordingly, approximately 100 g of sample should be ground to pass through sieve with 1 mm mesh, then mixed evenly using Millser (equipped with micronizer) (Iwatani Corporation) or equivalent product.

3. When using Erlenmeyer flask with stopper applied, secure to prevent stopper from unplugging during the heating procedure.

4. Shodex C18 M 4E (Showa Denko) or equivalent

«Summary of analysis methods»

This method is used in the analysis of gossypol contained in cottonseed and formula feed. Gossypol bonds easily with iron, and the phosphoric acid added to the extract is used to mask iron. The sample is loaded to the liquid chromatograph after extract is diluted with acetone – water (1 : 1), to be separated for quantification.

Figure 7.2-1. shows flow sheet of analysis method.

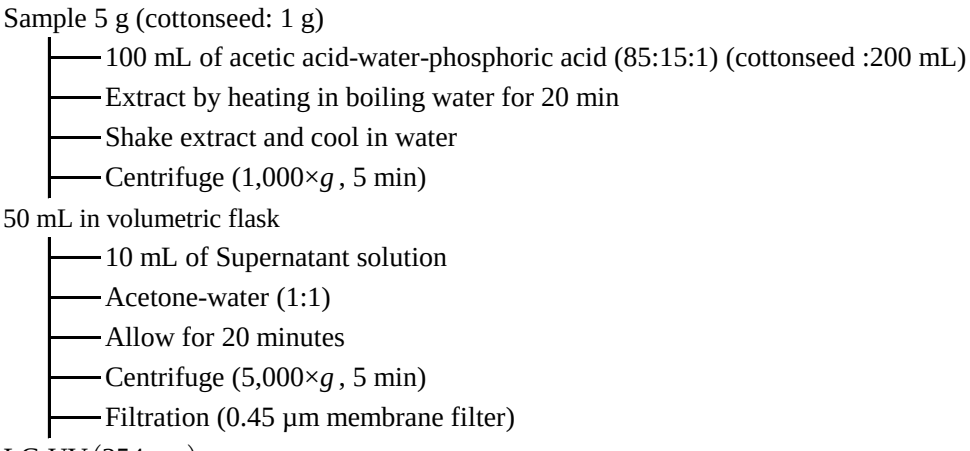


Figure 7.2-1 Liquid chromatography flow sheet for gossypol

References: Keisuke Aoyama: Journal of Food Safety, 49(4), 303 (2008)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Spike recovery (%) (Data (mg/kg))	Repeatability RSD (% or less)
Cottonseed	1,000~5,000	3	90.7-105.3	3.0
Cottonseed	Natural contamination	3	(5,490)	1.6
Formula feed for dairy cattle feed	58~580	3	90.8~95.5	2.8
Formula feed for beef cattle feed	58~580	3	95.6~96.8	2.7

• Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Data (mg/kg) (Spike recovery (%))	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Cottonseed	8	Natural contamination	6,090	3.3	4.4	1.01
Formula feed for dairy cattle feed	8	305	(87.0)	2.7	5.5	0.80

• Limit of quantification 5 µg/kg (Spike recovery rate and relative standard deviation)

• Limit of detection 2 µg/kg (*SN* ratio)

«Notes and precautions»

[1] Gossypol reference standard available from Sigma and other sources.

[2] Standard stock solution may be kept in frozen storage (−20 °C) for 120 days.

[3] Prepare prior to usage and promptly utilize.

[4] Under normal circumstances, the quantity of solvent does not deviate significantly in the heating process. If significant difference is observed, compensate with extracting solvent based on the difference of Erlenmeyer flask before and after the heating process.

[5] DISMIC-13HP (Toyo Roshi kaisha) or equivalent

[6] An example of chromatogram is shown in figure 7.2-2.

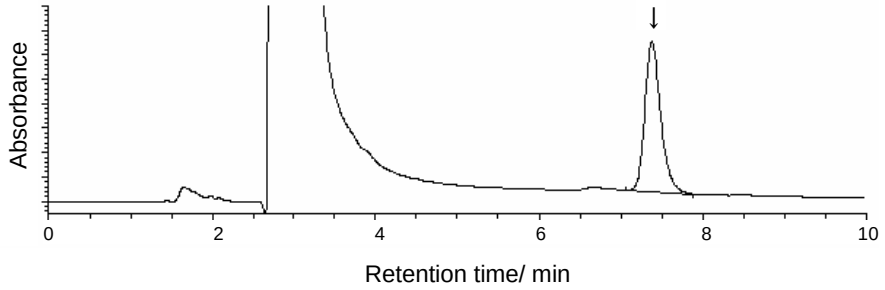
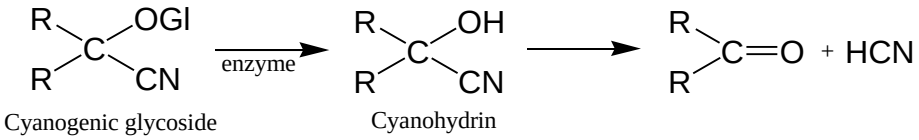


Figure 7.2-2 Chromatogram of gossypol standard solution (20 ng)
(Arrow indicates the peak of gossypol)

[7] Analysis should be conducted in lab illuminated with yellow lamps.

3 Hydrogen cyanide (Cyanogenic glycosides)



Cyanogenic glycoside　　Cyanohydrin

HCN　MW: 27.03　CAS No.: 74-90-8

[Summary of hydrogen cyanide]

Hydrogen cyanide (Cyanogenic glycosides) is generated by the decomposition of cyanogen glycoside contained in cassava, lima bean, burma bean, sorghum, sudangrass etc, not uncommonly causing poisoning in livestock.

Degradative enzyme acts on cyanogen glycoside in the process of mastication and digestion, resulting in the generation of cyanogen (hydrocianic acid) which is then absorbed by the livestock. As cyanogen strongly bonds with ferric iron, it bonds with the cytochrome oxidase within the body tissue of the livestock, inhibiting the activity of the enzyme. This results in respiratory inhibition, causing manifestation of toxicity including centrum paralysis. Because cyanogen obstructs the body tissue from taking in oxygen, the venous blood looks as red as arterial blood.

[Method listed in the Analytical Standards of Feeds]

1 Absorptiometric method [Analytical Standards of Feeds, Article 3.1, Chapter 7]

Scope of application: Cassava[1]

A. Reagent preparation

1) 0.1 mol/L of silver nitrate standard solution. Weigh 17 g of silver nitrate, place in a brown volumetric flask, add water for dissolve, further add water to the marked line to prepare 0.1 mol/L of silver nitrate standard solution. Standardize the concentration based on the following procedure. Standard solution to be preserved in brown bottles[2].

Weigh 1.461 g of sodium chloride (standard reagent) (to be heated for 40 to 50 minutes at 500 to 650 °C within a platinum crucible) into 250 mL volumetric flask, add water to dissolve the reagent and continue adding water to marked line. Transfer 25 mL of the solution accurately to Erlenmeyer flask, add 25 mL of water and 5 mL of dextrin hydrate solution (2 w/v%). Add several drops of uranin solution (0.2 w/v%), titrate with 0.1 mol/L standard silver nitrate solution up to the endpoint as the yellow-green fluorescence is replaced by faint ruby color. At this point, the concentration of the 0.1 mol/L standard silver nitrate solution is standardized.

1.461 g of sodium chloride corresponds to 25 mL of undiluted 0.1 mol/L silver nitrate standard solution.

2) *p*-Dimethylaminobenzylidenerhodanine solution. Dissolve 20 mg of *p*-dimethylaminobenzylidene-rhodanine in acetone to make up 100 mL.

3) Cyanide ion standard stock solution. Weigh 0.626 g of potassium cyanide into 250 mL volumetric

flask and add a small quantity of water to dissolve the reagent. Add 2.5 mL of sodium hydroxide solution (0.5 mol/L), further add water to the marked line to prepare cyanide ion standard stock solution and standardize concentration based on the following procedure:

Place 100 mL of standard stock solution accurately into 200 mL tall beaker, and add 0.5 mL of *p*-dimethylaminobenzylidenerhodanine solution. Then titrate with 0.1 mol/L silver nitrate standard solution up to the endpoint as the yellow color of the solution is replaced by red. Calculate concentration C (mg CN^-/mL) of the cyanide ion standard stock solution from the following equation:

$$C = \frac{a \times f \times 5.204}{100}$$

C : Cyanide ion standard stock solution (mg CN^-/mL)

a : 0.1 mol/L silver nitrate standard solution required for the titration (mL)

f : Coefficient for 0.1 mol/L silver nitrate solution

4) Cyanide ion standard solution. Place 100 mL of cyanide ion standard solution accurately into 250 mL volumetric flask, add 25 mL of sodium hydroxide solution (0.5 mol/L) and add water to marked line. Transfer 10 mL of this solution accurately into 100 mL volumetric flask, add water to marked line to prepare cyanide ion standard solution containing 1 μg of cyanide ion per 1 mL[*1].

5) Citrate buffer. Dissolve 128.1 g of citric acid monohydrate and 64.4 g of sodium hydroxide in water, and adjust volume to 1,000 mL.

At the time of use, dilute a certain quantity of this solution accurately 10-fold with water, and adjust pH to 5.9 with citric acid solution (2 w/v%) and sodium hydroxide solution (2 w/v%).

6) Phosphate buffer. Dissolve 3.40 g of monobasic potassium phosphate and 3.55 g of sodium hydrogenphosphate in water, and adjust volume to 1,000 mL.

7) Chloramine-T solution. Dissolve 0.62 g of sodium *p*-toluenesulfonchloramide trihydrate in water, and adjust volume to 50 mL. (Prepare at the time of use.)

8) Pyridine/pyrazolone solution. Dissolve 0.25 g of 3-methyl-1-phenyl-5-pyrazolone into 100 mL of water at 75 °C[*2]. Allow solution to cool to ambient temperature and add solution consisting of 0.02 g of bis (3-methyl-1-phenyl-5-pyrazolone) dissolved into 20 mL of pyridine (to be prepared at the time of use).

B. Preparation of sample solution

Weigh 10.0 to 15.0 g of analysis sample into a 500 mL Kjeldahl flask, add 100 mL of citrate buffer solution, seal the flask and allow to stand for 4 hours at 25 to 30 °C[*3]. Connect Kjeldahl flask to steam distillation apparatus with a receiver containing 25 mL of sodium hydroxide solution (0.5 mol/L). Distill solution until quantity of distillate reaches 100 mL[*4]. Add 1 drop of phenolphthalein solution (1+1), neutralize with acetic acid (1+8) solution and transfer to 200 mL volumetric flask using water, add water to the marked line to prepare the sample solution.

Transfer 5 mL of the sample solution accurately into 50 mL volumetric flask, add 5 mL of water followed by 10 mL of phosphate buffer solution and 0.25 mL of chloramine-T solution, then promptly seal the flask. Allow to settle for 5 minutes after gently mixing solution. Add 15 mL of pyridine/pyrazolone solution, then introduce water to the marked line of the flask. Allow to settle for 50 minutes at 25 to 30 °C after sealing

flask and gently mixing solution[3]. Then measure absorbance of the solution at 620 nm wavelength using a reference solution prepared as follows: place 10 mL of water into a 50 mL volumetric flask, and otherwise prepared in the similar way of the sample solution.

Concurrently place several amounts in the range of 0.5 to 9 mL of cyanide ion standard solution (1 μg CN^-/mL) into 50 mL volumetric flask, add water to make 10 mL. Measure absorbance of respective solution under same condition as sample solution, after preparing in the similar way of the sample. Prepare calibration curve from the obtained absorbance and calculate the amount of cyanide ion in the sample, multiply by 1.03 to obtain the amount of hydrogen cyanide in the sample.

* 1. Prepare at the time of use. Coefficient calculated for the standard stock solution shall be used for the standard solution.
2. Reagent need not dissolve completely.
3. Maintain stable temperature using constant temperature bath, etc.
4. Distillation speed to be 2 to 3 mL/min.

«Summary of analysis methods»

In this method, cyanogen compound in cassava is hydrolized to hydrogen cyanide, then hydrogen cyanide is steam-distilled, collecting the extract in alkaline solution and neutralized with acetic acid. Sample solution is then reacted with phosphate buffer solution, chloramine-T solution, and pyridine/pyrazolone solution to obtain the absorbance of blue color.

Alternately to this method and the titration method in next section, there are reports on recent approaches utilizing ion chromatography and head-space gas chromatography.

Flow of the analysis is indicated in figure 7.3-1.

Sample: 10.0 g
- 500 mL Kjeldahl flask
- 100 mL of citrate buffer (pH5.9)

Hydrolysis
- Stand for 4 hours

Steam distillation
- 25 mL of sodium hydroxide solution (0.5 mol/L) in receiver.
- Distillation: until distillate solution 100 mL <

Neutralization
- Neutralize with acetic acid (1+8), indicator (phenolphthalein)

200 mL volumetric flask
- Make up with water

50 mL volumetric flask
- Sample solution: 5 mL
- 5 mL of water, 10 mL of phosphate buffer, 0.25 mL of Chloramine-T solution
- Stand for 5 min
- 15 mL of pyridine/pyrazolone solution
- Make up with water
- Stand for about 50 min

UV measurement (620 nm)

Figure 7.3-1 Flow sheet of hydrogen cyanide absorbency method

Reference: Shigehiro Kai, Yuko Shirasawa: Research Report of Animal Feed, 32, 54 (2007)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (%)	Replicate	Average spike recovery (%)	Repeatability RSD (% or less)
Cassava (natural contamination concentration: approximately 3 mg/kg)	0.5~4.0	3	79.0~90.0	15.0

• Collaborative study

Sample type	No. of labs	Data (mg/kg)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Cassava	7	8.15	8.0	9.6	0.82
Cassava	8	3.18	8.0	14.9	1.10

• Limit of quantification: 2 µg/kg (standard deviation)

• Limit of detection: 0.7 µg/kg (standard deviation)

«Notes and precautions»

[1] Alternately known as tapioca, cassava is widely raised in the tropics, with the root mass of the plant utilized as feed. Raw cassava has a water content of approximately 70 %, but the water content for the so-called cassava meal produced by drying and grinding the root mass is approximately 15 %, with nitrogen-free extract making up for approximately 85 % of the weight. Major component of cassava

meal is starch.

Cassava contains toxic cyanide compounds although the content differs significantly among the varieties. Cyanide content in heated/dried products are lower than the content for raw cassava.

Analytical value of the hydrogen cyanide content in commercially available cassava meal is generally in the order of several 10 mg/kg.

[2] Commercially available silver nitrate standard solutions (0.1 mol/L) may be used.

[3] Color of solution changes from faint ruby to stable blue shifting through violet. Under these conditions, the color will stabilize for approximately 1 hour.

2 Silver nitrate titration method [Analytical Standards of Feeds, Article 3.2, Chapter 7]

Scope of application: Cassava

A. Reagent preparation

1) 0.01 mol/L silver nitrate standard solution. Prepare 0.01 mol/L silver nitrate standard solution in accordance with the specifications of section 3.1 A-1) and standardize concentration[1].

Dilute a certain amount of the standard stock solution accurately 10-fold with water, and prepare 0.01 mol/L silver nitrate standard solution prior to use.

2) *p*-dimethylaminobenzylidenerhodanine solution. Dissolve 20 mg of *p*-dimethylaminobenzylidene-rhodanine to acetone to make up 100 mL.

B. Quantification

Weigh accurately 10.0 to 15.0 g of analysis sample[2], place in a 500 mL round-bottom flask, add 200 mL of water and seal the flask tightly, and stand for 2 hours or more at 25 to 30 °C[3]. Connect the round-bottom flask to steam distillation apparatus with a receiver container containing 20 mL of sodium hydroxide solution (5 w/v%), and distill solution until quantity of distillate reaches 200 mL*4.

Add 0.5 mL of *p*-dimethylaminobenzylidenerhodanine solution[4] to distillate and standardize with 0.01 mol/L silver nitrate standard solution[5]. Calculate quantity of hydrogen cyanide [HCN] contained in sample using the following equation:

$$\text{Quantity of hydrogen cyanide contained sample (mg/kg)} = \frac{V \times f \times 0.54}{W} \times 10^3$$

V : Quantity of 0.01 mol/L silver nitrate standard solution required for standardization (mL)

f : 0.01 mol/L silver nitrate standard solution coefficient

W : Weight of sample used in analysis (g)

«Summary of analysis methods»

In this method, cyanogen compound contained in the sample is hydrolized to hydrogen cyanide in slightly acidic condition. Hydrogen cyanide is then steam-distilled, collecting extract in alkaline solution. Extract is titrated using silver nitrate standard solution to obtain the amount of hydrogen cyanide in the sample.

References: Satoshi Horii: Report or Livestock experiment station studies, 19, 63 (1969)

«Notes and precautions»

[1] Prepared as specified in A-1) of previous section.

[2] Due to its high starch content, cassava meal samples tend to gelatinize during the distillation procedure. The appropriate quantity is considered to be 10 to 15 g.

[3] Hydrocyanic acid is contained in cassava meal in the form of glycoside (linamarin, etc.). The glycoside is to be hydrolytically degraded into glucose, acetone and hydrogen cyanide with enzyme (linamarase) or weak-acid.

Hydrolysis is also enabled with pH 5.9 citrate buffer solution (Standard Methods of Analysis for Hygienic Chemistry) but the required acidity (pH 5.7 to 5.9) can be achieved with distilled water. In either method, sufficient level of hydrolysis is achieved by allowing solution to settle for over 2 hours.

Hydrolysis in the range of pH 5 to 6 will not affect analytical values. Hydrolysis at pH 4 results in analytical values 20 to 30 percent lower than the designated condition.

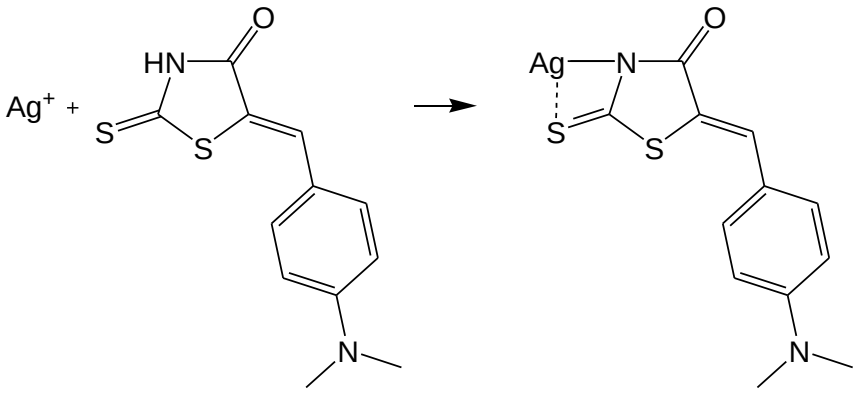
[4] Potassium iodide may be used as an alternative indicator to *p*-dimethylaminobenzylidenerhodanine but the determination of the endpoint is easier when using the latter.

Endpoint for the titration is when the yellow-green fluorescent is replaced by a faint ruby color.

CN^- within the sample solution generates $Ag(CN)_2^-$ by titrating with $AgNO_3$. At the endpoint of titration, the excess Ag^+ contained in the solution reacts with *p*-dimethylaminobenzylidenerhodanine and produces the red color.

Principle of titration

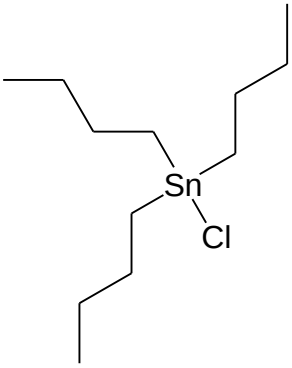
$$Ag^+ + 2CN^- \longrightarrow [Ag(CN)_2]^-$$



[5] Microburet shall be used in the titration process.

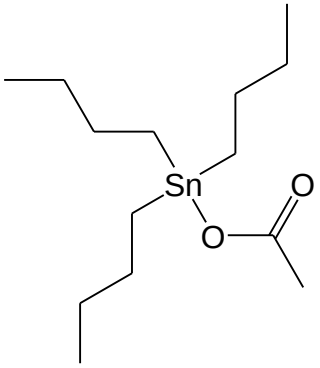
4 Tributyltin compounds

Tributyltin chloride



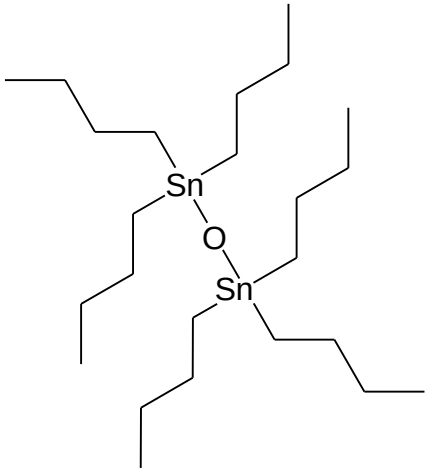
Tri-*n*-butyltin chloride
$C_{12}H_{27}ClSn$ MW: 325.5
CAS No.: 1461-22-9

Tributyltin acetate



Tri-*n*-butyltin acetate
$C_{14}H_{30}O_2Sn$ MW: 349.1
CAS No.: 56-36-0

Bis(tributyltin)oxide (TBTO)



Bis(tri-*n*-butyltin) oxide
$C_{24}H_{54}OSn_2$ MW: 596.1
CAS No.: 56-35-9

[Summary of Tributyltin compounds]

Tributyltin is a typical organotin with 14 types of compounds. Since 1960s, tributyltin saw widespread use as antifouling paint for ship bottom and coating material for fish cultivation nets to prevent the adhesion of crustacea and seaweed. The material was de-facto banned in Japan eventually due to concerns for ocean pollution and persistence in fishery products.

Accumulative property is not high with the exception of TBTO, but the compounds are recalcitrant and indicates toxicity including growth inhibition.

Ingestion of tin compounds are known to cause masculinizing in female *Thais clavigena* and other forms of snails, spurring studies of the substances as potential endocrine disrupting chemicals (EDC, so-called environment hormones).

[Method listed in the Analytical Standards of Feeds]

1 Gas chromatography[*1,2] [Analytical Standards of Feeds, Article 4.1, Chapter 7]

A. Preparation of reagent

1) Tributyltin chloride standard solution. Weigh accurately 0.1 g of tributyltin chloride [$C_{12}H_{27}ClSn$], place in a 100 mL volumetric flask, and add 5 mL of ethyl acetate to dissolve. Add hexane to marked line to prepare tributyltin chloride standard stock solution. (1 mL of the solution contains 1 mg of tributyltin chloride).

At the time of use, dilute a certain amount of the standard stock solution accurately with hexane to prepare tributyltin chloride standard solutions containing 0.125 to 2.0 µg of tributyltin chloride per 1 mL.

2) Extraction solvent. methanol – ethyl acetate – hydrochloric acid (11 : 10 : 1)

B. Quantification

Extraction. Weigh accurately 10.0 g of the analysis sample, place in a 200 mL stoppered Erlenmeyer flask, add 70 mL of extraction solvent and extract by shaking for 30 minutes. Place the 200 mL Erlenmeyer flask under a Büchner funnel and filter the extract with a filter paper (No. 5B) by suction. Then sequentially wash Erlenmeyer flask and residue with 30 mL of solvent, and filter the washings in a similar way to prepare sample solution subject to the liquid-liquid extraction.

Liquid-liquid extraction. Transfer sample solution to a 500 mL separating funnel A having contained 100 mL of sodium chloride solution (10 w/v%) and 50 mL of ethyl acetate – hexane (3 : 2) and shake vigorously for 5 minutes. After allowing solution to settle, transfer the water layer (lower layer) to an alternate 500 mL separating funnel B. Add 50 mL of ethyl acetate – hexane (3 : 2) to separating funnel B and shake vigorously for 5 minutes. After allowing solution to settle, transfer the ethyl acetate – hexane layer (upper layer) to the original separating funnel A. Add 150 mL of hexane to separating funnel A[1], gently shake separating funnel and allow solution to settle for 30 minutes. Then transfer ethyl acetate – hexane layer (upper layer) to a 500 mL stoppered Erlenmeyer flask. Dehydrate the ethyl acetate – hexane layer with appropriate quantities of sodium sulfate (anhydrous) and filter the solution into a 500 mL recovery flask with filter paper (No. 5A). Then sequentially wash Erlenmeyer flask and filter paper with small quantities of hexane, filter the washings into the recovery flask in the similar way.

Concentrate under reduced pressure to about 3 mL in a water bath at 40 °C or below, add 10 mL of hexane to the concentrated solution and repeat concentration. Repeat the procedure until the concentrated solution is free of the acetic acid odor[2]. Then dry up by nitrogen gas flow.

Add 10 mL of ethanol to dissolve the residue to prepare sample solution subject to column treatment.

Column treatment[3][4]. Wash anion-exchange minicolumn (1 g)[*3] with 10 mL of sodium hydroxide solution (0.2 mol/L), 20 mL of distilled water and 20 mL of ethanol. Attach cation-exchange minicolumn (1 g)[*4] washed with 20 mL of hydrochloric acid, 20 mL of distilled water and 20 mL of ethanol under the anion-exchange column.

Transfer sample solution to the minicolumn, allow to flow down at the rate of 1 mL/min until the surface of the solution reaches the top of the column packing material. After washing recovery flask originally containing the sample solution with small quantities of ethanol, add washings to the mini-column, allow to flow down in the similar way. Add 20 mL of the solvent to the minicolumn, and treat likewise.

Remove anion-exchange minicolumn and place a 100 mL separating funnel C[5] under the cation exchanger minicolumn. Add 15 mL of methanol – hydrochloric acid (11 : 1) to the cation-exchange minicolumn to elute tributyltin chloride.

Add 30 mL of water and 5 mL of hexane – cyclohexane[*5] (1 : 1) to separating funnel C, shake for 5 minutes and allow solution to settle. Transfer water layer (lower layer) to an alternate 100 mL separating funnel D, and the hexane – cyclohexane layer (upper layer) to a 50 mL stoppered Erlenmeyer flask.

Add 5 mL of hexane – cyclohexane (1 : 1) to separating funnel D and repeat above procedure. Discard water layer (lower layer) and transfer hexane – cyelohexane layer (upper layer) to the Erlenmeyer flask. Dehydrate the hexane – cyclohexane layer with an appropriate quantity of sodium sulfate (anhydrous)[6],

and filter the solution into a 50 mL pear-shaped flask with filter paper (No. 5A). Then sequentially wash Erlenmeyer flask and filter paper with small amounts of hexane – cyclohexane (1 : 1), and filter the washings into the pear-shaped flask in the similar way.

Concentrate under reduced pressure to about 1 mL in water bath at 40 °C or below, and transfer concentrate to a 50 mL stoppered test tube. Wash pear-shaped flask with small amounts of hexane – cyclohexane (1 : 1) and add the washings to the concentrate to prepare the sample solution subject to the propylation.

Propylation. Concentrate sample solution to less than 1 mL by nitrogen gas flow. Add 2 mL of *n*-propyl-magnesium bromide solution[7] and shake, allowing solution to settle for 30 minutes in water bath at 40 °C. The test tube is then cooled in ice-cold water for 10 minutes. Gradually add 10 mL of sulfuric acid (1+19) solution, decomposing the excess *n*-propyl-magnesium bromide.

Add 20 mL of water, 7 mL of ethanol[8] and 5 mL of hexane to the solution, shake and transfer the entire amount to a 100 mL separating funnel E[9]. Wash emptied test tube with small quantities of hexane, adding the washings to separating funnel E. Shake for 5 minutes and allow solution to settle. Transfer water layer (lower layer) to an alternate 100 mL separating funnel F, and the hexane layer (upper layer) to a 50 mL stoppered Erlenmeyer flask. Add 10 mL of hexane to separating funnel F, shake for 5 minutes and transfer hexane layer (upper layer) to the Erlenmeyer flask. Dehydrate the hexane layer with an appropriate quantity of sodium sulfate (anhydrous) and filter the solution into a 50 mL pear-shaped flask with a filter paper (No. 5A). Then sequentially wash Erlenmeyer flask and filter paper with small quantities of hexane, filtering the washings into the pear-shaped flask in the similar way. Concentrate under reduced pressure to about 1 mL in water bath at 40 °C or below, further concentrate by nitrogen gas flow*6 and transfer to a 2 mL volumetric flask. Wash emptied pear-shaped flask with small quantities of hexane, adding the washings to the volumetric flask. Add hexane to the marked line to prepare the sample solution subject to gas chromatography[10].

Propylation of standard solution. Place each 1 mL of respective tributyltin chloride standard solution accurately into a 50 mL stoppered test tube. Propylate standard solution in the similar way of sample solution toprepare standard solutions subject to gas chromatography.

Gas chromatography. Inject 2 μL[11] of the sample solution and respective standard solutions into a gas chromatograph and obtain chromatogram.

Example of measurement conditions:

Detector: Flame photometric detector (filter for detection of stannum)

Column: Fused silica capillary column (5 % diphenyl / 95 % dimethyl-polysiloxanecoating, inner diameter 0.32 mm, length 30 m, film thickness 0.25 μm)

Carrier gas: He (1.0 mL/min)

Makeup gas: N_2 (32 mL/min)

Hydrogen: 75 mL/min[12]

Dry air: 100 mL/min

Sample injection: splitless (60 s)

Injection port temperature: 240 °C

Column oven temperature: Initial temperature 80 °C (hold 2 min) → ramp 10 °C/min → 200 °C → ramp 30 °C/min → 230 °C (hold 10 min)

Detector temperature: 240 °C

Calculation. Calculate the peak height from obtained chromatogram[13] to prepare calibration curve and determine the amount of tributyltin in the sample as its chloride form.

* 1. Use pesticide analysis grade reagents for methanol, hexane, ethyl acetate and sodium sulfate (anhydrous).

2. Use trace analysis grade reagents for hydrochloric acid and sulphuric acid.

3. Bond Elut SAX (Varian, reservoir capacity 6 mL) or equivalent

4. Bond Elut SCX (Varian, reservoir capacity 6 mL) or equivalent

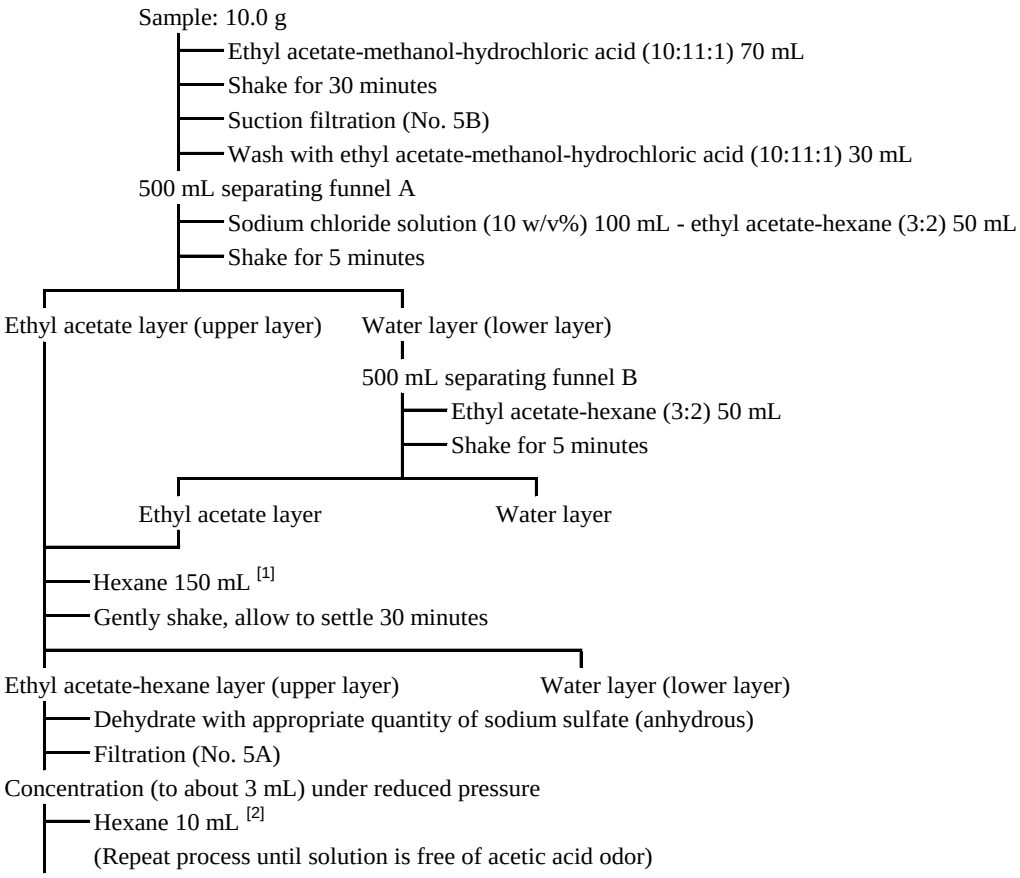
5. Cyclohexane shall be of liquid chromatography reagent grade or equivalent.

6. Do not let the sample dry to prevent the loss.

«Summary of analysis methods»

In this method, tributyltin compounds in sample are extracted as chloride form under the acidic environment of hydrochloric acid. The extract is purified by liquid-liquid extraction and anion-exchange/cation-exchange minicolumns, then tributyltin is propylated for quantification using a gas chromatograph equipped with flame photometric detector (tin detection filter).

Figure 7.4-1. shows flow sheet of analysis.



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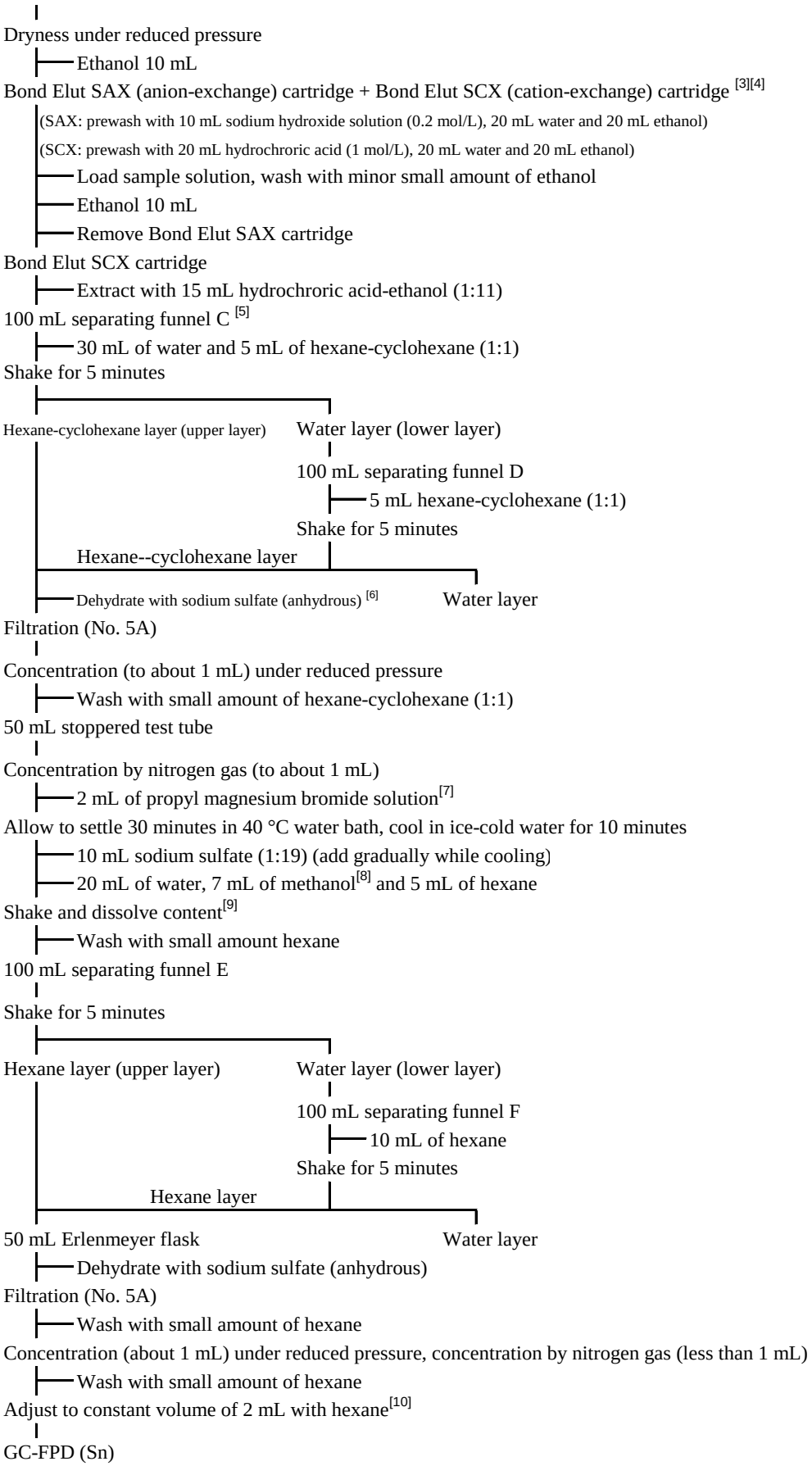


Figure 7.4-1 Flow sheet of analysis for tributyltin compounds

References: Masakazu Horikiri: Research Report of Animal Feed, 28, 59 (2003)
Health Survey Division, Environmental Health Department, Environment Agency: Chemical Substances Analysis Method Development Research Report, 1988, 80 (1989)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (μg/kg)	Replicate	Spike recovery (%)	Repeatability RSD (% or less)
Formula feed for adult chicken	50-500	3	93.5-93.9	4.3
Formula feed for lactating pig nurturing	50-500	3	100.8-103.9	7.1
Formula feed for fattening beef cattle	50-500	3	90.2-95.3	9.0

• Collaborative study

Sample type	No. of labs	Spike concentration (μg/kg)	Spike recovery (%)	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
Formula feed for lactating pig nurturing	6	50	91	8.9	11.7	0.53

• Limit of quantification: sample content: 5 μg/kg

«Notes and precautions»

[1] Due to high ethyl acetate content, solution cannot be dehydrated with anhydrous sodium sulfate. Dehydrate with anhydrous sodium sulfate after reducing water solubility to organic solvent by adding hexane.

[2] Presence of acetic acid generated by hydrolysis of ethyl acetate causes the decline of recovery rate by inhibiting adsorption to cation-exchange resin. Ethyl acetate may be removed by adding 10 mL of hexane and concentration under reduced pressure about three times.

[3] The use of vacuum manifold enables to raise flow rate up to 1 mL/min.

[4] Organic tin compounds are adsorbed by cation-exchange resin through ion-exchange reaction, but is not adsorbed by anion-exchange resin.

[5] Separating funnel may be substituted with stoppered test tube. In this case, the tributyltin chloride eluate is received in the stoppered test tube, add 30 mL of water and 5 mL of hexane – cyclohexane (1 : 1), shake for 5 minutes and allow solution to settle, and transfer hexane – cyclohexane layer (upper layer) to 50 mL stoppered Erlenmeyer flask using Pasteur pipette. Add 5 mL of hexane – cyclohexane to remaining water layer (lower layer), repeat procedure and allow solution to settle, transferring the hexane – cyclohexane layer (upper layer) to Erlenmeyer flask.

[6] Water remaining in concentrated solution may decompose *n*-propyl magnesium bromide, causing the decline of recovery rate. Solution should be completely dehydrated.

[7] Addition of acid may result in bumping. Carefully add one drop at a time with the solution cooled.

[8] Addition of methanol prevents large quantities of THF from contaminating the hexane extract.

[9] Subsequent procedures may be performed using only the stoppered test tube. In this case, add 20 mL of water, 7 mL of methanol and 10 mL of hexane to test tube, shake for 5 minutes and allow solution to settle. Transfer hexane layer (upper layer) to 50 mL stoppered Erlenmeyer flask using Pasteur pipette. Add 10 mL of hexane to remaining water layer (lower layer), shake for 5 minutes and allow

solution to settle, transferring the hexane layer (upper layer) to stoppered Erlenmeyer flask.

[10] Quantification will not be a problem in most cases, but in the event the determination is difficult due to interfering peaks and other issues, additionally perform purification using Florisil minicolumn. Wash Sep-Pak Plus Florisil cartridge with 10 mL of hexane, transfer GC sample solution to minicolumn using Pasteur pipette and flow down. Then add 10 mL of hexane – diethyl ether (99 : 1) to elute propyl tributyltin. Concentrate the entire eluate under reduced pressure in a water bath at 40 °C or below and adjust it to fixed volume of 2 mL with hexane to prepare the sample solution subject to the gas chromatography.

[11] Although specified injection volume is 2 µL, it should be reduced to 1 µL for the protection of equipment and analysis column if sufficient sensitivity is achieved.

[12] Optimum flow rate differs depending on model of GC. Sensitivity tends to increase when supply of hydrogen gas is increased in relation to the air flow.

[13] Figure 7.4-2 shows an example of chromatogram.

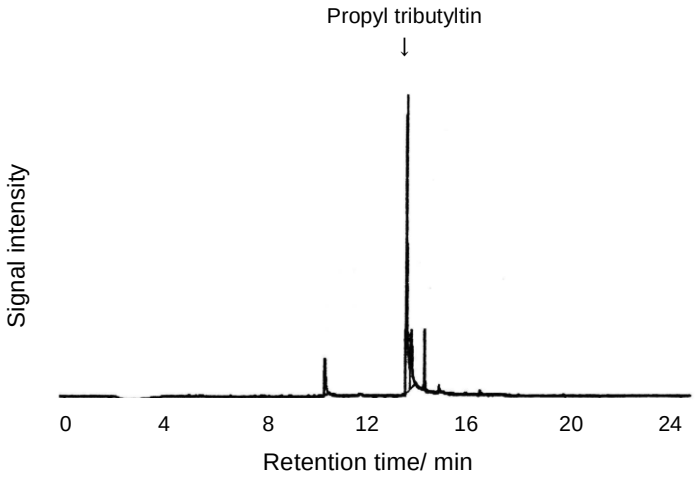
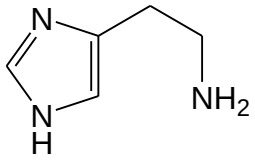


Figure 7.4-2 Chromatogram of formula feed for swine spiked with tributyltin chloride at 50 µg/kg

5 Histamine



2-(3*H*-imidazol-4-yl)ethanamine

$C_5H_9N_3$ MW: 111.145 CAS No.: 51-45-6

[Summary of histamine]

Histamine is generated by decarboxylation of histidine, an essential amino acid. Histamine is present in the somatic cells of most animals. However, the substance is known to cause food poisoning of allergic nature when ingested in large quantities. Significant quantities of histidine is contained in red-fleshed fish, and in the event the substance reacts with microbes capable of producing histidine decarboxylation enzymes (histamine production microbes), the resulting histamine may cause food poisoning. Furthermore, once produced, the histamine is not decomposed by heat processing.

This document provides for methods utilizing capillary electrophoresis and absorption spectrophotometry, but dedicated analyzers are recently made available on commercial basis (Histamine meter HM-505 - Central Kagaku Corp.) and applicable to rough analysis.

[Method listed in the Analytical Standards of Feeds]

1 Capillary electrophoresis[*1] [Analytical Standards of Feeds, Article 5.1, Chapter 7]

Scope of application: fish meal

A. Reagent preparation

Histamine standard solution. Weigh 82.8 mg of histamine dihydrochloride [$C_5H_9N_3 \cdot 2HCl$] in a 50 mL volumetric flask and dissolve in trichloroacetic acid solution (5 %), adding it to the marked line to prepare histamine standard stock solution (1 mL of the solution contains 1 mg of histamine).

At the time of use, dilute a certain quantity of the standard stock solution accurately with trichloroacetic acid solution (5 %), and prepare several histamine standard solutions containing 0.5 to 10 μg of histamine per 1 mL.

B. Quantification

Extraction. Weigh 10.0 g of analysis sample in a 200 mL stoppered Erlenmeyer flask, add 100 mL of trichloroacetic acid solution (5 %) and shake for 30 minutes to extract histamine. Transfer extract to a 50 mL stoppered centrifugal tube and centrifuge for 5 minutes at 1,500×*g*, diluting a certain amount of supernatant accurately with trichloroacetic acid solution (5 %). Introduce diluted solution to filter cup[*2] equipped with ultrafiltration membrane (molecular weight cut off: 30,000) and connected to plastic centrifugal tube (capacity: 1.5 mL)[1], perform centrifugal filtration at 5,000×*g* for 15 minutes to prepare sample solution subject to capillary electrophoresis[*3].

Capillary electrophoresis. Inject the sample solution and respective histamine standard solutions into the capillary electrophoresis, and extract electropherogram[*4].

Example of measurement conditions:

Column: Fused silica capillary column (inner diameter 75 μm, available length 56 cm, total length 64.5 cm)

Buffer: 0.05 mol/L sodium phosphate buffer (pH 2.5)[*5]

Voltage: +15 kV

Column temperature: 20 °C

Sample injection: Positive-pressure injection (5,000 Pa, 4 s)

Detector: UV spectrophotometer (wavelength 210 nm)

Column washing: Prior to the each injection of the sample solution and respective histamine standard solutions to the capillary electrophoresis system, wash the system for 2 minutes with water, 2 minutes with sodium hydroxide solution, again with water for 2 minutes, and finally with buffer for 3 minutes.

Calculation. Calculate the peak area from obtained electropherogram[2] to prepare calibration curve and determine the amount of histamine in the sample.

* 1. Water to be used in the process maintains a electrical conductivity of less than 5.6 μS/m (specific resistance of over 18 MΩ·cm).

2. Microcon YM-30 (Millipore Corporation) or equivalent

3. Supernatant of the filtrate obtained shall be used as the sample solution.

4. If sample yields inappropriate peak configuration, further dilute sample solution.

5. Agilent Technologies or equivalent

«Summary of analysis methods»

This analysis involves the extraction of histamine from fish meal using trichloroacetic acid solution (5 %), filtering the extract with ultrafiltration membrane (molecular weight cut off: 30,000) to quantify with a capillary electrophoresis system.

Figure 7.5-1.shows flow sheet of analysis method.

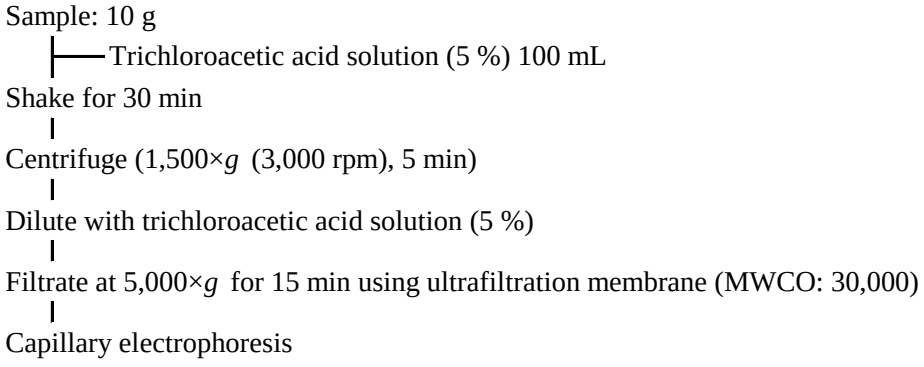


Figure 7.5-1 Flow sheet of histamine analysis

Reference: Koji Aoyama, Yuzo Ono, Eiichi Ishikuro: Research Report of Animal Feed, 28, 51 (2003)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Replicate	Spike recovery (%)	Repeatability RSD (% or less)
White fish meal	50-500	3	96.2-101.0	5.5
Atka mackerel meal	50-500	3	97.6-98.4	6.4
Japanese sand lance meal	50-500	3	93.3-99.9	5.8
Protein adjusted fish meal	50-500	3	99.4-103.8	4.2

• Collaborative study

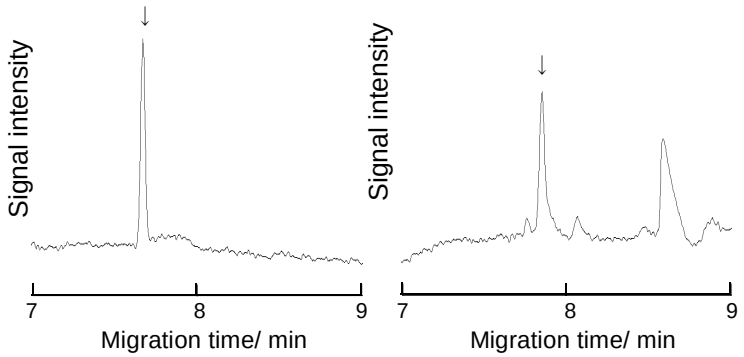
Sample type	No. of labs	Spike concentration (mg/kg)	Spike revovery (%) (Data (mg/kg))	Intra-lab repeatability RSD_r (%)	Inter-lab reproducibility RSD_R (%)	HorRat
Formula feed for lactating pig nurturing	7	Natural contamination	(245)	2.6	5.5	0.39
Formula feed for lactating pig nurturing	7	100	99.6	3.5	3.7	0.17

• Limit of quantification: 10 mg/kg in the sample

«Notes and precautions»

[1] Quantity of solution to be introduced to filter cup equipped with ultrafiltration membrane shall be several times more than required for the capillary electrophoresis.

[2] Figure 7.5-2. shows an example of electropherogram.



Standard solution Fish meal

Figure 7.5-2 Electropherogram of histamine for standard solution and fish meal (Arrows indicate the peaks of histamine)

2 Absoption spectrophotometry [Analytical Standards of Feeds, Article 5.2, Chapter 7]

A. Reagent preparation

1) Histamine hydrochloride standard solution. Weigh accurately 50 mg of histamine dihydrochloride [$C_5H_9N_3 \cdot 2HCl$], place in a 100 mL brown volumetric flask, add 50 mL of hydrochloric acid (0.1 mol/L) to dissolve. Then add the same solvent to the marked line to prepare histamine hydrochloride standard stock solution. (1 mL of the solution contains 0.5 mg of histamine dihydrochloride).

At the time of use, dilute a certain amount of the standard stock solution accurately with hydrochloric acid (0.1 mol/L), and prepare several histamine hydrochloride standard solutions containing 1.25 to 10

μg of histamine dihydrochloride per 1 mL.

2) Buffer

i) Acetate buffer[1] of 2 mol/L. Dissolve 115 mL of acetic acid and 40 g of sodium hydroxidein water, and adjust 1L.

ii) Acetate buffer[1] of 0.2 mol/L. Dilute a certain amount of acetate buffer of 2 mol/L 10-fold with water.

iii) Sodium carbonate buffer[2]. Dissolve 4 g of sodium carbonate and 2 g of sodium hydrogen carbonate in water, and adjust to 100 mL.

3) Coloring reagent. Dissolve 0.1 g of p-nitroaniline in hydrochloric acid (0.1 mol/L)[3], adjusting quantity of solution to 100 mL. Cool 15 mL of the solution with ice and add 0.1 mL of sodium nitrite solution (15 w/v%)[4]. (To be prepared prior to use.)

4) Neutral alumina. Desiccate neutral alumina for column chromatography (particle size: 63~200 μm (230~70 mesh))[*1] at 120 °C for 2 hours.

B. Quantification

Extraction. Place 20.0 g of analysis sample into blender cup. Add 70 mL of water and 30 mL of trichloroacetic acid solution[5] (8 w/v%), and homogenize for 5 minutes to extract. Then filter with a filter paper (No. 2) to prepare sample solution subject to column treatment I.

Column treatment I. Fill column tube (inner diameter: 14 mm) with 30 g of neutral alumina by dry process, and prepare column.

Transfer 50 mL of the sample solution to the column, discard 1 to 2 mL of the initial effluent and transfer 10 mL of the subsequent effluent accurately into a 50 mL beaker. Add 10 mL of 0.2 mol/L acetate buffer to the effluent for use as sample solution for the column treatment II procedure.

Column treatment II. Pour 3 g of ion-exchange resin[*2] suspended with water into column tube (caliber: 10 mm)[7], sequentially adding 5 mL of hydrochloric acid (1 mol/L), 5 mL of water and 5 mL of 2 mol/L acetate buffer to the column. Adjust column by flowing down until the surface of the solution reaches at 3 mm above the top of the resin.

Transfer sample solution to column, wash emptied beaker with small quantities of the 0.2 mol/L acetate buffer, adding the washings to the column. Flow down until the surface of the solution reaches at 3 mm above the top of the resin[8], then add 50 mL of the 0.2 mol/L acetate buffer and flow down in the similar way.

Place a 100 mL volumetric flask beneath the column. Add 30 mL of hydrochloric acid (0.2 mol/L) to the column to dissolve the histamine[9] and add hydrochloric acid (0.2 mol/L) to the marked line on the flask. This solution shall be used as the sample in the subsequent nitrous acid treatment.

Nitrous acid treatment and color development. Transfer 5 mL of the sample solution accurately into a test tube, add 0.5 mL of sodium nitrite solution (15 w/v%)[10], 0.5 mL of hydrochloric acid (0.1 mol/L) and 0.1 mL of potassium bromide (5 w/v%) then shake. Allow solution to react for 10 minutes in water bath at 60 °C, and stand to cool.

Add several drops of thymol blue reagent to the solution. Alkalize solution by adding sodium hydroxide solution (10 w/v%)[11] and depressurize in water bath at 50 °C for 5 to 10 minutes to volatilize

ammonia[12].

Adjust pH of residual solution to 5 to 6 with hydrochloric acid (0.1 mol/L)[13], add 1 mL of sodium carbonate buffer, shake and cool solution with ice for 10 minutes to allow color to develop[14].

Add 10 mL of ethyl acetate accurately into the colored solution, shake and allow to settle. Discard the water layer (lower layer) and wash ethyl acetate layer (upper layer) twice using 5 mL of sodium carbonate solution (1 w/v%) respectively to prepare sample solution subject to the measurement.

Concurrently, process respective histamine hydrochloride standard solutions in the similar way of the sample to prepare standard solutions subject to the measurement.

Measurement. Transfer sample solution into an alternate test tube, dehydrate with an appropriate quantity of sodium sulfate (anhydrous). Add 2 to 3 drops of ammonia water and shake. Promptly measure absorbance at wavelength of 550 nm with ethyl acetate as a reference.

Concurrently, measure absorbance for respective standard solutions in the similar way[15] of the sample solution.

Calculation. Prepare a calibration curve from the absorbance obtained for the standard solution and determine the amount of histamine [$C_5H_9N_3$] in the sample according to the following equation.

$$\text{Amount of histamine in the sample (mg/kg)} = A \times 0.603^{[16]} \times 10$$

A : Weight of histamine dihydrochloride obtained from the calibration curve (μg)

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

2. Amberlite CG-50 type-1 (Rohm and Haas) or equivalent

«Summary of analysis methods»

This quantification method involves the extraction of the histamine content in the sample using water added with trichloroacetic acid, refining solution in neutral alumina and anion exchange columns. The histamine is then determined in colorimetry by applying diazo reaction.

Due to the complexity of the procedure, liquid chromatography utilizing post-labeling method is generally used in recent analysis as an alternative method.

References: Atsuhi Tsuda: Nippon Suisan Gakkaishi, 25, 451 (1959)

Eiichi Ishikuro, Yoshitsugu Tanaka, Tetsuo Chihara: Research Report of Animal Feed, 7, 177 (1981)

«Notes and precautions»

[1] Adjust pH to 4.6 using acetic acid or sodium hydroxide.

[2] Seal for storage.

[3] Heating is needed as *p*-nitroaniline is difficult to dissolve.

[4] *p*-Nitroaniline is diazotized in acidic environment of hydrochloric acid by adding sodium nitrite.

[5] Used as protein coagulant.

[6] If difficult to filtrate, centrifuge sample prior to filtration.

[7] Stir with glass rod while pouring to prevent the mixing of air bubbles.

[8] Outflow to be maintained at rate of approximately 2 to 3 mL/min.

[9] In case of imported fish meal with high histamine content, dilute additionally about 20-fold, while in

case of formula feed and other samples with low histamine content, use a 50 mL volumetric flask to receive eluate.

[10] To be prepared at the time of use.

[11] Add sodium hydroxide until solution turns blue, but it is recommendable to apply an uniform quantity for usability in subsequent procedures.

[12] When using rotatory evaporators, employing test tubes of the uniform joint diameter will facilitate procedure. However, aspiration using a sucker through rubber caps is also acceptable.

[13] Procedure with highest potential for error within the entire process. Color of solution should be green, to be confirmed using universal pH test paper in the event the hue is uncertain. If solution turns yellow by excess hydrochloric acid, add diluted sodium hydroxide solution to compensate.

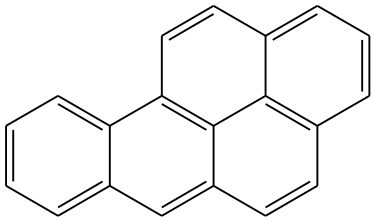
[14] Couple diazo compounds to generate coloring substance.

[15] Conduct procedures subsequent to the nitric acid treatment, skip the elimination of ammonia.

[16] Coefficient for the conversion of histamine dihydrochloride to histamine:

$$0.603 = \frac{C_5H_9N_3 (111)}{C_5H_9N_3 \cdot 2HCl \quad (184)}$$

6 3,4-Benzpyrene



Benzo[*a*]pyrene
$C_{20}H_{12}$ MW: 252.31 CAS No.: 50-32-8

[Summary of 3,4-benzpyrene]

Many polycyclic aromatic hydrocarbons indicate carcinogenic properties. Of these compounds, 3,4-benzpyrene has one of the strongest carcinogenicity. In the context of feed ingredients, 3,4-benzpyrene is contained in various types of yeast and fish meal, but the content is insignificant.

[Method listed in the Analytical Standards of Feeds]

1 Fluorescence analysis[*1] [Analytical Standards of Feeds, Article 6.1, Chapter 7]

Scope of application: Torula and other yeasts for feed

A Reagent preparation[1]

1) 3,4-Benzpyrene standard solution. Weigh accurately 20 mg of 3,4-benzpyrene [$C_{20}H_{12}$], place in a volumetric flask, add 50 mL of benzene to dissolve. Further add same solvent to the marked line to prepare 3,4-benzpyrene standard stock solution. (1 mL of the solution contains 0.2 mg of 3,4-benzpyrene)[1].

At the time of use, dilute a certain quantity of the standard stock solution with benzene accurately, prepare several benzpyrene standard solutions containing 5 to 30 µg of 3,4-benzpyrene per 1 mL.

2) Alumina. Wash alumina for column chromatography (particle size: 63 to 200 µm (230~70 mesh))[*2] with 5 times mass of hydrochloric acid (1 mol/L) then with water, desiccate for 2 to 3 hours at 110 °C. Mix with 1 v/w% equivalent of water.

B Quantification

Extraction. Weigh 100 g of analysis sample into extraction thimble[*3] (diameter 45 mm, height 150 mm) and extract for 8 hours with 500 mL of benzene using a large Soxhlet extraction apparatus. Then, concentrate the extract to almost dryness under reduced pressure in water bath at 40 °C[2].

Transfer the residue to a 300 mL separating funnel A using 100 mL of cyclohexane, add 100 mL of dimethylsulfoxide and shake. Allow solution to settle[3] and transfer dimethylsulfoxide layer (lower layer) to an alternative 300 mL separating funnel B.

Add 100 mL of cyclohexane to separating funnel B and repeat procedure, transferring dimethylsulfoxide layer to a 2 liter separating funnel C.

Add 100 mL of dimethylsulfoxide to separating funnel A and repeat procedure twice, introducing both

dimethylsulfoxide layers to separating funnel C.

Add 300 mL of cooled hydrochloric acid (1+4) solution to dimethylsulfoxide layer, followed by 400 mL of cyclohexane and shake solution. Transfer the dimethylsulfoxide – hydrochloric acid layer (lower layer) to a 1 liter separating funnel D while the cyclohexane layer (upper layer) is washed twice with 500 mL of warm water[4] and transfered into a 2 liter recovery flask.

Add 400 mL of cyclohexane to separating funnel D and shake. Discard dimethylsulfoxide – hydrochloric acid layer (lower layer) and wash cyclohexane layer (upper layer) twice with 500 mL of warm water. Transfer cyclohexane layer to the recovery flask and concentrate cyclohexane layer to approximately 30 mL under reduced pressure in a water bath at 40 °C. Dehydrate concentrate with 5 g of sodium sulfate (anhydrous) and filtrate into a 100 mL pear-shaped flask through a funeel stuffed with cotton wool[5]. Wash the emptied recovery flask and funnel with cyclohexane, transfer the washings to the flask. Concentrate filtrate to approximately 1 mL under reduced pressure in a water bath at 40 °C to prepare sample solution subject to column treatment.

Column treatment. Fill column tube (inner diameter: 20 mm) sequentially with 50 g of alumina and 15 g of sodium sulfate (anhydrous) by dry process to prepare column.

Place a 300 mL pear-shaped flask under the column and transfer sample solution[6] to the column. Wash emptied pear-shaped flask with small quantities of cyclohexane and add washings to the column. Allow solution to flow down until the surface of the solution reaches at 3 mm above the top of the column packing materials.

Sequentially add 100 mL of hexane – diethyl ether (1 : 1) and 100 mL of diethyl ether to the column to elute 3,4-benzpyrene, concentrating the eluate to approximately 0.5 mL under reduced pressure in a water bath at 30 °C to prepare sample solution[7] subject to two-step one-dimensional thin-layer chromatography.

Two-step one-dimensional thin-layer chromatography. Prepare two-step thin-layer plate[8] consisting of acetylated cellulose*4 and neutral alumina*5.

Apply entire quantity of sample solution onto thin alumina-layer in a band using microsyringe[9], concurrently applying 3,4-benzpyrene standard solution[10] to confirm retention factor of 3,4-benzpyrene.

Apply ethanol – diethyl ether – water (4 : 4 : 1) as developing solvent for 90 minutes to a height of 10 cm above the two-layer boundary zone and allow to air dry. Irradiate thin-layer with ultraviolet (365 nm)[11] and confirm zone of fluorescence indicating separated 3,4-benzpyrene on the acetylated cellulose thin-layer. Scrape off the zone and place within a 50 mL stoppered centrifugal tube[12]. Add 25 mL of methanol heated to 60 °C to the tube, centrifuge for 5 minutes at 1,500×*g* and transfer supernatant into a 100 mL pear-shaped flask.

Repeat procedure 3 times by introducing 25 mL each of methanol heated to 60 °C to the tube, respectively transferring the supernatant to the pear-shaped flask. Add 1 mL of *n*-hexadecane to supernatant and concentrate to approximately 1 to 2 mL under reduced pressure in a water bath at 60 °C. Transfer concentrate to 5 or 10 mL volumetric flask using benzene[13]. Add benzene to marked line on the flask to prepare sample solution subject to measurement[14].

Measurement[15]

1) Identification. Measure the fluorescence spectrum and excitation spectrum for the sample solution and 3,4-benzpyrene standard solution, compare the peak wavelength and fluorescence intensity to identify 3,4-benzpyrene in the sample solution.

2) Quantification. Measure fluorescence spectrum of sample solution in the vicinity of 405 nm upon excitation wavelength of 368 nm.

Concurrently measure fluorescence spectrum for respective 3,4-benzpyrene standard solutions under the same conditions. Prepare calibration curve by narrow baseline method[*6] [16] and determine the amount of 3,4-benzpyrene in the sample.

* 1. Solvent to be of nonluminescent reagent grade or equivalent.

2. Aluminiumoxid standardisiert Art. 1097 (Merck) or equivalent

3. No. 84 (Toyo Roshi Kaisha) or equivalent

4. Acetylcellulose for thin-layer chromatograph (Wako Pure Chemical Industries) or equivalent

5. Contain 9 % of calcium sulfate as binder. Aluminiumoxid G Art.1090 (Merck) or equivalent.

6. Designating fluorescence intensity at peak wavelength and at ±3.5 nm from the peak wavelength in the fluorescence spectrum as I_0, $I_{+3.5}$ and $I_{-3.5}$respectively, the generation of the calibration curve and the quantification of the sample solution is based on the value F obtained from the following equation:

$$F = I_0 - \frac{(I_{+3.5} + I_{-3.5})}{2}$$

«Summary of analysis methods»

In this method, 3,4-benzpyrene in the sample is extracted with benzene, purified by liquid-liquid extraction, column treatment and thin-layer chromatography, further quantified measuring the fluorescence spectrum in the vicinity of 405 nm upon excitation in wavelength 368 nm utilizing the properties of 3,4-benzpyrene to emit strong fluorescence.

Limit of quantification for the method is approximately 0.1 µg/kg of sample content .

Due to the complexity of the procedure, liquid chromatography is generally used in recent analysis as an alternative method (Standard Methods of Analysis for Hygienic Chemistry, etc.).

References: Hidetsuru Matsushita and Yoshio Esumi: Analytical Chemistry, 21, 1594 (1972)
Eiichi Ishikuro: Research Report of Animal Feed, 5, 50 (1979)

«Method validation»

• Spike recovery and repeatability

Sample type	Spike concentration (µg/kg)	Replicate	Spike recovery (%)	Repeatability RSD (% or less)
Yeast for feed	0.5-1	5	97.2-98.7	16.7

• Limit of quantification: 0.1 µg/kg in the sample

«Notes and precautions»

[1] Solvents of Guaranteed Reagent grade shall be used after rectification, with certain fractions of the

distillate subjected to the confirmation of nonluminescence in the range of 400 to 430 nm for excitation wavelength of 365 nm using a fluorospectrophotometer prior to conducting the analysis. If using commercially available products, benzene, cyclohexane and methanol shall be of fluorescence analysis grade (“Luminazohru”; Dojindo Laboratories) or liquid chromatograph grade, dimethyl sulfoxide (DMSO) shall be of ultraviolet absorption spectrophotometry grade (“Dotite Spectrosol”; Dojindo Laboratories) or equivalent, and diethyl ether shall be of residual pesticide/PCB testing grade respectively. Sodium sulfate (anhydrous) shall also be of fluorescence analysis grade.

[2] Concentrate to 1 to 2 mL.

[3] Shake it gently, as vigorous shaking may cause solution to emulsion.

[4] Sufficient results can be obtained by gently shaking.

[5] Filtrate with a funnel stuffed with cotton wool, wash funnel with small quantities of cyclohexane, adding washings to filtrate.

[6] After transferring entire quantity of extracted concentrate, wash pear-shaped flask with small quantities of cyclohexane using syringes and add washings to the column.

[7] When utilizing liquid chromatograph, use this sample solution.

Example of measurement conditions:

Detector: Excitation wavelength 384 nm, Fluorescence wavelength 406 nm

Column: μBondapak C_{18}

Eluent: Acetonitrile – water (7 : 3)

Flow rate: 1.5 mL/min

[8] Two-step applicators are available from Yazawa, Mitamura-Riken and other sources.

Two-step one-dimensional thin-layer chromatography is characterized by the superior resolution despite application of large quantities of samples. Alumina-G layer is a thin-layer dedicated to the application of sample solutions and is not involved in the separation. Accordingly, the specification of exact conditions for application of sample is unnecessary, allowing for the application of up to 1 to 2 mL.

Sample applied to alumina-G is easily dissolved in developing solution and transported to the two-step boundary, forming a narrow line 1 to 2 mm in width. The separation on the acetylated cellulose enables the attainment of high resolution.

Use two-step applicator to dispense alumina-G slurried with 10 % methanol into larger tank (16×20 cm) to be applied onto the plate glass (200×200 mm) to a thickness of 0.3 mm. Allow material to settle overnight and activate by subjecting to 110 °C for 1 hour. Use upon cooling off to ambient temperature within a desiccator.

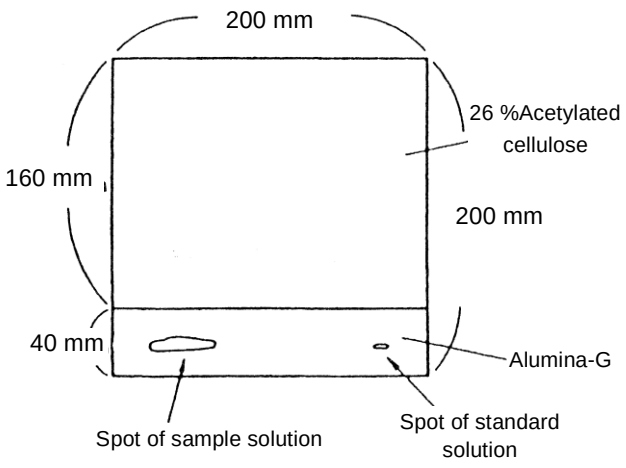


Figure 7.6-1 2-step 1-dimensional thin-layer plate

[9] Use syringe to wash pear-shaped flask with trace amount of diethyl ether, also applying the washings to the plate.

[10] Figure 7.6-1 shows the thin-layer plate of two-step one-dimentional.

[11] UV light (Products are same as MANASLU, TOSHIBA Corporation and IRIE SHOKAI or equivalent) can be used.

[12] Scratch off the fluorescence band by spurtle and/or spatula etc.

[13] Wash pear-shaped flask with small amounts of benzene, adding washings to the volumetric flask.

[14] Particles of thin-layer absorbents mixed in the sample solution may cause light scattering. In case the presence of suspended solids are confirmed, centrifuge sample prior to fluorescence measurement.

[15] Peak wavelengths and intensity of fluorescence/excitation spectrum for solutions tend to differ depending on lots of reagents. Accordingly, the solvents used for sample and standard solutions should be taken from the same lot.

Pattern of fluorescence/excitation spectrum is influenced by the slit width of spectrograph. Analysis should be conducted with the width of the fluorescence and excitation slits reduced as much as possible.

[16] Narrow baseline method

In order to determine height of characteristic fluorescence peak for the substance to be quantified, define line connecting two points respectively detached from the peak by 3.5 nm as the baseline (called the narrow baseline), measuring height of peak in relation to the line for quantification. (Figure 7.6-2)

$$F = I_{406.0} - \frac{1}{2}\left(I_{402.5} + I_{409.5}\right)$$

Value F is calculated for the preparation of the calibration curve to determine the concentration of the sample solution.

Calibration curve based on the value F indicates linearity in the range of 5 to 40 ng/mL. By altering sensitivity of fluorospectrophotometer, similar linearity will be achieved in the range of 1 to 20 ng/mL.

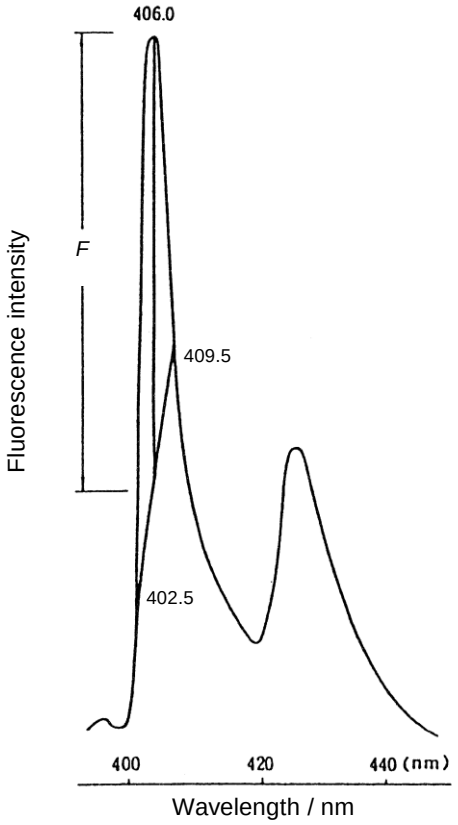


Figure 7.6-2 Spectrum of 3,4 - benzpyrene standard solution by narrow baseline method