Chapter 4　Inorganic constituents (including organic metal compounds)

【Summary of inorganic constituents】

In the context of livestock nutrition science, inorganic constituents are generally referred to as minerals (Inorganic elements) and occasionally includes mineral salts. Biological organisms are said to be composed of over 40 elements, and the term mineral widely covers miscellaneous elements other than the primary constituents, namely carbon, hydrogen, oxygen, and nitrogen.

Inorganic constituents contained in feed are both diverse and extremely numerous, although the number of constituents regarded to be essential in nutrition science can be narrowed down to approximately 20 items. Elements with relatively high requirements include calcium, magnesium, sodium, potassium, phosphorus, sulfur and chlorine. Trace elements or elements with relatively low requirements include iron, copper, zinc, manganese, cobalt, molybdenum, iodine and selenium. While inorganic constituents are vital components of the livestock physiology, functions assigned to each mineral differs. Primary constituents of the scleroid as represented by the skeletal structure of livestock consist of calcium, magnesium and phosphorus. Colligating with proteins and lipids, magnesium, phosphorus, sulfur and manganese serve as components in muscles, viscera and nerves. The elements calcium, magnesium, sodium, potassium, phosphorus and chlorine dissolves in body fluids, influencing the pH of the fluids and the activation of enzymes as well as the functions of the muscles and nerves. Enzymes, coenzymes, hormones and vitamins incorporate elements including iron, copper, zinc, manganese, cobalt, molybdenum, iodine and selenium.

Japanese livestock nutrition science views the minerals sodium, chlorine, calcium and phosphorus as elements with the potential of shortage. The shortage of these elements are supplemented in the form of sodium chloride, calcium carbonate and calcium phosphate mixed into mixed forage. Insufficiencies of other minerals including magnesium, iron, copper, zinc and manganese are resolved by augmenting the feed with mineral pre-mix additives containing these elements.

The contents of this chapter also spans over the hazardous chemical elements of cadmiumLead, mercury and arsenic, as well as chromium and selenium, hazardous when ingested excessively but causing disorders when deficient. Additionally, established methods of quantification for organic metal compounds are referenced within this chapter. Explanation of nitrate nitrogen, the causative agent for nitrate nitrogen poisoning in cattle is also provided within this chapter.

In order to quantify inorganic constituents, organic substances contained in the sample must first be degraded and removed. Roughly divided, the methods used in the degradation and removal of organic substances can be categorized into dry and wet degradation. Dry degradation is selected for phosphorus, copper and cadmium, typically involving several hours or overnight ashing of the sample in electrical furnaces. Wet degradation, on the other hand, is performed for samples incompatible with dry degradation and the preprocessing of mercury and arsenic which may volatilize if heated in electrical furnaces.

Additionally, microwave pretreatment equipment offering easier means of conducting accurate

preprocessing is gradually gaining popularity.

《Summarized table on the degradation and removal of organic content pertaining to the analysis of inorganic constituent of feed》

Dry degradation (inorganic constituent listed within the same column of the table can by quantified using the same sample solution.)

Name	Volume of sample	Heating temperature	Container
Magnesium, Iron, Copper, Zinc, Manganese, Cadmium, Lead	1-10 g	Less than 500 ℃	Borosilicate glass tall beaker
Chrome (atomic absorption photometry)	10 g	Less than 501 ℃	Borosilicate glass tall beaker
Potassium, Sodium	2-10 g	Less than 502 ℃	Platinum dish
Calcium, Phosphorus	2-10 g	550~600 ℃	Borosilicate glass tall beaker
Bromine	10 g	550~600 ℃	Nickel crucible

2 Wet degradation: Mercury, Arsenic, Selenium

Cadmium, Cd

【Summary of Cadmium】

Cadmium is yielded as a content of zinc ore, and is widely distributed within the natural environment. The substance sees applications as basic ingredients in pigment, chloroethylene, stabilization agent and galvanizing. Cadmium is viewed as the causative agent for the itai-itai disease that frequented in the watershed of Jintsu-gawa in Toyama prefecture.

Cadmium is found in most food products, with a relatively high concentration of 0.06 to 0.2 ppm in brown rice. Average Japanese is said to ingest 50% of the internal cadmium from rice and 11% from fishery products.

The Food Sanitation Act limits the acceptable content of cadmium and cadmium-based compounds to less than 1.0 ppm for brown rice (scheduled for reduction to 0.4 ppm). The Act prohibits the contamination of beverages by cadmium.

The cadmium content in typical feed ingredients are 0 to 0.5 ppm for cereals, 0.5 ppm for fish meal and about 3 ppm in yeast.

In a press release dated May 2nd, 2003, the Fisheries Agency announced the presence of high concentration of cadmium in the viscera among some mollusks and crustacea, and salted fish products using such mollusks and crustacea also indicated relatively high cadmium content (survey conducted on 1,336 samples of fish, crustacea and fishery products).

The cadmium content in primary fishery products are as indicated below: (Units: ppm)

Liver of Japanese flying squid	6.6 -96	(Avg. 33.9)
Bowel of Red snow crab	2.3 - 23	(Avg. 11.7)
Hepatopancreas of Scallop	1.3 -16	(Avg. 5.8)
Visceraof turban shell	1.2 -9.5	(Avg. 4.7)
Visceraof abalone	2.2 -5.6	(Avg. 3.1)
Hepatopancreas of Horsehair crab	0.8 -3.5	(Avg. 2.0)
Roe and Gonadsof Scallop	0.3-4.9	(Avg. 1.8)
Bloody clam	0.6-0.7	(Avg. 0.6)
Corbicula clam	0.03-0.8	(Avg. 0.4)
Japanese flying squid	0.03-1.3	(Avg. 0.3)

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

[Hazardous Substances guidelines for feed]

Formula feed and grass hay etc. : 1.0 ppm

Fish meal, meat meal, meat and bone meal : 2.5 ppm

【Method listed in the Feed Analysis Standards】

1 Solvent extraction method, Note 1 [Feed analysis standards, Chaper 4, Section 1, 12.1]

A Reagent preparation

Cadmium standard solution. Accurately measure 0.1 g of cadmium [Cd] into a tall beaker, add 50 mL of nitric acid (1:9) and heat to dissolve. Then boil solution to remove nitrogen oxide and allow to cool off. Transfer the solution to a 1,000 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as cadmium standard stock solution. (1 mL of the solution contains 0.1 mg of cadmium) [1].

Accurately dilute fixed quantities of the standard stock solution with hydrochloric acid (0.1 mol/l) [2] to prepare cadmium standard solutions containing 0.2 to 1 μg of cadmium per 1 mL of the solution prior to use.

B Preparation of the sample solution

Accurately pour 1 to 10 g of the analysis sample into a 100 mL tall beaker made of borosilicate glass [3] and carbonize through moderate heating. Then ashed by heating at temperatures at under 500 °C[4]. Add minor quantities of water and 10 mL of hydrochloric acid[5] to the residue, adjust quantity to 30 mL with additional water and allow to cool off after boiling for several minutes. Entire amount of the solution is then transferred to a 100 mL volumetric flask with water, adjusting the quantity by adding water to the marked line. Filter the solution using a paper filter (No. 6) to be used as the sample solution.

Concurrently perform the preparation procedure without the sample to produce blank test solutions[6].

C Quantification

Accurately fixed quantities of the sample solution (less than 10 μg of cadmiumLess than 30 mL in volume) into a 100 mL separating funnel containing 14 mL of phosphoric acid, add 5 mL of potassium iodide solution (68 w/v%) and adjust quantity to 50 mL with water. Shake flask gently and allow to settle for 5 minutes.

Pour 10 mL of 4-methyl-2-pentanone[7] into the separating funnel, acutely shake and allow solution to settle. Measure absorbance of 4-methyl-2-pentanone layer (upper layer) for wavelength 228.8 nm emitted by acetylene-air flame using an atomic absorption spectrometer [8].

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently conduct absorbance measurement for respective cadmium standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate the cadmium content of the sample.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis.

《Summary of analysis method》

The analysis sample is dry degraded by ashing, and wet degraded into a sample solution. The

solution is then imparted with strong acidity by adding either phosphoric or sulfuric acid (the described method utilizes phosphoric acid). Cadmium is iodized with potassium iodide, to be extracted using 4-methyl-2-pentanone (MIBK). The atomic absorption of the extract is measured at the wavelength of 228.8 nm. Concentration of the cadmium within the sample is calculated based on the calibration curve obtained for cadmium standard solutions.

Although direct measurement of the atomic absorption for the sample is difficult for solutions based on 0.1 mol/l hydrochloric acid, sensitivity improves for MIBK extracts and allows for measurement in stable conditions. Additionally, the measurement sensitivity is enhanced by a factor of 100 for flame-less atomic absorption equipment in comparison to the conventional flame method.

Feed analysis standard description progress 【0】New

《Analysis validation》

・Spike recovery and repeatability

1) Background adjustment method: D_2 lamp method

Sample type	Spike concentration (mg/kg)	Repeat	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for broiler	0.5-1.0	3	129.3~129.7	7.3
Formula feed for beef	0.5-1.0	3	127.3~131.3	4.8
Chicken meal	0.5-1.0	3	129.3~130.0	9.4
Feather Meal	0.5-1.0	3	122.0~123.3	4.3
Fish meal	0.5-1.0	3	121.0~131.3	3.3

2) Background adjustment method: Zeeman method

・Collaborative study

Sample type	No. of labs	Spike concentration (mg/kg)	Average spike recobery (Data(mg/kg))	Intra-lab repeatability RSDr(%)	Intra-lab repeatability RSD_R(%)	HorRat
Formula feed for dairy cows	6	0.5	107.2	2.7	2	0.11
Fish meal	6	Natural contamination	(0.486)	2.7	4.9	0.27

《Noes and precautions》

[1] Use of commercially available cadmium standard solutions 1,000 mg/l are acceptable.

[2] Concentration of commercially available hydrochloric acids (35 to 37 %) are approximately 11 mol/l, and requires preparation by diluting hydrochloric acid with 100X quantity of water (corresponding to hydrochloric acid (1:100)). However, the concentration of hydrochloric acid should be unified among the sample and standard solutions per se, and the use of hydrochloric acid (1 mol/l) is desirable.

[3] Although porcelain plates and other containers are acceptable, tall beaker made of pirex

glass is desirable upon consideration of the subsequent wet degradation procedure.

[4] Preliminary heating (carbonization) is difficult when tall beakers are employed. In this situation, the sample should directly be ashed using the electric furnace. Although the heating duration depends on the type and quantity of the analysis sample, the process generally requires several hours to an entire night. Occasionally, the ashing of samples may be insufficient even after an overnight ashing process (indicating large quantities of carbon residue). In this circumstance, the ashing process (dry ashing degradation) should be extended to completely convert the sample into ash.

[5] The use of hydrochloric acid may not be sufficiently effective to decompose the sample. In this circumstance, process sample with nitric acid-perchloric acid decomposition.

Likewise ash 10 to 20 g of analysis sample and add 5 mL respectively of nitric acid and perchloric acid. Cover container with watch-glass and allow sample to decompose to near-exsiccation. After cooling off sample, transfer to 100 mL volumetric flask using hydrochloric acid (0.1 mol/l) and adjust by adding water to the marked line. Filtrate solution with filter paper (No. 6) for use as sample solution in the analysis.

As residue carbon tends to absorb heavy metal, the carbon content of the sample must be completely degraded and removed.

Adequate precautions is required when adding perchloric acid as the substance is explosive in the absence of nitric acid.

[6] Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

[7] MIBK is toxic and requires the use of safety pipeters or dispensing burettes in handling the substance.

[8] MIBK extract is unstable, and must be promptly measured.

2 Simplified method[Note 1] 〔Feed analysis standards, Chapter 14, Section 1,12.2〕

A Reagent preparation

Cadmium standard solution. Accurately measure 0.1 g of cadmium [Cd] into a tall beaker, add 50 mL of nitric acid (1:9) and heat to dissolve. Then boil solution to remove nitrogen oxide and allow to cool off. Transfer the solution to a 1,000 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as cadmium standard stock solution. (1 mL of the solution contains 0.1 mg of cadmium) [1].

Accurately dilute fixed quantities of the standard stock solution with hydrochloric acid (0.1

mol/l) to generate multiple standard solutions containing 0.02 to 0.08 μg of cadmium per 1 mL when conducting measurement with low concentration samples, and 0.08 to 0.4 μg per 1 mL if using high concentration samples.

B Preparation of sample solution

Accurately pour 1 to 10 g of the analysis sample into a 100 mL tall beaker made of borosilicate glass [3] and carbonize through moderate heating. Then ashed by heating at temperatures at under 500 °C [4]. Add minor quantities of water and 10 mL of hydrochloric acid [5] to the residue, adjust quantity to 30 mL with additional water and allow to cool off after boiling for several minutes. Entire amount of the solution is then transferred to a 100 mL volumetric flask with water, adjusting the quantity by adding water to the marked line. Filter the solution using a paper filter (No. 6) to be used as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions [6].

C Quantification

Measure absorbance of wavelength 228.8 nm emitted by acetylene-air flame using an atomic absorption spectrometer.

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Then conduct absorbance measurement for respective cadmium standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate the cadmium content of the sample.

Measurement condition Example

Measured wavelength: 228.8 nm

Measurement method: Flame atomic absorption

Background correction: Continuous light source (D_2 lamp etc) method or Zeeman method

Following example of measurement conditions apply to background correction by continuous light source method (conditions applicable to Zeeman method indicated in parentheses).

Lamp current: 8 mA (9 mA)

Slit width: 0.5 nm (1.3 nm)

Burner height: 7 mm (5 mm)

Heating gas (Acetylene, flow rate: 1.8 l/min (2.0 l/min)

Supporting gas (air) flow rate: 15 l/min (15 l/min)

Note 1 Use atomic absorption analytical reagent grade acids in the analysis. .

《Summary of analysis method》

The analysis sample is dry degraded by ashing, and wet degraded into a sample solution using hydrochloric acid. The solution is then quantified with an atomic absorption spectrometer.

The performance of the atomic absorption spectrometer has improved significantly since the solvent extraction method described in section 1 was first included in the Feed analysis standards in 1977, allowing for the quantification of cadmium without the concentrate extraction procedure using 4-methyl-2-pentanone (MIBK).

Figure 4.1.12-1. shows flow sheet of the analysis method

Sample; 1-10 g
- Ashed at temperatures below 500 ᶜ

Residue
- Little water
- Hydrochloric acid 10 mL
- Add water to adjust to 30 mL
- Boil for several minutes, cool off

100 mL volumetric flask
- Transfer with water
- Add water to marked line
- Filtration (No. 6)

Atomic absorption spectrometer

Figure 4.1.12-1 Flow sheet of simplified analysis method for cadmium

References; Hisaaki Hiraoka, Yukiko Mori: Research Report of Animal Feed, 32, 4 (2007)
Feed analysis standard description progress 【28】New

《Analysis validation》

・Spike recovery and repeatability

1) Background adjustment method: D_2 lamp method

Sample type	Spike concentration (mg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Formula feed for broiler	0.5-1.0	3	107.7-110.0	8.3
Formula feed for beef	0.5-1.0	3	105.7-106.7	5.8
Chicken meal	0.5-1.0	3	107.7-108.7	6.0
Feather Meal	0.5-1.0	3	106.7-111.3	6.9
Fish meal	0.5-1.0	3	101.3-108.0	4.9

2) Background adjustment method: Zeeman method

Sample type	Spike concentration (mg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Formula feed for broiler	0.5-1.0	3	101.3-101.7	2.3
Formula feed for beef	0.5-1.0	3	101.3-102.0	3.0
Chicken meal	0.5-1.0	3	101.0-101.3	3.0
Feather Meal	0.5-1.0	3	102.7-103.3	1.1
Fish meal	0.5-1.0	3	100.7-104.0	5.8

・Collaborative study

Sample type	No. of labs	Spike concentration (%)	Spike recovery (%) (Data (mg/kg))	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
Formula feed for dairy cov	6	0.5	116.6	1.4	6.5	0.37
Fish meal	6	Natural contamination	(0.530)	4.8	12.8	0.73

・Quantification lower limit 0.1 mg/kg（SN ratio）

・Detection Lower limit: 0.05 mg/kg（SN ratio）

-To confirm precision of quantification at lower limit of quantification and lower limit of detection, results of the recovery test conducted is indicated as table 4.1.12-2.

The test results indicate the data obtained with an atomic absorption spectrometer for D_2 correction and Zeeman splitted spectrum correction respectively.

Table 4.1.12-2 Spike recovery test results for lower limit of detection and lower limit of quantification

Background correction		D_2 correction				Zeeman splitted spectrum correction			
Formura type		Formula feed for broiler		Fish Meal		Formula feed for broiler		Fish Meal	
Additive amount	Upper	Ave of quantitative value [a] (mg/kg)	SD [b] (mg/kg)	Ave of quantitative value [a] (mg/kg)	SD [b] (mg/kg)	Ave of quantitative value [a] (mg/kg)	SD [b] (mg/kg)	Ave of quantitative value [a] (mg/kg)	SD [b] (mg/kg)
	Lower	Ave. of recovery rate [a] (%)	RSD [c] (%)	Ave. of recovery rate [a] (%)	RSD [c] (%)	Ave. of recovery rate [a] (%)	RSD [c] (%)	Ave. of recovery rate [a] (%)	RSD [c] (%)
0.05		0.051	0.004	0.062	0.016	0.099	0.003	0.098	0.006
		103	7.9	123	26	96.7	7.5	110	13
0.1		0.019	0.015	0.112	0.017	0.099	0.003	0.098	0.006
		109	13	112	16	99.0	3.0	98.0	6.2

a) n-3: Average b) Standard deviation c) Relative standard deviation

《Notes and precautions》

[1] Use of commercially cadmium standard solutions 1,000 mg/l are acceptable.

[2] Although porcelain plates and other containers are acceptable, tall beaker made of pirex glass is desirable upon consideration of the subsequent wet degradation procedure.

[3] Preliminary heating (carbonization) is difficult when tall beakers are employed. In this situation, the sample should directly be ashed using the electric furnace. Although the heating duration depends on the type and quantity of the analysis sample, the process generally requires several hours to an entire night. Occasionally, the ashing of samples may be insufficient even after an overnight ashing process (indicating large quantities of carbon residue). In this circumstance, the ashing process (dry ashing degradation) should be extended to completely convert the sample into ash.

[4] The use of hydrochloric acid may not be sufficiently effective to decompose the sample. In this circumstance, process sample with nitric acid-perchloric acid decomposition.

Likewise ash 10 to 20 g of analysis sample and add 5 mL respectively of nitric acid and perchloric acid. Cover container with watch-glass and allow sample to decompose to near-exsiccation. After cooling off sample, transfer to 100 mL volumetric flask using hydrochloric acid (0.1 mol/l) and adjust by adding water to the marked line. Filtrate solution with filter paper (No. 6) for use as sample solution in the analysis.

As residue carbon tends to absorb heavy metal, the carbon content of the sample must be completely degraded and removed.

Adequate precautions is required when adding perchloric acid as the substance is explosive in the absence of nitric acid.

[5]Cover tall beaker with watch-glass

[6] Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

Chromium, Cr

【Summary of Chromium】

The toxicity of chromium is significantly higher in hexavalent chromium than in the trivalent form of the substance. Inflammation of skin, lungs and trachea caused by the attachment of chromium as well as ulcus and pulmonary cancer are well known consequences of subjection to the element. As the physiological activity of chromium in relation to the carbohydrate metabolism, especially the serum glucose level of animals became apparent in recent studies, and the usefulness of the substance is gradually being recognized. Trivalent chromium is a constituent of the glucose tolerance factor, facilitating the use of glucose though the secretion of insulin. The substance is said to support normal glycometabolism by lowering the blood sugar.

The beneficial properties of organic chromium in improving the reproduction performance of pigs is also evident. In this regard, it is assumed that the trivalent chromium acts on the pituritary gland and ovarium of the pigs, resulting in the increase in the number of child birth through the secretion of insulin.

Chromium is widely distributed in the soil, with a content of about 5 to 3,000 mg/kg (about 100 mg/kg on average), while the element is scarce in plants with a content of roughly 0.1 to 0.5 mg/kg. As with other trace elements, the chromium content in kernels is mostly distributed in the hull and the germ. Among feed ingredients, the chromium content is relatively high in brewing yeast and wheat.

Chromium is used as feed additive in some countries, and used as feed for pigs, cattle and sheep in the South African Republic, and chromium-rich yeast is used as additive to pig feed in Switzerland. Organic chromium (picoline acid chromium, etc) is added to feedstock in regions including US, Australia, eastern Europe, southeast Asia and Latin America. Chromium-rich yeast is added to mixed forage in Japan.

Digestibility is an important factor in the evaluation of feed nutritive value. Determined through digestion trials conducted on respective livestock, digestibility is conventionally measured using chromium oxide (Cr_2O_3) as the index substance. However, the notice of Consumer Product Safety Act No. 14272 issued by the Director General of the Food Safety and Consumer Affairs Bureau, MAFF, dated March 28th, 2008 admitted the use of acid insoluble ash (celite) for chicken, as well as celite and titanic oxide for pigs as substitute for chromium oxide as indicators.

【Method listed in the Feed Analysis Standards】

1 Atomic absorption spectrophotometry[Note 1]

Feed analysis standards, Chaper 4, Section1, 13.1

A Reagent preparation
1) Chromium standard solution Measure 0.283 g of potassium dichromate (standard reagent) pulverized with agate mortar and desiccated for 3 to 4 hours at 100 to 110 °C into 1,000 mL volumetric flask and dissolve in water, adding water to the marked line to prepare

chromium standard stock solution (1 mL of the solution contains 0.1 mg of chromium[Cr]) [1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare several chromium standard solutions containing 0.5 to 3 μg of chromium per 1 mL of the solution prior to use.

2) Extraction solvent Dissolve 3 mL of trioctylamine in 4-methyl-2-pentanone adjusting overall quantity of solvent to 100 mL.

B Preparation of sample solution

Accurately pour 10.0 g of the analysis sample into a 100 mL tall beaker made of borosilicate glass [2] and carbonize through moderate heating. Then ashed by heating at temperatures at under 500 °C [3]. Add minor quantities of water and 10 mL of hydrochloric acid to the residue, adjust quantity to 30 mL with additional water and allow to cool off after boiling for several minutes. Entire amount of the solution is then transferred to a 100 mL volumetric flask with water, adjusting the quantity by adding water to the marked line. Filter the solution using a paper filter (No. 6) to be used as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions[4].

C Quantification

Accurately fixed quantities of the sample solution (less than 30 μg of chromiumLess than 25 mL in liquid volume) into a 100 mL tall beaker, neutralize with sodium hydroxide solution (10 mol/l) [5], add 10 mL of sulfuric acid (1:17) and minor quantities of potassium permanganate (0.3 w/v%) and boil. If the red-violet hue of the solution dissipates during the process, add several drops of potassium permanganate (0.3 w/v%) to the solution. Repeat procedure until the red-violet hue is sustained during 5 minutes of boiling[6]. Allow solution to cool off.

Transfer the solution to a 100 mL separating funnel, adjust liquid volume to 50 mL, then add 10 mL of ammonium sulfate solution (40 w/v%) and 5 mL of sulfuric acid (1:17). Lightly shake funnel.

Accurately add 10 mL of extraction solvent to separating funnel, strongly shake for 5 minutes and allow solution to settle. Measure absorbance of extraction solvent layer[7] (upper layer) for wavelength 357.9 nm in acetylene-air flame using an atomic absorption spectrometer.

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently, place 10 mL of respective chromium standard solution in 100 mL separating funnels, adjust quantity to 50 mL with water, lightly shake after adding 10 mL of ammonium sulfate solution (40 w/v%) and 5 mL of sulfuric acid (1:17). Measure absorbance under the same conditions as the sample solution to generate a calibration curve for the calculation of the chromium content within the sample.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis.

《Summary of analysis method》

The analysis sample is dry degraded by ashing, and wet degraded into a sample solution. Chromium content of the solution is then oxidized into hexavalent chromium by subjecting to potassium permanganate within acidity environment of sulfuric acid. Trioctylamine-4-methyl-2-pentanone is then added to generate hexavalent chromium--trioctylamine complex. The complex is extracted and measured for atomic absorption at the wavelength of 357.9 nm, calculating the concentration of the chromium within the sample based on the calibration curve obtained from chromium standard solutions.

Figure 4.1.13-1. shows analysis method.

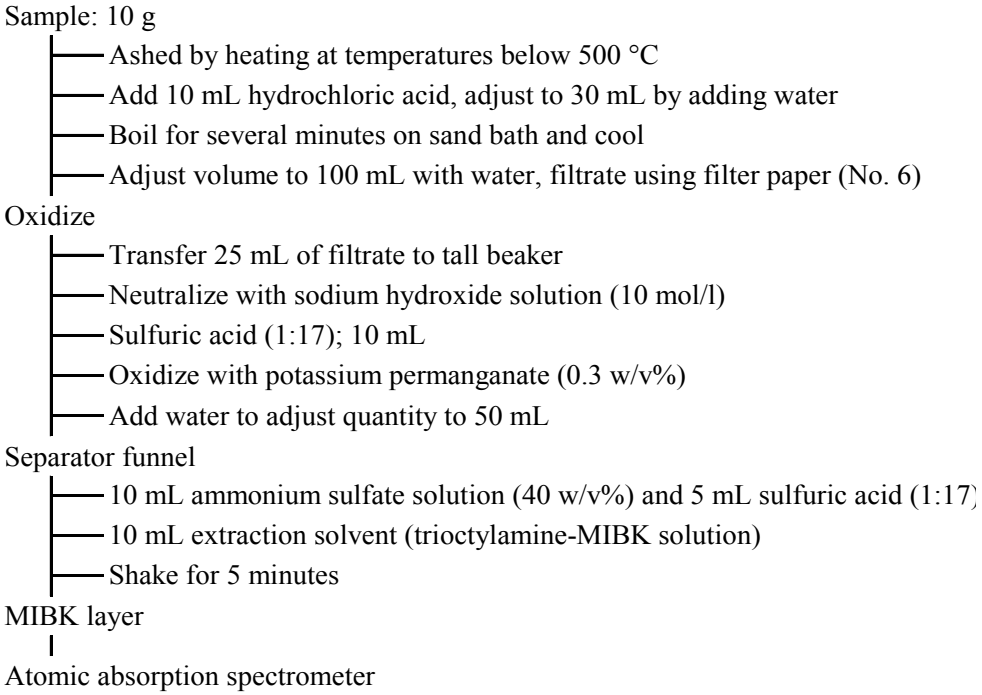


Figure 4.1.13-1 Analysis flow sheet of chromium contained in sample

Reference: Ryou Hashimoto, Jiro Harada: Research Report of Animal Feedm, 24,1 (1999)
Feed analysis standard description progress 【21】New

《Analysis validation》

・Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Repeat	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for piglet	5-20	3	94.3-96.3	8.9
Formula feed for beef	5-20	3	99.7-109.0	4.9
Fish meal	5-20	3	105.0-112.7	5.8

・Collaborative study

Sample type	No. of labs	Spike concentratio (mg/kg)	Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
Formula feed for dairy cows	6	10	102.0	3.8	7.8	0.35

・Quantification lower limit: Samples 1 mg/kg

《Notes and precautions》

[1] Use of commercially cadmium standard solutions 1,000 mg/l are acceptable.

[2] Although porcelain plates and other containers are acceptable, tall beaker made of pirex glass is desirable upon consideration of the subsequent wet degradation procedure.

[3] Preliminary heating (carbonization) is difficult when tall beakers are employed. In this situation, the sample should directly be ashed using the electric furnace. Although the heating duration depends on the type and quantity of the analysis sample, the process generally requires several hours to an entire night. Occasionally, the ashing of samples may be insufficient even after an overnight ashing process (indicating large quantities of carbon residue). In this circumstance, the ashing process (dry ashing degradation) should be extended to completely convert the sample into ash.

[4]Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

[5] Utilize pH meter.

[6] Hexavalent chromium is easily transformed into trivalent chromium, and therefore needs to be promptly subjected to solvent extraction and subsequent procedures upon oxidization into hexavalent chromium with potassium permanganate solution.

[7] In the event the extraction solvent layer is clouded, add 20 mL of ammonium sulfate solution to cleanse extraction solvent layer. Measurement should be initiated promptly upon extraction.

[8] As the sensitivity of the atomic absorption spectrometer is not exactly high in terms of chromium detection, measurement must be carefully conducted using appropriate settings for burner positioning, gas and air pressure, gas and air flow rate.

2 Absorption photometry Feed analysis standards, Chap4, Section1. 13-2

A Reagent preparation

1) Chromium standard solution Measure 0.283 g of potassium dichromate (standard reagent) pulverized with agate mortar and desiccated for 3 to 4 hours at 100 to 110 °C into 1,000 mL volumetric flask and dissolve in water, adding water to the marked line to prepare chromium standard stock solution (1 mL of the solution contains 0.1 mg of chromium [Cr]) [1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare several chromium standard solutions containing 0.5 to 3 μg of chromium per 1 mL of the solution prior to use.

2) 1,5-diphenylcarbonohydrazide solution Dissolve 0.5 g of 1,5- diphenylcarbonohydrazide with acetone, adjusting quantity to 100 mL [2].
3) Dilute acid Drop potassium permanganate solution (0.3 w/v%) into sulfuric acid (1:6) to tinge solution to faint ruby.

B Preparation of sample solution

Accurately measure 1 gl of the analysis sample into platinum crucible, carbonize by gradually applying heat, then ash at 500 °C, and cool off.

In the event ashing is insufficient, add 0.5 to 1 mL of sulfuric acid (1:1) and 4 to 5 mL of nitric acid to the platinum crucible, and evaporate to dryness by heating above sand bath. Continue process by adding 4 to 5 mL of nitric acid each time until the sample is completely decomposed. Then initially apply low heat , eventually increasing to 450 to 500 °C to ash sample. Allow sample to cool off before proceeding.

Mix 5 g of sodium carbonate and 0.5 g of sodium nitrate to the residue and gradually apply heat. Melt by subjecting to temperature of 600 °C for 15 minutes, and allow sample to cool off.

Add minor quantities of water to dissolve the condensate left in the crucible, pulverizing the solid residue using a glass rod. Use water to transfer the solution to a volumetric flask with capacity ranging from 100 to 500 mL depending on the chromium content of the sample, and adjust quantity by adding water to the marked line. Transfer the solution to a centrifugal tube and centrifuge at 150×*g* for 3 minutes to use supernatant solution as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions[3].

C Quantification

Pour fixed quantities of the sample solution (less than 50 μg of chromium) into a 50 mL volumetric flask, add dilute acid 2 mL in excess of neutralization quantity[4]. Add 1 mL of 1,5-diphenylcarbonohydrazide solution[5], and adjust quantity by adding water to the marked line of the flask. Allow solution to settle for 30 minutes[6]. Use solution to measure absorbance in the wavelength of 540 nm range with blank test solutions similarly prepared as the contrast solution.

Then conduct absorbance measurement for respective chromium standard solutions under the same condition as applied to the sample solution, generating a calibration curve [7] to calculate the chromium content of the sample.

《Summary of analysis method》

Oxidize chromium within sample using nitrate salt as the liquefacient, lixiviating the generated chromate with water. After separating and removing the ferrum and other elements,

add sulfuric acid to impart acidity to the solution. Tinge to develop pink hue to the solution by adding 1,5-diphenylcarbonohydrazide solution-Cr^{6+} reacts specifically with 1,5-diphenylcarbonohydrazide in acidic solution forming complexes-measuring the absorbance of the resulting solution in 540 nm range of wavelength. The absorption curve for the pink colored solution is as indicated in figure 4.1.13-2, with the absorption maximizing in the vicinity of 540 to 544 nm.

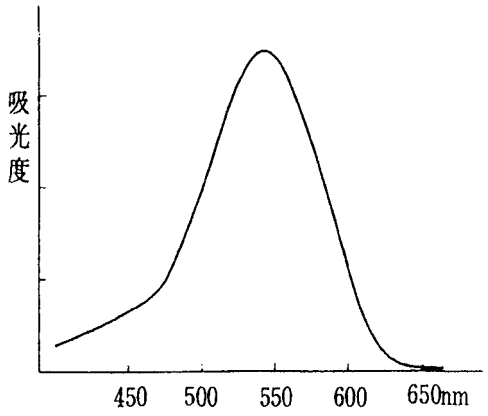


Figure 4.1.13-2 Absorption curve for reaction product between Cr^{6+} and diphenylcarbonohydrazide solution

Although few substances inhibit the reaction, Fe is one of the inhibitors.

Cr^{3+} precipitates as hydroxides in slightly acid to neutral environment, Cr^{6+} does not precipitate, even in alkaline environments. As ferrum and other elements precipitate as hydroxides in alkaline, these elements can be degraded and removed if the solution is alkalified after oxidizing the chromium into Cr^{6+}.

Additionally, Cr^{3+} does not color upon reaction with 1,5-diphenylcarbonohydrazide and must be converted into Cr^{6+} prior to measurement.

In recent times, the measurement is generally conducted with the easily implemented atomic absorption spectrophotometry.

Feed analysis standard description progress 【0】New

《Notes and precautions》

[1] Use of commercially chromium standard solutions 1,000 mg/l are acceptable.

[2]To be stored in refrigeration to prevent from decomposing.

[3] Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

[4]Although the acid concentration of approximately 0.1 mol/l is desirable after the color development, concentration in the range of 0.025 to 0.15 mol/l is acceptable. Deviation of the acid concentration may result in delays in color development and deterioration of absorbance.

[5] Shake promptly upon adding.

[6] Color development is relatively fast, and the pink hue is relatively stable, but it is recommendable to measure the absorbance within 3 hours of the color development.

[7] Reproducibility of the calibration curve is fairly good, although the curve has a tendency to indicate curvature when chromium is in abundance as portions of the element may be reduced, or color development is incomplete. Accordingly, measurement should be conducted with a Cr content in the range of 10 to 30 μg.

【Other analysis methods】

3 Absorption spectrophotometry (sodium peroxide melting method)

Scope of application Feed including chrome-tanned leather powder

A Reagent preparation

Chromium standard solution Measure 0.371 g of chrome oxide [Cr_2O_2] into platinum crucible, add 5 g of sodium peroxide (analytical grade), and melt at 600 °C for 5 minutes. Allow sample to cool off and transfer to 250 mL volumetric flask with water, then adjusting quantity by adding water to the marked line to use as chromium standard stock solution. (1 mL of the solution contains 1 mg of chromium [Cr]) [1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare chromium standard solutions containing 1 to 6 μg of chromium per 1 mL of the solution prior to use.

Concurrently prepar blank test solutions of standard it.

B Preparation of sample solution

Accurately measure 5 g of the analysis sample (corresponding to 0.5 to 4 mg in chromium [Cr] content) into platinum crucible, heat until content turns white or grey-white[2], and allow to cool off. Then add 2 g of sodium peroxide (analytical grade) and mix. Gradually apply heat, Increasing to melt sample at 600 °C for 5 minutes[3] and cool.

Add minor quantities of water to dissolve condensate within crucible and transfer to a 200 mL beaker with water. Allow solution to settle for 1 hour. Filtrate solution into a 250 mL volumetric flask using paper filter (No. 6). Cleanse with warm water and allow to cool off. Add water to marked line for use as the sample solution.

Concurrently prepar blank test solutions without sample.

C Quantification

Measure absorbance of sample solution in the vicinity of 370 nm wavelength using blank test solution as the contrast solution [5].

Concurrently measure the absorbance for respective chromium standard solutions and standard blank test solutions under the same conditions as the sample to generate a calibration curve allowing for the calculation of the chromium [Cr] content within the sample.

《Summary of analysis method》

Method utilizes sodium peroxide [Na_2O_2] as liquefacient for the sample. Absorbance is measured for the coloration of the sample solution attributed to CrO_4^{2-} ions leeching into the water from chromates generated in the alkaline resolution in the 370 nm range.

The absorption curve of CrO_4^{2-} ions is as indicated in figure 4.1.13-3, with absorption maximizing in the vicinity of approximately 370 to 375 nm.

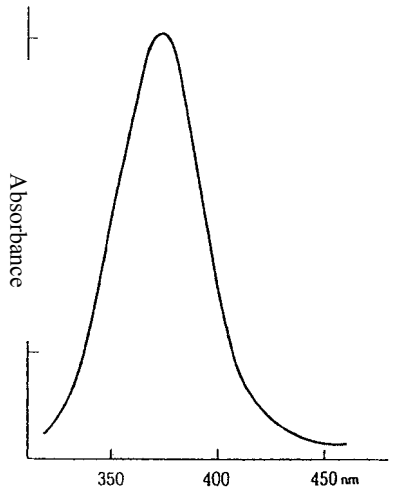


Figure 4.1.13-3 Absorption curve for CrO_4^{2-} ions

The method is suitable for application to samples relatively ding feeds containing chrome-tanned leather powder. It is used for the quantification of chrome oxide utilized as indicators in the evaluation of digestibility.

Reference; Kameoka, Yoshida, Kubota, Takahashi; Research Institute of Agricultural Technology Report (Animal Husbandry), 13, 67 (1957)

《 Notes and precautions》

[1] Use of commercially chromium standard solutions (1,000 mg/l) are acceptable.

[2] Completion of the ashing process may require a long time depending on the sample. However, the presence of residual carbon (partial insufficiency in ashing process) will be insignificant in the quantification results for chromium.

[3] Based on tests conducted to determine the melting conditions in temperatures ranging from 400 to 700 °C and heating time from 5 to 10 minutes, it was determined that temperatures should be maintained below 600 °C and extension of the processing time tended to decline the resulting values.

[4] Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

[5] Ion remains relatively stable within the sample solution, but may result in precipitation if allowed to settle for an entire day. This condition will not influence the quantification results as long as the measurement is taken after filtration.

Bromine, Br

【Summary of Bromine】

As with iodine, bromine is relatively abundant in fishery products. Excessive ingestion of bromine is said to cause nausea, vomition, abdominal pain, paralysis and other symptoms. Bromine also inhibits the absorption of iodine into the thyroid glans, triggering hypertrophy of the gland.
Acceptable levels of residual bromine in food stuff is specified for inorganic bromine deriving from methyl bromide contained in the fumigant for cereal.

《Reference value of bromine residue in cereals specified in Food Sanitation Act》
(As inorganic bromine)
Wheat, Barley and Rye: 50 ppm/ Corn: 80 ppm/ Other cereal: 50 ppm

【Method listed in the Feed Analysis Standards】

1 Ion chromatography[Note 1]

[Feed analysis standards, Chap4, Section 1, 14]

A Reagent preparation

1) Bromine standard solution Measure 0.149 g of potassium bromide into a 100 mL volumetric flask and add water to dissolve. Add water to marked line to prepare bromine standard stock solution. (1 mL of the solution contains 1 mg of bromine [Br]) [1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare bromine standard solutions containing 1 to 10 μg of bromine per 1 mL of the solution prior to use.

2) 2-aminoethanol solution Introduce 15 mL of 2-aminoethanol and 3 g of sodium hydroxide into brown ground stoppered Erlenmeyer flask and dissolve in 380 mL of ethanol to prepare solution.

B Quantification

Extraction Measure 10.0 g of analysis sample into nickel crucible, add 1.2 gl of sodium carbonate and mix elaborately[2], then add 10 mL of 2-aminoethanol solution and allow to settle for 1 hour[3]. Volatilize ethanol on heated plate. Gradually apply heat to carbonize sample and ash by subjecting to temperature of 550 to 600 °C overnight. Allow sample to cool off. Add 10 mL of water and 3 drops of hydrogen peroxide to residue[4] and boil. Transfer solution to a 50 mL volumetric flask with water and adjust quantity by adding water to the marked line. Filtrate solution using filter paper (No. 2).

Accurately measure 25 mL of the filtrate into a 50 mL tall beaker and adjust pH to 7[5] by adding acetic acid (1:5). Transfer solution to a 50 mL volumetric flask with water and adjust quantity by adding water to the marked line. Filtrate solution with membrane filter (pore diameter less than 0.5 μm) for use as sample solution to be subjected to ion chromatography

analysis.

Ion chromatography Inject 100 µl of sample solution and respective bromine standard solutions into ion chromatograph[Note 2] to obtain chromatogl [6].

Measurement conditions Example

Detector: Conductivity detector

Column: Strongly-basic anion exchanger column[Note 3]

Eluent: Sodium carbonate solution[Note 4]-Sodium hydroxide solution[Note 5] (1:1)

Removal solution: Add 0.84 mL sulfuric acid, 0.84 mL to 1 liter of water.

Flow rate: Eluant 2.0 mL/min, Removal solution 2.0 mL/min

Calculation Identify peak height from obtained chromatogl to generate calibration curve, calculating quantity of bromine content for sample.

Note 1 Water used in analysis should be of chemical analysis grade (JIS K0557 A2) or equivalent.

2 To be equipped with suppressor.

3 SAX1-205 (Yokogawa Electric Corporation) or equivalent[7].

4 Dissolve 0.212 g of sodium carbonate into water, adjust quantity to 1 liter.

5 Dissolve 0.400 g of sodium hydroxide into water, adjust quantity to 1 liter.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis. analysis.

《Summary of analysis method》

In this method, the bromine content of feed is converted to bromide using monoethanolamine as the catalyst. The sample is then ashed, extracting the bromine with water. Ion chromatograph equipped with suppressors is used to measure the overall bromine content of the sample.

Reference; Takeo Kozu, Toshikazu Komoriya, Tadashi Suhara: Research Report of Animal Feed, 16, 1 (1991)

Feed analysis standard description progress 【13】New

《Notes and precautions》

[1] Use of commercially bromine standard solutions (1,000 mg/l) are acceptable.

[2] Mix elaborately to prevent diversification of data.

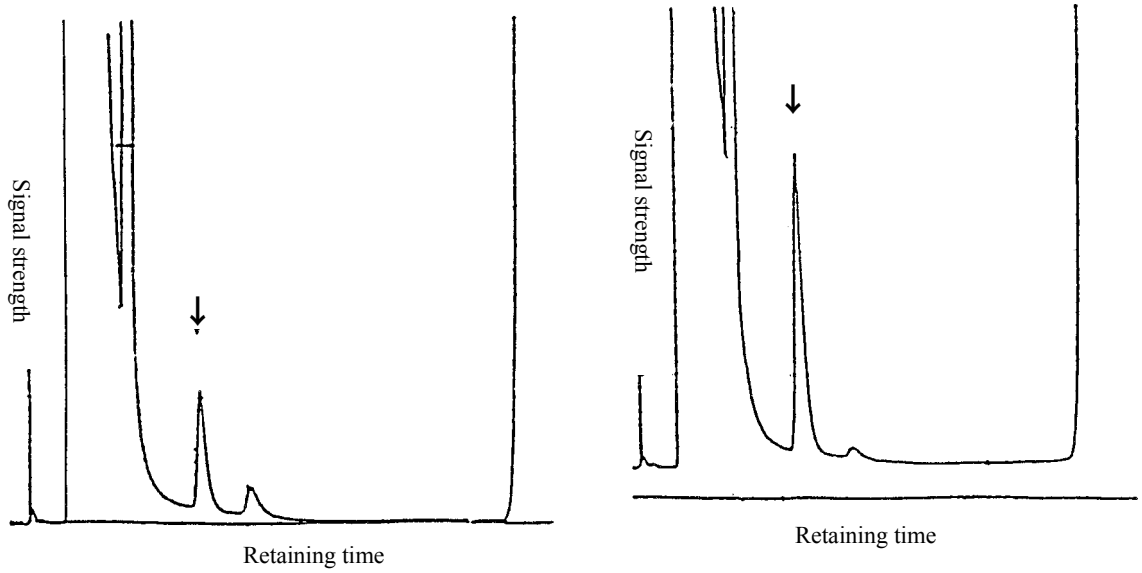
[3] Reaction time of 1 hour is sufficient.

[4] Added to prevent bromine from transforming into liberated bromine etc, thereby adversely affecting the analysis results.

[5] Improves segregation in chromatogl, providing for favorable recovery rates.

[6] An example of the chromatogl is indicated in figure 4.1.14-1.

[7] As the referenced product is currently obsolete, employ product with equivalent capabilities.



Formula feed for mature fowl (additive-free)

Formula feed for mature fowl (50 mg/kg of additive included)

Figure 4.1.14-1 Chromatogl of bromine in mixed forage
(Arrow indicates peak position of bromide)

Mercury, Hg

【Summary of Mercury】

Mercury is known as the causative agent for Minamata disease. Large fishes including tuna inherently contain mercury. For this reason, the mercury content of fish meal produced exclusively from tuna and other large fishes may exceed the allowable level of toxic substances.

Animal by-product feed such as fish meal contain naturally-derived mercury, and up to 0.15 ppm of the element may be detected.

The preliminary regulation value of mercury stipulated in the Food Sanitation Act is 0.4 ppm.

《Standards and criteria specified under the Feed Safety Law》

[Hazardous Substances guidelines for feed]

Formula feed and grass hay, etc.: 0.4 ppm

Fish meal, meat meal, meat and bone meal, : 1.0 ppm

【Method listed in the Feed Analysis Standards】

1 Hydride generation-Atomic absorption spectrophotometry[Note 1]

[Feed analysis standards, Chap4, Section 15]

A Reagent preparation

1) Mercury standard solution Measure 0.339 g of mercury chloride (II) [$HgCl_2$] into a 500 mL volumetric flask and add 5 mL of nitric acid (1:1) to dissolve. Add water to marked line to prepare mercury standard stock solution. (1 mL of the solution contains 0.5 mg of mercury [Hg]) [1].

Accurately dilute fixed quantities of the standard stock solution with nitric acid (1:70) [2] to prepare mercury standard solutions containing 2 to 10 μg of mercury per 1 mL of the solution prior to use.

2) Tin chloride solution Add 60 mL of sulfuric acid (1:20) to 10 g of tin chloride (II) dehydrate and apply heat while stirring to dissolve. Allow solution to cool off and adjust quantity to 100 mL by adding water [2].

B Preparation of sample solution

Accurately measure 1 gl of the analysis sample into a 100 mL volumetric flask[3], add 20 mg of vanadium pentoxide[4] and 10 mL of nitric acid to sufficiently wet sample. Allow solution to settle overnight[5].

Heat solution for 5 minutes on sand bath maintained below 200 °C and cool off. Add 10 mL of perchloric acid and cool[6], adding water to marked line of the volumetric flask for use as the sample solution.

Concurrently repeat preparation procedure under the same condition without introducing the sample to produce blank test solutions.

Repeat procedure for respective mercury standard solutions.

C Quantification

Accurately measure 20 mL of the sample solution[7] into a reduction container pre-installed with rotators [8] (Erlenmeyer flask with about 100 mL of capacity), add 2 mL of tin chloride solution and promptly attach to mercury analysis device. After stirring for 2 minutes[9], activate air pump to aerate[10]. Guide the emitted mercury vapor to the absorption cell (made of quartz glass, inner diameter 30 mmLength 100 to 300 mm) and measure absorbance for wavelength 253.7 nm[11].

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently conduct absorbance measurement 20 mL of respective mercury standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate the mercury content of the sample.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis.

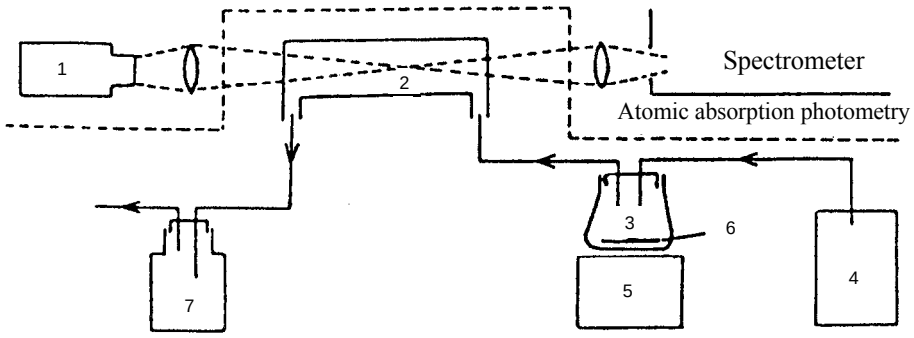
《Summary of analysis method》

Method quantifies the overall mercury content of the sample by flame-less atomic absorption spectrophotometry.

Sample is decomposed with nitric acid and perchloric acid to prepare sample solution, which is then reduced by adding stannous chloride solution, guiding the emitted mercury vapor to the absorption cell of the flame-less atomic absorption spectrometer, measuring the absorbance for wavelength 253.7 nm (Figure 4.1.15-1). As mercury is the only metal element vaporizing in ambient temperature, environment, obstruction by coexisting ions is negligible. Accordingly, no procedures for the removal of coexisting ions is required in the preparation phase of the sample solution.

Upon addition of the stannous chloride, the mercury compound is reduced into metal mercury through the following reaction, which in tern turns into mercury vapor.

$HgCl_2 + SnCl_2 \rightarrow Hg\uparrow + SnCl_4$



1 Hollow Cathode Lamp
2 Absorption cell
3 Hydride generation flaskm
4 Air pump
5 Magnetic stirrer
6 Stirrer peace
7 Mercury collection bottle

Hydride generation flask: 100 mL frosted glass Erlenmeyer flask

Mercury capturing solution: Saturated potassium permanganate solution (acidity by sulfuric acid)m

Figure 4.1.15-1 An example of mercury analysis device

Reference; Hiroshi Tuyakawa,: Research Report of Animal Feed, 5, 1 (1979)
Feed analysis standard description progress 【0】New

《Analysis validation》

・Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Formula feed (2 types)	0.4	3	95.2-97.0	2.1
Fish meal (Peruvian)	0.4	3	107.1	0.7
Fish meal (from Noth sea)	0.4	3	97.0	1.5
Rice bran & Cake	0.4	3	103.5	1.7
Meat cake	0.4	3	92.9	3.1

・Collaborative study

Sample type	No. of labs	Data (mg/kg)	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
Fish meal	6	0.81	3.9	9.5	1.2

《Notes and precautions》

[1] Use of commercially available mercury standard solutions (100 mg/l) diluted with sulfuric acid (1:10) is acceptable.

[2] Storage duration for the solution is 1 week. Re-create if solution clouds and turns yellow.

[3] Although JIS and other standards stipulate the use of devices equipped with reflux cooling tubes to prevent the loss of mercury upon decomposition of the sample, the method is unsuitable for the simultaneous processing of large quantities of sample. Alternately, a method of air-cooled reflux utilizing the neck portion of a volumetric flask in the wet degradation of the sample was indicated.

Immerse glass appliances used in the measurementin nitric acid (1:1) then cleansed with

ion-exchange water. Heat for 2 hours at 140 °C immediately prior to use.

[4] Is used as a catalyst.

[5] Foaming caused by the decomposition of nitric acid can be prevented by allowing solution to settle overnight. Leave samples overnight, especially in the case of samples abundant in lipids.

[6] Persistence of oily suspended matter will not affect quantification adversely. The absorbance does not change even if the decomposed solution is neglected for 24 hours.

[7] Iodine contained in iodine-rich feed additives and pre-mixes interfere with the reaction. In this situation, increase quantity of stannous chloride, solution used as reducing agent (to more than 10 times normal quantity) and reduce acquisition of sample solution to less than 5 mL.

[8] Use the same hydride generation flask and stirrer piece for the sequence of analysis.

[9] Agitate for 2 minutes at a fixed speed, then aerate after solution achieves the condition of vapor-liquid equilibrium.

[10] Utilize pump capable of circulating more than 1.5 liters of air per minute. Collodion to be applied to metal sections coming in contact with the sample gas.

Aquarium pumps used in raising goldfish may be used.

[11] Absorption cells shall be of quartz-glass, glass or plastic tubes equipped with quartz-glass at both ends, with an overall length of about 200 mm and a diameter of about 20 mm.

As mercury vapor may be partially absorbed by rubber or silicon rubber tubes, soft vinyl chloride tube are most suited for connection to the equipment.

The use of an atomic absorption spectrometer is not exactly necessary, and may be substituted with UV spectrophotometers capable of equipping mercury inclusion lamps or simplified mercury analyzers.

Commercially available as mercury analyzers from such manufacturers as Hiranuma-Sangyo Co. Ltd, Nippon Instruments,Co.,Ltd., Tokyo Koden Co.,Ltd. and Milestone General K.K.

Selenium, Se

【Summary of Selenium】

Previously regarded as problematic constituents causing intoxication in livestock when fed in excessive quantities, selenium is a substance recently in the process of appraisal as an essential mineral with bioactivation properties when supplied in trace amounts, capable of partially substituting the positive effects of vitamin E. Entry on selenium was newly added to the Japanese Standard Feed constituent Table.

Requirement of livestock for selenium is in the range of 0.1 to 0.3 mg/kg, and causes disorders when supplied in excess of 5 to 10 mg/kg. The small tolerance between deficiency and overabundance characterizes the element. Selenium content in coarse feed is under the influence of the selenium content in the soil used to cultivate the crop, and livestock bred in selenium-rich regions tend to suffer from excess symptoms, while those in selenium-poor regions often experience deficiencies.

The effectiveness of selenium-added feed has been established for some time in Europe and US, and addition of up to 0.3 mg/kg of inorganic selenium is permitted in the United States. In Japan, the use of the toxic inorganic selenium to feed is not authorized. Instead, yeast is encouraged to assimilate the element as organic selenium (in the form of selenomethionine), adding the selenium-enriched yeast as ingredients in mixed forage.

Mix ratio of selenium-enriched yeast into mixed forage is restricted to 0.3 mg/kg in selenium content. Additionally, manufacturers using selenium-enriched yeast are required to analyze the selenium content in the mixed forage products using the yeast as an ingredient at a frequency of once every 2 weeks for each of the feed using the ingredient.

【Method listed in the Feed Analysis Standards】

1. Fluorometry 注1 [Feed analysis standards, Chapter 4, Section 1.16]

A Reagent preparation

1) Selenium standard solution Accurately measure 0.5 g of selenium [Se] into a tall beaker, add 10 mL of nitric acid and heat in boiling water to dissolve. Add 2 mL of perchloric acid and apply heat until solution concentrates to approximately 2 mL. Add 5 mL of hydrochloric acid to the residue, heat for 5 minutes and allow to cool off. Transfer solution to a 500 mL volumetric flask using water, and adjust quantity by adding water to the marked line for use as the selenium standard stock solution, (1 mL of the solution contains 1 mg of selenium.)[1]

Accurately dilute fixed quantities of the standard stock solution with hydrochloric acid (0.1 mol/l) [2] to prepare selenium standard solutions containing 0.02 to 0.1 μg of selenium per 1 mL of the solution prior to use.

2) Diamino naphthalene solution Introduce 0.10 g of 2,3-diamino naphthalene[2] to a 200 mL

tall beaker, add 100 mL of hydrochloric acid (0.1 mol/l) and dissolve by heating in water maintained at 50 °C and allow solution to cool. Pour solution into separating funnel A, add 40 mL of cyclohexane and shake. Introduce water layer (lower layer) of the solution into separating funnel B, add 40 mL of cyclohexane, shake and filtrate water layer using paper filter (type 2) (preparation process to be shielded fromLight).

3) EDTA solution Dissolve 18.6 g of ethylene-diamine-tetra-acetic acid-dihydrogen-disodium-dihydrate into water, adjusting quantity to 500 mL.

B Preparation of sample solution

Accurately measure 2 g of the analysis sample into a 200 mL tall beaker, add 20 mL of nitric acid and 5 mL of perchloric acid. Cover beaker with watch-glass and allow to settle overnight.

Moderately apply heat to solution on sand bath and ignite after foaming has receded[3]. Allow solution to concentrate [4] to near-exsiccation and allow to cool off. Add 5 mL of hydrochloric acid to residue, apply heat to solution on sand bath for 5 minutes and allow to cool off. Transfer solution to a 100 mL volumetric flask with water, and adjust quantity by adding water to the marked line for use as the sample solution.

Concurrently perform the preparation procedure without the sample to produce blank test solutions[5]

C Quantification

Accurately introduce fixed quantities of the sample solution (corresponding to 0.2 to 1 μg of selenium) into a 100 mL tall beaker, add 5 mL of EDTA solution and adjust solution pH to 1.0 to 1.5 with hydrochloric acid (1:4) [6]. Transfer to 100 mL volumetric flask using hydrochloric acid 0.1 mol/l and adjust quantity by adding acid to the marked line. Filtrate solution with filter paper (No. 2).

Accurately measure 50 mL of the filtrate into a 100 mL tall beaker, add 5 mL of diamino naphthalene solution and shake[Note 2]. Apply heat for 20 minutes in water maintained at 50 °C and allow solution to cool off. Transfer solution to a 100 mL separating funnel using hydrochloric acid (0.1 mol/l). Accurately add 10 mL of cyclohexane, shake for 5 minutes and allow to settle. Discard water layer (lower layer) and add 25 mL of hydrochloric acid (0.1 mol/l) to residue. Discard water and add acid again. Transfer cyclohexane layer (upper layer) to stoppered test tube. Dehydrate cyclohexane layer with appropriate quantities of sodium sulfate (anhydrous), and filtrate using filter paper for use as sample solution.

Prepar blank test solutions and each selenium standard solution.

Using blank test solution as contrast, measure fluorescence intensity of sample solution for the excitation and fluorescence wavelength (wavelength 378 nm and 520 nm respectively) [7].

Concurrently conduct absorbance measurement for respective selenium standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate [8] the selenium content of the sample.

Note 1 Analysis requires the use of atomic absorption analytical reagent grade acids and nonluminescence reagent grade or equivalent quality solvents.
2 Subsequent procedures to be conducted shielded from the light.

《Summary of analysis method》

Established methods of quantification for selenium include atomic absorption analysis, fluorometric analysis, absorption spectroscopy, gas chromatography and liquid chromatography methods.

Descriptions provided pertain to the fluorometric analysis method adopted in the Standard Methods of Analysis for Hygienic Chemistry. Sample is wet degradated by nitric and perchloric acids, oxidizing selenium into selenic acid to repress volatility of the element. Selenic acid is then reduced to selenious acid by adding hydrochloric acid and applying heat. 2,3-diamino naphthalene is added, generating fluorescent substance which is then released into cyclohexane for measurement with fluorometer.

Reference; Ryuuji Koga, Hiroshi Kazui, Masanobu Kajii,: Research Report of Animal Feed, 15, 1 (1990)

Feed analysis standard description progress 【12】New

《Notes and precautions》

[1] Use of commercially selenium standard solutions (1,000 mg/l) are acceptable.

[2] Product available from sources including Wako Pure Chemical Industries, Kanto Kagaku, Sigma-Aldrich Japan, etc.

[3] In the event decomposition of organic material is insufficient when perchloric acid starts to emit white fume, add minor quantities of nitric acid after solution cools off and re-apply heat, repeating process until organic material is completely decomposed.

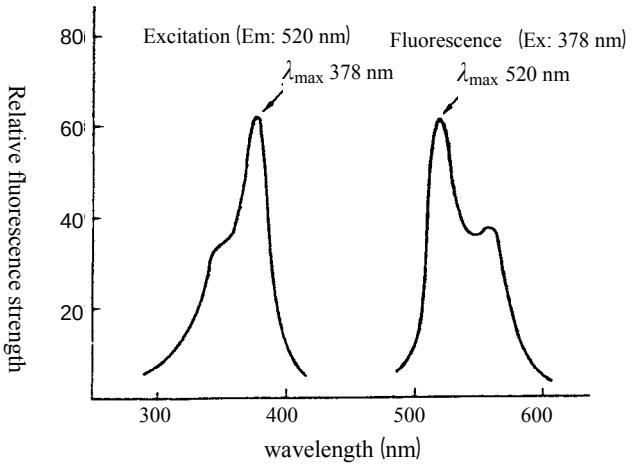


Figure 4.1.16-1 Excitation and fluorescence spectrum for selenium

[4] As nitric acid needs to be completely volatilized, concentrate solution to near-exsiccation levels.

When decomposing at 220 °C, the process requires about 8 to 10 hours to complete.

[5] Especially in quantifying trace elements, potential contamination from reagents should not

be disregarded. Accordingly, blank tests using blank test solutions prepared Concurrently with the sample solution are required. The use of precision analysis grade or atomic absorption analysis grade reagents are recommended.

[6] Use Hydrochloric acid (1:4) and ammonia water (1:1) as agents for pH adjustment.

[7] Results of fluorescence spectrum measurements taken for standard selenium solutions with cyclohexane as the contrast are as indicated in figure 4.1.16-1. Peaks were confirmed in the vicinity of 378 nm for excitation wavelength and 520 nm for fluorescence wavelength respectively.

[8] The calibration curve provided a linear line passing through the point of origin for selenium[Se] quantity in the range of 0.2 to 1.0 μg. The lower limit of quantification applicable to this method is determined to be approximately 0.05 mg/kg for sample quantity of 2 g.

[Other analysis method]

2 Hydride generation—atomic absorption spectrophotometry[Note 1]

A Reagent preparation

1) Selenium standard solution Accurately measure 1 gl of metal selenium into a 100 mL beaker, add 20 mL of nitric acid and apply heat to dissolve. Maintain heat until acid is almost completely volatilized, cool off and accurately quantify solution into 100 mL measuring flask with water for use as selenium standard stock solution. (1 mL of the solution contains 1 mg equivalent in selenium [Se]) [1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare selenium standard solutions containing 0.2 to 1 μg of selenium per 1 mL of the solution prior to use. (Prepare when it is needed)

2) Sodium borohydride reagent Dissolve 5 g of sodium hydroxide (special grade) and 10 g of boron sodium hydroxide (atomic absorption analysis grade) with water, adjust to 1 liter (to be prepared prior to use)[2].

B Preparation of sample solution

Accurately measure 2 g of the analysis sample into a 300 mL Kjeldahl flask, add 20 mL of nitric acid and moderately apply heat [3].

Heating solution after foaming recedes and cool off, add 5 mL of perchloric acid and apply heat until sample decomposes completely and an additional 15 minutes after the perchloric acid starts to emit white fume. Allow sample to cool off and wash interior of flask with minor quantities of water. Add 5mL of hydrochloric acid (1:1) and apply heat for 30 minutes in boiling water[4], then allow solution to cool. Transfer solution to a 100 mL volumetric flask and add water to the marked line. Filtrate using filter paper (No. 6) for use as the sample solution.

Concurrently perform the preparation procedure without the sample to produce blank test

solutions.

C Quantification

Successively supply a mixture of sodium borohydride reagent—hydrochloric acid (2:3) and the sample solution[5] to a hydride generation apparatus[6] linked to the atomic absorption spectrometer, combining the solutions and allowing to react. Introduce the generated selenium hydride to an absorption cell heated with flame[7] using argon as the carrier, measuring the absorbance at the wavelength of 196.0 nm [8].

Measure absorbance for the blank test solution following a likewise procedure, compensating the results obtained for the sample solution.

Concurrently conduct absorbance measurement 2.5 mL, 5 mL, 10 mL and 20 mL of selenium standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate the selenium content of the sample.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis. analysis.

《Summary of analysis method》

Hydrides generated through the reduction of selenium is introduced into a heated absorption cell to be measured using an atomic absorption spectrometer. Alternatively, the samples may be atomized by subjecting to argon—hydrogen flames, or measured using emission spectrography by introducing sample to inductive coupled plasma (ICP) torches.

《Notes and precautions》

[1] Use of commercially selenium standard solutions are acceptable.

[2] Solution maintains stability for approximately 1 week in refrigeration.

[3] Decompose after allowing to settle overnight in case of excessive foaming.

[4] Reduce selenium (VI) contained in sample solution to selenium (IV) by adding hydrochloric acid and heating. Generate hydride SeH_2 with sodium borohydride for introduction into the atomization unit.

[5] Selenium content within the range of 1 to 30 ng/mL is desirable.

[6] For details on the hydride generation apparatus, refer to figure 4.1.18-1 (p.124) describing 《Summary of analysis》 for Arsenic in section 18-1.

[7] To be heated to approximately 1,100 °C.

[8] Although elements with potential to inhibit the analysis based on the method specified are not sufficiently clarified, high concentrations of iron, copper and nickel are presumed to inhibit the reaction.

Conditions of measurement Example

Atomic absorption photometer: Model AA-6800; Shimadzu Seisakusho Ltd.

Hydride generation apparatus: Model HVG-1; Shimadzu Seisakusho Ltd.

Measurement method: Continuous flow method

Atomization unit: Heated absorption cell (heat supply by air—acetylene flame)
Carrier gas: Ar, 70 mL/min

3 Gas chromatography [1]

Subject of quantification Selenomethionine

Scope of application Selenium-enriched yeast

A Reagent preparation

1) Selenomethionine standard stock solution Accurately measure 5 mg of seleno-L-methionine into a 10 mL ground stoppered text tube and dissolve by adding 1 mL of hydrochloric acid (0.1 mol/l) for use as the selenomethionine standard stock solution. (1 mL of the solution contains 5 mg of selenomethionine.)

2) 5% cyanogen bromide solution Measure 0.5 g of cyanogen bromide into a 10 mL stoppered test tube and dissolve with 9.5 mL of hydrochloric acid (0.1 mol/l) to prepare solution.

B Preparation of the sample solution

Reaction Measure 50 mg of the sample into a centrifugal tube (capacity 1.5 mL) and add 500 µl of the 5% cyanogen bromide solution, moderately shake for 30 seconds and allow solution to settle overnight.

Extraction Add 100 µl of chloroform-1,2-dichlorobenzene [2] (2,000:1) mixture to centrifugal tube and shake moderately for 30 seconds, then centrifuge for 5 minutes at 200×*g*. Use the chloroformLayer (lower layer) of the solution as the sample solution for the gas chromatography analysis.

Reaction of standard stock solution Dispense selenomethionine standard stock solution into centrifugal tubes (capacity 1.5 mL) in quantities of 2 µl, 10 µl, 20 µl, 40 µl, 60 µl and 80 µl respectively with microsyringe, add 100 µl of the 5% cyanogen bromide solution to each of the tubes, moderately shake for 30 seconds and allow solutions to settle overnight. Add 100 µl of chloroform—1,2-dichlorobenzene (2,000:1) mixture to respective centrifugal tube and shake moderately for 30 seconds, then centrifuge for 5 minutes at 200×*g*. Extract the chloroformLayer (lower layer) of the solutions in preparative isolation, using solutions respectively containing 0.1 mg, 0.5 mg, 1 mg, 2 mg, 3 mg and 4 mg of selenomethionine per 1 mL of solution as the standard solutions.

Gas chromatography Inject 1 µl each of the sample and respective standard solutions into the gas chromatograph and generate chromatogl [3]. Compute peak area to obtain calibration curve, calculating the quantity of selenomethionine contained in the sample.

Measurement condition

Detector: Alkali flame ionization detector (NPD)

Column: DB-WAX (inner diameter 0,25 mm, length 30 m, film thickness 0.25 µm)

Carrier gas: He, 2.5 mL/min

Make up gas: He, 30 mL/min
Hydrogen: 4 mL/min
Dry air: 100 mL/min
Sample introduction method: Splitless
Temperature of sample introduction part: 140 °C
Temperature of detector: 110 °C
Column bath temperature： 150°C

《Summary of analysis method》

Methionine and selenomethionine react quantitatively to cyanogen bromide, respectively generating methyl thiocyanate (CH_3SCN) and selenocyanate methyl (CH_3SeCN). Gas chromatography is used to isolate and quantify these volatile reaction products.

Repeatability of the method is less than 14.3% (RSD) with the lower limit of detection determined to be approximately 200 mg/kg in selenomethionine.

Figure 4.1.16-2. shows flow of analysis method.

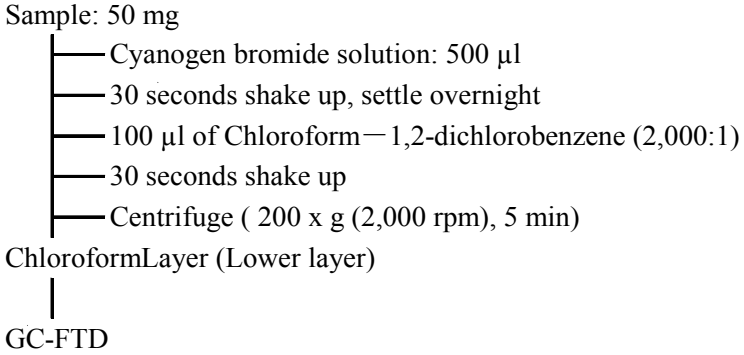


Figure 4.1.16-2 Flow sheet of gas chromatography analysis for selenomethionine

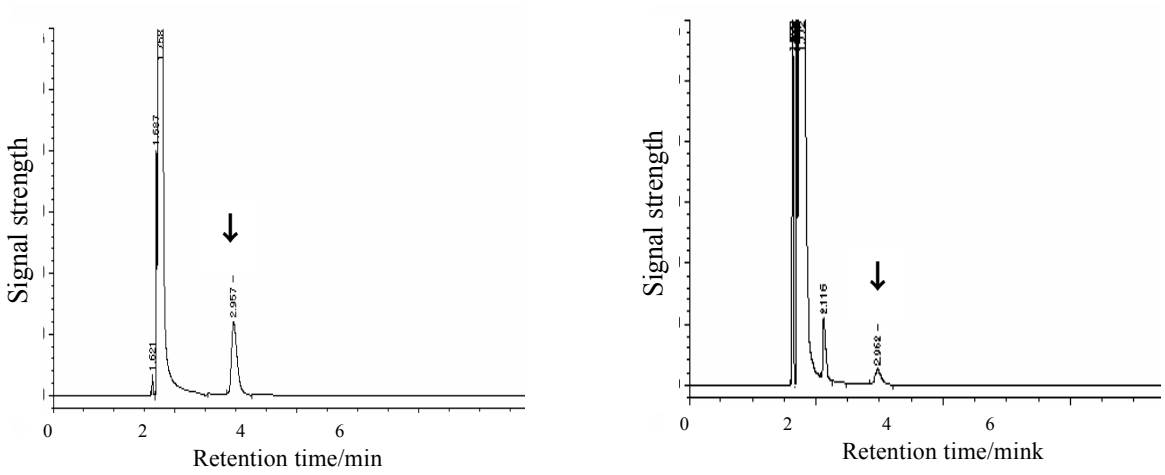
Reference: Yuzo Ono, Eiichi Ishiguro; Research Report of Animal Feed; 27. 243 (2002)

《Notes and precautions》

[1] Reagents used in the analysis are toxic. Procedure to be conducted using dry chambers, etc.

[2] Commercially available from sources including Sigma-Aldrich.

[3] Figure 4.1.16-3. shows an example of the chromatogl



Standard solution（500 ng）　　Sample solution

Figure 4.1.16-3　Chromatogl of selenium standard solution and sample solution (↓Arrow signifies peak position of selenocyanate methyl)

Lead, Pb

【Summary of lead】

Although lead is an essential trace element for livestock, it is normally supplied in abundance as naturally included mineral in feed with no need for supplement. Accordingly, the toxic properties of the element is of significance to livestock.

Upon absorption in organisms, lead affects the nerve center, resulting in motor disorder. Typical symptoms include convulsion, strokes and cattle exhibit rabid behavior when affected. Chronic toxicity includes symptoms of malnutrition, heightened attenuation and paralysis.

Lead ingested by livestock accumulates in the bones, liver and kidneys, with majority of lead poisoning indicating acute toxicity. The threshold of intoxication is approximately 30 mg/kg (feed content) and ingestion of 10 mg/kg (feed content) causes decline of fecurdation rate in domestic fowl.

Lead content in domestically-produced fish meal tends to be higher than imported fish meal.

《Standards and criteria specified under the Feed Safety Law》[Hazardous Substances guidelines for feed]

Formula feed and grass hay etc.: 3.0 ppm

Fish meal, meat meal, meat and bone meal, ： 7.5 ppm

【Method listed in the Feed Analysis Standards】

1 Solvent extraction－atomic absorption spectrophotometry[Note 1]

[Feed analysis standards, Chapter 4, Section 1. 17]

A Reagent preparation

Lead standard solution Accurately measure 0.1 g of lead [Pb]into a tall beaker, add 10 mL of nitric acid and 30 mL of water, heat to dissolve and cool. Transfer the solution to a 1,000 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as lead standard stock solution. (1 mL of the solution contains 0.1 mg of cadmium) [1].

Accurately dilute fixed quantities of the standard stock solution with hydrochloric acid (1 mol/l) [2] to prepare lead standard solutions containing 0.5 to 3 μg of lead per 1 mL of the solution prior to use.

B Preparation of the sample solution

Accurately pour 1 to 10 g of the analysis sample into a 100 mL tall beaker made of borosilicate glass [2] and carbonize through moderate heating. Then ashed by heating at temperatures at under 500 °C[3]. Add minor quantities of water and 10 mL of hydrochloric acid[4] to the residue, adjust quantity to 30 mL with additional water and allow to cool off after boiling for several minutes. Entire amount of the solution is then transferred to a 100 mL volumetric flask

with water, adjusting the quantity by adding water to the marked line. Filter the solution using a paper filter (No. 6) to be used as the sample solution.

Concurrently perform the preparation procedure without the sample to produce blank test solutions[5].

C Quantification

Accurately fixed quantities of the sample solution (less than 30 μg of lead, less than 30 mL in volume) into a 100 mL separating funnel containing 14 mL of phosphoric acid, add 5 mL of potassium iodide solution (68 w/v%) and adjust quantity to 50 mL with water. Shake flask gently and allow to settle for 5 minutes.

Pour 10 mL of 4-methyl-2-pentanone into the separating funnel, acutely shake and allow solution to settle. Measure absorbance of 4-methyl-2-pentanone layer (upper layer) for wavelength 283.3 nm emitted by acetylene-air flame using an atomic absorption spectrometer.

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently conduct absorbance measurement for respective cadmium standard solutions under the same condition as applied to the sample solution, generating a calibration curve to calculate the cadmium content of the sample.

Note 1 Use atomic absorption analytical reagent grade acids in the analysis. analysis.

《Summary of analysis method》

The analysis sample is ashed by dry degradation, then to be subjected to wet degradation for use as the sample solution. Phosphoric acid is added to provide a strong acidity to the solution. Lead is converted to iodide by adding potassium iodide, then to be extracted with 4-methyl-2-pentanone (MIBK). Atomic absorption spectrophotometry is conducted on the MIBK extract at the wavelength of 283.3 nm, calculating the concentration of lead based on the calibration curve obtained for lead standard solutions.

As lead iodide is significantly unstable in comparison with cadmium and copper iodide, the absorbance needs to be measured promptly upon extraction using MIBK.

The sensitivity of atomic absorption spectrophotometry for lead absorbance is not exactly adequate, requiring precise adjustment of the burner position, the pressure and flow rate of both gas and air corresponding to the equipment used in the analysis.

References; Yukiko Mori,: Research Report of Animal Feed, 33, 99 (2008)

Feed analysis standard description progress 【0】New

【31】Additional description; Quantification Lower limit, etc.

Spike recovery and repeatability

Sample type	Spike concentration (mg/kg)	Repeat	Average spike recovery (%)	Repeatability RSD (% or less)
Formula feed for pig	0.5-7.5	3	94.8~110	5.3
Formula feed for chicken	0.5-7.5	3	102~106	8.3
Chicken meal	0.5-7.5	3	94.3~104	5.6
Fish meal	1.5-7.5	3	86.3~108	7.9

・Collaborative study

Sample type	No. of lab.	Spike concentration (mg/kg)	Data		Average spike recovery (%)	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
			Before spike (mg/kg)	After spike (mg/kg)		(content after spike)		
Formula feed for chicken	6	3.0	ND	3.02	100.8	2.7	3.4	0.25
Fish meal	6	3.0	0.607	3.47	95.4	3.1	5.5	0.41

・Quantification Lower limit: Samples 0.5 mg/kg:

・Detection Lower limit: Samples 0.2 mg/kg:

《Notes and precautions》

[1] Use of commercially cadmium standard solutions 1,000 mg/l are acceptable.

[2]Although porcelain plates and other containers are acceptable, tall beaker made of pirex glass is desirable upon consideration of the subsequent wet degradation procedure.

[3] Preliminary heating (carbonization) is difficult when tall beakers are employed. In this situation, the sample should directly be ashed using the electric furnace. Although the heating duration depends on the type and quantity of the analysis sample, the process generally requires several hours to an entire night. Occasionally, the ashing of samples may be insufficient even after an overnight ashing process (indicating large quantities of carbon residue). In this circumstance, the ashing process (dry ashing degradation) should be extended to completely convert the sample into ash.

[4] The use of hydrochloric acid may not be sufficiently effective to decompose the sample. In this circumstance, process sample with nitric acid-perchloric acid decomposition.

Likewise ash 10 to 20 g of analysis sample and add 5 mL respectively of nitric acid and perchloric acid. Cover container with watch-glass and allow sample to decompose to near-exsiccation. After cooling off sample, transfer to 100 mL volumetric flask using hydrochloric acid (0.1 mol/l) and adjust by adding water to the marked line. Filtrate solution with filter paper (No. 6) for use as sample solution in the analysis.

As residue carbon tends to absorb heavy metal, the carbon content of the sample must be completely degraded and removed.

Adequate precautions is required when adding perchloric acid as the substance is explosive in the absence of nitric acid.

[5] Contamination from reagents becomes an issue, especially in the quantification of trace elements. Accordingly, blank test solutions shall be produced concurrently to the preparation of the sample solution for the performance of blank tests. It is desirable to use reagent for precision analysis or for atomic absorption analysis.

Arsenic, As

【Summary of Arsenic】

Arsenic is a substance exhibiting intermediate properties between metal and non-metal. The element is present as sulfides within minerals.

Although an essential trace element, arsenic is viewed as a toxic substance as evidenced by the “Morinaga arsenic milk incident”.

As seaweeds and crustacea has a naturally high concentration of inherent arsenic content, causing accumulation in fishes feeding upon these organisms. Occasionally, the concentration of arsenic in fish meal causes issues. However, excluding the hijiki species of seaweed (*hizikia fusiforme*), over 90% of the arsenic content in seaweed and fish meal consists of organic arsenics with relatively low toxicity.

Concerns were also raised for the arsenic content in rice straws. Concentration of arsenic in straw is presumably determined by the arsenic content of soil (generally in the downstream regions of mines and volcanic areas). According to a survey conducted in 2003, the minimum arsenic content of rice straw was 0.6 mg/kg, with a maximum of 6.8 mg/kg and a median of 2.5 mg/kg. Soil dressing and other counter-pollution measures are required for contaminated rice paddies indicating arsenic concentration of 415 mg per kilogl of soil.

Some organoarsenic compounds are beneficial in facilitating the growth in pigs and fowl, while the acute toxicity of the element causes abdominal pain, diarrhea and skin inflammation in livestock, as well as skin/hair damage and growth depression.

Arsenic compounds are absorbed from the mucosa and inhibits the cellular carbohydrate and lipid metabolism, also indicating toxication to degrade cellular respiration. Symptoms of chronic intoxication include collapse, malnutrition and sclerotization/desquamation of skin.

Inorganic arsenic (trivalent and pentavalent) typically possess toxicity significantly higher than organic arsenics, with trivalent arsenics (arsenious anhydride and arsenite) being more virulent than the pentavalent forms of the element. Primary organic forms of arsenic include the compounds methylarsinate, dimethylarsinic, acid, trimethylaluminum, arsenobetaine and arsenocholine.

Attempts are in progress to quantify various forms of inorganic and organic arsenic compounds individually, with successful results reported in solvent extraction-atomic absorption spectrometry, ultralow temperature collection-hydride generation-atomic absorption spectrophotometry, LC-ICP-MS and other methods.

《Standards and criteria specified under the Feed Safety Law》

[Hazardous Substances guidelines for feed]

Formula feed and grass hay etc (except rice straws) : 2.0 ppm

Rice straws fish meal, meat meal, meat and bone meal, : 7 ppm

【**Method listed in the Feed Analysis Standards**】

1. Hydride generation—atomic absorption spectrophotometry[Note 1]

[Feed analysis standards, Chapter 4, Section 1. 18.1]

Type of arsenic compound analyzed: Total arsenic

A Reagent preparation

1) General arsenic standard stock solution Measure 132 mg of diarsenic trioxide (standard reagent) (desiccated for 3 to 4 hours at 105 °C) into a beaker, add 5 mL of sodium hydroxide solution (4 w/v%) and 50 mL of water, apply heat to dissolve reagent and allow to cool off. Add 1 drop of phenolphthalein reagent to the solution, neutralize with sulfuric acid (1:10) and transfer to a 100 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as the general arsenic standard stock solution. (1 mL of the solution contains 1 mg in arsenic [As])[1].

Accurately dilute fixed quantities of the standard stock solution with water to prepare cadmium standard solutions containing 0.1 to 1 μg of total arsenic per 1 mL of the solution prior to use.

2) Sodium borohydride reagent: Dissove 5 g of sodium hydride and 10 g of sodium borohydride reagent to water, and adjust to 1 L. (Prepare when it is needed)

B Preparation of the sample solution

Accurately measure 2 g of the analysis sample into a 100 mL tall beaker [Note 2], add 10 mL of nitric acid and 5 mL of sulfuric acid, cover them with watch-glass, and allow to settle overnight.

Then moderately apply heat to solution for 30 minutes on sand bath[2] and ignite after foaming recedes[3] and allow to cool off. Add 5 mL of perchloric acid, cover with watch-glass again and heat to completely decompose sample[Note 3] and concentrate until liquid volume is reduced to less than 2 mL , then cool off. Add 5 mL of hydrochloric acid (arsenics analysis grade) (1:10) and 20 mL of water to residue and heat to dissolve [4], then transfer solution to a 100 mL volumetric flask. Add water to the marked line and filtrate using filter paper (No. 6) for use as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions.

C Quantification[Note 4]

Accurately transfer fixed quantities of the sample solution[5] into 100 mL volumetric flasks and sequentially add 10 mL of hydrochloric acid (arsenic analysis grade) and 10 mL of potassium iodide solution[6] (20 w/v%), then allow solution to settle for 15 minutes. Add water to the marked line for use as sample solutions for atomic absorption spectrophotometry analysis.

Successively supply a mixture of sodium borohydride reagent—hydrochloric acid (for analysis of arsenic) (2:3) and the sample solution to a arsenic hydride compound linked to the atomic absorption spectrometer, combining the solutions and allowing to react. Introduce the generated arsenic hydride to an absorption cell heated with flame[7] using argon as the carrier, measuring the absorbance at the wavelength of 193.7 nm [8].

Measure absorbance for the blank test solution following a likewise procedure, compensating the results obtained for the sample solution.

Concurrently process several solutions containing general arsenic standard stock solution in amounts ranging between 2.5 to 20 mL likewise to the sample solution, measuring the absorbance to generate a calibration curve, thus enabling the calculation of the total arsenic content of the sample[9].

Note 1 Unless otherwise specified, acids used in the analysis shall be of precision analysis grade.

2 Glass equipment used in the analysis shall be manufactured with non-arsenic borosilicate glass [10].

3 Ignite with sand bath maintained at 300 to 380 °C [2].
Remove from sand bath and cool off in the event the liquid volume decreases or discolor to brown-black, add 1 mL of nitric acid and re-apply heat on sand bath. When solution becomes clear with the progress of the decomposition (occasionally indicating faint yellowish or reddish hue) and no longer blackens, remove watch-glass and increase heat until sulfuric acid begins to emit white fumes to concentrate.

4 Quantities indicated for the hydrochloric acid to be added to the sample solution, the concentration and quantity of potassium iodide solution and the quantities provided in procedures subsequent to the addition of the sodium boron hydride reagent are all examples and should therefore be adjusted to suit the conditions required by the atomic absorption spectrometer used for the analysis.

《Summary of analysis method》

Sample is wet degraded with sulfuric, nitric and perchloric acids, reducing the pentavalent arsenic contents of the sample solution into hydrogenated product AsH_3 using sodium boron hydride. The resulting AsH_3 is then introduces to the atomizer unit (heated quartz cell) to measure the overall quantity of the arsenic by atomic absorption spectrophotometry of the atomized sample (refer to figure 4.1.18-1).

Although established alternative methods of overall arsenic quantification include the Gutzait method and silver diethyldithiocarbamate method, the specified hydride generation—atomic absorption spectrophotometry seems to be the most suitable in the context of feed analysis upon consideration of the arsenic concentration of 0.1 to 0.6 mg/kg in fish meal and 0 to 2 mg/kg in yeast.

However, in case of samples such as calcium phosphate with relatively high concentration of overall arsenic (approximately 10 mg/kg in calcium phosphate), silver diethyldithiocarbamate method is more eligible.

AS hot concentrated sulfuric acid and concentrated nitric acid are hazardous, appropriate safety precautions including the use of heat resistant gloves are required.

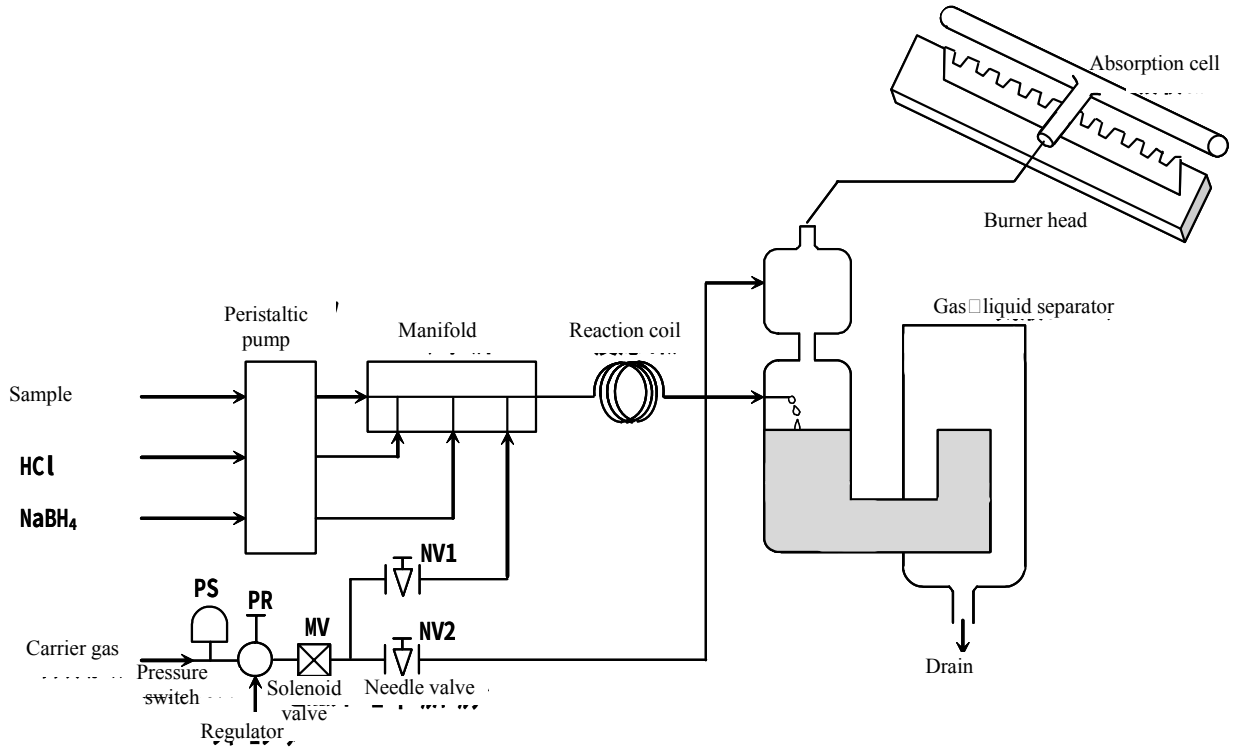


Figure 4.1.18-1 Configuration (example) of hydride generation apparatus

《Analysis validation》

・Collaborative study

Sample type	No. of labs	Data (mg/kg)	Intra-lab repeatability RSD_r (%)	Intra-lab reproducibility RSD_R (%)	HorRat
Fish meal (Import)	10	2.6	6.7	11.8	0.85
Fish meal (Export)	10	8.5	5.7	10.5	0.91

・Authenticated standard sample

Sample type	Authentication value (mg/kg)	Repeat	Data (mg/kg)	Relative standard deviation RSD (%)
Tuna meal (CRM-627)	4.8±0.3	3	4.8	2.1

《Notes and precautions》

[1] Use of commercially arsenic standard solutions (100 mg/l) are acceptable. Standard solution to be preserved in polyethylene bottles.

[2] Apply heat in the range of 170 to 220 °C. Sand bath (gas burner) may be substituted with heated plates.

As heated plates may not be capable of achieving the necessary 300 to 380 °C, confirm product setting prior to use.

[3] Carbonization of organic constituents by the sulfuric acid begins once the nitric acid is completely vaporized. As As^{5+} may be reduced to As^{3+} and volatilize in this condition, terminate application of heat when the generation of the nitrogen oxides (yellow-brown fume emission) recedes. Allow sample to cool off and proceed to perchloric acid processing.

Perchloric acid processing in the presence of significant quantities of organic substances may result in explosive reaction. Repeat the decomposition process using nitric acid prior to the addition of perchloric acid until the organic residue has sufficiently degraded. Addition of the nitric acid is necessary while the droplets of water collected at the center of the watch-glass drops into the sample solution with an audible noise. In case the sample solution requires extensive nitric acid introduction and decomposition processes, the same quantity of nitric acid must be added to the blank test solution and the reagent blank solution because arsenic deriving from reagents may be unnegligible.

After the receding of the white fumes generated by the perchloric acid, remove watch-glass and concentrate solution until the liquid volume is reduced to less than 2 mL (quantity of the sulfuric acid).

[4] If the residue is liquefied at the time the hydrochloric acid (1:10) and the water is added, the heating procedure is not necessary.

[5] Quantity of sample solution dispensed shall be within the range of 1 to 50 ng/mL in terms of arsenic [As] content.

Appropriate quantities of the sample solution to be siphoned into the reaction vessel of the peristaltic pump along with equivalent quantities of sodium tetrahydroborate reagent and hydrochloric acid (2:3).

[6] Add potassium iodide and convert pentavalent arsenic to trivalent, standardizing all arsenic content to trivalent.

[7] Absorption cell to be heated to approximately 1,000 °C.

[8] Influence of the mineral acid on the generation of arsenic hydride is insignificant for sulfuric acid and phosphoric acid up to concentration of 1 mol/l or higher. The presence of perchloric acid causes approximately 10 percent of adverse interference at concentration of 2 mol/l.

The presence of nitric acid ions has a significant influence, inhibiting the acquisition of the atomic absorption peak for 0.4 μg of arsenic when the concentration of nitric acid reaches 0.02 mol/l. This is presumably due to the oxidization of the arsenic hydride by the reduction product (nitrogen peroxide) of the nitric acid ion. Accordingly, the nitric acid ion content of the sample solution needs to be completely volatilized.

Measurement Conditions: Example

Atomic absorption photometer: Model AA-6800; Shimadzu Seisakusho Ltd.

Hydride generation apparatus: Model HVG-1; Shimadzu Seisakusho Ltd.

Measurement method: Continuous flow method

Atomization unit: Heated absorption cell (heat supply by air—acetylene flame)

Carrier gas: Ar, 70 mL/min

Procedures of operation may differ depending on the model utilized. For details, refer to the manual provided by the manufacturer.

[9] The calibration curve produced in this method indicates linearity in range corresponding to 2.5 µg/l to 20 µg/l of As content.

[10] Beakers, watch-glass and other glass equipment used for the analysis shall be of non-arsenic borosilicate glass (pirex #7740, vycol #7913, hariol 32 etc.)

2. Arsenic hydride generation--ultralow temperature collection—atomic absorption spectrophotometry analysis depending on type of sample

[Feed analysis standards, Chapter 4, section 2.2]

Type of arsenic compound analyzed: Inorganic arsenic, monomethyl arsenic, dimethyl arsenic trimethyl arsenic（4 types）

A Reagent preparation

1) Inorganic arsenic standard stock solution[1] Measure 132 mg of diarsenic trioxide (standard reagent) (desiccated for 3 to 4 hours at 105 °C) into a beaker, add 5 mL of sodium hydroxide solution (4 w/v%) and 50 mL of water, apply heat to dissolve reagent and allow to cool off. Add 1 drop of phenolphthalein reagent to the solution, neutralize with sulfuric acid (1:10) and transfer to a 100 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as inorganic arsenic standard stock solution. (1 mL of the solution contains 1 mg in arsenic [As]).

2) Monomethyl arsenic standard stock solution[2] Measure 18.7 mg of methylarsonic acid [CH_5AsO_3] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of solution by adding water to the marked line. for use as monomethyl arsenic standard stock solution. (1 mL of the solution contains 100 µg in arsenic [As]).

3) Dimethyl arsenic standard stock solution[2] Measure 18.4 mg of dimethylarsonic acid [CH_7AsO_2] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of solution by adding water to the marked line. for use as dimethyl arsenic standard stock solution. (1 mL of the solution contains 100 µg in arsenic [As]).

4) Trimethyl arsenic standard stock solution[2] Measure 18.2 mg of trimethylarsinoxide [C_3H_9AsO] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of

solution by adding water to the marked line. for use as trimethyl arsenic standard stock solution. (1 mL of the solution contains 100 μg in arsenic [As]).

5) Combined standard stock solution Mix fixed quantities of inorganic arsenic, monomethyl arsenic, dimethyl arsenic and trimethyl arsenic standard stock solutions prior to use, accurately diluting the solution with water to prepare several combined standard stock solutions respectively containing 1 to 5 ng of inorganic, monomethyl, dimethyl and trimethyl arsenics per 1 mL of the solution (to be prepared prior to use).
6) Sodium borohydride reagent: Dissove 1 g of sodium hydride and 20 g of sodium borohydride reagent to water, and adjust to 200 mL. (Prepare when it is needed)

B Preparation of the sample solution

Accurately measure 0.3 g of the analysis sample into a 100 mL tall beaker[Note 2], add 5 mL of nitric acid and cover with watch-glass. Moderately apply heat on sand bath for 60 minutes, and allow solution to cool. Add 2 mL of nitric acid and 2 mL of perchloric acid, cover beaker with watch-glass and concentrate on sand bath maintained at 200 to 250 °C until liquid volume is reduced to less than 1 mL[4]. Allow to cool off. Add 1 mL of hydrochloric acid (arsenic analysis grade) (1:10) to residue, transfer solution to a 50 mL volumetric flask with water, add water to the marked line for use as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions[6].

C Quantification

Introduce sodium boron hydride reagent, hydrochloric acid (arsenic analysis grade) (1:30) and sample solution to arsenic hydride generation system connected with atomic absorption spectrometer, mix solution[Note 3] and allow it to react. After collecting the generated arsenic hydride, methylarsinate, dimethylarsine and trimethylarsine in a tube cooled with liquid nitrogen, restore to ambient temperature and guide the vaporized arsenic hydride, methylarsinate, dimethylarsine and trimethylarsine to the absorption cell heated within a muffle kiln using helium as the carrier gas. Measure absorbance of the sample for the wavelength of 193.7 nm[5].

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently measure absorbance of various combined standard solutions treated likewise to the sample solution, generating a calibration curve based on the peak area or height to quantify the inorganic arsenic, monomethyl arsenic, dimethyl arsenic and trimethyl arsenic content of the sample.

Measurement condition Example

Injestion volume of sample：1 mL

Carrier gas: He（0.4 L/min）

Muffle kiln temperature：800 °C

Note 1 Unless otherwise specified, the acids used for the analysis shall be of precision analysis grade.

2 Glass equipment used in the analysis shall be manufactured with non-arsenic borosilicate glass.

3 Silicone oil solution (1%) to be used as antifoam agent in case of excessive foaming.

《Summary of analysis method》

The method is used to quantify the inorganic and organic arsenic content of feed with arsenic hydride generation--ultralow temperature collection—atomic absorption spectrophotometry analysis.

The flow of analysis method and overview of the analysis equipment are respectively indicated in figures 4.2.2-1 and 4.2.2-2.

0.3 g sample
- 5 mL Nitric acid
- Moderate heating for 60 minutes on sand bath
- Cooling
- 2 mL nitric acid and 2 mL perchloric acidc
- Heat and concentrate to less than 1 mL on sand bath at 200 to 250 °C
- 1 mL hydrochloric acid (1:10)
- Quantity adjustment to 50 mL with water

Measurement

Figure 4.2.2-1 Flow of analysis for arsenic by type of compound

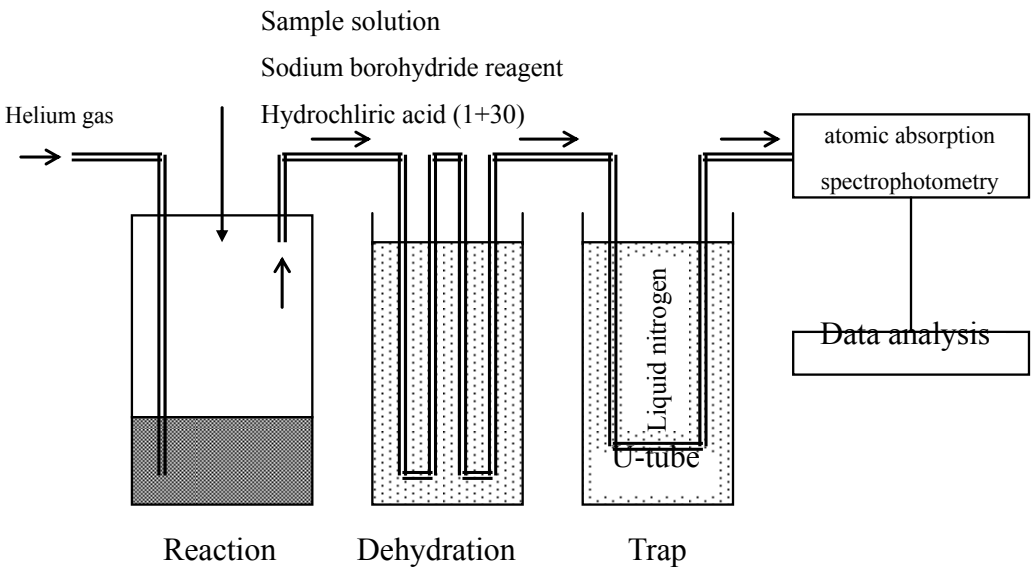


Figure 4.2.2-2 Overview of arsenic hydride generation--ultralow temperature collection—atomic absorption spectrophotometry analysis system

References; Satoru Yoshinaga, kotoharu Yagi, Eiichi Ishiguro, Kouji Taneike,: Research Report of Animal Feed, 31, 128 (2006)

Feed analysis standard description progress 【27】New

《Analysis validation》

・Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (mg/kg)	Repeat	Average spike recovery (%)	Repeatability RSD (% or less)
Inorganic arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	90.0-96.7	9.7
	Fish meal (Atka origin)	0.5-2.0	3	90.2-92.7	14.4
	Fish meal (Mackerel origin)	0.5-2.0	3	96.5-100.0	3.5
Monomethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	80.7-89.3	2.3
	Fish meal (Atka origin)	0.5-2.0	3	90.0-101.5	7.7
	Fish meal (Mackerel origin)	0.5-2.0	3	83.3-91.0	5.5
Dimethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	109.3-120.3	17.6
	Fish meal (Atka origin)	0.5-2.0	3	89.0-90.7	10.4
	Fish meal (Mackerel origin)	0.5-2.0	3	96.5-99.3	3.1
Trimethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	102.7-115.8	13.7
	Fish meal (Atka origin)	0.5-2.0	3	92.0-99.2	17.0
	Fish meal (Mackerel origin)	0.5-2.0	3	98.0-109.5	7.4

・Authenticated standard sample

Name of spiked component	Subject of measurement	Authentication value (mg/kg)	Data (mg/kg)
Tuna meal (BCR-627)	Dimethylated arsenic	0.15	0.16
	Arsenobetaine (measure as trimethylated arsenic	3.90	3.95

《Notes and precautions》

[1] Use of commercially inorganic arsenic standard solutions are acceptable.

[2] Available from Tri Chemical Laboratories Inc.

[3] Sample decomposition at temperatures exceeding 250 °C causes organic arsenics contained in the sample to decompose as well. Temperature management is facilitated by the use of electric heated plates.

[4] Residual nitric acid in sample solution may result in the detection of unwanted peak after the peak corresponding to trimethyl arsenic content. Accordingly, the nitric acid needs to be completely volatilized.

[5] Figure 4.2.2-3 shows an example of the chart.

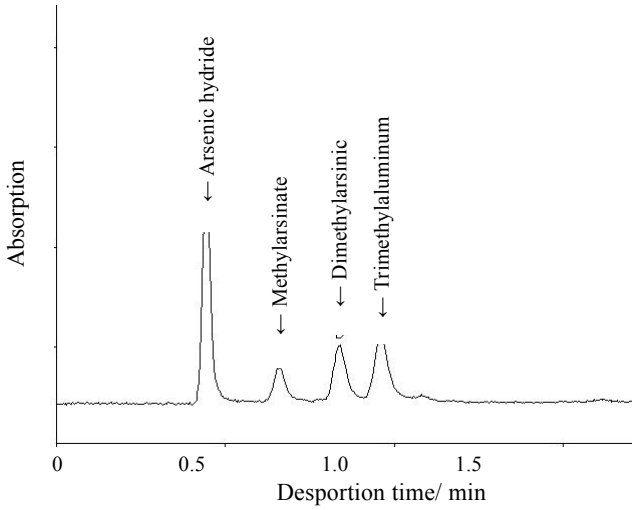


Figure 4.2.2-3 Chart indicating 0.5 mg/kg content of various types of arsenic compounds added to fish meal.

【Other analysis method】

3 Absorption photometry (silver diethyldithiocarbamate method)

Arsenic subject to analysis Total arsenic

A Reagent preparation

1) Arsenic standard solution Accurately dilute fixed quantities of arsenic standard stock solution with water, preparing an arsenic solution containing 1 μg of arsenic per 1 mL of solution.
2) Silver diethyldithiocarbamate solution Dissolve 0.5 g of silver diethyldithiocarbamate with 100 mL of pyridine[1], and place in refrigerated storage.
3) Tin chloride (II) solution Dissolve 15 g of tin chloride (II) in 100 mL of hydrochloric acid (1:1) (to be prepared prior to use)[2].
4) Potassium iodide solution Dissolve 200 g of potassium iodide in water, adjusting overall quantity to 1 liter (to be prepared prior to use).
5) Lead acetate cotton Immerse surgical cotton in 10% lead acetate solution and air dry.

B Preparation of the sample solution [3]

Accurately measure 2 g of the analysis sample into a 100 mL tall beaker, add 10 mL of nitric acid and 5 mL of sulfuric acid, cover them with watch-glass, and allow to settle overnight.

Then moderately apply heat to solution for 30 minutes on sand bath and ignite after foaming recedes and allow to cool off. Add 5 mL of perchloric acid, cover with watch-glass again and heat to completely decompose sample on sand bath. And concentrate until liquid volume is reduced to

less than 2 mL, then cool off. Add 5 mL of hydrochloric acid (arsenics analysis grade) (1:10) and 20 mL of water to residue and heat to dissolve. Then transfer solution to a 100 mL volumetric flask. Add water to the marked line and filtrate using filter paper (No. 6) for use as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions.

C Quantification[Note 4]

Accurately measure 10 mL of the sample solution into a arsenic hydride generation bottle[4], add10mL of hydrochloric acid, stir while adding 2 mL of potassium iodide solution and allow to settle for several minutes. Add 1 mL of tin chloride (II) solution[5], shake and allow solution to settle for 10 minutes. Add 2.5 g of zinc (arsenic analysis grade; diameter 1 to 1.5 mm) to solution, guiding generated gas to a glass tube lightly stuffed with lead acetate cotton and absorption tube accurately filled with 5 mL of silver diethyldithiocarbamate solution. Connect arsenic hydride generation bottle and glass tube to prevent escape of gas, allowing for the reaction to proceed for 45 minutes at approximately 25 °C.

Measure absorbance of color development solution within absorption tube at wavelength of 510 nm using blank test solution concurrently processed as the contrast solution.

Accurately measure 2 mL, 4 mL, 8 mL, and 16 mL of arsenic standard solution alternately, respectively adjusting quantity to 40 mL with water. The solutions should be processed likewise to the sample solution, using the obtained absorbance to generate a calibration curve, subsequently calculating the arsenic content based on the curve.

《Summary of analysis method》

Method measures the red-violet generated by the reaction between arsenic hydride (AsH_3, arsine) and silver diethyldithiocarbamate within a pyridine environment using a spectral photometer.

《Notes and precautions》

[1] Conduct process in drafted environment when handling pyridine to provide against the strong odor and toxicity of the substance.

[2] Store in a brown bottle.

[3] Subsection 1 Procedure is same as the hydride generation atomic absorption previously described.

Refer to the corresponding 《Notes and precautions》 for instructions.

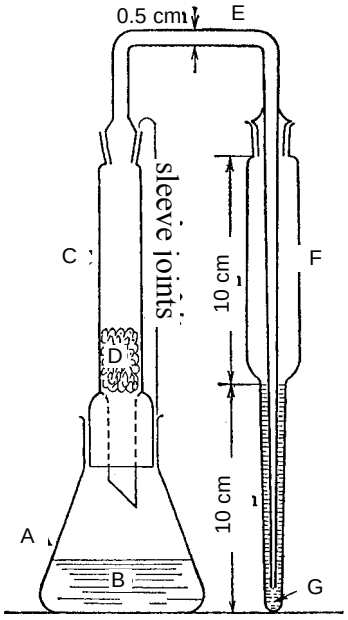
[4] Refer to figure 4.1.18-2.

Arsenic hydride generation bottle (A) should consist of a 100 mL Erlenmeyer flask attached with a glass tube (C) lightly stuffed with glass wool wetted in lead acetate (for the collection of H_2S), a glass duct tube (E) to the upper section of the flask, and leading to an absorption tube (G). In order to seal the glass equipment, apply a light coat of silicone grease to the sleeve

joints.

The required glass equipment complies with the provisions of section 61.1 of JIS standard K0102 “Industrial Waste Water Testing Method”, and commercially available from sources including Sibata Scientific Technology Ltd., Asahi Glass Co. ltd Science Product Dept., and Kiriyama Glass Co.

[5] For samples with large quantities of coexisting iron, add 1 gl of L-ascorbic acid.



A：Generatiom bottle (conocal flask)

B：Sample solution

C：Glass tube

D：Lead acetate glass wool

E：Glass pipe

F：Absorption tube

G：Silver dithyldithiocarbamate solution (5 mL)

Figure 4.1.18-2 Arsenic hydride (III) generation/collection equipment

Nitrite Nitrogen (NO_2^-)

Nitrate Nitrogen (NO_3^-)

【Summary of Nitrite Nitrogen and Nitrate Nitrogen】

Occasional poisoning is observed among cattle ingesting dried herbage rich in nitrite salts. While nitrite nitrogen is considered to be a direct causal substance basically resulting in acute intoxication, nitrate nitrogen is presumed to be an indirect causal substance with potential for chronic intoxication.

Corn, sorghum, sudangrass, italian ryegrass and rye has a tendency of accumulating nitrite salts in the leaves/ stems during the initial to intermediate stages of growth process. The nitrite salt content of these plants are reduced to nitrite nitrogen within cattle rumen through biodegration, eventually transforming into ammonia after being converted to hydroxyamine, to be utilized in the activity of the microorganisms.

However, in the event of excessive ingestion, nitric salts are taken into the bloodstream, causing oxygen deficiency and severe cases of nitric salt poisoning may result in deaths. Tolerance for nitrate nitrogen ingestion is specified to be 0.111 g/kg—body weight (daily consumption) or approximately 0.2% content in feed.

According to the monitoring inspection conducted by FAMIC on imported dried herbage, the nitrate nitrogen content of alfalfa hey was ND to 0.16% (detection rate 80%) and ND to 0.40% for sudangrass (detection rate 90%), although sudangrass with abnormally high concentration of 1.4% nitrate nitrogen content is not unprecedented in investigations conducted on the cause of cattle accidents.

《Method listed in the Feed Analysis Standards》

1 Simultaneous analysis of nitrite nitrogen and nitrate nitrogen by liquid chromatograph [Feed analysis standards, Chap4, Section 1.19.1 and 20. 1]

Subject of analysis : Nitrate nitrogen and Nitrite nitrogen

A Reagent preparation

1) Phosphate buffer solution Dissolve disodium hydrogenphosphate12 water 1.79 g, $12H_2O$ 1.79 g, 0.78 g of sodium dihydrogenphosphate dihydrate and 14.04 g of sodium perchlorate monohydrate into water, adjusting overall quantity to 1 liter.
2) Nitrite nitrogen standard stock solution Measure 0.493 g of sodium nitrite [$NaNO_2$] (desiccated for 3 to 4 hours at 105 °C) into a 100 mL volumetric flask and dissolve by adding water, adjusting quantity by adding water to the marked line for use as nitrite nitrogen standard stock solution. (1 mL of the solution contains 1 mg of nitrite nitrogen.)
3) Nitrate nitrogen standard stock solution, Measure 0.607 g of sodium nitrate [$NaNO_3$]

(desiccated for 3 to 4 hours at 105 °C) into a 100 mL volumetric flask and dissolve by adding water, adjusting quantity by adding water to the marked line for use as nitrite nitrogen standard stock solution. (1 mL of the solution contains 1 mg of nitrate nitrogen.)

4) Combined standard stock solution　Mix fixed quantities of nitrite nitrogen and nitrate nitrogen standard stock solutions prior to use, accurately diluting the solution with phosphate buffer to prepare several combined standard stock solutions respectively containing 0.5 to 20 ng of nitrate nitrogen and nitrite nitrogen per 1 mL of the solution

B　Quantification

Extraction　Accurately measure 5 g of sample into a 500 mL ground stoppered Erlenmeyer flask, add 250 mL of phosphate buffer solution[1] and shake for 20 minutes to extract[2]. Filtrate extract using filter paper (No. 5-A) and accurately dilute fixed quantities of the filtrate with phosphate buffer solution[3]. Filtrate solution with membrane filter (pore diameter: less than 0.5 μm) for use as the sample solution in the liquid chromatograph analysis.

Liquid chromatography　Inject 20 μl of sample solution and respective combined standard solutions into ion chromatograph to obtain chromatogl.

Measurement conditions; Example

Detector: UV absorption detector (wavelength: 210 nm)(4)

Column：Amino group modified vinyl alcohol polymer column (diameter4.6 mmLength 250 mm, particle size 5μm)

Eluent：Phosphate buffer

Flow rate：0.8 mL/min

Calculation　Identify peak height or area from obtained chromatogl to generate calibration curve, calculating quantity of nitrate nitrogen and nitrite nitrogen content for sample.

Note 1　Asahipak NH2P-50 4E（Manufactured by Showa Denko K.K.） or equivalent.

《Summary of analysis method》

Conventional means of nitrite nitrogen and nitrate nitrogen analysis included the colorimetric assay method (cadmium reduction method) and ion meter method. However, significant margins of error was evident among the methods, and were gradually replaced by the relatively precise liquid chromatograph analysis (LC) method.

Method enables the simultaneous measurement of nitrite nitrogen and nitrate nitrogen although nitrite nitrogen is rarely detected from imported herbage. Conventionally, colorimetric assay (cadmium reduction) and ion meter methods were utilized in the analysis of nitrate nitrogen with poor reproducibility. The simplified RQ flex test kit offers results relatively similar to analysis conducted by liquid chromatographs.

Reference;　Shigetaka Suzuki, Yuuji Shirai,: Research Report of Animal Feed, 20, 1 (1994)

Feed analysis standard description progress　【17】New

《Notes and precautions》

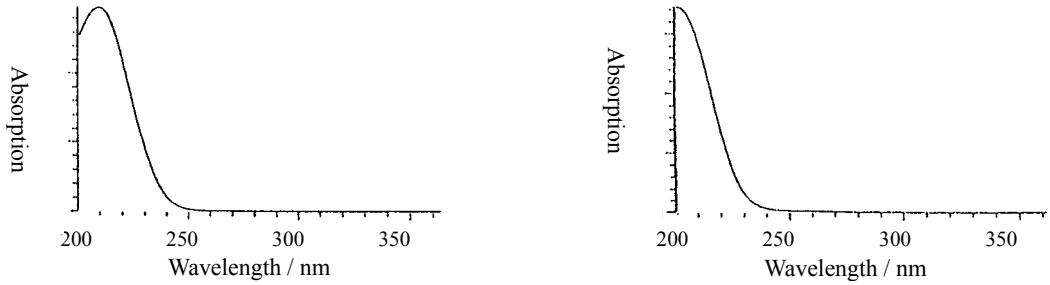
[1] Phosphate buffer solution are used in the extraction process because the buffer is also utilized as the eluant for the LC. However, extraction using water is equally acceptable.

[2] Shaking apparatus to be used.

[3] Typically diluted by about 10X to 100X depending on the nitrite/nitrate nitrogen content of the sample for use as the sample solution to be supplied to the liquid chromatograph.

[4] Absorption curves for nitrite and nitrate nitrogen are as indicated in figure 4.2.3-1.

[5] Figure 4.2.3-2. shows an example of the chromatogl



Nitrate nitrogen　　Nitrite nitrogen

Figure 4.2.3-1　Absorption curves for nitrite nitrogen and nitrate nitrogen

Measurement conditions;

Detector: UV absorption detector (wavelength: 217 nm)[4]

Column：Asahipak NI12p-50 4E (diameter4.6 mmLength 250 mm, particle size 5μm)

Guard Column: NI12p-50 4E (diameter4.6 mmLength 10 mm, particle size 5μm)

Eluent：Phosphate buffer

Flow rate：0.8 mL/min

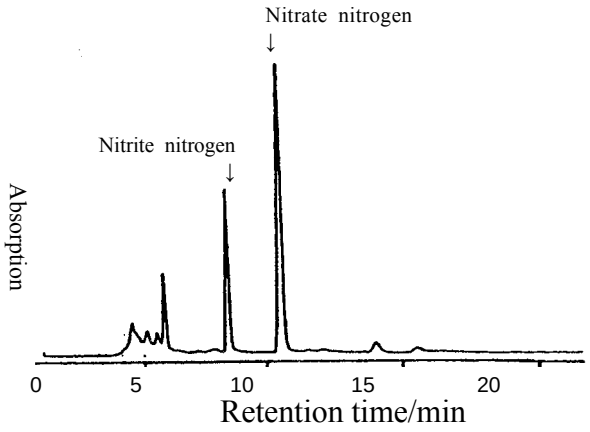


Figure 4.2.3-2　Chromatogl for nitrite nitrogen and nitrate nitrogen standard solutions

2 Simultaneous analysis of inorganic ions and organic acids by capillary electrophoresis [Feed analysis standards, Chapter 4, Section1.19.1 and 20. 2]

Subject of analysis : Cholorine, Nitrite nitrogen, Nitrate nitrogen, Propionic acid (Includes calcium propionate and sodium propionate), iso-valeric acid, *n*-valeric acid, Citric acid and Acetic acid (12 compounds)

Scope of application : Silage

A Reagent preparation

Organic acid/inorganic ion combined standard solution Measure reagents used in the preparation of standard stock solutions for the organic acids and inorganic ions indicated in the table below into individual 100 mL volumetric flasks, dissolve by adding water[Note 1] and adjust quantity of solutions within the flasks by adding water to the marked line for use as organic acid/inorganic ion standard stock solutions. (1 mL of respective solutions contain 2 mg of the organic acid and inorganic ion.)

Mix fixed quantity of respective organic acid/inorganic ion standard stock solution prior to use, accurately diluting solution with water to obtain several combined standard solutions containing 25 to 100 μg of respective organic acid/inorganic ion per 1 mL of individual solution.

Subject of analysis	Name of standard stock solution	Standard reagent	Sampled quantity
Chlorine	Chlorine standard stock solution	Sodium chloride〔NaCl〕	0.330 g
Nitrite nitrogen	Nitrite nitrogen standard stock solutio	Potassium nitrite〔KNO_2〕	0.370 g
Nitrate nitrogen	Nitrate nitrogen standard stock solutic	Potassium nitrate〔KNO_3〕	0.326 g
Propionic acid	Propionic acid standard stock solution	Sodium propionate〔$C_3H_5O_2Na$〕	0.259 g
Formic acid	Formic acid standard stock solution	Sodium formate〔CHO_2Na〕	0.296 g
iso-valeric acid	iso-valeric acid standard stock solutio	iso-sodium valerate〔$C_5H_9O_2Na$〕	0.243 g
n -valeric acid	*n* -valeric acid standard stock solution	*n* -sodium valerate〔$C_5H_9O_2Na$〕	0.243 g
Citric acid	Citric acid standard stock solution	Citric acid monohydrate〔$C_6H_8O_7 \cdot H_2$	0.218 g
Acetic acid	Acetic acid standard stock solution	Sodium acetate〔$C_2H_3O_2Na$〕	0.273 g
Lactic acid	Lactic acid standard stock solution	Sodium lactate〔$C_3H_5O_3Na$〕	0.249 g
n -hexanoic acid	*n* -hexanoic acid standard stock soluti	*n* -hexane sodium〔$C_6H_{11}O_2Na$〕	0.238 g
butyric acid	butyric acid standard stock solution	sodium *n* -butyrate〔$C_4H_7O_2Na$〕	0.250 g

B Quantification

Extraction Measure 10.0 g of the analysis sample into a 300 mL ground stoppered Erlenmeyer flask, add 100 mL of water and shake for 30 minutes to extract. Filtrate extract using filter paper (No. 5-A) for use as sample solution to be subjected to the column processing.

Column processing Introduce sample solution to a carboxy-methylated silica gel mini-column (360 mg)[Note 2] [2], douche tallow solution to run off[Note 3][3]. Accurately dilute fixed quantities of the effluent with water. The dilute solution is then transferred to a filter cup[Note 4] equipped with ultrafiltration membrane (molecular weight cutoff level: 30,000) and connected to a plastic centrifugal tube (capacity: 1.5 mL). Subject solution to centrifugal filtration at 5,000×*g* for 15 minutes, using the filtrate[Note 5] as sample solution for the capillary electrophoresis.

Capillary electrophoresis Introduce the sample solution and respective combined standard solutions

into the capillary electrophoresis system, obtaining electropherogls by means of indirect absorption photometry.

Measurement conditions Example

Column：Capillary column (caliber 50 μm, available length 104 cm, total length 112.5 cm)

Migration buffer fluid：2,6-pyridinedicarboxylic acid（30 mmol/L）、*n*-hexadecyltrimethylammonium bromide（0.5 mmol/L）mixed solution（pH 12.0）Note 6

Voltage：−30kV

Detector: Detector：UV absorptiometric detector（detection wavelength :350 nm, reference wavelength :275 nm)

Column bath temperature ：20 °C

Amount of sample introduction：Pressure injection method（5,000 Pa、6 s)

Column cleansing[4]: Cleanse column for at least 10 minutes with migration buffer fluid prior to the introduction of the sample solution and various combined standard solutions into the capillary electrophoresis system.

Calculation Identify peak height from obtained chromatogl to generate calibration curve, calculating quantity of bromine content for sample.

Note 1 Water used in the analysis shall maintain an electric conductivity of less than 5.6 μS/m (specific resistance of 18 MΩ·cm or above).

2 Sep-Pak Accell Plus CM Cartridge (manufactured by Waters) or equivalent.

3 0 to 2 mL of initial flow to be used.

4 Microcon YM-30 (manufactured by Millipore) or equivalent.

5 Use supernatant solution from the filtrate obtained s as the sample solution.

6 Introduce 0.501 g of 2,6-pyridinedicarboxylic acid into a 100 mL volumetric flask, add water and dissolve by supersonic treatment. Add 2 mL of sodium hydrate solution (1 mol/l) and adjust quantity by adding water to the marked line, then add 75 μl of *n*-hexadecyltrimethylammonium bromide and shake flask. Transfer the solution to a 200 mL beaker and adjust pH to 12.0 by adding sodium hydrate solution (1 mol/l).

《Summary of analysis method》

The method quantifies various organic acid and inorganic ion content of the silage Concurrently using capillary electrophoresis.

Feed analysis standard description progress 【26】New

《Analysis validation》

・Spike recovery and repeatability

Spike concentration	0.3%		0.6%	
Name of spiked component	Spike recovery（%）	Repeatability（% or less）	Spike recovery（%）	Repeatability（% or less）
Chlorine	99.9-110.5	6.3	103.5-108.9	2.8
Nitrite nitrogen	101.1-106.7	7.1	103.5-107.3	4.1
Nitrate nitrogen	99.0-109.6	5.0	98.0-100.4	4.9
Propionic acid	102.1-106.5	6.0	97.6-102.3	5.6
Formic acid	108.8-110.5	8.8	102.2-108.6	5.9
iso-valeric acid	103.5-107.9	5.5	99.2-102.5	5.5
n -valeric acid	99.3-107.0	4.0	100.3-103.3	4.5
Citric acid	101.0-109.4	8.2	97.6-104.5	5.4
Acetic acid	101.6-105.4	6.6	103.9-106.6	4.6
Lactic acid	101.8-106.6	5.9	97.1- 99.7	4.3
n -hexanoic acid	100.9-109.4	6.5	101.8-108.2	6.1
butyric acid	101.0-105.5	5.4	99.2-106.4	5.2

・Quantification lower limit:　For respective organic acidsand inorganic ions, Smples 0.02 %

《Notes and precautions》

[1] For details of capillary electrophoresis (CE) , refer to Section 1-6, Chlorine subsection 1, 《Notes and precautions》[1]（p.79） in Chapter 4 of this document.

[2] Mini-column shall not be pre-conditioned. Sample solution is to be introduced by dry degradation.

[3] No more than 2 mL of the solution should be isolated.

[4] In the event the condition of the column deteriorates, cleanse column using 0.1 mol/l sodium hydrate solution. Wash column with water for 10 minutes, then with 0.1 mol/l sodium hydrate solution for 5 to 10 minutes. Finally wash column with water for 20 minutes to wash away all traces of sodium hydrate from column.

[5] Negative peak presumably deriving from butyric acid follows the butyric acid peak on the electropherogl. Accordingly, the peak acid for the butyric acid should be calculated along the baseline ignoring the existence of the negative peak.

2 Arsenic hydride generation--ultralow temperature collection-atomic absorption spectrophotometry analysis depending on type of sample

[Feed analysis standards, Chapter 4, Section 2.2] Note 1

Type of arsenic compound analyzed: Inorganic arsenic, Monomethyl arsenic, Dimethyl arsenic, Trimethyl arsenic

A Reagent preparation

1) Inorganic arsenic standard stock solution[1] Measure 132 mg of diarsenic trioxide (standard reagent) (desiccated for 3 to 4 hours at 105 °C) into a beaker, add 5 mL of sodium hydroxide solution (4 w/v%) and 50 mL of water, apply heat to dissolve reagent and allow to cool off. Add 1 drop of phenolphthalein reagent to the solution, neutralize with sulfuric acid (1:10) and transfer to a 100 mL volumetric flask with water and adjust quantity by adding water to the marked line for use as inorganic arsenic standard stock solution. (1 mL of the solution contains 1 mg in arsenic [As]).

2) Monomethyl arsenic standard stock solution[2] Measure 18.7 mg of methylarsonic acid [CH_5AsO_3] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of solution by adding water to the marked line. for use as monomethyl arsenic standard stock solution. (1 mL of the solution contains 100 µg in arsenic [As]).
3) Dimethyl arsenic standard stock solution[2] Measure 18.4 mg of dimethylarsonic acid [CH_7AsO_2] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of solution by adding water to the marked line. for use as dimethyl arsenic standard stock solution. (1 mL of the solution contains 100 µg in arsenic [As]).
4) Trimethyl arsenic standard stock solution[2] Measure 18.2 mg of trimethylarsinoxide [C_3H_9AsO] into a 100 mL volumetric flask and dissolve with water, adjusting quantity of solution by adding water to the marked line. for use as trimethyl arsenic standard stock solution. (1 mL of the solution contains 100 µg in arsenic [As]).
5) Combined standard stock solution Mix fixed quantities of inorganic arsenic, monomethyl arsenic, dimethyl arsenic and trimethyl arsenic standard stock solutions prior to use, accurately diluting the solution with water to prepare several combined standard stock solutions respectively containing 1 to 5 ng of inorganic, monomethyl, dimethyl and trimethyl arsenics per 1 mL of the solution (to be prepared prior to use).
6) Sodium borohydride reagent: Dissove 1 g of sodium hydride and 20 g of sodium borohydride reagent to water, and adjust to 200 mL. (Prepare when it is needed)

B Preparation of the sample solution

Accurately measure 0.3 g of the analysis sample into a 100 mL tall beaker Note 2, add 5 mL of nitric acid and cover with watch-glass. Moderately apply heat on sand bath for 60 minutes, and allow solution to cool. Add 2 mL of nitric acid and 2 mL of perchloric acid, cover beaker with

watch-glass and concentrate on sand bath maintained at 200 to 250 °C until liquid volume is reduced to less than 1 mL[4]. Allow to cool off. Add 1 mL of hydrochloric acid (arsenic analysis grade) (1:10) to residue, transfer solution to a 50 mL volumetric flask with water, add water to the marked line for use as the sample solution.

Concurrently prepar procedure without the sample to produce blank test solutions[6].

C Quantification

Introduce sodium boron hydride reagent, hydrochloric acid (arsenic analysis grade) (1:30) and sample solution to arsenic hydride generation system connected with atomic absorption spectrometer, mix solution[Note 3] and allow it to react. After collecting the generated arsenic hydride, methylarsinate, dimethylarsine and trimethylarsine in a tube cooled with liquid nitrogen, restore to ambient temperature and guide the vaporized arsenic hydride, methylarsinate, dimethylarsine and trimethylarsine to the absorption cell heated within a muffle kiln using helium as the carrier gas. Measure absorbance of the sample for the wavelength of 193.7 nm[5].

Correct result based on the absorbance measurement conducted under the same condition for the blank test solution.

Concurrently measure absorbance of various combined standard solutions treated likewise to the sample solution, generating a calibration curve based on the peak area or height to quantify the inorganic arsenic, monomethyl arsenic, dimethyl arsenic and trimethyl arsenic content of the sample.

Measurement condition Example

Injestion volume of sample: 1 mL

Carrier gas: He (0.4 L/min)

Muffle kiln temperature: 800 °C

Note 1 Unless otherwise specified, the acids used for the analysis shall be of precision analysis grade.

2 Glass equipment used in the analysis shall be manufactured with non-arsenic borosilicate glass.

3 Silicone oil solution (1%) to be used as antifoam agent in case of excessive foaming.

《Summary of analysis method》

The method is used to quantify the inorganic and organic arsenic content of feed with arsenic hydride generation--ultralow temperature collection—atomic absorption spectrophotometry analysis.

The flow of analysis method and overview of the analysis equipment are respectively indicated in figures 4.2.2-1 and 4.2.2-2.

0.3 g sample
- 5 mL Nitric acid
- Moderate heating for 60 minutes on sand bath
- Cooling
- 2 mL nitric acid and 2 mL perchloric acidc
- Heat and concentrate to less than 1 mL on sand bath at 200 to 250 °C
- 1 mL hydrochloric acid (1:10)
- Quantity adjustment to 50 mL with water

Measurement

Figure 4.2.2-1 Flow of analysis for arsenic by type of compound

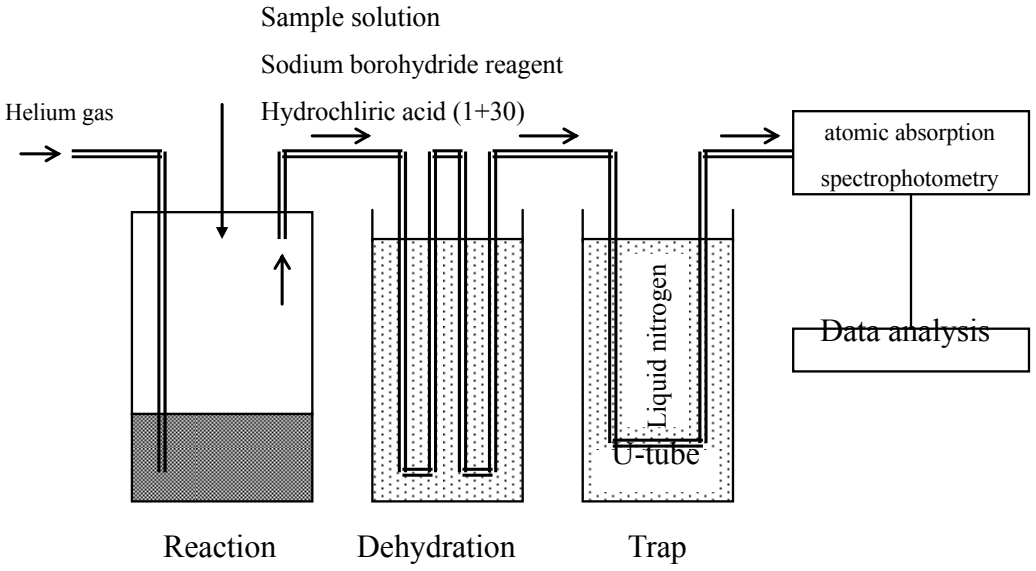


Figure 4.2.2-2 Overview of arsenic hydride generation--ultralow temperature collection—atomic absorption spectrophotometry analysis system

References; Satoru Yoshinaga, kotoharu Yagi, Eiichi Ishiguro, Kouji Taneike,: Research Report of Animal Feed, 31, 128 (2006)
Feed analysis standard description progress 【27】New

《Analysis validation》

・Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (mg/kg)	Repeat	Average spike recovery (%)	Repeatability RSD (% or less)
Inorganic arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	90.0-96.7	9.7
	Fish meal (Atka origin)	0.5-2.0	3	90.2-92.7	14.4
	Fish meal (Mackerel origin)	0.5-2.0	3	96.5-100.0	3.5
Monomethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	80.7-89.3	2.3
	Fish meal (Atka origin)	0.5-2.0	3	90.0-101.5	7.7
	Fish meal (Mackerel origin)	0.5-2.0	3	83.3-91.0	5.5
Dimethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	109.3-120.3	17.6
	Fish meal (Atka origin)	0.5-2.0	3	89.0-90.7	10.4
	Fish meal (Mackerel origin)	0.5-2.0	3	96.5-99.3	3.1
Trimethyl arsenic	Fish meal (Alaska Pollack origin)	0.5-2.0	3	102.7-115.8	13.7
	Fish meal (Atka origin)	0.5-2.0	3	92.0-99.2	17.0
	Fish meal (Mackerel origin)	0.5-2.0	3	98.0-109.5	7.4

・Authenticated standard sample

Name of spiked component	Subject of measurement	Authentication value (mg/kg)	Data (mg/kg)
Tuna meal (BCR-627)	Dimethylated arsenic	0.15	0.16
	Arsenobetaine (measure as trimethylated arsenic	3.90	3.95

《Notes and precautions》

[1] Use of commercially inorganic arsenic standard solutions are acceptable.

[2] Available from Tri Chemical Laboratories Inc.

[3] Sample decomposition at temperatures exceeding 250 °C causes organic arsenics contained in the sample to decompose as well. Temperature management is facilitated by the use of electric heated plates.

[4] Residual nitric acid in sample solution may result in the detection of unwanted peak after the peak corresponding to trimethyl arsenic content. Accordingly, the nitric acid needs to be completely volatilized.

[5] Figure 4.2.2-3 shows an example of the chart.

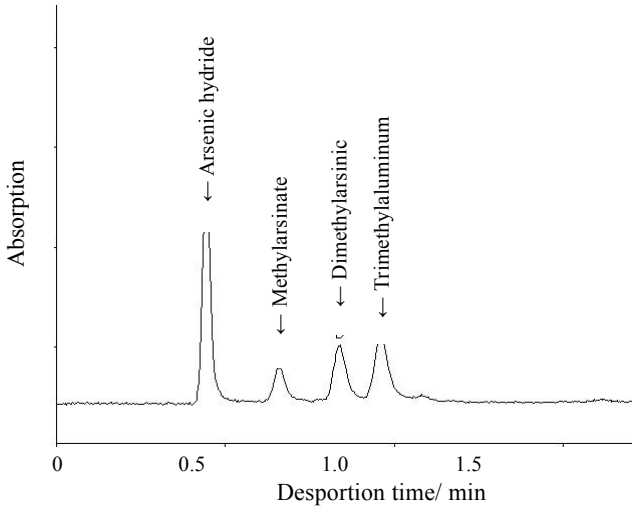


Figure 4.2.2-3 Chart indicating 0.5 mg/kg content of various types of arsenic compounds added to fish meal.