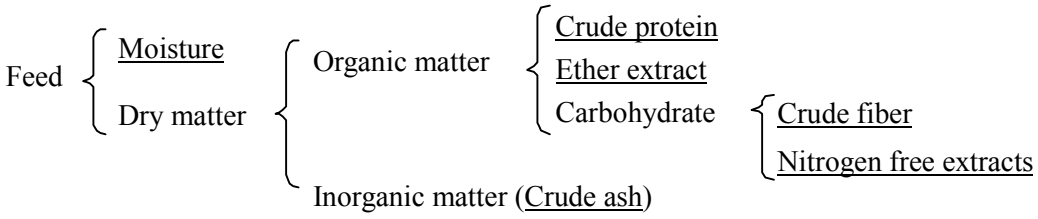
Chapter 3　Proximate composition and detergent fiber

[Summary of proximate composition]

Proximate composition is the term usually used in the field of feed/food and means the 6 components of moisture, crude protein, ether extract, crude fiber, crude ash and nitrogen free extracts, which are expressed as the content (%) in the feed, respectively.

The composition as the feed is classified as shown below:



The measured values of these 6 components in feed are important factors to understand the nature and the properties of the subject feed.

The 6 components in the feed and substances contained in them are listed in the table shown below:

Proximate compoition			Substances in respective composition
Moisture			Water, volatile substances
Dry matter	Organic matter	Crude protein	Pure protein, amino acids, non-protein compounds
		Crude fat (Ether extract)	Fat, complex lipid, sterols, fatty acids, fat-soluble dyes
		Crude fiber	Cellulose, hemicellulose, lignin
		Nitrogen free extracts	Soluble carbohydrate, hemicellulose, lignin, pectin, organic acids, tannin, water-soluble dyes
	Inorganic matter	Crude ash	Pure ash, organic residue, soil

Analysis methods for proximate composition was examined in the late 1950s mainly by the Department of Livestock Chemistry, National Institute of Agricultural Sciences (predecessor of Nutrition Department, National Institute of Animal Industry→ National Institute of Livestock and Grassland Science → National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization) and Analysis Branch, Feed Department, Livestock Industry Bureau (predecessor of Feed Inspection Station → Fertilizer and Feed Inspection Services → Food and Agricultural Materials Inspection Center).

At first, the methods were examined referring to Nougei-kagaku Jikkensho (Laboratory Manual for Agricultural Chemistry) and AOACI methods, etc., and were notified as “Standards for quantitative analysis testing of feeds” in October 1956, establishing analysis methods for feed testing. The methods were revised several times to be current analysis methods.

Due to the amendment of the Feed Safety Law in 1976, test methods for assays were newly defined (currently Note 3, Chapter 1 in “Official Specifications of Feeds” (Public Notice No. 756 of Ministry of Agriculture and Forestry, 1976), which includes analysis methods for calcium, phosphorus, water-soluble nitrogen and pepsin digestivity in addition to conventional proximate composition.

1. Moisture

[Summary of moisture]

The active ingredients from the view of feed nutrition are present in the part of dry matter (solid matter); therefore the level of moisture content is an important factor in both economy and storage. In summer at high temperature and humidity in Japan, the risk of putrefaction is predicted due to the proliferation of molds, etc., or self-digestion by enzymes in the feed when moisture in the feed is not less than about 15 %. For that reason, the moisture content in formula feed/mixed feed distributed in Japan is usually around 12-13 %.

As the assay for moisture in the feed measures loss on drying by heating at normal pressure as moisture, the result includes most of volatile substances other than H_2O. Therefore, it may be more appropriate to be referred to as volatile matter rather than moisture for accuracy.

Organic acids such as acetic acid and butyric acid in silage as well as ammonia and flavor components in feed materials are also vaporized and thus measured as moisture. Because the content of these in the feed is extremely low, there has hardly been a need to consider their influence on the measured value. However, in silage, etc., with high moisture content (low solid matter content), component contents per solid matter may be slightly affected depending on the content of volatile acids.

When the feed is spread on sheet and left at rest, moisture absorption or release proceeds, and under a constant relative humidity comes to equilibrium at the moisture content corresponding to the constant relative humidity (RH) (equilibrium moisture content), which indicates comparatively stable water content of the feed in the air-dry state. The equilibrium moisture content is different by the kind of the feed and is affected by hygroscopic materials such as salt if they are mixed in the feed. When the annual mean RH in Japan is presumed to be 65 %, the water content that is at equilibrium with it is in the range of 12-14 % for most of feed materials.

Precautions for the procedure of moisture assay are as follows:

(1) Check if the temperature display of the dryer indicates the predetermined temperature (it is needed to check if the thermometer is normal).

(2) Errors may be caused such as when the cooling time for the weighing dish is excessively long.

(3) Errors also may be caused by the location to place the weighing dish in the dryer; therefore it is needed to check the location in advance before use.

(4) A rough standard for the analysis value can be obtained when the standard sample (the sample with established analysis values distributed by the Conference for Feed Quality Improvement, etc.) is analyzed.

[Methods listed in the Analytical Standard of Feed]

1. Loss on drying[1] [Analytical Standard of Feed, Chapter 3, 1]

Measurement

Weigh accurately 2-5 g of an analysis sample[2], put it in an aluminum weighing dish[3] (dried and accurately weighed in advance)[4], dry it at 135±2 °C for 2 hours, let it stand to cool in a desiccator[5], and

then weigh accurately to calculate the moisture content in the sample.

However, the drying temperature should be 105±2 °C, and the drying time should be 3 hours[6] for fish soluble adsorption feed, molasses adsorption feed, gluten feed and corn distillers dried grains with soluble.

(Note) When it is difficult to grind the sample because of the high moisture content, prepare the analysis sample according to 2 (2) of Chapter 2, and then obtain the moisture content in the sample after preliminary drying by the assay method shown above, and calculate the moisture content in the original sample by the following formula:

$$\text{Moisture content (\%) in the original sample} = A + \frac{(100 - A) \times B}{100}$$

A : Moisture content (%) in the original sample after preliminary drying

B : Moisture content (%) in the sample after preliminary drying

«Summary of analysis method»

This is a method in which an analysis sample is heated with a temperature-controlled dryer and the loss is quantitated as moisture (loss on drying).

«Method validation»

· Results of proficiency testing in FY 2006

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)
Broiler starter	262	12.94	2.8
Fish meal	256	9.65	3.1

«Notes and precautions»

[1] The definition of moisture varies depending on the analysis subject or the analysis purpose; moisture by this analysis method mainly means water of adhesion, and loss on drying at normal pressure is designated as moisture for the reasons such as that most of the analysis subjects are organic matter and that it is easy to conduct the analysis.

The temperature distribution in the temperature-controlled dryer commonly used varies widely depending on the location, but the range is comparatively smaller in the temperature-controlled fan dryer with forced air-flow. However, care should be taken because a light sample may be blown off depending on the location to place the weighing dish.

Figure 3.1-1 Hand chopper

[2] Moisture in grains tends to be changed by grinding. In order to grind avoiding moisture change as much as possible, a device only for rough grinding is preferred, such as a hand chopper (manual roller mill) shown in Figure 3.1-1.

When a large amount of a sample is ground, the

procedure is accompanied by moisture change regardless of the grinding machine used that is commercially available.

Also, care should be taken for the storage of the sample after grinding.

[3] A grass weighing dish can also be used, but an aluminum weighing dish is more advantageous in that it is less fragile and lighter, shows better thermal conductivity, better airtightness, and is easier to handle.

Put the sample in the weighing dish, and put it in a dryer with the lid below or at the side of it. After drying for 2 hours, cover the container with the lid, and let it stand to cool in a desiccator. (It is recommended to use cotton work gloves, etc., because the dish is hot.)

A weighing dish of the shape and size as shown in Figure 3.1-2 is generally used. It is convenient to mark the lid and the dish with a number (the same number).

Additionally, a weighing dish stand as shown in Figure 3.1-3 is commercially available, which is convenient because it can be placed as is in a desiccator (Both commercially available from Sanshin Industrial.).

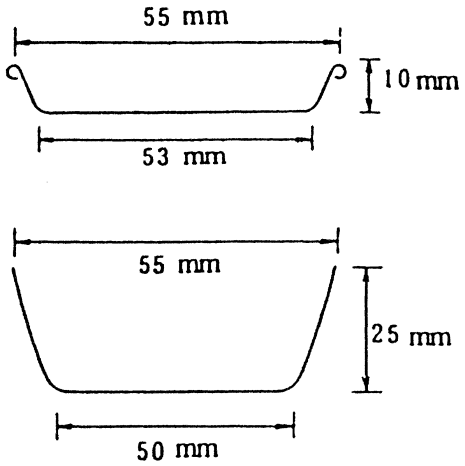


Figure 3.1-2 Aluminum weighing dish

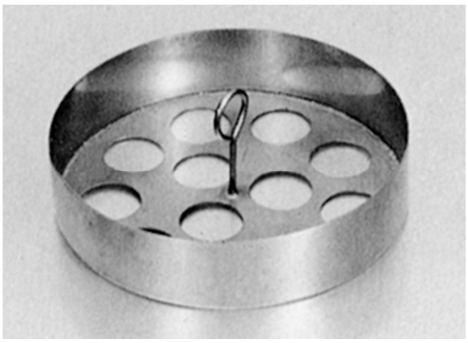


Figure 3.1-3 Weighing dish stand

[4] Collect and spread the sample, and weigh together with the lid.

[5] A desiccator of about 20-22 cm in the diameter of the platform is preferred.

Silica gel, calcium chloride (anhydrous), phosphorus pentoxide, or concentrated sulfuric acid, etc., can be used as a desiccant; however, use silica gel unless otherwise specified. Silica gel is supposed to be a common desiccant because it is easy to handle and regenerate; however, it should be dried again to be used when the blue color of cobalt salt added as the indicator of moisture absorption fades even if only slightly. Re-drying should be conducted at 130-140 °C for 2-3 hours.

Hygroscopicity is reduced by the adsorption of oil and fat, etc., to silica gel, and thus care should be taken. To minimize the analytical error due to cooling, it is recommended to keep the cooling duration in a desiccator to be constant (for 45 minutes for example), and to always put e.g., 8 weighing dishes in a desiccator. The number of weighing dishes to be contained in a desiccator is preferably not more than 10 because measurement errors are likely to occur between the start and the end of weighing when a large number of weighing dishes are contained in a desiccator.

[6] For fish soluble adsorption feed, molasses adsorption feed, gluten feed and corn distillers dried grains with soluble (DDGS), the method was modified as “drying at 105±2 °C, 3 hours” because there is a risk of vaporization or heat decomposition of volatile substances other than moisture if the

normal method is applied to them.

The assay of moisture in muciform feed such as fish soluble and molasses is usually conducted by the method shown below:

Weigh accurately 2 g of an analysis sample, put it in an aluminum weighing dish (put 10-20 g of sea sand* and a stirrer bar in it, dry, and weigh in advance), mix the sample and sea sand on a boiling water bath, and then dry for about 15 minutes stirring occasionally.

Then put it in a temperature-controlled dryer, dry at 105±2°C for 3 hours, let it stand to cool in a desiccator, and then weigh, to calculate the moisture content based on the loss.

* Use sea sand (silica sand) of 350-250 μm (60-80 mesh). Wash sea sand with water, heat in hydrochloric acid (1+1) for a few hours, wash with water until there is no acid, dry and store in a desiccator.

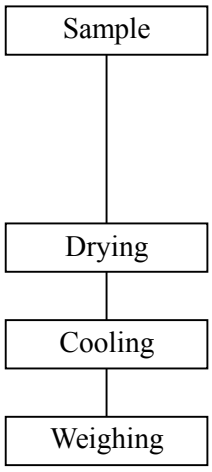
Toluene distillation or loss on heating at or 100 °C for 18 hours may be used for a sample with a high content of volatile components such as silage. For a highly viscous liquid, adsorb it on filter paper and dry to calculate the moisture content by the loss.

[Other analysis methods]

2. Moisture assay methods by international standards

ISO 6496 (1996) Animal feeding stuffs - Determination of moisture and other volatile matter content

Flow sheet of the analysis method



Weigh accurately about 5 g to the order of 1 mg, and put in a container*.

* Container of non-corrosive metal or glass with a sealable lid and with the surface area on which the analysis sample can be spread to be about 0.3 g/cm^2.

Dry at 103 °C for 4 hours using an electric dryer.

Desiccator

Weigh accurately to the order of 1 mg.

3. Distillation

Heat a sample in an organic solvent immiscible with water (such as toluene), distill water in the sample or the mixed vapor of water and the solvent, cool it and calculate moisture in the sample based on the volume of water separated from the solvent. This method is applicable to a thermostable sample that contains volatile components other than water as well as fat.

4. Kahl Fischer method

The method quantitates moisture in a substance utilizing the specific reaction of Kahl Fischer reagent, which contains iodine, sulfur dioxide and pyridine, with water under the presence of methanol, and is classified as the volumetric method and electrometric titration method.

The method is advantageous in that water alone can be quantitated when the sample contains volatile components other than moisture.

5. Heating furnace control method

A new type of thermogravitic analytical instrument that employs the heating furnace control method can measure 19 samples simultaneously and can quantitate ash content in addition to moisture.

Full-automatic moisture/ash analyzer TGA701 (manufactured by LECO (US), distributed by LECO Japan)

6. Other methods

When the sample contains thermostable components, dry under constant temperature and reduced pressure such as “60-70 °C, 26.7-33.3 kPa,” and the loss is obtained as the amount of moisture.

Also, a moisture meter that employs infrared radiation and can be used for measurement in the field is also used as a control analysis meter.

2. Crude Protein (CP)

[Summary of crude protein]

Crude protein is defined as the value obtained by quantitating nitrogen in a sample by the Kjeldahl method (in which nitrogen compounds in the sample is degraded by sulfuric acid to become ammonia, sodium hydroxide is added, steam distillation is conducted under the alkaline conditions, distilled ammonia is absorbed in acid and measured by titration) and multiplying the result by the factor 6.25 (6.38 for milk products). Therefore, crude protein includes ammonia, etc., that are not of protein origin.

Generally the nitrogen content of protein is 16 % on average; thus the inverse number of this (100/16 = 6.25) is used as the factor. However, as the factor is different between samples (5.83 for flour; 5.95 for rice), the crude protein of some feeds is different from the pure protein content; crude protein is measured to be excessively small in materials of milk product origin such as casein, and excessively large in flour and soybean.

Summary of the Kjeldahl method is as shown below:

Add concentrated sulfuric acid to a sample and heat, and then degradation and oxidation/reduction occur simultaneously to turn nitrogen into ammonia, which is present in the form of ammonium sulfate in concentrated sulfuric acid. Dilute it with water, add excess of concentrated sodium hydroxide and heat, and then ammonium sulfate is degraded into ammonia that is distilled. Transfer the ammonia into the sulfuric acid standard solution of a constant concentration, and titrate excess acid with the sodium hydroxide standard solution to obtain nitrogen content. The chemical reaction in the degradation by concentrated sulfuric acid is considered to be as follows:

(1) Organic compound is dehydrated and carbonized by sulfuric acid to produce carbon compounds.
(2) Carbon compounds are degraded into carbon and hydrogen by hydrolysis.
(3) Carbon and hydrogen are oxidized into carbon dioxide and water to be driven out.
(4) Sulfuric acid is reduced by a part of carbon into sulfurous acid, and carbon turns into carbon dioxide.
(5) Sulfurous acid reduces nitrogen into ammonia, and itself turns into sulfuric anhydride.
(6) Hydrogen produced by the degradation of carbon compounds facilitates the formation of ammonia.
(7) Ammonia formed immediately combines with sulfuric acid to become ammonium sulfate.
(8) When potassium sulfate is added as an oxidizing agent, the boiling point is elevated and the effect on organic compound becomes more active.

The combustion method is added to the Analytical Standard of Feed in 2006. The combustion method is also called the modified Dumas method and is employed as the AOACI method and the ISO (International Organization for Standardization) method, and is frequently used in foreign countries. The measurement principle is to combust a sample in oxygen at high temperature and to quantitate freed nitrogen by a thermal conductivity detector. The combustion method is characterized in the following points:

(1) Rapid quantitation is possible with measurement time of 5-10 minutes per sample.
(2) No draft is needed because there is no need to degrade a sample. Also, environmental load is smaller because deleterious or toxic substances such as concentrated sulfuric acid and copper sulfate are not used.

(3) The analysis instruments can be easily operated.

As the combustion method is used worldwide instead of the Kjeldahl method, it is supposed that the combustion method will also become the main stream of crude protein analysis in Japan.

[Methods listed in the Analytical Standard of Feed]

1. Kjeldahl method [Analytical Standard of Feed, Chapter 3, 2.1]

A. Reagent preparation

1) 0.1 mol/L sodium hydroxide standard solution[1]

Prepare a saturated solution[2] of sodium hydroxide, close the cap, leave at rest for not less than 10 days, and to 50 mL of the supernatant, add boiled and cooled water[3] to be 10 L to prepare the 0.1 mol/L sodium hydroxide standard solution. Moreover, standardize its concentration by the following procedure:

Weigh accurately 2-2.5 g of amidosulfuric acid (standard reagent) (dried in a desiccator (vacuum)[4] for 48 hours), put it in a 250-mL volumetric flask, add water to dissolve, and further add water up to the marked line to prepare the amidosulfuric acid standard solution. Transfer accurately 25 mL of the amidosulfuric acid standard solution into a 200-mL Erlenmeyer flask, add a few drops of bromothymol blue test solution[5], titrate with the 0.1 mol/L sodium hydroxide standard solution[6], and calculate the factor (f_1) of the 0.1 mol/L sodium hydroxide standard solution by the following formula:

$$f_1 = \frac{W \times 10^4}{V \times 97.10}$$[7]

W: Weight (g) of amidosulfuric acid in the amidosulfuric acid standard solution (25 mL) used for standardization

V: Volume (mL) of the 0.1 mol/L sodium hydroxide standard solution required for titration

2) 0.05 mol/L sulfuric acid standard solution[8]

Add 28 mL of sulfuric acid to 1 L of water gradually with stirring, let it stand to cool, and then add water to be 10 L to prepare the 0.05 mol/L sulfuric acid standard solution[9]. Moreover, standardize its concentration by the following procedure:

Transfer accurately 25 mL of the 0.05 mol/L sulfuric acid standard solution into a 200-mL Erlenmeyer flask, add a few drops of methyl red test solution[10], titrate with the 0.1 mol/L sodium hydroxide standard solution, calculate the factor (f_2) of the 0.05 mol/L sulfuric acid standard solution by the following formula:

$$f_2 = \frac{V \times f_1}{25}$$[11]

f_1: Factor of 0.1 mol/L sodium hydroxide standard solution

V: Volume (mL) of 0.1 mol/L sodium hydroxide standard solution required for titration

B. Sample solution preparation

Weigh accurately 1-5 g of an analysis sample, put it in a Kjeldahl flask, add 9 g of potassium sulfate and 1 g of copper sulfate (II) pentahydrate[12], further add 30-40 mL of sulfuric acid, and mix by shaking[13]. Heat it gradually[14], and then strongly after foaming subsides, and heat for not less than 2 hours after the

solution becomes clear[15], and then let it stand to cool. Transfer the solution with water into a 250-mL volumetric flask, and add water up to the marked line to be the sample solution.

C. Quantification[16]

1) Absorption by the sulfuric acid standard solution[17]

Transfer accurately a certain amount of the sample solution into a Kjeldahl flask, and add sodium hydroxide solution (50 w/v%) of a volume sufficient to turn the solution strongly alkaline[18]. Connect the flask to the steam distillation apparatus to which a receiver containing a certain amount[19] of 0.05 mol/L sulfuric acid standard solution in advance is attached, and distill until the distillate volume reaches about 120 mL[20].

Add a few drops of methyl red test solution[10] to the distillate, titrate with the 0.1 mol/L sodium hydroxide standard solution, and calculate the nitrogen [N] content by the following formula. Multiply it by 6.25 (6.38 for samples of milk products or milk replacer for calves which contain milk products not less than 50 %) to calculate the crude protein content in the sample.

$$\text{Nitrogen [N] content (\%)} = 1.40^{[21]} \times f_1 \times (V_1 - V_2) \times \frac{250^{[22]}}{V} \times \frac{100}{W} \times 10^{-3}$$

f_1 : Factor of 0.1 mol/L sodium hydroxide standard solution

V_1: Volume (mL) of 0.1 mol/L sodium hydroxide standard solution equivalent to the volume of 0.05 mol/L sulfuric acid standard solution in the receiver

V_2: Volume (mL) of 0.1 mol/L sodium hydroxide standard solution required for titration

V : Volume (mL) of the sample solution used for distillation

W: Weight (g) of the sample used for analysis

2) Absorption by boric acid solution[23]

Put a certain amount of boric acid solution (4 w/v%) into a receiver instead of the 0.05 mol/L sulfuric acid standard solution, and distill in the same way as 1).

Add a few drops of bromocresol green-methyl red test solution[24] to the distillate, titrate with the 0.05 mol/L sulfuric acid standard solution[25], and calculate the nitrogen [N] content by the following formula. Multiply it by 6.25 (6.38 for samples of milk products or milk replacer for calves which contain milk products not less than 50 %) to calculate the crude protein content in the sample.

$$\text{Nitrogen [N] content (\%)} = 1.40^{[26]} \times f_2 \times V_1 \times \frac{250^{[27]}}{V} \times \frac{100}{W} \times 10^{-3}$$

f_2 : Factor of 0.05 mol/L sulfuric acid standard solution

V_1: Volume (mL) of 0.05 mol/L sulfuric acid standard solution required for titration

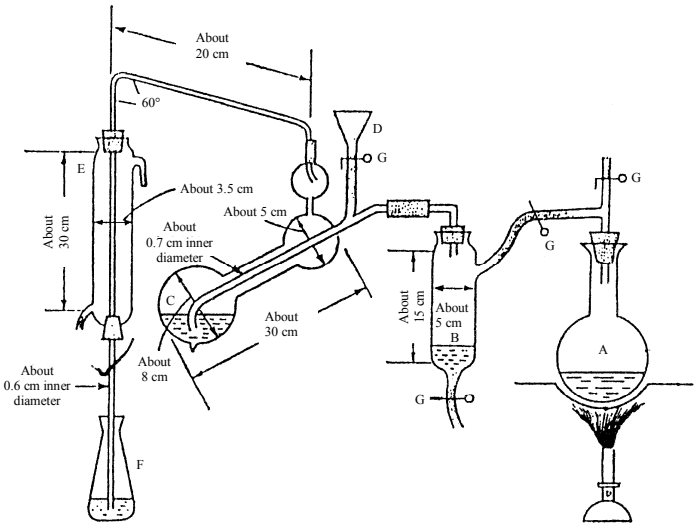
V : Volume (mL) of the sample solution used for distillation

W: Weight (g) of the sample used for analysis

«Summary of analysis method»

The method quantitates total nitrogen in a sample by the Kjeldahl method. However, nitrate nitrogen or nitrite nitrogen is not quantitated.

Generally, a macro-Kjeldahl nitrogen distillation apparatus by the indirect distillation method as shown in Figure 3.2-1 is used.



A : Round-bottom flask for steam generation
B : Trap for fluid waste collection
C : Distillation flask
D : Funnel for the addition of the sample solution and sodium hydroxide
E : Condenser (if condensation occurs on the outer surface, it is recommended to wrap the condenser with cloth to prevent dripping off.)
F : Receiver (200-mL Erlenmeyer flask)
G : Pinch cock

Figure 3.2-1 Nitrogen distillation apparatus

«Method validation»

· Results of proficiency testing in FY 2006

1) Absorption by sulfuric acid standard solution

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Broiler starter	31	24.23	1.7	0.8
Fish meal	28	63.14	2.7	2.1

2) Absorption by boric acid solution

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Broiler starter	56	24.24	1.9	0.8
Fish meal	55	63.23	1.3	0.6

«Notes and precautions»

[1] Connect the container containing the sodium hydroxide standard solution prepared with a soda lime tube or a bottle containing sodium hydroxide solution to avoid the entrance of carbon dioxide in the air; however, it is desirable to standardize it once every 2-3 months.

Commercially available 0.1 mol/L sodium hydroxide solution may as well be used.

[2] Because sodium hydroxide is highly hygroscopic and is likely affected by carbonic acid, theoretically it is difficult to obtain a solution of accurate concentration. Use saturated solution to avoid the effect of carbonic acid (concentrated sodium hydroxide solution contains little carbon dioxide).

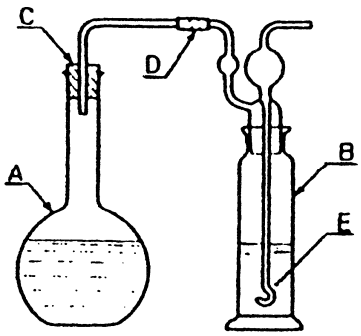
About 80 g of sodium hydroxide is soluble in 74 mL of water at 20 °C, thus it is recommended to

prepare saturated solution by adding slightly excessive sodium hydroxide and leave at rest to collect clear supernatant to be used (the concentration is about 20 mol/L at 20 °C).

[3] Use purified water that is boiled and then cooled to remove carbon dioxide. See JIS K 8001 "General rule for test methods of reagents."

Boil water in a flask for 15 minutes, and then shut out carbon dioxide in the air by attaching a gas washing bottle as shown in Figure 3.2-2 containing potassium hydroxide solution (25 w/v%) or a soda lime tube, and cool.

Prepare this water before use.



A : 1-L flat bottom flask
B : Gas washing bottle
C : Ground-joint stopper or silicon rubber stopper
D : Ground joint or silicon rubber tube
E : Potassium hydroxide solution (25 w/v%)

Figure 3.2-2 An example of cooling apparatus for water not containing carbon dioxide

[4] Vacuum by suction with a vacuum pump, etc. (not more than 2.0 kPa).

[5] See the appendix table for the preparation method.

[6] The endpoint is where the yellow color disappears and becomes greenish blue and the tone is maintained for not less than 30 seconds.

$$HO{\cdot}SO_2{\cdot}NH_2 + NaOH \rightarrow NaO{\cdot}SO_2{\cdot}NH_2 + H_2O$$

During titration, use the fixed range (such as the graduation range of 10-20 mL) of a burette.

[7] The number 97.10 means the molecular weight of amidosulfuric acid.

[8] Commercially available 0.05 mol/L sulfuric acid may as well be used.

[9] Connect the container containing the sulfuric acid standard solution prepared with a bottle containing dilute sulfuric acid to avoid the entrance of ammonia gas.

[10] Dissolve 0.1 g of methyl red in ethanol to be 100 mL. Filter the solution if needed.

Methyl red-methylene blue mixture test solution (dissolve 0.2 g of methyl red and 0.1 g of methylene blue respectively in ethanol (90 v/v%) to be 100 mL, and mix them.) may as well be used. When the mixture test solution is used for titration, the endpoint is where the red-purple color turns to blue and then to green.

[11] The number 25 in the calculation formula means the volume of the 0.05 mol/L sulfuric acid standard solution (25 mL) contained in the Erlenmeyer flask.

[12] Potassium sulfate and copper sulfate are used as degradation accelerators.

Degradation accelerators for Kjeldahl degradation includes (a) copper sulfate - potassium sulfate, (b) copper sulfate - selenium - potassium sulfate, (c) titanium dioxide - copper sulfate - potassium sulfate,

and (d) mercuric - potassium sulfate, etc. As a measure to prevent environmental pollution and in order to avoid troubles caused such as by the influence of degradation accelerators when the same sample solution is used for colorimetric determination of phosphorus, only copper sulfate - potassium sulfate were employed as degradation accelerators.

Copper sulfate is a catalyst to facilitate degradation, while potassium sulfate elevates the concentration of sulfuric acid and the boiling point as well as facilitates degradation via the following reactions:

$$K_2SO_4 + H_2SO_4 \rightarrow 2KHSO_4$$

$$2KHSO_4 \rightarrow K_2SO_4 + H_2O + SO_3$$

$$\left\{ \begin{array}{l} 2KHSO_4 \rightarrow K_2S_2O_7 + H_2O \\ K_2S_2O_7 \rightarrow K_2SO_4 + SO_3 \end{array} \right\}$$

[13] Degradation time may be further reduced by letting it stand to cool after adding sulfuric acid and heating while gradually adding about 1 mL of hydrogen peroxide solution (not less than 30 v/v%). Make sure to conduct degradation in a draft chamber.

[14] Care should be taken for samples with high oil content such as plant oil cake because it may foam violently and spill out of the Kjeldahl flask when heating is strong. When it foams strongly, stop heating and leave at rest for a while, and then heat again with lower heat. Additionally, it is recommended to add a small amount of paraffin to a sample that foams strongly.

[15] Because it is not appropriate in some cases to consider that degradation is completed when the solution becomes clear, it is needed to heat further.

Care should be taken so that the solution is not less than 10 mL because it is said that there is the loss of ammonia gas when the heating temperature is too high or the solution volume after degradation is too small.

Crude protein is degraded by sulfuric acid and turns into the form of $(NH_4)_2SO_4$.

[16] The two methods, absorption by the sulfuric acid standard solution and absorption by boric acid solution, are listed in the Analytical Standard of Feed as the quantification methods for crude protein.

[17] This method is frequently used in fertilizer analysis, food analysis and plant analysis, etc.

[18] It is judged by the emergence of the blue color of copper oxide.

[19] When 10 mL of the 0.05 mol/L sulfuric acid standard solution is used, distillation can be conducted according to the rough standards shown below based on the crude protein content in the sample:

Not more than 15 %	2 g-250 mL-50 mL
35 %	2 g-250 mL-25 mL
45 %	2 g-250 mL-20 mL
60 %	2 g-250 mL-10 mL

[20] At first ammonia is generated in a large amount; care should be taken to avoid the loss of ammonia by controlling the amount of steam introduced.

[21] One milliliter (1 mL) of the 0.1 mol/L sodium hydroxide standard solution corresponds to 1.40 mg of nitrogen.

[22] The value corresponds to the case when the degradation solution of the analysis sample is accurately 250 mL.

[23] This method is widely used in the Japanese Pharmacopoeia, JIS, and food analysis, etc. The method titrates ammonia with a strong acid when the ammonia (NH_3) generated from an alkalinized sample solution is passed through dilute boric acid solution and completely is dissociated as shown in the following formula:

$$NH_3 + H_3BO_3 + H_2O \rightarrow H_3BO_3(OH)^- + NH_4^+$$

The method is advantageous in that it is not needed to strictly define the concentration of the boric acid solution or the amount to take the boric acid solution, because the amount of boric acid is not directly involved in titration.

In addition, it is convenient to add methyl red - bromocresol green test solution in the boric acid solution in advance.

Prepare by dissolving 400 g of boric acid, 100 mL of 0.1 % bromocresol green solution in ethanol, and 70 mL of 0.1 % methyl red solution in ethanol in 10 L of water.

Ammonia is absorbed by boric acid, which is a weak acid. Ammonia can be sufficiently captured at the boric acid concentration of not less than 3 %; however, the concentration is stipulated as 4 % including the margin of safety.

Additionally, care should be taken for the boric acid solution in the receiver not to exceed 40 °C. Ammonia absorption decreases at high temperature, leading to loss.

[24] Dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 180 mL of ethanol. Add water to be 200 mL.

[25] The endpoint is when the green color disappears and changes into slightly grayish blue and then to slightly grayish red purple.

[26] One milliliter (1 mL) of the 0.05 mol/L sulfuric acid standard solution corresponds to 1.40 mg of nitrogen.

[27] The value corresponds to the case when the degradation solution of the analysis sample is accurately 250 mL.

2. Combustion method [Analytical Standard of Feed, Chapter 3, 2.2] *1

Quantification

Weigh 100-500 mg[2] of an analysis sample*2[1][3], put it in a nitrogen (protein) analyzer,*3[4] and run the analyzer to obtain the response peak of nitrogen gas with a detector.

Similarly, weigh the reagent for calibration curve preparation*4 accurately, and put it in the analyzer to obtain the response peak of nitrogen gas with a detector. Calculate the area from the response peak obtained to prepare the calibration curve, calculate the nitrogen [N] amount in the sample, and multiply the nitrogen [N] amount by 6.25 (6.38 for samples of milk products or milk replacer for calves which contain milk products not less than 50 %) to be the crude protein content in the sample.

Requirements for the analysis instrument

i) Capable of thermolysis of a sample in oxygen gas (purity not less than 99.9 %), maintaining the temperature in the reactor at 870 °C at the minimum

ii) Capable of separation of free nitrogen gas from the other combustion products

iii) Equipped with the system to convert nitrogen oxide (NO_x) into nitrogen gas (N_2), or capable of measuring nitrogen as NO_2

iv) Capable of measuring nitrogen gas with a thermal conductivity detector

* 1. For samples with high nitrate nitrogen content such as Sudan grass, it is quantitated as higher crude protein, and thus measure nitrate nitrogen [N] content separately and subtract the value.
2. All the amount of the analysis sample is screened through a net sieve of 0.5-mm mesh.
3. Use the instrument according to the combustion method, and measure under the conditions appropriate for the instrument.
4. Use reagents specified for the nitrogen (protein) analyzer used, such as disodium dihydrogen ethylenediaminetetraacetate dihydrate, DL-aspartic acid, etc.

«Summary of analysis method»

The method is a quantification method using an automatic analyzer applying the Dumas method, in which a sample is degraded by combustion at high temperature and released nitrogen gas is quantitated by a thermal conductivity detector (TCD) for crude protein in feeds.

The method is advantageous in that the analysis time is reduced and that facilities such as a draft are not needed.

The schematic diagram of the analyzer is shown in Figure 3.2-3.

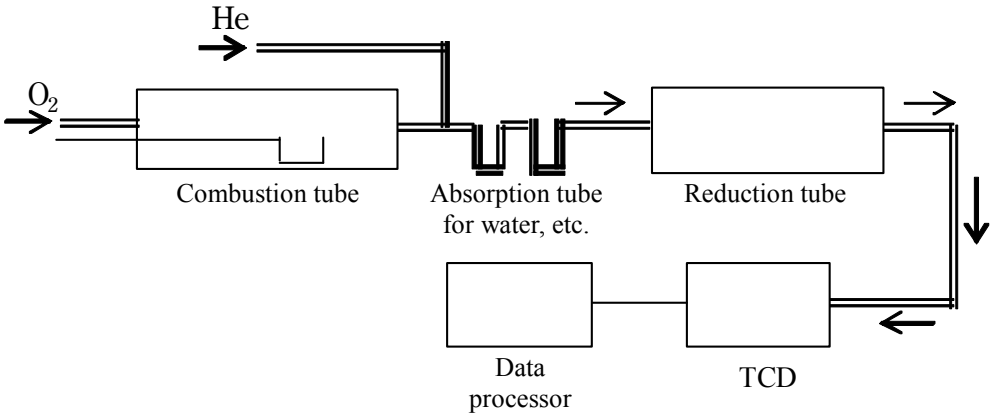


Figure 3.2-3 Schematic diagram of the analyzer by the combustion method

«Method validation»

· Collaborative study

Sample type	Number of laboratories	Measured value (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Cattle formula feed	11	13.5	1.5	1.9	0.5
Pig formula feed	11	19.3	0.5	1.8	0.8
Chicken formula feed	11	19.6	0.3	1.7	0.8
Fish meal (imported fish meal)	11	67.9	0.7	0.7	0.6
Fish meal (modified fish meal)	11	63.1	0.2	0.6	0.5
Milo	11	9.4	1.0	3.5	0.9
Bran	11	16.2	0.9	2.8	1.1
Soybean meal	11	50.2	0.2	0.7	0.5
Alfalfa hay	11	18.3	0.8	2.8	1.2
L-Lysine hydrochloride	11	95.1	0.3	1.3	1.3

«Notes and precautions»

[1] Particle size is set as 0.5 mm because the measured value may vary in a sample that passed a net sieve of 1-mm mesh; however, 1 mm will do in some samples.

[2] The sampling amount should be adjusted according to the nitrogen content in the sample and to the specifications of the analyzer used.

[3] Sampling boats to load a sample are made of quartz, ceramic, etc.

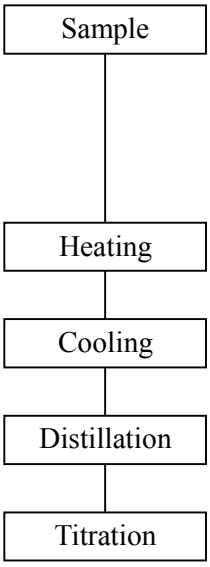
[4] Currently available nitrogen (protein) analyzers include SUMIGRAPH NC-220F (Sumika Chemical Analysis Service), Nitrogen/Protein Analyzer Type TruSpec N (LECO, distributed by LECO Japan), Dumatherm (Gerhardt, distributed by Gerhardt Japan), JM3000N (J-Science Lab), vario EL III full automatic element analyzer (elementar, distributed by DKSH Japan), and FLASH 2000 CHNS-O (Thermo scientific, distributed by AMCO).

[Other methods]

3. Crude protein quantification by international standards

ISO 5983 (1997) Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Kjeldahl method

Flow sheet of the analysis method



0.5-2.0 g (800-mL Kjeldahl flask)
← 15 g of potassium sulfate
← 0.3 g of copper (II) oxide or 0.9-1.2 g of copper (II) sulfate tetrahydrate
← Add 25-40 mL of sulfuric acid and, depending on the sample, a defoaming agent (paraffin)

After the sample solution becomes clear, heat for 2 hours.

← Add 250-350 mL of water and dissolve.
Put 25 mL of 0.1 mol/L or 0.05 mol/L sulfuric acid in a trapping flask, add 100-150 mL of water, and add 2-3 drops of mixed indicators (MR and MB).
← Put 100 mL of 33 % sodium hydroxide solution in a flask, and connect it to the distiller.
Titrate with 0.1 mol/L or 0.05 mol/L sodium hydroxide standard solution.
(When boric acid is used as the trapping solution, titrate with 0.05 mol/L or 0.125 mol/L sulfuric acid standard solution.)

4. Analysis with automated instruments

Recently, automated instruments have been developed to accelerate quantification procedures applying the Kjeldahl method by degradation with sulfuric acid.

(1) SuperKjel automatic nitrogen/protein analyzer (Actac)
(2) Kjeltec auto system (FOSS Tecator, distributed by Foss Japan)
(3) Kjeldahl method nitrogen/protein analyzer VAP series (Gerhardt, distributed by Gerhardt Japan)

In the field of crude feed, near-infrared spectrophotometers have been becoming popular.

3. Ether Extract (EE) or crude fat

[Summary of ether extract]

A sample is extracted with ether using the Soxhlet extractor, .to obtain the extract as ether extract.

Ether extract contains, in addition to fat, oil-soluble dyes (such as chlorophyll and carotenoids), wax, free fatty acids, lecithin, cholesterin, and phospholipids, etc.

Generally, ether extract of oil meal is around 1 % and the major component is oil and fat, therefore the energy value is high. Ether extract in cereals and bran is 4-5 % and the major component is oil and fat but contains a large amount of contaminants, therefore the energy value is less than that of oil meal; however, it is characterized in that it contains a lot of oil-soluble vitamins such as carotene and tocopherol.

Also, when feeds are stored for a long period, a phenomenon is observed in that moisture does not change while ether extract decreases gradually. This is because unsaturated fatty acids contained in feeds are oxidatively polymerized absorbing oxygen in the air and becomes insoluble in ether.

[Methods listed in the Analytical Standard of Feed]

1. Diethyl ether extraction [Analytical Standard of Feed, Chapter 3, 3.1]

Quantification

Weigh accurately 2-5 g of an analysis sample, put it in a filter paper thimble*1[1] (22 mm in diameter, 90 mm in height)[2], put absorbent cotton on it pushing lightly,[3] and dry at 95-100 °C for 2 hours[4].

Transfer the thimble into a Soxhlet extractor[5], connect to a fat weighing bottle (dried at 95-100 °C, cooled in a desiccator, and accurately weighed in advance), add diethyl ether, and extract for 16 hours*2.

Then, remove the filter paper thimble, and collect diethyl ether. Remove the fat weighing bottle[6] to evaporate diethyl ether[7], dry at 95-100 °C for 3 hours, let it stand to cool in a desiccator[8], and weigh accurately to calculate the ether extract content in the sample.

* 1. No. 84 (Toyo Roshi) or equivalents

2. Apparatus with an equivalent extraction effect[9] can be used.

(Note) When it is difficult to grind the sample due to high fat content, prepare the analysis sample according to 2 (3) of Chapter 2, and obtain the ether extract content in the original sample as follows.

To the volumetric flask containing diethyl ether used in the preliminary extraction, add diethyl ether to the marked line, transfer accurately a certain amount of it to a fat weighing bottle (dried at 95-100 °C, cooled in a desiccator, and accurately weighed in advance), and obtain the ether extract content in the original sample after preliminary extraction according to the quantification method shown above.

Then, obtain the ether extract content in the sample after preliminary extraction by the quantification method shown above, and calculate the ether extract content in the original sample by the following formula:

$$\text{Ether extract content (\%) in the original sample} = A + \frac{(100 - B) \times C}{100}$$

A : Ether extract content (%) obtained from the original sample after preliminary extraction

B : Loss (%) by preliminary extraction

C : Ether extract content (%) in the sample after preliminary extraction

«Summary of analysis method»

Put a sample in a filter paper thimble, load it on a Soxhlet extractor, add diethyl ether, heat and circulate to collect diethyl ether-soluble components in a fat weighing bottle below, evaporate diethyl ether, dry, and weigh the weight of the extract.

«Method validation»

· Results of proficiency testing in FY 2006

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)
Broiler starter	180	5.82	5.7

«Notes and precautions»

[1] Select according to the size of the extractor tube. Use the thimble of 22×90 mm when the apparatus shown in [5] below is used.

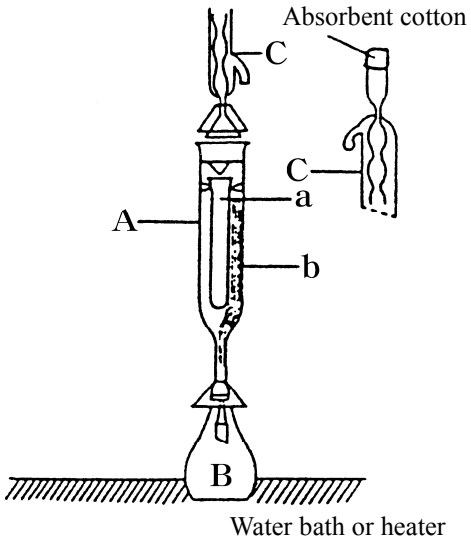
[2] Place absorbent cotton on the bottom of the filter paper thimble and put an analysis sample on it, so that the removal of the analysis sample after extraction will be easier. It is convenient to use a glass funnel of about 10 mm in the diameter of the stem to transfer the analysis sample into the filter paper cylinder.

[3] Absorbent cotton is stuffed on the analysis sample in order to infiltrate diethyl ether into the whole sample and to prevent release of the sample from the top of the filter paper thimble.

[4] Drying for a long time may cause evaporation of lower free fatty acids or oxidation of unsaturated fatty acids in some samples, while insufficient drying may cause extraction of water-soluble substances such as sugars into the diethyl ether extract, resulting in a higher measured value.

[5] The Soxhlet extractor (Figure 3.3-1) circulates diethyl ether by the siphon principle.

Adjust the water bath or heater so that 2-3 drops per second of diethyl ether dribbles from the condenser (at the level of 16-20 circulations per hour).



A Length about 280 mm
Diameter in the middle about 45 mm
B Volume about 150 mL
a Length about 110 mm
Diameter about 30 mm
b The top of the siphon part should be about 30 mm lower than the top of a.
C Length about 280 mm
Diameter about 40 mm

Adjust temperature so that 2-3 drops per second of diethyl ether dribbles from the condenser (usually about 60 °C).

Figure 3.3-1 Soxhlet extractor

[6] Dust or dirt on the hand may be adhered on the outer surface of the weighing bottle; wipe the bottle with gauze, etc., before weighing.

[7] Remaining diethyl ether causes danger in a dryer; therefore evaporate diethyl ether by putting the fat weighing bottle in a water bath.

[8] The cooling time in a desiccator should be the same as in the pre-drying.

[9] Recently, automated Soxhlet apparatus is also used in which extraction time is shortened and the solvent amount is reduced.

2. Acid degradation/diethyl ether extraction [Analytical Standard of Feed, Chapter 3, 3.2]

Scope of application: Expanded feeds, formula feeds using fat powder material (only formula feeds for milk replacer for calves in the milk-feeding period and formula feeds for piglets in the in the milk-feeding period), formula feeds for dairy cows using fatty acid calcium salt material, soy oil foots and rapeseed oil foots

Quantification

Weigh accurately 2 g of an analysis sample, put it in a 100-mL beaker[*1], add 2 mL of ethanol[1], mix with a glass bar to moisten the sample, then add 20 mL of hydrochloric acid (4+1), cover with a watch glass, heat in a water bath at 70-80 °C with occasional stirring for 1 hour[2], and then let it stand to cool.

Transfer the content of the said beaker into a 200-mL separatory funnel A[*1], wash the beaker sequentially with 10 mL of ethanol and 25 mL of diethyl ether, and add the washing to the separatory funnel A. Further add 75 mL of diethyl ether to the separatory funnel A, mix by shaking[3] and leave at rest. Collect the diethyl ether layer (upper layer) with a pipette, etc.[4], and transfer it to a 300-mL separatory funnel B containing 20 mL of water in advance.

Add 50 mL of diethyl ether to the separatory funnel A, repeat the same procedure twice, and collect each diethyl ether layer with a pipette, etc., and add it to the separatory funnel B.

Shake the separatory funnel B to mix, and leave at rest, and discard the water layer (lower layer). Further add 20 mL of water to the separatory funnel B, and repeat the same procedure twice. Filter the diethyl ether layer[5] with a funnel that is stuffed with absorbent cotton and a proper amount, not less than 10 g, of sodium sulfate (anhydrous) in advance into a fat weighing bottle or a 300-mL recovery flask (dried at 95-100 °C, cooled in a desiccator, and accurately weighed in advance).

Then, recover diethyl ether in the said fat weighing bottle using a Soxhlet extractor, or in the said recovery flask using a rotary evaporator[6]. Remove the fat weighing bottle or the recovery flask to evaporate diethyl ether, dry at 95-100 °C for 3 hours, let it stand to cool in a desiccator, and weigh accurately to calculate the ether extract content in the sample.

* 1. If a Mojonnier flask[7] is available, use it instead of the beaker and the separatory funnel A; check in advance the amount of diethyl ether needed for extraction.

«Summary of analysis method»

In this method, a sample from which fat is not directly extracted completely with diethyl ether is hydrolyzed using hydrochloric acid solution to disperse free fat in the sample, and then is subjected to liquid-liquid extraction with diethyl ether. A process to wash the diethyl ether layer with water is added in order to prevent higher measured value caused by water-soluble substances contained in the diethyl ether layer other than ether extract.

The scope of application of the method is specified to avoid confusion in analytical testing.

The flow sheet of the analysis method is shown in Figure 3.3-2.

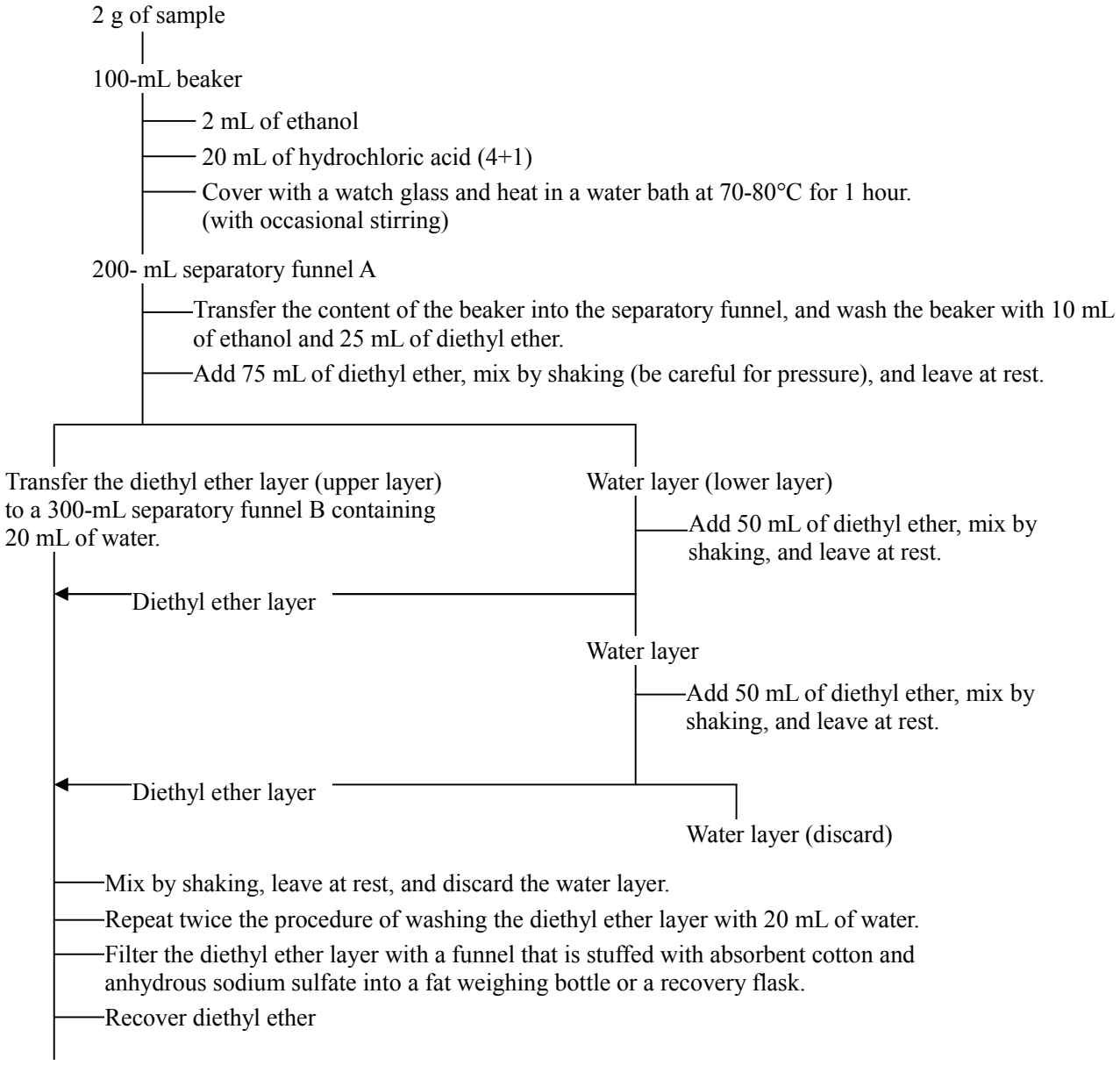


Figure 3.3-2 Flow sheet of acid degradation/diethyl ether extraction

«Notes and precautions»

[1] Ethanol is added to prevent the solidification of the sample when hydrochloric acid is added.

[2] The solution turns from brown to blackish brown along with degradation.

[3] Pressure in the separatory funnel increases immediately; release pressure sufficiently before shaking.

[4] Care should be taken because a plus error is caused by the transfer of the blackish brown water layer to the separatory funnel B.

[5] White solid content may be left on the funnel in samples with a high content of hydrogenated fat; filter it in with heating to avoid the precipitation of the white solid content.

[6] An error may be caused by ethanol remaining in the fat weighing bottle or the recovery flask;

evaporate ethanol by heating with a water bath or purging with nitrogen gas.

[7] A Mojonnier flask has the shape shown in Figure 3.3-3, and is made so that it can be centrifuged and the upper layer can be collected only by tilting.

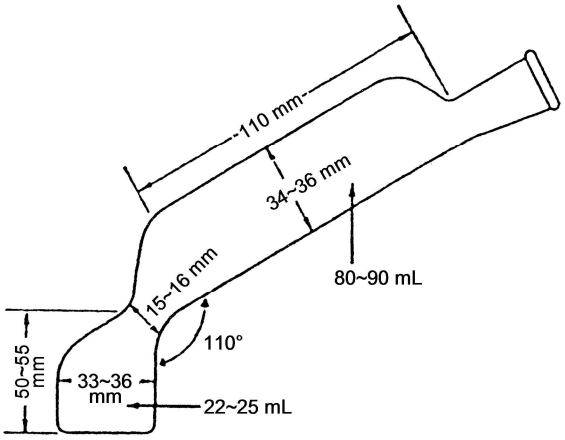


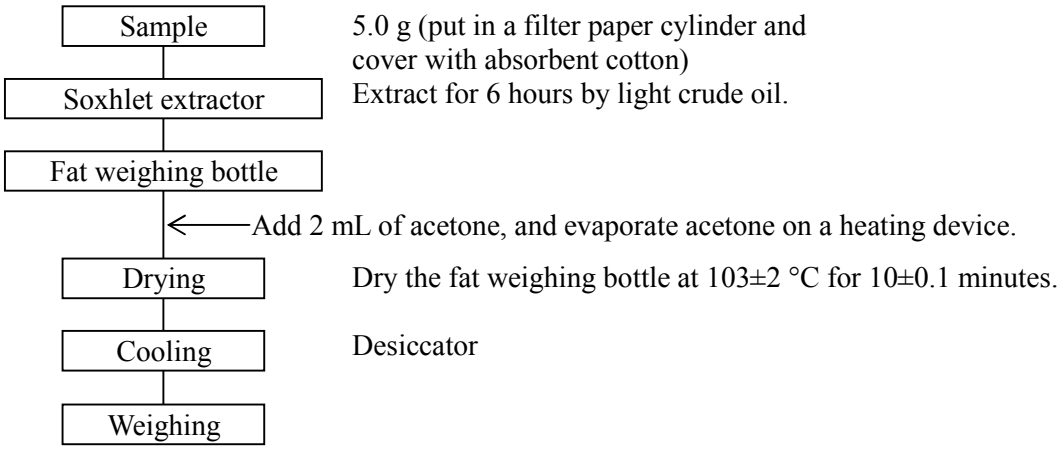
Figure 3.3-3 Mojonnier flask

[Other analysis methods]

3. Ether extract quantification by international standards

ISO 6492 (1999) Animal feeding stuffs - Determination of fat content

Flow sheet of the analysis method



4. Analysis with automated instruments

The following instruments are commercially available, which are considered to be equivalent to the Soxhlet extractor in 1:

(1) Soxtest SER (Actac)

(2) Soxtec (FOSS Tecator, distributed by Foss Japan)

(3) Soxtherm (Gerhardt, distributed by Gerhardt Japan)

These automated instruments are characterized in that analysis time can be completed in about 1/5 of the

time for the method by the conventional Soxhlet extractor, that less amount of solvent is used, and that solvent can also be collected automatically.
Moreover, Hydrotherm (Gerhardt, distributed by Gerhardt Japan) was developed as an instrument to automate acid degradation in 2 before an ordinary Soxhlet extractor or an automated extractor.
However, data should be compared with the data from the conventional Soxhlet extractor before using a newly released instrument without history of use.

4. Crude Fiber (CF)

[Summary of crude fiber]

A sample is boiled sequentially with dilute acid and then with dilute alkali, and then sequentially washed with ethanol and diethyl ether, and the residue is subtracted by its ash, and the result is defined as crude fiber. Crude fiber is primarily measured to comprehend indigestible parts in feeds, and is consisted mainly of a part of lignin, pentosan, chitin, etc., in addition to cellulose.

These compounds are collectively called as fiber; however, the sum of their individually measured contents is significantly different from the crude fiber content obtained by the method shown above, and the former is always larger. This is because a part of lignin and hemicellulose is dissolved during the boiling procedure, and the percentage of dissolution varies depending on the feed type and thus is not constant.

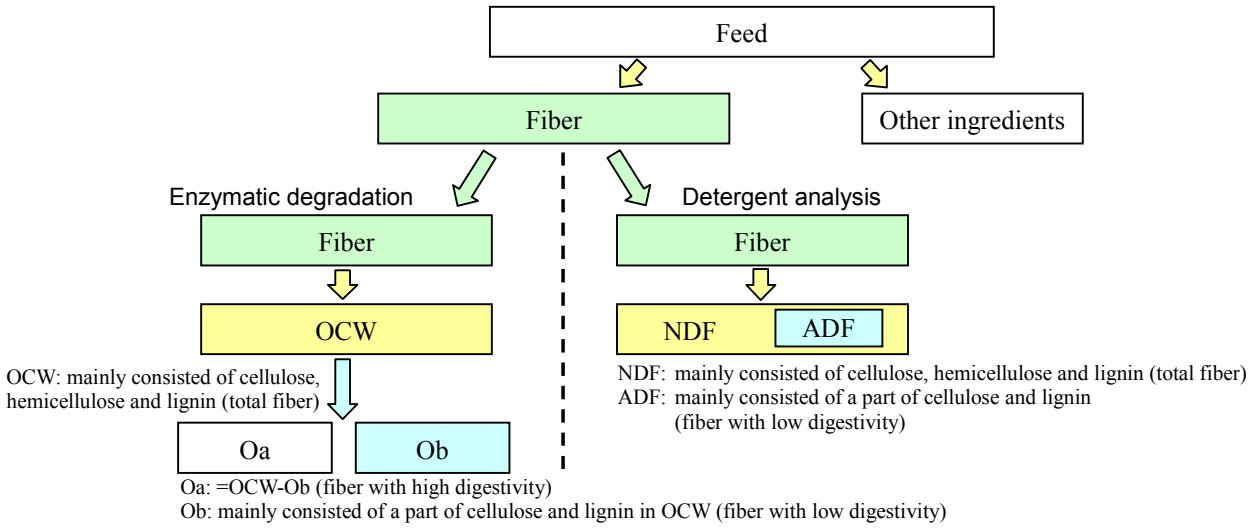
Crude fiber clearly corresponds only to feeds of plant origin considering the component compounds; however, a small amount of it is contained in feeds of animal origin. This is because organic residue that is not dissolved by acid/alkali boiling is observed in feeds of animal origin, and the residue is chitin and some of scleroprotein (albuminoid), which are completely different from so-called crude fiber in content.

There is a close relationship between the crude fiber content and the nutrition value of the feed, and generally the higher the crude fiber content is, the lower the nutrition value is.

On the other hand, fiber is an important nutrient for ruminant livestock. Fiber that was previously in the scope of feed analysis was only crude fiber of general nutrients, which was insufficient for the evaluation of fiber. This is because plant fiber is mainly consisted of cell wall which is comprised of substances such as cellulose, hemicellulose, lignin and pectin, but crude fiber quantitated in general analysis does not include hemicellulose, etc. To measure fiber as accurate as possible, detergent analysis using detergents was developed in Europe and the US, while enzymatic analysis using enzymes was developed in Japan led by the National Institute of Animal Industry.

In detergent analysis, a feed is heated using a neutral detergent and is separated into soluble and insoluble parts. Organic matter in the insoluble part corresponds to cell wall, and is defined as neutral detergent fiber (NDF). The “low digestivity” fraction in cell wall is measured by heat treatment with an acidic detergent, and is defined as acid detergent fiber (ADF).

In enzymatic analysis, starch and protein are degraded by enzymes and is separated into soluble and insoluble parts. Organic matter in the insoluble part corresponds to cell wall, and is defined as organic cell wall (OCW). The “indigestible” fraction after the degradation of starch and protein is measured by degrading cellulose with enzymes, and is defined as fiber with low digestivity or organic b fraction (Ob).



[Methods listed in the Analytical Standard of Feed]

1. Standing method [Analytical Standard of Feed, Chapter 3, 4-1)]

Quantification

Weigh accurately 2-5 g of an analysis sample, put it in a 500-mL tall beaker, add 50 mL of sulfuric acid (1+34), and further add water to be 200 mL.

Then, cover the tall beaker with a watch glass or a condenser, boil for 30 minutes[1] while supplying moisture that is evaporated, add 300 mL of water and leave at rest overnight, aspirate[2] the supernatant, add water again to be 200 mL, and subject to the same procedure[3].

To the residue (acid-insoluble fraction), add 50 mL of sodium hydroxide solution (5 w/v%), add water to be 200 mL, cover with a watch glass or a condenser, and subject to the same procedure as acid treatment[4].

Filter the residue (acid/alkali-insoluble fraction) with filter paper (No.5A) (placed in an aluminum weighing dish[5], dried at 135±2 °C for 2 hours, cooled in a desiccator, and accurately weighed in advance)[6]. Wash the residue on the filter paper with hot water until the alkaline reaction of filtrate disappears[7], further wash 2-3 times each sequentially with a small amount of ethanol and diethyl ether[8], and then air-dry for 3-4 hours.

Then put the acid/alkali-insoluble fraction with the filter paper in the said weighing dish, dry at 135±2 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately, and calculate the amount of the acid/alkali-insoluble fraction in the sample. Transfer the residue in the weighing dish to a crucible (heated at 550-600 °C for 2 hours, cooled in a desiccator, and accurately weighed in advance). Heat this gently to be charred, then heat at 550-600 °C for 2 hours to incinerate, let it stand to cool in a desiccator, and accurately weigh to obtain the ash content in the sample[9].

Subtract the ash content from the amount of the acid/alkali-insoluble fraction to calculate the crude fiber content in the sample.

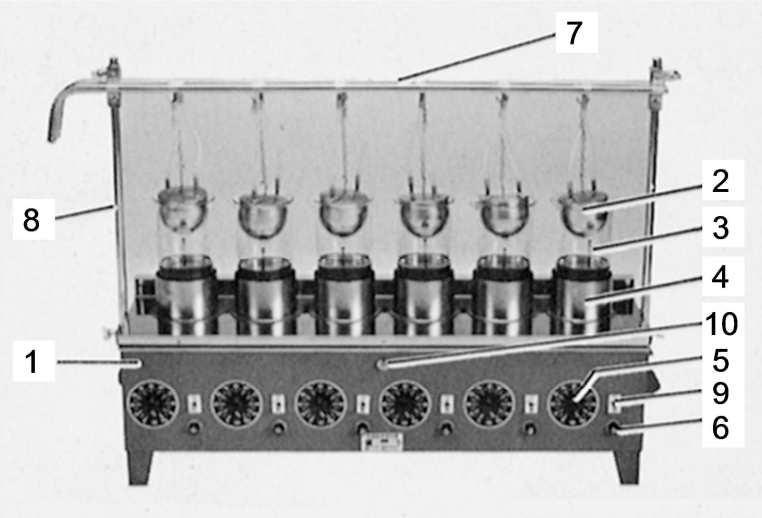
«Summary of analysis method»

Treat a sample sequentially with sulfuric acid (1+34), sodium hydroxide solution (5 w/v%), ethanol and

ether, and weigh the residue (designated as A).

Then, incinerate the residue and weigh (designated as B), and calculate A-B as crude fiber. Crude fiber contains most of cellulose and a part of hemicellulose, lignin and pectin.

The instrument shown in Figure 3.4-1 is commercially available from Sanshin Industrial as an instrument for the quantification of crude fiber.



1 Main unit
2 Float (stainless hemisphere)
3 Crude fiber beaker
4 Heater (inside)
5 Voltage regulator
6 Fuse
7 Cooler pipe
8 Support post
9 Switch
10 Pilot lamp

Figure 3.4-1 Instrument for crude fiber quantification

«Method validation»

· Results of proficiency testing in FY 2006

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)
Broiler starter	30	2.79	8.1

«Notes and precautions»

[1] If the content adhered to the inner wall of the tall beaker, drop it off so that the content is continuously treated with sulfuric acid. Make sure to keep the acid concentration constant by adding hot water when moisture is evaporated.

[2] When an aspiration tube with a filter plate (G1) is used, as shown in Figure 3.4-2, wash off adhered matter on the glass filter plate after the aspiration procedure with water into the tall beaker.

[3] Conduct the procedure of boiling, leaving at rest, and then aspirating the supernatant.

[4] Bumping often occurs when boiling with sodium hydroxide solution; this can be prevented by adding boiling stones weighed in advance to the solution.

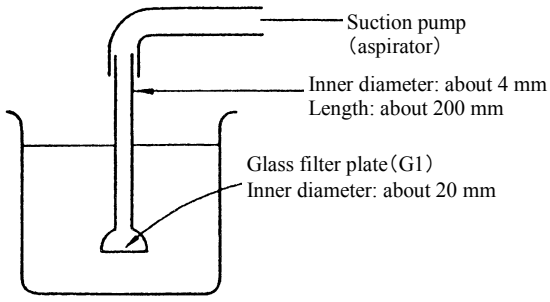


Figure 3.4-2 Aspiration tube with a filter plate (example)

In addition, make sure to keep the alkali concentration constant by adding hot water when moisture is evaporated.

[5] See 1. Moisture, 1 **«Notes and precautions»** [3] in this chapter.
Write the number of aluminum weighing dish on filter paper with a pencil, etc.

[6] For fish meal and feather meal, etc., filter immediately because filtration becomes difficult at lower temperature when the solution is left after treatment.

[7] Check that there is no alkaline reaction with litmus paper or pH test paper.

[8] The major objectives are the reduction of air-dry time and defatting.

[9] Obtain according to the quantification method for crude ash (5. Crude ash, 1 in this chapter).

2. Filtration method [Analytical Standard of Feed, Chapter 3, 4-2)]

Quantification

Weigh accurately 2-5 g of an analysis sample, put it in a 500-mL tall beaker, add 50 mL of sulfuric acid (1+34), further add water to be 200 mL, cover with a watch glass or a condenser, boil for 30 minutes while supplying moisture that is evaporated, and then filter the residue with 0.045-mm stainless wire mesh[1], and wash with hot water.

Transfer the residue (acid-insoluble fraction) to the said tall beaker using 130-140 mL of water, add 50 mL of sodium hydroxide solution (5 w/v%) 50 mL, and further add water to be 200 mL.

Then, cover the tall beaker with a watch glass or a condenser, and boil for 30 minutes while supplying moisture that is evaporated.

Filter the residue (acid/alkali-insoluble fraction) with filter paper (No.5A) (placed in an aluminum weighing dish, dried at 135±2 °C for 2 hours, cooled in a desiccator, and accurately weighed in advance). Wash the residue on the filter paper with hot water until the alkaline reaction of filtrate disappears, further wash 2-3 times each sequentially with a small amount of ethanol and diethyl ether, and then air-dry for 3-4 hours.

Then put the acid/alkali-insoluble fraction with the filter paper in the said weighing dish, dry at 135±2 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately, and calculate the amount of the acid/alkali-insoluble fraction in the sample. Transfer the residue in the weighing dish to a crucible (heated at 550-600 °C for 2 hours, cooled in a desiccator, and accurately weighed in advance). Heat this gently to be charred, then heat at 550-600 °C for 2 hours to incinerate, let it stand to cool in a desiccator, and accurately weigh to obtain the ash content in the sample.

Subtract the ash content from the amount of the acid/alkali-insoluble fraction to calculate the crude fiber content in the sample.

«Summary of analysis method»

The principle of this analysis method is the same as the standing method in 1. in this section. In the standing method, there is a step of standing overnight after boiling with sulfuric acid, while filtration with stainless wire mesh or nylon paper is conducted in this method to speed up the analysis.

«Method validation»

· Results of proficiency testing in FY 2006

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)
Broiler starter	120	2.73	12

«Notes and precautions»

[1] Formerly nylon filter paper (Nylon Paper #1025 from Tokushu Paper Mfg) was used, but the product is discontinued; thus, the method is modified to use stainless wire mesh of 0.045 mm (325 mesh) (Figure 3.4-3) which has a filtering effect similar to the nylon paper employed in the AOACI method. This product is manufactured by Sanshin Industrial.

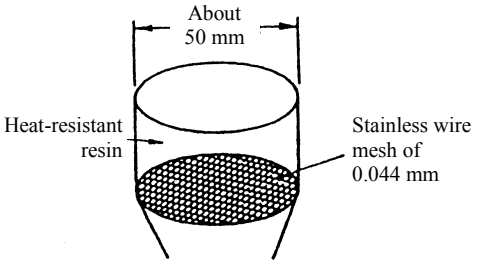


Figure 3.4-3 Stainless wire mesh

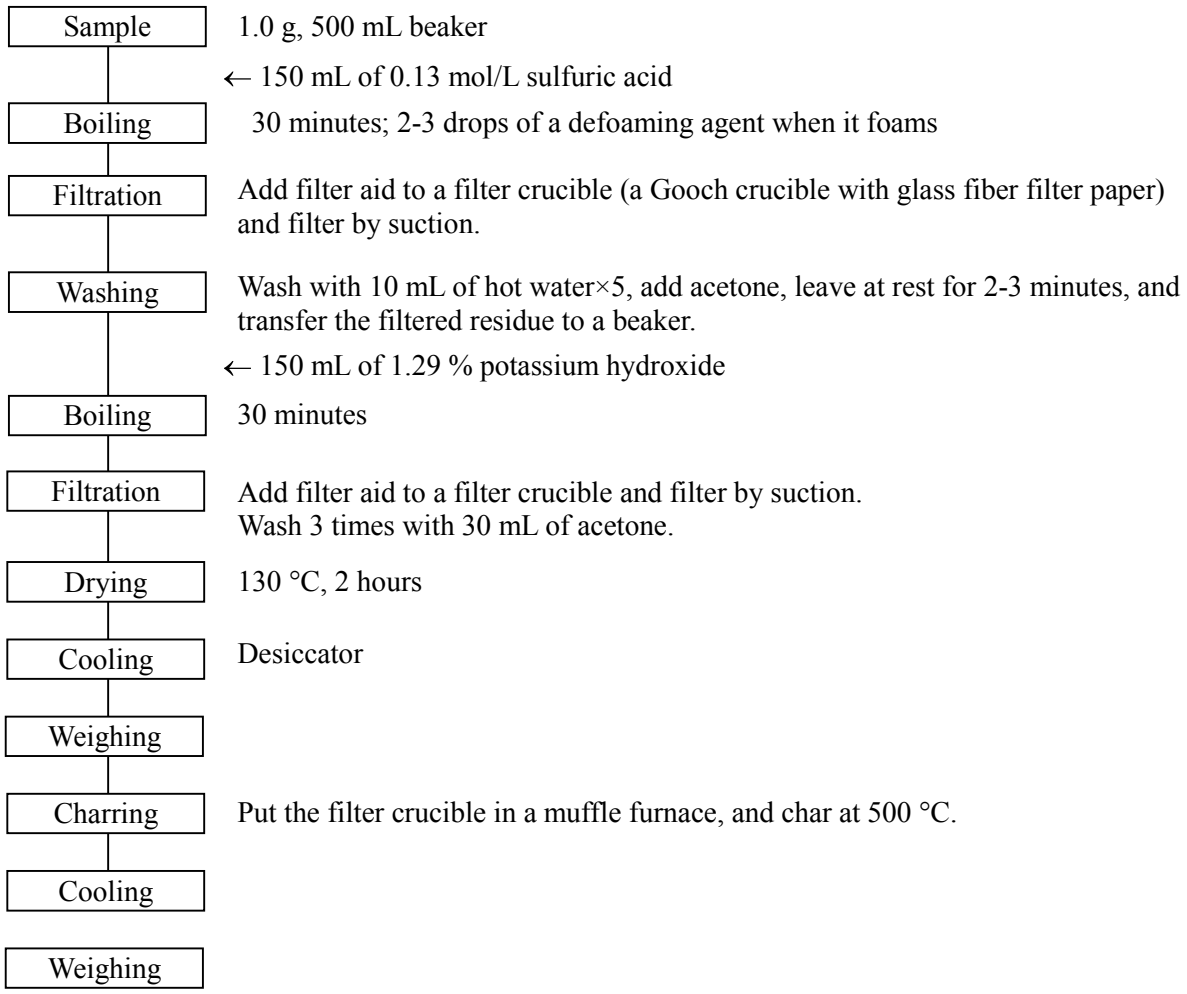
Another product equivalent to the previously used nylon paper has been developed and commercially available from Sanshin Industrial by the name of Rayon Paper C-7000.

[Other analysis methods]

3. Crude fiber quantification by international standards

ISO 6865 (2000) Animal feeding stuffs - Determination of crude fiber content

Flow sheet of the analysis method



4. Analysis with automated instruments

Recently, automated instruments for crude fiber have been becoming fairly popular. Types commercially available in Japan are shown below:

(1) Fibertest FIWE (Actac)

(2) Fibertec, FiberCap (FOSS Tecator, distributed by Foss Japan)

(3) Fiber analyzer SA-120 (ANKOM, distributed by Sanshin Industrial)

(4) Fibretherm FT12 (Gerhardt, distributed by Gerhardt Japan)

(1) and (2) automate the manual procedure of the analysis method in 2. in this section, and can treat 3 or 6 samples simultaneously. High-speed filtration and washing can be conducted by a built-in suction pump, significantly reducing analysis time.

(3) and (4) automate reagent injection and washing, with samples loaded in filter bags.

All the instruments can also analyze ADF, NDF, cellulose, hemicellulose, lignin, etc.

Comparison with manual analysis should be conducted before using these instruments.

5. Heat-stable *α*-amylase-treated neutral detergent fiber (aNDF and aNDFom)

[Summary of heat-stable *α*-amylase-treated neutral detergent fiber]

Neutral detergent fiber (NDF) was developed as an analysis method for total fiber in grass. Various modification have been done later to be applicable to feed crop other than grass, and several modified methods have been introduced. Therefore, there are many kinds of analysis methods, and results by different treatment methods have all been reported as the NDF value. For that reason, NDF may differ for the same sample between different laboratories, thus the handling of the analysis value is a problem. With that, heat-stable *α*-amylase-treated neutral detergent fiber (hereinafter referred to as aNDF) was developed as an analysis method that is commonly applicable to all feed crop. Today the Nutrient Requirements of Dairy Cattle (NRC) used in the United States employs aNDF instead of previously used NDF, while AOAC International and ISO standardize the aNDF analysis method. These aNDF methods use heat-stable *α*-amylase and sodium sulfite commonly for all feeds.

Substances soluble in neutral detergent are digested by bacteria in the digestive organs of livestock. Therefore, NDF is the part that is not digestible or is slowly digested by livestock, and corresponds to nonsoluble fiber. Its major components are cellulose, hemicellulose and lignin.

[Methods listed in the Analytical Standard of Feed]

1. Heat-stable *α*-amylase-treated neutral detergent fiber

[Analytical Standard of Feed, Chapter 3, 5]

A. Reagent preparation

1) Neutral detergent solution[*1][1]

Weigh 18.6 g of disodium ethylenediaminetetraacetate dihydrogen dihydrate, 6.8 g of sodium tetraborate decahydrate, and 4.6 g of disodium hydrogen phosphate, put in a 1-L volumetric flask, and dissolve by the addition of 500 mL of water[2]. To this solution, add 30.0 g of sodium *n*-dodecyl sulfate, 10 mL of triethylene glycol and 250 mL of water, mix, and further add water up to the marked line of the volumetric flask to prepare neutral detergent solution[3].

Check that the pH is in the range of 6.95-7.05 before use.

2) Amylase stock solution

Put 1 mL of heat-stable *α*-amylase[*2] in a 10-mL amber volumetric flask, dissolve by the addition of water, and further add water up to the marked line to prepare the amylase stock solution[*3].

3) Amylase solution

Check in advance the sufficient volume *a* μL of the amylase stock solution to add by a confirmatory test[*4]. Transfer the volume of the amylase stock solution to take *b* μL calculated by the following formula to a 100-mL amber volumetric flask, and add water up to the marked line to prepare the amylase solution.

Volume of amylase stock solution to take *b* (μL) =

sufficient volume of amylase stock solution to add *a* (μL) ×50

4) Iodine solution

Dissolve 2.0 g of potassium iodide and 1.0 g of iodine in water to be 100 mL.

B. Quantification

Weigh accurately 0.5 g[4] of an analysis sample, put it in a 500-mL tall beaker[5], add 0.5 g of sodium sulfite*5 and 50 mL of neutral detergent solution, and then cover the tall beaker with a watch glass or a condenser, and heat until it boils by a fiber boiler heated in advance. Immediately after the start of boiling, add 2 mL of the amylase solution to the tall beaker, and boil for 1 hour supplying evaporating moisture[6]. Remove the tall beaker from the fiber boiler, further add 2 mL of the amylase solution to the tall beaker, mix by shaking mildly, and then leave at rest for 60 seconds. Filter by suction the content of the tall beaker using a glass filter*6 (heated at 520-550 °C for 2-5 hours, incubate at 150 °C for 2 hours[7], cooled in a desiccator, and weighed accurately in advance). Wash the residue in the glass filter (neutral detergent-insoluble fraction) with 40 mL each of hot water for 3 times, wash further with 10-20 mL of acetone for 3-4 times, and then air-dry until the acetone odor disappears.

Then, dry the said glass filter at 135±2 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately to calculate the content (%) of neutral detergent-insoluble fraction (aNDF) in the sample.

Moreover, heat the said glass filter at 520-550 °C for 2-5 hours to incinerate neutral detergent-insoluble fraction, incubate at 150 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately to obtain the ash content (%) in the sample.

Subtract the ash content obtained in advance from the neutral detergent-insoluble fraction content to calculate the heat-stable α-amylase-treated neutral detergent fiber (aNDFom) content (%) in the sample.

* 1. Store neutral detergent fiber at room temperature.

2. α-Amylase A3306, α-Amylase A3403 (both Sigma) or equivalents

3. Store the amylase stock solution at 2-8 °C.

4. Confirmation test[8][9]

Weigh accurately 0.5 g of coarsely ground hominy (corn kernel endosperm)[10] (that passes a sieve of 1-mm mesh), put it in a 500-mL tall beaker; prepare 6 sets of this. Add 50 mL each of the neutral detergent solution to tall beakers, cover the tall beakers with a watch glass or a condenser, and heat by a fiber boiler heated in advance. Immediately after the start of boiling, add amylase stock solution 25, 50, 100, 200 and 400 µL (which contain 125, 250, 500, 1000 and 2000 units, respectively, when the enzyme activity of the heat-stable α-amylase is 50000 unit/mL) to the tall beakers, respectively. Do not add the amylase stock solution to a set of the tall beaker to be the blank test solution. Boil them accurately for 10 minutes. Remove the tall beakers from the fiber boiler, further add the amylase solution of the same amount as the previous step to the tall beakers, mix by shaking mildly, and then leave at rest for 60 seconds. Filter the content of the tall beakers using glass fiber filter paper (GA-100 (Toyo Roshi) or equivalents) into 100-mL beakers[11], cool in an ice bath for 5 minutes to be 1 °C or less, and return to normal temperature in an incubator at 20 °C. Place the beakers on white paper, add 0.5 mL of the iodine solution quickly, leave at rest for 90 seconds, and check the color of the solution within 30 seconds. It is judged that the volume of the

amylase stock solution added is sufficient when the solution is colorless to yellow, and insufficient when the solution is purple to pinkish light brown[12]. If the volume added was judged as insufficient at 400 µL of the amylase stock solution, conduct the same test using increased volume to add, and check the sufficient volume of the amylase stock solution to add.

5. Weigh immediately before use.
6. P2 (pore size 40-100 µm, Foss Tecator) or equivalents

«Summary of analysis method»

In this method, feeds are boiled and treated with heat-stable *α*-amylase simultaneously in a solution in which a neutral detergent is dissolved to remove starch and quantitate neutral detergent fiber. *α*-amylase-treated NDF is described as aNDF, while NDF without *α*-amylase treatment is described as NDF. NDF that does not contain ash is described as NDFom.

When the method is applied to feed rice, starch degradation may not be sufficient with the optimal amylase amount obtained by the confirmatory test depending on the rice cultivar. If amylase is changed from the usual method to the amount that can degrade starch sufficiently, it is required to describe as such in the analysis result.

Flow sheet of the analysis method is shown in Figure 3.5-1.

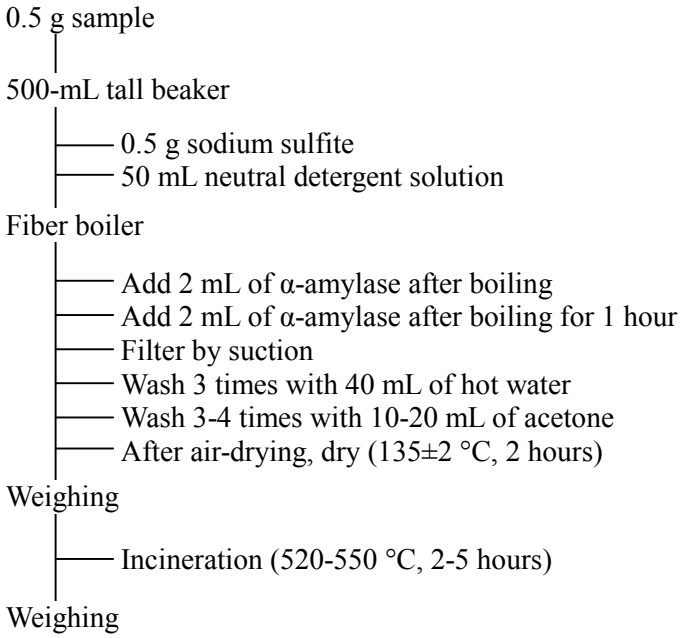


Figure 3.5-1 Flow sheet of the measurement of heat-stable *α*-amylase-treated neutral detergent fiber

«Method validation»

· Repeatability

Sample type \ Enzyme	Number of repeats	A3306 Measured value (%)	A3306 Repeatability RSD_r (%)	A3403 Measured value (%)	A3403 Repeatability RSD_r (%)
Corn	4	7.8	3.9	7.9	2.8
Barley	4	22.6	6.3	22.0	4.2
Wheat	4	9.9	4.9	10.5	5.1
Rye	4	15.4	2.1	15.0	5.3

· Collaborative study

Sample type	Number of laboratories	Measured value (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)
Alfalfa	10	38.8	1.2	1.8
Cattle formula feed	10	14.6	3.9	5.3

«Notes and precautions»

[1] If it is stored in a cold place, warm it to 25 °C before use, and the solution should be uniform to be used.

[2] If it cannot be dissolved, dissolve by heating.

[3] When the pH is not within the range, adjust using hydrochloric acid and sodium hydroxide. If the pH is different by not less than 0.5, prepare newly the neutral detergent solution.

[4] Defat with acetone when the fat content in the sample is not less than 5 %. Acetone defatting should be conducted as shown below:

Weigh accurately 0.5 g of an analysis sample, put it in a glass filter, add 40 mL of acetone to immerse, and filter while mixing by stirring for 2-3 times. Repeat the procedure 3 times. On the third filtration, air-dry at room temperature until the residual acetone odor disappears, transfer to a tall beaker using 50 mL of the neutral detergent solution, and the add 0.5 g of sodium sulfite. The glass filter used here should be used in filtration after boiling.

[5] When a fiber extractor is used, weigh an analysis sample in a glass filter, further add 0.5 g of sodium sulfite, load on the fiber extractor, and then add 50 mL of the neutral detergent solution.

[6] If the content adhered to the inner wall of the tall beaker, drop it off so that the content is continuously treated with the neutral detergent solution. Make sure to keep the neutral detergent solution concentration constant by adding hot water when moisture is evaporated. When a fiber extractor is used, use back pressure so that the content adhered to the inner wall of the glass filter is treated by the neutral detergent solution.

[7] This is because the rapid temperature decreasing in the glass filter from high temperature (520-550 °C) to room temperature causes distortion between the glass part and the filter part of the glass filter.

[8] Check every 6 months; and conduct when the lot is different.

[9] When the method is applied to high-starch feeds such as feed rice, it may not be sufficient with the

optimal amylase amount obtained by the confirmatory test depending on the cereal cultivar.

[10] Coarsely ground hominy (corn kernel endosperm) is distributed for a charge by the Committee of Feed Quality Improvement.

There may be changes in the distribution method, etc.; see the website of FAMIC for the purchase procedure.

[11] If the blank test solution cannot be filtered, the supernatant can be used.

[12] Examples of solution colors are shown below:

1-3: Purple to pinkish light brown (insufficient)

4-6: Colorless to yellow (sufficient)

6. Acid detergent fiber (ADF and ADFom)

[Summary of acid detergent fiber]

The analysis method for acid detergent fiber (ADF) comprehends the fiber fraction in cattle feed, measuring fractions with no or low digestivity in fiber. The major components of ADF are cellulose and lignin.

[Methods listed in the Analytical Standard of Feed]

1. Acid detergent fiber [Analytical Standard of Feed, Chapter 3, 6]

A. Reagent preparation

Acid detergent solution*1

To 1 L of sulfuric acid (1+37), add 20 g of cetyltrimethylammonium bromide and dissolve.

B. Quantification

Weigh accurately 1 g of an analysis sample, put it in a 500-mL tall beaker[1], add 100 mL of the acid detergent solution, and then cover the tall beaker with a watch glass or a condenser, and boil. After boiling for 1 hour supplying evaporating moisture[2], filter by suction the content of the tall beaker using a glass filter*2 (heated at 520-550 °C for 2 hours, incubated at 150 °C for 2 hours[3], cooled in a desiccator, and weighed accurately in advance). Wash the residue in the glass filter (acid detergent-insoluble fraction) with hot water sufficiently, wash further with 10-20 mL of acetone for 3-4 times, and then air-dry until the acetone odor disappears.

Then, dry the said glass filter at 135 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately to calculate the content (%) of acid detergent-insoluble fraction (ADF) in the sample.

Moreover, heat the said glass filter at 520-550 °C for 2 hours to incinerate acid detergent-insoluble fraction, incubate at 150 °C for 2 hours, let it stand to cool in a desiccator, and then weigh accurately to obtain the ash content (%) in the sample.

Subtract the ash content from the acid detergent-insoluble fraction content obtained above to calculate the acid detergent fiber (ADFom) content (%) in the sample.

* 1. Store the acid detergent solution at room temperature.

2. P2 (pore size 40-100 μm, Foss Tecator) or equivalents

«Summary of analysis method»

In this method, feeds are boiled within an acid treatment solution in which a detergent is dissolved to quantitate acid detergent fiber (ADF). ADF that does not contain ash is described as ADFom.

Flow sheet of the analysis method is shown in Figure 3.6-1.

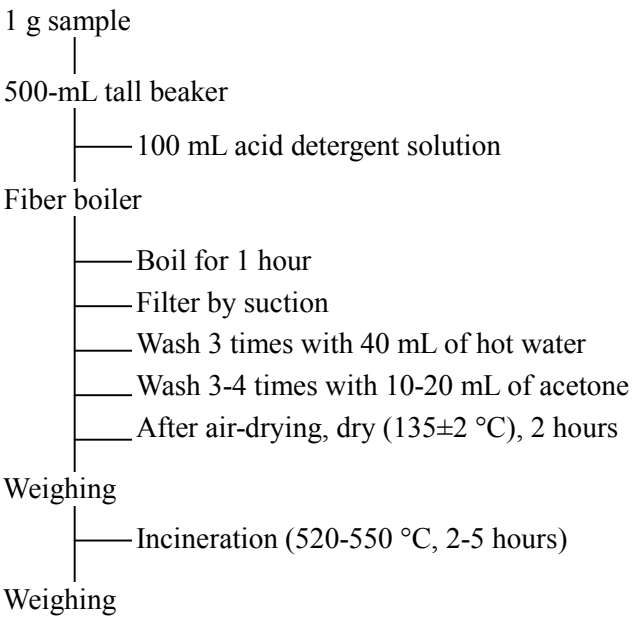


Figure 3.6-1 Flow sheet of the analysis method for acid detergent fiber

«Method validation»

· Repeatability

Sample type	Number of repeats	Measured value	Repeatability
		(%)	RSD_r (%)
Corn	4	2.3	1.2
Corn gluten feed	4	9.6	0.8
Milo	4	4.6	5.1
Barley	4	4.3	4.7
Wheat	4	2.6	5.0
Rye	4	2.9	2.3
Rapeseed oil meal	4	18.1	5.3
Alfalfa	4	22.6	1.7
Ryegrass	4	34.0	1.6
Italian ryegrass straw	4	37.6	1.6
Corn silage	4	24.8	1.5
Grass silage	4	33.4	2.9
Cow formula feed	4	6.3	3.4

· Collaborative study

Sample type	Number of laboratories	Measured value (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)
Alfalfa	8	30.2	3.1	3.7
Cattle formula feed	8	5.0	3.9	4.7

«Notes and precautions»

[1] When a fiber extractor is used, weigh an analysis sample in a glass filter, load on the fiber extractor, and then add 100 mL of the acid detergent solution.

[2] If the content adhered to the inner wall of the tall beaker, drop it off so that the content is

continuously treated with the acid detergent solution. Make sure to keep the acid detergent solution concentration constant by adding hot water when moisture is evaporated. When a fiber extractor is used, use back pressure so that the content adhered to the inner wall of the glass filter is treated by the acid detergent solution.

[3] This is because the rapid temperature decreasing in the glass filter from high temperature (520-550 °C) to room temperature causes distortion between the glass part and the filter part of the glass filter.

7. Crude Ash (CA)

[Summary of crude ash]

A sample is incinerated by heating to be crude ash.

When a sample is incinerated without special treatment, there always is contamination with charred organic matter, resulting in a blackish color. Therefore, it cannot be considered as pure ash (inorganic salts), and is referred to as crude ash.

Ash in feeds is useful for judging nutritional characteristics of the feed because ash has generally constant element composition by feed material type as long as the feed does not contain earth and sand, etc. Ash content in feeds of plant origin is not very good as an nutritional indicator because: it varies widely; silicate accounts for a large percentage of ash; and the other element composition is apt to vary depending on soil and fertilizers.

[Methods listed in the Analytical Standard of Feed]

1. Ignition [Analytical Standard of Feed, Chapter 3, 7]

Quantification

Weigh accurately 2-5 g of an analysis sample and put it in a crucible[1] (heated at 550-600 °C for 2 hours, cooled in a desiccator, and then weighed accurately in advance). Heat this gently to be charred[2] , then ash at 550-600 °C for 2 hours, let it stand to cool in a desiccator[3], and accurately weigh to calculate the crude ash content in the sample.

«Summary of analysis method»

Crude ash is defined as the weight of a sample measured after heating at 550-600 °C for 2 hours to incinerate in a crucible.

«Method validation»

· Results of proficiency testing in FY 2006

Sample type	Number of laboratories	Measured value (%)	Inter-laboratory reproducibility RSD_R (%)
Broiler starter	256	4.90	2.9
Fish meal	252	17.06	1.8

«Notes and precautions»

[1] The container for incineration can be any of ceramic, platinum, and Pyrex; however, when crude ash is to be used for other elemental analysis after quantification, a platinum container is preferred depending on the purpose. A ceramic crucible can be about 40 mm in inner diameter, and about 37 mm in height.

A crucible stand shown in Figure 3.7-1 is convenient that it can be placed as is in a desiccator (with a stopcock on the top). This product is commercially available from Sanshin Industrial.

[2] For feeds with high sugar content or feeds of animal origin, the sample may be expanded and

overflow out of the crucible; heat carefully.

[3] The cooling time should be the same as in cooling in the pre-heating.

Figure 3.7-1 Crucible stand

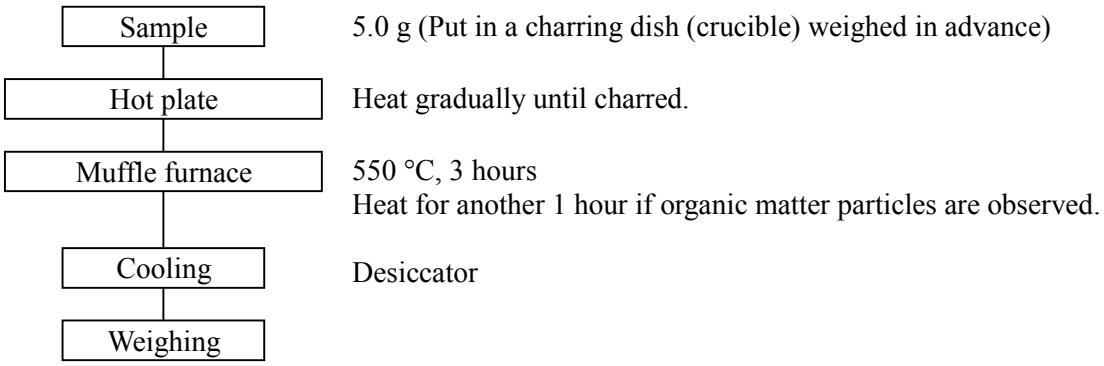
Made of stainless: convenient for the transport of crucibles taken out of a muffle furnace

[Other analysis methods]

2. Crude ash quantification by international standards

ISO 5984 (2002) Animal feeding stuffs - Determination of crude ash

Flow sheet of the analysis method



8. Nitrogen Free Extracts (NFE)

[Summary of nitrogen free extracts]

Subtract the contents (%) of the following 5 ingredients, moisture, crude protein, ether extract, crude fiber and crude ash, from the whole feed, and the result is shown as nitrogen free extracts (NFE) (%).

The content of NFE is those that are not contained in the 5 ingredients shown above, and the major ingredients are soluble carbohydrates such as starch and sugars, as well as organic acids and lignin. NFE is an important nutrient as an energy source for animals.

[Methods listed in the Analytical Standard of Feed]

1. Calculation of Nitrogen free extracts [Analytical Standard of Feed, Chapter 3, 8]

Quantification

Calculate the nitrogen free extracts content by the following formula:

Nitrogen free extracts content (%) = 100 − { moisture content (%) + crude protein content (%) + ether extract content (%) + crude fiber content (%) + crude ash content (%) }