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**Animal feeding stuffs — Determination of  
amylase-treated neutral detergent fibre  
content (aNDF)**

*Aliments des animaux — Détermination du contenu en fibre détergente  
neutre traitée à l'amylase*

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Case postale 56 • CH-1211 Geneva 20  
Tel. + 41 22 749 01 11  
Fax + 41 22 749 09 47  
E-mail [copyright@iso.org](mailto:copyright@iso.org)  
Web [www.iso.org](http://www.iso.org)

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## Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16472 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

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# Animal feeding stuffs — Determination of amylase-treated neutral detergent fibre content (aNDF)

**WARNING** — The use of this International Standard may involve the use of hazardous materials, operations and equipment. This International Standard does not purport to address all the safety risks associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of local regulatory limitations prior to use.

## 1 Scope

This International Standard specifies methods for the determination of amylase-treated neutral detergent insoluble fibrous residue content in all types of animal feed.

It includes a gravimetric routine method and a reference method.

## 2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6498, *Animal feeding stuffs — Preparation of test samples*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

### 3.1

#### **amylase-treated neutral detergent fibre content aNDF content**

mass fraction of insoluble fibre residues determined by the procedure specified in this International Standard

**NOTE** The aNDF content is expressed as a percentage by mass.

## 4 Principle

Neutral detergent (ND) solution and heat-stable alpha-amylase are used to dissolve the easily digestible proteins, lipids, sugars, starches and pectins in feeds, leaving an insoluble fibrous residue that is primarily cell wall components of plant materials (cellulose, hemicellulose and lignin) and indigestible nitrogenous matter in animal products.

## 5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

**5.1 Sodium sulfite**, anhydrous ( $\text{Na}_2\text{SO}_3$ ).

**5.2 Dried hominy corn** (corn grits, raw), ground to pass through a 1 mm screen in a cutter mill.

**5.3 Iodine solution**, containing 2 g of potassium iodide and 1 g of iodine in 100 ml of water.

Store the solution in an amber or opaque bottle.

**5.4 Heat-stable alpha-amylase**, as a solution or a water extract of lyophilised enzyme powder (approx. 1 g of powder extracted in 100 ml of water).

EXAMPLE Termamyl 120 I from Novo Enzymes or equivalent.

Standardize the heat-stable alpha-amylase solution or enzyme powder extract so that two additions of 2 ml will remove starch from 0,5 g of raw corn starch (5.2). For a detailed procedure on standardizing heat-stable alpha-amylase solution, see Annex B.

### 5.5 Neutral-detergent (ND) solution

Pour between 400 ml and 500 ml of water into a 1 l flask. Add 4,0 g of sodium hydroxide (NaOH), 14,6 g of EDTA, 4,56 g of sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), and 6,81 g of sodium borate decahydrate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ ) and mix until dissolved (heat if necessary). The NaOH and EDTA may be replaced with 18,6 g of disodium EDTA.

Under a safety hood, add 30 g of sodium lauryl sulfate and, after dissolution, add 10 ml of triethylene glycol (anti-foaming aid). Add water to about 950 ml and mix. Adjust the pH to between 6,95 and 7,05 with concentrated hydrochloric acid (HCl) or sodium hydroxide (NaOH) and dilute to 1 000 ml with water. If the pH is off the range by more than 0,5, discard the solution.

Store the ND solution at room temperature. If precipitation occurs, warm the solution to 25 °C and mix before use. Record the date the ND solution was prepared, the pH measurements and any adjustments in a reagent log book.

## 6 Apparatus

Usual laboratory apparatus and, in particular, the following.

**6.1 Analytical balance**, capable of weighing to the nearest 0,1 mg, with a readability of 0,1 mg.

**6.2 Cyclone mill** with 2 mm screen, or **cutter mill** with 1 mm screen, capable of grinding samples to obtain a geometric mean particle size of 220  $\mu\text{m}$  to 260  $\mu\text{m}$

**6.3 Refluxing apparatus**, with individual heating units and cold water condensers that fit 600 ml flasks.

Any conventional apparatus suitable for crude fibre determinations is acceptable. Calibrate the heating unit settings so that 50 ml of water boils within 4 min to 5 min when using cold water condensers. A Fibertec type apparatus may be used and should boil 50 ml of water within 10 min.

**6.4 Fritted-disc Gooch crucibles**, coarse porosity (pore size 40  $\mu\text{m}$  to 60  $\mu\text{m}$ ) crucibles, high-form, 40 ml to 50 ml capacity, or P2 (pore size 40  $\mu\text{m}$  to 100  $\mu\text{m}$ ), 26 ml to 28 ml capacity.

Clean new crucibles and ash at 500 °C for 1 h. Clean crucibles after each use by ashing at 500 °C for 3 h, removing ash, inverting in a detergent solution and sonicating for 7 min to 10 min. Rinse crucibles in hot water, and soak in water at room temperature for at least 30 min. Fit the top of each crucible with a rubber stopper fitted with a port that is connected to a trap and vacuum line. Back-flush each crucible with water, by repeatedly plunging and removing the bottom of the crucible into water to create a vigorous rinsing action.

Occasionally check the filtration rate as follows. Fill each crucible with 50 ml of distilled water (25 ml for Fibertec P2 crucibles) and record the time required to drain completely without vacuum (should be 180 s  $\pm$  60 s for Gooch or 75 s  $\pm$  30 s for P2). If the drain time is < 100 s (or < 30 s for P2), discard the crucible. If it is < 120 s (or < 45 s for P2), check for cracks in the fritted disc. If the filtration takes > 240 s (or > 105 s for P2), clean the crucible with acid or alkaline cleaning solution (see Reference [1]). If cleaning does not improve the filtration rate, discard the crucible.

Instead of P2 crucibles, stainless-steel metal crucibles with a 90  $\mu$ m aperture stainless-steel metal sieve may also be used.

#### 6.5 Vacuum filter manifold (e.g. Fibertec type), that allows adequate soaking of fibrous residues.

The manifold should provide a vacuum-tight seal with the crucible to reduce foam formation in vacuum lines. Use thick-walled vacuum tubing to connect the manifold to a trap (4 l to 18 l) and vacuum source. A vacuum reservoir (18 l) between the trap and vacuum source is recommended to ensure adequate vacuum capacity to remove the foam.

#### 6.6 Boiling water supply

Use a continuous boiling water generator as described in Reference [1] or a suitable alternative. The apparatus shall be capable of supplying boiling water (> 95 °C) in a quantity sufficient for all samples being washed at one time, through a nozzle producing a fine stream (flow rate of 35 ml to 40 ml per 10 s; a 2,5 ml disposable plastic pipette tip makes an acceptable nozzle). A fine nozzle minimizes the water needed to transfer particles to the crucible, but provides the water pressure needed to remove residues attached to the side of the flask. It is critical that water is boiling when added to the crucibles, especially for samples containing starches, pectic substances, mucilages or glyco-proteins. For Fibertec type apparatus, use a syringe with a cone-spray nozzle to rinse the condensers and a 60 ml disposable syringe with 12 gauge needle that is 10 cm in length to dislodge any residues adhering to the condensers.

## 7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

## 8 Preparation of test sample

Prepare the test samples in accordance with ISO 6498.

For sample storage and ease of grinding, samples should be air-dry (about 90 % dry matter)

Dry wet samples at < 60 °C to prevent creation of artefact fibre. The amount of residue after extraction is affected by the particle size of the sample. Grind representative samples to obtain a geometric mean particle size of 220  $\mu$ m to 260  $\mu$ m (see 6.2).

Grinding segregates the sample, with highest fibre content material passing out of the grinder last. Do not discard material in the grinder, combine it with material in the grinder receptacle. Mix the ground sample by placing it on a square sheet of paper (approximately 40 cm  $\times$  40 cm) creased along both diagonals. Lift two

opposite corners of the sheet to slide the sample in towards the central crease. Spread the sheet out flat again, turn it through 90° and lift the other two corners. Repeat 11 times. Transfer the sample to a suitable container.

NOTE Wet samples can be analysed for aNDF; however this is not a routine approach because it is difficult to grind the samples to the equivalent particle size as above.

## 9 Procedure

### 9.1 Procedure for traditional method as described in Reference [1]

#### 9.1.1 Test portion

Dry the empty crucibles at  $105\text{ °C} \pm 1\text{ °C}$  for 4 h then weigh them. Record the empty crucible mass for samples ( $m_c$ ) or blanks ( $m_b$ ) to the nearest 0,000 1 g.

Mix the material thoroughly and weigh  $1\text{ g} \pm 0,001\text{ g}$  of air-dry feed, or the equivalent amount of wet test sample ( $m_s$ ), into a crucible or refluxing flask, depending on preliminary defatting.

Inhomogeneous samples that need grinding shall be dried (see Clause 8). Only wet samples that can easily be homogenized may be weighed in directly.

If results are to be reported on a dry matter basis, weigh a second sample at the same time for determination of the dry matter.

Include an in-house reference sample and two blanks for the first 20 to 30 samples in a run, and add one reference and one blank for each additional 20 to 30 samples.

#### 9.1.2 Preliminary defatting

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Samples containing > 5 % fat should be pre-extracted. Those with > 10 % fat shall be pre-extracted to remove the fat.

To pre-extract with acetone, put a test portion into a crucible and weigh it. Place it on the filter manifold and extract four times with 40 ml to 50 ml of acetone (allow material to soak at least 5 min and stir three times during each soaking). Apply vacuum to remove traces of acetone, air-dry for 10 min to 15 min to ensure that all traces of acetone are removed and transfer to a reflux flask. Use the same crucible to collect the fibre residue for the test sample after ND extraction.

If a filtering aid is used, it shall be dried and weighed with the crucible, then transferred to another container before the test sample is weighed into the crucible and extracted with acetone. Replace the filtering aid in the crucible before filtration of fibre residues after ND extraction.

#### 9.1.3 Digestion

Add  $0,5\text{ g} \pm 0,1\text{ g}$  of sodium sulfite (5.1) using a graduated scoop and  $50\text{ ml} \pm 5\text{ ml}$  of ND solution (5.5) to each refluxing flask and swirl (this is critical for starchy feeds that stick to the bottom during refluxing). Do not add the ND and sodium sulfite to samples more than 60 min before refluxing.

Heat to boiling within 4 min to 5 min, add 2 ml of standardized amylase solution (5.4), resuspend any particles stuck to the bottom or sides, and swirl.

Reflux for 60 min at a rate that creates vigorous particle movement, but not excessive foaming that would carry particles up the side of the flask. Samples may foam vigorously for 1 min to 2 min (do not reduce the temperature of the heating unit). Rinse the sides of the flask with a minimum amount of ND solution, using a bottle with a fine nozzle, 5 min to 10 min after adding the amylase, and rinse as needed to resuspend particles on the side of the flask (twice max.).



#### 9.1.4 Filtration

Remove the extracted sample from the heating unit and allow particles to settle for 30 s to 60 s. Before transfer, observe the mixture to determine if lipid globules are present on the surface or if the solution is milky, which indicates a high-fat material that should be rerun after acetone pre-extraction (9.1.2).

Place a Teflon stirring rod in the crucible and preheat by adding 40 ml of boiling water for 30 s to 60 s. Remove the water with vacuum and immediately decant the top 30 ml to 40 ml of the solution from the flask, keeping the flask inverted over the crucible. Use minimum vacuum to evacuate excess liquid and close vacuum before residue becomes dry.

NOTE Excessive vacuum and evacuating to dryness causes some samples to clog the crucible and so not wash properly.

Rinse all unattached particles into the crucible using a fine stream of boiling water. Fill the crucible half-full with hot water. Add 2 ml of working amylase solution (5.4) and stir.

React with amylase for a minimum of 45 s to 60 s while scraping particles from the bottom and sides of the reflux flask using a rubber policeman. Evacuate the amylase solution and transfer any remaining residue from the reflux flask into the crucible with 20 ml to 30 ml boiling water. Two rinses are usually sufficient. After transferring residues from the flask, fill the crucible three-quarters full with boiling water and soak for 3 min.

Evacuate the water, add 40 ml to 50 ml of boiling water, soak for 3 min to 5 min, and repeat. If residues are difficult to filter after the first soak, add an additional 2 ml of working amylase solution. If residues appear translucent and become more difficult to filter with each additional soaking, eliminate the third water soak. If plugged, the crucible may be back-flushed by removing it from the filter manifold and reinserting it.

Evacuate the water, refill the crucible with 40 ml to 50 ml acetone, stir to disperse particles, soak for 3 min to 5 min, and repeat. Rinse the stirring rod to remove any attached fibre particles. Do not evacuate water completely from the fibre residues with vacuum before adding the acetone. Excessive drying clumps the residues and makes particle dispersion in acetone difficult, which hampers acetone extraction.

Apply a vacuum to dry the sample. Remove the crucible from the manifold and air dry for 10 min to 60 min to remove acetone.

#### 9.1.5 Drying

Dry crucibles at  $105\text{ °C} \pm 1\text{ °C}$  for a minimum of 8 h. Leave to cool in the dessicator and weigh to the nearest 0,000 1 g ( $m_{ce}$  and  $m_{be}$ ).

#### 9.1.6 Ashing

Ignite the crucible with the residue in a furnace at  $500\text{ °C} \pm 20\text{ °C}$  for 5 h or until carbon-free. Leave to cool in the dessicator and weigh to the nearest 0,000 1 g ( $m_{ca}$  and  $m_{ba}$ ).

### 9.2 Determination using Fibertec-type apparatus

#### 9.2.1 Test portion

Add the filtering aid to the P2 crucible, dry at  $105\text{ °C} \pm 1\text{ °C}$  for 2 h to 4 h and weigh to the nearest 0,000 1 g ( $m_c$  or  $m_b$ ). Mix material thoroughly and weigh  $0,5\text{ g} \pm 0,050\text{ g}$  of air-dry feed, or an equivalent amount of wet test sample ( $m_s$ ), into the crucible.

If results are to be reported on a dry matter basis, weigh a second sample at the same time for determination of the dry matter.

Include an in-house reference sample and two blanks for the first 20 to 30 samples in a run, and add one reference and one blank for each additional 20 to 30 samples.

### 9.2.2 Preliminary defatting

Generally samples with unknown fat contents should be pre-extracted. Those with > 10 % fat shall be pre-extracted to remove the fat.

Place the crucible on the cold-extraction unit and extract four times with 20 ml to 30 ml of acetone (allow the material to soak for at least 5 min and stir three times during each soaking). Apply vacuum to remove traces of acetone, air-dry for 10 to 15 min to ensure that all traces of acetone are removed.

### 9.2.3 Digestion

Start up the Fibertec-type apparatus, following the instructions of the manufacturer.

Add  $0,5 \text{ g} \pm 0,1 \text{ g}$  of sodium sulfite and  $50 \text{ ml} \pm 5 \text{ ml}$  of ND solution (5.5) to each crucible and mix using back-pressure (this is critical for starchy feeds that stick to the bottom during refluxing). Do not add the ND and sodium sulfite to samples more than 60 min before refluxing. Add 2 ml of standardized amylase solution (5.4) and heat to boiling within 10 min. Use back pressure to mix the amylase with the ND solution and the sample.

Boil for 60 min. Samples may foam vigorously for 1 min to 2 min (do not reduce the temperature of the heating unit). Rinse the sides of the flask with a minimum amount of ND, using a bottle with fine nozzle, 5 min to 10 min after adding the amylase, and rinse as needed to resuspend particles on the side of the flask (twice max.).

### 9.2.4 Filtration

Before the initial filtration, observe the mixture to determine if lipid globules are present on the surface or if the solution is milky, which indicates a high-fat material that should be rerun after acetone pre-extraction (9.2.2).

Evacuate the solution without allowing residues to become dry. Use minimum vacuum to evacuate excess liquid, but close vacuum before residue becomes dry.

NOTE 1 Excessive vacuum and evacuating to dryness causes some samples to clog the crucible and so not wash properly.

Add 30 ml of hot water (80 °C) and 2 ml of standardized amylase solution (5.4). Use back-pressure to mix the amylase in the initial water soak. Remove amylase-water soak after a minimum of 60 s of reaction.

NOTE 2 Crucibles can be removed from the hot to the cold filtration unit for the remaining hot water soaks for samples that are easy to filter. This allows the next set of samples to begin ND extraction on the hot filtration unit. Samples that are difficult to filter can be washed on the Fibertec heating unit with heat reduced to minimize particle agitation.

Add 30 ml of hot water, soak for 3 min to 5 min, and remove the water. If residues are difficult to filter after the first soak, add an additional 2 ml of amylase solution. If residues appear translucent and become more difficult to filter with each additional soaking, eliminate the third water soak. If plugged, the crucibles may be back-flushed using minimum back-pressure.

Do not evacuate the water completely from the fibre residues with vacuum before the acetone wash. Excessive drying clumps the residues and makes particle dispersion in acetone difficult, which hampers acetone extraction.

Move crucibles to the cold extraction unit. Fill crucibles with 30 ml of acetone and use minimum back-pressure to disperse the particles. Soak for 3 min to 5 min and evacuate. Repeat the acetone wash.

Dry the residue under vacuum, remove the crucible from the manifold and air-dry for 10 min to 60 min to remove acetone.

### 9.2.5 Drying

Dry the crucibles at  $105\text{ °C} \pm 1\text{ °C}$  for a minimum of 8 h. Leave to cool in the dessicator and weigh to the nearest 0,000 1 g ( $m_{ce}$  and  $m_{be}$ ).

### 9.2.6 Ashing

Ignite the crucible with the residue in a furnace at  $500\text{ °C} \pm 20\text{ °C}$  for 5 h or until carbon-free. Leave to cool in the dessicator and weigh to the nearest 0,000 1 g ( $m_{ca}$  and  $m_{ba}$ ).

## 9.3 Modifications for specific types of samples

**9.3.1** If the extracted ND solution appears milky and opaque and filtration is slow during transfer of residues or after the first water soaking, a high starch content is suspected. Add an additional treatment with 2 ml of amylase during the second water soaking. Shorten soaking times to the minimum to keep the soaking solutions as hot as possible ( $> 85\text{ °C}$ ).

**9.3.2** If the residue clogs the crucible during transfer and additional amylase does not improve filtration, the feed material may contain proteinaceous, gum or mucilage residues (as is the case with meat products and some oil seed meals). Preheating the crucible with boiling water is crucial for filtering these materials. The best filter aid for these materials is 12 g to 15 g (6 g to 8 g for Fibertec P2) of silica sand (sand, cristobalite, acid-purified, 40 mesh to 200 mesh, Fluka Cat. No. 84880 or equivalent)<sup>1)</sup>. The gummy substances in these feed materials will stick to sand particles, which prevents them from clogging the fritted disc and allows the residues to be washed. All filter aids shall be added to the crucibles (including blanks) before the initial masses are recorded.

**9.3.3** If the fibre residue has a glossy, translucent sheen and filtration becomes more difficult with each water soaking, pectic substances are suspected. Preheat the crucible with boiling water and transfer residues as quickly as possible without settling when removed from the reflux unit. Reduce all soaking times to the minimum to maintain a temperature of  $> 85\text{ °C}$  to prevent cooling and jelling of pectin in the crucible. The following filter aids may improve filtration (in order of preference): 12 g to 15 g (6 g to 8 g for Fibertec P2) silica sand, 0,25 g (0,15 g for Fibertec P2) glass wool and glass microfibre mats (4,25 cm Whatman GF/D or equivalent)<sup>1)</sup>.

**9.3.4** If fat globules are observed floating on the surface of the ND or wash water and the sample is difficult to filter, or if the sample is known to contain  $> 10\%$  fat, it should be pre-extracted with acetone or ether (see 9.1.2 or 9.2.2).

**9.3.5** If the sample contains fine particles, flocculant precipitates, dirt (fine clay) or faecal matter, but not pectic substances or starch, increase the settling time to a maximum of 2 min after removal from the refluxing unit and use a filter aid in the crucible. Filter aids (in order of preference) include: glass microfibre mats, ceramic fibre, 12 g to 15 g of silica sand, and 0,25 g of glass wool. Microfibre mats can be gently scraped to renew the surface during filtration.

**9.3.6** If all other modifications fail, reduce the test sample amount to 0,3 g and repeat the analysis with a filter aid in the crucible. Reducing the sample amount will magnify the effects of weighing errors and increase variation in results. Sometimes reducing the sample amount and increasing the ND amount to 70 ml to 100 ml is beneficial. If the fibre content is  $< 1,5\%$ , do not reduce the sample amount; if filtration is not possible, report results as "difficult to analyse, fibre content  $< 1,5\%$ ".

**9.3.7** Do not add acetone before all the rinse water has been removed. Although this will occasionally improve the filtering, it does not remove detergent or detergent solubles from residues. Adding acetone before water washing is complete will give inflated fibre content values.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.