

Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

ANNEX III

METHODS OF ANALYSIS TO CONTROL THE COMPOSITION OF FEED MATERIALS AND COMPOUND FEED

A. DETERMINATION OF MOISTURE

1. Purpose and Scope

This method makes it possible to determine the moisture content of feed. In case of feed containing volatile substances, such as organic acids, it is to be observed that also significant amount of volatile substances are determined together with the moisture content.

It does not cover the analysis of milk products as feed materials, the analysis of mineral substances and mixtures composed predominantly of mineral substances, the analysis of animal and vegetable fats and oils or the analysis of the oil seeds and oleaginous fruit.

2. Principle

The sample is desiccated under specified conditions which vary according to the nature of the feed. The loss in weight is determined by weighing. It is necessary to carry out preliminary drying when dealing with solid feed which has high moisture content.

3. Apparatus

- 3.1. Crusher of non-moisture-absorbing material which is easy to clean, allows rapid, even crushing without producing any appreciable heating, prevents contact with the outside air as far as possible and meets the requirements laid down in 4.1.1 and 4.1.2 (e.g. hammer or water cooled micro-crushers, collapsible cone mills, slow motion or cog wheeled crushers).
- 3.2. Analytical balance, accurate to 1 mg.
- 3.3. Dry containers of non-corrodible metal or of glass with lids ensuring airtight closure; working surface allowing the test sample to be spread at about 0,3 g/cm².
- 3.4. Electrically heated isothermal oven (± 2 °C) properly ventilated and ensuring rapid temperature regulation⁽¹⁾.
- 3.5. Adjustable electrically heated vacuum oven fitted with an oil pump and either a mechanism for introducing hot dried air or a drying agent (e.g. calcium oxide).
- 3.6. Desiccator with a thick perforated metal or porcelain plate, containing an efficient drying agent.

4. Procedure

N.B. The operations described in this section must be carried out immediately after opening the packages of samples. Analysis must be carried out at least in duplicate.

4.1. Preparation

4.1.1. *Feed other than those coming under 4.1.2 and 4.1.3*

Take at least 50 g of the sample. If necessary, crush or divide in such a way as to avoid any variation in moisture content (see 6).

4.1.2. *Cereals and groats*

Take at least 50 g of the sample. Grind into particles of which at least 50 % will pass through a 0,5 mm mesh sieve and will leave no more than 10 % reject on a 1 mm round-meshed sieve.

4.1.3. *Feed in liquid or paste form, feed predominantly composed of oils and fats*

Take about 25 g of the sample, weigh to the nearest 10 mg, add an appropriate quantity of anhydrous sand weighed to the nearest 10 mg and mix until a homogeneous product is obtained.

4.2. Drying

4.2.1. *Feed other than those coming under 4.2.2 and 4.2.3*

Weigh a container (3.3) with its lid to the nearest 1 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container, without its lid, in the oven preheated to 103 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible. Leave to dry for four hours reckoned from the time when the oven temperature returns to 103 °C. Replace the lid on the container, remove the latter from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg.

For feed composed predominantly of oils and fats, dry in the oven for an additional 30 minutes at 130 °C. The difference between the two weighings must not exceed 0,1 % of moisture.

4.2.2. *Cereals, flour, groats and meal*

Weigh a container (3.3) with its lid to the nearest 0,5 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the crushed sample and spread evenly. Place the container, without its lid, in the oven preheated to 130 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible. Leave to dry for two hours reckoned from the time when the oven temperature returns to 130 °C. Replace the lid on the container, remove the latter from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg.

4.2.3. Compound feed containing more than 4 % of sucrose or lactose: feed materials such as locust beans, hydrolysed cereal products, malt seeds, dried beet chips, fish and sugar solubles; compound feed containing more than 25 % of mineral salts including water of crystallisation.

Weigh a container (3.3) with its lid to the nearest 0,5 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container, without its lid, in the vacuum oven (3.5) preheated to between 80 °C and 85 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible.

Bring the pressure up to 100 Torr and leave to dry for four hours at this pressure, either in a current of hot, dry air or using a drying agent (about 300 g for 20 samples). In the latter instance, disconnect the vacuum pump when the prescribed pressure has been reached. Reckon drying time from the moment when the oven temperature returns to 80 °C to 85 °C. Carefully bring the oven back to atmospheric pressure. Open the oven, place the lid on the container immediately, remove the container from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg. Dry for an additional 30 minutes in the vacuum oven at 80 °C to 85 °C and reweigh. The difference between the two weighings must not exceed 0,1 % of moisture.

4.3. Preliminary drying

4.3.1. *Feed other than those coming under 4.3.2*

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Solid feed with a high moisture content which makes crushing difficult must be subjected to preliminary drying as follows:

Weigh 50 g of *uncrushed* sample to the nearest 10 mg (compressed or agglomerated feed may be roughly divided if necessary) in a suitable container (e.g. a 20 × 12 cm aluminium plate with a 0,5 cm rim). Leave to dry in an oven from 60 °C to 70 °C until the moisture content has been reduced to between 8 % and 12 %. Remove from the oven, leave to cool uncovered in the laboratory for one hour and weigh to the nearest 10 mg. Crush immediately as indicated in 4.1.1 and dry as indicated in 4.2.1 or 4.2.3 according to the nature of the feed.

4.3.2. Cereals

Grain with a moisture content of over 17 % must be subjected to preliminary drying as follows:

Weigh 50 g of unground grain to the nearest 10 mg in a suitable container (e.g. a 20 × 12 cm aluminium plate with a 0,5 cm rim). Leave to dry for 5 to 7 minutes in an oven at 130 °C. Remove from the oven, leave to cool uncovered in the laboratory for two hours and weigh to the nearest 10 mg. Grind immediately as indicated in 4.1.2 and dry as indicated in 4.2.2.

5. Calculation of results

The moisture content (X), as a percentage of the sample, is calculated by using the following formulae:

5.1. Drying without preliminary drying

$$X = \frac{(m - m_0)}{m} \times 100$$

where:

m = initial weight, in grammes, of the test sample,
 m₀ = weight, in grammes, of the dry test sample.

5.2. Drying with preliminary drying

$$X_p = \left[\frac{(m_2 - m_0) \times m_1}{m_2} + m - m_1 \right] \times \frac{100}{m} = 100 \times \left(1 - \frac{m_1 \times m_0}{m \times m_2} \right)$$

where:

m = initial weight, in grammes, of the test sample,
 m₁ = weight, in grammes, of the test sample after preliminary drying,
 m₂ = weight, in grammes, of the test sample after crushing or grinding,
 m₀ = weight, in grammes, of the dry test sample.

5.3. Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed 0,2 % of the absolute value of moisture.

6. Observation

If crushing proves necessary and if this is seen to alter the moisture content of the product, the results of the analysis of the components of the feed must be corrected on the basis of the moisture content of the sample in its initial state.

B. DETERMINATION OF MOISTURE IN ANIMAL AND VEGETABLE FATS AND OILS

1. Purpose and scope

This method makes it possible to determine the water and volatile substances content of animal and vegetable fats and oils.

2. Principle

The sample is dried to constant weight (loss in weight between two successive weighings must be less than or equal to 1 mg) at 103 °C. The loss in weight is determined by weighing.

3. Apparatus

- 3.1. Flat-bottomed dish, of a corrosion-resistant material, 8 to 9 cm in diameter and approximately 3 cm high.
- 3.2. Thermometer with a strengthened bulb and expansion tube at the top end, graduated from approximately 80 °C to at least 110 °C, and approximately 10 cm in length.
- 3.3. Sand bath or electric hot-plate.
- 3.4. Desiccator, containing an efficient drying agent.
- 3.5. Analytical balance.

4. Procedure

Weigh out to the nearest mg approximately 20 g of the homogenised sample into the dry, weighed dish (3.1) containing the thermometer (3.2). Heat on the sand bath or hot-plate (3.3), stirring continuously with the thermometer, so that the temperature reaches 90 °C in about 7 minutes.

Reduce the heat, watching the frequency with which bubbles rise from the bottom of the dish. The temperature must not exceed 105 °C. Continue to stir, scraping the bottom of the dish, until bubbles stop forming.

In order to ensure complete elimination of moisture, reheat several times to 103 °C ± 2 °C, cooling to 93 °C between successive heatings. Then leave to cool to room temperature in the desiccator (3.4) and weigh. Repeat this operation until the loss in weight between two successive weighings no longer exceeds 2 mg.

N.B. : An increase in the weight of the sample after repeated heating indicates an oxidation of the fat, in which case calculate the result from the weighing carried out immediately before the weight began to increase.

5. Calculation of results

The moisture content (X), as a percentage of the sample, is given by the following formula:

$$X = (m_1 - m_2) \times \frac{100}{m}$$

where:

- | | |
|-------|---|
| m | = weight, in grammes, of the test sample, |
| m_1 | = weight, in grammes, of the dish with its contents before heating, |
| m_2 | = weight, in grammes, of the dish with its contents after heating. |

Results lower than 0,05 % must be recorded as 'lower than 0,05 %'.

Repeatability

The difference in moisture between the results of two parallel determinations carried out on the same sample must not exceed 0,05 %, in absolute value.

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C. DETERMINATION OF THE CONTENT OF CRUDE PROTEIN

1. Purpose and scope

This method makes it possible to determine the crude protein content of feed on the basis of the nitrogen content, determined according to the Kjeldahl method.

2. Principle

The sample is digested by sulphuric acid in the presence of a catalyst. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in a measured quantity of sulphuric acid, the excess of which is titrated with a standard solution of sodium hydroxide.

Alternatively, the liberated ammonia is distilled into an excess of boric acid solution, followed by titration with hydrochloric acid or sulphuric acid solution.

3. Reagents

- 3.1. Potassium sulphate.
- 3.2. Catalyst: copper (II) oxide CuO or copper (II) sulphate pentahydrate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.
- 3.3. Granulated zinc.
- 3.4. Sulphuric acid, $\rho_{20} = 1,84$ g/ml.
- 3.5. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,25$ mol/l.
- 3.6. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,1$ mol/l.
- 3.7. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,05$ mol/l.
- 3.8. Methyl red indicator; dissolve 300 mg of methyl red in 100 ml of ethanol, $\sigma = 95\% - 96\%$ (v/v).
- 3.9. Sodium hydroxide solution (Technical grade may be used) $\beta = 40$ g/100 ml (m/v: 40 %).
- 3.10. Sodium hydroxide, standard volumetric solution $c(\text{NaOH}) = 0,25$ mol/l.
- 3.11. Sodium hydroxide, standard volumetric solution $c(\text{NaOH}) = 0,1$ mol/l.
- 3.12. Granulated pumice stone, washed in hydrochloric acid and ignited.
- 3.13. Acetanilide (m.p. = 114 °C, N-content = 10,36 %).
- 3.14. Sucrose (nitrogen free).
- 3.15. Boric acid (H_3BO_3).
- 3.16. Methyl red indicator solution: dissolve 100 mg methyl red in 100 ml ethanol or methanol.
- 3.17. Bromocresol green solution: dissolve 100 mg bromocresol green in 100 ml ethanol or methanol.
- 3.18. Boric acid solution (10 g/l to 40 g/l depending on the apparatus used).

When colorimetric end-point detection is applied, methyl red and bromocresol indicators must be added to the boric acid solutions. If 1 litre of the boric acid solution is prepared, before adjusting to volume, 7 ml methyl red indicator solution (3.16) and 10 ml bromocresol green solution (3.17) shall be added.

Dependent on the water used, the pH of the boric acid solution might differ from batch to batch. Often an adjustment with a small volume of alkali is necessary to obtain a positive blank.

Note : The addition of about 3 ml to 4 ml of NaOH (3.11) into 1 litre of 10 g/l boric acid usually gives good adjustments. Store the solution at room temperature and protect the solution from light and sources of ammonia fumes during storage.

3.19. Hydrochloric acid standard volumetric solution $c(\text{HCl}) = 0,1 \text{ mol/l}$.

Note: Other concentrations of volumetric solutions (3.5, 3.6, 3.7, 3.10, 3.11, and 3.19) can be used, if this is corrected for in the calculations. The concentrations shall always be expressed to four decimal places.

4. Apparatus

Apparatus suitable for performing digestion, distillation and titration according to the Kjeldahl procedure.

5. Procedure

5.1. Digestion

Weigh 1 g of the sample to the nearest 0,001 g and transfer the sample to the flask of the digestion apparatus. Add 15 g of potassium sulphate (3.1), an appropriate quantity of catalyst (3.2) (0,3 to 0,4 g of copper (II) oxide or 0,9 to 1,2 g of copper (II) sulphate pentahydrate), 25 ml of sulphuric acid (3.4) and if required, a few granules of pumice stone (3.12) and mix.

Heat the flask moderately at first, swirling from time to time if necessary until the mass has carbonised and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Heating is adequate if the boiling acid condenses on the wall of the flask. Prevent the sides from becoming overheated and organic particles from sticking to them.

When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

5.2. Distillation

Add carefully enough water to ensure complete dissolution of the sulphates. Allow to cool and then add a few granules of zinc (3.3), if required. Proceed according to 5.2.1 or 5.2.2.

5.2.1. Distillation into sulphuric acid

Place in the collecting flask of the distillation apparatus an exactly measured quantity of 25 ml of sulphuric acid (3.5) or (3.7) depending on the presumed nitrogen content. Add a few drops of methyl red indicator (3.8).

Connect the digestion flask to the condenser of the distillation apparatus and immerse the end of the condenser in the liquid contained in the collecting flask to a depth of at least 1 cm (see observation 8.3). Slowly pour 100 ml of sodium hydroxide solution (3.9) into the digestion flask without loss of ammonia (see observation 8.1). Heat the flask until the ammonia has distilled over.

5.2.2. Distillation into boric acid

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Where titration of the ammonia content of the distillate is performed manually, the procedure mentioned below applies. Where the distillation unit is fully automated to include titration of the ammonia content of the distillate, follow the manufacturer's instructions for operation of the distillation unit.

Place a collecting flask containing 25 ml to 30 ml of the boric acid solution (3.18) under the outlet of the condenser in such a way that the delivery tube is below the surface of the excess boric acid solution. Adjust the distillation unit to dispense 50 ml of sodium hydroxide solution (3.9). Operate the distillation unit in accordance with the manufacturer's instructions and distil off the ammonia liberated by the addition of the sodium hydroxide solution. Collect distillate in the boric acid receiving solution. The amount of distillate (time of steam distillation) depends on the amount of nitrogen in the sample. Follow the instructions of the manufacturer.

Note: In a semi-automatic distillation unit, the addition of excess sodium hydroxide and the steam distillation are performed automatically.

5.3. Titration

Proceed according to 5.3.1 or 5.3.2.

5.3.1. Sulphuric acid

Titrate the excess sulphuric acid in the collecting flask with sodium hydroxide solution (3.10 or 3.11) depending on the concentration of the sulphuric acid used, until the end-point is reached.

5.3.2. Boric acid

Titrate the contents of the collecting flask with the hydrochloric acid standard volumetric solution (3.19) or with the sulphuric acid standard volumetric solution (3.6) using a burette and read the amount of titrant used.

When colorimetric end-point detection is applied, the end-point is reached at the first trace of pink colour in the contents. Estimate the burette reading to the nearest 0,05 ml. An illuminated magnetic stirrer plate or a photometric detector may aid visualisation of the end-point.

This can be done automatically using a steam distiller with automatic titration.

Follow the manufacturers' instructions for operation of the specific distiller or distiller/titrator.

Note : When an automatic titration system is used, titration begins immediately after distillation starts and the 1 % boric acid solution (3.18) is used.

Where a fully automatic distillation unit is employed, the automatic titration of the ammonia can also be carried out with end-point detection using a potentiometric pH system.

In this case an automatic titrator, with a pH-meter is used. The pH-meter shall be calibrated properly in the range of pH 4 to pH 7 following normal laboratory pH-calibration procedures.

The pH end-point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point).

5.4. Blank test

To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using 1 g of sucrose (3.14) in place of the sample.

6. Calculation of results

Calculations are performed according to 6.1 or 6.2.

6.1. Calculation for titration according to 5.3.1

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_0 - V_1) \times c \times 0,014 \times 100 \times 6,25}{m}$$

where:

- V_0 = is the volume (ml) of NaOH (3.10 or 3.11) used in the blank test,
 V_1 = is the volume (ml) of NaOH (3.10 or 3.11) used in the sample titration,
 c = is the concentration (mol/l) of sodium hydroxide (3.10 or 3.11),
 m = is the weight (g) of sample.

6.2. Calculation for titration according to 5.3.2

6.2.1. Titration with hydrochloric acid

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_1 - V_0) \times c \times 1,4 \times 6,25}{m}$$

where:

- m = is the weight (g) of the test portion,
 c = is the concentration (mol/l) of the standard volumetric solution of the hydrochloric acid (3.19),
 V_0 = is the volume (in ml) of hydrochloric acid used for the blank test,
 V_1 = is the volume (in ml) of hydrochloric acid used for the test portion.

6.2.2. Titration with sulphuric acid

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_1 - V_0) \times c \times 2,8 \times 6,25}{m}$$

where:

- m = is the weight (g) of the test portion,
 c = is the concentration (mol/l) of the standard volumetric solution of sulphuric acid (3.6),
 V_0 = is the volume (in ml) of sulphuric acid (3.6) used for the blank test,
 V_1 = is the volume (in ml) of sulphuric acid (3.6) used for test portion.

7. Verification of the method

7.1. Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

- 0,2 % in absolute value, for crude protein contents of less than 20 %,
- 1,0 % relative to the higher value, for crude protein contents from 20 % to 40 %,
- 0,4 % in absolute value, for crude protein contents of more than 40 %.

7.2. Accuracy

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Carry out the analysis (digestion, distillation and titration) on 1,5 to 2,0 g of acetanilide (3.13) in the presence of 1 g of sucrose (3.14); 1 g acetanilide consumes 14,8 ml of sulphuric acid (3.5). Recovery must be at least 99 %.

8. Observations

- 8.1. Apparatus may be of the manual, semi-automatic or automatic type. If the apparatus requires transference between the digestion and distillation steps, this transfer must be carried out without loss. If the flask of the distillation apparatus is not fitted with a dropping funnel, add the sodium hydroxide immediately before connecting the flask to the condenser, pouring the liquid slowly down the side.
- 8.2. If the digest solidifies, recommence the determination using a larger amount of sulphuric acid (3.4) than that specified above.
- 8.3. For products with a low nitrogen content, the volume of sulphuric acid (3.7) to be placed in the collecting flask may be reduced, if necessary, to 10 or 15 ml and made up to 25 ml with water.
- 8.4. For routine analysis, alternative methods of analysis can be applied for the determination of crude protein but the Kjeldahl method described in this Part C is the reference method. The equivalence of the results obtained with the alternative method (e.g. DUMAS) compared to the reference method must be demonstrated for each matrix individually. As the results obtained with an alternative method, even after having verified the equivalency, might deviate slightly from the results obtained with the reference method, it is necessary to mention in the analytical report the method of analysis used for the determination of crude protein.

D. DETERMINATION OF UREA

1. Purpose and scope

This method makes it possible to determine the level of urea in feed.

2. Principle

The sample is suspended in water with a clarifying agent. The suspension is filtered. The urea content of the filtrate is determined after the addition of 4-dimethylaminobenzaldehyde (4-DMAB) by measuring the optical density at a wavelength of 420 nm.

3. Reagents

- 3.1. Solution of 4-dimethylaminobenzaldehyde: dissolve 1,6 g of 4-DMAB in 100 ml of 96 % ethanol and add 10 ml of hydrochloric acid ($\rho_{20} 1,19$ g/ml). This reagent keeps for a maximum period of two weeks.
- 3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ and 3 g of glacial acetic acid. Make up to 100 ml with water.
- 3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide, $\text{K}_4 \text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$. Make up to 100 ml with water.
- 3.4. Active carbon which does not absorb urea (to be checked).
- 3.5. Urea, 0,1 % solution (w/v).

4. Apparatus

- 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
- 4.2. Test tubes: 160 × 16 mm with ground-glass stoppers.
- 4.3. Spectrophotometer.
5. Procedure
- 5.1. Analysis of sample

Weigh out 2 g of the sample to the nearest mg and place with 1 g of active carbon (3.4) in a 500 ml volumetric flask. Add 400 ml of water and 5 ml of Carrez solution I (3.2), mix for approximately 30 seconds and add 5 ml of Carrez solution II (3.3). Mix for 30 minutes in the tumbler. Make up to volume with water, shake and filter.

Remove 5 ml of the transparent colourless filtrates, place in test tubes with ground-glass stoppers, add 5 ml of 4-DMAB solution (3.1) and mix. Place the tubes in a water bath at 20 °C (+/- 4 °C). After 15 minutes measure the optical density of the sample solution with the spectrophotometer at 420 nm. Compare with the blank test solution of the reagents.

- 5.2. Calibration curve

Remove volumes of 1, 2, 4, 5 and 10 ml of the urea solution (3.5), place in 100 ml volumetric flasks and make up the volume with water. Remove 5 ml from each solution, add 5 ml of 4-DMAB solution (3.1) to each of them, homogenise and measure the optical density as shown above in comparison with a control solution containing 5 ml of 4-DMAB and 5 ml of water free from urea. Plot the calibration curve.

6. Calculation of results

Determine the amount of urea in the sample using the calibration curve.

Express the result as a percentage of the sample.

7. Observations

- 7.1. In the case of contents of urea exceeding 3 %, reduce the sample to 1 g or dilute the original solution so that there are not more than 50 mg of urea in 500 ml.
- 7.2. In the case of low contents of urea, increase the sample as long as the filtrate remains transparent and colourless.
- 7.3. If the sample contains simple nitrogenous compounds such as amino acids, the optical density shall be measured at 435 nm.

E. DETERMINATION OF VOLATILE NITROGENOUS BASES

I. BY MICRODIFFUSION

1. Purpose and scope

This method makes it possible to determine the content of volatile nitrogenous bases, expressed as ammonia, in feed.

2. Principle

The sample is extracted with water and the solution clarified and filtered. The volatile nitrogenous bases are displaced by microdiffusion using a solution of potassium carbonate, collected in a solution of boric acid and titrated with sulphuric acid.

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3. Reagents

- 3.1. Trichloroacetic acid, solution 20 % (w/v).
- 3.2. Indicator: dissolve 33 mg of bromocresol green and 65 mg of methyl red in 100 ml of 95 %-96 % (v/v) of ethanol.
- 3.3. Boric acid solution: in a 1 litre graduated flask dissolve 10 g of boric acid in 200 ml of 95 %-96 % (v/v) ethanol and 700 ml of water. Add 10 ml of indicator (3.2). Mix and, if necessary, adjust the colour of the solution to light red by adding a solution of sodium hydroxide. 1 ml of this solution will fix a maximum of 300 µg of NH₃.
- 3.4. Saturated potassium carbonate solution: dissolve 100 g of potassium carbonate in 100 ml of boiling water. Leave to cool, filter.
- 3.5. Sulphuric acid 0,01 mol/litre.

4. Apparatus

- 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
- 4.2. Glass or plastic Conway cells (see diagram).
- 4.3. Microburettes graduated in 1/100 ml.

5. Procedure

Weigh 10 g of sample to the nearest 1 mg and place with 100 ml of water in a 200 ml graduated flask. Mix or stir in the tumbler for 30 minutes. Add 50 ml of trichloroacetic acid solution (3.1), make up to volume with water, shake vigorously and filter through a pleated filter.

Using a pipette, introduce 1 ml of boric acid solution (3.3) into the central part of the Conway cell and 1 ml of the sample filtrate into the crown of the cell. Cover partially with the greased lid. Drop 1 ml of saturated potassium carbonate solution (3.4) quickly into the crown and close the lid so that the cell is airtight. Turn the cell carefully rotating it in a horizontal plane so that the two reagents are mixed. Leave to incubate either for at least four hours at room temperature or for one hour at 40 °C.

Using a microburette (4.3), titrate the volatile bases in the boric acid solution with sulphuric acid (3.5).

Carry out a blank test using the same procedure but without a sample to be analysed.

6. Calculation of results

1 ml of H₂SO₄ 0,01 mol/litre corresponds to 0,34 mg of ammonia.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed:

- 10 %, in relative value, for ammonia contents of less than 1,0 %,
- 0,1 %, in absolute value, for ammonia contents of 1,0 % or more.

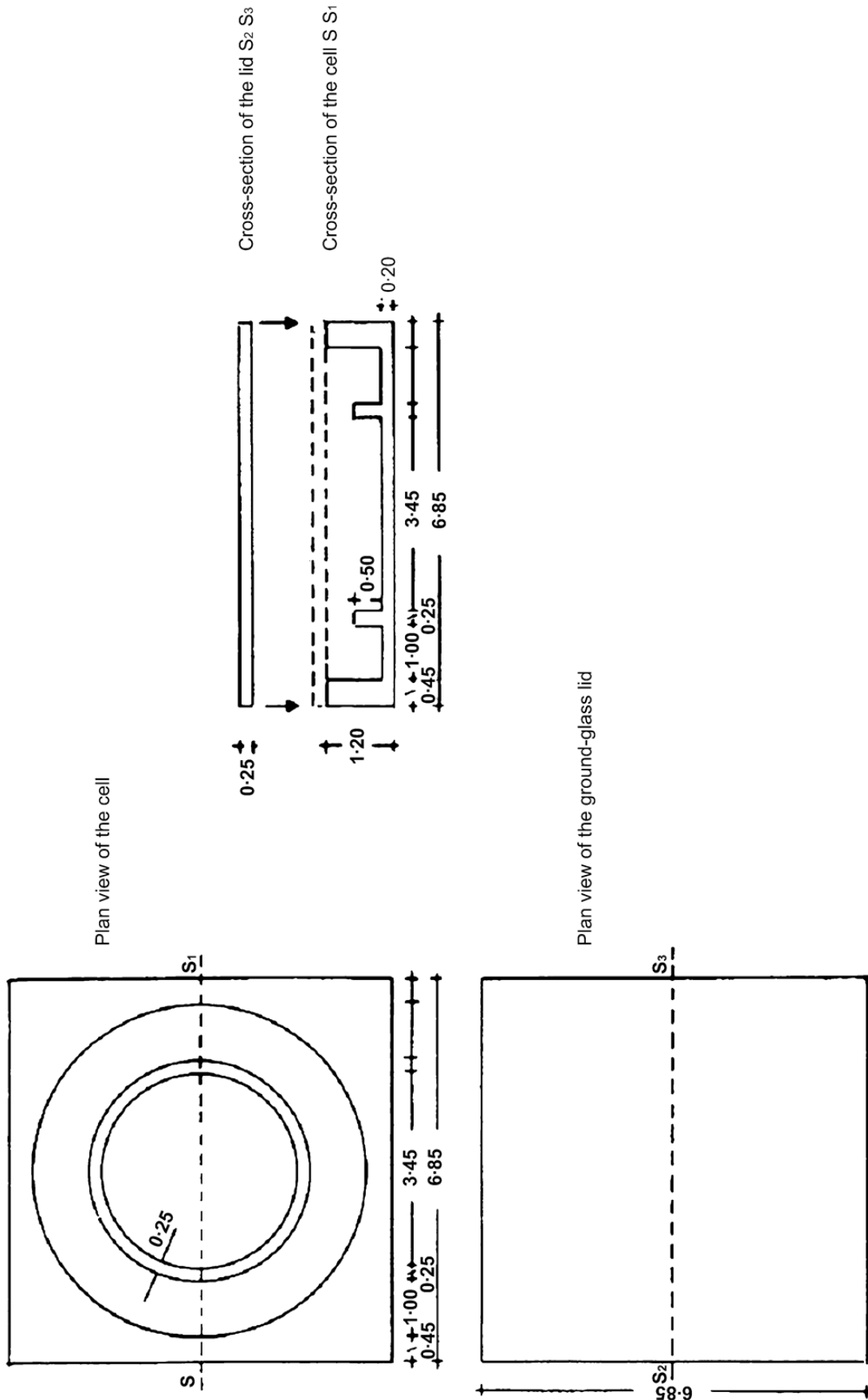
7. Observation

If the ammonia content of the sample exceeds 0,6 %, dilute the initial filtrate.

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CONWAYscale 1/1
CELL

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II. BY DISTILLATION

1. Purpose and Scope

This method makes it possible to determine the content of volatile nitrogenous bases, expressed as ammonia, in fish-meal containing practically no urea. It is applicable only to ammonia contents of less than 0,25 %.

2. Principle

The sample is extracted with water and the solution clarified and filtered. The volatile nitrogenous bases are displaced at boiling point by adding magnesium oxide and collected in a specific quantity of sulphuric acid, the excess of which is back-titrated with a solution of sodium hydroxide.

3. Reagents

- 3.1. Trichloroacetic acid, solution 20 % (w/v).
- 3.2. Magnesium oxide.
- 3.3. Anti-foaming emulsion (e.g. silicone).
- 3.4. Sulphuric acid 0,05 mol/litre.
- 3.5. Sodium hydroxide solution 0,1 mol/litre.
- 3.6. Methyl red solution 0,3 % in 95 %-96 % (v/v) ethanol.

4. Apparatus

- 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
- 4.2. Distilling apparatus of the Kjeldahl type.

5. Procedure

Weigh 10 g of the sample to the nearest 1 mg and place with 100 ml of water in a 200 ml graduated flask. Mix or stir in the tumbler for 30 minutes. Add 50 ml of trichloroacetic acid solution (3.1), make up to volume with water, shake vigorously and filter through a pleated filter.

Take a quantity of clear filtrate appropriate for the presumed content of volatile nitrogenous bases (100 ml is usually suitable). Dilute to 200 ml and add 2 g of magnesium oxide (3.2) and a few drops of anti-foaming emulsion (3.3). The solution must be alkaline to litmus paper; otherwise add some magnesium oxide (3.2). Proceed according to 5.2 and 5.3 of the method of analysis for the determination of the crude protein content (Part C of this Annex).

Carry out a *blank test* using the same procedure but without a sample to be analysed.

6. Calculation of results

1 ml of H₂SO₄ 0,05 mol/litre corresponds to 1,7 mg of ammonia.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed, in relative value, 10 % of ammonia.

F. DETERMINATION OF AMINO ACIDS (EXCEPT TRYPTOPHANE)

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

1. Purpose and scope

This method makes the determination possible of free (synthetic and natural) and total (peptide bound and free) amino acids in feed, using an amino acid analyser. It is applicable to the following amino acids: cyst(e)ine, methionine, lysine, threonine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, tyrosine and valine.

The method does not distinguish between the salts of amino acids and it cannot differentiate between D and L forms of amino acids. It is not valid for the determination of tryptophan or hydroxy analogues of amino acids.

2. Principle

2.1. Free amino acids

The free amino acids are extracted with diluted hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The filtered solution is adjusted to pH 2,2. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin with photometric detection at 570 nm.

2.2. Total amino acids

The procedure chosen depends on the amino acids under investigation. Cyst(e)ine and methionine must be oxidised to cysteic acid and methionine sulphone respectively prior to hydrolysis. Tyrosine must be determined in hydrolysates of unoxidised samples. All the other amino acids listed in paragraph 1 can be determined in either the oxidised or unoxidised sample.

Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. The oxidised or unoxidised sample is hydrolysed with hydrochloric acid (3.20) for 23 hours. The hydrolysate is adjusted to pH 2,2. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline).

3. Reagents

Double distilled water or water of equivalent quality must be used (conductivity < 10 µS).

- 3.1. Hydrogen peroxide, w (w/w) = 30 %.
- 3.2. Formic acid, w (w/w) = 98 %-100 %.
- 3.3. Phenol.
- 3.4. Sodium disulphite.
- 3.5. Sodium hydroxide.
- 3.6. 5-Sulfosalicylic acid dihydrate.
- 3.7. Hydrochloric acid, density approximately 1,18 g/ml.
- 3.8. tri-Sodium citrate dihydrate.
- 3.9. 2,2'-Thiodiethanol (thiodiglycol).
- 3.10. Sodium chloride.
- 3.11. Ninhydrin.

- 3.12. Light petroleum, boiling range 40-60 °C.
- 3.13. Norleucine, or other compound suitable for use as internal standard.
- 3.14. Nitrogen gas (< 10 ppm oxygen).
- 3.15. 1-Octanol.
- 3.16. Amino acids.
- 3.16.1. Standard substances listed under paragraph 1. Pure compounds containing no water of crystallisation. Dry under vacuum over P₂O₅ or H₂SO₄ for 1 week prior to use.
- 3.16.2. Cysteic acid.
- 3.16.3. Methionine sulphone.
- 3.17. Sodium hydroxide solution, c = 7,5 mol/l:
Dissolve 300 g NaOH (3.5) in water and make up to 1 litre.
- 3.18. Sodium hydroxide solution, c = 1 mol/l:
Dissolve 40 g NaOH (3.5) in water and make up to 1 litre.
- 3.19. Formic acid — phenol solution:
Mix 889 g formic acid (3.2) with 111 g water and add 4,73 g phenol (3.3).
- 3.20. Hydrolysis mixture, c = 6 mol HCl/l containing 1 g phenol/l:
Add 1 g phenol (3.3) to 492 ml HCl (3.7) and make up to 1 litre with water.
- 3.21. Extraction mixture, c = 0,1 mol HCl/l containing 2 % thiodiglycol: Take 8,2 ml HCl (3.7), dilute with approximately 900 ml water, add 20 ml thiodiglycol (3.9) and make up to 1 litre with water, (do not mix 3.7 and 3.9 directly).
- 3.22. 5-Sulfosalicylic acid, β = 6 %:
Dissolve 60 g 5-sulfosalicylic acid (3.6) in water and make up to 1 l with water.
- 3.23. Oxidation mixture (Performic acid — phenol):
Mix 0,5 ml hydrogen peroxide (3.1) with 4,5 ml formic acid-phenol solution (3.19) in a small beaker. Incubate at 20-30 °C for 1 hour in order to form performic acid, then cool on an ice-water bath (15 min.) before adding to the sample.
Caution: Avoid contact with skin and wear protective clothing.
- 3.24. Citrate buffer, c = 0,2 mol Na⁺/l, pH 2,2:
Dissolve 19,61 g sodium citrate (3.8), 5 ml thiodiglycol (3.9), 1 g phenol (3.3) and 16,5 ml HCl (3.7) in approximately 800 ml water. Adjust pH to 2,2. Make up to 1 litre with water.
- 3.25. Elution buffers, prepared according to conditions for the analyser used (4.9).
- 3.26. Ninhydrin reagent, prepared according to conditions for the analyser used (4.9).
- 3.27. Standard solutions of amino acids. These solutions shall be stored below 5 °C.

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3.27.1. Stock standard solution of amino acids (3.16.1).

c = 2,5 µmol/ml of each in hydrochloric acid.

May be obtained commercially.

3.27.2. Stock standard solution of cysteic acid and methionine sulphone, c = 1,25 µmol/ml.

Dissolve 0,2115 g cysteic acid (3.16.2) and 0,2265 g methionine sulphone (3.16.3) in citrate buffer (3.24) in a 1 litre graduated flask and make up to mark with citrate buffer. Store below 5 °C for not more than 12 months. This solution is not used if the stock standard solution (3.27.1) contains cysteic acid and methionine sulphone.

3.27.3. Stock standard solution of the internal standard e.g. norleucine, c = 20 µmol/ml.

Dissolve 0,656 g norleucine (3.13) in citrate buffer (3.24) in a graduated flask and make up to 250 ml with citrate buffer. Store below 5 °C for no more than 6 months.

3.27.4. Calibration solution of standard amino acids for use with hydrolysates, c = 5 nmol/50 µl of cysteic acid and methionine sulphone and c = 10 nmol/50 µl of the other amino acids. Dissolve 2,2 g sodium chloride (3.10) in 100 ml beaker with 30 ml citrate buffer (3.24). Add 4,0 ml stock standard solution of amino acids (3.27.1), 4,0 ml stock standard solution of cysteic acid and methionine sulphone (3.27.2) and 0,5 ml stock standard solution of internal standard (3.27.3) if used. Adjust pH to 2,2 with sodium hydroxide (3.18).

Transfer quantitatively to a 50 ml graduated flask and make up to the mark with citrate buffer (3.24) and mix.

Store below 5 °C for not more than 3 months.

See also observation 9.1.

3.27.5. Calibration solution of standard amino acids for use with hydrolysates prepared according to paragraph 5.3.3.1 and for use with extracts (5.2). The calibration solution is prepared according to 3.27.4 but omitting sodium chloride.

Store below 5 °C for not more than 3 months.

4. Apparatus

- 4.1. 100 or 250 ml round bottomed flask fitted with a reflux condenser.
- 4.2. 100 ml borosilicate glass bottle with screw cap with rubber/teflon liner (e.g. Duran, Schott) for use in the oven.
- 4.3. Oven with forced ventilation and a temperature regulator with an accuracy better than ± 2 °C.
- 4.4. pH-meter (three decimal places).
- 4.5. Membrane filter (0,22 µm).
- 4.6. Centrifuge.
- 4.7. Rotary vacuum evaporator.
- 4.8. Mechanical shaker or magnetic stirrer.

4.9. Amino acid analyser or HPLC equipment with ion exchange column, device for ninhydrin, post column derivatisation and photometric detector.

The column is filled with sulfonated polystyrene resins capable of separating the amino acids from each other and from other ninhydrin-positive materials. The flow in the buffer and ninhydrin lines is provided by pumps having a flow stability of $\pm 0,5\%$ in the period covering both the standard calibration run and the analysis of the sample.

With some amino acid analysers hydrolysis procedures can be used in which the hydrolysate has a sodium concentration of $c = 0,8$ mol/l and contains all the residual formic acid from the oxidation step. Others do not give a satisfactory separation of certain amino acids if the hydrolysate contains excess formic acid and/or high sodium ion concentrations. In this case the volume of acid is reduced by evaporation to approx. 5 ml after the hydrolysis and prior to pH adjustment. The evaporation shall be performed under vacuum at 40°C maximum.

5. Procedure

5.1. Preparation of the sample

The sample is ground to pass through a 0,5 mm sieve. Samples high in moisture must be either air-dried at a temperature not exceeding 50°C or freeze dried prior to grinding. Samples with a high fat content shall be extracted with light petroleum (3.12) prior to grinding.

5.2. Determination of free amino acids in feed and premixtures

Weigh to the nearest 0,2 mg an appropriate amount (1-5 g) of the prepared sample (5.1), into a conical flask and add 100,0 ml of extraction mixture (3.21). Shake the mixture for 60 min. using a mechanical shaker or a magnetic stirrer (4.8). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a 100 ml beaker.

Add 5,0 ml of sulfosalicylic acid solution (3.22), with stirring and continue to stir with the aid of magnetic stirrer for 5 min. Filter or centrifuge the supernatant in order to remove any precipitate. Place 10,0 ml of the resulting solution into a 100 ml beaker and adjust the pH to 2,2 using sodium hydroxide solution (3.18), transfer to a volumetric flask of appropriate volume using citrate buffer (3.24), and make up to the mark with the buffer solution (3.24).

If an internal standard is being used add 1,0 ml of internal standard (3.27.3) for each 100 ml final solution and make up to the mark with the buffer solution (3.24).

Proceed to the chromatography step according to paragraph 5.4.

If the extracts are not being examined the same day, they must be stored below 5°C .

5.3. Determination of total amino acids

5.3.1. Oxidation

Weigh to the nearest 0,2 mg from 0,1 to 1 g of the prepared sample (5.1) into:

- a 100 ml round-bottomed flask (4.1) for open hydrolysis (5.3.2.3) or,
- a 250 ml round-bottomed flask (4.1) if a low sodium concentration is required (5.3.3.1) or,
- a 100 ml bottle fitted with a screw cap (4.2), for closed hydrolysis (5.3.2.4).

The weighed sample portion must have a nitrogen content of about 10 mg and a moisture content not exceeding 100 mg.

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Place the flask/bottle in an ice-water bath and cool to 0 °C, add 5 ml of oxidation mixture (3.23) and mix using a glass spatula with a bent tip. Seal the flask/bottle containing the spatula with an air-tight film, place the ice-water bath containing the sealed container in a refrigerator at 0 °C and leave for 16 hours. After 16 hours remove from the refrigerator and decompose the excess oxidation reagent by the addition of 0,84 g of sodium disulphite (3.4).

Proceed to 5.3.2.1.

5.3.2. Hydrolysis

5.3.2.1. Hydrolysis of oxidised samples

To the oxidised sample prepared according to 5.3.1 add 25 ml of hydrolysis mixture (3.20) taking care to wash down any sample residue adhering to the sides of the vessel and the spatula.

Depending on the hydrolysis procedure being used, proceed according to 5.3.2.3 or 5.3.2.4.

5.3.2.2. Hydrolysis of unoxidised samples

Weigh into either a 100 ml or a 250 ml round-bottom flask (4.1) or a 100 ml bottle fitted with a screw cap (4.2), to the nearest 0,2 mg, from 0,1 to 1 g of the prepared sample (5.1). The weighed sample portion must have a nitrogen content of about 10 mg. Add carefully 25 ml of hydrolysis mixture (3.20) and mix with the sample. Proceed according to either 5.3.2.3 or 5.3.2.4.

5.3.2.3. Open hydrolysis

Add 3 glass beads to the mixture in the flask (prepared in accordance with 5.3.2.1 or 5.3.2.2) and boil with continuous bubbling under reflux for 23 hours. On completion of hydrolysis, wash the condenser down with 5 ml of citrate buffer (3.24). Disconnect the flask and cool it in an ice bath.

Proceed according to 5.3.3.

5.3.2.4. Closed Hydrolysis

Place the bottle containing the mixture prepared in accordance with 5.3.2.1 or 5.3.2.2 in an oven (4.3) at 110 °C. During the first hour in order to prevent a build up of pressure (due to the evolution of gaseous substances) and to avoid explosion, place the screw cap over the top of the vessel. Do not close the vessel with the cap. After one hour close the vessel with the cap and leave in the oven (4.3) for 23 hours. On completion of hydrolysis, remove the bottle from the oven, carefully open the cap of the bottle and place the bottle in an ice-water bath. Leave to cool.

Depending on the procedure for pH adjustment (5.3.3), quantitatively transfer the contents of the bottle to a 250 ml beaker or a 250 ml round-bottom flask, using citrate buffer (3.24).

Proceed according to 5.3.3.

5.3.3. Adjustment of pH

Depending on the sodium tolerance of the amino acid analyser (4.9) proceed according to 5.3.3.1 or 5.3.3.2 for the pH adjustment.

5.3.3.1. For Chromatographic Systems (4.9) requiring a low sodium concentration

It is advisable to use an internal stock standard solution (3.27.3) when amino acid analysers requiring a low sodium concentration are employed (when the acid volume has to be reduced).

In this case add 2,0 ml of the internal stock standard solution (3.27.3) to the hydrolysate before the evaporation.

Add 2 drops of 1-octanol (3.15) to the hydrolysate obtained in accordance with paragraph 5.3.2.3 or 5.3.2.4.

Using a rotary evaporator (4.7) reduce the volume to 5-10 ml under vacuum at 40 °C. If the volume is accidentally reduced to less than 5 ml the hydrolysate must be discarded and the analysis recommenced.

Adjust the pH to 2,2 with sodium hydroxide solution (3.18) and proceed to paragraph 5.3.4.

5.3.3.2. *For all other Amino Acid Analysers (4.9)*

Take the hydrolysates obtained in accordance with 5.3.2.3 or 5.3.2.4 and partly neutralise them by carefully adding with stirring, 17 ml of sodium hydroxide solution (3.17), ensuring that the temperature is kept below 40 °C.

Adjust the pH to 2,2 at room temperature using sodium hydroxide solution (3.17) and finally sodium hydroxide solution (3.18). Proceed to 5.3.4.

5.3.4. *Sample solution for chromatography*

Quantitatively transfer the pH adjusted hydrolysate (5.3.3.1 or 5.3.3.2) with citrate buffer (3.24) to a 200 ml graduated flask, and make up to the mark with buffer (3.24).

If an internal standard has not already been used, add 2,0 ml of internal standard (3.27.3) and make up to the mark with citrate buffer (3.24). Mix thoroughly.

Proceed to the chromatography step (5.4).

If the sample solutions are not being examined the same day they must be stored below 5 °C.

5.4. *Chromatography*

Before chromatography bring the extract (5.2) or hydrolysate (5.3.4) to room temperature. Shake the mixture and filter a suitable amount through a 0,22 µm membrane filter (4.5). The resulting clear solution is subjected to ion exchange chromatography, using an amino acid analyser (4.9).

The injection may be performed manually or automatically. It is important that the same quantity of solution $\pm 0,5$ % is added to the column for the analysis of standards and samples except when an internal standard is used, and that the sodium:amino acid ratios in the standard and sample solutions are as similar as is practicable.

In general the frequency of calibration runs depends on the stability of the ninhydrin reagent and the analytical system. The standard or sample is diluted with citrate buffer (3.24) to give a peak area of the standard of 30 %-200 % of the sample amino acid peak area.

The chromatography of amino acids will vary slightly according to the type of analyser employed and resin used. The chosen system must be capable of separating the amino acids from each other and from the ninhydrin-positive materials. In the range of operation the chromatographic system must give a linear response to changes in the amounts of amino acids added to the column.

During the chromatography step the valley:peak height ratios mentioned below apply, when an equimolar solution (of the amino acids being determined) is analysed. This equimolar solution must contain at least 30 % of the maximum load of each amino acid which can be accurately measured with the amino acid analyser system (4.9).

For separation of threonine-serine the valley:peak height ratio of the lower of the two overlapping amino acids on the chromatogram must not exceed 2:10. (if only cyst(e)ine,

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methionine, threonine and lysine are determined, insufficient separation from adjoining peaks will adversely influence the determination). For all other amino acids the separation must be better than 1:10.

The system must ensure that lysine is separated from 'lysine artifacts' and ornithine.

6. Calculation of results

The area of the sample and standard peaks is measured for each individual amino acid and the amount (X), in g amino acid per kg sample, is calculated as follows:

$$X = \frac{A_{sc} \times M \times V}{B_{sc} \times m \times 1000}$$

If an internal standard is used multiply by:

$$\frac{D}{C}$$

A	= peak area, hydrolysate or extract
B	= peak area, calibration standard solution
C	= peak area, internal standard in hydrolysate or extract
D	= peak area, internal standard, calibration standard solution
M	= molar weight of the amino acid being determined
c	= concentration of standard in $\mu\text{mol/ml}$
m	= sample weight (g) (corrected to original weight if dried or defatted)
V	= ml total hydrolysate (5.3.4) or ml calculated total dilution volume of extract (6.1)

Cystine and cysteine are both determined as cysteic acid in hydrolysates of oxidised sample, but calculated as cystine ($\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2$, M 240,3 g/mol) by using M 120,15 g/mol (= 0,5 x 240,3 g/mol).

Methionine is determined as methionine sulphone in hydrolysates of oxidised sample, but calculated as methionine by using M of methionine: 149,21 g/mol.

Added free methionine is determined after extraction as methionine, for the calculation the same M is used.

6.1. The total dilution volume of extracts (F) for determination of free amino acids (5.2) is calculated as follows:

$$F = \frac{100 \text{ ml} \times (10 \text{ ml} + 5 \text{ ml})}{10 \text{ ml}} \times \frac{V}{10}$$

V = Volume of final extract

7. Evaluation of the method

The method has been tested in an intercomparison made at international level in 1990 using four different feed (mixed pig feed, broiler compound, protein concentrate, premixture). The results, after elimination of outliers, of mean and standard deviation are given in the tables in this point:

MEANS IN G/KG

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	6,94 n = 15	3,01 n = 17	3,27 n = 17	9,55 n = 13
n	= Number of participating laboratories.			

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Broiler Compound	9,31 n = 16	3,92 n = 18	5,08 n = 18	13,93 n = 16
Protein Concentrate	22,32 n = 16	5,06 n = 17	12,01 n = 17	47,74 n = 15
Premixture	58,42 N = 16	—	90,21 n = 16	98,03 n = 16

n = Number of participating laboratories.

7.1. Repeatability

The repeatability expressed as ‘within laboratory standard deviation’ of the abovementioned intercomparison is given in the tables below:

WITHIN LABORATORY STANDARD DEVIATION (S_R) IN G/KG

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	0,13 n = 15	0,1 n = 17	0,11 n = 17	0,26 n = 13
Broiler Compound	0,2 n = 16	0,11 n = 18	0,16 n = 18	0,28 n = 16
Protein Concentrate	0,48 n = 16	0,13 n = 17	0,27 n = 17	0,99 n = 15
Premixture	1,3 N = 16	—	2,19 n = 16	2,06 n = 16

n = Number of participating laboratories.

COEFFICIENT OF VARIATION (%) FOR WITHIN LABORATORY STANDARD DEVIATION (S_R)

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	1,9 n = 15	3,3 n = 17	3,4 n = 17	2,8 n = 13
Broiler Compound	2,1 n = 16	2,8 n = 18	3,1 n = 18	2,1 n = 16
Protein Concentrate	2,7 n = 16	2,6 n = 17	2,2 n = 17	2,4 n = 15
Premixture	2,2 n = 16	—	2,4 n = 16	2,1 n = 16

n = Number of participating laboratories.

7.2 Reproducibility

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The results for between laboratory standard deviation by the abovementioned intercomparison are given in the table below:

BETWEEN LABORATORY STANDARD DEVIATION (S_R) IN G/KG

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	0,28 n = 15	0,3 n = 17	0,23 n = 17	0,3 n = 13
Broiler Compound	0,48 n = 16	0,34 n = 18	0,55 n = 18	0,75 n = 16
Protein Concentrate	0,85 n = 16	0,62 n = 17	1,57 n = 17	1,24 n = 15
Premixture	2,49 n = 16	—	6,2 n = 16	6,62 n = 16

n = Number of participating laboratories.

COEFFICIENT OF VARIATION (%) FOR BETWEEN LABORATORY STANDARD DEVIATION (S_R)

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	4,1 n = 15	9,9 n = 17	7,0 n = 17	3,2 n = 13
Broiler Compound	5,2 n = 16	8,8 n = 18	10,9 n = 18	5,4 n = 16
Protein Concentrate	3,8 n = 16	12,3 n = 17	13,0 n = 17	3,0 n = 15
Premixture	4,3 n = 16	—	6,9 n = 16	6,7 n = 16

n = Number of participating laboratories.

8. Use of reference materials

The correct application of the method shall be verified by making replicate measurements of certified reference materials when available. Calibration with certified amino acid calibration solution is recommended.

9. Observations

9.1. Because of differences between amino acid analysers the final concentrations of the calibration solutions of standard amino acids (see 3.27.4 and 3.27.5) and of the hydrolysate (see 5.3.4) shall be taken as a guideline.

The range of linear response of the apparatus has to be checked for all amino acids.

The standard solution is diluted with citrate buffer to give peak areas in the middle of the range.

- 9.2. Where high performance liquid chromatographic equipment is used to analyse the hydrolysates, the experimental conditions must be optimised in accordance with the manufacturer's recommendations.
- 9.3. By applying the method to feed containing more than 1 % chloride (concentrate, mineral feeds, supplementary feeds) underestimation of methionine could occur and special treatment has to be done.

G. DETERMINATION OF TRYPTOPHAN

1. Purpose and scope

The method makes the determination possible of the total and free tryptophan in feed. It does not distinguish between D- and L- forms.

2. Principle

For the determination of the total tryptophan, the sample is hydrolysed under alkaline conditions with saturated barium hydroxide solution and heated to 110 °C for 20 hours. After hydrolysis internal standard is added.

For the determination of free tryptophan, the sample is extracted under mild acidic conditions in the presence of internal standard.

The tryptophan and the internal standard in the hydrolysate or in the extract are determined by HPLC with fluorescence detection.

3. Reagents

- 3.1. Double distilled water or water of equivalent quality must be used (conductivity < 10 µS/cm).
- 3.2. Standard substance: tryptophan (purity/content ≥ 99 %) dried under vacuum over phosphorous pentoxide.
- 3.3. Internal standard substance: α-methyl-tryptophan (purity/content ≥ 99 %), dried under vacuum over phosphorous pentoxide.
- 3.4. Barium hydroxide octa-hydrate (care shall be taken not to expose the Ba(OH)₂ · 8 H₂O excessively to air in order to avoid formation of BaCO₃, which could disturb the determination) (see observation 9.3).
- 3.5. Sodium hydroxide.
- 3.6. Ortho-phosphoric acid, w (w/w) = 85 %.
- 3.7. Hydrochloric acid, ρ₂₀ 1,19 g/ml.
- 3.8. Methanol, equivalent to HPLC grade.
- 3.9. Light petroleum, boiling range 40-60 °C.
- 3.10. Sodium hydroxide solution, c = 1 mol/l:

Dissolve 40,0 g NaOH (3.5) in water and make up to 1 litre with water (3.1).

- 3.11. Hydrochloric acid, c = 6 mol/l:

Take 492 ml HCl (3.7) and make up to 1 litre with water.

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3.12. Hydrochloric acid, $c = 1 \text{ mol/l}$:

Take 82 ml HCl (3.7) and make up to 1 litre with water.

3.13. Hydrochloric acid, $c = 0,1 \text{ mol/l}$:

Take 8,2 ml HCl (3.7) and make up to 1 litre with water.

3.14. Ortho-phosphoric acid, $c = 0,5 \text{ mol/l}$:

Take 34 ml ortho-phosphoric acid (3.6) and make up to 1 litre with water (3.1).

3.15. Concentrated solution of tryptophan (3.2), $c = 2,5 \text{ } \mu\text{mol/ml}$:

In a 500 ml volumetric flask dissolve 0,2553 g tryptophan (3.2) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at $-18 \text{ }^\circ\text{C}$ for a maximum of 4 weeks.

3.16. Concentrated internal standard solution, $c = 2,5 \text{ } \mu\text{mol/ml}$:

In a 500 ml volumetric flask dissolve 0,2728 g α -methyl-tryptophan (3.3) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at $-18 \text{ }^\circ\text{C}$ for a maximum of 4 weeks.

3.17. Calibration standard solution of tryptophan and internal standard:

Take 2,0 ml concentrated solution of tryptophan (3.15), and 2,0 ml of concentrated internal standard (α -methyl-tryptophan) solution (3.16). Dilute with water (3.1) and methanol (3.8) to approximately the same volume and to approximately the same concentration of methanol (10 %-30 %) as the finished hydrolysate.

This solution must be prepared freshly before use.

Protect from direct sunlight during preparation.

3.18. Acetic acid

3.19. 1,1,1-trichloro-2-methyl-2-propanol.

3.20. Ethanolamine w (w/w) > 98 %.

3.21. Solution of 1 g 1,1,1-trichloro-2-methyl-2-propanol (3.19) in 100 ml methanol (3.8).

3.22. Mobile phase for HPLC: 3,0 g acetic acid (3.18) + 900 ml water (3.1) + 50,0 ml solution (3.21) of 1,1,1-trichloro-2-methyl-2-propanol (3.19) in methanol (3.8) (1g/100ml). Adjust pH to 5,0 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1).

4. Apparatus

4.1. HPLC equipment with a spectrofluorometric detector.

4.2. Liquid chromatographic column, 125 mm x 4 mm, C_{18} , 3 μm packing, or equivalent.

4.3. pH-meter.

4.4. Polypropylene flask, capacity 125 ml, with wide neck and screw cap.

4.5. Membrane filter, 0,45 μm .

4.6. Autoclave, 110 (± 2) $^\circ\text{C}$, 1,4 ($\pm 0,1$) bar.

4.7. Mechanical shaker or magnetic stirrer.

4.8. Vortex mixer.

5. Procedure

5.1. Preparation of samples

The sample is ground to pass through a 0,5 mm sieve. Samples high in moisture must be either air-dried at a temperature not exceeding 50 °C or freeze dried prior to grinding. Samples with high fat content shall be extracted with light petroleum (3.9) prior to grinding.

5.2. Determination of free tryptophan (extract)

Weigh to the nearest 1 mg an appropriate amount (1-5 g) of the prepared sample (5.1), into a conical flask. Add 100,0 ml hydrochloric acid, (3.13) and 5,0 ml concentrated internal standard solution (3.16). Shake or mix for 60 min. using a mechanical shaker or a magnetic stirrer (4.7). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a beaker. Add 5 ml ortho-phosphoric acid (3.14). Adjust the pH to 3 using sodium hydroxide (3.10). Add sufficient methanol (3.8) to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water to a volume necessary for the chromatography (approx. the same volume as the calibration standard solution (3.17)).

Filter a few ml of the solution through a 0,45 µm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to paragraph 5.4.

Protect standard solution and extracts against direct sunlight. If it is not possible to analyse the extracts the same day, the extracts may be stored at 5 °C for a maximum of 3 days.

5.3. Determination of total tryptophan (hydrolysate)

Weigh to the nearest 0,2 mg from 0,1 to 1 g of the prepared sample (5.1) into the polypropylene flask (4.4). The weighed sample portion shall have a nitrogen content of about 10 mg. Add 8,4 g barium hydroxide octa-hydrate (3.4) and 10 ml water. Mix on a vortex mixer (4.8) or magnetic stirrer (4.7). Leave the teflon coated magnet in the mixture. Wash down the walls of the vessel with 4 ml water. Put on the screw cap and close the flask loosely. Transfer to an autoclave (4.6) with boiling water and steam for 30-60 minutes. Close the autoclave and autoclave at 110 (± 2) °C for 20 hours.

Before opening the autoclave reduce the temperature to just under 100 °C. In order to avoid crystallisation of Ba(OH)₂ · 8 H₂O, add to the warm mixture 30 ml water which is at room temperature. Shake or stir gently. Add 2,0 ml concentrated internal standard (α-methyl-tryptophan) solution (3.16). Cool the vessels on water/ice bath for 15 minutes.

Then, add 5 ml ortho-phosphoric acid (3.14). Keep the vessel in the cooling bath and neutralise with HCl (3.11) whilst stirring and adjust the pH to 3,0 using HCl (3.12). Add sufficient methanol to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water to the defined volume necessary for the chromatography (for example 100 ml). The addition of methanol shall not cause precipitation.

Filter a few ml of the solution through a 0,45 µm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to paragraph 5.4.

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Protect standard solution and hydrolysates against direct sunlight. If it is not possible to analyse the hydrolysates the same day, they may be stored at 5 °C for a maximum of 3 days.

5.4. HPLC determination

The following conditions for isocratic elution are offered for guidance; other conditions may be used, provided they yield equivalent results (see also observations 9.1 and 9.2):

Liquid chromatographic column (4.2):	125 mm x 4 mm, C ₁₈ , 3 µm packing or equivalent
Column temperature:	Room temperature
Mobile phase (3.22):	3,0 g acetic acid (3.18) + 900 ml water (3.1) +50,0 ml solution (3.21) of 1,1,1-trichloro-2-methyl-2-propanol (3.19) in methanol (3.8) (1 g/100 ml). Adjust pH to 5,0 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1)
Flow rate:	1 ml/min.
Total run time:	approx. 34 min.
Detection wavelength:	excitation: 280 nm, emission: 356 nm.
Injection volume	20 µl

6. Calculation of results

The amount of tryptophane (X), in g per 100g sample, is calculated as follows:

$$X = \frac{A \times B \times V_1 \times c \times V_2 \times M}{C \times D \times V_3 \times 10\,000 \times m}$$

- A = peak area of internal standard, calibration standard solution (3.17)
 B = peak area of tryptophan, extract (5.2) or hydrolysate (5.3)
 V₁ = volume in ml (2 ml) of concentrated tryptophan solution (3.15) added to the calibration solution (3.17)
 c = concentration in µmol/ml (= 2,5) of concentrated tryptophan solution (3.15) added to calibration solution (3.17)
 V₂ = volume in ml of concentrated internal standard solution (3.16) added at the extraction (5.2) (= 5,0 ml) or to the hydrolysate (5.3) (= 2,0 ml)
 C = peak area of internal standard, extract (5.2) or hydrolysate (5.3)
 D = peak area of tryptophan, calibration standard solution (3.17)
 V₃ = volume in ml (= 2,0 ml) of concentrated internal standard solution (3.16) added to calibration standard solution (3.17)
 m = sample weight in g (corrected to original weight if dried and/or defatted)
 M = molar weight of tryptophan (= 204,23 g/mol)

7. Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10 % relative to the highest result.

8. Results of a collaborative study

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

An EC collaborative study (4th intercomparison) was arranged in which three samples were analysed by up to 12 laboratories to certify the method for hydrolysis. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 1 Pig feed	Sample 2 Pig feed supplemented with L-tryptophan	Sample 3 Feed concentrate for pigs
L	12	12	12
n	50	55	50
Mean [g/kg]	2,42	3,4	4,22
s_r [g/kg]	0,05	0,05	0,08
r [g/kg]	0,14	0,14	0,22
CV_r [%]	1,9	1,6	1,9
S_R [g/kg]	0,15	0,2	0,09
R [g/kg]	0,42	0,56	0,25
CV_R [%]	6,3	6,0	2,2

L = number of laboratories submitting results
n = number of single results retained eliminating outliers (identified by Cochran, Dixon outlier test)
 s_r = standard deviation of repeatability
 S_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
 CV_r = coefficient of variation of repeatability, %
 CV_R = coefficient of variation of reproducibility, %

Another EC collaborative study (3rd intercomparison) was arranged in which two samples were analysed by up to 13 laboratories to certify the method for extraction of free tryptophan. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 4 Wheat and soya mixture	Sample 5 Wheat and soya mixture (= sample 4) with added tryptophan (0,457g/kg)
L	12	12
n	55	60
Mean [g/kg]	0,391	0,931
s_r [g/kg]	0,005	0,012
r [g/kg]	0,014	0,034
CV_r [%]	1,34	1,34
S_R [g/kg]	0,018	0,048

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R [g/kg]	0,05	0,134
CV _R [%]	4,71	5,11

L	= number of laboratories submitting results
n	= number of single results retained after eliminating outliers (identified by Cochran, Dixon outlier test)
s _r	= standard deviation of repeatability
S _R	= standard deviation of reproducibility
r	= repeatability
R	= reproducibility
CV _r	= coefficient of variation of repeatability, %
CV _R	= coefficient of variation of reproducibility, %

Another EC intercomparison study was arranged in which four samples were analysed by up to 7 laboratories with the aim of a tryptophan certification for hydrolysis. The results are given below. Replicate (5) analyses were performed on each sample.

	Sample 1 Mixed pig feed(CRM 117)	Sample 2 Low fat fish meal(CRM 118)	Sample 3 Soybean meal(CRM 119)	Sample 4 Skimmed milk powder(CRM 120)
L	7	7	7	7
n	25	30	30	30
Mean [g/kg]	2,064	8,801	6,882	5,236
s _r [g/kg]	0,021	0,101	0,089	0,04
r [g/kg]	0,059	0,283	0,249	0,112
CV _r [%]	1,04	1,15	1,3	0,76
S _R [g/kg]	0,031	0,413	0,283	0,221
R [g/kg]	0,087	1,156	0,792	0,619
CV _R [%]	1,48	4,69	4,11	4,22

L	= number of laboratories submitting results
n	= number of single results retained after eliminating outliers (identified by Cochran, Dixon outlier test)
s _r	= standard deviation of repeatability
S _R	= standard deviation of reproducibility
r	= repeatability
R	= reproducibility
CV _r	= coefficient of variation of repeatability, %
CV _R	= coefficient of variation of reproducibility, %

9. Observations

- 9.1. Following special chromatographic conditions may give better separation between tryptophan and α -methyl-tryptophan.

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Isocratic elution followed by gradient column cleaning:

Liquid chromatographic column:	125 mm x 4 mm, C ₁₈ , 5 µm packing or equivalent		
Column temperature:	32 °C		
Mobile phase:	A: 0,01 mol/l KH ₂ PO ₄ /méthanol, 95+5 (V+V). B: methanol		
Gradient program:	0 min.	100 % A	0 % B
	15 min.	100 % A	0 % B
	17 min.	60 % A	40 % B
	19 min.	60 % A	40 % B
	21 min.	100 % A	0 % B
	33 min.	100 % A	0 % B
Flow rate:	1,2 ml/min.		
Total run time:	approx. 33 min.		

9.2. The chromatography will vary according to the type of HPLC and column packing material used. The chosen system must be capable of giving baseline separation between the tryptophan and the internal standard. Moreover it is important that degradation products are well separated from the tryptophan and the internal standard. Hydrolysates without internal standard shall be run in order to check the base line under the internal standard for impurities. It is important that the run time is sufficiently long for the elution of all the degradation products, otherwise late eluting peaks may interfere with subsequent chromatographic runs.

In the range of operation, the chromatographic system shall give linear response. The linear response shall be measured with a constant (the normal) concentration of the internal standard and varying concentrations of tryptophan. It is of importance that the size of both the tryptophan and internal standard peaks are within the linear range of the HPLC/fluorescence system. If either the tryptophan and/or the internal standard peak(s) is (are) too small or too high the analysis shall be repeated with another sample size and/or a changed final volume.

9.3. *Barium hydroxide*

With age barium hydroxide becomes more difficult to dissolve. This results in an unclear solution for the HPLC determination, which may produce low results for tryptophan.

H. DETERMINATION OF CRUDE OILS AND FATS

1. Purpose and scope

This method is for the determination of crude oils and fats in feed. It does not cover the analysis of oil seeds and oleaginous fruit.

The use of the two procedures described below depends on the nature and composition of the feed and the reason for carrying out the analysis.

1.1. Procedure A — Directly extractable crude oils and fats

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

This method is applicable to feed materials of plant origin, except those included within the scope of Procedure B.

1.2. Procedure B — Total crude oils and fats

This method is applicable to feed materials of animal origin and to all compound feeds. It is to be used for all materials from which the oils and fats cannot be completely extracted without prior hydrolysis (e.g. glutens, yeast, potato proteins and products subjected to processes such as extrusion, flaking and heating).

1.3. Interpretation of results

In all cases where a higher result is obtained by using Procedure B than by Procedure A, the result obtained by Procedure B shall be accepted as the true value.

2. Principle

2.1. Procedure A

The sample is extracted with light petroleum. The solvent is distilled off and the residue dried and weighed.

2.2. Procedure B

The sample is treated under heating with hydrochloric acid. The mixture is cooled and filtered. The residue is washed and dried and submitted to the determination according to Procedure A.

3. Reagents

3.1. Light petroleum, boiling range: 40 to 60 °C. The bromine value must be less than 1 and the residue on evaporation less than 2 mg/100 ml.

3.2. Sodium sulfate, anhydrous.

3.3. Hydrochloric acid, $c = 3 \text{ mol/l}$

3.4. Filtration aid, e.g. Kieselguhr, Hyflo-supercel.

4. Apparatus

4.1. Extraction apparatus. If fitted with a siphon (Soxhlet apparatus), the reflux rate shall be such as to produce about 10 cycles per hour; if of the non-siphoning type, the reflux rate shall be about 10 ml per minute.

4.2. Extraction thimbles, free of matter soluble in light petroleum and having a porosity consistent with the requirements of point 4.1.

4.3. Drying oven, either a vacuum oven set at $75 \pm 3 \text{ °C}$ or an air-oven set at $100 \pm 3 \text{ °C}$.

5. Procedure

5.1. Procedure A (see point 8.1)

Weigh 5 g of the sample to the nearest 1 mg, transfer it to an extraction thimble (4.2) and cover with a fat-free wad of cotton wool.

Place the thimble in an extractor (4.1) and extract for six hours with light petroleum (3.1). Collect the light petroleum extract in a dry, weighed flask containing fragments of pumice stone⁽²⁾.

Distil off the solvent. Dry the residue maintaining the flask for one and a half hours in the drying oven (4.3). Leave to cool in a desiccator and weigh. Dry again for 30 minutes to ensure that the weight of the oils and fats remains constant (loss in weight between two successive weighings must be less than or equal to 1 mg).

5.2. Procedure B

Weigh 2,5 g of the sample to the nearest 1 mg (see point 8.2), place in a 400 ml beaker or a 300 ml conical flask and add 100 ml of hydrochloric acid (3.3) and fragments of pumice stone. Cover the beaker with a watch glass or fit the conical flask with a reflux condenser. Bring the mixture to a gentle boil over a low flame or a hot-plate and keep it there for one hour. Do not allow the product to stick to the sides of the container.

Cool and add a quantity of filtration aid (3.4) sufficient to prevent any loss of oil and fat during filtration. Filter through a moistened, fat-free, double filter paper. Wash the residue in cold water until a neutral filtrate is obtained. Check that the filtrate does not contain any oil or fats. Their presence indicates that the sample must be extracted with light petroleum, using Procedure A, before hydrolysis.

Place the double filter paper containing the residue on a watch glass and dry for one and a half hours in the air oven (4.3) at 100 ± 3 °C.

Place the double filter paper containing the dry residue in an extraction thimble (4.2) and cover with a fat-free wad of cotton wool. Place the thimble in an extractor (4.1) and proceed as indicated in the second and third paragraphs of point 5.1.

6. Expression of result

Express the weight of the residue as a percentage of the sample.

7. Repeatability

The difference between the results of two parallel determinations carried out on the same sample by the same analyst shall not exceed:

- 0,2 %, in absolute value, for contents of crude oils and fats lower than 5 %,
- 4,0 % relative to the highest result for contents of 5 % to 10 %,
- 0,4 %, in absolute value, for contents above 10 %.

8. Observations

8.1. For products with a high content of oils and fats, which are difficult to crush or unsuitable for drawing a homogeneous reduced test sample, proceed as follows.

Weigh 20 g of the sample to the nearest 1 mg and mix with 10 g or more of anhydrous sodium sulfate (3.2). Extract with light petroleum (3.1) as indicated in point 5.1. Make up the extract obtained to 500 ml with light petroleum (3.1) and mix. Take 50 ml of the solution and place in a small, dry, weighed flask containing fragments of pumice stone. Distil off the solvent, dry and proceed as indicated in the last paragraph of point 5.1.

Eliminate the solvent from the extraction residue left in the thimble, crush the residue to a fineness of 1 mm, return it to the extraction thimble (do not add sodium sulfate) and proceed as indicated in the second and third paragraphs of point 5.1.

Calculate the content of oils and fats as a percentage of the sample by using the following formula:

$$(10m_1 + m_2) \times 5$$

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where:

- m_1 = weight in grams of the residue after the first extraction (aliquot part of the extract),
 m_2 = weight in grams of the residue after the second extraction.

- 8.2. For products low in oils and fats the test sample may be increased to 5 g.
- 8.3. Pet foods containing a high content of water may need to be mixed with anhydrous sodium sulfate prior to hydrolysis and extraction as per Procedure B.
- 8.4. In paragraph 5.2 it may be more effective to use hot water in place of cold water to wash the residue after filtration.
- 8.5. The drying time of 1,5 h may need to be extended for some feed. Excessive drying shall be avoided as this can lead to low results. A microwave oven can also be used.
- 8.6. Pre-extraction by Procedure A prior to hydrolysis and re-extraction by Procedure B is recommended if the crude oil/fat content is greater than 15 %. To some extent this depends on the nature of the feed and the nature of the oil/fat in the feed.

I. DETERMINATION OF CRUDE FIBRE

1. Purpose and scope

This method makes it possible to determine fat-free organic substances in feed which are insoluble in acid and alkaline media and are conventionally described as crude fibre.

2. Principle

The sample, defatted where necessary, is treated successively with boiling solutions of sulphuric acid and potassium hydroxide of specified concentrations. The residue is separated by filtration on a sintered-glass filter washed, dried, weighed and ashed within a range of 475 to 500 °C. The loss of weight resulting from ashing corresponds to the crude fibre present in the test sample.

3. Reagents

- 3.1. Sulphuric acid, $c = 0,13$ mol/l.
- 3.2. Anti-foaming agent (e.g. n-octanol).
- 3.3. Filter aid (Celite 545 or equivalent), heated at 500 °C for four hours (8.6).
- 3.4. Acetone.
- 3.5. Light petroleum boiling-range 40 to 60 °C.
- 3.6. Hydrochloric acid, $c = 0,5$ mol/l.
- 3.7. Potassium hydroxide solution, $c = 0,23$ mol/l.

4. Apparatus

- 4.1. Heating unit for digestion with sulphuric acid and potassium hydroxide solution, equipped with a support for the filter crucible (4.2) and provided with an outlet tube with a tap to the liquid outlet and vacuum, possibly with compressed air. Before use each day preheat the unit with boiling water for five minutes.

- 4.2. Glass filter crucible with fused sintered glass filter plate pore size 40-90 µm. Before first use, heat to 500 °C for a few minutes and cool (8.6).
- 4.3. Cylinder of at least 270 ml with a reflux condenser, suitable for boiling.
- 4.4. Drying oven with thermostat.
- 4.5. Muffle furnace with thermostat.
- 4.6. Extraction unit consisting of a support plate for the filter crucible (4.2) and with a discharge pipe with a tap to the vacuum and liquid outlet.
- 4.7. Connecting rings to assemble the heating unit (4.1), crucible (4.2) and cylinder (4.3) and to connect the cold extraction unit (4.6) and crucible.

5. Procedure

Weigh out 1 g of the prepared sample to the nearest 1 mg and place it in the crucible (4.2), (see observations 8.1, 8.2 and 8.3) and add 1 g of filter aid (3.3).

Assemble the heating unit (4.1) and the filter crucible (4.2), then attach the cylinder (4.3) to the crucible. Pour 150 ml of boiling sulphuric acid (3.1) into the assembled cylinder and crucible and if necessary add a few drops of anti-foaming agent (3.2).

Bring the liquid to the boil within 5 ± 2 minutes and boil vigorously for exactly 30 minutes.

Open the tap to the discharge pipe (4.1) and, under vacuum, filter the sulphuric acid through the filter crucible and wash the residue with three consecutive 30 ml portions of boiling water, ensuring that the residue is filtered dry after each washing.

Close the outlet tap and pour 150 ml boiling potassium hydroxide solution (3.7) to the assembled cylinder and crucible and add a few drops of anti-foaming agent (3.2). Bring the liquid to boiling point within 5 ± 2 minutes and boil vigorously for exactly 30 minutes. Filter and repeat the washing procedure used for the sulphuric acid step.

After the final washing and drying, disconnect the crucible and its contents and reconnect it to the cold extraction unit (4.6). Apply the vacuum and wash the residue in the crucible with three consecutive 25 ml portions of acetone (3.4) ensuring that the residue is filtered dry after each washing.

Dry the crucible to constant weight in the oven at 130 °C. After each drying cool in the desiccator and weigh rapidly. Place the crucible in a muffle furnace and ash to constant weight (loss in weight between two successive weightings must be less than or equal to 2 mg) at 475 °C to 500 °C for at least 30 minutes.

After each heating cool first in the furnace and then in the desiccator before weighing.

Carry out a blank test without the sample. Loss of weight resulting from ashing must not exceed 4 mg.

6. Calculation of results

The crude fibre content as a percentage of the sample is given by the expression:

$$X = \frac{(m_0 - m_1) \times 100}{m}$$

where:

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- m = weight of sample in g,
 m₀ = loss of weight after ashing during the determination, in g,
 m₁ = loss of weight after ashing during the blank test, in g.

7. Repeatability

The difference between two parallel determinations carried out on the same sample must not exceed:

- 0,6 % in absolute value for crude fibre contents lower than 10 %,
- 6 % relative to the higher result, for crude fibre contents equal to or greater than 10 %.

8. Observations

- 8.1. Feed containing more than 10 % crude fat must be defatted prior to analysis with light petroleum (3.5). Connect the filter crucible (4.2) and its contents to the cold extraction unit (4.6) and apply vacuum and wash the residue with three consecutive 30 ml portions of light petroleum, ensuring that the residue is dry. Connect the crucible and its contents to the heating unit (4.1) and continue as described under 5.
- 8.2. Feed containing fats which cannot be extracted directly with light petroleum (3.5) must be defatted as shown in 8.1 and defatted once more after boiling with acid. After boiling with acid and the subsequent washing connect the crucible and its contents to the cold extraction unit (4.6) and wash three times with 30 ml acetone followed by three further washings with 30 ml portions of light petroleum. Filter under vacuum until dry and continue the analysis as described under 5, beginning with potassium hydroxide treatment.
- 8.3. If the feed contains over 5 % of carbonates, expressed as calcium carbonate, connect the crucible (4.2) with the weighed sample to the heating unit (4.1). Wash the sample three times with 30 ml hydrochloric acid (3.6). After each addition let the sample stand for about one minute before filtering. Wash once with 30 ml water and then continue as described under 5.
- 8.4. If an apparatus in the form of a stand is used (several crucibles attached to the same heating unit) no two individual determinations on the same sample for analysis may be carried out in the same series.
- 8.5. If after boiling it is difficult to filter the acidic and basic solutions, use compressed air through the discharge pipe of the heating unit and then continue filtering.
- 8.6. The temperature for ashing shall not be higher than 500 °C in order to extend the lifetime of the glass filter crucibles. Care must be taken to avoid excessive thermal shock during heating and cooling cycles.

J. DETERMINATION OF SUGAR

1. Purpose and scope

This method makes it possible to determine the amount of reducing sugars and total sugars after inversion, expressed as glucose or where appropriate as sucrose, converting by the factor 0,95. It is applicable to compound feed. Special methods are provided for other feed. Where necessary, lactose shall be measured separately and taken into account when calculating the results.

2. Principle

The sugars are extracted in dilute ethanol; the solution is clarified with Carrez solutions I and II. After eliminating the ethanol, the quantities before and after inversion are determined by the Luff-Schoorl method.

3. Reagents

- 3.1. Ethanol solution 40 % (v/v) density: 0,948 g/ml at 20 °C, neutralised to phenolphthalein.
- 3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate $Zn(CH_3COO)_2 \cdot 2H_2O$ and 3 g of glacial acetic acid. Make up to 100 ml with water.
- 3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide $K_4Fe(CN)_6 \cdot 3H_2O$. Make up to 100 ml with water.
- 3.4. Methyl orange, solution 0,1 % (w/v).
- 3.5. Hydrochloric acid 4 mol/litre.
- 3.6. Hydrochloric acid 0,1 mol/litre.
- 3.7. Sodium hydroxide solution 0,1 mol/litre.
- 3.8. Luff-Schoorl reagent:

Stirring carefully, pour the citric acid solution (3.8.2) into the sodium carbonate solution (3.8.3). Add the copper sulphate solution (3.8.1) and make up to 1 litre with water. Leave to settle overnight and filter.

Check the concentration of the reagent thus obtained (Cu 0,05 mol/litre; Na_2CO_3 1 mol/litre), see (5.4) last paragraph. The solution's pH shall be approximately 9,4.

- 3.8.1. Copper sulphate solution: dissolve 25 g of copper sulphate, $CuSO_4 \cdot 5H_2O$, free from iron, in 100 ml of water.
- 3.8.2. Citric acid solution: dissolve 50 g of citric acid, $C_6H_8O_7 \cdot H_2O$ in 50 ml of water.
- 3.8.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate in approximately 300 ml of warm water. Leave to cool.
- 3.9. Sodium thiosulphate solution 0,1 mol/litre.
- 3.10. Starch solution: add a mixture of 5 g of soluble starch in 30 ml of water to 1 litre of boiling water. Boil for three minutes, leave to cool and if necessary add 10 mg of mercuric iodide as a preservative.
- 3.11. Sulphuric acid 3 mol/litre.
- 3.12. Potassium iodide, solution 30 % (w/v).
- 3.13. Granulated pumice stone boiled in hydrochloric acid, washed in water and dried.
- 3.14. 3-methylbutan-1-ol.

4. Apparatus

Mixer (tumbler): approximately 35 to 40 r.p.m.

5. Procedure

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5.1. Extraction of sample

Weigh 2,5 g of the sample to the nearest mg and place in a 250 ml volumetric flask. Add 200 ml of ethanol (3.1) and mix in the tumbler for one hour. Add 5 ml of Carrez solution I (3.2) and stir for approximately 30 seconds. Add 5 ml of Carrez solution II (3.3) and again stir for one minute. Make up to volume with ethanol (3.1), homogenise and filter. Remove 200 ml of the filtrate and evaporate to approximately half volume in order to eliminate most of the ethanol. Transfer the evaporation residue quantitatively to a 200 ml volumetric flask using warm water, cool, bring up to volume with water, homogenise and filter if necessary. This solution will be used to determine the amount of reducing sugars and, after inversion, of total sugars.

5.2. Determination of reducing sugars

Using a pipette, remove not more than 25 ml of the solution containing less than 60 mg of reducing sugars expressed as glucose. If necessary, make up to 25 ml with distilled water and determine the content of reducing sugars by the Luff-Schoorl method. The result is expressed as the percentage content of glucose in the sample.

5.3. Determination of total sugars after inversion

Using a pipette take 50 ml of the solution and transfer to a 100 ml volumetric flask. Add a few drops of methyl orange solution (3.4) then, carefully and stirring continuously, add hydrochloric acid (3.5) until the liquid turns a definite red. Add 15 ml of hydrochloric acid (3.6), immerse the flask in a fast boiling water bath and keep there for 30 minutes. Cool rapidly to approximately 20 °C and add 15 ml of sodium hydroxide solution (3.7). Make up to 100 ml with water and homogenise. Remove not more than 25 ml containing less than 60 mg of reducing sugars expressed as glucose. If necessary, make up to 25 ml with distilled water and determine the content of reducing sugars by the Luff-Schoorl method. The result is expressed as the percentage of glucose or, where appropriate, sucrose, by multiplying by the factor 0,95.

5.4. Titration by the Luff-Schoorl method

Using a pipette, take 25 ml of Luff-Schoorl reagent (3.8) and transfer to a 300 ml Erlenmeyer flask; add exactly 25 ml of the clarified sugar solution. Add 2 granules of pumice stone (3.13), heat, stirring by hand, over a free flame of medium height and bring the liquid to the boil in approximately two minutes. Place the Erlenmeyer immediately on an asbestos-coated wire gauze with a hole approximately 6 cm in diameter under which a flame has been lit. The flame shall be regulated in such a way that only the base of the Erlenmeyer is heated. Fit a reflux condenser to the Erlenmeyer flask. Boil for exactly 10 minutes. Cool immediately in cold water and after approximately five minutes titrate as follows:

Add 10 ml of potassium iodide solution (3.12) and immediately afterwards (carefully, because of the risk of abundant foaming), add 25 ml of sulphuric acid (3.11). Titrate with sodium thiosulphate solution (3.9) until a dull yellow colour appears, add the starch indicator (3.10) and complete titration.

Carry out the same titration on an accurately measured mixture of 25 ml of Luff-Schoorl reagent (3.8) and 25 ml of water, after adding 10 ml of potassium iodide solution (3.12) and 25 ml of sulphuric acid (3.11) without boiling.

6. Calculation of results

Using the table establish the amount of glucose in mg which corresponds to the difference between the values of the two titrations, expressed in mg of sodium thiosulphate 0,1 mol/litre. Express the result as a percentage of the sample.

7. Special procedures

- 7.1. In the case of feed which are rich in molasses and other feed which are not particularly homogeneous, weigh out 20 g and place with 500 ml of water in a 1 litre volumetric flask. Mix for one hour in the tumbler. Clarify using Carrez 1 (3.2) and II (3.3) reagents as described under 5.1, this time however using four times the quantities of each reagent. Bring up to volume with 80 % ethanol (v/v).

Homogenise and filter. Eliminate the ethanol as described under 5.1. If there is no dextrinised starch, bring up to volume with distilled water.

- 7.2. In the case of molasses and feed materials which are rich in sugar and almost starch-free (carobs, dried beetroot cassettes etc.), weigh out 5 g, place in a 250 ml volumetric flask, add 200 ml of distilled water and mix in the tumbler for one hour, or more if necessary. Clarify using Carrez I (3.2) and II (3.3) reagents as described under 5.1. Bring up to volume with cold water, homogenise and filter. In order to determine the amount of total sugars, continue as described under 5.3.

8. Observations

- 8.1. In order to prevent foaming it is advisable to add (irrespective of the volume) approximately 1 ml of 3-methylbutan-1-ol (3.14) before boiling with Luff-Schoorl reagent.
- 8.2. The difference between the content of total sugars after inversion, expressed as glucose, and the content of reducing sugars, expressed as glucose, multiplied by 0,95, gives the percentage content of sucrose.
- 8.3. In order to determine the content of reducing sugars, excluding lactose, two methods may be adopted:
- 8.3.1. For an approximate calculation, multiply by 0,675 the lactose content established by a different method of analysis and subtract the result obtained from the content of reducing sugars.
- 8.3.2. For an accurate calculation of reducing sugars, excluding lactose, the same sample must be used for the two final determinations. One of the analyses is carried out on part of the solution obtained under 5.1, the other on part of the solution obtained during the determination of lactose by the method laid down for that purpose (after fermenting the other types of sugar and clarifying).

In both cases the amount of sugar present is determined by the Luff-Schoorl method and calculated in mg of glucose. One of the values is subtracted from the other and the difference is expressed as a percentage of the sample.

Example:

The two volumes taken correspond, for each determination, to a sample of 250 mg.

In the first case 17 ml of sodium thiosulphate solution 0,1 mol/litre corresponding to 44,2 mg of glucose is consumed; in the second, 11 ml, corresponding to 27,6 mg of glucose.

The difference is 16,6 mg of glucose.

The content of reducing sugars (excluding lactose), calculated as glucose, is therefore:

$$\frac{4 \times 16,6}{10} = 6,64 \%$$

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

TABLE OF VALUES FOR 25 ML OF LUFF-SCHOORL REAGENT

ml of Na₂ S₂ O₃0,1 mol/litre, two minutes' heating, 10 minutes' boiling

Na ₂ S ₂ O ₃ 0,1 mol/ litre	Glucose, fructose invert sugarsC ₆ H ₁₂ O ₆		LactoseC ₁₂ H ₂₂ O ₁₁		MaltoseC ₁₂ H ₂₂ O ₁₁		Na ₂ S ₂ O ₃ 0,1 mol/ litre
	ml	mg	difference	mg	difference	mg	
1	2,4	2,4	3,6	3,7	3,9	3,9	1
2	4,8	2,4	7,3	3,7	7,8	3,9	2
3	7,2	2,5	11,0	3,7	11,7	3,9	3
4	9,7	2,5	14,7	3,7	15,6	4,0	4
5	12,2	2,5	18,4	3,7	19,6	3,9	5
6	14,7	2,5	22,1	3,7	23,5	4,0	6
7	17,2	2,6	25,8	3,7	27,5	4,0	7
8	19,8	2,6	29,5	3,7	31,5	4,0	8
9	22,4	2,6	33,2	3,8	35,5	4,0	9
10	25,0	2,6	37,0	3,8	39,5	4,0	10
11	27,6	2,7	40,8	3,8	43,5	4,0	11
12	30,3	2,7	44,6	3,8	47,5	4,1	12
13	33,0	2,7	48,4	3,8	51,6	4,1	13
14	35,7	2,8	52,2	3,8	55,7	4,1	14
15	38,5	2,8	56,0	3,9	59,8	4,1	15
16	41,3	2,9	59,9	3,9	63,9	4,1	16
17	44,2	2,9	63,8	3,9	68,0	4,2	17
18	47,1	2,9	67,7	4,0	72,2	4,3	18
19	50,0	3,0	71,7	4,0	76,5	4,4	19
20	53,0	3,0	75,7	4,1	80,9	4,5	20
21	56,0	3,1	79,8	4,1	85,4	4,6	21
22	59,1	3,1	83,9	4,1	90,0	4,6	22
23	62,2		88,0		94,6		23

K. DETERMINATION OF LACTOSE

1. Purpose and scope

This method makes it possible to determine the level of lactose in feed containing more than 0,5 % of lactose.

2. Principle

The sugars are dissolved in water. The solution is subjected to fermentation by the yeast *Saccharomyces cerevisiae* which leaves the lactose intact. After clarification and filtration the lactose content of the filtrate is determined by the Luff-Schoorl method.

3. Reagents

- 3.1. Suspension of *Saccharomyces cerevisiae*: suspend 25 g of fresh yeast in 100 ml of water. The suspension will keep for a maximum period of one week in a refrigerator.
- 3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate, Zn (CH₃ COO)₂ 2H₂O and 3 g of glacial acetic acid. Make up to 100 ml with water.

- 3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide $K_4Fe(CN)_6 \cdot 3H_2O$. Make up to 100 ml with water.
- 3.4. Luff-Schoorl reagent:
- Stirring carefully, pour the citric acid solution (3.4.2) into the sodium carbonate solution (3.4.3). Add the copper sulphate solution (3.4.1) and make up to 1 litre with water. Leave to settle overnight and filter. Check the concentration of the reagent thus obtained (Cu 0,05 mol/litre; Na_2CO_3 1 mol/litre). The solution's pH shall be approximately 9,4.
- 3.4.1. Copper sulphate solution: dissolve 25 g of copper sulphate $CuSO_4 \cdot 5H_2O$, free from iron, in 100 ml of water.
- 3.4.2. Citric acid solution: dissolve 50 g of citric acid $C_6H_8O_7 \cdot H_2O$ in 50 ml of water.
- 3.4.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate in approximately 300 ml of warm water. Leave to cool.
- 3.5. Granulated pumice stone boiled in hydrochloric acid, washed in water and dried.
- 3.6. Potassium iodide, solution 30 % (w/v).
- 3.7. Sulphuric acid 3 mol/litre.
- 3.8. Solution of sodium thiosulphate 0,1 mol/litre.
- 3.9. Starch solution: add a mixture of 5 g of soluble starch in 30 ml of water to 1 litre of boiling water. Boil for three minutes, leave to cool, and if necessary add 10 mg of mercuric iodide as a preservative.

4. Apparatus

Water bath with thermostat set at 38-40 °C.

5. Procedure

Weigh 1 g of the sample to the nearest mg and place this portion of the sample in a 100 ml volumetric flask. Add 25 to 30 ml of water. Place the flask in a boiling water bath for 30 minutes and then cool to approximately 35 °C. Add 5 ml of yeast suspension (3.1) and homogenise. Leave the flask to stand for two hours in a water bath, at a temperature of 38-40° C. Cool to approximately 20 °C.

Add 2,5 ml of Carrez solution I (3.2) and stir for 30 seconds, then add 2,5 ml of Carrez solution II (3.3) and again stir for 30 seconds. Make up to 100 ml with water, mix and filter. Using a pipette, remove an amount of filtrate which does not exceed 25 ml and which preferably contains from 40 to 80 mg of lactose and transfer it to a 300 ml Erlenmeyer flask. If necessary, make up to 25 ml with water.

Carry out a blank test in the same way with 5 ml of yeast suspension (3.1). Determine the lactose content according to Luff-Schoorl, as follows: add exactly 25 ml of Luff-Schoorl reagent (3.4) and two granules of pumice stone (3.5). Stir by hand-while heating over a free flame of medium height and bring the liquid to the boil in approximately two minutes. Place the Erlenmeyer immediately on an asbestos-coated wire gauze with a hole approximately 6 cm in diameter under which a flame has been lit. The flame shall be regulated in such a way that only the base of the Erlenmeyer is heated. Fit a reflux condenser to the Erlenmeyer flask. Boil for exactly 10 minutes. Cool immediately in cold water and after approximately five minutes titrate as follows:

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Add 10 ml of potassium iodide solution (3.6) and immediately afterwards (carefully, because of the risk of abundant foaming) add 25 ml of sulphuric acid (3.7). Titrate with sodium thiosulphate solution (3.8) until a dull yellow colour appears, add the starch indicator (3.9) and complete titration.

Carry out the same titration on an accurately measured mixture of 25 ml of Luff-Schoorl reagent (3.4) and 25 ml of water, after adding 10 ml of potassium iodide solution (3.6) and 25 ml of sulphuric acid (3.7) without boiling.

6. Calculation of results

Using the attached table, establish the amount of lactose in mg which corresponds to the difference between the results of the two titrations, expressed in ml of sodium thiosulphate 0,1 mol/litre.

Express the result of anhydrous lactose as a percentage of the sample.

7. Observation

For products containing more than 40 % of fermentable sugar, use more than 5 ml of yeast suspension (3.1).

TABLE OF VALUES FOR 25 ML OF LUFF-SCHOORL REAGENT

ml of Na₂ S₂ O₃0,1 mol/litre, two minutes' heating, 10 minutes' boiling

Na ₂ S ₂ O ₃ 0,1 mol/litre	Glucose, fructose invert sugars C ₆ H ₁₂ O ₆		Lactose C ₁₂ H ₂₂ O ₁₁		Maltose C ₁₂ H ₂₂ O ₁₁		Na ₂ S ₂ O ₃ 0,1 mol/litre
	ml	mg	difference	mg	difference	mg	
1	2,4	2,4	3,6	3,7	3,9	3,9	1
2	4,8	2,4	7,3	3,7	7,8	3,9	2
3	7,2	2,5	11,0	3,7	11,7	3,9	3
4	9,7	2,5	14,7	3,7	15,6	4,0	4
5	12,2	2,5	18,4	3,7	19,6	3,9	5
6	14,7	2,5	22,1	3,7	23,5	4,0	6
7	17,2	2,6	25,8	3,7	27,5	4,0	7
8	19,8	2,6	29,5	3,7	31,5	4,0	8
9	22,4	2,6	33,2	3,8	35,5	4,0	9
10	25,0	2,6	37,0	3,8	39,5	4,0	10
11	27,6	2,7	40,8	3,8	43,5	4,0	11
12	30,3	2,7	44,6	3,8	47,5	4,1	12
13	33,0	2,7	48,4	3,8	51,6	4,1	13
14	35,7	2,8	52,2	3,8	55,7	4,1	14
15	38,5	2,8	56,0	3,9	59,8	4,1	15
16	41,3	2,9	59,9	3,9	63,9	4,1	16
17	44,2	2,9	63,8	3,9	68,0	4,2	17
18	47,1	2,9	67,7	4,0	72,2	4,3	18
19	50,0	3,0	71,7	4,0	76,5	4,4	19
20	53,0	3,0	75,7	4,1	80,9	4,5	20
21	56,0	3,1	79,8	4,1	85,4	4,6	21
22	59,1	3,1	83,9	4,1	90,0	4,6	22
23	62,2		88,0		94,6		23

L. DETERMINATION OF STARCH POLARIMETRIC METHOD

1. Purpose and scope

This method makes it possible to determine the levels of starch and of high molecular weight starch degradation products in feed for the purpose of checking compliance with the declared energy value (provisions in Annex VII) and [F1Regulation (EC) No 767/2009].

Textual Amendments

F1 Words in Annex 3 Pt. L point 1 substituted (31.12.2022) by The Food and Feed (Miscellaneous Amendments) Regulations 2022 (S.I. 2022/1351), regs. 1(1), 16(5)

2. Principle

The method comprises two determinations. In the first, the sample is treated with dilute hydrochloric acid. After clarification and filtration the optical rotation of the solution is measured by polarimetry.

In the second, the sample is extracted with 40 % ethanol. After acidifying the filtrate with hydrochloric acid, clarifying and filtering, the optical rotation is measured as in the first determination.

The difference between the two measurements, multiplied by a known factor, gives the starch content of the sample.

3. Reagents

3.1. Hydrochloric acid, solution 25 % (w/w) density: 1,126 g/ml.

3.2. Hydrochloric acid. solution 1,13 % (w/v)

The concentration must be checked by titration using a sodium hydroxide solution 0,1 mol/litre in the presence of 0,1 % (w/v) methyl red in 94 % (v/v) ethanol. For the neutralisation of 10 ml, 30,94 ml of NaOH 0,1 mol/litre is needed.

3.3. Carrez solution I: dissolve 21,9 g of zinc acetate $Zn(CH_3COO)_2 \cdot 2H_2O$ and 3 g of glacial acetic acid in water. Make up to 100 ml with water.

3.4. Carrez solution II: dissolve 10,6 g of potassium ferrocyanide $K_4 Fe(CN)_6 \cdot 3H_2O$ in water. Make up to 100 ml with water.

3.5. Ethanol, solution 40 % (v/v), density: 0,948 g/ml at 20 °C.

4. Apparatus

4.1. 250 ml Erlenmeyer flask with standard ground-glass joint and with reflux condenser.

4.2. Polarimeter or saccharimeter.

5. Procedure

5.1. Preparation of the sample

Crush the sample until it is fine enough for all of it to pass through a 0,5 mm round-meshed sieve.

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5.2. Determination of the total optical rotation (P or S) (see observation 7.1)

Weigh 2,5 g of the crushed sample to the nearest mg and place in a 100 ml graduated flask. Add 25 ml of hydrochloric acid (3.2), shake to obtain even distribution of the test sample and add a further 25 ml of hydrochloric acid (3.2). Immerse the flask in a boiling water bath shaking vigorously and steadily for the first three minutes to prevent the formation of agglomerates. The quantity of water in the water bath must be sufficient for the bath to remain at boiling point when the flask is introduced into it. The flask must not be taken out of the bath whilst being shaken. After exactly 15 minutes, remove from the bath, add 30 ml of cold water and cool immediately to 20 °C.

Add 5 ml of Carrez solution I (3.3) and shake for approximately 30 seconds. Then add 5 ml of Carrez solution II (3.4) and shake again for approximately 30 seconds. Make up to volume with water, mix and filter. If the filtrate is not perfectly clear (which is rare), repeat the determination using a larger quantity of Carrez solutions I and II, for example 10 ml.

Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharimeter.

5.3. Determination of the optical rotation (P' or S') of substances soluble in 40 % ethanol

Weigh 5 g of the sample to the nearest mg, place in a 100 ml graduated flask and add about 80 ml of ethanol (3.5) (see observation 7.2). Leave the flask to stand for 1 hour at room temperature; during this time, shake vigorously on six occasions so that the test sample is thoroughly mixed with the ethanol. Make up to volume with ethanol (3.5), mix and filter.

Pipette 50 ml of the filtrate (corresponds to 2,5 g of the sample) into a 250 ml Erlenmeyer flask, add 2,1 ml of hydrochloric acid (3.1) and shake vigorously. Fit a reflux condenser to the Erlenmeyer flask and immerse the latter in a boiling water bath. After exactly 15 minutes, remove the Erlenmeyer flask from the bath, transfer the contents to a 100 ml graduated flask, rinsing with a little cold water, and cool to 20 °C.

Clarify using Carrez solutions I (3.3) and II (3.4), make up to volume with water, mix, filter and measure the optical rotation as indicated in the 2nd and 3rd paragraphs of 5.2.

6. Calculation of results

The starch content (%) is calculated as follows:

6.1. Measurement by polarimeter

$$\text{Starch content (\%)} = \frac{2\,000(P - P')}{[\alpha]_{20}^D}$$

- P = Total optical rotation in angle degrees
 P' = Optical rotation in angle degrees of the substances soluble in 40 % (V/V) ethanol
 $[\alpha]_{20}^D$ = Specific optical rotation of pure starch. The numerical values conventionally accepted for this factor are the following:

+185,9°:	rice starch
+185,7°:	potato starch
+184,6°:	maize starch
+182,7°:	wheat starch

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+181,5°:	barley starch
+181,3°:	oat starch
+184,0°:	other types of starch and starch mixtures in compound feed

6.2. Measurement by saccharimeter

$$\text{Starch content (\%)} = \frac{2\,000}{[\alpha]_{20}^D} \times \frac{(2\,N \times 0,665) \times (S - S')}{100} - \frac{26,6\,N \times (S - S')}{[\alpha]_{20}^D}$$

S = Total optical rotation in saccharimeter degrees

S' = Optical rotation in saccharimeter degrees of the substances soluble in 40 % (v/v) ethanol

N = weight (g) of saccharose in 100 ml of water yielding an optical rotation of 100 saccharimeter degrees when measured using a 200 mm tube

16,29 g for the French saccharimeters

26,0 g for the German saccharimeters

20,0 g for mixed saccharimeters.

$[\alpha]_{20}^D$ = Specific optical rotation of pure starch (see 6.1)

6.3. Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed 0,4 in absolute value for a starch content lower than 40 % and 1 % relative for starch contents equal to or greater than 40 %.

7. Observations

7.1. If the sample contains more than 6 % of carbonates, calculated in terms of calcium carbonate, they must be destroyed by treatment with an exactly appropriate quantity of dilute sulphuric acid before determination of the total optical rotation.

7.2. In the case of products with a high lactose content, such as powdered milk serum or skimmed milk powder, proceed as follows after adding 80 ml of ethanol (3.5). Fit a reflux condenser to the flask and immerse the latter in a water bath at 50 °C for 30 minutes. Leave to cool and continue the analysis as indicated in 5.3.

7.3. The following feed materials, where they are present in significant amounts in feed, are known to give rise to interferences when determining the starch content by the polarimetric method and thereby incorrect results could be yielded:

- (sugar) beet products such as (sugar)beet pulp, (sugar) beet molasses, (sugar) beet pulp — molassed, (sugar) beet vinasse, (beet) sugar,
- citrus pulp,
- linseed; linseed expeller; linseed extracted,
- rape seed; rape seed expeller; rape seed extracted; rape seed hulls,
- sunflower seed; sunflower seed extracted; sunflower seed, partially decorticated, extracted,
- copra expeller; copra extracted,
- potato pulp,
- dehydrated yeast,

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- products rich in inulin (e.g. Chips and meal of Jerusalem artichokes),
- greaves.

M. DETERMINATION OF CRUDE ASH

1. Purpose and Scope

This method makes it possible to determine the crude ash content of feed.

2. Principle

The sample is ashed at 550 °C; the residue is weighed.

3. Reagents

Ammonium nitrate, solution 20 % (w/v).

4. Apparatus

4.1. Hot-plate.

4.2. Electric muffle-furnace with thermostat.

4.3. Crucibles for ashing made of silica, porcelain or platinum either rectangular (approx. 60 × 40 × 25 mm) or circular (diameter: 60 to 75 mm, height: 20 to 40 mm).

5. Procedure

Weigh out to the nearest mg approximately 5 g of the sample (2,5 in the case of products which have a tendency to swell) and place in a crucible for ashing which has first been heated at 550 °C, cooled down and tared. Place the crucible on the hot-plate and heat gradually until the substance carbonises. Ash according to 5.1 or 5.2.

5.1. Put the crucible into the calibrated muffle furnace set at 550 °C. Keep at this temperature until white, light grey or reddish ash is obtained which appears to be free from carbonaceous particles. Place the crucible in a desiccator, leave to cool and weigh immediately.

5.2. Put the crucible into the calibrated muffle-furnace set at 550 °C. Ash for 3 hours. Place the crucible in a desiccator, leave to cool and weigh immediately. Ash again for 30 minutes to ensure that the weight of the ash remains constant (loss in weight between two successive weightings must be less than or equal to 1 mg).

6. Calculation of results

Calculate the weight of the residue by deducting the tare.

Express the result as a percentage of the sample.

7. Observations

7.1. The ash of *substances which are difficult to ash* must be subjected to an initial ashing of at least three hours, cooled and then a few drops of 20 % solution of ammonium nitrate or water added to it (carefully, to avoid dispersal of the ash or the formation of lumps). Continue calcining after drying in the oven. Repeat the operation as necessary until ashing is complete.

7.2. In the case of *substances resistant to the treatment* described under 7.1, proceed as follows: after ashing for three hours, place the ash in warm water and filter through a

small, ash-free filter. Ash the filter and its contents in the original crucible. Place the filtrate in the cooled crucible, evaporate until dry, ash and weigh.

- 7.3. In the case of *oils and fats*, weigh accurately a sample of 25 g in a suitably sized crucible. Carbonise by setting light to the substance with a strip of ash-free filter paper. After combustion, moisten with as little water as possible. Dry and ash as described under 5.

N. DETERMINATION OF ASH WHICH IS INSOLUBLE IN HYDROCHLORIC ACID

1. Purpose and Scope

This method makes it possible to determine the level in feed of mineral substances which are insoluble in hydrochloric acid. Two methods can be used, depending on the nature of the sample.

- 1.1. *Method A*: applicable to organic feed materials and to most compound feed.
- 1.2. *Method B*: applicable to mineral compounds and mixtures and to compound feed, whose content in substances insoluble in hydrochloric acid, as determined by Method A, is greater than 1 %.

2. Principle

- 2.1. *Method A*: the sample is ashed, the ash boiled in hydrochloric acid and the insoluble residue filtered and weighed.
- 2.2. *Method B*: the sample is treated with hydrochloric acid. The solution is filtered, the residue ashed and the ash thus obtained treated in accordance with Method A.

3. Reagents

- 3.1. Hydrochloric acid 3 mol/litre.
- 3.2. Trichloroacetic acid, solution 20 % solution (w/v).
- 3.3. Trichloroacetic acid, solution 1 % (w/v).

4. Apparatus

- 4.1. Hot plate.
- 4.2. Electric muffle-furnace with thermostat.
- 4.3. Crucibles for ashing made of silica, porcelain or platinum, either rectangular (approx. 60 × 40 × 25 mm) or circular (diameter: 60 to 75 mm, height: 20 to 40 mm).

5. Procedure

5.1. Method A

Ash the sample using the method described for the determination of crude ash. Ash obtained from that analysis may also be used.

Place the ash in a 250 to 400 ml beaker using 75 ml of hydrochloric acid (3.1). Bring slowly to the boil and boil gently for 15 minutes. Filter the warm solution through an ash-free filter paper and wash the residue with warm water until the acid reaction is no longer visible. Dry the filter containing the residue and ash in a tared crucible at a temperature of not less than 550 °C and not more than 700 °C. Cool in a desiccator and weigh.

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5.2. Method B

Weigh 5 g of the sample to the nearest mg and place in a 250 to 400 ml beaker. Add 25 ml of water and 25 ml of hydrochloric acid (3.1) successively, mix and wait for effervescence to cease. Add a further 50 ml of hydrochloric acid (3.1). Wait for any release of gas to cease then place the beaker in a boiling water bath and keep it there for 30 minutes or longer, if necessary, in order to hydrolyse thoroughly any starch which may be present. Filter while warm through an ash-free filter and wash the filter in 50 ml of warm water (see observation 7). Place the filter containing the residue in a crucible for ashing, dry and ash at a temperature of not less than 550 °C and not more than 700 °C. Place the ash in a 250 to 400 ml beaker using 75 ml of hydrochloric acid (3.1); continue as described in the second subparagraph of 5.1.

6. Calculation of results

Calculate the weight of the residue by deducting the tare. Express the result as a percentage of the sample.

7. Observation

If filtration proves difficult recommence the analysis, replacing the 50 ml of hydrochloric acid (3.1) by 50 ml of 20 % trichloroacetic acid (3.2) and washing the filter in a warm solution of 1 % trichloroacetic acid (3.3).

O. DETERMINATION OF CARBONATES

1. Purpose and Scope

This method makes it possible to determine the amount of carbonates, conventionally expressed as calcium carbonate, in most feed.

However in certain cases (for example, with iron carbonate) a special method must be used.

2. Principle

The carbonates are decomposed in hydrochloric acid; the carbon dioxide released is collected in a graduated tube, and its volume compared with that released under the same conditions by a known quantity of calcium carbonate.

3. Reagents

3.1. Hydrochloric acid, density 1,1 g/ml.

3.2. Calcium carbonate.

3.3. Sulphuric acid, approximately 0,05 mol/litre, coloured with methyl red.

4. Apparatus

Scheibler-Dietrich apparatus (see diagram) or equivalent apparatus.

5. Procedure

According to the sample's carbonate content, weigh a portion of the sample as shown below:

- 0,5 g for products containing from 50 % to 100 % of carbonates, expressed as calcium carbonate,
- 1 g for products containing from 40 % to 50 % of carbonates, expressed as calcium carbonate,
- 2 to 3 g for other products.

Place the portion of the sample in the special flask (4) of the apparatus, fitted with a small tube of unbreakable material containing 10 ml of hydrochloric acid (3.1), and connect the flask to the apparatus. Turn the three-way cock (5) so that the tube (1) connects with the outside. Using the mobile tube (2), which is filled with coloured sulphuric acid (3.3) and connected to the graduated tube (1), bring the level of the liquid up to the zero mark. Turn the cock (5) in order to connect up tubes (1) and (3) and check that the level is at zero.

Run the hydrochloric acid (3.1) slowly over the portion of the sample, tilting the flask (4). Make the pressure equal by lowering the tube (2). Shake the flask (4) until the release of carbon dioxide has stopped completely.

Restore pressure by bringing the liquid back to the same level in tubes (1) and (2). After a *few minutes*, when the volume of gas has become constant, take the reading.

Carry out a control test in the same conditions on 0,5 g of calcium carbonate (3.2).

6. Calculation of results

The content of carbonates, expressed as calcium carbonate, is calculated by using the formula:

$$X = \frac{V \times 100}{V_1 \times 2m}$$

where:

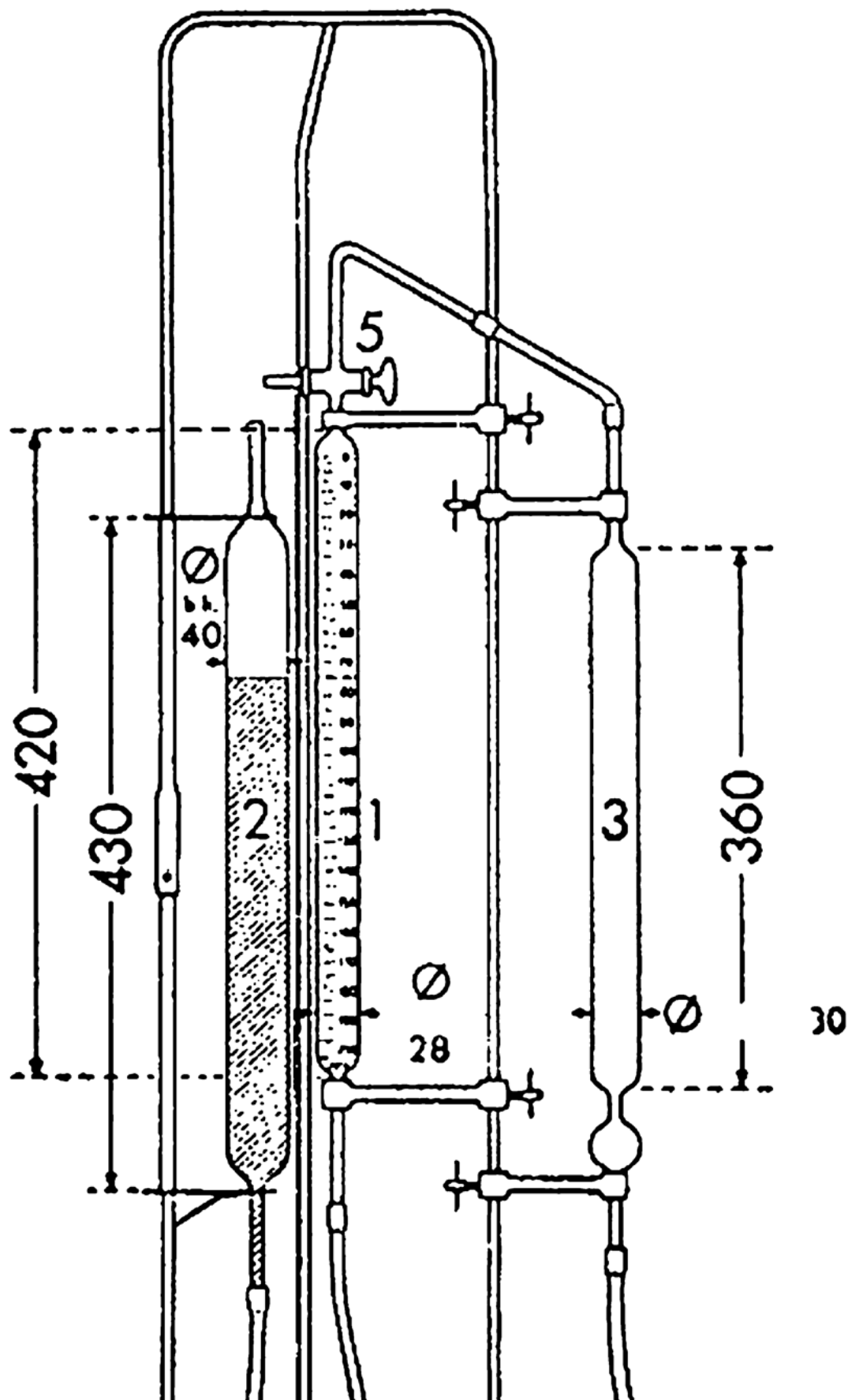
X	=	% (w/w) of carbonates in the sample, expressed as calcium carbonate
V	=	ml of CO ₂ released by the portion of the sample.
V ₁	=	ml of CO ₂ released by 0,5 g of CaCO ₃ .
m	=	weight, in grammes, of the portion of the sample.

7. Observations

- 7.1. When the portion of the sample weighs more than 2 g, first place 15 ml of distilled water in the flask (4) and mix before beginning the test. Use the same volume of water for the control test.
- 7.2. If the apparatus used has a different volume from that of the Scheibler-Dietrich apparatus, the portions taken from the sample and from the control substance and the calculation of the results must be adapted accordingly.

Changes to legislation: There are currently no known outstanding effects for the
Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

SCHEIBER-DIETRICH APPARATUS FOR THE DETERMINATION OF CO₂



P. DETERMINATION OF TOTAL PHOSPHORUS PHOTOMETRIC METHOD

1. Purpose and Scope

This method makes it possible to determine the content of total phosphorus in feed. It is particularly appropriate for the analysis of products low in phosphorus. In certain cases (product rich in phosphorus), a gravimetric method may be used.

2. Principle

The sample is mineralised, either by dry combustion (in the case of organic feed) or by acid digestion (in the case of mineral compounds and liquid feed), and placed in an acid solution. The solution is treated with molybdovanadate reagent. The optical density of the yellow solution thus formed is measured in a spectrophotometer at 430 nm.

3. Reagents

3.1. Calcium carbonate.

3.2. Hydrochloric acid, $\rho_{20} = 1,1$ g/ml (approx 6 mol/litre).

3.3. Nitric acid, $\rho_{20} = 1,045$ g/ml.

3.4. Nitric acid, $\rho_{20} = 1,38$ to 1,42 g/ml.

3.5. Sulphuric acid, $\rho_{20} = 1,84$ g/ml.

3.6. Molybdovanadate reagent: mix 200 ml of ammonium heptamolybdate solution (3.6.1), 200 ml of ammonium monovanadate solution (3.6.2) and 134 ml of nitric acid (3.4) in a 1 litre graduated flask. Make up to volume with water.

3.6.1. Ammonium heptamolybdate solution: dissolve in hot water 100 g of ammonium heptamolybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$). Add 10 ml of ammonia (density 0,91 g/ml) and make up to 1 litre with water.

3.6.2. Ammonium monovanadate solution: dissolve 2,35 g of ammonium monovanadate NH_4VO_3 in 400 ml of hot water. Stirring constantly, slowly add 20 ml of dilute nitric acid (7 ml of HNO_3 (3.4) + 13 ml of H_2O) and make up to 1 litre with water.

3.7. Standard solution of 1 mg phosphorus per ml: dissolve 4,387 g of potassium dihydrogen phosphate KH_2PO_4 in water. Make up to 1 litre with water.

4. Apparatus

4.1. Silica, porcelain or platinum ashing crucibles.

4.2. Electric muffle-furnace with thermostat set at 550 °C.

4.3. 250 ml Kjeldahl flask.

4.4. Graduated flasks and precision pipettes.

4.5. Spectrophotometer.

4.6. Test tubes about 16 mm in diameter, with stoppers graded to a diameter of 14,5 mm; capacity: 25 to 30 ml.

5. Procedure

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

5.1. Preparation of the solution

According to the nature of the sample, prepare a solution as indicated in 5.1.1 or 5.1.2.

5.1.1. Usual procedure

Weigh 1 g or more of the sample to the nearest 1 mg. Place the test sample in a Kjeldahl flask, add 20 ml of sulphuric acid (3.5), shake to impregnate the substance completely with acid and to prevent it from sticking to the sides of the flask, heat and keep at boiling point for 10 minutes. Leave to cool slightly, add 2 ml of nitric acid (3.4), heat gently, leave to cool slightly, add a little more nitric acid (3.4) and bring back to boiling point. Repeat this procedure until a colourless solution is obtained. Cool, add a little water, decant the liquid into a 500 ml graduated flask, rinsing the Kjeldahl flask with hot water. Leave to cool, make up to volume with water, homogenise and filter.

5.1.2. *Samples containing organic substances and free from calcium and magnesium dihydrogen phosphates*

Weigh about 2,5 g of the sample to the nearest 1 mg in an ashing crucible. Mix the test sample until completely merged with 1 g of calcium carbonate (3.1). Ash in the oven at 550 °C until white or grey ash is obtained (a little charcoal does not matter). Transfer the ash into a 250 ml beaker. Add 20 ml of water and hydrochloric acid (3.2) until effervescence ceases. Add a further 10 ml of hydrochloric acid (3.2). Place the beaker on a sand bath and evaporate until dry to make the silica insoluble. Redissolve the residue in 10 ml of nitric acid (3.3) and boil on the sand bath or hot plate for 5 minutes without evaporating until dry. Decant the liquid into a 500 ml graduated flask, rinsing the beaker several times with hot water. Leave to cool, make up to volume with water, homogenise and filter.

5.2. Development of coloration and measurement of optical density

Dilute an aliquot part of the filtrate obtained by 5.1.1 or 5.1.2 to obtain a phosphorus concentration of not more than 40 µg/ml. Place 10 ml of this solution in a test tube (4.6) and add 10 ml of molybdovanadate reagent (3.6). Homogenise and leave to stand for at least 10 minutes at 20 °C. Measure the optical density in a spectrophotometer at 430 nm against a solution obtained by adding 10 ml of the molybdovanadate reagent (3.6) to 10 ml of water.

5.3. Calibration curve

From the standard solution (3.7) prepare solutions containing respectively 5, 10, 20, 30 and 40 µg of phosphorus per ml. Take 10 ml of each of these solutions and add thereto 10 ml of molybdovanadate reagent (3.6). Homogenise and leave to stand for at least 10 minutes at 20 °C. Measure the optical density as indicated in 5.2. Trace the calibration curve by plotting the optical densities against the corresponding quantities of phosphorus. For concentrations between 0 and 40 µg/ml, the curve will be linear.

6. Calculation of results

Determine the amount of phosphorus in the test sample by using the calibration curve.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed:

— 3 %, relative to the higher result, for phosphorus contents of less than 5 %,

— 0,15 % in absolute value, for phosphorus contents of 5 % or more.

Q. DETERMINATION OF CHLORINE FROM CHLORIDES

1. Purpose and Scope

This method makes it possible to determine the amount of chlorine in chlorides which are soluble in water, conventionally expressed as sodium chloride. It is applicable to all feed.

2. Principle

The chlorides are dissolved in water. If the product contains organic matter it is clarified. The solution is slightly acidified with nitric acid and the chlorides precipitated in the form of silver chloride by means of a solution of silver nitrate. The excess silver nitrate is titrated with a solution of ammonium thiocyanate, by Volhard's method.

3. Reagents

- 3.1. Solution of ammonium thiocyanate 0,1 mol/litre.
- 3.2. Solution of silver nitrate 0,1 mol/litre.
- 3.3. Saturated solution of ammonium ferric sulphate $(\text{NH}_4)\text{Fe}(\text{SO}_4)_2$.
- 3.4. Nitric acid, density: 1,38 g/ml.
- 3.5. Diethyl ether.
- 3.6. Acetone.
- 3.7. Carrez I solution: dissolve in water 21,9 g of zinc acetate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ and 3 g of glacial acetic acid. Make up to 100 ml with water.
- 3.8. Carrez II solution: dissolve in water 10,6 g of potassium ferrocyanide $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$. Make up to 100 ml with water.
- 3.9. Active carbon, free from chlorides and not absorbing them.

4. Apparatus

Mixer (tumbler): approximately 35 to 40 r.p.m.

5. Procedure

5.1. Preparation of the solution

According to the nature of the sample, prepare a solution as shown under 5.1.1, 5.1.2 or 5.1.3.

At the same time carry out a *blank test* omitting the sample to be analysed.

5.1.1. *Samples free from organic matter*

Weigh to the nearest mg a sample of not more than 10 g and containing not more than 3 g of chlorine in the form of chlorides. Place with 400 ml of water in a 500 ml volumetric flask at approximately 20 °C. Mix for 30 minutes in the tumbler, bring up to volume, homogenise and filter.

5.1.2. *Samples containing organic matter, excluding the products listed under 5.1.3.*

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

Weigh approximately 5 g of the sample to the nearest mg and place with 1 g of active carbon in a 500 ml volumetric flask. Add 400 ml of water at approximately 20 °C and 5 ml of Carrez solution I (3.7), stir for 30 seconds then add 5 ml of Carrez solution II (3.8). Mix for 30 minutes in the tumbler, bring up to volume, homogenise and filter.

5.1.3. *Cooked feed, flax cakes and flour, products rich in flax flour and other products rich in mucilage or in colloidal substances (for example, dextrinated starch)*

Prepare the solution as described under 5.1.2 but do not filter. Decant (if necessary centrifuge), remove 100 ml of the supernatant liquid and transfer to a 200 ml measuring flask. Mix with acetone (3.6) and bring up to volume with this solvent, homogenise and filter.

5.2. Titration

Using a pipette, transfer to an Erlenmeyer flask from 25 ml to 100 ml of the filtrate (according to the assumed chlorine content) obtained as described under 5.1.1, 5.1.2 or 5.1.3. The aliquot portion must not contain more than 150 mg of chlorine (Cl). Dilute if necessary to not less than 50 ml with water, add 5 ml of nitric acid (3.4), 20 ml of saturated solution of ammonium ferric sulphate (3.3) and two drops of ammonium thiocyanate solution (3.1) transferred by means of a burette filled up to the zero mark. Using a burette, transfer the silver nitrate solution (3.2) in such a way that an excess of 5 ml is obtained. Add 5 ml of diethyl ether (3.5) and shake hard to coagulate the precipitate. Titrate the excess silver nitrate with the ammonium thiocyanate solution (3.1) until the reddish-brown tint has lasted for one minute.

6. Calculation of results

The amount of chlorine (X), expressed as % sodium chloride is calculated by using the following formula:

$$X = \frac{5,845 \times (V_1 - V_2)}{m}$$

where:

V_1 = ml of silver nitrate solution 0,1 mol/l added
 V_2 = ml of ammonium thiocyanate solution 0,1 mol/l used for titration
 m = weight of sample.

If the blank test indicates that silver nitrate solution 0,1 mol/l has been consumed deduct this value from the volume ($V_1 - V_2$).

7. Observations

- 7.1. Titration may also be carried out by potentiometry.
- 7.2. In the case of products which are very rich in oils and fats, first de-fat with diethyl ether or light petroleum.
- 7.3. In the case of fish-meal, titration may be carried out by Mohr's method.

Changes to legislation: There are currently no known outstanding effects for the
Commission Regulation (EC) No 152/2009, ANNEX III. (See end of Document for details)

- (1) For the drying of cereals, flour, groats and meal, the oven must have a thermal capacity such that, when pre-set at 131 °C, it will return to that temperature in less than 45 minutes after the maximum number of test samples have been placed inside to dry simultaneously. Ventilation must be such that, when as many samples of common wheat as it can contain are dried for two hours, the results differ from those obtained after four hours of drying by less than 0,15 %.
- (2) Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

Changes to legislation:

There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX III.