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COMMISSION REGULATION (EC) No 121/2008

of 11 February 2008

laying down the method of analysis for the determination of starch content in preparations of a kind used in animal feeding (CN code 2309)

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Article 1

By derogation from Article 1 of Directive 72/199/EEC, the starch content by weight of preparations of a kind used in animal feeding within the meaning of CN code 2309 shall be determined by the enzymatic analytical method laid down in the Annex to this Regulation in cases where the following feed materials are present in significant amounts:

(a) (sugar) beet products such as (sugar) beet pulp, (sugar) beet molasses, (sugar) beet pulp-molassed, (sugar) beet vinasse, (beet) sugar;

(b) citrus pulp;

(c) linseed; linseed expeller; linseed extracted;

(d) rape seed; rape seed expeller; rape seed extracted; rape seed hulls;

(e) sunflower seed; sunflower seed extracted; sunflower seed, partially decorticated, extracted;

(f) copra expeller; copra extracted;

(g) potato pulp;

(h) dehydrated yeast;

(i) products rich in inulin (e.g. chips and meal of Jerusalem artichokes);

(j) greaves;

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(k) soya products.

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Article 2

This Regulation shall enter into force on the twentieth day following that of its publication in the *Official Journal of the European Union*.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

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ANNEX

ENZYMATIC METHOD FOR THE DETERMINATION OF THE STARCH CONTENT IN PREPARATIONS USED IN ANIMAL FEEDING USING HIGH PRESSURE LIQUID CHROMATOLOGY (HPLC)

1. **Scope**

This method describes the enzymatic determination of the starch content in animal feeding. The starch content is derived from the quantitative determination of glucose after enzymatic breakdown of the present starch into glucose. All measured glucose is considered to be derived from the starch, present in the sample.

2. **Definitions**

In this method, the content of starch and its high molecular mass breakdown products, insoluble in 40 % ethanol, is determined. The starch content is expressed in % (m/m).

3. **Principle**

The samples are homogenised by milling. The sample is washed with 40 % ethanol to eliminate soluble sugars and soluble products of starch decomposition.

The enzyme thermostable alpha-amylase is added to the suspension. This enzyme breaks down the starch at 100 °C in smaller chains, whether or not the starch is fully in solution.

Large lumps of starch are broken down very slowly. Therefore it is necessary that the samples should be wholly solved or should be present in the form of a suspension containing very small solid parts.

Then the second enzyme amyloglucosidase is added, which hydrolyses the degraded glucose chains into glucose at 60 °C.

After clarification of the liquid whereby the present proteins, fats and residues are discarded after filtration, a clear solution is obtained which may be used for HPLC.

Separation of the present sugars is done by HPLC.

4. **Reagents and other materials**

Use reagents of recognized analytical grade and demineralized water.

4.1. Ethanol 40 % vol. in water

4.2. Glucose, min 99 %

4.3. Solution of Amyloglucosidase (1,4-alpha-D-Glucan glucohydrolase) from Aspergillus niger (enzyme activity > 5 000 U/ml). Storage at ca. 4 °C

Alternatively, also amyloglucosidase powder can be used.

4.4. Thermostable alpha-amylase (1,4-alpha-D-Glucan-glucanohydrolase). Storage at ca. 4 °C

4.5. Zinc acetate dihydrate, p.a.

4.6. Potassium hexacyanoferrate (II) ($K_4[Fe(CN)]_6.3H_2O$), extra pure.

4.7. Sodium acetate anhydrous, p.a.

4.8. Glacial acetic acid, 100 % (v/v)

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4.9. Sodium acetate buffer (0,2 mol/l).

Weigh 16,4 gram sodium acetate (4.7) into a beaker glass. Dissolve in water and rinse into a volumetric flask of 1 000 ml. Dilute to the mark with water and adjust the pH to 4,7 with acetic acid (by use of a pH-meter (5.11)). This solution may be used for max 6 months with storage at 4 °C.

4.10. Amyloglucosidase solution (enzyme activity > 250 U/ml)

Prepare a solution of 5 ml of amyloglucosidase-solution (4.3) or 660 mg amyloglucosidase powder in a final volume of 100 ml by using sodium acetate buffer (4.9). Prepare instantaneously.

4.11. Reference solution

Prepare solutions of glucose in water, as conventionally used in the HPLC analysis.

4.12. Reagent for clarification (Carrez I)

Dissolve 219,5 gram zinc acetate (4.5) in water in a beaker glass. Rinse into a volumetric flask of 1 000 ml, add 30 ml acetic acid (4.8). Mix thoroughly and make up with water. This solution may be used for max 6 months while stored at ambient temperature.

Other clarification reagents, equivalent to Carrez solution, may be used.

4.13. Reagent for clarification (Carrez II)

Dissolve 106,0 gram potassium hexacyanoferrate (II) (4.6) in water in a beaker glass. Rinse into a volumetric flask of 1 000 ml. Mix thoroughly and make up with water. This solution may be used for max. 6 months while stored at ambient temperature.

Other clarification reagents, equivalent to Carrez solution, may be used.

4.14. Mobile phase

A mobile phase is prepared which is conventionally used in the HPLC analysis of sugars. In case of using an aminopropyl silicagel column, e.g., a common mobile phase is a mixture of HPLC graded water and acetonitrile.

5. **Apparatus**

5.1. Standard laboratory glassware

5.2. Centrifuge giving 1 000 g or more (calculated at the centre of the tube)

5.3. Glass centrifuge tubes of 100 ml

5.4. Magnetic stirrer

5.5. Magnetic rods

5.6. Fluted filters, e.g., 185 mm

5.7. Syringe filters, 0,45 µm, suitable for aqueous solutions

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5.8. Sample vials suitable for the HPLC autosampler

5.9. 100 ml volumetric flasks

5.10. Plastic syringes, 5 and 10 ml

5.11. pH-meter

5.12. Water bath with thermostat, adjustable to 60 °C and 100 °C

5.13. Heating plates with magnetic stirrers

5.14. HPLC Apparatus

5.14.1. Pump, fit for zero-pulsation

5.14.2. Autosampler

5.14.3. Column and pre-column, suitable for analysis of sugars

5.14.4. Column oven, temperature range between ambient and 40 °C

5.14.5. Detector, suitable for analysis of sugars, e.g., Refractive Index Detector

5.14.6. Integration system

6. **Procedure**

6.1. General

Samples are analysed in singular.

6.2. Preparation of the sample for several types of products

The product is homogenised by milling.

6.3. Sample portion

The starch content is estimated with help from the ingredient declaration. The amount of sample (weighed to 0.1 mg accuracy) can be estimated from:

$$\text{amount of sample (g)} = \frac{\text{volume of volumetric flask (100 ml)}}{\text{estimated starch content (\%)}}$$

6.4. Blank determination

The blank is determined by performing a complete analysis (as described in 6.5), without adding sample. The result of the blank determination is used in the calculation of the starch content (7.1).

6.5. Analysis

6.5.1. Preparation of the samples

Mix the sample by shaking or stirring. The chosen test portion (6.3) is weighed into a centrifuge tube (5.3) and 50 ml 40 % ethanol (4.1) is added. Stir in a magnetic stirrer for 20 minutes at ambient temperature. Leave the magnetic rod in the tube and centrifuge for five minutes. Aspirate carefully and remove the liquid phase (e.g., with a Pasteur pipette). Repeat this extraction procedure with two times 25 ml ethanol (4.1). Transfer the residue into a volumetric flask of 100 ml (5.9) with circa 70 ml water. After dissolving or suspending, add 100 microliter of

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thermostable alpha-amylase (4.4) and heat at 100 °C for 1 hour e.g., in a water bath (5.12). Cool to 60 °C in a water bath, and add 5 ml of a solution of amyloglucosidase (4.10). Place flask for 30 min in a water bath at 60 °C. Cool to ambient temperature, clarify the sample by adding 1 ml Carrez I (4.12), shake and then add 1 ml Carrez II (4.13). Carrez I and II may be added before or after cooling. Dilute to the mark with water, homogenize and filter the solution through a fluted filter (5.6). Collect the sample extract.

6.5.2. Processing of the sample extracts

Filter the extracts through a disc filter (5.7) with a syringe (5.10) that has been preflushed with the extract. Collect the filtrates in vials (5.8).

Note: The disc filter can be used several times. It should be flushed with the next extract to prevent contamination of the previous extract.

6.6. Chromatography

HPLC is performed as conventionally for analysis of sugars. Because the samples are extracted with ethanol/water, glucose is the main sugar to be analysed. If the HPLC analysis shows traces of maltose, then this may indicate incomplete conversion of starch.

7. Calculation and expression of results

7.1. Calculation of the HPLC results

From the results of the HPLC analysis, the content (%, m/m) of glucose is calculated. The enzymatic solution of amyloglucosidase (4.3) is stabilised with glucose. Furthermore, thermostable alpha-amylase (4.4) is stabilised with sucrose, which can partially be converted into glucose by invertase-activity of amyloglucosidase. Therefore, the glucose concentration (%, m/v) measured should be corrected for the glucose concentration (%, m/v) in the blank. The glucose content (%, m/m) corrected for the blank is then calculated from the corrected glucose concentration, the sample weight and the calibration with reference solutions (4.11).

7.2. Calculation of the starch content

The starch content (%, m/m) is calculated from the glucose content (%, m/m) after correction for the blank.

$$\text{Starch content} = 0{,}9 * \text{Glucose corrected}$$

8. Precision

8.1. Inter-laboratory test

Details of an inter-laboratory test on the precision of the method are summarized in 8.4.

8.2. Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the repeatability limit of 1,1 % (m/m). The repeatability limit was derived from the pooled results of an inter-laboratory test (see 8.4).

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8.3. Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the reproducibility limit of 3,7 % (m/m). The reproducibility limit was derived from the pooled results of an inter-laboratory test (see 8.4).

8.4. Results of inter-laboratory test

An inter-laboratory test was carried out in 2005 and 2006 with participation of the European Customs laboratories. This test was performed in accordance with ISO 5725 and the IUPAC protocol (W. Horwitz, Pure and Applied Chemistry, vol. 67, 1995, p. 331-343). The precision data are given in the table below.

Statistical results of the inter-laboratory study

	Sample				
	1	2	3	4	5
Number of laboratories after eliminating outliers	25	26	26	25	24
Number of accepted results	50	52	52	50	48
Mean starch content (%, m/m)	31,2	14,4	25,1	12,9	27,8
Repeatability standard deviation s_r (%, m/m)	0,4	0,3	0,6	0,2	0,3
Repeatability limit r (%, m/m)	1,1	0,8	1,7	0,7	0,9
Reproducibility standard deviation s_R (%, m/m)	1,7	0,8	1,7	0,9	1,3
Reproducibility limit R (%, m/m)	4,8	2,2	4,7	2,5	3,7

Samples

1: dry dog food
2: dry cat food
3: dry cat food (sample 2) with addition of starch
4: dry cat food (sample 2) with addition of beet pulp
5: commercial pet food