May 2, 2007

NorFor
Nordic Feed Evaluation System

Starch analysis by enzymatic colorimetric method

*Spectrophotometric method**

Principle

* Starch is gelatinized in a boiling water bath and simultaneous partly degraded with thermostable α-amylase (E.C. 3.2.1.1.)

* A complete degradation of starch oligomers into glucose is performed by the use of amyloglucosidase (E.C. 3.2.1.3.)

* The released amount of glucose is determined quantitatively using glucose oxidase (E.C. 1.1.3.4.) according to the following reaction :

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$2H_2O_2 + \text{p-hydroxy benzoic acid} + 4 \text{ amino antipyrine} \xrightarrow{\text{POD}}$$

$$\text{Quinone-imin-colour} + 4H_20$$

Usually it is not necessary to extract free sugars from cereals, as the concentration of free glucose is very low (0.1-0.5%). Furthermore, the glucose oxidase measures glucose specifically. However, samples containing more than 4% sugar must be extracted with 80% ethanol.

If the fat content exceeds 8%, the samples have to be extracted with acetone.

References:

Bach Knudsen, K.E. (1997). Animal Feed Science and Technology **67**, 319-338.
Bach Knudsen, K.E. et al. (1987), J. Cereal Science **6**, 173-186.

* Inter-changeable with the method using plate count reader

1. Apparatus

10 ml plastic tubes
50 ml plastic centrifuge tubes (Greiner) with tight-fitting lids
(in case of acetone extraction: 50 ml glass tubes)
Autopipette, 30 ml
Whirlmixer
Water bath, 100 °C
Water bath, 60 °C
Centrifuge 3000 rpm (2270 g)
Eppendorf pipette (enzymes, GODPOD and standards)
Finnpipette 200-1000 µl (dilutions and sampling for glucose analysis)
Finnpipette 1-5 ml (dilutions and sampling for glucose analysis)
Spectrophotometer, Spectronic 2000, 510nm
Timer
Magnets

For preparation of buffers and standards:
1000 ml volumetric flasks
100 ml volumetric flasks

2. Reagents

Water : Millipore grade

2.1 Enzymes (to be kept in refrigerator)

2.1.1 Thermostable Alfa-amylase, EC 3.2.1.1, 120 000 U
Megazyme Ltd. E-BLAAM, 53,7 U/mg. Ltd, Ireland

2.1.2 Amyloglucosidase
Megazyme International, catalogue no E-AMGDF, 140.000 U, 3260 U/ml.

2.1.3 Glucose oxidase kit (GODPOD).
Megazyme International, catalogue no K-GLUC.

2.1.3.1 Glucose Reagent Buffer
1 M potassium dihydrateorthophosphate ($KH_2PO_4,8H_2O$)
200 mM para-hydroxide benzoic acid (HOC_6H_4COOH)
0,4 % Sodium azide

2.1.3.2 Glucose analysis reagent (per bottle):
Glucose oxidase ≥ 12.000 U
Peroxidase ≥ 650 U
4-amino antipyrine 0,4 mmol.

2.2 Chemicals

2.2.1 Acetone
2.2.2 Ethanol, 96 %
2.2.3 Glucose ($C_6H_{12}O_6$), Merck 8337
2.2.4 Glacial acetic acid (CH_3COOH), Merck 63
2.2.5 Sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$), Merck 6267.
2.2.6 Ethanol, 99%

2.3 Solutions

2.3.1 Glucose oxidase (GODPOD).

Dilute 50.0 ml (1 bottle) glucose reagent buffer (2.1.3.1) with water to 1 litre in a volumetric flask.
Dilute 1 vial glucose determination reagent (2.1.3.2) in the buffer.
Must be kept in a brown bottle. Can be kept for three months in refrigerator, > 12 months in the freezer.

2.3.2 80% (v/v) Ethanol

833 ml 96% ethanol pr 1 l.

2.3.3 Acetate buffer, 0.1 M, pH = 5.0

	Merck no	1 l	2 l	5 l
CH_3 COOH (ml)	63	2.088	4.176	10.440
CH_3 COONa•$3H_2O$ (g)	6267	8.641	17.282	43.206

The pH value of the buffer solution is adjusted with HCl or NaOH.

2.3.4 Amyloglucosidase solution, 1087 U/ml

Per 1 ml amyloglucosidase (2.1.2) 2 ml acetate buffer is added (2.3.3). Must be kept in refrigerator for maximum 1 week.

2.4 Starch standard

Wheat starch, Sigma, catalogue no S 5127.

2.5 Glucose standard

A standard solution consisting of 500 mg $C_6H_{12}O_6$ (2.2.3) (dried in a vacuum oven for 4 hrs at 50°C) is made in 1000 ml with water. This mixture is left in the refrigerator overnight. When the solution has reached room temperature solutions are made by weighing out 6, 12, 16, 20, 24, 32, and 42 g from the stock solution, respectively, and dilute them with *weighing* with water up to 100 g. From these solutions 1.5 ml is taken by an Eppendorf pipette and put into plastic tubes, which are packed in sets with one of each concentration and freezed at –20°C. The concentration of $C_6H_{12}O_6$ is about (µg/ml): 0, 30, 60, 80, 100, 120, 170, and 210. The concentration must be calculated *accurately* by each new standard graph.

3. Procedure

3.1. The sample is finely grounded into particle size < 0.5 mm.

3.2. The dry matter content is determined after grinding.

3.3. Weigh out about 150 mg of the sample (100 mg pure starch) in a 50 ml screw-capped centrifuge tube, 150 mg barley standard, and 100 mg starch standard (2.4). Put a magnet in each centrifuge tube. All samples must be analysed in duplicate. For samples with a low sugar and fat content, go directly to item 3.6.

3.4. **Samples having a sugar content >4%** are extracted with 80% ethanol (2.3.2). The samples are added 40 ml 80% ethanol. Mix the samples thoroughly on magnet stirrer for 15 minutes. Centrifuge for 10 minutes at 3000 rpm. Remove the supernatant by suction and collect the spillage. Repeat the procedure one time with 80 % ethanol and afterwards one time with 99% ethanol. Dry the samples in the fume cupboard.

3.5. **Samples with a fat content of >8%** are extracted with acetone (2.2.1). The sample, which is weighed in a **glass tube**, is added 40 ml acetone, mixed on a magnet stirrer for 15 minutes og centrifuged for 10 minutes at 3000 rpm. Remove the supernatant by suction and collect the spillage. The proceduren is repeated two times. The sample is dried in the fume cupboard.

3.6. Add 30 ml acetate buffer (2.3.3) with a calibrated auto-pipette.

3.7. Immediately before the sample is put into a boiling water bath, 100 µl Thermostable Alpha-amylase (2.1.1) is added, and mixed on a whirl mixer.

3.8. Tightly cap the tubes. Leave the samples in the boiling water bath for 60 minutes from when the water is boiling again. Mix the samples carefully three times during incubation.

3.9. Remove the samples from the water bath, cool to about 40° C, and add 200 µl amyloglucosidase solution (2.3.4).

3.10. Mix on a whirl mixer and transfer the tubes into a water bath at 60° C to be inculbated for 2 hours. The tubes are mixed after one hour.

3.11. Put the sample in a boiling water bath for 10 minutes to inactivate the enzymes.

3.12. Mix on magnet stirrer and centrifuge the tubes (3000 rpm for 10 minutes).

3.13. Dilutions are made with calibrated pipettes. Wheat starch and barley controls are diluted 30 times with water. Include a blank sample for all dilutions.

Samples with a starch content of <4% should be analysed undiluted.

Samples with a starch content of 4-15% should be diluted 10 times.
Samples with a starch content of >16% should be diluted 30 times.

3.14. 2 x 500 µl of samples and standards are put into 10 ml tubes and mixed with 3 ml glucose oxidase. Incubate in a 40° C water bath for 20 minutes. Then, take the samples and measure absorbance at wavelength 510 nm.

4. Calculation

Dilution factor (DF):

DF= Total volume (ml)/ volume of aliquot for dilution (ml)

Glucose **(µg/ml)** according to standard curve **= (X abs-abs blind)*m+b**
Where as **m** is slope and **b** is the intercept.

% starch (as is);

$$\frac{\text{DF*glucose according to std. curve*(buffer(ml)+amylase(ml)+amyloglucosidase(ml))*0,9*1000µl/ml*100}}{\text{Sample amount (mg)*1000µg/mg}}$$

Conversion factor (glucose to starch) is a multiplation by 0,9 (162/180).

% starch (dry matter) = (% starch as is)/ (% dry matter) *100

Average % (dry matter) = (result of determination a + result of determination b)/2

Deviation % = (result of determination a + result of determination b)/average %*100

Variance limits

The deviation between duplicate determinations must be <3.5%
The starch standard must not be lower than 92%.
The barley standard must not lower than 51 %.
For samples with starch contents < 5% the deviation in absolute values should be less than 0.5 %.

5. Safety regulations

Hazard and safety sentences are enclosed.

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Hazard and safety sentences:

R: Risk/hazard, S: Safety precautions

Pure compounds:

Name	R-sentences	S-sentences	Labeling	Waste group
Acetone, 100 %	11	1-9-16-23-33	F	C
Ethanol, 99 %	11	2-7-16	F	C
Acetic acid, 100%	10-35	1/2-23-26-45	C	H

R og S-sentences:

Compound	R	R-Sentences	S	S-Sentences	Symbol	Waste
Acetone, 100%	11	Extremely flammable	1	Keep under lock	F	C
			9	Keep container in a well-ventilated place		
			16	Keep away from sources of ignition No smoking		
			23	Do not inhale fumes		
			33	Take precautionary measures against static discharges		

Compound	R	R-Sentences	S	S-entences	Symbol	Waste
Ethanol, 99%	11	Extremely flammable	2	Keep out of childrens' reach	F	C
			7	Keep container tightly closed		
			16	Keep away from sources of ignition No smoking		

Compound	R	R-Sentences	S	S-entences	Symbol	Waste
Glacial acetic acid	10	Flammable	1/2	Keep under lock and out of childrens' reach	C	H
	35	Very corrosive	23	Do not inhale fumes		
			26	If contact with the eyes, flush at once carefully with plenty of water and get medical attention immediately		
			45	By an accident or indisposition, get medical attention immediately; show the label if possible.		

Reagents:

Compound	R	R-sentences	S	S-sentences	Symbol	Waste
80 % Ethanol	11	Very flammable	2	Keep out of childrens' reach	F	C
			7	Keep container tightly closed		
			16	Keep away from ignition sources – No smoking		

Gloves: Best N-DEX nitril (blue)