**STANDARD OPERATION PROCEDURE**

**Faculty of Biosciences, NMBU**

**Method name: Water soluble carbohydrates (WSC)**

BIOVIT No: Arb1014

**1. Introduction**

The samples are extracted into an acetate buffer at room temperature overnight before being filtrated. Sucrose and fructans in the extract are hydrolyzed using sulfuric acid. The monosaccharides are then converted to glucose-6-phosphate (G-6-F) and fructose-6-phosphate (F-6-F) by an enzymatic method. G-6-F is further oxidized by NADP+ which is then reduced to NADPH. The amount of NADPH formed in this reaction corresponds to the amount of D-glucose. Then F-6-F is converted to G-6-F which is further oxidized by NADP + and leads to another increase in NADPH level. This increase corresponds to the amount of D-fructose. The absorbance of NADPH before and after the reaction is measured spectrophotometrically. The increase in absorbance is directly proportional to the glucose and fructose concentration. The analysis gives the total sum of monosaccharides, sucrose and fructans as one result.

The results have shown satisfactory agreement with a similar method used at SLU (R2 = 0.89, 0.99 and 0.98 for raw, freeze-dried and oven-dried samples, respectively).

**2. Reagents**

0.05 M Acetate buffer pH 5.0:

* **Solution 1**: 13.61 g Sodium acetate\*3H2O is dissolved in 2 L water (RO)
* **Solution 2**: Dilute 3,003 g acetic acid in 1 L water (RO).
* Mix solution 1 and solution 2 to pH 5.0 (ratio approx. 2: 1)
* Add 280 mg Calcium Chloride (CaCl2) per 1L solution

0,074M H2SO4:

* Add 4.1 mL of concentrated sulfuric acid (H2SO4) to approximately 800 mL water in 1 L volumetric flask
* Fill up to 1 liter with distilled water (RO)

Kit:

Megazyme, D-Fructose/D-Glucose assay kit, K-FRUGL (Megazyme, Wicklow, Ireland).

**3. Risk assessment**

Sulfuric acid (H₂SO₄) is highly corrosive. In case of spillage: Rinse immediately with plenty of water. Acetic acid is highly corrosive and flammable. Avoid inhalation of vapors. In case of spillage: Rinse immediately with plenty of water. Spills of large quantities on the floor - cover with absorbent before cleaning with water.

**4. Equipment**

* Scale
* Ultra Torax *(if samples are not grinded)*
* Magnets
* Water bath
* 250 mL Erlenmeyer flasks (alternatively 150 mL)
* Folding filter
* 10 mL test tube with cap
* Eppendorf tube
* Disposable cuvettes 1cm - PMMA macro (cat.no.VWR: 634-0677)
* UV-VIS Spectrophotometer

**5. Special remarks**

Analysis in freeze-dried or oven-dried, grinded samples has shown to give 60-80% of the level one gets in non-dried (raw samples) of grass and silage, the reason for this is unknown. Level differences between raw and dried samples were insignificantly larger for oven drying than freeze-drying, and clearly larger for silage samples (60-80% of the raw sample) than for grass samples (77-81% of the raw sample). The sample material can alternatively be extracted in boiling water. This has given about the same level as extraction in acetate buffer for raw samples, but somewhat lower level for freeze-dried and oven-dried samples. This is especially true for silage. Solution in acetate buffer appears to give a more stable reading on the spectrophotometer.

This procedure is suitable for samples with expected WSC values ​​between 1-25%. If the values ​​are expected to be higher/lower, the weighed amount and/or volume of buffer must be adjusted.

**6. Sample material**

The analysis is suitable for fresh, pre-dried or frozen grass/silage, feed or other feed products.

**7. Work procedure**

A: Raw samples

1a) Cut 2-3 g sample into as small pieces as possible with scissors and weigh in about 1 g of the finely cut sample

2a) Transfer the sample to a 250 mL Erlenmeyer flask

3a) Add 80 mL of 0.05 M Acetate Buffer

4a) Homogenize with Ultra Tourax (w/large knife) until it becomes a smooth solution

5a) Use 20 mL Acetate Buffer to rinse sample residue out of the knife

B: Pre dried samples

1b) Weigh in about 1 g of sample

2b) Transfer the sample to a 150- or 250-mL Erlenmeyer flask

3b) Add 100 mL of 0.05 M Acetate Buffer

Common to all sample types

 6) Add a magnet and place the samples on magnetic stirrer for 5 min

 7) Cover the samples with parafilm

 8) Allow to extract overnight (approx. 1 day) at room temperature

 9) The next day, the samples are filtered with a folding filter

10) Pour only a small amount of the sample into the filter and discard the first 10 mL of the filtrate

11) The rest of the filtrate is collected in a suitable container (e.g. 150 mL Erlenmeyer flask)

12) Label Eppendorf tubes and 10 mL reagent tubes w/cap

13) Have about 1 mL of sample in the Eppendorf tube and freeze (in case of reanalysis)

14) Pipette 0.5 mL into the test tube (this is the sample to be hydrolyzed in the next step)

*Here the sample can be frozen at -20 ° C until the hydrolysis*

Hydrolysis

15) 0.5 mL of sample is added 0.5 mL of 0.074 M H2SO

16) The samples are placed in a water bath at 80 °C for 70 min

 (Poly- and disaccharides in the extract are now hydrolyzed to glucose and fructose)

Info about "D-fructose & D-glucose kit" from megazyme

* The kit is enough to about 110 samples and contains:
* Bottle 1: Used as is (stable> 2 years at 4 ° C)
* Bottle 2: Dissolve the contents in 12 mL of distilled water (stable> 1 year at 4 ° C)

 *Can be divided and frozen at -20 ° C (stable> 2 years)*

* To read water-soluble sugar (WSC = glucose + fructose), transfer all the contents of bottle 4 to bottle 3 (gently shake the bottles before opening)

 Mix gently and store upright (stable> 2 years at 4 ° C)

* Bottle 5 is used as is, and only as a control if the spectrophotometer is suspected to be inaccurate (stable> 2 years at 4 ° C)

*Note: It is also possible to read glucose and fructose separately, but then bottles 3 and 4 should NOT be mixed and should be read one more time. Se “assay procedure” part A for more info.*

Reading on spectrophotometer

*Retrieved from "assay procedure" - Part B: Manual assay procedure; total reducing sugars:*

17) Turn on spectrophotometer (instrument goes through a series of self-tests) + printer

18) Press «**GOTO WL**» on front panel - enter «**340**», press «ok»

19) Use 1 cm cuvette (disposable PMMA macro/VWR)

20) Put a cuvette with distilled water in the first position, close the lid and press «AUTOZERO» on the front panel

21) Pipette water, sample, solution 1 and solution 2 into a new cuvette (See table below for details)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Blank** | **Sample** |
| Distilled water (25 °C)SampleSolution 1Solution 2 | 2.10 mL---------0.10 mL0.10 mL | 2.00 mL0.10 mL0.10 mL0.10 mL |

22) Put the lid on the cuvette and turn several times to mix

23) Repeat steps 21 and 22 for all samples including a blank (without sample)

24) Wait about 3 minutes

25) Put the cuvette down in the first position and close the lid

26) Press START / STOP on the front panel (enters a new image)

27) Check that it says "photometric" at the top and that the wavelength is 340 nm

28) Read absorbance (A1) by pressing «START / STOP»

29) The results are printed continuously, but note down the absorbance as well

30) After all the samples have been read for the first time: add 40 µL of mixture 3 & 4 in all the cuvettes (see table below for details)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Blank** | **Sample** |
| Mixture 3 & 4 | 0.04 mL | 0.04mL |

31) Put the lid on the cuvette and turn several times to mix

32) Repeat steps 30 and 31 for all samples + blank

33) Wait 10 minutes \*

34) Read absorbance (Atotal) by pressing «START / STOP»

35) Write down the absorbance

36) Turn off the spectrophotometer + printer when all the samples have been read

*\* If the reaction has not stopped after 10 min, continue reading at 2 min intervals until the absorbance is stable over 2 min.*

*(If the reaction continues to increase, this may be due to color compounds or enzymes in the sample. These interferences may be removed during sample preparation.)*

**8. Calculation**

All equations are inside the worksheet for WSC (requisition sheet).

Determine the absorbance difference (Atotal-A1) for both blanks and samples.

Subtract the absorbance difference in the blank from the absorbance difference in the sample.

These are: ∆AD-glucose + D-fructose.

The value of ∆AD-glucose + D-fructose should as a rule be at least 0.100 absorbance units to achieve reliable results.

The concentration of D-glucose and D-fructose is calculated as follows:

C= V **\* MW \*** ∆A(g/L)

**ε \* d \* v**

Where:

V = final volume = 2.34 mL

MW = molecular weight of D-glucose or D-fructose = 180.16 g / mol

ε = Coefficient of extension for NADPH at 340nm = 6300 (1\*x mol -1 \*cm -1)

d = light path (cm) = 1

v = sample volume (mL) = 0.1

Concentration of D-glucose + D-fructose (total sugar):

C = 2,34 \* 180,16 \* ∆ AD-glucose D-fructose = 0,6692 \* ∆AD-fructose (g/l)

 6300 \* 1 \* 0,1

*If the sample is diluted, the result must be multiplied by the dilution factor!*

*(normal dilution = 1: 1 with acid). This is inside the excel sheet, but only applies to the samples, NOT the standard since the standard has not been hydrolyzed.*