NEWFEED

Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes

Grant Agreement number: 2013, Call 2020 Section 1 Farming IA

Orange-based ingredient feeding strategy

Deliverable number 2.5

Work Package 2	Optimization of the Valorisation and Feeding strategies
Task 2.x	Case study 2: Orange peel-based ingredients for dairy sheep
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Foreword

The work described in this report was developed under the project NEWFEED: Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes (Grant Agreement number: 2013/ Call 2020 Section 1 Farming IA). If you wish any other information related to this report or the NEWFEED project please visit the project web-site (www.newfeed-prima.eu) or contact:

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PU	Public	X
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Executive Summary

This deliverable provides the orange-based ingredient feeding strategy including the orange peels nutritional value and in-vitro digestibility. This process is included in the second case study developed in the project:

Use of orange peels for dairy sheep feedstuff.

The analysis of the data obtained will allow the detection of the most suitable process to increase the nutritional value of the ingredient and, therefore, will help to increase the percentage of inclusion in the validation trials with ruminants in WP3.

Determining the factors that affect the ingredient nutritional value is of vital importance to define the industrial process and to valorise as much ingredient as possible.

The second case study evaluates the use of orange peels as a second generation ingredient for dairy sheep feed. This case study is led by NTUA and will be validated with real feedstock and under optimised conditions.

Table of Contents

Table of Figures

Table of Tables

1. Introduction

The overall objective of the project is to develop alternative feed ingredients using a circular economy approach through the conversion of food by-products into high-value secondary animal feed.

The purpose of this Deliverable is to define the orange peel-based ingredient feeding strategy including its nutritional value and in-vitro digestibility. This process is included in the second case study developed in the project:

- ✓ The second case study evaluates the use of orange peels as a second generation ingredient for dairy sheep feed. This case study is led by NTUA and will be validated with real feedstock and under optimised conditions.

The analysis of the data obtained will allow the definition of the feeding strategy and the percentage of inclusion for the feeding trials that will be developed in WP3 to ensure the successful valorisation of these by-products as alternative ingredients for animal feed.

2. Methodology

2.1 Chemical analysis

2.1.1 Determination of total solids and moisture in biomass

Introduction

Biomass samples can contain large and varying amounts of moisture, which can change quickly when exposed to air. To be meaningful, the results of chemical analyses of biomass are typically reported on a dry weight basis.

Parts of this procedure are similar to ASTM E1756-01 and T412 om-02.

The following procedure describes the methods used to determine the amount of solids or moisture present in a solid or slurry biomass sample. It also covers the determination of dissolved solids in a liquor sample.

Apparatus

- Convection drying oven, with temperature control of 105 ± 3°C
- Analytical balance, accurate to 0.1 mg
- Desiccator containing dessicant
- Convection drying oven, with temperature control of 105 ± 3°C

Materials

- Aluminum pans, made to fit infrared moisture analyzer if necessary
- Glass fiber pads for liquor samples
- 0.2 µm pore size filters, either large syringe filters with syringes or 50 mm filter units, for liquor samples only

Procedure

Solid samples usually require 0.5 to 2 grams, slurry samples require 2-5 grams, and liquor samples require 10 mL, per duplicate. Liquor samples should be filtered through a 0.2 µm pore size filter prior to analysis.

Pre-dry aluminum weighing dishes by placing them in a 105 ± 3ºC drying oven for a minimum of four hours. Cool the dishes in a desiccator. Using gloves or tweezers to handle the dishes, weigh a pre-dried dish to the nearest 0.1 mg. Record this weight.

Thoroughly mix the sample and then weigh out an appropriate amount to the nearest 0.1 mg, into the weighing dish. Liquor samples should be passed through a 0.2 µm filter prior to analysis. Record the weight of the sample plus weighing dish. Analyze each sample in duplicate, at minimum.

Place the sample into a convection oven at 105 ± 3ºC for a minimum of four hours. Remove the sample from the oven and allow it to cool to room temperature in a desiccator. Weigh the dish containing the oven-dried sample to the nearest 0.1mg and record this weight.

Place the sample back into a convection oven at 105 ± 3ºC and dry to constant weight. Constant weight is defined as ± 0.1% change in the weight percent solids upon one hour of re-heating the sample. Overnight drying is usually required for very wet or liquid samples [1].

Calculations

Calculate the percent total solids for a liquor sample, on a 105ºC dry weight basis as follows:

$$\%\ \boldsymbol{Total\ Solids} = \frac{Weight_{dry\ pan\ plus\ dry\ sample} - Weight_{dry\ pan}}{Weight_{sample\ as\ received}} \cdot 100 \qquad \textit{Equation 1}$$

The percent moisture can also be calculated:

$$\%\ \boldsymbol{Moisture} = 100 - \frac{Weight_{dry\ pan\ plus\ dry\ sample} - Weight_{dry\ pan}}{Weight_{sample\ as\ received}} \cdot 100 \qquad \textit{Equation 2}$$

2.1.2 Determination of volatile mass and ash in biomass

Introduction

The amount of inorganic material in biomass, either structural or extractable, should be measured as part of the total composition. Structural ash is inorganic material that is bound in the physical structure of the biomass, while extractable ash is inorganic material that can be removed by washing or extracting the material.

This procedure is substantially similar to ASTM Standard Method Number E1755-01 “Standard Method for the Determination of Ash in Biomass”.

This test method covers the determination of ash, expressed as the percentage of residue remaining after dry oxidation at 550 to 600°C. All results are reported relative to the 105°C oven dry weight of the sample.

Apparatus

- Muffle furnace, equipped with a thermostat, set to 575 + 25 °C
- Analytical balance, accurate to 0.1 mg.
- Desiccator containing desiccant
- Ashing crucibles, 50 mL, porcelain, silica, or platinum
- Porcelain markers, high temperature, or equivalent crucible marking method
- Ashing burner, ignition source, tongs, and clay triangle with stand
- Convection drying oven, with temperature control of 105 ± 3°C, optional

Procedure

Using a porcelain marker, mark an appropriate number of crucibles with identifiers, and place them in the muffle furnace at 575 +25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator. If using a furnace set to 575 + 25 °C, cool for a specific period of time, one hour is recommended. Record the cool time. Weigh the crucibles to the nearest 0.1 mg and record this weight.

Place the sample back into the muffle furnace at 575 ± 25°C and dry to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

Weigh 0.5 to 2.0 g, to the nearest 0.1 mg, of a test specimen into the tared crucible. Record the sample weight. If the sample being analyzed is a 105°C dried test specimen, the sample should be stored in a desiccator until use.

Ash the samples using a muffle furnace set to 575 + 25 °C for 24 + 6 hours. When handling the crucible, protect the sample from drafts to avoid mechanical loss of sample.

Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.

Place the sample back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible. When allowing samples to cool in a desiccator, it is necessary to maintain the initial cool time [2].

Calculations

Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content.

$$ODW = \frac{Weight_{air\ dry\ sample} \cdot \%\ Total\ solids}{100} \qquad \textit{Equation 3}$$

where:

ODW_{sample} = weight of sample in milligrams

Calculate and record the percentage ash on an ODW basis.

$$\%\ Ash = \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{ODW_{sample}} \cdot 100 \qquad \textit{Equation 4}$$

$$\%\ Volatile\ Solids = 100 - \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{ODW_{sample}} \cdot 100 \qquad \textit{Equation 5}$$

2.1.3 Determination of extractives in biomass

Introduction

It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material, among others. Inorganic material in the water soluble material may come from both the biomass and any soluble material that it is associated with the biomass, such as soil or fertilizer. Some biomass may require both extraction steps, while other biomass may only require exhaustive ethanol extraction.

This method is similar to ASTM Standard Test Method E 1690 “Determination of Ethanol Extractives in Biomass” for extraction procedures for isolation and characterization of extractives.

This procedure covers the determination of soluble non-structural materials in a biomass sample. The results are reported, on a dry weight basis, as a weight percentage of the biomass. Extractives

percentages are measured and used to convert compositions from an extractives-free basis to and as-received basis.

Apparatus

- Analytical balance, accurate to 1 mg or 0.1 mg
- Medium to large capacity oven set to 105 + 5 °C for glassware drying
- Vacuum oven set to 40 + 2 °C or drying oven set to 45 + 2 °C
- Apparatus for extraction, either Soxhlet or automatic
- Heating mantles, suitable for 500 mL boiling flasks
- Rotary evaporator with trap and water bath set to 40 + 5 °C

Reagents and materials

Reagents

- Water, HPLC grade
- Ethyl alcohol, 190 proof, USP grade

Materials

- Materials necessary for extraction
- Boiling flasks, round bottom, 500 mL capacity, 24/40 joint, equal to the number of extractions desired
- Teflon boiling chips or stir bars (stir bars may only be used with heating mantles equipped with stirring capacity)
- Cellulose filter paper, medium porosity, of appropriate size
- Buchner funnels, for paper diameter 70 mm or larger
- 200 mL volumetric flasks,
- Desiccator(s) containing desiccant, of a volume large enough to accommodate appropriate glassware

Procedure

Prepare the sample for extraction.

The moisture content of a biomass sample can change rapidly when exposed to air. Weigh samples for total solids determination at the same time as the samples for the extractives determination to avoid errors due to changes in humidity.

Prepare the apparatus for extraction.

Dry boiling flasks and other relevant glassware in a 105 + 5 °C drying oven for a minimum of 12 hours. Remove the glassware and allow it to come to room temperature in a desiccator. Add boiling stones (or stir bars if using heating mantles with stirring capacity) to the flasks, label clearly, and record the oven dry weight (ODW) to the nearest 0.1 mg.

Add 2-10 g of sample to a tared extraction thimble. Record the weight to the nearest 0.1 mg. The amount of sample necessary will depend on the bulk density of the biomass. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube. If the biomass height does exceed the siphon height, incomplete extraction will occur. Label the top edge of the thimble with a pencil.

Assemble the Soxhlet apparatus. Add a 250 mL bump trap between the receiving flask and the Soxhlet tube to control foaming if necessary. Insert the thimble into the Soxhlet tube.

Analyze the sample for water extractives

Add 190 + 5 mL of HPLC grade water to the tared receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 4-5 siphon cycles per hour.

Reflux for 6-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate. In some biomass, the reflux time is usually around eight hours, and any remaining water soluble material will be extracted during the ethanol extraction.

When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature.

If a successive ethanol extraction is to be performed, leave the thimble in the Soxhlet extractor, removing as much residual water from the Soxhlet tube as possible. If an ethanol extraction is not necessary, remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh HPLC grade water. Allow the solids to dry using vacuum filtration or air dry [3].

Calculations

Calculate the amount of extractives in the sample, on a percent dry weight basis.

$$\%\ \boldsymbol{Extractives} = \frac{\boldsymbol{Weight_{flask\ plus\ extractives} - Weight_{flask}}}{\boldsymbol{ODW_{sample}}} \cdot \mathbf{100} \qquad \textit{Equation 6}$$

2.1.4 Determination of nitrogen in biomass

Introduction

The Kjeldahl method is used to determine the nitrogen content in organic and inorganic samples. For longer than 100 years the Kjeldahl method has been used for the determination of nitrogen in a wide range of samples. The determination of Kjeldahl nitrogen is made in foods and drinks, meat, feeds, cereals and forages for the calculation of the protein content. Also the Kjeldahl method is used for the nitrogen determination in wastewaters, soils and other samples. It is an official method and it is described in different normatives such as AOAC, USEPA, ISO, DIN, Pharmacopeias and different European Directives. The Kjeldahl procedure involves three major steps: digestion, distillation and titration.

Apparatus

- Analytical balance, accurate to 1 mg or 0.1 mg
- Medium to large capacity oven set to 105 + 5 °C for glassware drying
- Distillation apparatus (Gerhard Vapodest 30s): 500- to 900-ml Kjeldahl digestion flask connected to distillation trap by rubber stopper; distillation trap connected to condenser with low-S tubing (outlet of condenser tube should be <4 mm in diameter)
- Digestion apparatus (Gerhard Kjeldatherm KB / KBL)

Reagents and materials

Reagents

- Salicylic acid/ Concentrated sulfuric acid solution
- Catalyst mixture (K_2SO_4 - $CuSO_4 \cdot 5H_2O$ - TiO_2)
- Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5\ H_2O$)
- Boric acid solution

- Concentrated sulfuric acid (H_2SO_4; 95% to 98%, nitrogen-free)
- 0.5 N hydrochloric or sulfuric acid standard solution
- Methyl red indicator solution
- Concentrated NaOH solution
- 0.1 N NaOH standard solution

Materials

- 500- to 900-ml Kjeldahl digestion flasks
- 8 to 14 mesh alumina boiling stones (Thomas Scientific)
- 500-mL or equivalent size titration beaker
- 25-mL burette

Procedure

Digestion process

In a digested tube, the weighed amount of solid sample is quantitatively transferred, and then 20 mL of the salicylic acid solution in sulfuric acid are added. The tube is shaken until the acid is thoroughly mixed with the solid sample. The mixture is left for several hours (or overnight). Then 2.5 g of sodium thiosulfate are added through a dry funnel to the bottom of the tube and the mixture is heated to 170±10˚C in the special Gerhard Kjeldatherm KB / KBL digestion apparatus for 30 minutes (until foaming stops). Then, the tube is cooled and 5.5 g of catalyst mixture are added.

The tube is placed back into the digestion apparaus where it is gently heated to boiling conditions for 2 hours at 400±10 ˚C. The tube is allowed to cool (10 min/300±10 ˚C, 10 min/200 ±10 ˚C).

NOTE: The temperature of the solution should not exceed 400˚C. The mixture is boiled gently for up to 5 hours, so that the sulfuric acid liquefies about 1/3 of the way up the neck of the tube. In most cases 2 h of boiling is sufficient.

At the end of the digestion, the tube is allowed to cool and transferred to the Vapodest distillation apparatus.

At least one blank sample (20 mL of salicylic acid/sulfuric acid solution, 2.5 g of sodium thiosulfate and 5.5 g of catalyst mixture) is prepared at the same time and follows the same digestion, distillation and titration process.

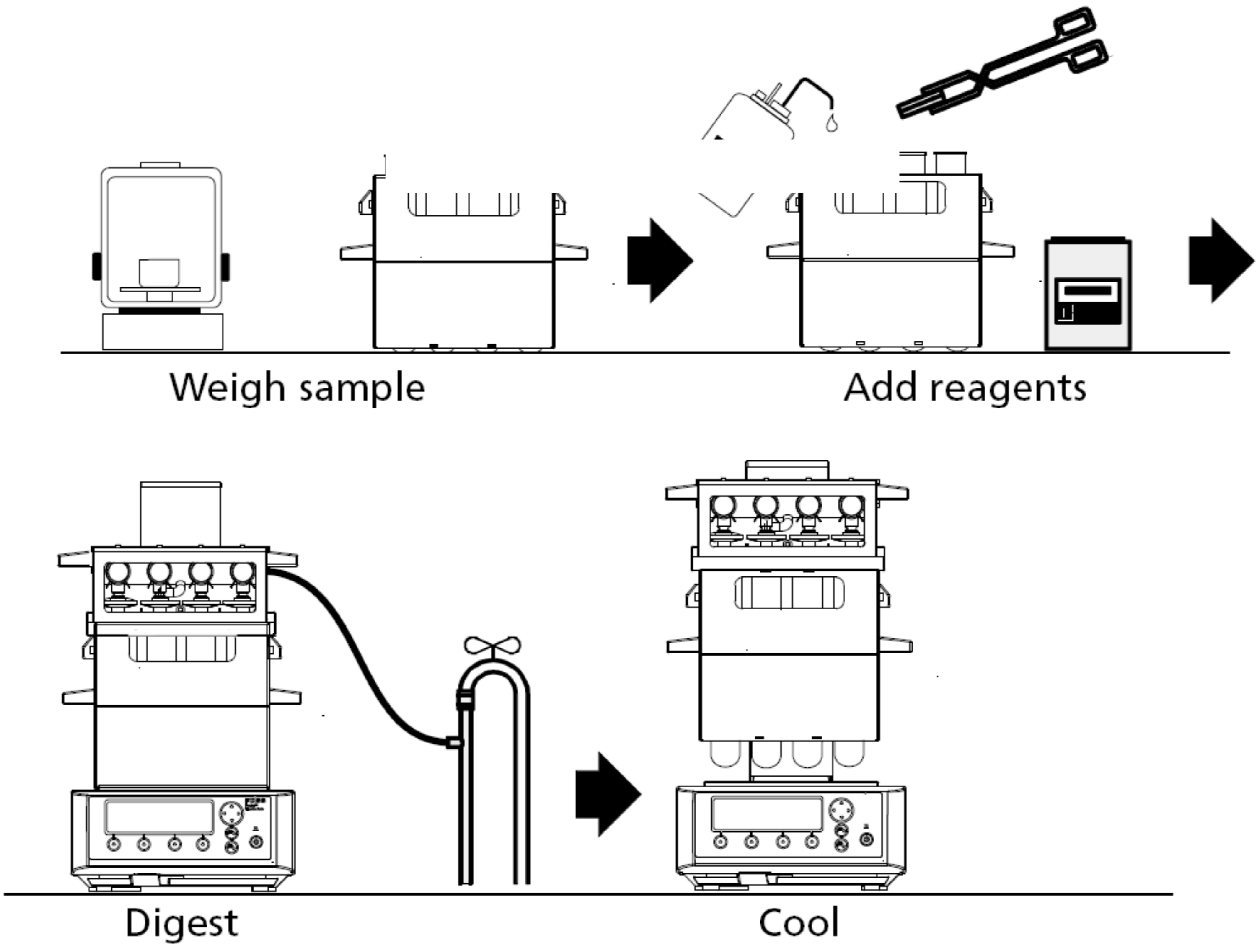

Figure 1. Digestion process

Distillation process

By opening the distillation apparatus, the tap water connected to the instrument should be also opened, so that there is flow in the system and the vapors formed are cooled down. When starting the still, it takes a quarter of an hour to warm up. There are four programs in the device:

00 TEST: Initial cleaning of the device

01 NITROGEN: Nitrogen determination process. 100 mL of 40% w/w sodium hydroxide solution and 100 mL of deionized water are automatically added. Distillation immediately starts capturing ammonia, which is released in the alkaline environment, from 25 mL of 2% w/v boric acid solution also containing an indicator.

02 CLEAN: Performed after each sample analysis for cleaning due to contamination

03 KATH: Performed at the end of all analyzes for the final cleaning of the device

The steps of the procedure are the following:

An initial cleaning of the device is carried out with the program 00 TEST.

The tube with the digested sample is attached to the distillation apparatus.

25 mL of boric acid and 3-4 drops of indicator are added to a 250 mL conical flask and the flask is placed under the condenser of the distillation apparatus.

The distillation program 01 NITROGEN is carried out.

The collected distillate in the conical flask is transferred to the titration unit.

The distillation program 02 CLEAN is carried out.

At the end of all distillations, the 03 KATH distillation program is carried out.

The collected distillate in the conical flask is titrated with the sulfuric acid to the end point with a color change from green to violet.

2.1.5 Determination of proteins in biomass

Introduction

Biomass used as a feedstock for conversion to fuels and chemicals may contain protein and other nitrogen containing materials. These constituents are measured as part of a comprehensive biomass analysis. Protein in biomass is difficult to measure directly. In many cases the nitrogen content of the biomass sample is measured by combustion or Kjeldahl methods and the protein content is estimated using an appropriate Nitrogen Factor (NF). There are published methods that recommend using an NF of 6.25 for all types of biomass except wheat grains where an NF of 5.70 is recommended [4].

2.1.6 Determination of structural carbohydrates and lignin in biomass

Introduction

Carbohydrates and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis. Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be removed using extraction or washing steps. Lignin is a complex phenolic polymer.

Parts of this procedure are substantially similar to ASTM E1758-01 "Standard Method for the Determination of Carbohydrates by HPLC."

This procedure is suitable for samples that do not contain extractives. Thus, extractives should be removed prior to this procedure. It uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into acid insoluble material and acid soluble material. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolyzed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. Protein may also partition into the liquid fraction.

Interferences

This procedure has been optimized for the particle size range specified in LAP "Preparation of Samples for Biomass Compositional Analysis". Deviation to a smaller particle size may result in a low bias in carbohydrate content (and consequent high lignin bias) due to excessive carbohydrate degradation. Deviation to a larger particle size may also result in a low bias in carbohydrate content (and consequent high lignin bias) due to incomplete hydrolysis of polymeric sugars to monomeric sugars.

Samples containing extractives are not suitable for this procedure. Extractives will partition irreproducibly, resulting in a high lignin bias. Furthermore, samples with an ash content above 10 wt % may not be suitable for this procedure, as the sample may contain soil or other minerals that will interfere with appropriate acid concentrations and may catalyze side reactions. Samples with a moisture content above 10 wt % may also not be suitable for this procedure, as the excess moisture will interfere with appropriate acid concentrations. Samples should be dried (air-dried or oven dried at less than 40°C) prior to this procedure.

Samples containing protein will bias the acid insoluble lignin high unless the protein is accounted for in the gravimetric determination of acid insoluble material. An independent nitrogen analysis is required to estimate the protein content of the residue. The protein estimate is then subtracted from the acid insoluble residue measurement.

Apparatus

- Analytical balance, accurate to 0.1 mg.
- Convection drying oven, with temperature control of 105 ± 3°C.
- Muffle furnace, equipped with a thermostat, set to 575 ± 25 °C or equipped with optional ramping program.
- Water bath, set at 30 ± 3 °C.
- Autoclave, suitable for autoclaving liquids, set to 121 ± 3 °C.
- Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles.
- Desiccator containing desiccant.
- HPLC system equipped with refractive index detector and suitable columns.
- UV-Visible spectrophotometer, diode array or single wavelength, with high purity quartz cuvettes of pathlength 1 cm.
- Automatic burette, optional.

Reagents and Materials

Reagents

- Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C)
- Calcium carbonate, ACS reagent grade.
- Water, purified, 0.2 µm filtered.
- 7.1.4 High purity standards: D-cellobiose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose.
- 7.1.5 Second set of high purity standards, as listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS).

Materials

- Pressure tubes, minimum 90 mL capacity, glass, with screw on Teflon caps and o-ring seals.
- Teflon stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes.
- Filtering crucibles, 25 mL, porcelain, medium porosity.
- Bottles, wide mouth, 50 mL.
- Filtration flasks, 250 mL.
- Erlenmeyer flasks, 50 mL.
- Adjustable pipettors, covering ranges of 0.02 to 5.00 mL and 84.00 mL.
- pH paper, range 4–9.
- Disposable syringes, 3 mL, fitted with 0.2 µm syringe filters.

Procedure

Prepare the sample for analysis and hydrolyze.

Place an appropriate number of filtering crucibles in the muffle furnace at 575 ± 25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator and cool for a specific period of time, one hour is recommended. Weigh the crucibles to the nearest 0.1 mg and record this weight. It is important to keep the crucibles in a specified order, if they are not marked with identifiers.

Place the crucible back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

Weigh 300.0 ± 10.0 mg of the sample into a tared pressure tube. Record the weight to the nearest 0.1 mg. Each sample should be analyzed in duplicate, at minimum.

Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

Place the pressure tube in a water bath set at 30 ± 3 °C and incubate the sample for 60 ± 5 minutes. Using the stir rod, stir the sample every 5 to 10 minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.

Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 ± 0.04 mL deionized water using an automatic burette. Dilution can also be done by adding 84.00 ± 0.04 g of purified water using a balance accurate to 0.01 g. Screw the Teflon caps on securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

The volume of the 4% solution will be 86.73 mL.

Prepare a set of sugar recovery standards that will be taken through the remaining hydrolysis and used to correct for losses due to destruction of sugars during dilute acid hydrolysis.

Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C, usually the liquids setting. After completion of the autoclave cycle, allow the hydrolyzates to slowly cool to near room temperature before removing the caps.

Analyze the sample for acid insoluble lignin as follows.

Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.

Transfer an aliquot, approximately 50 mL, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. Acid soluble lignin determination must be done within 6 hours of hydrolysis.

Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible. Rinse the solids with a minimum of 50 mL fresh deionized water. Hot deionized water may be used in place of room temperature water to decrease the filtration time.

Dry the crucible and acid insoluble residue at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.

Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.

Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.

Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the

nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight.

Analyze the sample for acid soluble lignin as follows.

On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.

Using the hydrolysis liquor aliquot obtained, measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer. Dilute the sample as necessary to bring the absorbance into the range of 0.7–1.0, recording the dilution. Deionized water or 4% sulfuric acid may be used to dilute the sample, but the same solvent should be used as a blank. Record the absorbance to three decimal places. Reproducibility should be ± 0.05

Calculate the amount of acid soluble lignin present using Equation 9.

Analyze the sample for structural carbohydrates

Prepare a series of calibration standards containing the compounds that are to be quantified. Use a four point calibration. A fresh set of standards is not required for every analysis.

Using the hydrolysis liquor obtained, transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.

Use calcium carbonate to neutralize each sample to pH 5–6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5–6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.

Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 µm filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate

HPLC conditions:

Injection volume: 10–50 µL, dependent on concentration and detector limits

Mobile phase: HPLC grade water, 0.2 µm filtered and degassed

Flow rate: 0.6 mL/minute

Column temperature: 80–85 °C

Detector temperature: as close to column temperature as possible

Detector: refractive index

Run time: 35 minutes [5].

Calculations

Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis.

$$\%\ \boldsymbol{AIR} = \frac{\boldsymbol{Weight_{crucible\ plus\ AIR} - Weight_{crucible}}}{\boldsymbol{ODW_{sample}}} \cdot \mathbf{100} \qquad \textit{Equation 7}$$

$$\%\ \boldsymbol{AIL} = \frac{(\boldsymbol{Weight_{crucible\ plus\ AIR} - Weight_{crucible}}) - (\boldsymbol{Weight_{crucible\ plus\ ash} - Weight_{crucible}}) - \boldsymbol{Weight_{protein}}}{\boldsymbol{ODW_{sample}}} \cdot \mathbf{100} \qquad \textit{Equation 8}$$

where:

$Weight_{protein}$ = Amount of protein present in the acid insoluble residue. This measurement is only necessary for biomass containing high amounts of protein.

Calculate the amount of acid soluble lignin (ASL) on an extractives free basis.

$$\%\ \boldsymbol{ASL} = \frac{\boldsymbol{UV_{abs} \cdot Volume_{filtrate} \cdot Dilution}}{\boldsymbol{\varepsilon \cdot ODW_{sample} \cdot Pathlength}} \cdot \mathbf{100} \qquad \textit{Equation 9}$$

where:

UVabs = average UV-Vis absorbance for the sample at appropriate wavelength

$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 86.73 mL

$$\boldsymbol{Dilution} = \frac{\boldsymbol{Volume_{sample} + Volume_{diluting\ solvent}}}{\boldsymbol{Volume_{sample}}} \qquad \textit{Equation 10}$$

ε = Absorptivity of biomass at specific wavelength

ODW_{sample} = weight of sample in milligrams

Pathlength = pathlength of UV-Vis cell in cm.

Calculate the total amount of lignin on an extractives free basis.

$$\%\ \boldsymbol{Lignin_{ext\ free}} = \%\ \boldsymbol{AIL} + \%\ \boldsymbol{ASL} \qquad \textit{Equation 11}$$

Calculate the total lignin value to an as received basis, if necessary:

$$\%\boldsymbol{Lignin_{as\ received}} = \left(\%\ \boldsymbol{Lignin_{ext\ free}}\right) \cdot \frac{\boldsymbol{(100 - \%Extractives)}}{\mathbf{100}} \qquad \textit{Equation 12}$$

where:

% Extractives = percent extractives in the prepared biomass sample.

Create a calibration curve for each analyte to be quantified using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.

Calculate and record the amount of each calibration verification standard recovered following HPLC analysis.

$$\%\ \boldsymbol{CVS\ recovery} = \frac{\boldsymbol{conc.detected\ by\ HPLC,mg/mL}}{\boldsymbol{known\ conc.of\ standard,mg/mL}} \cdot \mathbf{100} \qquad \textit{Equation 13}$$

For the sugar recovery standards, calculate the amount of each component sugar recovered after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis. Average any replicate ($\%R_{sugar}$) values obtained for each individual sugar and report $\%R_{avg,\ sugar}$.

$$\%\ \boldsymbol{R_{sugar}} = \frac{\boldsymbol{conc.detected\ by\ HPLC,mg/mL}}{\boldsymbol{known\ conc.of\ sugar\ before\ hydrolysis,mg/mL}} \cdot \mathbf{100} \qquad \textit{Equation 14}$$

Use the percent hydrolyzed sugar recovery values calculated in the previous step to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis.

$$\boldsymbol{C_x} = \frac{\boldsymbol{C_{HPLC} \cdot dilution\ factor}}{\%\ \boldsymbol{R_{ave.sugar}}/\mathbf{100}} \qquad \textit{Equation 15}$$

where:

C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL

$\%\ R_{ave.\ sugar}$ = average recovery of a specific SRS component

$C_x = C_{cor.\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis.

Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or 162/180) for C-6 sugars (glucose, galactose, and mannose).

$$\boldsymbol{C_{anhydro} = C_{corr} \cdot Anhydro\ correction} \qquad \text{Equation 16}$$

2.1.7 Determination of starch in biomass

Introduction

Starch, a non-crystalline glucose polymer, is often found in biomass feedstock that contains grain. This is a procedure that is based on the Megazyme Total Starch Assay (amyloglucosidase/α-amylase method). Extraction of the biomass is recommended prior to the starch assay to remove any nonstructural free glucose. Failure to remove free glucose will artificially elevate the starch content of the biomass sample. If this procedure is performed in conjunction with carbohydrate (cellulose and hemicelluloses) determination, the contribution of glucose from starch will be included in the total glucose value.

Apparatus

- Grinding mill: centrifugal, with 12-tooth rotor and 0.5 mm sieve, or similar device.
- Bench centrifuge: capable of holding 101 x 65 mm polypropylene tubes, with rating of approx. 3250 rcf (~ 4000 rpm)
- Microfuge centrifuge: capable of 13000 rpm.
- Spectrophotometer: capable of operating at 510 nm, (10 mm path length).
- Analytical balance: 0.1 mg readability, accuracy and precision.
- Thermostatted water bath: set at 50°C.
- Boiling water bath: with tube rack.
- Magnetic stirrer
- Magnetic stirring bars
- Vortex mixer
- Pipettors: capable of delivering 100 µL or 1.0 mL
- Dispensers to dispense 4 mL and 10 mL of 100 mM sodium acetate buffer (pH 5.0)
- Disposable polypropylene tube: 13 mL, 101 x 16.5 mm
- Disposable 2.0 mL polypropylene microfuge tubes
- Glass test tubes
- Digestion tubes

Reagents

- Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM): Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L and store the buffer at 4°C. Stable for > 6 months at 4°C.
- Sodium acetate buffer (200 mM, pH 4.5) plus calcium chloride (5 mM): Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 60 mL is required). Adjust the volume to 1 L. Stable for > 6 months at 4°C.
- Sodium acetate buffer (600 mM, pH 3.8) plus calcium chloride (5 mM): Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 1600 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Add 1.48 g of calcium chloride dihydrate and dissolve. Adjust the volume to 2 L with distilled water. Stable for > 12 months at room temperature.
- Sodium hydroxide solution (1.7 M): Add 68 g NaOH to 900 mL of deionised water and dissolve by stirring. Adjust the volume to 1 L. Store in a sealed container. Stable for > 2 years at room temperature.
- Ethanol (~ 50% and ~ 80% v/v): Add 500 mL of either ethanol (95% v/v) to 500 mL of distilled water. ~80% v/v: Add 800 mL of either ethanol (95% v/v) to 200 mL of distilled water. Store in a 1 L Duran bottle. Stable for > 4 years at room temperature.

Procedure

Mill sample to pass a 0.5 mm screen.

Accurately weigh ~ 100 mg of test sample, in duplicate (one as a sample blank) into Corning culture tubes (16 x 120 mm). Record the exact weight. Tap the tube so that sample drops to the bottom of the tube.

To both of the tubes, add 10 mL of sodium acetate buffer (100 mM, pH 5) plus calcium chloride (5 mM). Stir the tubes vigorously on a vortex mixer for 5 sec.

To one of the tubes (sample tube), add 0.1 mL of undiluted thermostable α-amylase with a 5 mL tip. To the second tube (sample blank) add 0.1 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM).

Vortex the tubes for 3 sec, cap the tubes loosely and immediately transfer them to a boiling water bath and start the timer. After approx. 2 min, tighten the caps and mix the tube contents vigorously on a vortex mixer. After further 5 and 10 min, vortex the tube contents again for 5 sec and return the tubes to the boiling water bath. After 15 min (from addition of α-amylase), remove tubes from the boiling water bath and mix the contents vigorously for 5 sec on a vortex mixer. Place the tubes in a water bath at 50°C and allow them to equilibrate to temperature over 5 min.

To one of the tubes (the sample tube), add 0.1 mL of undiluted AMG (3300 U/mL) and vortex for 3 sec. To the second tube (the sample blank) add 0.1 mL of sodium acetate buffer (100 mM pH 5.0) plus calcium chloride (5 mM). Incubate the tubes at 50°C for 30 min with no further mixing.

Remove the tubes from the water bath and allow them to cool to room temperature over 10 min. Invert the tubes a few times to ensure condensed water on the inside of the lid is mixed with liquid in the tube.

Transfer 2.0 mL of each solution (sample and sample blank) to microfuge tubes and centrifuge the tubes at 13000 rpm for 5 min. Accurately transfer a 1.0 mL aliquot of the supernatants to 12 x 120 mm tubes containing 4 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) and mix the contents.

Accurately transfer duplicate 0.1 mL aliquots of each sample to the bottoms of 16 x 120 mm glass test tubes. Also transfer a single 0.1 mL aliquot of sample blanks to a 16 x 120 mm glass test tube.

Add 3.0 mL of GOPOD reagent and incubate the solutions at 50°C for 20 min and measure absorbance against the reagent blank at 510 nm.

Concurrently incubate:

Glucose controls: 0.1 mL of glucose standard solution (1.0 mg/mL) plus 3.0 mL of GOPOD reagent, in quadruplicate.

Reagent Blank: 0.1 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) with 3.0 mL of GOPOD reagent in duplicate [6].

Calculations

Calculations for solid samples:

$$\textbf{Starch}, \% = \Delta A \cdot F \cdot \frac{EV}{0.1} \cdot D \cdot \frac{1}{1000} \cdot \frac{100}{W} \cdot \frac{162}{180} = \Delta A \cdot F \cdot EV \cdot \frac{D}{W} \cdot 0.90$$ *Equation 17*

where:

ΔA = absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank (only where a sample blank is determined).

F = factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose).

EV = sample extraction volume [10.2 mL for procedure].

0.1 = volume of sample analysed.

D = further dilution of sample solution (either undiluted, or diluted 5-fold or 11-fold)

1/1000 = conversion from mg to mg.

100/W = conversion to 100 mg sample; W = sample weight in mg.

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

$$\textbf{Starch}\ \%\ w/w\ (\textbf{dry wt. basis}) = \textbf{Starch}\ \%\ w/w\ (\textbf{as is}) \cdot \frac{100}{100 - \textbf{moisture content}\ (\%\ w/w)}$$

Equation 18

2.1.8 Determination of glucose in liquid samples

Introduction

The method of glucose determination is based on the conversion of glucose into a red product, which is the result of the successive action of the enzymes oxidase and glucose peroxidase. Initially, glucose is converted to gluconic acid by oxidase with simultaneous production of hydrogen peroxide. Subsequently, hydrogen peroxide in the presence of aminophenazone and a phenolic derivative by the action of peroxidase is converted to a red product which has a maximum absorption of 510 nm. The 2 reactions mentioned are presented below (GOD = oxidase, POD = peroxidase):

$$\text{Glucose} \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Aminophenazone} + \text{Phenolic derivative} \xrightarrow{\text{POD}} \text{Product (of red colour)}$$

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of glucose.

Reagents and Materials

Reagents

A commercial kit by BIOSIS S.A. (https://www.biosis.com.gr/) is used that includes a sugar reagent R1 and a standard glucose solution R4 (100mg/dL).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

To determine the free glucose in the samples, the samples are initially filtered and then the filtrates are properly diluted. Then, 2 mL of working solution R1 and 0.1 mL of filtrate from each diluted solution are added to test tubes. To prepare the blank, 2 mL of working solution and 0.1 mL of deionized water are added to a test tube. The test tubes are then placed for incubation in a water bath at 37 °C for 15 minutes. At the end of the incubation period, each test tube is stirred in the Vortex-Genie 2 device and then its absorbance is measured on a Spectroquant Pharo 300 Merck photometer, at a wavelength of 510 nm. The photometer is zeroed out with deionized water before starting the measurement of the samples.

The presence of glucose in the sample is evident by the coloring of the solution from colorless to light pink when the sample is added. If the sample is not coloured, this would mean that either the sample does not contain glucose or that the dilution is very high.

Calculations

Free glucose is calculated by the following equation:

$$\textbf{\textit{Free glucose}}\ (\%\,{}^{w}/_{w}) = \frac{0{,}71 * \textbf{\textit{ABS}}_{mean} * D * (Vtot) * 10^{-3}}{\textbf{\textit{initial solid}}} * \mathbf{100} \qquad \textit{Equation 19}$$

where:

ABSmean = the average absorption

D = the dilution (D = 1 when the solution remains undiluted, D = 10 when the dilution is 1:10)

Vtot = the total volume of the filtrate

initial solid = the initial mass of the sample.

2.1.9 Determination of total reducing sugars in liquid samples

Introduction

Carbohydrates are classified into 3 categories, which are analyzed below:

1. **Monosaccharides:** These are the carbohydrates that can not be hydrolyzed further in order to give a simpler poly-hydroxy unit aldehyde or ketone. Monosaccharides can be classified according to the number of carbon atoms and the active group of their molecule in trioses (with 3 carbon atoms), tetroses (with 4 carbon atoms), pentoses (with 5 carbon atoms), hexoses (with 6 carbon atoms) etc. Also, if the monosaccharide has an aldehyde group in the molecule, it belongs to the group of aldoses and if it has a ketone group it belongs to the group of ketoses. There are about 20 monosaccharides in nature and most commonly contain 5 (pentoses) or 6 (hexoses) carbon atoms: glucose, fructose, ribose, arabinose, xylose etc.

2. **Oligosaccharides:** These are the carbohydrates that during their hydrolysis can yield 2 to 10 units of monosaccharides. Depending on the number of monosaccharides yielding by hydrolysis, they are divided into disaccharides, trisaccharides, tetra-saccharides, etc. Typical examples of disaccharides are sucrose which when hydrolyzed gives an equimolar mixture of glucose and fructose and maltose which when hydrolyzed gives 2 molecules of glucose.

3. **Polysaccharides:** These are the carbohydrates that during their hydrolysis produce large number of monosaccharides. Examples of polysaccharides are starch, cellulose, hemicellulose, glycogen etc.

In addition, carbohydrates can be categorized as reducing or non-reducing. Reducing carbohydrates are those that can function as reducing agents due to the presence of free aldehyde or ketone group in their molecule. This property is used by the DNS method to quantify sugars in a solution. Reduction of 5- dinitrosalicylic acid leads to the formation of a 3-amino-5-nitro-salicylic acid product which shows an absorption maximum at 540 nm.

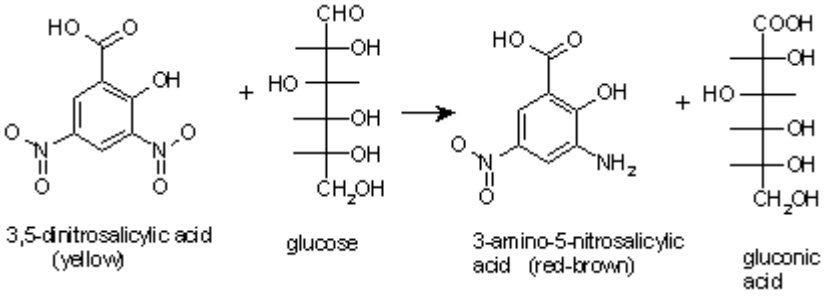

Figure 2. Reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitro-salicylic acid in the presence of glucose.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of total reducing sugars (TRS).

Reagents and materials

Reagents

- 3,5- dinitrosalicylic acid solution.
- NaOH solution.

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.

- Whatman filter papers.

Procedure

Total reducing sugars are determined by the 3,5-dinitrosalicylic acid method. Reduction of 3,5-dinitrosalicylic acid (yellow) leads to the formation of a 3-amino-5-nitro-salicylic acid product (orange-yellow) which shows an absorption maximum at 540 nm. 250µL of each sample (diluted or not) is added to test tubes along with 250µL DNS. The test tubes are boiled for 15 min to incubate and destroy any microorganisms that have grown and consumed a certain amount of sugar. Immediately after heating, the test tubes are removed from the water bath and then 2 mL of water are added. Finally, the absorptions at a wavelength of 540nm are recorded from which, the concentrations of reducing sugars are calculated.

Calculations

The measured absorptions are introduced into the proper calibration curves. More specifically:

*TRS (mg/L) = 1.6058 * ABSmean * D* *Equation 20*

where:

ABSmean: the average absorption

D: The dilution performed in the solution (D = 1 when the solution is not diluted, D = 5 when the dilution is 1: 5 and D = 10 when the dilution is 1:10).

2.1.10 Determination of ethanol in liquid samples

Introduction

Ethanol is ubiquitous in its natural occurrence, and thus its quantitative determination is not only important in the manufacture of intoxicating wines, beers and spirits, but also for low-alcohol and non-alcoholic beverages, fruit juices and a range of other foodstuffs, including chocolates, sweets, jam, honey, vinegar and dairy products. A large range of non-foods also contain significant quantities of ethanol, such as cosmetics and pharmaceuticals.

Alcohol dehydrogenase (ADH) catalyses the oxidation of ethanol to acetaldehyde coupled with the reduction of nicotinamide-adenine dinucleotide (NAD^+).

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

The amount of NADH formed in this reaction pathway is stoichiometric with the amount of ethanol. It is the NADH which is measured by the increase in absorbance at 340 nm.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of ethanol.

Reagents and materials

Reagents

A commercial kit by Megazyme (https://www.megazyme.com/) is used that includes the following reagents:

- Reagent 1 (120 mL) Contains sodium azide (0.02% w/v) as a preservative.
- Reagent 2 (30 mL) Contains sodium azide (0.02% w/v) as a preservative.
- Ethanol Standard (5 mL, 5 mg/mL).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

The following procedure is used to determine ethanol:

Place 2 mL of Reagent 1 in a glass vial and add 0.1 mL of the sample.

In another glass vial prepare the blank sample. 2 mL of Reagent 1 ethanol are placed in a glass vial and 0.1 mL of deionized water is added.

After 3 minutes, add 0.5 mL of Reagent 2 to the glass vials and leave to stand.

After 7 minutes the samples are photometered at 340nm, after stirring in the Vortex Genie 2 stirrer and resetting the photometer with deionized water.

Calculations

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{ethanol}$. The value of $\Delta A_{ethanol}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of ethanol can be calculated as follows:

$$c = \frac{V \cdot MW}{\varepsilon \cdot d \cdot v} \cdot \Delta A \quad \left(\frac{g}{L}\right) \qquad \textit{Equation 21}$$

where:

V = final volume (mL)

MW = molecular weight of ethanol (g/mol)

ε = extinction coefficient of NADH at 340 nm = 6300 (l x mol^{-1} x cm^{-1})

d = light path (cm)

v = sample volume (mL)

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

2.1.11 Determination of total organic carbon

Introduction

Total Organic Carbon (TOC) is a measure of the total amount of carbon in organic compounds in pure water and aqueous systems. TOC has become an important parameter used to monitor overall levels of organic compounds present. This has happened despite the lack of any direct quantitative correlation between total organic carbon and the total concentration of organic compounds present and reflects the importance of having an easy-to-measure, general indicator of the approximate level of organic contamination. It also reflects the appeal of a parameter which has a name which

sounds more fundamental than it is. In many cases, the TOC is used as an on-going monitor of change or lack of change in organic content.

Apparatus

A SHIMADZU TOC-VCSH TOC analyser is used in order to quantify the concentrations of total, organic and inorganic carbon in liquid samples. The carbon content of solid samples is quantified by applying the Solid Sample Module (SSM5000A).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Porcelain capsules.
- Whatman filter papers.

Procedure

The operation principle of the method is based on the calculation of the amount of carbon dioxide produced resulting from the oxidation of carbonaceous compounds present in the test solutions. The samples enter the combustion tube of the apparatus and the total carbon contained in the sample is oxidized at 720^{o}C to form carbon dioxide. A carrier gas is then utilized which carries the various combustion products along with the carbon dioxide. In the case of this instrument (SHIMADZU TOC-VCSH), ultra-clean air was used as the carrier gas. With the help of ultra-clean air, the combustion products are transferred to a dehumidifier in order to remove the moisture while at the same time all the gases produced during the initial combustion are cooled. The carrier gas then passes through a halogen trap and ends up in the NDIR (Non Dispersive Infra Red gas analyzer) cell, where carbon dioxide is detected. It is worth noting that in the way described above the total carbon present in the samples, organic and inorganic, is measured. Inorganic carbon is in the form of carbon dioxide and is not bound to organic compounds. Therefore, when the measurement of inorganic carbon (IC) is required, then the instrument automatically removes the IC from the total carbon (TC) and thus calculates the organic carbon that was initially present in the sample.

The experimental TOC determination process of solid samples starts by weighing a 40 mg sample, which is ground to a size of 1 mm and dried at 80°C. These samples are placed in porcelain capsules. The capsules are then placed in the special oven of the instrument where the sample are heated to 900ºC. At this temperature the organic carbon is burned and its exhaust gases are carried to the TOCVCSH central unit. The mass of organic carbon (mg) of CO_2 produced during combustion is calculated in this unit.

Calculations

The instrument provides the concentrations of TC, TOC or TIC of the liquid samples and just the dilutions made should be taken into account.

The (%) percentage of organic carbon in the solid samples is given by the following formula:

$$\boldsymbol{TOC}\ (\%) = \frac{\frac{m_1}{M_1} + \frac{m_2}{M_2}}{2} \cdot \mathbf{100} \qquad \textit{Equation 22}$$

where:

m_1: the measured mass of organic carbon of the first sample (mg)

m_2: the measured mass of organic carbon of the second sample (mg)

M_1: the initial mass of the first sample (mg)

M_2: the initial mass of the second sample (mg).

2.1.12 Determination of volatile fatty acids

Introduction

Volatile fatty acids (VFA) are the important intermediates indicating the stability and performance of fermentation process but they are also the product of delignification process. In anaerobic digestion, VFA accumulation reflects a kinetic uncoupling between acid formers and consumers and is typical of the stress situation caused by hydraulic or organic overloading, sudden temperature variations, or the presence of toxic or inhibitory compounds, among other factors. Thus, the monitoring of their concentration is of crucial importance.

In an acidic medium lower fatty acids (VFA) react with a diole to form fatty acid esters, which are subsequently converted into hydroxamic acids with hydroxylamine. These in turn react with iron(III) ions to form red complexes that are determined photometrically.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of volatile fatty acids.

Reagents and materials

Reagents

A commercial kit (Spectroquant Volatile Organic Acids Test 1018909) by Merck (https://www.merckmillipore.com/) is used that includes Reagents OA-1 to OA-5.

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

The samples must be analyzed immediately after sampling. Otherwise they should be stored at 4 °C for less than 24 hours.

The pH must be within the range 2 - 12.

Turbid samples must be filtered.

Pipette 0.75 mL Reagent OA-1 into a clean round cell.

Add 0.50 mL Reagent OA-2 with pipette.

Add 0.50 mL of pretreated or diluted sample with pipette, close the cell tightly, and mix.

Heat the cell at 100 °C in the preheated thermoreactor for 15 min, then cool to room temperature under running water.

Add 1.0 mL Reagent OA-3 with pipette.

Add 1.0 mL Reagent OA-4 with pipette, close the cell tightly, and mix.

Add 1.0 mL Reagent OA-5 with pipette, close the cell tightly, and mix.

A transient turbidity or precipitate may form.

Leave to stand for 1 min (reaction time), then measure the sample in the photometer.

Calculations

The instrument provides the concentration of volatile fatty acids as mg/L of acetic acid and just the dilutions made should be taken into account.

2.1.13 Determination of fiber and lignin contents (Van Soest analysis)

Introduction

The Van Soest analysis aims to identify fractions (polymer classes) of cell wall components, which are as free as possible from other feed components (e.g. proteins) by removing the cellular content with appropriate solutions.

This method is applicable to grains, feeds, forages, and all fiber-bearing materials. The Neutral Detergent Fiber (NDF), at first, determines the residue remaining after digesting in a detergent solution. These fiber residues are predominantly hemicelluloses, cellulose and lignin. Then, a follow-up procedure aims to determine the Acid Detergent Fiber (ADF), which is the fiber cellulose and lignin residue (cellulose and lignin) remaining after processing with acid detergent. Last, the Acid Detergent Lignin (ADL) can then be quantified using sulfuric acid extraction and ashing to determine the residue lignin.

Apparatus

- Electronic analytical balance with the least accuracy of 0.1mg.
- Desiccator.
- Autoclave, able to achieve 100°C at 10-25 psi.
- Furnace at 550°C.

- Oven at 105°C.

Reagents and materials

Reagents

- Distilled De-Ionized water (DDI water)
- Purified acetone
- NDS:

In 1 L distilled H_2O,

- Add 30.0 g Sodium dodecyl sulfate, USP ($NaC_{12}H_{25}SO_4$),
- Add 18.61g of EDTA disodium salt, dehydrated,
- Add 6.81 g Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$),
- Add 4.56 g Sodium phosphate dibasic, anhydrous (Na_2HPO_4) or 5.72 g $Na_2HPO_4 \cdot H_2O$,
- Add 10.0 ml Triethylene glycol ($C_6H_{14}O_4$),
- Check pH ranges from 6.9 to 7.1. Agitate and heat to aid the solution.
- 1N H_2SO_4:
- Prepare in a fume hood.
- Add approximately 500 mL of DDI water in a 1L volumetric flask followed by 28 mL concentrated H_2SO_4.
- Allow the solution to cool in a water bath before bringing it to volume with Distilled De-Ionized (DDI) water.
- ADS:
- Add 20 g of Cetyl-trimethylammonium bromide (CTAB) to 1 L of 1N H_2SO_4. Agitate and heat to aid the solution process.
- 72% Sulfuric Acid (H_2SO_4)
- Na_2SO_3.

Materials

- Fiber filter bags.
- Heat Sealer.
- 50 mL, 500 mL, and 1 L beakers.
- Chemical resistant marker.
- 250 mL Borosilicate glass bottles (BORO bottles).

- Crucibles, large enough to hold 40-50 mL liquid.
- Forceps.

Procedure

Weigh and record the weight of each empty filter bag (W1). It is not necessary to pre-dry filter bags. Any moisture will be accounted for by the blank bag correction.

Weigh 0.45-0.55 g of prepared sample (W2) directly in the filter bag. Avoid placing the sample on the upper 4mm of the bag. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top.

Weigh one blank bag and include it in the run to determine blank bag correction (**C**).

Next, the samples are placed into BORO bottles of 250 mL volume with 100 mL of NDS and 0.5 g/50mL NDS of Na_2SO_3. The process of heating (NDF extraction) takes place in an autoclave (ISOLAB Laborgerate) for 75 minutes at 100°C.

After the samples are cooled to ambient temperature, rinse with hot DDI water to remove NDS (About 8-10 washouts for 5 minutes). During the incubation, stir and pressurize the samples with the help of forceps. If pH levels are above 6, stop the rinsing process.

Then the bags are placed in a 250 mL beaker with enough acetone to soak the bags for 5 - 10 minutes. Remove the samples and wait till the evaporation of acetone to place them into the oven at 105°C for 2-4 hours. *CAUTION: Do not place bags in the oven until the acetone has completely evaporated. Extended drying times or too high temperatures can compromise the bag's filtration media.*

Remove the filter bags from the oven and immediately place them directly into a collapsible desiccant pouch and flatten them to remove any air. Cool to ambient temperature and weigh the filter bags (**W3**). Calculate blank bag correction using weight loss of a blank bag upon NDF procedure (**C1**). At this point, the NDF content can be calculated.

Afterwards, place the fiber bags in BORO bottles of 250 mL volume and repeat the previous steps, while using the ADS Solution instead of the NDS Solution. In addition, the processing time is 60 minutes instead of 75. In the last step weigh the filter bags (W4) and calculate blank bag correction using the weight loss of a blank bag upon ADF procedure (C2), to determine the ADF content.

After the ADF extraction, the dry bags/samples are placed into 100 mL beakers along with a sufficient quantity (approximately 20 – 30 mL) of 72% H_2SO_4 to cover the bags.

CAUTION: Bags must be completely dry and at ambient temperature before adding concentrate acid to prevent heat generation by the acid and water reaction and affect the results.

Place weight inside the beaker to submerge the bags. Agitate bags at the beginning and at 30-minute intervals for 3 hours, by pushing and lifting the weight up and down approximately 30 times.

After 3 hours, pour off H_2SO_4 and rinse with warm water to remove all acid. Repeat rinses until the pH is neutral.

Rinse with approximately 250 ml of pure acetone for 3 minutes to remove water and let it evaporate. *CAUTION: Do not place bags in the oven until acetone is completely evaporated.*

Place bags in the oven at 105°C for 3 – 4 hours.

Remove bags from the oven and cool in a desiccator. Flatten it to remove air and weigh the pouch (**W5**). Calculate blank bag correction using weight loss of a blank bag upon sulfuric acid extraction (**C3**).

Then, add bags to a dry crucible and weigh them before (**W6**) placing them in the furnace at 550°C for 3 hours. Cool the crucibles to ambient temperature post and weigh (**W7**). Calculate blank bag ash correction using weight loss of a blank bag upon ignition (**C4**). Finally, calculate the ADL content.

Calculations

The Neutral Detergent Fiber (NDF), the Acid Detergent Fiber (ADF) and the Acid Detergent Lignin (ADL) contents can be calculated as follows:

$$\%\ \boldsymbol{NDF} = \frac{(\boldsymbol{W3}-(\boldsymbol{W1}*\boldsymbol{C1})}{\boldsymbol{W2}} * \mathbf{100} \qquad \textit{Equation 23}$$

$$\%\ \boldsymbol{ADF} = \frac{(\boldsymbol{W4}-(\boldsymbol{W1}*\boldsymbol{C2})}{\boldsymbol{W2}} * \mathbf{100} \qquad \textit{Equation 24}$$

$$\%\ \boldsymbol{ADL} = \frac{(\boldsymbol{W5}-(\boldsymbol{W1}*\boldsymbol{C3})) - (\boldsymbol{W6}-\boldsymbol{W7})-(\boldsymbol{W1}*\boldsymbol{C4})}{\boldsymbol{W2}} * \mathbf{100} \qquad \textit{Equation 25}$$

where,

W1: Empty fiber filter bag

W2: Initial dry sample weight prior processing

W3: Dried sample weight after NDF extraction process

W4: Dried sample weight after ADF extraction process

W5: Dried sample weight after sulfuric acid extraction process

W6: Dried sample weight prior ashing process in crucible

W7: Sample weight after ashing process

C1: NDF extraction blank bag correction

C2: ADF extraction blank bag correction

C3: Sulfuric acid extraction blank bag correction

C4: Ash blank bag correction.

2.2 In vitro digestibility

Procedure

4 in vitro series were set up for the determination of in-vitro digestibility of the samples. In each series, ruminal fluid was collected from one multiparous Latxa ewe slaughtered for this production purpose. Before slaughtering, ewes were fed a basal diet (80% meadow hay and 20% compound feed) for 3 weeks and had free access to fresh water and feed. Ruminal fluid was collected before the morning feeding and filtered through four layers of cheesecloth into a volumetric flask. Then, it was diluted in culture medium in a 1:4 ratio (ruminal fluid and phosphate-bicarbonate buffer, respectively) under anaerobic conditions according to Menke et al. [7].

Approximately 500 mg of orange peel samples were weighed into 125 mL serum bottles. Each sample was incubated in 50 mL of culture medium in triplicate, the bottles were crimp sealed and incubated at a constant temperature (39°C) in the incubator for 24 hours. Gas production was released at 2, 4, 6 and 22 hours post-inoculation to avoid the pressure in the bottle headspace exceeding 48 kPa, as suggested by Theodorou et al. [8]. After 24 hours of incubation, the bottles were put in the fridge for 20 min to stop fermentation for subsequent sampling of short chain fatty acids (SCFA) and in vitro organic matter digestibility (IVOMD) determination.

In vitro organic matter digestibility, in the short term in vitro trial was calculated as described by Pell and Schofield [9], where 45 mL of a neutral detergent solution was added to each bottle and warmed at 105 °C for 1 h; then, the bottles were cooled, filtered through glass filter crucibles (Porosity 2)

and washed with distilled water, ethanol and acetone. The remaining sample was dried at 100 °C overnight and then burned in a muffle furnace at 500 °C to obtain true IVOMD values.

The analysis of the SCFA (acetic, propionic, butyric, isobutyric, valeric and isovaleric) of rumen samples was performed by gas chromatography using a flame ionization detector.

3. Ingredients nutritional value characterization

According to Deliverable 2.2, the final animal feedstuff should be formulated by mixing the solid residue of orange peels after the saccharification process under the optimum conditions (50°C, 24h, 7.5% solids loading, Pectinex 25µL/g TS, CellicCTec3 25 µL/g TS) with the harvested yeast cultivated aerobically on orange peels hydrolysate (30°, 24h, orange peels hydrolysate as sugar source, nutrients addition, pH =5, pH and DO control).

According to the results of the experimental trials and the achieved yields, a mass balance of the applied treatment train is presented in Figure 3.

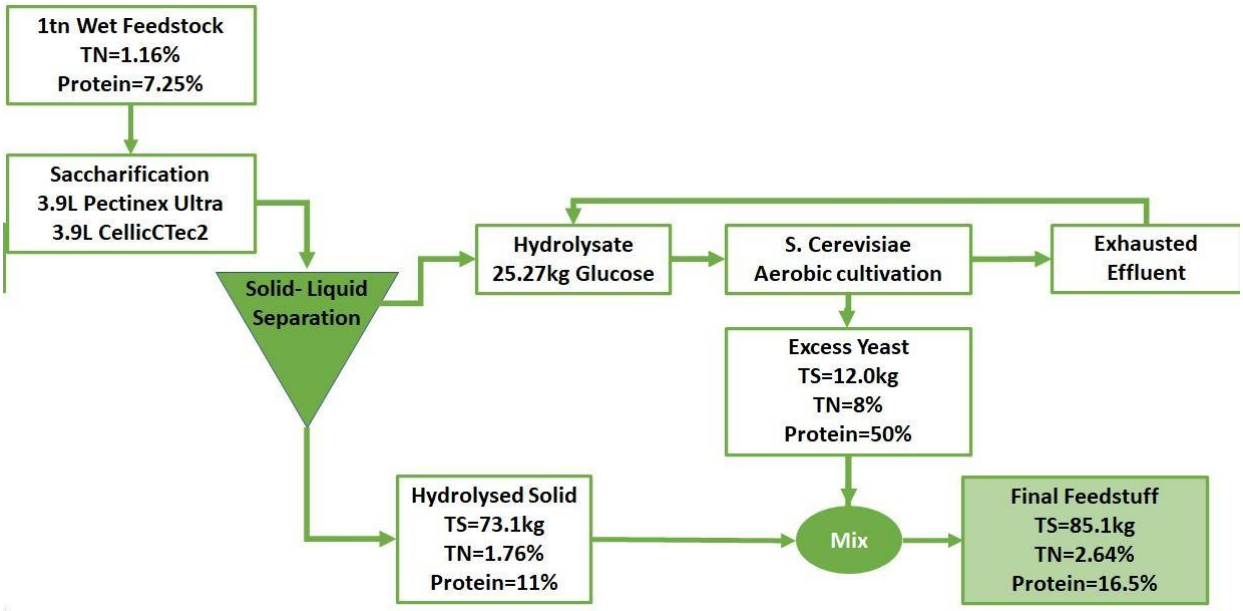

Figure 3. Flow chart of implemented strategy including mass balances

It is evident that the implementation of this strategy could lead to a final feedstuff with elevated protein content (16.5%). According to the presented mass balances and in line with a zero waste discharge concept, the mixing of excess yeast and hydrolysed solid (14% yeast) is the final step for the formulation of the animal feedstuff prior to drying.

In this context, samples of feedstuff prepared in the laboratory under the optimum conditions descripted above were formulated and characterized in physicochemical and nutritional terms (Table 1).

Table 1. Composition of dried unprocessed orange peels and feedstuff prepared under the optimum conditions of the implemented strategy

Parameter	Dried unprocessed orange peels	Feedstuff prepared under the optimum conditions
TS (%)	91.27	94.78
Moisture (%)	8.73	5.22
ASH (%)	4.81	5.03
VS (%)	95.19	94.97
Oil (%)	2.71	2.25
TN(%)	1.15	2.36
Crude Protein (%)	7.18	14.75
Cellulose (%)	20.58	6.80
Hemicellulose (%)	24.62	17.94
Acid Insoluble Residue (%)	12.98	18.92
Ether extract (%)	3.57	2.96
Neutral detergent fibre (NDF) (%)	38.32	28.54
Acid detergent fibre (ADF) (%)	29.66	17.81
Lignin Acid Detergent (ADL) (%)	5.24	6.43
In vitro organic matter digestibility (IVOMD) (%)	72.7	89.5

It is worth noticing that the feedstuff prepared under the optimum conditions of the implemented strategy presented higher (23.11%) in vitro organic matter digestibility and almost doubled protein content.

4. Feeding strategy

The dietary trial is going to include ewes of the Chios breed. The Chios breed is a high yielding Greek sheep breed and the mean body weight (BW) of Chios ewes has been reported to be 60 kg [10]. Experimental diets will be offered after weaning, starting on the 43rd day of lactation. At that time, ewes will be on their peak lactation stage, thus their diet must cover, both maintenance and high lactation needs.

Taking into account the results stated in "3. Ingredients value characterization", apart from the control diet, formulated for the needs of an adult Chios breed ewe producing 2L of milk per day, the inclusion of the following feedstuffs will be evaluated: Dried unprocessed orange peels (Experimental Material A-EMA) and Feedstuff prepared under the optimum conditions of the implemented strategy (Experimental Material B-EMB).

For the duration of the acclimation period and the main experiment, ewes will be offered 1.5kg of a basal concentrate (Table 2), 1 kg of alfalfa hay and 0.2kg of wheat straw daily, with a concentrate-to-forage ratio of 55.5:44.7. The concentrate for treatment (Control) will not be supplemented with any experimental material, while that for treatments EMA and EMB will contain 110g of supplemented experimental feedstuff per kg (as mixed basis), respectively (Table 2). Diets will be formulated to meet nutrient requirements of sheep for lactation [11] and ewes will be fed twice daily, after milking. Prior to the onset of the experimentation, ratio formulation will be updated according to the composition of raw materials and as lactation proceeds, according to the nutritional needs of the ewes. The nutritional composition of control and experimental diets is presented at Table 3.

Table 2. Basal concentrate composition (g/kg as mixed)

Ingredient composition	Diet		
	Control	EMA	EMB
Corn grain (ground)	300	300	300
Barley grain (ground)	200	200	200
Wheat bran	200	120	120
Soyabean meal	110	110	110
Sunflower meal	150	120	120
Experimental feedstuff	0	110	110
Limestone	5	5	5
Monocalcium phosphate	5	5	5
Salt	5	5	5
Vitamin & mineral premix	25	25	25
Total	1000	1000	1000

Table 3. Chemical composition of basal concentrate (g/kg of dry matter)

Chemical composition	Diet		
	Control	EMA	EMB
Dry matter (as fed)	836.75	841.70	845.56
Crude protein	148.43	140.57	148.90
Crude fat	19.12	21.56	20.89
Crude fibre	61.67	73.35	58.19
Neutral detergent fibre	153.21	171.44	160.68
Acid detergent lignin	74.59	94.34	81.30
Acid detergent fibre	3.61	8.68	9.99
Ash	25.70	27.52	27.76

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