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APPENDICES

APPENDICES A
PUBLICATIONS

- Buaphan Sirirat, V. Pattarajinda, M.A. Froetschel, M. Duangjinda, and Y. Opatpatanakit 2008. Effects of replacing dietary starch with sugar on nutrient digestibility and gas production technique. In: The Proceedings of the 13th Animal Science Congress, Asian-Asustralasian Association of Animal production Societies, September 22-26, Hanoi, Vietnam.
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- Sirirat Buaphan, Virote Pattarajinda, Mark. A., Froetschel, Monchai Duangjinda, and Yanin Opatpatanakit. 2010. Quantifying *in vitro* microbial protein synthesis response to soluble carbohydrate feeding. In: The Proceedings of RGJ-Ph.D. Congress XI on "Research Towards Sustainability". April 1-3, 2010. Jomtien Plam Beach Resort, Pattaya, Chonburi, Thailand. pp. 319.
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APPENDICES B
LAB PROCEDURE

***In Vitro* Digestibility using the DAISY^{II} Incubator
(ANKOM Method)**

1. Reagents

(a) Buffer Solution A:	g/l
KH ₂ PO ₄	10.0
MgSO ₄ ·7H ₂ O	0.5
NaCl	0.5
CaCl ₂ ·2H ₂ O	0.1
Urea (reagent grade)	0.5

(b) Buffer Solution B:	g/l
Na ₂ CO ₃	15.0
Na ₂ S ₉ H ₂ O	1.0

(c) Neutral Detergent Solution

2. Apparatus

- (a) ANKOM DAISY^{II} Incubator
- (b) Filtration device - F57 Filter Bags.
- (c) Impulse bag sealer - 1915/1920 Heat Sealer.
- (d) Thermos
- (e) ANKOM^{200/220} Fiber Analyzer

3. Procedure

Preparation of Filter Bags and Sample: Pre-rinse F57 filter bags in acetone for three to five minutes and completely air-dry. The acetone rinse removes a surfactant that inhibits microbial digestion. Weigh each F57 filter bag and record weight (W_1). Zero the balance and weigh 0.25g of sample (W_2) directly into filter bag. NOTE: For 48 hr studies a sample size of 0.5 g is acceptable. Heat seal bag closed and place in the digestion jar (up to 25 samples per jar). Samples should be evenly

distributed on both sides of the digestion jar divider. Include at least one sealed blank bag for correction factor (C_1).

Preparation of (combined) Buffer Solution: (For each digestion jar)

a) Pre-warm at 39°C both buffer solutions (A & B). In separate container add ~266 ml of solution B to 1330 ml of solution A (1:5 ratio). The exact amount of A to B should be adjusted to obtain a final pH of 6.8 at 39°C. No further adjustment of pH is necessary. Add 1600 ml of combined A/B mixture to each digestion jar.

b) Place the digestion jars with samples and buffer solution into Daisy^{II} Incubator and turn on heat and agitation switches. Allow temperature of digestion jars to equilibrate for at least twenty to thirty minutes.

Preparation of Inoculum and Incubation:

1. Preheat two 2 l thermos bottles by filling with 39°C water. Empty heated water just prior to collection of rumen inoculum. Using the appropriate collection procedure, remove at least 2000 ml of rumen inoculum and place in thermos. Include approximately two "fistfuls" of the fibrous mat from the rumen with your collection in one thermos.

2. Preheat a blender by filling with 39°C water. Empty the heated water just prior to pouring the rumen inoculum from the thermos into the blender. Purge the blender container with CO₂ gas and blend at a high speed for 30 seconds. The blending action serves to dislodge microbes that are attached to the mat and assure a representative microbial population for the *in vitro* fermentation. Filter the blended digesta through four layers of cheesecloth into a five-liter flask (pre-heated 39°C). Filter the remaining rumen fluid in the other thermos through four fresh layers of cheesecloth into the same five-liter flask. NOTE: Allow for extra cheesecloth around the edges to facilitate squeezing contents of filtered mat. The flask should be continually purged with CO₂ and continued during the transfer of the inoculum.

3. Remove one digestion jar from the Daisy^{II} Incubator and add the 400ml of inoculum to the buffer solution and samples. Purge the digestion jar with CO₂ gas for thirty seconds and secure lid.

4. Repeat process for all digestion jars to be used. NOTE: Do not allow CO₂ gas to bubble through the buffered inoculum, rather use the CO₂ to form a gaseous blanket over the contents of the jar.

5. Incubate for 48 hours. The DAISY^{II} Incubator will maintain a temperature of 39.5°C ± 0.5. If temperature of jars varies greater than one degree then move incubator to a warmer location or place blanket or similar insulator over incubator.

6. At completion of incubation, remove jars and drain fluid. Rinse bags thoroughly with cold tap water until water is clear. Use a minimum of mechanical agitation.

7. When determining True Digestibility it is necessary to remove microbial debris and any remaining soluble fractions using Neutral Detergent Solution. After rinsing the bags in water place them in the ANKOM^{200/200} Fiber Analyzer and follow the procedure for determining NDF. Record the post *in vitro* NDF weight as WB3B. NOTE: Bags can be stored in the refrigerator or freezer until NDF determinations can be performed.

4. Calculations:

$$\% \text{ IVDMD} = 100 - (W_3 - (W_1 \times C_1)) \times 100 \div (W_2 \times \text{DM})$$

Where: W₁ = Bag tare weight

W₂ = Sample weight

W₃ = Final bag weight after In Vitro and sequential ND treatment

C₁ = Blank bag correction (final oven-dried weight ÷ original blank bag weight)

5. References

Robinson, P. H., M. Campbell, Mathews, and J. G. Fade. 1999. Influence of storage time and temperature on in vitro digestion of neutral detergent fibre at 48 h, and comparison to 48 h in sacco neutral detergent fibre digestion. *Anim. Feed Sci. Technol.* 80:257-266.

Neutral Detergent Fiber in Feeds (Filter Bag Technique)

1. Apparatus

1. Analytical Balance (capable of weighing down to 0.1 mg).
2. Oven: capable of maintaining a temperature of $102 \pm 2^\circ\text{C}$.
3. Digestion instrument: capable of performing the digestion at $100 \pm 0.5^\circ\text{C}$ and maintaining a pressure of 10-25 psi). The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction (ANKOM200, 65 rpm agitation, ANKOM Technology).
4. Filter bags: constructed from chemically inert and heat resistant filter media), capable of being heat sealed closed and able to retain 25 micron particles while permitting rapid solution penetration (F57, ANKOM Technology).
5. Heat sealer: sufficient for sealing the filter bags closed to ensure complete closure (1915, ANKOM Technology).
6. Desiccator pouch: collapsible sealable pouch with desiccant inside that enables the removal of air from around the filter bags (MoistureStop Weigh Pouch, ANKOM Technology).
7. Marking pen: solvent and acid resistant (F08, ANKOM Technology).

2. Reagents

1. Neutral Detergent Solution, add 30.0 g Sodium dodecyl sulfate, USP; 18.61g Ethylenediaminetetraacetic disodium salt, dihydrate; 6.81 g Sodium borate; 4.56 g Sodium phosphate dibasic, anhydrous; and 10.0 ml Triethylene glycol, in 1 l distilled H_2O . Check pH range to 6.9 to 7.1. Agitate and heat to aid solution.

2. Alpha-amylase (Heat-stable bacterial alpha amylase: activity = 17,400 Liquefon Units / ml) (FAA, ANKOM Technology).
3. Sodium sulfite (Na_2SO_3 , anhydrous).

3. Preparation of sample

Grind samples in a centrifugal mill with a 2 mm screen or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

4. Procedure

1. Use a solvent resistant marker to label the filter bags. Weigh filter bag (W_1) and zero balance. Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction.
2. Weigh 0.45-0.55 g of prepared sample (W_2) directly in filter bag. Avoid placing the sample on the upper 4 mm of the bag.
3. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top. Use sufficient heat to completely seal the filter bag and allow enough cool time (2 sec) before removing the bag from the heat sealer.
4. Weigh one blank bag and include in run to determine blank bag correction (C_1).
5. Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the weight on top to keep it submerged. Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cold water and exhaust.
6. When processing 24 sample bags, add 1900- 2000 ml of ambient ND solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of ND solution (use minimum of 1500 ml to ensure Bag Suspender is covered). Add 20 g (0.5 g/50ml of ND solution) of sodium sulfite and 4.0 ml of alpha-amylase to the solution in the vessel.

7. Turn Agitate and Heat on and confirm agitation. Set timer for 75 min and close lid. 9. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid. The solution in the vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the lid.

8. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900ml of (70-90°C) rinse water and 4.0 ml of alpha-amylase to the first and second rinses. Turn Agitate on and rinse for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat hot water rinses a total of three times.

9. When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker and add enough acetone to cover bags and soak for 3-5 min. 12. Remove bags from acetone and place on a wire screen to air-dry. Completely dry in oven at 102±2°C (most ovens will complete drying within 2-4 hrs). Do not place bags in the oven until acetone has completely evaporated.

10. Remove bags from oven, place directly into a desiccator container. Cool to ambient temperature and weigh bags (W_3).

5. Calculations

$$\% \text{ NDF (as-received basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Where: W_1 = Bag tare weight

W_2 = Sample weight

W_3 = Dried weight of bag with fiber after extraction process

C_1 = Blank bag correction (final oven dried weight ÷ original blank bag weight)

6. Notes

Caution: Powdered chemicals will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical. Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

7. References

Robinson, P. H., M. Campbell, Mathews, and J. G. Fade. 1999. Influence of storage time and temperature on in vitro digestion of neutral detergent fibre at 48 h, and comparison to 48 h in sacco neutral detergent fibre digestion. *Anim. Feed Sci. Technol.* 80:257-266.

Acid Detergent Fiber in Feeds (Filter Bag Technique)

1. Apparatus

1. Analytical Balance (capable of weighing 0.1 mg).
2. Oven, maintaining a temperature of $102 \pm 2^\circ\text{C}$.
3. Digestion instrument (capable of performing the digestion at $100 \pm 0.5^\circ\text{C}$ and maintaining a pressure of 10-25 psi). The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction (ANKOM²⁰⁰, 65 rpm agitation, ANKOM Technology).
4. Filter bags (F57, ANKOM Technology).
5. Heat sealer: sufficient for sealing the filter bags closed to ensure complete closure (1915, ANKOM Technology).
6. Desiccator.
7. Marking pen (solvent and acid resistant; F08, ANKOM Technology).

2. Reagents

Acid Detergent Solution: add 20g cetyl trimethylammonium bromide (CTAB) to 1 l of 1N H₂SO₄ previously standardized (premixed chemical solution available from ANKOM). Agitate and heat to aid solution.

3. Preparation of sample

Grind samples in a centrifugal mill with a 2 mm screen or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

4. Procedure

1. Use a solvent resistant marker to label the filter bags. Weigh filter bag (W_1) and zero balance.

2. Weigh 0.45-0.55 g of prepared sample (W_2) directly in filter bag. Avoid placing the sample on the upper 4 mm of the bag.

3. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top.

4. Weigh one blank bag and include in run to determine blank bag correction factor (C_1).

5. Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top to keep it submerged.

6. When processing 24 sample bags, add 1900-2000 ml of ambient temperature AD solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of AD solution (use minimum of 1500 ml to ensure Bag Suspender is covered).

7. Turn Agitate and Heat ON and confirm agitation. Set timer for 60 min and close lid.

8. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid.

9. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900-2000ml of (70-90°C) rinse water. Turn Agitate on and rinse for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat 5 min. hot water rinses a total of three times or until water is neutral pH.

10. When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker and add enough acetone to cover bags and soak for 3-5 min.

11. Remove bags from acetone and place on a wire screen to air-dry. Completely dry in oven at $102 \pm 2^\circ\text{C}$ (most ovens will complete drying within 2-4 hrs). Do not place bags in the oven until acetone has completely evaporated.

12. Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags.

5. Calculations

$$\% \text{ ADF (as-received basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Where: W1 = Bag tare weight

W2 = Sample weight

W3 = Dried weight of bag with fiber after extraction process

C1 = Blank bag correction factor (final oven dried weight ÷ original blank bag weight t)

6. References

Robinson, P. H., M. Campbell, Mathews, and J. G. Fade. 1999. Influence of storage time and temperature on in vitro digestion of neutral detergent fibre at 48 h, and comparison to 48 h in sacco neutral detergent fibre digestion. *Anim. Feed Sci. Technol.* 80:257-266.

Determination of Soluble Crude Protein in feedstuffs

1. Principle

The dried and milled sample is extracted with a borate-phosphate buffer pH 6.75 at 39°C for 1 hour. After centrifugation the soluble crude protein (SP) in the supernatant is determined using Kjeldahl or other suitable methods for total nitrogen determination. For silage samples the content of ammonium nitrogen should also be determined as a correction for loss of crude protein as ammonium nitrogen during drying is needed in the calculation of SP.

2. Reagents Only use reagents of recognized analytical grade

2.1 $\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$

2.2 $\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$

2.3 Water: Distilled or deionised water

2.4 Borate-phosphate buffer (modified from Licitra et al., 1996), pH 6.75 ± 0.05 . Dissolve in 900 ml of water 12.2 g of sodium dihydrogen phosphate and 8.91 g of sodium tetraborate. Check the pH with a pH-meter and if necessary adjust pH. Dilute in a 1000 ml volumetric flask to the mark with water. Prepare fresh buffer solution daily

2.5 Sulfuric acid, $\rho_{20} 1.84$ g/ml

2.6 Catalyst: Kjeltabs CF 5 g ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: Approximately 0.10 g Cu/tablet, Thompson & Capper Ltd.) or equivalent.

2.7 Titrant for the auto-burette in the Kjelttech apparatus (0.1 mol/l HCl).

3. Apparatus

3.1 Analytical balance capable of weighing to the nearest 1 mg

3.2 Centrifuge test tubes, 50 ml, with lid

3.3 Dispenser or pipette 50 ± 0.5 ml

3.4 Water bath, thermostated at $39 \pm 0.5^\circ\text{C}$ (or incubating chamber, $39 \pm 0.5^\circ\text{C}$)

3.5 Glass rods

3.6 Centrifuge suitable for the centrifuge tubes and capable of spinning at 3000 g (values of g is given for the bottom of the test tubes)

3.7 Pipette 20 ± 0.2 ml

3.8 Kjeldahl equipment or other equipment for total nitrogen determination in liquids

3.9 Heating block suitable for digestion of the samples

3.10 pH-meter, calibrated and capable of measuring pH to the nearest 0.01 pH unit

4. Sample preparation

The samples are dried as specified for the NorFor samples ($< 60^\circ\text{C}$) and ground on a hammer mill to pass a 1 mm sieve.

5. Procedure

5.1 Weigh approximately 1.5 g of the test sample to nearest 1 mg in a centrifuge tube (See note 6.1).

5.2 Add 50 ± 0.5 ml borate-phosphate buffer, pre-heated to $39\text{ }^{\circ}\text{C}$ in water bath, to the samples (See note 6.2)

5.3 A blank sample of 50 ml borate-phosphate buffer should be included in each series of samples

5.4 To hydrate the sample, mix the sample gently with e.g. a glass rod. Then put the lid on the tube and shake the sample thoroughly.

5.5 Incubate in a water bath or an incubating chamber at $39 \pm 0.5\text{ }^{\circ}\text{C}$ for $1\text{ h} \pm 5$ minutes. Shake the tubes manually every 15th minute.

5.6 Centrifuge the tubes at $3000 \times g$ for 10 min (see note 6.3).

5.7 Pipette 20 ± 0.2 ml of the supernatant and transfer to Kjeldahl tubes.

5.8 Add salt/catalyst and the volume of sulphuric acid to the tubes according to the standard procedure in the lab. Some feed samples foam extensively when the acid is added. Foaming during digestion in the Kjeldahl analysis can be reduced if the acidified samples are allowed to stand at room temperature for 1-2 hours or overnight.

5.9 Increase the temperature of the digester stepwise, to prevent foaming of the samples. Do not include the time it takes to reach working temperature in the total digestion time.

5.10 Analyse the nitrogen content by Kjeldahl distillation.

5.11 Calculate the content of soluble crude protein

6. Notes

6.1 In the procedure 1.5 g sample and 50 ml buffer is recommended. If using the common 50 ml centrifuge tubes from Falcon, NUNC, Greiner etc the tube will be very full when using 50 ml of buffer and the shaking might be a problem. The recommendation is then to use 1.2 g sample and 40 ml of buffer. Depending on the facilities in the lab other multiples of this sample:buffer ratio could be used, e.g. 3 g of sample and 100 ml buffer.

6.2 Analytical steps 5.2-5.8 (sulfuric acid addition) should be performed in sequence without interruption.

6.3 Some insoluble particles (containing trapped air), particularly from forages may float on the surface after centrifugation, but if the supernatant is carefully pipetted the insoluble matter will not cause contamination. The particles may be removed with a spoon or a paper tissue. If still a problem the supernatant can carefully be poured into a beaker through a tea-strainer and then pipetted.

7. Calculations

The content of soluble crude protein per kg crude protein is calculated according to:

$$\frac{(V_1 - V_0) \times c \times 14.007 \times 6.25 \times V_2 \times 1000}{m \times CP \times V_3}$$

Where:

V_0 = volume (ml) of HCl used for titration of blank sample

V_1 = volume (ml) of HCl used for titration of sample

V_2 = volume (ml) of buffer added in step 5.2

V_3 = volume (ml) of extract pipetted in step 5.7

c = concentration of titrant (mol/l)

m = sample size (g)

14.007 = MW for nitrogen (g)

6.25 = Factor for conversion of nitrogen content to crude protein

8. References

- Krishnamoorthy, U. T., V. Muscato, C. J. Sniffen, and P. J. Van Soest. 1982. Nitrogen fractions in selected feedstuffs. *J. Dairy Sci.* 65: 217-225.
- Licitra, G., T.M., Hernandez, P.J., Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. *Anim. Feed Sci. Technol.* 51: 347-358.

Sugar (Reducing sugar invert sugar and sucrose) in food

1. Principle: sugars having free aldehyde and/or ketone groups can react as a weak reducing agent and are termed reducing sugar. In boiling alkaline solution of copper salt (such as Fehling's solution) with a solution of reducing sugar, copper (II) can be reduced to copper (I) and the red precipitate of cuprous oxide is formed. The weight of reducing sugar is equivalent to the weight of cuprous oxide, can be obtained from Munson-Walker table. Sucrose is a disaccharide and can be hydrolyzed with acid to yield two monosaccharides, glucose and fructose. These monosaccharides are reducing sugars.

2. Modification

This method is modified as follows.

2.1 For inversion, using 5 ml conc HCl to hydrolyze (in water bath) at 65-70 °C for 15 min.

2.2 Use carrez I and carrez II solution in place of saturated lead acetate solution as clarifying agent.

2.3 Use 40 % sodium hydroxide solution in place of concentrated sodium hydroxide solution.

2.4 Volume of test solution is 250 ml in place of 200 ml.

3. Reagents

3.1 Copper sulfate solution (Fehling A solution): dissolve 34.639g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water, dilute to 500 ml with distilled water in volumetric flask and filter through filter paper Whatman no. 41.

3.2 Alkaline tartrate solution (Fehling B solution): dissolve 173 g potassium sodium tartrate. $4\text{H}_2\text{O}$ (Rochelle salt) and 50 g sodium hydroxide in distilled water, dilute to 500 ml with distilled water in volumetric flask, let stand for 2 days and filter through filter paper Whatman no. 541.

3.3 Fehling's solution is prepared by mixing equal volumes of Fehling A solution and Fehling B solution immediately before use.

3.4 Hydrochloric acid, conc.

3.5 Carrez I solution: dissolve 3.60 g $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ in 100 ml distilled water.

3.6 Carrez II solution: dissolve 7.20 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml distilled water.

3.7 Sodium hydroxide, 40 % (w/v).

3.8 Ethanol, 95 % (v/v)

4. Apparatus

4.1 Analytical balance, accurate to 0.1 mg.

4.2 Beaker, 50 and 400 ml.

4.3 Volumetric flasks, 100 and 250 ml.

4.4 Erlenmeyer flask, 250 ml.

4.5 Pipettes, 5 and 25ml.

4.6 Graduated cylinder, 50 ml.

4.7 Suction flask, 500ml.

4.8 Sintered crucible.

4.9 Watch glass.

4.10 Hot plate

4.11 Filter paper Whatman no. 41 and 541.

4.12 Electrical air oven.

4.13 Desiccator with effective desiccant.

5. Preparation of test sample

Solid sample: bring sample to room temperature, grind the laboratory sample and mix until homogeneous.

6. Procedure

6.1 Preparation of test solution.

6.1.1 Weigh sample (depends on the amount of reducing sugar) accurately in 50 ml beaker.

6.1.2 Quantitatively transfer to 250 ml volumetric flask and distilled water to about 2/3 volume.

6.1.3 Add 5 ml Carrez I solution and add 5 ml Carrez II solution. Shake vigorously after each addition then to volume.

6.1.4 Filter through filter paper Whatman no. 41 into 250 ml Erlenmeyer flask; discard the first 5 ml filtrate. Collect the filtrate for procedure (6.2 and 6.3).

6.2 Determination of reducing sugar.

6.2.1 Pipette 25 ml test sample solution (6.1.4) into 400ml beaker.

6.2.2 Add 25 ml distilled water and 50 ml Fehling's solution (3.3).

6.2.3 Cover with watch glass and keep on hot plate.

6.2.4 Heat to boil within 4 min, and continue boiling for exactly 2 min.

6.2.5 Filter through sintered crucible (previously dried in air oven at $100 \pm 2^\circ\text{C}$ for 30 minutes, cooled and weighed) by using suction and wash precipitate of cuprous oxide thoroughly with hot distilled water till alkaline free. Then rinse with about 5ml ethanol (3.8).

6.2.6 Dry sintered crucible with precipitate in air oven $100 \pm 2^\circ\text{C}$ for 30 min, cool in desiccator and weigh (W_1).

6.2.7 Carry on the blank as 10.2.1 to 10.2.6 by using distilled water in place of sample (W_2).

6.2.8 Calculate reducing sugar content as dextrose (W_3) from the weigh of Cu_2O precipitate obtained ($W_1 - W_2$) and Munson-Walker table.

6.2.9 Calculate reducing sugar content as invert sugar (W_4) from the weight of Cu_2O precipitate obtained ($W_1 - W_2$) and Munson-Walker table.

6.3 Determination of invert sugar.

6.3.1 Pipette 50 ml test sample solution (6.1.4) into 100 ml volumetric flask.

6.3.2 Add 5 ml conc. HCl little by little with shaking, hydrolyze at $65 - 70^\circ\text{C}$ for 15 min in water bath (with continuously shake 3 min and let stand for 12 min).

6.3.3 Cool in cold distilled water bath after hydrolyzation finished.

6.3.4 Neutralize with sodium hydroxide (3.7) and dilute to volume.

6.3.5 Pipette 50 ml sample solution into 400 ml beaker.

6.3.6 Add 50 ml Fehling's solution (3.3), cover with watch glass and heat on hot plate to boil within 4 min. Continue boiling for exactly 2 minutes.

6.3.7 Filter through sintered crucible (previously dried in air oven at $100 \pm 2^\circ\text{C}$ for 30 minutes, cooled and weighed) by using suction and wash precipitate of cuprous oxide thoroughly with hot distilled water till alkaline free. Then rinse with about 5 ml ethanol.

6.3.8 Dry sintered crucible with precipitate in air oven at $100 \pm 2^\circ\text{C}$ for 30 min, cool in desiccator and weight (W_5).

6.3.9 Calculate total sugar content as invert sugar (W_6) from the weight of Cu_2O precipitate obtained ($W_5 - W_2$) and Munson-Walker table.

7. Calculations

7.1 Reducing sugar as dextrose, g/100 g

$$= \frac{W_3 \times 250 \times 100}{25 \times \text{weight of sample}}$$

Where:

W_3 = weight of dextrose from Muson-Walker table (g).

7.2 Total sugar as invert sugar, g/100 g

$$= \frac{W_6 \times 100 \times 250 \times 100}{50 \times 50 \times \text{weight of sample}}$$

Where:

W_6 = weight of invert sugar from Muson-Walker table (g).

7.3 Sucrose, g/100 g

$$= \frac{(W_6 - W_4) \times 100 \times 250 \times 100 \times 0.95}{50 \times 50 \times \text{weight of sample}}$$

Where:

W_4 = weight of reducing as invert sugar from Muson-Walker table (g).

W_6 = weight of invert sugar from Muson-Walker table (g).

8. References

AOAC. 2000. Official Method of Analysis of AOAC international 17th ed (method 925.35 (B)), Gaithersburg, MD, p.17.

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