Standard Operating Procedure
for
Hazardous Chemicals

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Building and rooms: Davison Life Sciences Building. Lab B310

Chemical(s)	Acetic acid glacial, acetonitrile, calcium chloride, citric acid, ethanol, fructose, glucose, p-hydroxybenzoic acid hydrazite (p-HBAH), 1-naphthol, sodium acetate, sodium bisulfite, sodium citrate, sodium hydroxide (NaOH), sucrose, sulfuric acid
Process	**Starch and sugar analysis**
Specific Hazards *referred to MSDSs for more detailed information*	Acetonitrile and ethanol: Extremely flammable. Vapor may cause flash fire. Severely irritating to eyes. NaOH: Corrosive liquid, may cause burns to skin and eyes upon contact and to mucous membranes if inhaled or ingested. Acetic acid and sulfuric acid: Corrosive, inhalation hazard. Avoid skin contact, serious burns may result. p-HBAH is an irritant to skin, respiratory system and eyes.
Personal protective equipment	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist
Engineering/ventilation controls	: chemical fume hood : emergency shower and eyewash accessible
Special handling procedures and storage requirements	Store acetic acid, acetonitrile, ethanol in flammable cabinet, away from oxidizers, underneath the chemical hood in Rm B310. Store NaOH in general area away from oxidizers, acids and flammable reagents. Store all the other buffers on bench top.
Spill and accident procedures *for hazardous chemicals only*	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. Eye exposure: Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
	Small (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 2L): Evacuate the room, notify PIs and call 2-5801 to request emergency spill assistance from the Environmental Safety Division.
Waste disposal	Acetonitrile: Collect and label as hazardous waste according to the SOP for Hazardous Waste Disposal. Small quantities of diluted acids and bases may be disposed of down the drain.
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: Raja Payyavula, 8/17/2008.
Reviewed by	Name/date:

Starch and Sugar analysis

Chemicals:

- Acetic acid, glacial
- Calcium chloride
- Glucose
- Ethanol
- p-hydroxy benzoic acid hydrazite (p-HBAH)
- Sodium acetate
- Sodium hydroxide (NaOH)
- Starch
- Trisodium citrate (sodium citrate)

Enzymes:

- α-amylase
- Amyloglucosidase

Equipment:

- Hot water bath
- Plate reader

Buffers:

- *Sodium acetate buffer pH 5.1:* Prepare 0.5 M **Sodium acetate trihydrate** solution and adjust pH with **glacial acetic acid**.
- *Alkaline dilutent:* 0.5 M **trisodium citrate**, 0.1 M **calcium chloride** and 0.5 M **sodium hydroxide**.
- p-*HBAH solution*: Prepare 33 mM **p-HBAH** (5 mg/mL) in alkaline diluent. Make fresh p-HBAH solution each day.

Heating with ethanol removes soluble sugars, phenolics and lipids and also increases gelatinization which allows the accessibility of the starch polymer to hydrolyzing agent. Dilute alkali and α-amylase split the polymer into amylose and amylopectin. Amyloglucosidase helps in complete digestion of the long chain molecules to glucose. Glucose is then estimated calorimetrically by using p-HBAH and the % of starch is calculated.

Procedure:

1. Extract the dry sample (20 mg) three times with 5 ml of 80% **ethanol**, by boiling the samples in a 95 °C water bath for 10 min each.
2. After each extraction, centrifuge the tubes at 2,500 rpm for 5 min, and the supernatants of the three extractions combined for soluble sugar analysis. Use the residues for starch analysis.
3. Heat the pellet with 2 ml of 0.1 N **NaOH** in a 50 °C water bath for 30 min with intermittent mixing.

4. After neutralizing with 2.5 ml of 0.1 N **acetic acid**, add 0.5 ml of 0.05M **sodium acetate buffer** (pH 5.1) and 200 U of ***α-amylase*** and 1 U of ***amyloglucosidase***.
5. Incubate the combined solution for 48 h in a 50 °C water bath.
6. Following starch hydrolysis, add hydrolysate to 5 mL of 33 mM **p-HBAH** solution in a 20 mL boiling tube and heat in a boiling water bath for 4 min.
7. Cool the contents rapidly to room temperature and read the absorbance at 415 nm.
8. Develop standard curve with known amounts of glucose and estimate the glucose levels in the samples comparing to standard curve.
9. If estimating only glucose levels in a sample, follow the procedure from step 6.

References:

- Chow P.S and Landhausser S.M. 2004. A method for routine measurements of total sugar and starch content in woody plant tissues. Tree Physiol 24, 1129 –1136
- Fox G, Logue S, Harasymow S, Taylor H, Ratcliffe M, Roumeliotis S, Onley K, Tansing P, Ferguson R, Glennie-Holmes M, Inkerman A, Tarr A, Evans B, Panozzo J, Osman A and Smith A. Standardisation Of Diastatic Power Method For Barley Breeding Programs. Proceedings of the 10th Australian Barley Technical Symposium, Canberra, ACT, Australia 16-20 September 2001.
- Rose R, Rose C.L, Omi S.K, Forry K.R, Durall D.M, and Bigg W.L. 1991. Starch Determination by Perchloric Acid vs Enzymes: Evaluating the Accuracy and Precision of Six Colorimetric Methods *J. Agric. Food Chem.* WI, *39,* 2-1 1

Soluble sugar analysis:

The ethanol extracts from the plant samples are used for soluble sugar analysis.

Chemicals:

- Acetonitrile
- Citric acid
- Ethanol
- 1-naphthol
- Sodium bisulfate
- Sodium citrate
- Sulfuric acid

Buffers and reagents:

- ***1-naphthol - sulfuric acid reagent*** *(1X)- prepared by mixing 1-naphthol (5 g) with ethanol (33 mL) and then adding sulfuric acid (20 mL), ethanol (127 mL), and water (13 mL) and mixing. This solution is light-sensitive and should be stored covered with aluminum foil.*
- ***Citrate buffer (*****pH 4.8)** 0.5 M stock – *23 ml of 0.1 M citric acid and 27 ml of 0.1 M sodium citrate to 50 ml of water. Adjust the pH with citric acid or NaOH*

Equipment:

- 10 X 20 cm Merck silica gel 60 C F_{254} HPTLC silica gel plates.
- Camag twin-trough HPTLC chamber
- HPTLC imaging system
- Hot oven

Impregnation of the plates with salts especially with sodium bisulfite and citrate buffer provides better separations of sugars by TLC as compared to un-impregnated plates or impregnated plates with other salts such as monobasic potassium phosphate. Acetonitrile provides several advantages such as separation in short time, quick drying and producing compact zones of sugar spots as compared to other solvents such as methanol and ethyl acetate. Better detection of sugar spots was observed using 1-naphthol reagent than other reagents tested.

Procedure:

1. Spray the HPTLC plates with 0.10 M **sodium bisulfate** solution and thoroughly dry by heating with a hair-drier set on high heat for 2 min

   Note: cover the sample loading area with aluminum foil to prevent the spray

2. Spray the plates again with 10 mM **citrate buffer** (pH 4.8), and heat again for 2 min. Spray till the plate begins to appear wet.
3. Heat the plates at 100°C for 1 h and stored in a desiccators until use.
4. Spot the samples and standards on HPTLC plate
5. Develop the plates three times* with **acetonitrile:ddH2O** (85:15) in a CAMAG - HPTLC development chamber that had been equilibrated with the mobile phase for 10-15 min.

   ** Run the mobile phase to a distance of 7 cm, then remove and dry the plate and re run the mobile phase again for 7 cm. Repeat one more time. Use fresh solvent for each run. Approximately 13 mL of solvent will be required for each development.*

6. After the third development, dry the plate thoroughly and detect the sugar spots as blue - purple zones by spraying with **1-naphthol-sulfuric acid** reagent
7. After spraying, heat the plate for ca 5-10 min at 100-110°C in an oven.
8. Quantify sugars by reflectance scanning at 515 nm using TLC scanner by developing standard curves for each sugar.

References:

- Gomori G. (1955) Preparation of buffers. Methods Enzymol. 1**:**138-146
- Lee K.Y, Nurok D and Zlatkis A, (1979) Determination of glucose, fructose and sucrose in molasses by high-performance thin-layer chromatography. J. Chromatogr., 174, 187
- Sherma J, Zulick D.L (1996) Determination of fructose, glucose, and sucrose in beverages by high-performance thin layer chromatography. Acta Chromatographica 6:7-13