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# Practical Note

# Nutritional Biochemistry
# (BCH 445 )

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# 1. INTRODUCTION

## 1.4 Preparation of Laboratory Samples

The food material within the sample selected from the population is usually heterogeneous, *i.e.,* its properties vary from one location to another. Sample heterogeneity may either be caused by variations in the properties of different units within the sample (*inter-unit* variation) and/or it may be caused by variations within the individual units in the sample (*intra-unit* variation). The units in the sample could be apples, potatoes, bottles of ketchup, containers of milk etc. An example of inter-unit variation would be a box of oranges, some of good quality and some of bad quality. An example of intra-unit variation would be an individual orange, whose skin has different properties than its flesh. For this reason it is usually necessary to make samples *homogeneous* before they are analyzed, otherwise it would be difficult to select a representative *laboratory sample* from the *sample.* A number of mechanical devices have been developed for homogenizing foods, and the type used depends on the properties of the food being analyzed (*e.g.,* solid, semi-solid, liquid). Homogenization can be achieved using mechanical devices (*e.g.,* grinders, mixers, slicers, blenders), enzymatic methods (*e.g.,* proteases, cellulases, lipases) or chemical methods (*e.g.,* strong acids, strong bases, detergents).

Once the sample has been made homogeneous, a small more manageable portion is selected for analysis. This is usually referred to as a *laboratory sample,* and ideally it will have properties which are representative of the population from which it was originally selected. Sampling plans often define the method for reducing the size of a sample in order to obtain reliable and repeatable results.

## 1.5 Preventing Changes in Sample

Once we have selected our sample we have to ensure that it does not undergo any significant changes in its properties from the moment of sampling to the time when the actual analysis is carried out, *e.g.,* enzymatic, chemical, microbial or physical changes. There are a number of ways these changes can be prevented.

*Enzymatic Inactivation*

Many foods contain active enzymes they can cause changes in the properties of the food prior to analysis, *e.g.,* proteases, cellulases, lipases, etc. If the action of one of these enzymes alters the characteristics of the compound being analyzed then it will lead to erroneous data and it should therefore be inactivated or eliminated. Freezing,

drying, heat treatment and chemical preservatives (or a combination) are often used to control enzyme activity, with the method used depending on the type of food being analyzed and the purpose of the analysis.

*Lipid Protection*

Unsaturated lipids may be altered by various oxidation reactions. Exposure to light, elevated temperatures, oxygen or pro-oxidants can increase the rate at which these reactions proceed. Consequently, it is usually necessary to store samples that have high unsaturated lipid contents under nitrogen or some other inert gas, in dark rooms or covered bottles and in refrigerated temperatures. Providing that they do not interfere with the analysis antioxidants may be added to retard oxidation.

*Microbial Growth and Contamination*

Microorganisms are present naturally in many foods and if they are not controlled they can alter the composition of the sample to be analyzed. Freezing, drying, heat treatment and chemical preservatives (or a combination) are often used to control the growth of microbes in foods.

*Physical Changes*

A number of physical changes may occur in a sample, *e.g.*, water may be lost due to evaporation or gained due to condensation; fat or ice may melt or crystallize; structural properties may be disturbed. Physical changes can be minimized by controlling the temperature of the sample, and the forces that it experiences.

**1.6** Sample Identification

Laboratory samples should always be labeled carefully so that if any problem develops its origin can easily be identified. The information used to identify a sample includes: a) Sample description, b) Time sample was taken, c) Location sample was taken from, d) Person who took the sample, and, e) Method used to select the sample. The analyst should always keep a detailed notebook clearly documenting the sample selection and preparation procedures performed and recording the results of any analytical procedures carried out on each sample. Each sample should be marked with a *code* on its label that can be correlated to the notebook. Thus if any problem arises, it can easily be identified.

**1.7** Sources of Error

There are three common sources of error in any analytical technique:

*Personal Errors (Blunders)*

These occur when the analytical test is not carried out correctly: the wrong chemical reagent or equipment might have been used; some of the sample may have been spilt; a volume or mass may have been recorded incorrectly; etc. It is partly for this reason that analytical measurements should be repeated a number of times using freshly prepared laboratory samples. Blunders are usually easy to identify and can be eliminated by carrying out the analytical method again more carefully.

*Random Errors*

These produce data that vary in a non-reproducible fashion from one measurement to the next *e.g.*, instrumental noise. This type of error determines the standard deviation of a measurement. There may be a number of different sources of random error and these are accumulative (see "Propagation of Errors").

*Systematic Errors*

A systematic error produces results that consistently deviate from the true answer in some systematic way, *e.g.*, measurements may always be 10% too high. This type of error would occur if the volume of a pipette was different from the stipulated value. For example, a nominally 100 $cm^3$ pipette may always deliver 101 $cm^3$ instead of the correct value.

To make accurate and precise measurements it is important when designing and setting up an analytical procedure to identify the various sources of error and to minimize their effects. Often, one particular step will be the largest source of error, and the best improvement in accuracy or precision can be achieved by minimizing the error in this step.

EXPERIMENT 1

## 2. Determination of Benzoate Estimation

Sodium benzoate ($C_6H_5COO^-Na^+$, MW=144) is a commonly used preservative which is added to fruit juices to prevent the growth of microorganisms. When added in high concentration, it affects the taste of the juice. Sodium benzoate is usually permitted at a concentration of up to 1.3 grams per litre of juice.

The benzoate anion is not soluble in non-polar solvents because of its negative charge. However, in acid solution, benzoate acid is formed. This is neutral and quite non-polar. Moreover, it is soluble in non-polar solvents, into which it may be extracted at acidic pH, i,e well below the pKa of the carboxyl group, which is 4.20. In this experiment, benzoic acid is extracted into chloroform, which is then removed by evaporation. After dissolving the residue in 50% (v/v) neutralized ethanol, the benzoic acid is titrated with 0.05M sodium hydroxide, using phenolphthalein as an indicator

### **2.1** Objectives**:**

To estimate the concentration of benzoate in a given sample of soft drink.

### **2.2** Principle**:**

The benzoate anion is insoluble in non-polar solvents. However, in acid solution, benzoic acid is neutral and quite nonpolar. Moreover it is soluble in non-polar solvents, into which it may be extracted at acidic pH. In this experiment, Benzoic acid is separated from a known quantity of the sample by saturating with NaCl and then acidifying with dilute HCl and extracting with chloroform. The chloroform layer is made mineral acid free and the solvent is removed by evaporation. The residue is dissolved in neutral alcohol and the amount of benzoic acid is determined by titration against standard alkali.

### **2.3** Materials and Methods**:**

#### 2.3.1 Materials**:**

1. Electric steam bath

2. Conical flasks.
3. Volumetric flasks
4. Graduated flasks of 250 ml capacity
5. Separating funnels of 250 ml capacity
6. Whatman no. 4 filter paper.
7. Sodium Chloride (AR).
8. Chloroform
9. HCL (dil. 1 + 3)
10. Sodium hydroxide (10%)
11. Standard NaOH solution (0.05N)
12. Saturated Sodium Chloride solution.

2.3.2 Preparation of beverages and liquid products:

**1.** Mix the sample thoroughly and transfer 100 ml of the sample into a 250 volumetric flask.
**2.** Make alkaline to litmus paper with 10% NaOH solution (add 5 ml)
**3.** Make up to volume with saturated NaCl solution. Shake thoroughly and let it stand for 2 hours.
**4.** Filter the sample and use the filtrate for determination.

2.3.3 Method**:**

1. Pipette 100 ml to 200 ml of the filtrate into a 250 ml separatory funnel.
2. Neutralize to litmus paper using HCI (dil) and add 5 ml excess.
3. Extract carefully with 40, 30, 30 and 20 ml portions of chloroform.
4. Transfer the combined chloroform extract in to a separating funnel and wash it free from mineral acid by shaking gently and rinsing with water.
5. Drain off the water phase.
6. Dry the chloroform layer over anhydrous sodium sulphate and distil off the solvent.
7. Remove the last traces of the solvent under a current of air at room temperature.
8. Dissolve residue in 30-50 ml of alcohol neutralised to phenolphthalein and titrate with 0.05 N NaOH.

**2.4** Results and Calculation**:**

1. Calculate the benzoic acid contents as follows:

$$\text{Benzoic acid (ppm) mg/l} = \frac{122 \times \text{titre} \times \text{dilution} \times 1000 \times \text{NaOH}}{\text{Weight of sample}}$$

2. Determine the concentrations sodium benzoate in your soft drink sample in units of ppm (mg/l).

**1.8** Discussion

Compare your results with the total sodium benzoate content in the whole can of the tested soft drink.

**2.5** RFERENCES**:**

A.O.A.C 17th edn , 2000, Official Method 963.19 Benzoic acid in Foods Titrimeric Method).

EXPERIMENT 2

## 3. Determination of Nitrate and Nitrite in Foods

Sodium and potassium salts of nitrate and nitrite are added mainly to preserve meat and meat products such as cured meat and meat pickles.

**3.1** Principle**:**

The sample is clarified with alumina cream and the amount of nitrate present determined by allowing it to diazotise arsenilic acid and coupling the diazonium salt with n-1-naphthylethylene diamine. The colour so formed is extracted into n-butanol and the absorbance is measured at 545 nm. An aliquot of the sample is mixed with spongy cadmium in order to reduce any nitrate present and the nitrite so produced is determined in the same way. The amount of nitrate present is then calculated by subtracting the nitrite from the total.

**3.2** Objectives**:**

To estimate the amount of added nitrite in (Mortadella) cured meat

**3.3** Materials and Methods**:**

3.3.1 Reagents**:**

a) Distilled or deionized but a blank must be carried out to check that satisfactory quality for the preparation of the spongy cadmium.
b) Alumina Cream: Prepare a saturated solution of potassium aluminum sulphate and add ammonia slowly with stirring until pH is 7.0.
c) Sulfanilamide: 0.1% in 5M HCl.
d) N-naphthylethylene diamine dihydrochloride 0.1% in distilled water.
e) Buffer pH, 9.6: Prepare 0.7 M $NH_4Cl$ (37.45 gm/l) in distilled water and add 0.88 ammonia until the pH is 9.6.
f) Spongy cadmium: Place zinc rods in 20 % aqueous cadmium sulphate solution and leave for 3 or 4 hrs. Separate the precipitated cadmium, wash twice with distilled water and then macerate with water for 2-3 minutes Activate by shaking with 2M HCl and then wash at least 5 times with distilled water, keep the cadmium under distilled water and prepare freshly for each batch of determination.

g) Standard nitrite solution: Weigh out 0.4783 gm of $NaNO_2$ and dilute to one litre. Dilute 10 times to get 10 mgs /1 of nitrite nitrogen.

h) n- butanol

3.3.2 Procedure**:**

1. Mix the sample thoroughly by macerating or homogenizing and weigh 5 gm into a 150 ml beaker.
2. Add 50 ml water and heat to 80ºC stirring gently. Maintain at 80ºC for 10 min.
3. Add 20 ml alumina cream and transfer gently to a 100 ml volumetric flask. Cool and dilute to volume with water.
4. Mix and filter through Whatman No.4 filter paper rejecting the first 10 ml of filtrate. The filter paper must be previously washed with at least 100 ml of hot water to remove the small amounts of nitrate that' it may have contained

3.3.3 (a) Determination of nitrite**:**

1. Pipette 1 ml of filtrate into a test tube.
2. Add 0.2 ml of' sulfanilamide solution and mix. Leave for 5 minutes.
3. Add 0.2 ml of napthylethylenediamine solution. Mix and leave for 10 minutes. If the solution is clear, dilute to 5 ml with water and read the absorbance at 538 nm.

   If the solution is cloudy, extract with n-butanol using 2.0, 1.5 and then 0.5 ml. Pass the butanol extracts through a small cotton pledged in a funnel into a dry 5 ml calibrated flask and dilute to volume with n – butanol. Read the absorbance at 545 nm in a 1 cm cell.

3.3.4 (b) Determination of nitrate**:**

1. Pipette 10 ml of filtrate into a small stoppered conical flask.
2. Add 5 ml of buffer solution and one gram of wet cadmium.
3. Stopper the flask and shake for 5 min.
4. Filter the solution through a washed filter paper into a 50 ml volumetric flask rinsing the cadmium and the filter paper with 5ml water.
5. Determine the nitrite in the filtrate as given above starting at “add 2 ml of sulphanilic acid solution”.

3.3.5 (c) Preparation of standard curve**:**

2. Pipette into a series of 50 ml volumetric flasks dilute standard solution of $NaNO_2$ containing 2-15 µg of nitrite nitrogen.
3. Develop the colour as given in the procedure for nitrite.
4. Read the absorbance and plot standard curve.

**4.4 Results and Calculation**

| **Tube No.** | **$NaNO_2$** (µg) | **Solution (c) (0.2 ml)** | **Solution (d) (0.2 ml)** | **$H_2O$** |
|---|---|---|---|---|
| 1 | 0 | | | |
| 2 | 2 | | | |
| 3 | 4 | | | |
| 4 | 6 | | | |
| 5 | 8 | | | |
| 6 | 10 | | | |
| Sample | | | | |

From the graph calculate the nitrite content before and after reduction and calculate the nitrate content by subtraction.

| **Tube No.** | **$NaNO_2$ Std Concentration** | **Abs at 545 nm** |
|---|---|---|
| 1 | 0 | |
| 2 | 2 | |
| 3 | 4 | |
| 4 | 6 | |
| 5 | 8 | |
| 6 | 10 | |
| sample | | |

**4.5** Discussion

Comment on the values obtained for FFA determination for each sample. What can you say about the samples based on these values?

**4.6** RFERENCES**:**

F.A.O Manuals of Food Quality Control 14 / 2 , 1980, P. 22

EXPERIMENT 3

# 4. Liquid Chromatographic (HPLC) Determination of Caffeine in Cola Soft Drinks

**4.1** Principle**:**

Food additives such as caffeine, saccharin, and benzoate can be quantified in beverages by liquid chromatography on u-Bondapack-C18 column using acetic acid (20%) buffered to pH 3.0 with saturated sodium acetate and modified by adding 0.2% isopropanol. The concentration of the additive in the sample is determined by measuring the peak height monitored at 254 nm using a UV absorbance detector.

4.2 Objectives

To estimate the concentration of caffeine in beverages by reverse phase high performance liquid chromatography.

**4.3** Materials and Methods**:**

**4.3.1** Apparatus and Glass ware**:**

a. Liquid Chromatograph equipped with 6000 A solvent delivery system injector,
b. u-Bondapak C18 column,
c. UV. 254 nm detector sensitivity adjustable from 0.02 – 0.05 AUFS.
d. Chart recorder
**e.** Beaker, pipette, flasks

**4.3.2** Reagents**:**

Mobile Phase: 20% acetic acid (v/v) buffered to pH 3.0 with saturated sodium acetate solution modify with 0-2% isopropanol. De-gas prior to use

**4.3.3** Standards and Sample Preparation

1. Standard solutions: Prepare individual standard solutions from standard compound to get following concentrations-sodium saccharin: 0.5 mg/ml, caffeine: 0.05 mg/ml and sodium benzoate: 0.5 mg/ml use solutions to determine sensitivity for detector response and retention times of individual standards.

**2.** Mixed standard solution: Prepare solution containing 0.5 mg/ml sodium, saccharin 0.05 mg/ ml caffeine and 0.5 mg/ml of sodium benzoate. Use this solution to optimise LC conditions for complete resolution and to quantify.

3. Carbonated beverages: Decarbonate by agitation. If free of particulate matter, inject directly.
4. Beverages containing particulate matter: Filter through Millipore filter (0.45 μm) discarding first few ml filtrate. If large amount of particulate matter is present, centrifuge prior to filtration.

**4.3.4** Analysis Method

1. Inject known volume (10 μl) of mixed or individual standard solution in duplicate. Peak heights should agree within ± 2.5%.
2. Inject known volume of prepared unknown sample in duplicate.
3. Measure peak heights of standards and sample components.

4.4 Results and Calculation

| | **Conc. (mg/ml)** | **Volume injected** | **Peak Height** |
|---|---|---|---|
| Standard 1 | | | |
| Standard 2 | | | |
| Standard 3 | | | |
| Sample 1 | | | |
| Sample 2 | | | |

Calculate the amount of sample using the following formula:

% compound = $C_1 X (H/H_1) X V_1/V X 0.1$

Where $C_1$ = concentration of standard in mg/ml

H and $H_1$ = average peak heights of sample and standard respectively.

V and $V_1$ = volume injected in μl of sample and standard respectively.

**4.5 Discussion:**

Comment on the values obtained for the amount of caffeine in beverages sample. Compare your results with amount mentioned on the beverage can.

4.6 **REFERENCES:**

Pearson's Composition and Analysis of Foods 9th edn., 1991, p.373

EXPERIMENT 4

## 5. Crude Fibre Digestion and Analysis

### 5.1 Introduction

Dietary fibre is defined as plant polysaccharides that are indigestible by humans, plus lignin. The major components of dietary fiber are cellulose, hemicellulose, pectin, hydrocolloids and lignin.

The basis of many fiber analysis techniques is therefore to develop a procedure that mimics the processes that occur in the human digestive system. The crude fiber method gives an estimate of indigestible fiber in foods. It is determined by sequential extraction of a defatted sample with 1.25% $H_2SO_4$ and 1.25% NaOH. The insoluble residue is collected by filtration, dried, weighed and ashed to correct for mineral contamination of the fiber residue.

### 5.2 Objectives

1. To determine solubility properties of dietary fibres at different temperatures
2. To determine the amount of crude fibre in whole flour.

### 5.3 Materials and Methods:

**5.3.1** Chemicals

1. Dilute Sulphuric acid – 1.25 % (w / v ), accurately prepared
2. Sodium hydroxide Solution – 1.25 % (w / v), accurately prepared
3. Ethanol – 95 % (v / v)

4. Petroleum ether.

CAUTION*: Always add acid to water in making solutions of acid. Do not add water to acid. Wear goggles when working with these solutions.*

### 5.3.2 Glassware and Equipments

a. Beaker graduated cylinders, round bottom flasks, Rreflux condenser**,** soxhlet extractor, and thimbles,
b. Crucible, Thermometer, and Timer,
c. Analytical balance, heating mantle, Drying oven,

### 5.3.3 Samples

Cellulose, hemicellulose, pectin, guar, agar and xanthan gum,

## 5.4 PHYSICAL PROPERTIES OF DIETARY FIBRES

### 5.4.1 Determination of solubility at room temperature:

Accurately weigh a known amount of each type of fiber (2 g will suffice). Add the fiber to beakers containing 100 ml of distilled water, 1.0 N Sulfuric Acid, or 1.0 N Sodium Hydroxide and stir carefully. Let stand for 24 hours.

Filter the solutions through preweighed, dry filter papers and rinse with distilled water. Carefully place filter papers on a tray and dry overnight in a drying oven. Determine the amount of solubility of the fibers by gravimetric analysis (i.e. by weighing).

### 5.4.2 Fibre solubility at 60ºC and 100ºC:

Repeat the experiment above except heat the solutions to 60ºC and 100ºC gently for 1 hour. Beakers should be covered with watch glasses to prevent loss of solvent or spattering of samples. Electric hot plates or paraffin baths are better suited for this than are bunsen burners. Carefully monitor temperature and watch for signs of gel formation. Determine the amount of solubility by filtering and gravimetric analysis as before.

All experiments should be performed in triplicate and results reported as means + standard deviation.

### 5.4.3 Determination of Crude Fibre

1. Weigh accurately about 2 gm ground sample into a thimble (e.g whole flour)
2. Extract for about 1 hour with petroleum ether in a soxhlet extractor.
3. Transfer the material in the thimble to a 1 litre flask.
4. Take 200 ml of dilute sulphuric acid in a beaker and bring it to boil.
5. Transfer the whole of the boiling acid to the flask containing fat free material and immediately connect the flask to a water cooled reflux condenser and heat so that the contents of the flask begin to boil within 1 minute.
6. Rotate the flask frequently, taking care to keep the material from remaining on the sides of the flask and out of contact with the acid. Continue boiling for exactly 30 minutes.
7. Remove the flask and filter through fine linen ( about 18 threads to a cm ) or through a coarse acid washed, hardened filter paper held in a funnel and wash with boiling water until the washings are no longer acid to litmus paper.
8. Bring some quantity of sodium hydroxide solution to boil under a reflux condenser. Transfer the residue on the filter into the flask with 200 ml of boiling sodium hydroxide solution.
9. Immediately connect the flask with the reflux condenser and boil for exactly 30 minutes.
10. Remove the flask and immediately filter through the linen or filter paper.
11. Thoroughly wash the residue with hot water and transfer to a gooch crucible prepared with a thin but compact layer of asbestos.
12. Wash the residue thoroughly first with hot water and then with about 15 ml of ethanol and with 3 successive washings of petroleum ether.
13. Dry the gooch crucible and contents in an air oven at 105°C for 3 hours. Cool and weigh.
14. Repeat the process of drying for 30 minutes, cooling and weighing until the difference between two consecutive weighings is less than 1 mg.
15. Incinerate the contents of the gooch in a muffle furnace at 550°C until all carbonaceous matter is burnt.
16. Cool the gooch crucible in a dessicator and weigh

**5.5** Results and Calculations**:**

Calculate the amount crude fibre using the following formula:

Crude fibre (on dry basis) = 100 ( W1 – W2 ) x 100

Percent by weight W 100 – M

Where W1 = wt of gooch crucible + contents + asbestos before ashing

W2 = wt of gooch crucible + ash and asbestos after ashing

W = wt of sample taken for test

M = Percent moisture content.

**5.6 Discussion:**

Comment on the values obtained for the crude fibre content in the flour sample. What is the importance of crude fibres for the human digestive system?

**5.7 REFERENCES**

1. Roehrig, K. Carbohydrate Biochemistry and Metabolism. Westport, CT: The AVI Publishing Co. 1984.

EXPERIMENT 5

## 6. Ascorbic Acid Determination

### 6.1 Introduction

Vitamins are a group of small molecular compounds that are essential nutrients in many multi-cellular organisms, and humans in particular. The name "vitamin" is a contraction of "vital amine", and came about because many of the first vitamins to be discovered were members of this class of organic compounds. And although many of the subsequently discovered vitamins were not amines, the name was retained. In this exercise you will be studying vitamin C, also known as ascorbic acid.

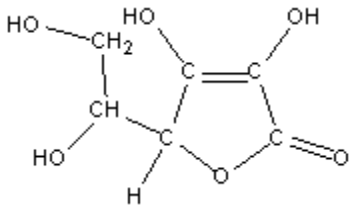



**Figure 1.** Structure of vitamin C (ascorbic acid).

Ascorbic acid ($C_6H_8O_6$) is a water-soluble vitamin, whose structure is shown in Fig. 1. Vitamin C is easily oxidized, and the majority of its functions *in vivo* rely on this property. It plays a key role in the body's synthesis of collagen and norepinephrine by keeping the enzymes responsible for these processes in their active reduced form. Vitamin C may also play a role in detoxifying by-products of respiration. Occasionally during respiration $O_2$ is incompletely reduced to superoxide ion ($O_2^-$) instead of being reduced completely to its -2 oxidation state (as in $H_2O$). Normally an enzyme called superoxide dismutase converts $O_2^-$ to $H_2O_2$ and $O_2$, but in the presence of $Fe^{2+}$ the hydrogen peroxide may be converted into the highly-reactive hydroxyl radical (•OH). The hydroxyl radical can initiate unwanted and deleterious chemistry within a cell when it removes a hydrogen atom (H•) from an organic compound to

form $H_2O$ and a new, potential more reactive free radical. Ascorbic acid can donate a hydrogen atom to a free radical, and thus stop these reactions from occuring.

The human body cannot produce ascorbic acid, and so it must be obtained entirely through one's diet. A vitamin C deficiency in humans results in the disease called scurvy, whose symptoms include hemorrhaging (especially in the gums), joint pain and exhaustion. In its final stages scurvy is characterized by a profound exhaustion, diarrhea, and then pulmonary and kidney failure, which result in death. A very small daily intake of vitamin C (10-15 mg/day for an adult) is required to avoid deficiency and stave off scurvy. However, there has been, and continues to be, vigorous debate on what the optimum daily intake of vitamin C is.

Fruits, vegetables, and organ meats (e.g., liver and kidney) are generally the best sources of ascorbic acid; muscle meats and most seeds do not contain significant amounts of ascorbic acid. The amount of ascorbic acid in plants varies greatly, depending on such factors as the variety, weather, and maturity. But the most significant determinant of vitamin C content in foods is how the food is stored and prepared. Since vitamin C is easily oxidized, storage and the cooking in air leads to the eventual oxidation of vitamin C by oxygen in the atmosphere. In addition, ascorbic acid's water-solubility means that a significant amount of vitamin C present in a food can be lost by boiling it and then discarding the cooking water.

**6.2 Principle:**

Besides being an important vitamin, ascorbic acid is a sugar acid and a powerful reducing agent. The ascorbic acid content in fruits and vegetables can be estimated by macerating the sample with stabilising agents such as 20 % metaphosphoric acid. The most common assay of ascorbic acid is based on its oxidation to dehydroascorbic acid by the redox dye 2,6-dichlorophenol indophenol which is blue in neutral solution and pink in acid. The titration is carried out in acid conditions and at the end point the dye appears rose pink.

**6.3 Objectives**

To estimate the amount of vitamin C in fruit Juices.

## 6.4 Materials and Methods:

### 6.4.1 Apparatus

50 ml Burette, Pipette, Magnetic stirrer and stirring bars, and 250 ml amber glass bottle.

### 6.4.2 Chemicals

a. Sodium 2,6-dichloroindophenol
b. Sodium bicarbonate (NaHCO3)
c. Acetic acid (glacial) (C2H4O2)
d. Ascorbic acid (C6H8O6)

### 6.4.3 Reagents

A. Dye solution (0.5%): Dissolve 0.042 g of NaHCO3 in distilled water and then add 0.050 g of sodium 2,6-dichloroindolphenol, shake vigorously. When dye dissolves, make up to 200 ml. Filter into an amber glass bottle and stored capped in a refrigerator. The solution is good until it fails to give a distinct endpoint.

B. Stabilization Solution - 10% acetic acid. This will keep in the refrigerator for 7 - 10 days.

C. Ascorbic acid standard solution (1 mg/ml): Accurately weigh 0.100 g of ascorbic acid into a 100-ml volumetric flask. Immediately before use, dissolve in 100 ml of acid stabilization solution.

Fruit juice samples (2 ml) are to be analysed.

### 6.4.4 Method

a. Standardize in triplicate the indophenol solution by rapidly titrating against 2 ml aliquots of standard ascorbic acid solution plus 5 ml of stabilization solution until a distinct rose-pink persists for at least 5 s. Express standardized indophenol solution as mg ascorbic acid equivalent per 1 ml of dye.

b. Titrate in triplicate 2 ml aliquots of the fruit juices (which should contain between 10- 100 mg ascorbic acid/100 m) plus 5 ml extracting solution using the standardized indophenol solution.

**6.5 Results and Calculations:**

| **Sample** | **Titration volume (1)** | **Titration volume (2)** | **Titration volume (3)** | **Average titration volume** |
|---|---|---|---|---|
| Blank | | | | |
| Standard | | | | |
| Fruit juice | | | | |

Take the average of the three titrations, subtract a blank titration (no food sample), and calculate the ascorbic acid as mg / 100 ml by the following formula:

**[ (mean sample titration volume – blank titration volume) X (standardized mg AA/ ml dye) X 100/2]**

Comment on the results obtained for the concentration of ascorbic acid in your fruit juice sample.

**6.6 REFERENCE**

1. Official Methods of Analysis. 1999. 16th Edition, 5th Reversion, AOAC International, Gaithersburg, MD, method 967.21.

EXPERIMENT 6

## 7. Determination of Crude Protein

by the Kjeldahl Method

### 7.1 Introduction

Proteins are the major structural components of many natural foods, often determining their overall texture, *e.g.*, tenderness of meat or fish products. Isolated proteins are often used in foods as ingredients because of their unique functional properties, *i.e.*, their ability to provide desirable appearance, texture or stability. Typically, proteins are used as gelling agents, emulsifiers, foaming agents and thickeners. Many food proteins are enzymes which are capable of enhancing the rate of certain biochemical reactions. These reactions can have either a favorable or detrimental effect on the overall properties of foods. Food analysts are interested in knowing the total concentration, type, molecular structure and functional properties of the proteins in foods.

7.2 **Principle**:

This method enables the determination of all protein nitrogen (N) and most non-protein N in all types of foods.

The Kjeldahl method involves:

**7.2.1 Digestion:** Digestion of the sample in concentrated sulphuric acid with a catalyst such as copper, selenium, titanium, or mercury. This results in the conversion of susceptible nitrogenous compounds to ammonium sulphate.

N (food) □ $(NH_4)_2SO_4$ (1)

**7.2.2 Neutralization:** The resultant solution is then neutralised with excess caustic soda. Ammonia is liberated by steam distillation and captured in boric acid. The borate formed is titrated with dilute sulphuric acid. Depending on equipment chosen, titration can be fully automated or carried out manually.

**7.2.3 Titration:** The nitrogen content is then estimated by titration of the ammonium borate formed with standard sulfuric or hydrochloric acid, using a suitable

indicator to determine the end-point of the reaction. The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen that was in the original food (Equation 3).

$(NH_4)_2SO_4 + 2\ NaOH \rightarrow 2NH_3 + 2H_2O + Na_2SO_4$ (2).

$NH_3 + H_3BO_3$ (boric acid) $\rightarrow NH_4^+ + H_2BO_3^-$ (borate ion) (3)

$H_2BO_3^- + H^+ \rightarrow H_3BO_3$ (4)

A blank sample is usually ran at the same time as the material being analyzed to take into account any residual nitrogen which may be in the reagents used to carry out the analysis. Once the nitrogen content has been determined it is converted to a protein content using the appropriate conversion factor:

% Protein = F X %N.

**7.3 Objectives**

To determine the protein content of a sample of milk using the indirect method of Kjeldahl

**7.4 Materials and Methods:**

**7.4.1 Equipment**:

1. Fume hood with neutralising tank for acidic vapours
2. Block digester, capable of attaining 420$^oC$ and digesting 20 samples at a time.
3. Steam distillation apparatus.
4. Analytical electronic balance, accurate to 0.1 mg
5. Digestion tubes and digestion tube stand
6. Forceps, scoop, and spatula,
7. Variable dispenser – 25 ml volume
8. Conical flasks, Glass 250 ml
9. Digital burette
10. Protective gloves

### 7.4.2 Reagents:

1. Digestion acid - concentrated, commercial grade sulphuric acid, (98 - 99% purity - SG 1.84).
2. Catalyst - Kjeltabs, (containing 3.5 g $K_2SO_4$ and 0.035 g Se) or Kjeltabs TCT (containing 3.0 g $K_2SO_4$, 0.105 g $CuSO_4$ and 0.105 g TiO).
3. Sodium Hydroxide solution, typically 35 - 45% w/v for use in steam distillation apparatus (refer operating instructions for required concentration). To prepare 6 litres of stock concentrate 45% NaOH solution, proceed as follows:
   (a) Weigh 2.7 kg of commercial grade NaOH into a plastic container.
   (b) Transfer 6 litres of deionised water to a suitably sized stainless steel bucket.
   (c) Slowly add the NaOH to the water.
   (d) Stir to dissolve.
   (e) Allow to cool and transfer to a suitable plastic reservoir.
4. Boric Acid/Indicator Solution, 4% w/v. To prepare 10 litres of solution:
   (a) Weigh 400 g of commercial grade boric acid and transfer to an appropriately sized electric blender bowl. Add enough deionised water to ¾ fill the bowl. Cover and blend on 'high' for about 1 minute.
   (b) Transfer the slurry to a stainless steel bucket (capacity > 10 l) ¾ filled with deionized water. Stir to dissolve to a clear solution.
   (c) Add 100 ml of bromocresol green solution (0.1g bromocresol green in 100 ml of ethanol).
   (d) Add 70 ml of methyl red solution (0.1g methyl red in 100 ml of ethanol).
   (e) Add deionised water to make to the 10 litre mark and continue to stir until all crystalline material has dissolved.
   (f) Transfer to a suitable container.
5. Sulphuric Acid - approximately 0.1000 N, standardized to 0.0001N.

### 7.4.3 Safety Precautions:

- Sulphuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.
- Handle concentrated acid and alkali safely. Wear heavy rubber gloves and face

shield to protect against concentrated acid or alkali. Use effective fume removal device to protect against acid fumes, alkali dusts and vapours.

- Always add concentrated sulphuric acid or sodium hydroxide pellets to water, not *vice versa*.
- The sulphur dioxide fumes produced during digestion are hazardous to inhale.
- Digests must be cool before dilution water is added to avoid a violent reaction. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

**7.5 Procedure:**

**7.5.1 Step1: Digestion**

1- Weigh (to 0.1mg) about 1g of well mixed sample into a tared scoop. Transfer to a digestion tube without loss. Record sample number, digestion tube number and weight of sample.

2- Weigh a second sub-sample for laboratory dry matter determination.

3- Place digestion tubes in a fume hood. Add two (2) Kjeltabs to each tube then add concentrated sulphuric acid. Usual volume is 15-18 ml but actual volume will depend on digestion system selected. Mix carefully by swirling the tube by hand.

4- Place tubes in block digester preheated to 420$^{o}$C. (Digester must be equipped with an exhaust system and/or placed in an acid fume hood).

5- Digest for 45 - 60 minutes.

**Note:** Actual temperature and time of digestion vary with equipment. For example, some block digestion systems reduce temperature to 390$^{o}$C after the initial 10 minutes and then digest for a further 50 minutes.

6- When time has elapsed, remove tubes from the digester block and let cool for at least 20 minutes in a fume hood. Time will depend upon airflow around tubes. Do not cool to point of solidification or loss of Nitrogen will occur. Rapid solidification is an indication of insufficient acid or excessive digestion times.

7- When cool, direct a rapid spray or stream of deionised water to the bottom of each tube to dissolve the acid digest completely. Add about 50 ml of water.

### 7.5.2 Step 2: Distillation and Titration

1- Place NaOH in alkali tank of steam distillation unit. Make sure that sufficient NaOH (about 50 ml) is dispensed from the unit to neutralise all of the acid in the tube before distillation starts.
2- Place a 250 ml conical titration flask containing 25 ml 4% boric acid/indicator solution on the receiving platform. Ensure the tube from the condenser reaches below the surface of the boric acid.
3- Attach digestion tube containing diluted, cooled digest to steam distillation unit.
4- Dispense appropriate volume of NaOH solution.
5- Steam distil until 100-125 ml distillate collects or the automatic process is completed.
6- Remove the receiver flask, rinse the condenser tip with deionised water and place on the bench ready for titration. Put the next sample into distillation unit and proceed from Step 8.
7- While the next sample is distilling, titrate the distillate from the previous sample to a neutral grey with 0.1N $H_2SO_4$. Record the volume of titrant ($H_2SO_4$) used (VS) to the nearest 0.01ml. Titrate the reagent blank (VB) similarly. The colour change is to a neutral grey.
8- When all the samples have been distilled and distillate has been titrated, empty the digestion tubes and flasks down the sink with copious amounts of water. Wash tubes and flasks as appropriate.
9- Shut down the distillation unit according to manufacturer's instructions.

**7.6** Results and Calculations**:**

For standard $H_2SO_4$ titrant, results are calculated using the equation:

- **% Nitrogen (%N)** = $$\frac{(V_S - V_B) \text{ X N } (H_2SO_4) \text{ X } 14.007 \text{ X } 100}{W \text{ X } 1000}$$

Where:

**VS** = Volume, in ml, of standard $H_2SO_4$ required to titrate sample

**VB** = Volume, in ml, of standard $H_2SO_4$ required to titrate blank

**N ($H_2SO_4$)** = Normality of the acid titrant

**14.007** = equivalent weight of Nitrogen

**W** = sample weight in grams

**% Crude Protein (%CP) = %N X 6.25** and

**% Nitrogen Dry Matter basis (%N DM) = %N X [100 / Lab DM%]**

Where:

Lab DM% = Percent Laboratory Dry Matter of the sample analysed.

Calculate the grams of nitrogen in your sample, then the N% and finally the protein % using the suitable conversion factor.

**7.7 Discussion:**

Comment on the values obtained for total proteins in your sample.

**Notes:**

- Reagent proportions, heat input and digestion time are critical factors - Do not change. *Choose the settings for temperature and length of digestion, which give a 98% recovery of N from an EDTA standard*.
- Ratio of salt to acid (wt:vol) should be 1:1 at end of digestion with proper temperature control.
- Digestion may be incomplete at lower ratio and nitrogen may be lost at higher ratio.
- The protein content may be determined by multiplying the nitrogen value by the following factors:

| | |
|---|---|
| Proteins general | (x 6.25) |
| Milk proteins | (x 6.38) |
| Foodstuffs | (x 6.25) |
| Soya products | (x 6.00) |
| Wheat flour | (x 5.70) |

**7.8 REFERENCES**

**3.** **S**audi standard. Methods for the chemical analysis of cheese. 1977, SSA, 70/1397 H.

4. FEEDTEST, DPI Vic, Hamilton, Victoria - Method Manual, Method 2.6 Determination of Crude Protein - Kjeldahl Method.

EXPERIMENT 7

## 8. Estimation of casein in milk

### 8.1 Introduction:

Casein is the main protein in milk and is present at a concentration of about 35g/litre. It is actually a heterogeneous mixture of phosphorous containing proteins and not a single compound. It is 85 to 90% of total protein in milk.

### 8.2 Objective:

To determine the concentration of casein in milk.

### 8.3 Materials and Methods:

#### 8.3.1 Chemicals:

a. Casein
b. Sodium carbonate
c. Sodium hydroxide
d. Sodium potassium tartarate
e. Sodium dodecyl sulphate (SDS)
f. Copper sulphate ( $CuSO_4.5H_2O$)
g. Folin-Ciocalteu Reagent

#### 8.3.2 Solutions:

a. Casein standard (100 μg/ml)
b. Reagent A
c. Reagent B
d. Reagent C

#### 8.3.3 Materials:

Milk (keep in fridge before the experiment)

#### 8.3.4 Equipment and glassware:

1. Refrigerated centrifuge & tubes
2. Spectrophotometer
3. Test tubes
4. Glass cuvettes

### 8.3.5 Preparation of solutions:

Casein standard (100 µg/ml): Dissolve 0.01g casein in water and make up to 100 ml with water.

**Reagent A**:

Dissolve; 2 g $Na_2CO_3$ (2%), 0.4 g NaOH (0.4%), 0.16 g sodium, potassium tartarate ( 0.16%), 1g SDS (1%) in water and make up to 100 ml.

Store at room temperature.

**Reagent B**:

4% $CuSO_4.5H_2O$, Dissolve 0.4g $CuSO_4.5H_2O$ in a little volume of water and make up to 10 ml. Store at room temperature.

**Reagent C**:

100 parts of reagent A + 1 part reagent B. Take 100 ml reagent A and add 1ml reagent B.

**Folin-Ciocalteu reagent**:

Dilute commercial reagent by 1: with water. Prepare fresh.

## 8.4 Procedure

### Preparation of fat-free milk:

1. Centrifuge cold milk at 0 - 4°C for 30 min. at 4,000 rpm. Quickly remove the lipid layer from the top.
2. Dilute fat-free milk 1: 1000
3. Pipette 0.1ml milk into a 100ml volumetric flask and make up to the mark with distilled water.
4. Set up 8 tubes as follows:
5. A

| Tube | Water (ml) | Casein standard ( ml) | Diluted milk (ml) |
|---|---|---|---|
| A(blank) | 1.0 | - | - |
| B | 0.8 | 0.2 | - |
| C | 0.6 | 0.4 | - |
| D | 0.4 | 0.6 | - |
| E | 0.2 | 0.8 | - |
| F | - | 1.0 | - |
| G | - | - | 1.0 |
| H | - | - | 1.0 |

ent C to all tubes. Mix and let stand at room temp. for 15 min.

6. Add 0.3 ml of Folin-Ciocalteu reagent. (Add this reagent to one tube at a time and immediately after adding it mix well).
7. Let the tubes stand at room temperature for 45 min.
8. Read absorbance at 660 nm against the blank.

**8.5 Results and Calculations:**

| Tube | Casein concentration (µg/ml) | $A_{660}$ |
|---|---|---|
| A | 0 | |
| B | 20 | |
| C | 40 | |
| D | 60 | |
| E | 80 | |
| F | 100 | |
| G | | |
| H | | |

9. Plot a standard curve for absorbance at 660 nm against casein concentration (µg/ml). From the standard curve obtain the concentration of casein in the diluted milk.

**Average casein concentration in diluted milk = (µg/ml).**

**Calculate the concentration of casein in the original milk (g/litre)**

$$\textbf{Concentration in original milk} = \frac{\textbf{Average conc. in diluted milk} \times 10^3 \times 10^3}{106}$$

REFERENCES


- Neide K.K. et al., Determination of total proteins in cow milk powder samples: a comparative study between the Kjeldahl method and spectrophotometric methods. J. Food Anal. 2003, 16: 507-516.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193, 265–275.

EXPERIMENT 8

# 9. DETERMINATION OF MOISTURE AND SUCROSE IN BEE HONEY

## 9.1 INTRODUCTION AND PRINCIPLE

Natural bee honey contains from 21 to 23% moistures (water) and hence higher moisture content indicates the addition of water as an adulterant. In order to determine the moisture content, the sample may be dried under vacuum for a sufficient length of time (i.e, until no further apparent weight loss occurs). The difference in weight before and after drying may then be taken to represent water content.

In practice, water is not completely removed from the sample, even when drying at elevated temperatures. The use of vacuum drying prevents the loss of volatile materials, which occurs at elevated temperatures and introduces a positive error into the determination of moisture content.

Natural bee honey also contains from 6 to 10% sucrose. The sucrose content may be increased artificially by addition of sucrose or decreased either by the addition of other sugars or components of bee honey or by hydrolysis (inversion) of the sucrose. Since sucrose is made up of glucose and fructose, its hydrolysis produces an equimolar mixture of these two monosaccharides (invert sugar). Glucose and fructose are both reducing sugars, whereas sucrose is non-reducing. Hence determination of reducing sugar content both before and after hydrolysis of the sucrose may be used to determine its content in a sample of bee honey.

The method which is used to determine reducing sugar content in this experiment is the method of Lane and Eynon, in which the reducing sugars are determined titrimetrically. The principle upon which the method is based is the ability of reducing sugars, when heated in alkaline solution, to reduce cupric ($Cu^{2+}$) salts, which are blue, to cuprous oxide ($Cu_2O$), which forms a red precipitate. In the Lane and Eynon method, the sample containing the reducing sugars is added from a burette to a vigorously boiling standard volume of Fehling's solution. Near the end-point, mthylene blue indicator is added to the boiling solution and the titration is completed, adding the sample dropwise, until the blue colour of the indicator disappears, indicating a slight excess of reducing sugar. The titration is then repeated twice, each time adding the bulk of the sample immediately and completing the titration quickly. The average of these latter two titrations is then used for purposes of calculation.

## 9.2 OBJECTIVES:

1. To determine the moisture (water) content of samples of bee honey.
2. To determine the sucrose content of bee honey.

## 9.3 Materials and Methods:

### 9.3.1 MATERIALS

#### 9.3.1.1 Determination of moisture content:

1. Shallow steel evaporating dishes equipped with glass stirrers.
2. Ground pumice stone.
3. Vacuum drier.
4. Desiccator.

#### *9.3.1.2* Determination of sucrose content

1- Fehling's A solution: Dissolve 69.28 g of analytical grade copper sulphate pentahydrate ($CuSO_4.5H_2O$) in distilled water and make the volume up to one litre in a volumetric flask. (Keep the solution for one day before titration) Fehling's B solution: Dissolve 346 g of sodium potassium tartrate ($C_4H_4KNaO_6.4H_2O$ MW= 282.23) and l00 g of sodium hydroxide in distilled water and make the volume up to one litre in a volumetric flask. Filter through asbestos layers or glass wool.

2- Standard invert sugar solution (5g/l): Weigh. accurately 4.75g of pure sucrose into a flask and add 5 ml of concentrated hydrochloric acid. Dilute with distilled water to a volume of about l00 ml and store for several days at room temperature to complete the acid hydrolysis of the sucrose (7 days at 12-15°C or 3 days at 20-25°C). Then dilute to exactly one litre with distilled water in a volumetric flask.

3- Methylene blue indicator -Dissolve 2g of methylene blue in distilled water and make the volume up to one litre.

4- Alumina cream: Prepare a cold saturated solution of alum $[K_2SO_4Al_2SO_4)_3.24H_2O]$ in distilled water. Add ammonium hydroxide with constant stirring until the solution is alkaline to litmus. Let the precipitate settle and wash it by decantation with distilled water until the wash water gives only a slight test for sulphate with barium chloride

solution. Pour off the excess water and store the residual cream in a stoppered bottle.

5- 6.34 N hydrochloric acid.

6- 5 N sodium hydroxide.

### 9.3.2 METHODS

#### *9.3.2.1* Determination of moisture content

1. Place approximately 10 g of ground pumice stone in a shallow steel evaporating dish containing a small glass stirrer and dry for 2 hours in an oven at 105°C.
2. After cooling in a desiccators, weigh, add 5 g of sample and then re-weigh to obtain the sample weight accurately. Mix the sample with the pumice, spreading over the bottom of the dish.
3. Dry under vacuum (310-320 mm mercury) for 3 hours. The air flow should be 100 $cm^3$ $min^{-1}$ and the air should be passed over activated alumina and barium oxide.
4. Repeat the drying procedure for a further 15 minutes and weigh again.
5. Continue to dry by this method until constant weight is reached i.e. no further weight loss occurs upon drying for 15 minutes.
6. Use the final weight to calculate the moisture content of the sample from the weight loss and express your result as moisture per 100 grams of sample.

#### 9.3.2.2 Determination of sucrose content

1. Using an analytical balance. weigh exactly about 25 g of the well-mixed sample of honey and transfer it quantitatively to a 100 ml volumetric flask, using distilled water. Add 5 ml of alumina cream and dilute to volume with distilled water. Mix thoroughly and filter. THE QANTITY OF HONEY TATAKEN FOR DILUTION SHOULD BE DETERMINED BY A DEMONSTRATOR BEFORE THE EXPERIMENT.
2. Pipette equal (5 ml) quantities of Fehling's A and Fehling's B solutions into a 250 ml conical flask and swirl to mix the contents.
3. Place the diluted honey sample solution in a 50 ml burette.

4. Add to the contents of the flask about l0 ml of distilled water, some powdered pumice and about 15 ml of the diluted honey sample solution from the burette.
5. Heat the contents of the flask until they boil and then add 1ml of 0.2% (w/v) methylene blue indicator.
6. Complete the titration with the diluted honey sample solution, taking the disappearance of the blue colour to indicate the end-point.
7. Standardize the mixed Fehling's solution by titration of 5 ml of Fehling's A solution and 5 ml of Fehling's B solution with standard invert sugar solution (5 g/l). If the titration volume is too small or too large, adjust the concentration of the invert sugar solution accordingly.
8. Calculate the reducing sugar content in the sample taking into consideration the concentration of the standard solution and the dilution factor for the sample honey.
9. For the determination of sucrose content, take exactly 50 ml of the diluted honey solution (from step "1" above) and place it in a 100 ml volumetric flask, together with 25 ml of distilled water.
10. Heat gently in a water bath at about 60°C and add l0 ml of 6.34N hydrochloric acid. leaving the solution at this temperature for about 10 minutes to ensure hydrolysis of sucrose.
11. Cool the solution for 15 minutes and then neutralize it with 5 N sodium hydroxide using litmus paper.
12. Cool the solution again and make the volume up to 100 ml with distilled water. Note that this procedure constitutes a further 2X dilution of the sample.
13. Determine the reducing sugar content as before (steps "2" - "6" above).

**9.4 Results and Calculations:**

Calculate the sucrose content 1n the sample as follows:

**Sucrose content = 0.95 (S - R)**

where R is the reducing sugar content in the undiluted sample before inversion and S is the reducing sugar content in the undiluted sample after inversion.

**9.5 Discussion**

- Comment on the values obtained for sucrose in your honey sample. What can you say about your sample compared to natural bee honey values?
- Comment on the values obtained for moisture base on natural bee honey moisture content.

## 9.6 REFERENCES

1- Saudi Standard "Honey" , ( 1978 ), SSA 101/1978 .
2- Saudi Standard "Methods of Testing for Honey", (1978). SSA 102/1978.
3- Lees. R. "Food Analysis", (1975), Leonard Hill Books.

## 9.7 SAUDI STANDARD FOR BEE HONEY

Honey is the sweet substance produced by honey bees from the nectar of blossoms or from the secretions of living parts of plants. which they collect. transform and combine with specific substances and store in honey combs. The main requirements of bee honey are:

Free from impurities  insects or grains of sand. Free from objectionable flavour, fermentation or effervescence.

Not heated to such an extent as to inactivate greatly or completely the natural enzymes –it contains, e.g. diastase activity.

Free from any additives.

Having moisture content from 21 to 23%.

Having sucrose content from 6 to 10% and water-insoluble solids from 0.1 to 0.5%.

EXPERIMENT 9

## 10. Determination of Antioxidant Activities using the DPPH Radical Scavenging Assay

### 10.1 Introduction:

Fruits and vegtables contain a wide variety of free-radical scavenging molecules, including phenolic compounds, carotenoids, and vitamins. Free radicals are normally produced by a number of mechanisms including aerobic metabolism but are unstable and highly reactive due to their unpaired electrons. There is increasing evidence that free radicals and reactive oxygen species (ROS) can induce oxidative damage in biomolecules such as lipids, proteins, and nucleic acids, leading to human diseases such as atherosclerosis, cancer, diabetes mellitus, and neurodegenerative disorders. Interestingly, diets rich in plant-based products such as fresh vegetables and fruits, wine, and tea, have been found to protect humans from these degenerative diseases.

The antioxidant properties may be determined by monitoring the reaction of the species with a model free radical, such as diphenyl-1-picrylhydrazyl or DPPH. DPPH (see Figure 1) is a “stable” free radical because of delocalization of the spare electron throughout the molecule. This delocalization gives rise to a deep violet color when DPPH is dissolved in methanol, with an absorption maximum at 515 nm. When a solution of DPPH is mixed with an antioxidant that can donate a hydrogen atom to the free radical, it gives rise to the reduced form of the DPPH molecule, with a subsequent loss of the violet color (although a residual pale yellow may remain due to the picryl group present on the DPPH molecule).

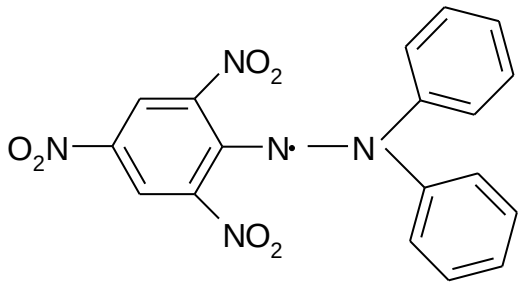


**Fig. 1: DPPH**

## 10.2 Objectives:

Tea remains one of the most popular beverages world-wide and previous research has shown that tea contains a variety of phenolic compounds which are potent antioxidants.

## 10.3 Principles

In this method, we will use a colorimetric method, the Folin-Ciocalteu assay, to quantify the total phenolic content of tea samples. The Folin-Ciocalteu method involves complexation of phenolic compounds in a sample with a molybdate reagent to form a colored complex that absorbs at 735 nm. Gallic acid is used to produce a standard curve and final phenolic concentrations in the sample are reported in mg/L gallic acid equivalents (GAE).

## 10.4 Materials and Methods:

### 10.4.1 Materials:

1- Green tea powder
2- Black tea powder
3- Folin Ciocalteu phenol reagent
4- Gallic acid: prepare 1000 mg/l in ethanol
5- Ascorbic acid: Prepare 100 mg/l.
6- 20% Sodium carbonate
7- 1,1-diphenyl-2-picrylhydrazyl (DPPH) (1 mM in ethanol)
8- Ethanol
9- UV/VIS spectrophotometer
10- Hot plate

### 10.4.2 Preparation of tea samples

1- Brew about 2 g of tea sample in 250 ml boiling distilled water for 5 min.
2- Cool to room temperature and filter your using Whatman No. 2 filter paper.

### 10.4.3 Determination of total phenolic content

1. Add 50 µl of tea extract to a 2 ml amber vial with 450 µl of distilled water and 250 µl of Folin-Ciocalteu reagent.
2. Add 1.25 ml of 20% $Na_2CO_3$ to the amber vial with caution. Shake and allow it to incubate for 20 min at room temperature.
3. The absorbance may then be measured at 735 nm versus a water/$Na_2CO_3$ blank. Gallic acid should be used as the analytical standard over a concentration range from 50 to 500 mg/l (note that if the absorbance of tea sample does not fall on your gallic acid calibration curve, you may need to dilute your sample).

### 10.4.4 Determination of DPPH radical scavenging

1. Mix 1 ml of 1 mM DPPH solution in ethanol with 3 ml of tea extract solution (choose the same extract concentration for each of your tea samples).
2. Mix vigorously and incubate for 30 minutes in the dark.
3. The absorbance may then be measured at 517 nm versus an ethanol /water blank.
4. Ascorbic acid should be used as a positive control over a concentration range from 10 – 50 mg/l.

### 10.5 Results and Calculations:

**Determination of total phenolics**

Set up different concentrations of Gallic acid as follows**:**

| Tube | Gallic acid standard ( ml) | Folin-Ciocalteu reagent (ml) | Dist. Water (ml) | $Na_2CO_3$ (ml) |
|---|---|---|---|---|
| A (blank) | - | 0.25 | 1 | 1.25 |
| B | 0.05 | 0.25 | 0.950 | 1.25 |
| C | 0.10 | 0.25 | 0.900 | 1.25 |
| D | 0.20 | 0.25 | 0.800 | 1.25 |
| E | 0.30 | 0.25 | 0.700 | 1.25 |

| | | | | |
|---|---|---|---|---|
| F | 0.40 | 0.25 | 0.600 | 1.25 |
| **Black tea** | **0.05** | 0.25 | 0.950 | 1.25 |
| **green tea** | **0.05** | 0.25 | 0.950 | 1.25 |

**Radical scavenging activity:**

| | **Absorbance at 517 nm** |
|---|---|
| **DPPH without tea extract** | |
| **DPPH containing tea extract** | |

The antioxidant activity is expressed as percent DPPH scavenging and is calculated as:

$$\% \text{ DPPH scavenging} = [(A_{control} - A_{extract})/A_{control}] \times 100$$

Where:

$A_{control}$ is the absorbance of the sample without tea extract and

$A_{extract}$ is the absorbance of the sample containing the tea water extract.

## 10.6 Discussion:

- Does a correlation exist between total phenolic content and antioxidant activity for your tea samples? Explain.

- Based on your results, make a final conclusion about which tea is likely to have the greatest antioxidant health benefits

## 10.7 REFERENCES

RONALD L. PRIOR, XIANLI WU, AND KAREN SCHAICH. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. J. Agric. Food Chem. 2005, 53, 4290-4302

EXPERIMENT 10

## 11. Qualitative Tests on Fats and Oils

### Saponification Number

### 11.1 Introduction and Principles

On refluxing with alkali, triacylglycerols (fatty acid esters) are hydrolyzed to give glycerol and potassium salts of fatty acids (soap). Such process is known as, *Saponification* . The saponification equation is shown below:

Triacylglycerol + 3 KOH $\longrightarrow$ Glycerol + 3 Fatty acid salts of potassium (soap)

The saponification value is the number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1g of fat. The lipid is first extracted and then dissolved in an ethanol solution which contains a known excess of KOH. This solution is then heated so that the reaction goes to completion. The unreacted KOH is then determined by adding an indicator and titrating the sample with HCl. The saponification value gives an indication of the nature of the fatty acids constituent of fat and thus, depends on the average molecular weight of the fatty acids constituent of fat. The saponification number is then calculated from a knowledge of the weight of sample and the amount of KOH which reacted. The smaller the saponifications number the larger the average molecular weight of the triacylglycerols. Some saponification values:
Butter - 210 to 235, Cod liver oil - 171 to 189, Olive oil - 190 to 194, Peanut oil - 186 to 194, Cottonseed oil - 191 to 198. These values are given as a range and therefore a number of determinations of your unknown should be carried out to determine the range to enable you to identify your oil (only if it is pure).

### 11.2 Objectives:

To determine the saponification value for a sample of fat or oil

### 11.3 Materials and Methods:

#### 11.3.1 Materials:

1. Fats and oils (olive oil, coconut oil, sesame oil, and butter)
2. Fat solvent (equal volumes of 95% ethanol and ether)

3. Alcholic KOH (0.5 M)
4. Reflux condenser.
5. Boiling water bath.
6. Phenolphethalein.
7. Hydrochloric acid (0.5 mol/liter)
8. Burettes (10 ml and 25 ml)
9. Conical flasks (250 ml)

**11.4 Procedure:**

1. Accurately weight 1g of fat or oil in a small beaker and dissolve it in about 3 ml of the fat solvent.
2. Quantitatively transfer the contents of the beaker to a 250 ml conical flask by rinsing the beaker three times with a further milliliters of solvent.
3. Add 25 ml of alcoholic KOH and attach to a reflux condenser .
4. Set another reflux condenser as blank with everything present except the fat.
5. Heat both flasks on a boiling water bath for 30 min.
6. Leave to cool to room temperature and titrate with 0.5 mol/liter HCl and use phenolphthalein as indicator. Until the pink color disappears.
7. Record your readings as **T** ml for test and **B** ml for blank.

## 11.5 Results and Calculations:

The difference between the blank and the test reading gives the number of milliliters of KOH required to saponify 1g fat. You can use this formula to calculate the saponification value:

1 ml (0.5 N HCl ) = 28.05 mg KOH

( B-T ) = S

$$\text{saponification value (S)} = \frac{(B-T) \times 28.05}{\text{Wt. of fat (1g}} = \quad \text{mg KOH/1g}$$

11.6 **Discussion:**

Comment on the values obtained for the saponification value for triglyceride sample. What can you say about the samples based on these values?

## 11.7 REFERENCES

1. Aynon. (1964), Official and Tentative Methods of the American Oil Chemists' Society, 2nd. edn., American Oil Chemists' Society, Chicago.
2. Aynon. (1954), International Union of Pure and Applied Chemistry, "Standard Methods for the Analysis of Oils and Fats", Paris, France.

EXPERIMENT 11

## 12. Determination of Acid Value

### 12.1 Introduction:

The acid value is defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in one gram of fat. It is a relative measure of rancidity as free fatty acids are normally formed during decomposition of oil glycerides. The value is also expressed as per cent of free fatty acids calculated as oleic acid.

### 12.2 Objectives:

To determine the concentration of fatty acids that are removed from a triacylglycerols (lipid) due to hydrolysis

### 12.3 Principle:

The value is a measure of the amount of fatty acids which have been liberated by hydrolysis from the glycerides due to the action of moisture, temperature and/or lypolytic enzyme lipase.

The acid value is determined by directly titrating the oil/fat in an alcoholic medium against standard potassium hydroxide/sodium hydroxide solution.

### 12.4 Materials and Methods:

#### 12.4.1 Reagents:

1. Ethyl alcohol :- Ninety-five per cent alcohol or rectified spirit neutral to phenolphthalein indicator.
2. Phenolphthalein indicator solution :- Dissolve one gram of phenolphthalein in 100 ml of ethyl alcohol.
3. Standard aqueous potassium hydroxide or sodium hydroxide solution (0.1 or 0.5 N). The solution should be colorless and stored in a brown glass bottle.

#### 12.4.2 Procedure:

1. Mix the oil or melted fat thoroughly before weighing.
2. Weigh accurately about 5 to 10 g of cooled oil sample in a 250 ml conical flask and add 50 ml to 100 ml of freshly neutralised hot ethyl alcohol and about one ml of phenolphthalein indicator solution.

3. Boil the mixture for about five minutes and titrate while hot against standard alkali solution shaking vigorously during the titration.

   The weight of the oil/fat taken for the estimation and the strength of the alkali used for titration shall be such that the volume of alkali required for the titration does not exceed 10 ml.

## 12.5 Results and Calculations:

Acid value = 56.1(V x N) / W

Where V = Volume in ml of standard potassium hydroxide or sodium hydroxide used

N = Normality of the potassium hydroxide solution or Sodium hydroxide solution; and W = Weight in g of the sample

The acidity is frequently expressed as free fatty acid for which calculation shall be

Free fatty acids as oleic acid per cent by weight W = 28.2 (V x N) / W

Acid value = Percent fatty acid (as oleic) x 1.99.

## 12.6 Discussion

- Comment on the values obtained for FFA determination for oil sample. What can you say about the samples based on these values?

12.7 **References**:

1. Handbook of Food Analysis (Part XIII)-1984 Page 67
2. IUPAC 2.201(1979) / I.S : 548 (Part 1 ) – 1964, Methods of Sampling and Test for Oils and Fats )

EXPERIMENT 12

## 13. Determination of Iodine Number

### 13.1 Introduction:

The amount of unsaturation in fat samples is often determined industrially and is termed the iodine number of the fat. The iodine value (*IV*) gives a measure of the average *degree of unsaturation* of a lipid: the higher the iodine value, the greater the number of C=C double bonds. By definition the iodine value is expressed as the grams of iodine absorbed per 100g of lipid. One of the most commonly used methods for determining the iodine value of lipids is "Wijs method".

### 13.2 Principle:

The lipid to be analyzed is weighed and dissolved in a suitable organic solvent, to which a known excess of iodine chloride is added. Some of the ICl reacts with the double bonds in the unsaturated lipids, while the rest remains: The amount of ICl that has reacted is determined by measuring the amount of ICl remaining after the reaction has gone to completion. The amount of ICl remaining is determined by adding excess potassium iodide to the solution to liberate iodine, and then titrating with a sodium thiosulfate ($Na_2S_2O_3$) solution in the presence of starch to determine the concentration of iodine released:

R-CH=CH-R + ICl (excess) □ R-CHI-CHCl-R + ICl (remaining)

ICl (reacted) =ICl (excess) – ICl (remaining).

ICl (remaining) + 2KI □ KCl + KI + $I_2$

$I_2$ + starch + $2Na_2S_2O_3$ (blue) □ 2NaI + starch + $Na_2S_4O_6$ (colorless)

Iodine itself has a reddish brown color, but this is often not intense enough to be used as a good indication of the end-point of the reaction. For this reason, starch is usually used as an indicator because it forms a molecular complex with the iodine that has a deep blue color. Initially, starch is added to the solution that contains the iodine and the solution goes a dark blue. Then, the solution is titrated with a sodium thiosulfate solution of known molarity. While there is any $I_2$ remaining in the solution it stays blue, but once all of the $I_2$ has been converted to $I^-$ it turns colorless. Thus, a change in solution appearance from blue to colorless can be used as the end-point of the titration. The concentration of C=C in the original sample can therefore be calculated by measuring the amount of sodium thiosulfate needed to complete the titration. The higher the degree of

unsaturation, the more iodine absorbed, and the higher the iodine value. The iodine value is used to obtain a measure of the average degree of unsaturation of oils, and to follow processes such as hydrogenation and oxidation that involve changes in the degree of unsaturation. The sample titration (T) gives a measure of the amount of (iodine) remaining in solution after halogenation.

## 13.3 Objectives:

To determine the iodine number for fats and oils.

## 13.4 Materials and Methods:

### 13.4.1 Reagents

1. The halogenation reagent:- Pyridine sulphate dibromide in glacial acetic acid is made as follows:
   8 ml of pyridine and 5.5 ml concentrated sulphuric acid are separately added to 20 ml of glacial acetic acid. A 2.5 ml aliquot of bromine is then added to another 20 ml portion of the volume made up to 1 litre with glacial acetic acid giving an approximately 0.1 M solution of Bromine.
2. 10% aqueous solution of potassium iodide.
3. 1% aqueous solution of starch.
4. 0.1 mol/l standard thiosulphate solution.
5. various fats to be assigned.

## 13.5 Procedure

1. To 2.0 ml of the fat, add 25 ml of chloroform to dissolve the material.
2. Pipette 5 ml sample of this solution into two separate Erlenmeyer flasks.
3. To a third flask add 5 ml of chloroform to form a blank. Cover with aluminum foil, shake and allow to stand for 15 minutes.
4. Add 7.5 ml of potassium iodide KI solution to each flask and titrate the liberated iodine using the thiosulphate solution.
5. Titrate the test solution until a light brown colour is obtained then add 5 drops of starch indicator solution immediately and titrate to a colourless end point.
7. Record the total volume of the thiosulphate required to reach the end point.

### 13.6 Results and Calculations:

The titration of a blank (B) gives a measure of the total amount of halogenating reagent present initially.

Subtraction of the values (B) from (T) indicates the quantity of reagent utilized in the halogenation of a 5 ml sample of all from an original 27 ml volume. The difference C between the blank thiosulphate titer B and the sample titer T is the thiosulphate equivalent of iodine which combined with the fat. This titer volume is used in calculations.

1ml (0.1N) = 0.0127 g I2

C ml = Y g $I_2$ reacted with the unsaturated bonds in the oil used

$$Y = \frac{C \times 0.0127}{1} = \quad g\ I2$$

27 ml of oil solution contain = 2 ml oil

5ml = X ml oil

$$X = \frac{5 \times 2}{27} = 0.37 \text{ ml oil in } 27 \text{ ml}$$

Meaning that the 5 ml of the test sample contain 0.37 ml oil. and to convert this amount from milliliters to grams this formula is used:

W = V x d Where, W weight in gm, V volume in milliliters, D density of oil = 0.39 g/ml

By substitution, W = 0.3441 g oil

Since, 0.3441 g oil = Y g I2

100 g oil = Z

$$Z = 100 \times \frac{Y}{0.3441}$$ = % the iodine number.

## 13.7 Discussion:

Comment on the values obtained for iodine value determination for your sample.

Why it is important to know the iodine number of fats and oils?

## 13.8 REFERENCES

3. Aynon. (1964), Official and Tentative Methods of the American Oil Chemists' Society, 2nd. edn., American Oil Chemists' Society, Chicago.
4. Aynon. (1954), International Union of Pure and Applied Chemistry, "Standard Methods for the Analysis of Oils and Fats", Paris, France.

EXPERIMENT 13

## 14. Determination of Peroxide values in Fats and oils

### 14.1 **Introduction**:

Foods which contain high concentrations of unsaturated lipids are particularly susceptible to lipid oxidation. Lipid oxidation is one of the major forms of spoilage in foods, because it leads to the formation of off-flavors and potentially toxic compounds. Lipid oxidation is an extremely complex process involving numerous reactions that give rise to a variety of chemical and physical changes in lipids. Two types of lipid oxidation cause the most concern. These are oxidative rancidity and hydrolytic rancidity.

**Hydrolytic Rancidity:** Hydrolytic rancidity results in the formation of free fatty acids and soaps (salts of free fatty acids) and is caused by either the reaction of lipid and water in the presence of a catalyst or by the action of lipase enzymes.

**Oxidative Rancidity**: Oxidative rancidity results from more complex lipid oxidation processes. The processes are generally considered to occur in three phases: an initiation or induction phase, a propagation phase, and a termination phase. In complex systems, the products of each of these phases will increase and decrease over time, making it difficult to quantitatively measure lipid oxidation. During the initiation phase, molecular oxygen combines with unsaturated fatty acids to produce hydroperoxides and free radicals, both of which are very reactive. For this phase to occur at any meaningful rate, some type of oxidative initiators must also be present, such as chemical oxidizers, transition metals (i.e., iron or copper), or enzymes (i.e., lipoxygenases). Heat and light also increase the rate of this and other phases of lipid oxidation. The reactive products of this initiation phase will, in turn, react with additional lipid molecules to, form other reactive chemical species. The propagation of further oxidation by lipid oxidation products gives rise to the term “auto-oxidation” that is often used to refer to this process. In the final, termination phase of lipid oxidation, relatively uncreative compounds are formed including hydrocarbons, aldehydes, and ketones.

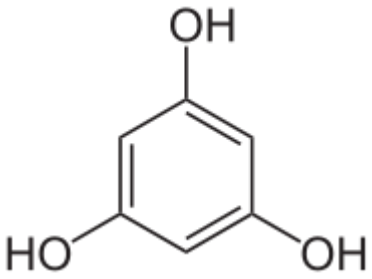
### 14.2 Assay Principle:

Auto oxidation at fatty acid double bonds occur by reaction with molecular oxygen present in the atmosphere, causing the formation of labile peroxides. These are capable of oxidizing the added potassium iodide, KI, with the liberation of molecular

iodine,$I_2$ The lodine may be determined by titration with standard sodium thiosulphate solution using starch as an indicator. While iodine is present in excess, a purple starch-iodine complex is formed. At the end point the solution becomes colorless.

$$I_2 + 2Na_2S_4O_3 \longrightarrow Na_2S_4O_6 + 2NaI$$

It is generally accepted that the first compounds formed by oxidation of oil are hydroperoxides. The usual method of hydroperoxide assessment is by determination of the Peroxide value, which is reported in units of millimole of hydroperoxide per kilogram of oil (or expressed as milliequivalents of iodine per kilo of oil and fat). The Lea value is defined as the number of millimoles of peroxide oxygen per kilogram of fat or oil and is therefore numerically half of the peroxide value. The peroxides formed during autooxidation are unstable and decompose into free radicals. These initiates chain reaction which lead eventually to decomposition of the fatty acid into various low molecular weight aldehyde and ketones. One carbonyl compound which is formed early in the process is epihydrinaldehyde. The latter reacts with phloroglucinol (1,3,5-trihydroxybenzene) to give a red colour in acid solution. This reaction is the basis of the qualitative Kreis-kerr test. The procedure may be made semi quantitative by preparing various dilution of the sample and determining the degree of dilution required to give a negative kries-kerr test (no red colour)




Phloroglucinol

## 14.3 Materials and Methods:

### *14.3.1* Materials

#### *14.3.1.1* Determination of peroxide value

1. Stout-walled test tube (approx. 27 x 2 cm)
2. $Co_2$ cylinder

3. KI powder, analytical grade
4. Rubber stoppers for the above test tubes, each stopper having two inlet glass tubes, each of which contains a glass stopcock.
5. Electric water bath
6. 2:1 (v/v) mixture of glacial acetic acid and chloroform.

#### 14.3.1.2 Kris- Kerr test

1. 1% phloroglucinol in ether solution
2. concentrated hydrochloric acid

### 14.3.2 Method

**A. Determination of peroxide value**

1. Weigh out accurately 1g of the melted and well-mixed sample into test tube (1 above) and add to it finely- powdered potassium iodine of analytical grade.
2. Add about 20 ml of the glacial acetic acid/chloroform mixture and shake well to dissolve the fat.
3. Stopper the tube with a bored rubber stopper (4 above).
4. Pass $CO_2$ through this solution for 10 minutes.
5. Open the stopcocks and place the tube in a boiling water bath until all chloroform vapours have escaped and then close the stopcocks and cool rapidly.
6. Titrate the liberated iodine with 0.002 M sodium thiosulphate (freshly prepared) using 1% starch solution (aqueous) as indicator.
7. Carry out blank experiment using the same reagents but without the sample.
8. Express the results as ml 0.002 M sodium thiosulphate as per gram of sample.

**B. Kreis- Kerr test**

1. To 1g of melted sample add a similar amount of concentrated HCl.
2. Add about 1ml of 1% phloroglucinol in either.
3. A slowly- developing red color indicates that the sample of fat or oil is in the early stages of rancidity.

## 14.4 Discussion

1. Make a table for various peroxide values for different samples of fat and oil, and discuss your result
2. How reliable is the above peroxide value test and describe various other methods used for determining Rancidity of fat and oil.
3. Draw your own conclusion about the nutritional and health hazards involved due to the rancidity of fats and oil.

## 14.5 REFERENCES

5. Aynon. (1964), Official and Tentative Methods of the American Oil Chemists' Society, 2nd. edn., American Oil Chemists' Society, Chicago.
6. Aynon. (1954), International Union of Pure and Applied Chemistry, "Standard Methods for the Analysis of Oils and Fats", Paris, France.

EXPERIMENT 14

## 15. ASH DETERMINATION

### 15.1 **Introduction**:

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. The three main types of analytical procedure used to determine the ash content of foods are *dry* ashing, *wet* ashing and *low temperature plasma dry* ashing.

**Dry Ashing:** Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600$^{o}$C. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. Although most minerals have fairly low volatility at these high temperatures, some are volatile and may be partially lost, *e.g.,* iron, lead and mercury. If an analysis is being carried out to determine the concentration of one of these substances then it is advisable to use an alternative ashing method that uses lower temperatures. There are a number of different types of crucible available for ashing food samples, including quartz, Pyrex, porcelain, steel and platinum. Selection of an appropriate crucible depends on the sample being analyzed and the furnace temperature used. The most widely used crucibles are made from porcelain because it is relatively inexpensive to purchase, can be used up to high temperatures (< 1200$^{o}$C) and are easy to clean. Porcelain crucibles are resistant to acids but can be corroded by alkaline samples, and therefore different types of crucible should be used to analyze this type of sample. In addition, porcelain crucibles are prone to cracking if they experience rapid temperature changes. A number of dry ashing methods have been officially recognized for the determination of the ash content of various foods (AOAC Official Methods of Analysis). Typically, a sample is held at 500-600$^{o}$C for 24 hours.

**Wet Ashing:** Wet ashing breaks down and removes the organic matrix surrounding the minerals so that they are left in an aqueous solution. A dried ground food sample is usually weighed into a flask containing strong acids and oxidizing agents (*e.g.,* nitric, perchloric and/or sulfuric acids) and then heated. Heating is continued until the organic matter is completely digested, leaving only the mineral oxides in solution.

The temperature and time used depends on the type of acids and oxidizing agents used. Typically, a digestion takes from 10 minutes to a few hours at temperatures of about 350$^{o}$C. The resulting solution can then be analyzed for specific minerals.

**15.2 Objectives:**

To determine the ash content of different varieties of date fruits.

**15.3 Materials and Methods:**

**15.3.1 Apparatus**

(1) Dish – Flat bottomed having a surface area of at least 15 cm$^2$.

(2) Muffle Furnace - regulated at 550 ± 25°C

(3) Filter Paper – ashless, medium fine.

**15.3.2 Materials:**

Different varieties of date fruits.

**15.3.3 Procedure**

Weigh to the nearest 0.001 gm about 2 gm of the prepared sample into the tared dish. Pour about 2 ml of ethanol on the material and ignite it. When the ethanol is burnt off, heat the dish carefully over a small flame to char the material. Then ignite in a muffle furnace at 550°C for 2 hours. Cool and wet the ash with a few drops of water, evaporate carefully to dryness and heat in the muffle furnace for a further 1 hour. If the wetting shows the ash to be carbon free, remove the dish to a dessicator, allow to cool and weigh without delay. If the wetting shows presence of carbon, repeat the wetting and heating until no specks of carbon are visible and ignite in the muffle furnace for 1 hour after the disappearance of carbon. If carbon is still visible, leach the ash with hot water, filter through ashless filter paper, wash the filter paper thoroughly, transfer the filter paper and contents to ashing dish, dry and ignite in muffle furnace at 550°C until the ash is white. Cool the dish, add the filterate and evaporate to dryness on a water bath. Heat in muffle furnace again, cool in a dessicator and weigh as previously. Heat again in the muffle furnace for 1 hour, cool and weigh. Repeat these operations until the difference in mass between two successive weighings is less than 0.001 gm. Record the lowest mass.

## 15.4 Results and Calculations:

Total ash on (dry basis) % by wt = $\frac{(W_2 - W)}{W_1 - W} \times 100 \times \frac{100}{100 - M}$

Where:

W = wt in gm of empty dish,

W1 = wt in gm of dish + sample,

W2 = wt in gm of dish + total ash,

M = Percent moisture content.

## 15.5 Discussion

Make a table for the ash content for different dates samples and discuss your result.

## 15.6 **REFERENCES**:

Quality Control Methods for Medicinal Plant Materials. World Health Organization (1998).

EXPERIMENT 15

## 16. DETERMINATION OF COPPER IN DATES

By ATOMIC ABSORPTION SPECTROSCOPY (AAS)

### 16.1 Introduction:

Atomic absorption spectroscopy provides one of the most useful and convenient means for the determination of metallic elements in solution from a wide variety of samples. The sample must be solubilized in aqueous or other solvents in order that it can be aspirated into the flame of the atomic absorption spectrophotometer. An alternative to this solubilization process is the use of the graphite or carbon rod furnace which is gaining wide acceptance for solid sampling.

The analysis of the metallic elements in foods such as beverages, eg. juices, tea, coffee etc, is easily accomplished by direct aspiration into the atomic absorption flame or direct injection into the graphite furnace. However, foods such as animal and plant tissues, and fluids with considerable solids must be treated to either decompose the solids or extract the metals. Ashing and/or wet digestion are the most commonly used methods. Ashing is usually performed at temperatures less than 500°C and digestion is usually accomplished by mixtures of nitric, sulphuric and perchloric acids.

Note: All glassware must be cleaned thoroughly prior to use to remove any contaminants.

### 16.2 Objectives:

To determine the concentration of copper in different varieties of Date fruits.

### 16.3 Materials and Methods:

#### 16.3.1 Preparation of Copper Standards

**Stock Copper Solution**:

A. 100 ppm solution of copper metal.
B. By dilution (with distilled water) of the 100 ppm solution supplied, use **ONLY** a burette and volumetric flasks to prepare 100 ml each of 0, 1, 2, 3, and 4 ppm Cu solutions..

### 16.3.2 Materials

1. Ashed samples of Dates
2. Moisten the ash with 10 ml of 8 M nitric acid and evaporate on a steam bath to near dryness.
3. Transfer the moist render to a 50 ml volumetric flask and dilute to the mark with distilled water. Filter ready for analysis by AAS.
4. Aspirate the Cu standards in turn, note the absorbance readings and construct a calibration graph of absorbance *versus* Cu concentration.
5. Aspirate the tea samples and determine the copper content (in mg/kg) of the tea leaves given to you.

### 16.4 Results and Calculations:

Be sure to use the appropriate dilution factor when converting the final concentration (ppm or mg/L) obtained from your graph to the mass of Cu in the original Dates samples (mg/kg).

## 16.5 Discussions:

Comment on the copper content of various Dates samples (mg/kg dry weight) determined by dry ashing.

## 16.6 REFERENCES

Vandecasteek C. and Block C. B. 1997. Modern methods for trace element determination, Wiley, Chichester, England.

## *APPENDIX*

**COPPER (Cu):** Atomic Wt. 63.54

Aqueous: Copper metal foil or wire (99.99%),

Non-aqueous: Copper 4-cyclohexylbutyrate

Preparation of 1000 µg/mL standard: Dissolve 1.0000 g of copper in 50 mL of 6M nitric acid and dilute to 1 litre to give 1000 µg/mL Cu.

**ATOMIC ABSORPTION**

Lamp current: 3.0 mA