CHAPTER 3

MATERIALS AND METHODS
3.1 Introduction

The physico-chemical studies were conducted on honey samples collected from Indian hive bee *Apis cerana* F. colonies from different regions of the northwest Himalayas and adjoining states consisting of Himachal Pradesh, Uttar Pradesh, Jammu and Kashmir, Punjab, and Haryana. The first three states are predominantly hilly states whereas Punjab and Haryana have predominantly plain areas.

The northwest Himalayas lies between 33° to 39° N latitude and 73° to 81° E longitude with total area of 2,22,336 square kilometres and altitude ranging from 300 to 8,000 metres above mean sea level. Beekeeping is widespread in the region with potential honey producing areas (Verma, 1990).

3.2 Collection of Honey Samples

Honey samples were collected from Indian hive bee, *A. cerana* colonies from hive and wild from different places in the northwest Himalayas having different altitudes, latitudes and climatic conditions. Places of collection of honey samples are shown in Table 3.1 and the name of places, altitudes, latitudes and longitudes are summarised in Figure 3.1.

The collection were made during the major honey flow season (i.e. May- June and September -October) of years 1992 and 1993. All the samples were machine extracted and were bottled and brought to the laboratory for investigations. The sample
were warmed for 20 minutes by keeping them in a water bath maintained at 50º C. These were then strained through a single thickness of fine cloth of 4 meshes per mm length. In this way, the samples were free from extraneous matters such as beeswax pieces, scum, surface dirt and were stored in air tight bottles at room temperature.

Table 3.1 Physiographic details of places of collection of the honey samples from the states of the northwest Himalayas of India

<table>
<thead>
<tr>
<th>State</th>
<th>S No</th>
<th>Place</th>
<th>Altitude (m)</th>
<th>Latitude °N</th>
<th>Longitude °E</th>
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<tr>
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<td>Karsog</td>
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85
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S.No = Sample Number
Figure 3.1  Places of honey samples collection in the northwest Himalayas and adjoining states of India.
3.3 Methods of analysis

The following physico-chemical properties of the collected honey samples were studied in the present investigations:

1. Colour
2. Refractive index
3. Moisture content
4. Specific gravity
5. Viscosity
6. Surface tension
7. Electrical conductivity
8. pH value
9. Ash
10. Mineral content
11. Determination of reducing sugars content
12. Levulose
13. Dextrose
14. Hydroxymethylfurfuraldehyde content
15. Determination of acidity
16. Lactone content.

The various techniques employed for the above physico-chemical studies are described below:
3.3.1 Colour

Visual observations of colours of different samples were made. The samples were cleaned, heated in water bath up to 40° C so that all samples were in liquid form and then colour of various samples were compared with the various colour charts given by Kornerup and Wancher (1978), Soni (1993).

3.3.2 Refractive Index / Moisture content

\[
\text{Refractive index} = \frac{\text{Velocity of light in medium a}}{\text{Velocity of light in medium b}}
\]

The refractive index was measured with the help of Abbe’s refractometer (Anderson and Perold, 1964; Mahajan, 1984).

3.3.2.1 Procedure

The prism box was opened and cleaned thoroughly with the help of distilled water and acetone in order to remove any material sticking on the prisms. Then the prisms were allowed to dry. Few drops of conductivity water were introduced between the prisms with the help of a dropper and prism were pressed tightly against each other. Then light from the electric lamp was reflected with the help of reflecting mirror into the prism box. The prism box was rotated by moving the lever in a proper direction until the light shade boundary appeared in the field of view. The final adjustment was made so that light shade boundary passed exactly through the centre of the cross wires. The refractive index of water at 20° C was read directly from the scale and compared with the literature value. The error of the instrument, if any, was calculated by using the water as the standard liquid.
Similar procedure was repeated for different honey samples which were already placed in the thermostat set at a particular temperature. The value of refractive index thus obtained were used to calculate the moisture content by using the standard equation as below.

\[
\text{Percentage moisture} = 400 (1.5380 - n_{20}) - 0.35
\]

where \( n_{20} \) is the refractive index of sample at 20° C. Readings were taken at 25° C, 35° C, and 45° C.

3.3.3 Specific Gravity measurements

Specific gravity measurements were made by using the specific gravity bottle of 25 ml capacity (Perti and Pandey, 1967, Hussein, 1989).

3.3.3.1 Procedure

The capillary of the bottle had a small bore to check any evaporation loss and stopper were fitted tightly in the mouth of the bottle. Specific gravity bottle was thoroughly cleaned with chromic acid and dried in an oven until a constant weight was obtained. The bottle was then filled with the conductivity water (triple distilled over alkaline KMnO₄) and placed in a water thermostat set at the required temperature. When the bottle attained the equilibrium temperature (after about an hour), it was taken out of the thermostat and its outer surface was wiped out carefully by means of a cloth. Weight of bottle and its contents was taken with the help of an electrical balance. The bottle as rinsed and dried in an oven. The same procedure was repeated for the different honey samples and the specific gravity was calculated as follows:

\[
\frac{\text{Weight of the sample}}{\text{Weight of same volume of water}} \times \text{density of water at that temperature}
\]
3.3.4 Viscosity Measurements

All liquids exhibit a characteristic property of flowing under an applied force which could even be the force of their own weight. When a liquid flows through a tube, the liquid layer in contact with the wall of the tube is stationary, whereas, that in the centre has maximum velocity. The intermediate layers have the gradation of velocities on account of relative motions of different layers, each layer experiences a functional force and behaves as if it is being dragged in the backward direction and work is to be done to maintain the flow. It is this resistance or friction which resists the relative motion of its part i.e. the flow of the liquid which is known as viscosity. In a streamlined motion, the dragging force due to internal resistance is proportional to the viscosity gradient, \( du / dx \), and to the area of contact A between the moving layers of the liquid. Thus, the retarding force is

\[
F \times A \frac{du}{dx} = \eta A \frac{du}{dx}
\]

The proportionately constant \( \eta \) has a characteristic value for a liquid at a specified temperature and is known as the coefficient of viscosity. It may be defined as the force required to maintain a velocity difference of unity between the two layers of the liquid, unit distance apart and having a unit area of contact. When a force of one dyne can maintain a velocity difference of 1 cm/sec between two layers of the liquid 1 cm apart, the velocity of liquid is said to be one poise.
3.3.4.1 Viscosity calculations

There are number of experimental methods for the determination of viscosity of liquids for example- flow through capillary tube, torque or rotating cylinder, fall of solid spheres through the liquids and flow of liquid through an aperture in a plate. In the present investigations, the method involving flow of liquid through capillary tube was used which is described below:

The volume $V_1$ of a liquid flowing through a capillary tube, which passes a section of the tube in time “$t$” seconds is given by Poiseuille’s equation:

$$V = \frac{\pi r^4 t}{8 \eta l}$$

where $r$ is the radius of the tube, $l$ is the length and $P$ is the pressure difference at the two ends of the tube and $\eta$ is the coefficient of viscosity.

Thus it can be seen that determination of absolute viscosity of a liquid by flow method is difficult and laborious one. The difficulties can be overcome by measuring the relative viscosity of the liquid with respect to water or any other standard liquid. this involves the measurement of time of flow for equal volumes of the two liquids through the same capillary under pressures due to their own weight and densities of the liquids.

the instrument which is most conveniently and frequently used for measurement of viscosity is the Ostwald’s viscometer.

3.3.4.2 The Ostwald’s viscometer

It consists of a U-tube with two bulbs D and E, one in each limb, the latter of which is larger than the former. The left hand limb is essentially a pipette with two
definite marked A and B above and below the bulbs and capillary C about 10 cms long. A definite volume of the liquid, which depends upon the capacity of the viscometer is introduced into the larger bulb using a graduated pipette. The liquid from the left hand limb is sucked just above the upper mark A and then allowed to flow under its own weight. The time of flow of the liquid from the mark A to the mark B is noted with the help of stop watch. Now if h is the mean difference of levels of the liquid in two limbs and d is the density of the liquid, then the force causing the liquid to flow down is hdg, whereas the force which resists the flow depends upon the dimensions of capillary and viscosity of the liquid. Thus the time of flow of liquid from mark A to B for a given capillary is directly proportional to the viscosity and inversely proportional to the driving force, that is

\[ t_1 \propto \frac{\eta_1}{h \cdot d_1 \cdot g} \]  

(2)

where \( \eta_1 \) is the coefficient of viscosity of liquid.

If \( t_2 \) is the time of flow of another liquid of same volume from mark A to B then,

\[ t_2 \propto \frac{\eta_2}{h \cdot d_2 \cdot g} \]  

(3)

where \( d_2 \) is the density of second liquid. From equation (2) and (3), it can be seen that:

\[ \frac{t_1}{t_2} = \frac{\eta_1 \cdot d_2}{\eta_2 \cdot d_1} \]  

(4)

The absolute viscosity can thus be determined by measuring \( t_1, t_2, d_1, d_2 \) and knowing the viscosity of the standard liquid. In the first case conductivity water has
been used as the standard liquid and viscosity of various honey samples has been determined by using the relation (4).

The present experiments were carried out by keeping the Ostwald’s viscometer in a water thermostat maintained at the required temperature, in which the water was allowed to attain the equilibrium temperature. After about an hour, it was taken out and dried in the oven. The same procedure was repeated for different samples and the viscosity was calculated using equation (4) (Mahajan, 1984).

3.3.5 Surface Tension

The surface tension may be defined as the force in dynes along the surface acting at right angle to a line 1 cm long drawn in the surface. It is represented by $\gamma$.

3.3.5.1 Measurement

Static and dynamic methods are generally used for the determination of surface tension of liquids. Among the static, capillary use method (single tube, double tube), are the most important, whereas drop fall method and ring or tension balance method fall under the latter type. Due to accuracy of results the dynamic methods are preferred over the static ones. In the present investigation, drop number method has been used (Mahajan, 1984).

3.3.5.2 Drop number method

In this method, a fixed volume of the liquid is obtained as freely falling drops from the end of the capillary tube and the number of drops formed are counted. If the same volume of standard liquid (water in the present investigation) is allowed to flow through the same capillary and the number of drops is again counted.
Where $r_1$ and $r_2$ are the surface tensions of the test liquid and the reference liquid respectively, $v_1$ and $v_2$ the volume and $d_1$ and $d_2$ the test and reference densities.

If $V$ is the volume of each liquid delivered and $n_1$ and $n_2$ are the number of drops counted for liquid 1 and 2 respectively, then

$$\frac{r_1}{r_2} = \frac{v_1 d_1}{v_2 d_2}$$

Thus determination of the number of drops of some definite volume of the given liquid and a reference liquid and their densities enable us to calculate the surface tension of the liquid provided the surface tension of reference liquid is known. Traube’s stagmometer (drop pipette) has been used for the measurement of surface tension in the present investigations. The Traube’s stagmometer is essentially a pipette with a capillary at the lower end, the end is flattened out to increase the dropping surface which is carefully ground. Above and below the bulb, the stem of the instrument carries two marks A and B.

### 3.3.5.3 Procedure

The stagmometer was rinsed thoroughly with chromic acid and then with distilled water several times. It was then washed with acetone and dried in an oven. A piece of clean rubber tubing with a screw clip was attached to the top of the
stalagmometer, to control the number of drops falling. The flattened end of the instrument was dipped in the distilled water which was used as the reference liquid and sucked through the rubber tubing till the liquid level rose above the mark A. The screw clip was closed, so that the liquid meniscus does not fall below the mark A. Now the stalagmometer was mounted on a stand in a vertical plane, inside an air thermostat, set at the required temperature. The liquid was allowed to attain the equilibrium temperature and the screw clip was adjusted so that number of drops were countable. The number of drops of the liquid were counted from mark A to mark B.

The same procedure was applied for the best honey samples and the surface tension was calculated by using the relation:

\[
\frac{r_1}{r_2} = \frac{n_2 d_1}{n_1 d_2}
\]

at the specified temperature.

The density of the honey samples was determined with the help of specific gravity bottle as already described before.

3.3.6 Electrical conductivity

Solutions of electrolytes (acids, bases, and salts in water) conduct electricity, like the metallic conductors in the solution. The passage of electric current is due to the migration of positively and negatively charged particles known as cations and anions respectively in appropriate directions. It is the valence migration velocity and concentration of ions which determine the strength of the current flowing through a solution of an electrolyte.
Ohm’s law is obeyed by electrolytic conductors

\[ I = \frac{E}{R} \]

where \( E \) is the potential difference between the ends of the conductors and is measured in volts, \( R \) is the resistance of the conductor and is measured in ohms, \( I \) is the current and \( E \) is the current strength measured in amperes. The reciprocal of resistance \( R \) is called the conductivity and is measured in reciprocal of ohm i.e. mho or ohm\(^{-1}\).

3.3.6.1 Specific Conductivity

The specific resistance of a conductor is defined as

\[ R = \frac{1}{a} \]

where \( l \) is the length (cms) and \( a \) is the cross sectional area of the conductor of resistance \( R \). The specific resistance may thus be defined as the resistance in ohms offered by a cube of solutions (or metallic conductor) of 1 cm length to the passage of electricity through phases. The reciprocal of specific resistance is known as specific conductivity or conductance and is usually represented by \( K \). The units in which it is measured are mho/cm.

3.3.6.2 Measurement of Conductivity

Conductivity is a reciprocal of the resistance which can be determined using the principle of Wheatstone bridge circuit. In the present investigations the conductivity has been measured by using a Toshniwal conductivity bridge at 50 cycles/sec. The cell used for the purpose is a simple dip the cell, which is quite convenient to use. The cell is made up of Pyrex glass and electrodes of platinum of sufficient thickness enclosed in glassed to ensure the rigidity (Vorwohl, 1964).
3.3.6.3 Platinisation of electrodes

Platinisation of electrodes is done to decrease the effect of polarisation, as although alternative current is employed to eliminate the polarisation but it does occur to some extent when smooth platinum electrodes are not used. In order to decrease the effect of polarisation, the electrodes are coated with finely divided platinum black. This coating increases the effective area of the electrodes for the current discharge and thereby reduce the local current density. The platinisation was done as described below. The cell was first thoroughly cleaned with an acid and then with distilled water. The electrodes were dipped in platinising solution consisting of platinic chloride and lead acetate. Then current was passed for about half an hour, reversing it every 30 seconds till a moderately thick coat of platinum black was formed on the electrodes. The cell was removed from the platinising solution and dipped in dilute solution of sulphuric acid and current passed for about 15 minutes, reversing it every minute. Then the electrodes were washed thoroughly with water and distilled water.

3.3.6.4 Cell constant

If R is resistance of a solution measured by a cell with electrodes of cross sectional area ‘a’ cm² and 1 cm apart, the specific conductivity of the solution is given by:

\[ \frac{1}{L} \frac{K}{R} = \frac{K}{R} \]

\[ R a \]

where \( \frac{1}{a} \) = K is known as cell constant
The cell constant i.e. the ratio 1/a cannot be obtained from geometrical dimensions of
the cell, for both l and a are not accurately known. It is, therefore, necessary to
calibrate the cell with a solution of known specific conductivity.

In the present case, 0.1 N and 0.01 N KCl solution in conductivity water were
prepared for the measurement of cell constant of the given cell. Suppose k is the specific
conductivity of 0.1 N or 0.01 N KCl solution, K1 is the conductivity measured by the
cell under use, then the cell constant is given by

\[ K = \frac{k}{k1} \]

The value of cell constant thus obtained was 0.174. The specific conductivity
was calculated by using the following relation:

Specific conductance \( k = \) observed conductance \( \times \) cell constant

All the honey samples were first placed in a thermostat set at the required
temperature to attain the constant temperature and then all measurements were carried
out. The fluctuation of the temperature was within ± 0.1 °C.

3.3.7 pH measurements

pH is defined as the negative log of hydrogen ion concentration i.e. pH = - log [H⁺]

For all practical purposes, the pH of aqueous solutions lies between 0-14. With
acidic range from 0-7 and alkaline from 7-14, whereas the pH 7 is neutral value.

3.3.7.1 Procedure

pH measurements during the present investigations was done by means of a
Toshniwal pH meter having glass pH electrodes and single rod assemblies (Lin et al.,
1977). Shake the electrode gently to ensure that the internal buffer solution covers the
whole membrane and no air bubbles are entrapped. Single rod assemblies should be filled with the appropriate electrolyte to a height of about 1 cm below the filling point. Wash off any salt film present on the exteriors of single rod assemblies using distilled water. Avoid wiping as this may cause slow response and erroneous results. Soak electrodes in water for some hours (preferably overnight). Electrodes which have a slow response due to drying of membrane or use under extreme conditions may be reactivated by dipping them in 2% hydrofluoric acid for 5-10 seconds and immediately washing with distilled water. This drastic treatment should however be applied only as a last resort as it reduces the life of the electrodes after conditioning the electrodes as above, if it is not to be used it should be kept in distilled water. To prevent the entry of water through the diaphragm of the reference electrode of single rod assemblies the stopper of the filling opening should be removed or perforated with a pin.

3.3.7.2 Calibration of a pH measuring system

The electrode system was immersed in a buffer solution of known pH, say 7. The pointer of the pH meter was brought to the value of 7 by means of the asymmetry potential adjustment. The electrode system was then removed from the known buffer solution and washed with distilled water. Buffer solution of pH 4 was prepared by dissolving 1 tablet of pH 4 in 100 ml of distilled water. The pointer of the pH meter was brought to the value 4 by means of slope adjustment. The electrode system was now remove from the buffer solution and dipped in distilled water and immersed in another freshly prepared know buffer solution of say 9 pH. The pointer of the measuring instrument should not have indicated the value, if it did so, the electrode system was functioning correctly. Now the different honey samples were diluted in the ratio 1:1
with distilled water. The electrode was immersed in the solution and pH was measured at different temperature.

3.3.8 Determination of mineral content (ash)

The mineral content in honey samples were determined by ashing the honey samples and different major minerals constituents were determined by photometry and absorption spectrophotometer.

3.3.8.1 Ignition of the honey (Ash)

For ash, 5 to 10 gms of honey samples were weighed accurately in a silica crucible and a few drops of pure olive oil were added to prevent spattering. This mixture was heated over a low flame or were ignited in a muffle furnace at 525°C till white ash is obtained and then crucible was cooled and weighed.

\[
\text{Ash percentage by mass} = \frac{M_2 - M}{M_1 - M} \times 100
\]

3.3.8.2 Minerals

Ash obtained above was dissolved in 2 ml of 6 N HCl and made it to a specific volume in a volumetric flask and then filtered.

Phosphate (PO₄) in the aliquot was determined by Vanado-molybdophosphoric yellow colour method (Jackson, 1967; Mahajan, 1984). Potassium content was analysed by flame photometry method. Calcium (Ca), Magnesium (Mg), Iron (Fe), Manganese (Mn) and Zinc (Zn) in the digest were determined on Pye-unicam SP-1900 Atomic Absorption Spectrophotometer (Ivanov and Chervenakov, 1984).
3.3.9 Determination of reducing sugar content

3.3.9.1 Principles of the method

The method was a modification of the procedure involving the reduction of Soxhlet's modification of Fehling's solution by titration at the boiling point against a solution of reducing sugars in honey using methylene blue as an internal indicator. The maximum accuracy for this type of determination was attained by ensuring that the reduction of Fehling's solution during the standardisation step and in the determination of the reducing sugars in the honey solution were carried at constant volume. A preliminary titration was, therefore, essential to determine the volume of water to be added before the determinations were carried out to satisfy the requirement (Vorwohl et. al., 1989).

3.3.9.2 Reagents

Soxhlet's modification of Fehling's solution

Solution A: Dissolved 69.28 g copper sulphate pentahydrate (CuSO₄·5H₂O; Mol.Wt. = 249.71) in distilled water and the volume was made to 1 litre. The solution was kept for one day before the titration was done.

Solution B: Dissolved 346 g Sodium Potassium tartrate (C₄H₄KNaO₆·4H₂O; Mol. Wt. = 282.23) and 100 g Sodium hydroxide (NaOH) in distilled water to one litre and filtered through asbestos.

Standard invert sugar solution (10 g/l. aq.)

9.5 gms. pure sucrose was weighed accurately and added to 5 ml hydrochloric acid (Ca 36.5% w/w pure HCl) and diluted with water to 100 ml. This acidified solution was
stored for several days at room temperature (3 days at 20° to 25° C), and then diluted to 1 litre. (Acidified 1 % invert sugar remains stable for several months). A suitable volume of this solution was neutralised with 1N Sodium hydroxide solution (40 gm/l) immediately before use and then diluted to the required concentrations (2 gm/l) for the standardisation.

*Methylene blue solution*
Dissolved 2 g in distilled water and diluted to 1 litre.

*Alumina cream*
Prepared cold saturated solution of alum (K₂SO₄.Al₂(SO₄)₃.2H₂O in water. Added ammonium hydroxide with constant stirring until solution was alkaline to litmus and precipitate were allowed to settle and washed by decantation with water until wash-water gave only slight test for sulphate with barium chloride solution. Poured off excess water and stored residual cream in stoppered bottle.

3.3.9.3 Procedure

*Preparation of test sample*

a) Transferred an accurately weighed sample of approximately 25 g from the homogenised honey to 100 ml volumetric flask, added 5 ml alumina cream dilute to volume with water at 20° C and filter.

b) Diluted 10 ml of this solution to 500 ml with distilled water (diluted honey solution).

*Standardisation of the modified Fehling’s solution*
Standardised the modified Fehling’s solution A so that exactly 5 ml (pipette), when mixed with approximately 5 ml of Fehling’s solution B, would react completely with 0.05 g invert sugar solution (2 g/l).
The total volume of the added reactants at the completion of the reduction titration must be 35 ml. This was made up by the addition of a suitable volume of water before the titration commences. Since the compositional criteria of the honey standard specify that there should be more than 60% reducing sugars (calculated as invert sugar) a preliminary titration was necessary to establish the volume of water to be added to a given sample to ensure the reduction is carried out at a constant volume. This volume of water to be added was calculated by subtracting the volume of diluted honey solution consumed in the preliminary titration (x ml) from 25 ml.

Pipette out 5 ml Fehling’s solution A into a 250-ml Erlenmeyer flask and added approximately 5 ml Fehling’s solution B. Added 7 ml distilled water, a little powdered pumice or other suitable antidumping agent, followed by about 15 ml diluted honey solution from a burette. Heated the cold mixture to boiling over a wire gauge, and maintained moderate ebullition for 2 min. Added 1 ml 0.2% aqueous methylene blue solution whilst still boiling and complete the titration within a total boiling time of 3 minutes, by repeated small additions of diluted honey solution until the indicator is decolorized. It was the colour of the supernatant liquid that must be observed. Noted the total volume of diluted honey used (x ml).

3.3.9.4 Determination

Calculated the amount of added water necessary to bring the total volume of the reactants at the completion of the titration to 35 ml by subtracting the preliminary titration (x ml) from 25 ml.
Pipette 5 ml *Fehling's solution A* into a 250 Erlenmeyer flask and add approximately 5 ml *Fehling's solution B*

Added (25-x) ml distilled water, a little powdered pumice or other suitable honey solution volume determined in the preliminary titration. Heated the cold mixture to boiling over a wire gauze and maintain moderate ebullition for 2 min. Added 1.0 ml 0.2% methylene blue solution whilst still boiling and complete the titration within a total boiling time 3 min by repeated small additions of diluted honey solution until the indicator is decolorized. Noted the total volume of diluted honey solution (y ml). Duplicated the titrations and agreed within 0.1 ml.

### 3.3.9.5 Calculation and expression of results

\[
C = \frac{2000}{W \cdot y}
\]

\( C = \) g invert sugar per 100 g honey (%)

\( W = \) weight of honey sample taken

\( y = \) volume of diluted honey solution consumed in determination (ml)

### 3.3.10 Hydroxymethylfurfuraldehyde (HMF) test (Fiehe's Test)

The satisfactory test for the presence of invert sugar syrup in honeys depends indirectly upon the formation of a characteristics product, 5-hydroxymethyl furfural (HMF), while boiling the sugar solution with acid.

#### 3.3.10.1 Qualitative Fiehe's test

About 5 gm of honey was ground with ether in a mortar. The ether extracts were taken up in a porcelain dish, the ether evaporates of and the residue is treated with
a large drop of resorcinol solution (gm in 100 ml of HCl of Specific gravity 1.19). A cherry red colour indicates positive Fiehe’s test and the presence of HMF (Phadke and Nair, 1968). With pure honey orange to pink colour disappearing rapidly developed as the colour intensity in this test depended on the quantity of HMF in the sample.

3.3.10.2 Quantitative test

5 grams of honey ground in a mortar with ether three times, each time the ether was decanted and the combined extracts are evaporated to dryness at room temperature. The residue was taken up in distilled water filtered and resorcinol solution added. The colour intensity was then estimated in a colorimeter with green filter 540 μ. The HMF content was then read off from the standard graph prepared with different concentrations of pure HMF (Schade et al., 1958; Jimenez et al. 1994).

Preparation of Standard Curve

Standard aqueous solutions are prepared with hydroxymethylfurfural (HMF), the concentration of which should be checked in the standard solutions spectrophotometrically. The standard solutions should be in the range of 0 to 50 mg/litre, since higher concentrations are not usually found in honeys unless they have been severely heated.

To 3 ml. of each standard solution add 3 ml. of ethanol and mix. Then add 3 ml. of the resorcinol solution and mix thoroughly by shaking. Allow the mixture to stand at room temperature for exactly 30 minutes after the addition of resorcinol; then determine the color intensity against distilled water with the colorimeter fitted with a no. 54 green filter. (The colour complex shows maximum absorption at 490 nm). Plot the colorimeter readings against the concentrations of the standard solutions (μg HMF/ml). Beer’s law
is obeyed to a concentration of at least 175 µg. HMF/ml (Schade et al., 1958; Jimenez et. al., 1994).

3.3.11 Determination of acidity

3.3.11.1 Reagents

Sodium hydroxide 0.1 N (carbonate-free)

Phenolphthalein indicator 1 % (m/v) in ethanol, neutralised.

Distilled water made carbon dioxide free by boiling and subsequent cooling.

3.3.11.2 Procedure

Preparation of test sample

Honey (10.0 g) was weighed accurately and dissolved in 75 ml distilled water.

Titration

The test samples were titrated against carbohydrate-free 0.1 N sodium hydroxide solution using 4-5 drops of neutralised phenolphthalein indicator to a pH of 8.3. The end-point colour should persist for 10 sec. For darkly coloured samples, a smaller weight should be taken. Immediately 10 ml of 0.1 NaOH was added to the solution and without delay the solution is titrated with 0.1 N HCl to pH 8.3. First titration represents the free acidity and second represents the lactone content (White, Petty and Hager, 1958)

3.3.11.3 Calculation and expression of results

The result is expressed as millieq. (milli equivalents) acid/kg honey and is calculated as follows:
Acidity = 10 v,

where v = the number of ml 0.1N NaOH used in the neutralisation of 10 g honey.

**Statistical analysis of data**

Physico-chemical data was analysed statistically by using “Microsoft Excel 5” package to calculate the standard deviation, correlation coefficient (r) and probability values (P). Abbreviated titles of periodicals in the references were indicated according to “SERIAL SOURCES FOR BIOSIS DATA BASE TM 1994 VOLUME.”