3. MATERIALS AND METHODS

The present study “Nutritional, storage and value addition studies on raw and heat processed honey” was carried out in the Department of Food Science and Nutrition, College of Home Science, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur. Honey means the food derived entirely from the work of bees operating upon the nectar of flowers and other sweet exudation of plants. In India, the consumption of honey is mainly restricted to medicinal purposes. Honey crystallizes during storage if not properly processed or pasteurized. Crystallized honey is considered to be adulterated with sugar. The proper processing of honey can overcome the problem of crystallization. Although raw honey is the best honey, but its processing is needed to meet the market requirements.

3.1 Materials

The honey samples (Ripened and Unripened) required for the present study were procured from the local market and Bee Keeping Research Station, of CSKHPKV Nagrota Bhagwan, Distt Kangra, H.P. The other ingredients required for the research study and preparation of food products were purchased from the local market.

3.2 Processing and packaging of honey

Ripened and unripened honey samples were processed at 60\(^0\), 70\(^0\) and 80\(^0\)C for 12, 24, 36 and 48 hrs. The sample of raw and processed honey were packaged in different packaging materials (glass jars, plastic jars and polythene pouches) and analysed fresh and after every 3.0, 6.0, 9.0 and 12.0 months of storage intervals for chemical, microbiological and sensory evaluation. Figure 3.1 indicates the steps followed in processing of honey.
Honey

Ripened

Unripened

Processed

Water bath

60°C 70°C 80°C

12, 24, 36 and 48hrs

Packaged

Glass jars Plastic jars Polypack pouches

Analyzed fresh and after 3, 6, 9 and 12 months of storage
3.3  Chemical analysis of honey

The samples of honey viz ripened and unripened after giving different treatments were analyzed fresh and after 3.0, 6.0, 9.0 and 12 months storage intervals for the following parameters.

3.3.1 Moisture Content (AOAC, 1990)

Weighed samples (5.0 g) in triplicate were dried for eight hours in a hot air oven at 105°C in pre-weighed crucibles. The crucibles were transferred immediately to desiccators, cooled and weighed. The loss in weight represented the moisture content of the samples.

$$\text{Moisture Content (\%)} = \frac{\text{Loss in weight (g)}}{\text{Weight of sample (g)}} \times 100$$

3.3.2 Ash Content (AOAC, 1990)

The samples (5 g) were weighed in pre-weighed crucibles and charred on a hot plate and then placed in a muffle furnace at 600°C for 4 hours. From the weight of residue left in crucible, the total ash content was calculated as follows:

$$\text{Ash Content (\%)} = \frac{\text{Weight of residue after ashing (g)}}{\text{Weight of sample (g)}} \times 100$$

3.3.3 pH (AOAC, 1990)

The pH was estimated with the help of a Elico pH meter. The equipment was switched on to warm up at least 30 minutes before use. The temperature of the solution to be tested was accurately measured and the temperature control
at this temperature was set. The instrument was standardized with a buffer solution of pH 7 and the operating instructions were followed. Sample 10ml approximately was taken so that the knob was dipped properly and readings were recorded.

### 3.3.4 Total Soluble Solids (Gould 1978)

The total soluble solids (TSS) in the sample were determined with the help of a Abbe type Refractometer and the values were expressed as degree Brix (°B). A temperature correction was also applied when the temperature was above or below 25°C (Appendix-I).

### 3.3.5 Titrable Acidity (Gould, 1978)

Two ml sample was taken and diluted to 25 ml with distilled water. An aliquot of 5 ml in triplicate was titrated against 0.1 N sodium hydroxide to a faint pink colour by using phenolphthalein as an indicator.

\[
\text{Acidity (\%)} = \frac{\text{Titre} \times \text{Normality of alkali} \times \text{Volume made up} \times \text{Equivalent wt. of acid}}{\text{Aliquot} \times \text{Weight of sample} \times 100} \times 10
\]

### 3.3.6 Crude protein (AOAC, 1990)

Weighed sample (0.5 g) was digested with nitrogen free sulphuric acid (20 ml) using 10 g digestion mixture containing potassium sulphate and copper sulphate (9:1). The contents were cooled and transferred to 100 ml volumetric flask. The volume was made up to the mark with distilled water and mixed. Measured aliquot (10.0 ml) was taken in a distillation flask followed by the addition of 40.0 percent sodium hydroxide. Liberated ammonia was trapped in HCL (0.01N) containing methyl red indicator and then titrated with (0.01 N) NaOH. Nitrogen present in the sample was used to calculate per cent crude
protein by using a factor of 6.25. The digestion and distillation of the samples were done with the help of Kjel Plus (Pelican Bio-1 innovations).

\[
\text{Nitrogen (\%)} = \frac{\text{Titre value} \times 0.00014 \times \text{Vol. made}}{\text{Aliquot taken (ml)} \times \text{Weight of sample (g)} \times 100}
\]

\[
\text{Crude Protein (\%)} = \% \text{Nitrogen} \times 6.25
\]

### 3.3.8 Diastatic activity (AOAC, 1990)

The following standard reagents were used for the determination of diastatic activity of honey samples.

**Iodine stock solution:** Dissolved 8.80 g resublimed iodine in 30-40 ml water containing 2.2 g potassium iodide, and diluted to 1 litre with distill water.

**Iodine solution:** (0.0007 N): Dissolved 20 g potassium iodide and 5.00 ml iodine solution (a) in water and diluted to 500 ml. Prepared fresh every second day.

**Acetate buffer solution:** (pH 5.3, 1.59 M). Dissolved 87g sodium acetate. Dissolved 87 g sodium acetate in 400 ml water, added 10.5 ml acetic acid in 400 ml water and diluted to 500 ml, with water. Adjusted pH to 5.30 with sodium acetate or acetic acid if necessary.

**Sodium chloride solution:** (0.5 M): Dissolved 5g sodium chloride in water and diluted to 500 ml.

**Starch solution:** Weighed 2 g starch and mixed in 90 ml water in 250 ml Erlenmeyer flask. Rapidly brought to boiling point, swirled solution as much as possible. Reduced the heat and boiled gently for 3 minutes, and allowed cool to room temperature. Transferred to 100 ml volumetric flask and volume made to 100ml.
**Standardisation:** Pipetted 5 ml starch solution into 10 ml water in a volumetric flask and mixed well. Pipetted 1 ml of this solution into several 50 ml graduates containing 10 ml dilution solution. This is standard dilution for starch preparation used. Repeat when changing starch source.

**Determination:** Weighed 5 g sample into 20 ml beaker, dissolved in 10 – 15 ml water and 2.5 ml buffer solution and transferred to 25 ml volumetric flask containing 1.5 ml sodium chloride solution. Diluted to volume. Pipetted 5 ml starch solution into side arm of reaction tube and 10 ml sample solution into bottom of tube. Place tube in water bath for 15 minutes at 40 ± 0.2°C then mixed contents by tilting tube back and forth several times. Started stopwatch.

At 5 minute, removed aliquot with 1 ml serological pipet and added rapidly to 10 ml dilution solution in 50 ml flask. Mixed dilution to previously determined volume, and determination A in photometer. Noted time from mixing starch and honey to addition of aliquot to I as reaction time. Continued taking 1 ml aliquots at intervals until A value of <0.235 was obtained.

<table>
<thead>
<tr>
<th>Absorbance</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.70</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>0.65</td>
<td>20 – 25</td>
</tr>
<tr>
<td>0.60</td>
<td>15-18</td>
</tr>
<tr>
<td>0.55</td>
<td>11-13</td>
</tr>
<tr>
<td>0.50</td>
<td>9 – 10</td>
</tr>
<tr>
<td>0.45</td>
<td>7 – 8</td>
</tr>
</tbody>
</table>

Plotted A (absorbance) against time (min) on rectilinear paper; drew straight line through starting A and as many points as possible. From graph, determination time dilution reaction-I mixture reaches A of 0.235. Divide 300 by this time to obtain diastase number (DN).

**3.3.9 Energy:** (O’shea and Magwire, 1962)
Reagents:

1.5 N potassium dichromate solution: Dissolved 73.54 g of potassium dichromate in 100 ml of warm distilled water and volume made up to 1 litre.

0.15 N sodium thiosulphate solution: Dissolved 37.23 g of sodium thiosulphate in 100 ml of warm distilled water and volume was made to 1 litre.

Potassium iodide sodium bicarbonate solution: 100 g of potassium iodide and 32 g of sodium bicarbonate were dissolved in distilled water and diluted to 500 ml. Prepared fresh everytime.

Concentrated sulphuric acid.

Procedure: 1g sample was oxidised with 20 ml of 1.5 N potassium dichromate solution and 40 ml of concentrated sulphuric acid for 90 minutes. The volume was made to 250 ml with distilled water. To a 2.5 ml aliquot, 10 ml of potassium iodide sodium bicarbonate solution was added in a dark, avoiding exposure to light for 25 minutes. The contents were diluted with 50 ml distilled water and iodide liberated was titrated against 0.15 N sodium thiosulphate using starch as an indicator to light green end point. The duplicate blanks were also run for each set. The amount of 1.5 N potassium dichromate used for oxidizing the sample was calculated by subtracting the above reading from the blank. The energy value was calculated by following equation:

\[
\text{Energy (Kcal/100g)} = \frac{\text{ml of 1.5 N potassium dichromate used for Oxidising 1g of sample}}{\text{Oxidising coefficient}} \times 100
\]

Oxidising coefficient = 23.39 = 0.069 P + 0.00026 P²

Where, P = True Protein

3.3.10 NPN or True protein (O’shea and Magwire, 1962).

Reagents:
TCA (10%) solution

Conc. $\text{H}_2\text{SO}_4$

Procedure: 500 mg sample in triplicate was extracted which 10 ml ice cold 10% TCA and centrifuged at 10,000 rpm for 5 minutes. The pipettes were washed twice with TCA (10%). An aliquot of 25 ml was taken and digested with concentration sulphuric acid for estimation of N. nitrogen content was determined by micro-kjeldhal method (AOAC, 1990).

$$\text{TP} = (\text{Crude protein nitrogen} – \text{NPN} \times 6.25 \text{ nitrogen}).$$

3.3.11 Sugars (Ranganna, 1995)

Sugars in all products were estimated by Lane and Eynon’s method reported by Ranganna (1995) and describe as follows:

Reagents:

Fehling A

Fehling B

Methylene blue

Neutral lead acetate: Dissolved 25 mg of lead acetate in water and added water to solution (45%) and volume made 500 ml with water.

Potassium oxalate: Dissolved 110 g of potassium oxalate (K$_2$C$_2$O$_4$.H$_2$O) in water and solution (22%) made the volume to 500 ml.
**Preparation of extract:** Weighed sample of 10 g dissolved in water and made the volume made to 250 ml in a conical flask. Added 2 ml of lead acetate solution, shaked well, and kept for 10 minutes. Necessary amount of potassium oxalate was added to remove the excess of lead and filtered through Whatman filter paper No. 1. The filtrate was used for the estimation of reducing sugars.

**3.3.11.1 Reducing Sugars**

In a conical flask, 5 ml each of Fehling’s solution A and B were taken. Took the sugar extract in burette and titrated against boiling Fehling’s solution by using methylene blue as an indicator. The end point was indicated by the appearance of brick red precipitates.

\[
\text{Reducing sugars (\%)} = \frac{\text{mg of invert sugar} \times \text{Dilution}}{\text{Titre} \times \text{wt. of sample (g)} \times 1000} \times 100
\]

**Standard invert sugar solution:** Weighed 9.5 mg sucrose (AR) in to a 1.0 L volumetric flask. Added 100 ml of water and 5 ml concentrated HCl. Allowed it to stand for 3 days at room temperature for inversion and then made upto mark by adding water. Factor for Fehling’s solution was determined by titrating equal amounts of Fehling's A and B with invert sugar by using methylene blue indicator and the end point was indicated by the complete discoloration of the indicator.

\[
\text{Factor for Fehling’s solution} = \frac{\text{Titre} \times 2.5}{1000}
\]

\[
\text{mg of invert sugar} = \frac{\text{g of invert sugar} \times 1000}{\text{Factor for Fehling’s solution}}
\]

**3.3.11.2 Total Sugars**

A measured amount (50 ml) of the extract was taken in a 100 ml volumetric flask to which 1.0 ml concentrated HCl was added and kept for
hydrolyzation overnight at room temperature. Next day, the solution was neutralized with saturated NaOH solution followed by a drop of phenolphthalein, finally the volume was made up to the mark with distilled water. This solution was then titrated against Fehling’s A and B as was done previously in case of reducing sugars. Titre was used to calculate the per cent total sugar using the formulae.

\[
\text{Total sugars (\%)} = \frac{\text{mg of invert sugar} \times \text{Dilution}}{\text{Titre (after inversion)} \times \text{Wt. of Sample (g)}} \times 100
\]

Non-reducing Sugars (\%) = \[\text{Total Sugar (\%)} - \text{Reducing Sugar (\%)}\] \times 0.95

3.3.12 Fructose: (AOAC 1990)

Fructose is one of the factors responsible for granulation. A minimum 1.0 % has been prescribed under I.S.I. standards (I.S.I, 1977). The importance of this ratio lies in the prediction of granulation, closer the ratio more the tendency of honey to granulate.

Reagents:

Shaffer – Somogyi Carbonate 50 reagent, 5g KI: Dissolve 25 g each of anhydrous sodium carbonate and sodium potassium tartrate 4H₂O (Rochelle salt) in 500 ml water in 2 litre beaker. Added while stirring 75 ml of solution of 100 g copper sulphate 5H₂O/L. Added 20 g sodium bicarbonate dissolve and add 5g potassium iodide.

Transfer solution to 1 litre volume flask, add 250 ml 0.100N potassium iodate (3.567g dissolved and dilution to 1 litre), dilution to volume and filter through glass. Keep overnight before use.

Iodide Oxalate solution: Dissolved 2.5 g potassium iodide and 2.5 g potassium oxalate in water and diluted to 100 ml. Prepared fresh weekly.

Thiosulphate standard solution (O.005N) :
Boiled gently for 5 minutes. Transferred while hot to storage bottle previously cleaned with hot chromic acid cleaning solution and rinsed with boiled warm water. Heat-resistant, bottle was used. Stored solution in a dark and cool place. Care was taken not to return unused portions to stock bottle (more dilution solutions are less stable and should be prepared just before use).

Accurately weighed 0.20 – 0.23 g potassium dichromate (K₂Cr₂O₇) (NBS SRM 136 dried 2 hour at 100⁰). Placed in flask. Dissolved in 80 ml chlorine free water containing 2 g potassium iodide. Added with swirling, 20 ml 1N HCl. Immediately placed in dark for 10 minutes. Titrated with sodium thiosulfate solution above, using starch solution as an indicator.

Starch indicator: Rub 2.5 g solution starch and 10 mg mercuric iodide red (H₉I₂) in little water. Dissolved in 500 ml boiling water.

Iodine solution: (0.05N) Dissolved 13.5 g pure iodine in solution of 3 g potassium iodide in 25 ml water. Diluted to 250 ml.

Sodium sulfite solution: Dissolved 1g sodium sulfite in 100 ml water. Made fresh daily.

Bromocresol green solution.

Column: A Column with 22 mm od X 370 mm long, with 1L spherical section and 35/20 spherical joint at top was used. Adsorbent used was 1+1 mixture of Darco G-60 charcoal and rapid filter aid. (Celite 545 or Dicalite 4200).

Preparation of fractions: Washed column with 250 ml water and decanted any supernatant. Passed 20 ml solvent 1 through column, and discarded. Dissolved 1g sample in 10 ml solvent 1 in 50 ml beaker. Transferred sample onto column, and forced into column. Used 15 ml solvent 1 to rinse beaker and funnel, and added to column. Collected all eluate, beginning with sample introduction, in 250
ml volumetric flask. Added 50 ml solvent 1, in 250 ml total (fraction 1, monosaccharides).

**Procedure:** Pipetted 20 ml fraction 1 into 200 ml volumetric flask. Added 40 ml 0.05N 1 solution by pipet; then with vigorous mixing, added 25 ml 0.1N NaOH over 30 sec period, and immediately placed flask in 18 ± 0.1°C water bath. Exactly 10 minutes after alkali addition, added 5ml 1NH₂SO₄ and removed from bath. Neutralized with sodium sulphite solution, using 2 drops starch solution, near end point. Added 5 drops bromocresol green and neutralized solution with 1N NaOH. Diluted to volume and added 5 ml aliquots of Shaffer Somogyi reagent and made duplicate blanks. Subtract titre value from blank.

% Fructose = 500 (titre X 0.1150) – 0.0915] X 100/mg sample

Fructose correction for glucose determination = f.c. = [(titer X 0.1150) + 0.0915] X 40.

### 3.3.13 Glucose (AOAC, 1990)

**Reagents:**

- **Sodium thiosulfate solution** (0.05N): Prepared from standardized stock 0.1N solution (as in fructose).
- **0.1N NaOH**
- **2NH₂SO₄**
- **Starch solution**

**Procedure:** Pipetted 20 ml fraction 1 (as in fructose) in duplicate 250 ml erlenmeyers flasks. Evaporated to dryness on steam bath in air current. Added 20 ml water pipet in 20 ml 0.1N NaOH, as in fructose determination. Immediately placed in 18 ± 0.1°C water bath. Exactly 10 minutes after alkali addition, added 5 ml 2NH₂SO₄, removed from bath, and titrated with 0.05 N sodium thiosulphate, using starch solution as an indicator.
Made duplicate blanks, using water, subtracted titre value from that of blank.

\[
\text{Glucose (\%)} = 56.275 \times (\text{titre} - (0.01215 \times f.c.)) \times 100/\text{mg sample}.
\]

### 3.3.14 Minerals

Weighed 1 g sample and added 25 ml of diacid mixture containing nitric acid and perchloric acid in (1:5) proportions and kept overnight. Next day, it was digested on hot plate till 1-2 ml solution was left in the flask. Further, the estimation was performed in accordance with instrument setting standardization and reading with reference to manufacturer’s specifications. The Fe, Mg, Mn, Zn and Cu were estimated in Atomic Absorption Spectrophotometer manufactured by Perkin Elmer Analysts 400, Na and K were estimated in Flame Photometer manufactured by Mediflame 127 and P was determined by Colorimetric method.

### 3.4 Microbiological Evaluation

**Standard plate count**: Microbiological evaluation was carried out by pour plate technique described by Harrigan and Mc Lance (1966), using standard plate count agar medium.

The media had the following composition.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar agar</td>
<td>15g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.0g</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>23.5g</td>
</tr>
</tbody>
</table>

Preparation of Standard Plate Count (SPC) agar media:

23.5g of SPC agar media was dissolved in 1 litre distilled water and heated to boil the contents. The pH of the media was adjusted to 7±0.2.
Sterilization: The petri plates and pipettes were sterilized in oven at 180°C for 2 hours. The blanks and the media were sterilized in an autoclave at 15 lb pressure per square inch for 15 minutes.

Weighing of Sample: The samples of honey were weighed under aseptic conditions. The beakers/other materials used were sterilized with ethanol to avoid any contamination.

Dilutions: 10g of the sample was added to 90ml of water in the form of water blank. It is now 10\(^{-1}\) dilution. 1ml of the same after proper shaking was transferred, using sterilized pipettes into 9ml water blank. It became 10\(^{-2}\) dilution and the process was repeated until 10\(^{-4}\) dilution obtained.

Plating of the samples: 1ml of the appropriate dilution of the honey was inoculated in petri plates under aseptic conditions in a laminar flow. The molten media at about 45°C was poured into each plate, aseptically. About 15ml of the media was used in each plate. When the plating was completed and the media had set completely, the petri plates were inverted and incubated at 30±1°C for 48 hours. The observations on the growth of colonies of micro-organisms, were recorded after 24 and 48 hours. For each dilution, 2 plates were used.

   Average count of 2 plates was recorded and expressed as number of Colony Forming Units (CFU) /g. The typical colonies were examined by simple staining technique using saffronine stain for differentiation of bacteria, yeasts or actinomycetes.

3.5 Organoleptic Evaluation
Sensory evaluation depends upon the responses given by different sense organs such as eyes, taste buds of tongue and olfactory lobes of the nostril. Here, the method suggested by Gould (1978) was adopted for organoleptic evaluation of honey and honey-based products. The prepared squashes were evaluated organoleptically. A minimum of 10 judges were selected at random giving due consideration to age and sex. Each panel member was asked to evaluate the product with respect to colour, flavour and texture on the prescribed form as shown in Appendix–II.

### 3.6 Preparation of products

#### 3.6.1 Standardization of products

The different honey-based formulations for preparation of RTS, squash, jam, biscuits and toffee were standardized in the laboratory. The different levels of TSS of products viz. RTS, squash and jam were adjusted for the preparation of acceptable quality of honey-based value-added products. The substitutions of sugar with honey in baking (biscuits) and confectionery (toffees) were also adjusted in the formulations for preparations of acceptable quality of biscuits and toffee. The steps followed during standardization and preparation of honey and mango RTS, squash, jam, biscuits and toffee are illustrated in figures 3.2, 3.3, 3.4, 3.5 and 3.6, respectively.
Figure 3.2 Steps followed in the preparation of Mango RTS prepared by substituting sugar with honey
Mango
↓
Washing
↓
Peeling
↓
Pulping
(25% pulp + water)
↓
Addition of sugar, honey and citric acid
[Sugar:Honey (100:0, 0:100, 50:50]
↓
Filtering
↓
Preservative
↓
Bottling
↓
Corking
↓
Cooling
↓
Storage
Figure 3.3 Steps followed in the preparation of Mango Squash prepared by substituting sugar with honey

Mango and seabuckthorn pulp (50:50)

↓

Cooking

↓

Addition of sugar, honey and citric acid

(Sugar:Honey (100:0, 0:100, 50:50, 25:75 and 75:25)

↓

Cooling

↓

Packaging

↓

Storage

Figure 3.4 Steps involved for the preparation of jam prepared by substituting sugar with honey

Beat ghee, sugar and honey

↓

Sieve maida, sodium-bi-carbonate, baking powder and salt

↓

Add maida slowly and slowly

↓

Knead dough

↓

Cut it into shapes

↓

Bake in oven 200°C for 15-20 minutes
Figure 3.5 Steps involved for the preparation of biscuits by substituting sugar with honey

Fruits (seabuckthorn) (Firm and ripe)
  ↓
Washing
  ↓
Pulping
  ↓
Pulp concentrated to about half of its volume
  ↓
Continuous stirring
  ↓
Addition of sugar, honey, glucose and edible fat
  ↓
TSS 80 B
  ↓
Adding milk powder dissolved in little water
  ↓
Spread mass uniformly in thick layer
  ↓
Cut pieces into suitable size
  ↓
Wrap in a butter paper
  ↓
Storage

Figure 3.6 Steps involved for the preparation of Fruit Toffee prepared by substituting sugar with honey
3.7 Economics of the product

Per unit cost of the preparations was calculated on a standard bulk basis. The per unit cost determined reflected the costs of ingredients at the local retail price.

3.8 Statistical analysis

The data generated on various parameters were subjected to analysis of variance technique completely randomized design (Appendix-III) to derive statistical inferences.