CHAPTER 2

METHODOLOGY

"Let food be thy medicine and medicine be thy food."

Hippocrates (Greek Physician)

2.1. Introduction

In this chapter, various methodologies have been described for the processing of raw ingredients followed by estimation of nutrient composition, sensory profiling, shelf life and microbial count. Gamma radiations have been used to enhance shelf life of perishable products. GM Counter and NaI (TI) scintillation detectors have been used for the detection of radiation counts.

2.2. Procurement and processing of raw ingredients

Milling by-products include bran, husk and broken parts of the grains. Regarding the present study required milling by-products of legumes (Chickpea and moong bean) were procured from the local dhal mill of Mahendergarh city, Haryana (India). Milling by-products of cereals (Rice and wheat) are collected from rice and wheat milling industries. Fruit peels were collected from the juice vendors of the local market. Procured ingredients were processed to obtain the flour. By-products were separately washed under running tap water properly. Washed by-products were distributed evenly over bloating paper placed over separate drying trays. The trays containing samples was kept in hot air oven at 60ºC for 6 hrs. for moisture removal. Laboratory grinder was used to grind the dried materials followed by sieving using a 200 µm sieve. Obtained flours of different by-products were stored in a polyethylene bag for further use at room temperature (Figure 2.2). For the fruit peels, they were washed properly followed by blanching for 10 min. and soaked in water for 30 min. Soaked peels were kept on the bloating paper placed over drying tray and dried at 55ºC for 5-6 hrs. to make the peels moisture free. Dried peels were grinded and sieved

to obtain the flour. For further use, the flours were stored in a polyethylene bag and kept at room temperature (Figure 2.3).

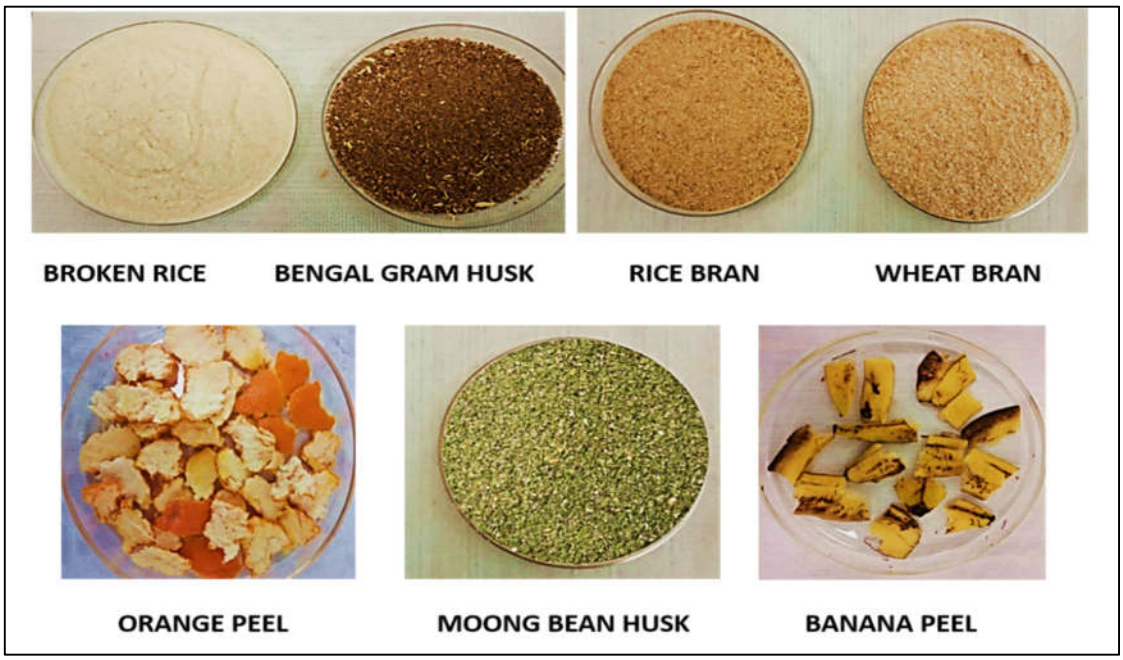

Figure 2. 1: Raw materials utilized to formulate value added food products

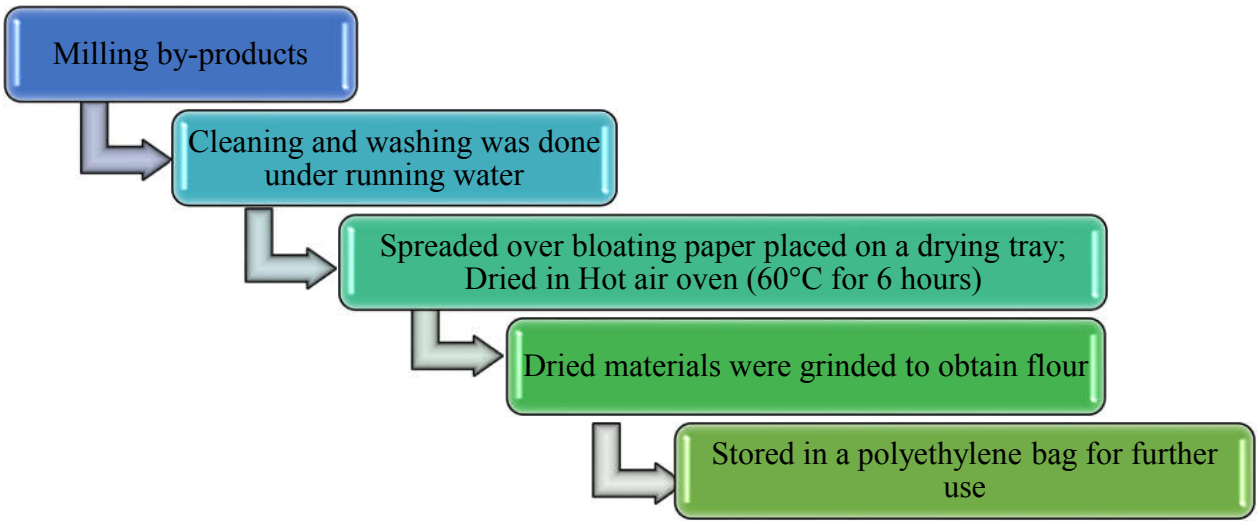

Figure 2. 2: Processing of milling by-products for value added products formulation

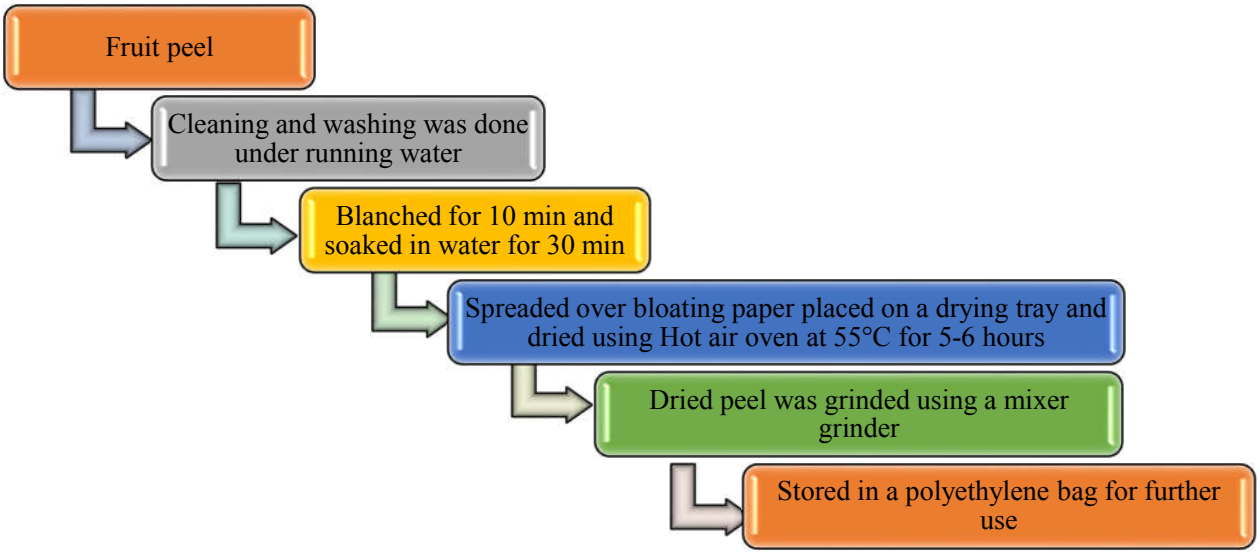

Figure 2. 3: Processing of fruit peels for value added products formulation

2.3. Formulation of value-added products using agricultural by-products

Different types of value-added products have been developed in the present study using agricultural by-products which are milling by-products and fruit peels. The formulated products include baked products, fermented products, health drink and beverage, snack products including roasted products, confectionary products.

Baked products are known over ages worldwide. The art of baking had emerged early in the Roman Empire. Around 300 BC, baking was considered as a profession. Afterwards, the trend of baked products began in German, London, Paris. In 1928, pre-cut bread was introduced in Missouri. After World War II, novel bread formulation method was emerged with enhanced flavour and taste (Ashokkumar, 2009). Apart from bread, other popular baked products are cookies and biscuits. Cookies were discovered in 7th Century AD Persia. Later the product become popular in Europe due to Muslim Conquest of Spain. During the arrival of Mughals and Persians in India, Indian baked products came into light (Ashokkumar, 2009). The popularity of bakery products like bread, cookies continued with the Dutch, Danes, French, British empire. Use of yeast in dough fermentation for bread leavening was first done in ancient Egypt (Steinkraus, 1995).

Fermentation is considered as a culinary treatment to the microbial systems. It is believed to be one of the oldest practices in human evaluation, dating back to 10,000 BCE with the preservation of milk from cattle, sheep, camel, goat. Fermented products like buttermilk, cheese, yogurt, kefir improves gut health. Fermentation of cereals and legumes started from 500-1000 AD to formulate nutrient rich diet (Steinkraus, 1995). In 1970 first ingestible probiotics came in light. Metchnikoff identified benefits of fermented milk and found *Bulgarian bacillus*, which was later named *Lactobacillus bacillus*, unable to survive in human digestive system. Later on, *Lactobacillus acidophillus* was found which can survive in human gut in active form (Steinkraus, 1995). Consumption of fermented food showed benefits in health improvement and prevention of diseased conditions like metabolic syndrome, bowel problems, cardiovascular disease, hyperglycemic condition, cancer, immune malfunction, inflammation (Egounlety and Aworh, 2003). Besides these popular product formulations, formulation and consumption of health drink and beverages were also in practice from ancient time. Tea was discovered in China in 2737 B.C. In 1901, first energy drink was developed in Scotland which was named *Im-Bru* (Ashokkumar, 2009). Later in 1960s, Japan, South Korea started to develop energy drinks. The United States introduced energy drink in early 1985 and since then consumption as well as acceptance of energy drink among consumer got increased. Formulation of nutrient rich health drink is quite popular among consumers worldwide due to its health beneficial properties. Apart from these, other common products include the roasted products, snacks and confectionaries. In Asian countries, especially in India, *papad* is very popular and common roasted product, made from different ingredients like rice, lentils, potato, fruits with different flavours. Madurai, India is the origin of the product *papad* (Khedkar *et al.,* 2016). Confectionery is also a well-known type of products. Although these products are rich in sugar and carbohydrate, value-added confectionery products are also being developed (Goldstein and Mintz, 2015).

Due to their popularity and higher consumer acceptance, products like bakery products, fermented products, roasted products, confectionary, health drink and beverages

have been formulated using milling by-products and fruit peels in the present study followed by the estimation of the nutrients, sensory profiling, shelf life and microbial load.

2.4. Measurement of Physico-chemical properties and nutrient composition

Proximate analysis of raw food samples is determined to get the information regarding nutritional composition of the sample. Proximate composition includes ash, moisture, crude fat, crude fibre, crude protein, sugars, acidity. Sum total of these components are deducted from 100 to estimate available carbohydrates other than sugars. Other than proximate composition, physical properties like bulk density, water and oil absorption capacity and other nutrients present in the raw sample are also necessary to evaluate to understand its suitability for further use in food formulation. Nutrients other than proximate composition includes minerals, dietary fibre, antioxidants, antinutrient.

2.4.1. Physical parameters

2.4.1.1. Bulk density

Bulk density, also known as volumetric density, depicts the physical property of powder or granular form of solids. Powder is a mixture of particle gas with intraparticle voids along with interparticle spaces. Hence, determination of mass of bulk solid per unit volume gives the bulk density. There are three types of bulk density viz., aerated, tap and poured (*López and Goyanes, 2017).* It is the measurement of heaviness of flour indicating appropriateness of the flour for product development and application in food industry. Determination of bulk density also can be helpful to understand and correlate other physical parameters such as water absorption capacity, porosity (Subramania and Viswanathan, 2007).

Estimation of Bulk density is calculated by taking known amount of raw ingredient in a 100 ml cylinder and tapped gently on table till to obtain a settled volume (AACC, 2000).

$$\text{Bulk density (g/ml)} = \frac{\textbf{Weight of the sample}}{\textbf{Volume of the sample in cylinder}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{ Eq. (2.1)}$$

2.4.1.2. Water absorption capacity

Water absorption capacity (WAC) is the measurement of water holding capacity of a certain material. This physical parameter of food product helps to understand the quality of the product. Estimated capacity of flour helps to understand suitability of the sample for product formulation. It indicates consistency in dough making. High protein containing flour also exhibits higher water absorption capacity. Hence, determination of water absorption capacity is necessary for better understanding of food products and formulation.

For the estimation of WAC (Singh and Singh, 1991), 25 ml distilled water was added to taken 3 g of raw ingredient in a pre-weighed centrifuge tubes followed by a stirring using vortex mixer. Then the tubes were kept at water bath to incubate for 30 min followed by centrifugation for 30 min at 3000 rpm. WAC is the number of grams of water absorbed per gram of sample.

Figure 2. 4: Centrifuge used for physico-chemical property estimation and bioactive compound analysis

2.4.1.3. Oil absorption capacity

Oil absorption capacity (OAC) is the difference between the weight of flour before and after absorbed in oil. For the baked products like bread, higher absorption of oil in flour enhances the flavour as well as texture of the product. Besides product quality, based upon

the oil absorption capacity of certain flour, storage stability and probability of rancidity during storage can be predicted (Ubbor and Akobundu, 2009).

In a pre-weighed centrifuge tube 0.5 g of by-product was mixed with 6 ml oil *using vortex mixer* for one minute so that the oil disperses in the sample completely**.** It was then kept for 30 min in a water bath followed by centrifugation at 3000 rpm for 30 min. The supernatant that is the separated oil was separated followed by the draining of residual oil in the tubes by inverting them for few min. OAC is also the g of oil absorbed per g of the sample.

2.4.2. Measurement of Proximate composition

The proximate analysis was done according to AOAC (2000) in triplicate.

2.4.2.1. Moisture

Water content in food sample remains in different forms such as free water that is dispersing medium for colloids or as solvent for crystalloids. It can be adsorbed on the surface or in chemical combination with other elements present in the sample. Determination of water content that is the moisture present in sample can be helpful to find the storage stability of the same. Higher moisture content indicates higher chances of microbial contamination. Moisture can be analysed using various methods like oven drying, vacuum oven drying, immiscible solvent distillation method, physical and chemical method.

Hot air oven method has been followed in the present study to determine moisture content. The oven, also known as air circulating oven, is generally used for dry heating to sterilize or eliminate water content in sample. This process of dry heat sterilization through hot air oven is established by Louis Pasteur. The range of temperature can be in between 50 to 300ºC. The working principle of hot air oven is based upon convection and conduction.

Figure 2. 5: Hot air oven for moisture determination

For the determination of the moisture of the products, 2g of each samples were kept in hot air oven at 55° C for 6 hours. Moisture was measured as:

$$\left[\frac{\text{weight of dish with moisture containing sample} - \text{weight of dish with dried sample}}{\text{weight of dish with moisture containing sample} - \text{weight of the empty dish}}\right] X100$$

Eq. (2.2)

2.4.2.2.Ash

Ash content in food sample indicates inorganic matter present in the sample after burning of the organic matter. High ash content with low alkalinity in some cases depicts presence of adulterants. Whereas, acid soluble ash indicates presence of silicious matter. For complete churning, sample is ignited or digested using concentrated acid.

In the present study, samples were churned using muffle furnace, also known as muffle oven. It is used for the isolation of inorganic matter through destruction of organic matter. This oven works based upon the heating by conduction, convection. There occurs no combustion in the temperature control system leading to the uniform temperature control.

Figure 2. 6: Muffle furnace for the determination of ash

2 g of each sample was taken in dried and weighed crucibles and kept in muffle furnace (Thermo Scientific) for 5 hours at 550 ° C. Incinerated samples were then placed in the desiccator and weighed down after its temperature goes to the room temperature.

$$\left[\frac{\textbf{weight of crucible and dried sample} - \textbf{weight of the crucible}}{\textbf{weight of crucible and raw sample} - \textbf{weight of the crucible}}\right] \boldsymbol{X100} \quad \text{.....Eq. (2.3)}$$

2.4.2.3.Crude Protein

Protein is estimated using Kjeldahl method where amount of reduced nitrogen (NH_2 and NH) is estimated. Nitrogenous compounds present in the sample are converted to ammonium sulphate by boiling the sample with concentrated Sulphuric acid (H_2SO_4). Later during the digestion of the sample, converted ammonium sulphate is decomposed with alkali treatment using Sodium Hydroxide (NaOH). Produced ammonia is collected in boric acid solution for titration. This method was established by Johan Kjeldahl in 1883.

However, this method does not evaluate true protein content. The estimated nitrogen content includes both protein and nonprotein nitrogen. This method is not appropriate for the sample containing nitrogen in nitro and azo groups as these nitrogen does not convert into ammonium sulphate.

Figure 2. 7: Kjeldahl method for protein estimation using FOSSTM KjeltecTM

Reagents used for the crude protein estimation are-

i. 0.01 N Hydrochloric Acid

ii. 40% Boric acid: The solution was prepared by dissolving 400g Boric Acid powder in distilled water. The volume was made up to one litre.

iii. 40% Sodium Hydroxide (NaOH): 400g of carbonate free Sodium Hydroxide was dissolved in one litre distilled water.

iv. Copper Sulphate Catalyst was prepared by mixing Copper Sulphate ($CuSO_4$:$5H_2O$) and Potassium Sulphate (1:9).

v. 2 g Methyl Red and 1g Methylene Blue or Bromo-Cresol Green were dissolved in one litre of Ethanol which was stored in dark brown bottle for further use.

vi. Potassium Sulphate (K_2SO_4) and Copper Sulphate ($CuSO_4$) were used as Digestion Mixture.

Crude protein estimation is carried out through three steps: Digestion, Distillation, and Titration. 0.5 g of sample was taken into tubes with 12ml H_2SO_4 and Digestion Mixture. 0.8g $CuSO_4$ and 7g K_2SO_4 were used to prepare the digestion mixture. With continuous connection of water flow, digestion was processed for 1 hr at 420°C followed by distillation using Boric Acid and NaOH in Kjeldahl Apparatus (FOSSTM KjeltecTM). The obtained distillate (bluish green) was titrated with 0.1 M HCl solution where green colour converts into permanent pink.

%Total nitrogen= Eq. (2.4)

$$\frac{[\mathbf{100}\ \boldsymbol{X}\ (\textbf{volume of standard acid used in titration} - \textbf{volume of standard acid used in blank})\boldsymbol{X}\ \textbf{normality of acid}\ \boldsymbol{X}\ \mathbf{14}]}{(\textbf{weight of the sample}\ \boldsymbol{X}\ \mathbf{1000})}$$

2.4.2.4.Crude Fat

Crude fat, also known as ether extractives is an important parameter of food sample. This ether soluble extract can be produced using the Soxhlet extraction method. Crude fat in food depicts presence of triglycerides, phospholipids, sterols, essential oils, fat soluble pigments which is extractable in ether. This extraction method using Soxhlet extractor was established in 1879 by Franz von Soxhlet. This method is generally used when the sample exerts limited solubility in solvent.

In the study, 2 g moisture free sample was shifted into thimbles for the extraction and were placed in a Soxhlet. It was connected to a round bottom flask which contained 250 ml petroleum ether. The extraction continued for 16 hours 30 min on an electrothermal extraction unit. After extraction, the flask was removed and the petroleum ether was evaporated. The flask with the fat was heated in an oven at 103°C for 1 hour followed by cooling in a desiccator, and then recorded the weight of the flask.

Figure 2. 8: Soxhlet method for fat extraction from food products

$$\frac{(Weight\ of\ flask\ \&\ fat)-(Weight\ of\ empty\ flask)}{Weight\ of\ taken\ sample}\ X\ 100 \quad \text{.....Eq. (2.5)}$$

2.4.2.5. Crude fibre

Crude fibre is the organic matter of the food which remains as residue after digestion of the food under standardized conditions of acid and alkali treatment. Crude fibre includes cellulose with lignin. In the present study, crude fibre was estimated using Fibraplus fibre extraction system. The system is designed for the treatment of acid and alkali digestion. It enables suction and filtration under vacuum conditions with filter aspirator pumps and manifold system. Fat free sample is taken for fibre extraction in Fibraplus. Presence of fat creates the clog in the system. For the removal of clogging of the sample, the system also generates reverse stream of air pressurized by electrically operated air pump.

Figure 2. 9: Fibraplus for fibre extraction from food products

Fat free sample was taken in round bottom flask, and 200 ml 1.25% H_2SO_4 was added to it. After boiling the sample mixture for 30 min, it was filtered followed by the washing of the residue with boiling water. The residue was boiled again with 200 ml 1.25% NaOH and filtered. The residue kept in Gooch crucible followed by washing with 15 ml 10% HCl and filtered. The weight of the crucible with the content were noted and dried overnight at 105°C. Weight of the same was again noted after the drying and kept in a muffle furnace for ashing.

$$\left[\frac{X-Y}{W}\right] \mathbf{X\ 100} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{Eq. (2.6)}$$

X- weight of crucible and dried sample before ashing, Y- weight of the crucible and sample after ashing, W- weight of the sample used in the fat determination.

Figure 2. 10: Water bath for the estimation of biochemical analysis

2.4.2.6. Total Carbohydrates

Carbohydrate is considered as the major source of energy which contribute to 60-70& total required calorie. Starch, lactose, sucrose, fructose, and glycogen are easily digested carbohydrates. Whereas, cellulose, hemicellulose, and pectin are not easily digested. The carbohydrate content was evaluated by sum up the values of other determinations viz., fat, protein, moisture, ash, fibre (100-the sum of the other determinations) (AOAC, 2000).

2.4.3. Sugars

Sample preparation- 0.5g sample was added to 25ml of 80% Ethanol in a flask and heated for 30min with occasional stirring. After cooling down, centrifugation of the extract at 8000 rpm was carried out for 15 min. The above mentioned procedure was done twice followed by the evaporated on a boiling water bath. At last the residue was dissolved to make up the volume to 50 ml by distilled water.

Total soluble sugar was determined according to the method given by Yemm and Willis (1954).

Reagents used for the estimation were:

1. 25 mg glucose in 100 ml distilled water (250µg glucose per ml)

2. Freshly prepared Anthrone (0.2%) in H_2SO_4 (70%) was prepared 30 to 40 mins prior the use.

10 ml Anthrone solution was taken in a chilled test tube and placed in ice cold water. 1 ml of the sugar extraction of the sample was diluted to 10 ml and 1 ml of this diluted solution was added to the taken anthrone reagent followed by cooling for 3-5 mins. Then they were mixed vigorously and kept in a boiling water bath for 10 mins and cooled in chilled water. The absorbance was recorded at 625 nm by using UV-VIS spectrophotometer**.**

Total Soluble Sugars (g/100g) = $\frac{\mathbf{C\ X\ V\ X\ 100}}{\boldsymbol{W\ X\ V1}}$……..Eq. (2.7)

C = concentration of glucose from curve (µg)**,** V = volume of extract made**,** W = weight of sample taken**,** V_1= volume of aliquot taken.

For Reducing sugar estimation, one ml copper reagent (Copper reagent A: Potassium Sodium Tartarate, Sodium Carbonate, Sodium Bicarbonate, and anhydrous Sodium Sulphate; Copper reagent B: Copper Sulphate and HCl) was added to one ml sample extraction and then kept in boiling water bath for 20 min. followed by mixing of one ml arsenomolybdate reagent and diluted to 25 ml. Arsenomolybdate reagent was prepared by dissolving 25g Ammonium molybdate in 450 ml distilled water with continuous heating followed by addition of 21ml concentrated H_2SO_4 with stirring. 25 ml Sodium hydrogen arsenate (3g Sodium hydrogen arsenate + 25 ml distilled water) was added to this mixture. The final solution was kept at 37°C for 24 hours prior to use in a brown bottle in refrigerator. The addition of Aresenomolybdate reagent gives a quick appearance stable blue colour. The amount of reducing sugar was then determined by referring to the glucose standard curve. Reducing sugar was determined by the method of Somogyi (1945) and data was recorded at 520 nm using UV- VIS spectrophotometer. **Non-reducing sugar** was determined as the difference between the amount of total sugar and reducing sugar. For the **starch estimation**, 6.5 ml Perchloric acid (52%) treated extracted sugar residue was diluted with 20 ml distilled water followed by centrifugation. The collected supernatant was diluted with 5 ml distilled water and the above mentioned

procedure was repeated. The final supernatant was filtered and one ml of this filtrate was diluted with 15 ml distilled water. One ml of this diluted solution was subjected to glucose estimation. Starch was calculated by ($Starch = Glucose * 0.9$).

2.4.4. Soluble and insoluble dietary fibre

Dietary fibre is another important element for the proper biological functioning. Incorporation of fibre in diet improve various diseased conditions. Although this resists digestion in human gut, get fermented partially or completely by gut microbiota. Produced by-products after fermentation of dietary fibre helps in lowering risk of breast cancer, type 2 diabetes, diseases of colon, metabolic syndromes, cardiovascular diseases along with improvement of bowel movement, insulin sensitivity of the peripheral glucose-utilizing tissues (liver, fatty tissue). Dietary fibre is associated with plant phytochemicals like polyphenols, lignans, carotenoids, isoflavones.

Dietary fibre of defatted samples were carried out by the enzymatic method given by Furda (1981). 2g defatted sample was digested using 200ml of 0.005N HCl followed by boiling for 20 minutes. After cooling down, 0.3g of disodium EDTA was added and pH was set to 5.0-6.5 with phosphate buffer. After the extraction with minimal degradation, overnight incubation with 10mg bacterial alpha-amylase and bacterial protease was carried out. It was filtered and insoluble residue was washed with water, alcohol, acetone and dried at 70°C overnight. Concentrated HCl was added to the filtrate followed by slow addition of four volumes of ethanol and incubated for 1 hr. It was filtered and the residue was washed with 75% ethanol, acetone and dried at 70°C overnight. The filtrate was acidified with conc. HCl (pH 2-3) which be likely to facilitate rapid precipitation of polysaccharides. Four volumes of ethanol were added to this suspension and stand for 1 hr. followed by filtration and washing with 75% ethanol and acetone and dried at 70°C in oven overnight. The dried residue was weighed for the soluble dietary estimation.

2.4.5. Minerals

Minerals are essential elements for the proper development and growth of body. Calcium, phosphorus, iron, potassium, zinc are essential minerals for health. They help to maintain

bone health, immune system and manage the problem of heart, nervous system, respiration and other biological activities. Estimation of mineral availability in food is necessary. Various methods are used to evaluate the mineral content. In this study, UV-VIS spectrophotometer has been used to detect the mineral contents present in the developed value-added products.

Ash of sample was dissolved in 25 ml of 20% HCl followed by filtration and diluted to the 50ml with deionized water with proper mixing. The analysis for the available iron and phosphorus in the formulated products were done following spectrophotometer method (AOAC, 2000).

Figure 2. 11: UV-VIS Spectrophotometer for the biochemical estimation

Phosphorus present in food products, forms phosphomolybdate complex during the estimation process (**Chen *et al.*, 1956**) and then reduced to the complex molybdenum blue. 6N H_2SO_4, 2.5% Ammonium molybdate and 10% Ascorbic acid (1:2:1:1 v/v) were mixed to prepare fresh reagent solution. 4 ml of this solution was mixed to 1ml extract and incubated for 90 min at 37°C. Absorbance was recorded at 820 nm.

Available iron was converted to ferric using saturated Potassium persulphate (oxidizing agent). Later it was treated with 3N Potassium thiocyanate (Wong, 1928).

	Blank (ml)	Standard (ml)	Sample (ml)
Standard Iron solution (1 ml= 0.1 mg of Fe)	0.0	1.0	0.0
Sample ash solution	0.0	0.0	5.0
Water	5.0	4.0	0.0
Conc. H_2SO_4	0.5	0.5	0.5
Potassium persulphate	1.0	1.0	1.0
Potassium thiocyanate	2.0	2.0	2.0
Make up volume to 15 ml with water			

Table 2 1: Reaction mixture preparation for iron estimation

Absorbance was taken at 480 nm by using UV-VIS spectrophotometer.

$$\text{Iron (mg/100 g)} = \frac{OD\ of\ sample\ X\ 0.1\ X\ Total\ volume\ of\ ash\ solution\ X\ 100}{OD\ of\ standard\ X\ 5\ X\ Weight\ of\ sample\ taken\ for\ ashing} \text{ ..Eq. (2.8)}$$

However, **available calcium** was determined by acid digestion followed by the titration method. Calcium was precipitated as Calcium Oxalate and dissolved in hot dilute H_2SO_4. Titration was carried out with standard Potassium permanganate (0.01 N). 10 ml of saturated ammonium oxalate solution with 2 drops of Methyl Red indicator was added to 20-100 ml of ash solution and maintained the pH 5.0. The solution was heated to boiling point and kept overnight at room temperature followed by filtration next day. The precipitate in filter paper was washed with hot dilute H_2SO_4 (1+4) followed by washing with hot water and titration to first permanent pink colour.

Calcium (mg/100 g) =

$$\frac{Titre\ X\ Normality\ of\ Potassium\ permanganate\ X\ 2\ 0X\ Total\ volume\ of\ ash\ solution\ X\ 100}{ml\ of\ ash\ solution\ taken\ for\ estimation\ X\ Weight\ of\ the\ sample\ taken\ for\ ashing} \text{ …Eq. (2.9)}$$

2.4.6. Antioxidants

Antioxidants prevent the damage caused by free radicals. Hence, they are also known as free-radical scavengers. Antioxidants mange the oxidative stress, generated due to excessive production free radicals. Oxidative stress can increase the risk of cancer, immune malfunction, Parkinson's disease, respiratory problems, inflammatory conditions. Presence of antioxidants in such conditions, neutralize free radicals and improve overall health

condition. Various methods are available to detect antioxidant activity. In the present study, DPPH activity and phenol content have been measured.

According to the method of Xu et al. (2007), extracted samples were kept in 4ºC. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (Hudec *et al.,* 2007) was determined by taking 0.4 ml 2.5 mg/L DPPH solution and 7.6 ml of extracted solution followed by incubation for 30 min at room temperature. Absorbance was taken at 517 nm.

$$\frac{[(A_{Control} - A_{Sample}) X\ 100]}{A_{Control}} \quad \text{.....Eq. (2.10)}$$

Where, $A_{Control}$= absorbance for the control and A_{Sample}= absorbance for the sample

Due to requirement of relatively less time, this method is generally followed to check the antioxidant activity present in foods (Rahman *et al.,* 2015).

2.4.7. Total phenolic activity

Total phenolic activity (Singleton *et al.,* 1965) was evaluated through Gallic acid calibration standards and was absorbance was taken at 750 nm. 5ml Folin-Ciocalteu reagent (1N) was added to 1ml of extracted sample and incubated for 8 min at room temperature. 15 ml of Na_2CO_3 solution was added followed by incubation of 2 hrs. at room temperature to further monitor the reading.

2.4.8. In vitro digestibility of Protein and Starch

Incorporation of nutrients in diet is necessary for proper development and functioning of body. After food consumption, food gets processed leading to absorption of nutrients in body. The amount of absorbed nutrients and that of nutrients present in food is not same. Hence, it is necessary to estimate availability of nutrients after digestion. Therefore, in vitro conditions are created to evaluate the availability of nutrients.

In this study, In vitro protein and starch, digestibility was examined by the modified methods of Mertz et al. (1983) and Singh et al. (1982) respectively. 250 mg of sample and 20 ml of pepsin reagent in a tube was incubated at 37ºC for 3 h. After cooling down, 5 ml of TCA (50 %) was added followed by the centrifugation at 10,000 rpm for 10 min and filtered. Digested protein in the sample was determined.

Protein digestibility (%) = (Digested protein/ total protein) X 100 ……Eq. (2.11)

50 mg fat free sample along with 1.0 ml 0.2 M phosphate buffer (pH 6.9) and 0.5 ml pancreatic amylase were incubated at 37ºC for 2 h. Then 2 ml dinitrosalicylic reagent was added and heated for 5 min in a boiling water bath. After cooling, the solution was made to 25 ml with distilled water and filtered. Dinitrosalicylic reagent was added before addition of the enzyme solution.

In vitro starch digestibility= concentration from graph (mg)/ weight of sample (g).Eq.(2.12)

2.4.9. Antinutrient

Besides nutrient composition, presence of antinutrient in raw ingredients and food products can result in various health conditions. Higher level of antinutrient impairs minerals absorption in body. Plant sources are generally considered as rich source of antinutrient. Although antinutrient lowers the nutrient absorption in body, presence of certain amount of the antinutrient also helps in improvement of some health conditions. Phytate in higher concentration has been found to lower the iron absorption in body. But the lower amount of phytate has been found to exert positive effect in diabetic condition. Hence estimation of antinutrient in food is crucial. In the present study, after the treatment with nitric acid (0.5 M) and centrifugation at 800 rpm for 15mins, the extracted sample was used to check phytic acid level in the products following the method described by Davies and Reid (1979) at 465 nm against iso-amyl alcohol taken as blank. Trypsin inhibitor activity (TIA) was determined according to the modified method of Roy and Rao (1971). Sample extraction was done with trichloroacetic acid (TCA) at 10000 rpm for 10 minutes to further check the TCA soluble proteins in the supernatant according to the method of Lowry et al. (1951). TIA is expressed as trypsin inhibitor units (TIU)/mg. The amount of trypsin enzyme that converts one mg of casein to TCA soluble proteins (37ºC for 20 minutes; pH 7.6) is known as one unit of trypsin or TIU.

2.4.10. Determination of Vitamin C

Vitamin C, also known as Ascorbic acid, is a water soluble vitamin and generally found in citrus fruits. This essential nutrient helps to prevent scurvy and is required for various enzymatic function, immune system. It acts as antioxidant and lowers of risk of diseased conditions like cancer, dementia, cardiovascular problems, neuro-malfunction, skin diseases. This is the first vitamin to be produced chemically. In the present study, Vitamin C available in the food sample was analysed by the titration method with 2,6-dichlorophenol- indophenol dye until observed a faint pink endpoint (AOAC, 2000)

2.5. Measurement of Shelf life evaluation of the preferred formulations

The shelf life of the formulated products was checked at the interval of 0, 15, 30, 45, 60, 75, and 90 days.

2.5.1. Sensory evaluation of products

Besides food formulation and nutrient composition estimation, evaluation of sensory profile of the developed food products is a crucial aspect. The sensory evaluation viz., appearance, colour, texture, flavour acceptance of the products and overall acceptance for quality were evaluated using 9-points hedonic rating scale (Peryam and Pilgrim 1957) where, 1 point depicts the lowest acceptance and 9 points depicts the highest acceptance. The points were given by a Panel consisting 10 judges. They were informed in advance that they would be evaluating cereal bran and legume husk based fermented products. Panellists were said to wash their mouths with water before the examination to minimize residual effect.

2.5.2. Free fatty acid

Free fatty acid is generated from triacylglycerol due to cleavage of ester bonds by action of lipase, moisture and high temperature. It can act as pro-oxidants in oils and enhance the rate of hydroperoxide decomposition leading to further oxidation and generation of foul taste, flavour. Hence free fatty acid value is considered as indicator for quality of oil or oil containing products. In the present study, Free fatty acid level in the food products were estimated according to AOAC (2000). Food samples along with 50 ml of neutralized Isopropyl alcohol was titrated against 0.25N NaOH for 30 seconds for the quantification.

2.5.3. Peroxide value

Thirty ml of acetic acid and chloroform mixture was added to dissolve the product samples (AOAC, 2000). After treatment of sample with 0.5 ml saturated potassium iodide solution, it was titrated slowly against 0.01 N Sodium Thiosulphate followed by another titration until release of all iodine from chloroform layer.

2.6. Determination of Microbial load

Growth of microorganisms was analysed to detect microbiological safety of freshly prepared products (Rico *et al.,* 2019).

Figure 2. 12: Laminar flow for the microbial load count estimation

Total Plate Count of microbial growth in food products or raw ingredients gives the count of all heterotrophic bacteria that grow in aerobic as well as microaerophilic condition. Total number of viable microorganisms under aerobic conditions i.e. the Total Plate Count was done by using method IS 5402 with a slight alteration. Incubation temperature and duration were 30°C and 72 hrs. respectively. Yeast and Mold count was done by using method IS 5403. Plates with sample dilution were incubated at 25°C for 3 to 5 days. One gram of the formulated products was suspended in 10 ml sterile saline that is 0.9% and mixed properly using a vortex mixer to get appropriate dilutions for microbial analysis. Coliform bacteria are facultative anaerobic, gram-negative, non-spore-forming

rods which ferment lactose and generates acid and gas. These bacteria are believed to be good indicator as their presence in food indicates the suitability of pathogen growth. Lactose fermenting coliform microorganism growth (Coliform Count) was determined by using Violet Red Bile Agar and incubated at 35°C for 48 hrs. (IS 5401).

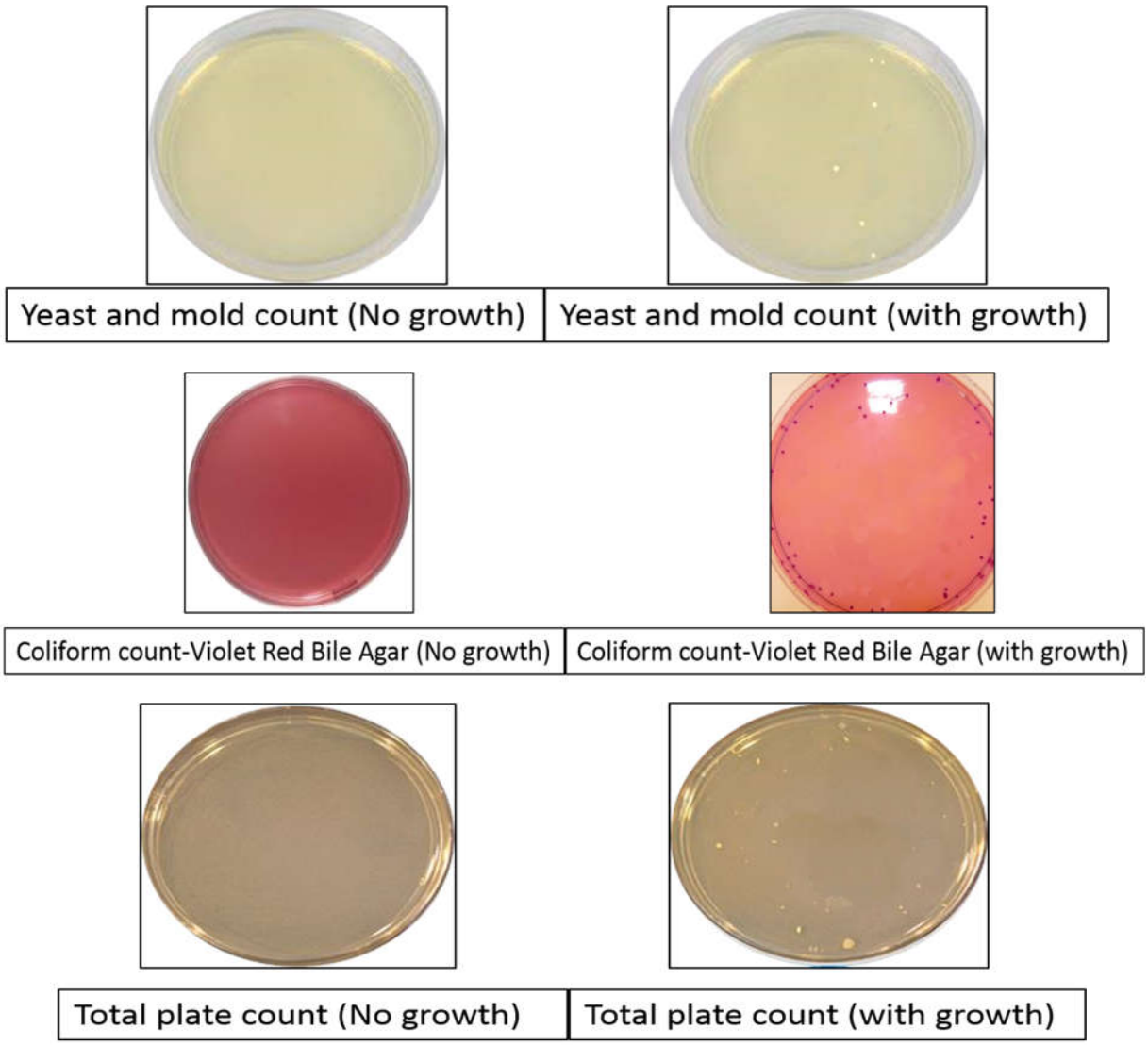

Figure 2. 13: Microbial load determination: Total Plate Count, Yeast and Mold Count, Coliform Count

2.7. Statistical analysis

Based upon the completely random design data was analysed using MS Office Excel (2016) in triplicates where the analysed data was presented as means ± standard error.

Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey HSD Test ($p < 0.05$).

2.8. Enhancement of shelf life using Gamma radiation

Ionizing radiation is a phenomenon where radiation energy is utilized to sterilize or preserve materials, destroy microorganisms, parasites, insects and pests. The emitted energy can remove bound electrons from an atomic or molecular structure resulting in ionization. The major ionizing radiation used for industrial purpose, especially in food industry is electromagnetic gamma rays (γ ray). These rays obtained from radioactive source of Cobalt 60 (^{60}Co) and Caesium 137 (^{137}Cs), are channelled by an electron accelerator to form a stream of high-speed electrons driven to foods (Munir and Federighi, 2020). Among all other sources, common radioactive sources used for food purpose are ^{137}Cs and ^{60}Co. ^{60}Co generally emits radiation and two photons of energy that is 1.17 and 1.33 MeV (Munir and Federighi, 2020). In food industry, ^{60}Co is mostly used. ^{60}Co is an artificial radio nucleotide which was produced in nuclear reactors from Cobalt 59, a non-radioactive metal. The use of radioactive treatments is based on the duration of the exposure of radioactive source to the material. ^{137}Cs is also used for food purpose, although its use is limited to laboratory purposes. The half-lives of the radioactive sources, ^{60}Co and ^{137}Cs are 5.26 and 30.17 years respectively (Munir and Federighi, 2020).

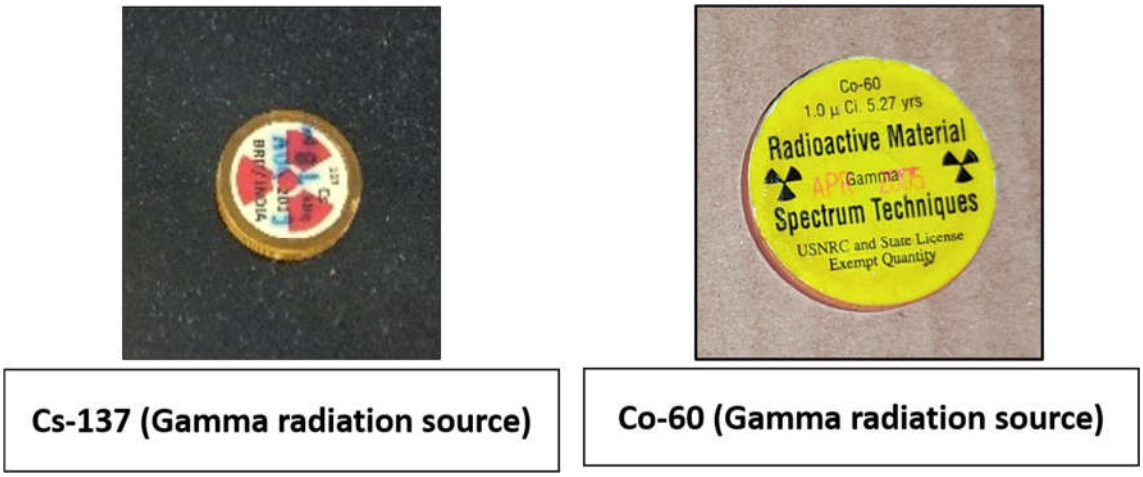

Figure 2. 14: Gamma sources- Caesium-137 & Cobalt-60 to irradiate food products

2.8.1. Attenuation Co-efficient determination

After exposure of any material to radioactive source, the interaction between the material and the source occurs in three possible ways viz., Photo-absorption, Compton effect, and Pair production. Their probability of occurrence per unit length of path traversed characterizes them and the sum of such probabilities is known as Linear Attenuation Coefficient. The value of linear attenuation coefficient increases with increase in atomic number of the material medium and decreases with the energy of incident photon.

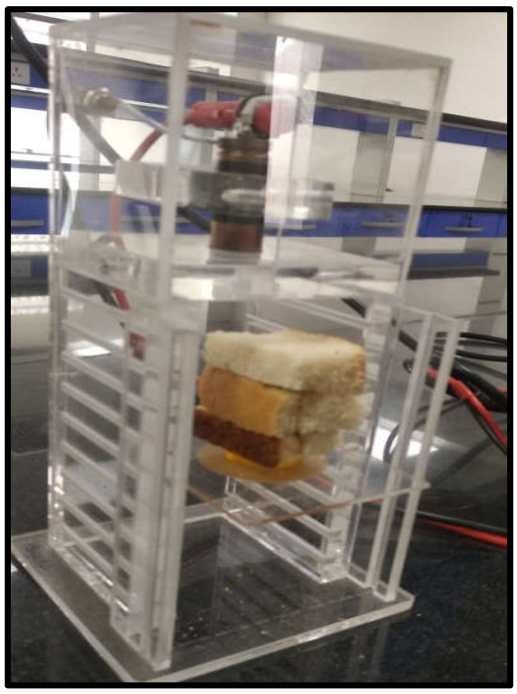

Figure 2. 15: Attenuation coefficient measurement of the food product using GM counter

During the measurement of attenuation coefficient using GM Counter, Background count (n_b) was recorded first at time interval of 90 seconds and operating voltage 550 volts followed by measurement of counts of the radioactive source only (n_0), placed in the 2nd groove. Then one sample (absorber) (n_c) was placed just above the source and the count was recorded to check the attenuation property of the same. Thickness of the sample was also measured. Hence, the intensity of radiation to material's thickness can be linked as follows:

$$I = I_o e^{-\mu x}$$

$$\boldsymbol{\ln I = \ln I_o - \mu x} \quad \text{.....Eq. (2.13)}$$

Where 'I_o' is the intensity without any absorber; 'I' is the intensity by placing an absorber; 'μ' is the attenuation coefficient.

Effect of gamma radiation on any absorber depends on the energy of the gamma radiation as well as on the composition of the absorber. Attenuation Coefficient and absorbed energy reflects the interaction of radiation with the absorber. Attenuation Coefficient describes the relative decrement in the intensity of radiation passing through the absorber. If the absorber is relatively transparent, we will get small values of attenuation coefficient whereas large values of attenuation coefficient reflect the opacity of the absorber.

The mass attenuation coefficients of different samples of Bran Breads and Multibran Cookies are determined using scintillation detector system combined with 1024 channels multichannel analyser (MCA). ^{137}Cs and ^{60}Co point source having present day activity 0.3477μC and 0.12μC respectively. The calibration of detector was performed using test radioactive source. The counting time with and without absorber was set to 1000 seconds in order to minimize statistical error, counting timing and other experimental conditions were kept same to obtain initial and final intensity. Stability of detection system was checked by channels representing energy peak positions with and without absorber. No shift in the Peak confirms the stability of the detector. Samples were made to expose with gamma sources ^{137}Cs and ^{60}Co for different intervals of time. The attenuation coefficient is the reflection of the internal structure and composition. The quantity of energy absorbed by any food item is related to their attenuation coefficient. Therefore, measurement of attenuation coefficient is very important quantity. Small value of linear attenuation coefficient indicate that material is transparent, while a larger value of attenuation coefficient indicates greater opacity (Ghosh and Das, 2014).

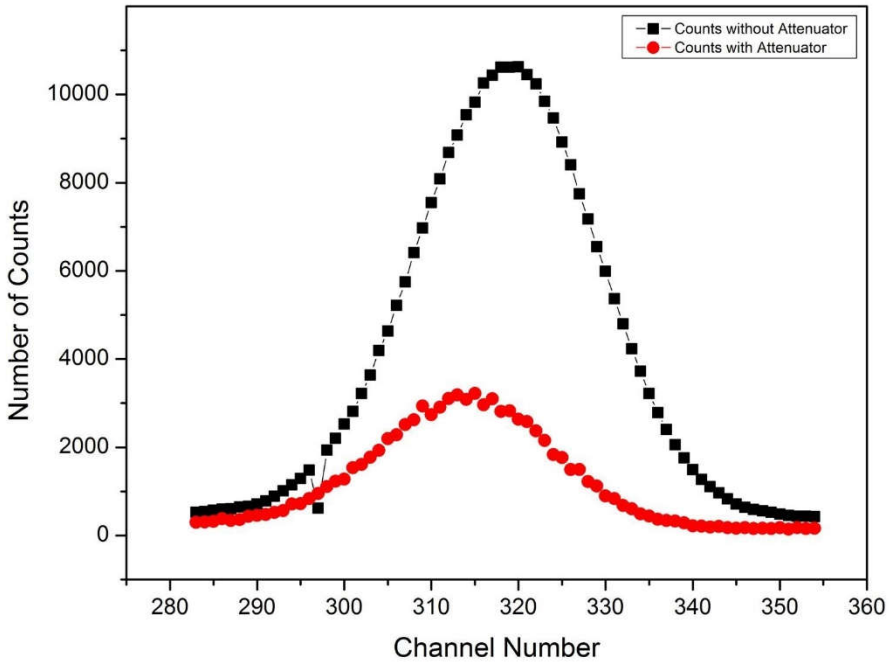

Figure 2. 16: A typical spectrum of ^{137}Cs with and without Attenuation by Bran Bread sample.

2.8.2. Irradiation of the formulated products

In the present study initially we have estimated the background due to the local environmental conditions. Gamma radiations effects structural as well as chemical composition in different food materials (Nayak *et al.,* 2006). It has been observed that the free radical formation results in biochemical changes in food.

^{137}Cs and ^{60}Co gamma sources were used to improve the shelf life of the products (Bran Bread, Gram Pak, Bran Paneer). The samples were placed at a height of 7 cm below the radioactive source inside a box container (Figure 2.16**)** for different time durations viz., 24 hours, 48 hours, 72 hours at room temperature. After the exposure, irradiated samples and non-irradiated samples (Control) were stored at room temperature. Present counts in the irradiated products were measured until the count became equal to the background count i.e. the zero count. The counts were detected using GM Counter and Na(I)-TI Detector. The evaluation of the shelf life of irradiated and control products was made from the observation of the visual aspects. The samples were observed continuously each

passing day till mold started growing on the samples and that was considered as initiation of the spoilage.

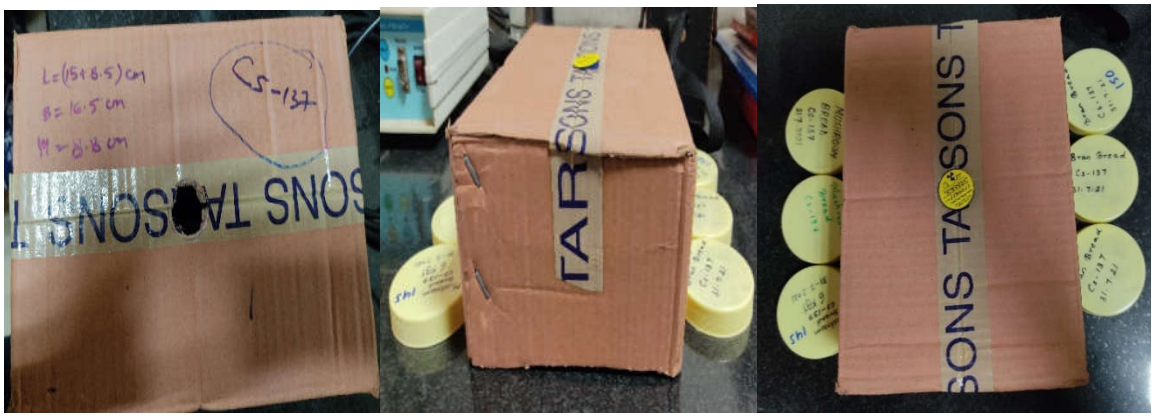

Figure 2. 17: Exposure technique to irradiate food samples using gamma source

2.8.3. Detection of counts using GM Counter

GM Counter is a type of nuclear detector, capable of detecting different nuclear radiations viz., alpha particle, beta particle and gamma Radiations and also in some cases neutron particle through some indirect methods.

Figure 2. 18: GM Counter to screen the radiation counts.

This counter consists of a Geiger-Muller tube which is a hollow and cylindrical shaped metallic tube. This tube consists of some Gas Mixture (Primary Gas; Figure 2.18) and mixture of compounds of alcohol (Quenched Gas).

This metallic cylinder is connected to the negative end of the high tension battery and acts like Cathode. Through the centre of this tube, there is a metallic (Tungsten) electrode, acting as an anode, connected to a load resistance (R) and is further connected to the positive terminal of that same high voltage battery. Across the load resistance (R), there is also an electronic setup, capable of determining any kind of potential drop across the load resistance (R) and measuring the current pulse associated with that.

Gas Mixture
90% Ar. 10% CH_4 (P-10)
95% Ar. 5% CH_4 (P-5)
100% CH_4 (Methane)
100% C_3H_8 (Propane)
96% He. 4% Isobutane
75% Ar. 15% Xe. 10% CO_2
69.4% Ar. 19.9% Xe. 10.7% CH_4
64.6% Ar. 24.7% Xe. 10.7% CO_2
90% Xe. 10% CH_4
95% Xe. 5% CO_2

Figure 2. 19: Gas mixtures along with their percentage concentration that can be taken as a primary gas **(Knoll, 2010)**

When an alpha or beta particle enters into this kind of medium, it basically leads to ionization. In the meantime, some external nuclear particle viz., an alpha particle comes into a material medium and collides with the molecules of the material medium. It transfers energy to the molecules of the material medium. Electrons, in the outermost shells of these molecules and atoms, absorb some of the energy. If the energy is sufficient enough, these electrons will become free and results in creation of positive ion and a free electron. So, the effect of an external nuclear particle is basically to ionize the gas inside this kind of detector. It ends up getting a positive ion and the negative electron due to the ionization caused by some kind of an external nuclear particle. GM Counters are having battery of creating potential of 1000 to 3000 volts and under this potential difference, the electron will experience acceleration towards the Central Wire (Anode) and the positive ion towards the metallic tube (Cathode).

2.8.4. Detection of counts using Na(I)-TI Scintillation Detector

Scintillation counter detects and measures ionizing radiation based upon the excitation effect of incident radiation on a scintillating material. It detects the resultant light pulses. There are many varieties of inorganic as well as organic scintillators which mainly differ based upon the light yield and decay time (Kharzheev, 2015). The most commonly utilized inorganic scintillators are NaI(Ti), CsI(Ti), BaF_2, BGO, $PbWO_4$, and LSO:Ce. Among other scintillators, sodium iodide NaI(Ti) exhibits the highest yield that is 40000 photons per 1 MeV of energy loss. It is hygroscopic in nature (need air-tight enclosure to operate). The spectral sensitivity of bi-alkaline PMTs goes with the spectral composition of maximum 415 nm of its light. The principle of scintillation counter denotes operation of the counter through emission of light as an ionizing particle passes through the luminescent material of the counter. Then the emitted light is first collected followed by conversion and guided to the photodetector (PD) (Kharzheev, 2015). The most central features of the scintillators are light yield and collection, time resolution, attenuation length, stability characteristics, radiation hardness, ability to adapt the emitted light to the spectral sensitivity of the PD followed by transmission of light to PD. These counters are considered as efficient instruments in many experiments, especially the field of nuclear physics. They are easy to operate and calibrate, reliable (Kharzheev, 2015).

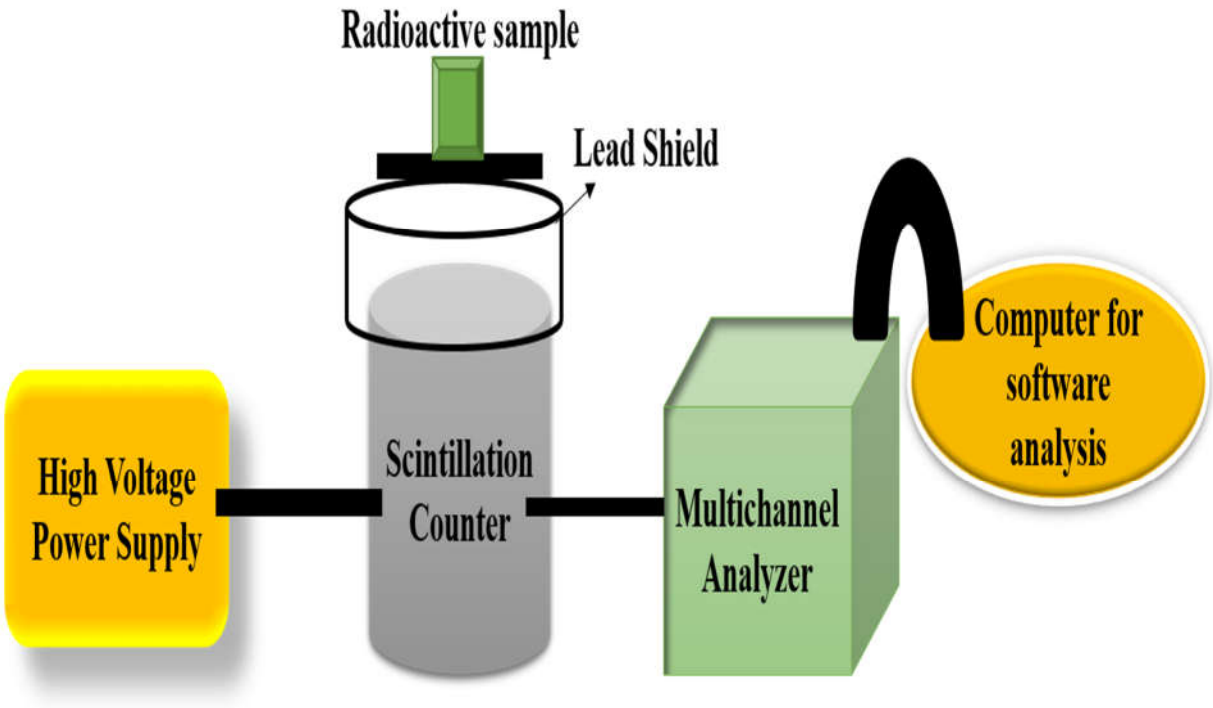

Figure 2. 20: Schematic presentation of scintillation counter

Figure 2. 21: Scintillation Detector for the detection of radiation counts

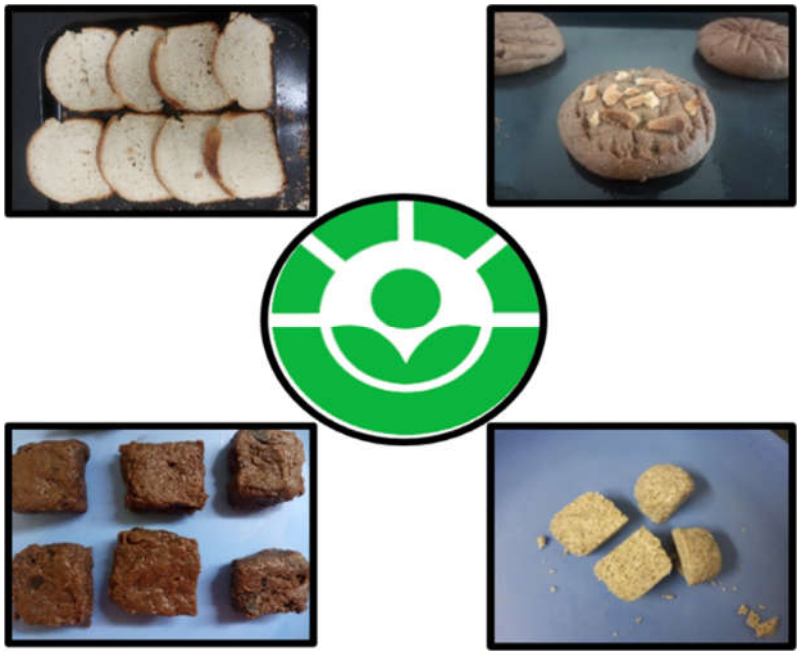

Figure 2. 22: Food irradiation technology to enhance the shelf life of the products

2.9. Popularization of the acceptable product and transfer the technology regarding their preparation and utilization

In collaboration with KrishiVigyan Kendra, Mahendergarh, training program was conducted to popularize the acceptable product along with the transfer of the process for their preparation and utilization to women of local adapted villages.

2.10. Summary

In summary, we discussed the details of methods to process raw by-products, value added product formulation followed by estimation of nutrient composition, shelf life study, sensory evaluation in this chapter. In the following chapters, we shall present the detailed analysis of various value added products and their nutrient composition along with their storage stability at different storage conditions. Apart from this, microbial load count also will be discussed. The effect of gamma radiation exposure using ^{137}Cs and ^{60}Co upon the improvement of shelf life of the developed products will also be disclosed. Dissemination of the technology and popularization of the formulated products will be further discussed in the successive chapters.